UNIVERSIDADE FEDERAL DE VIÇOSA

Influence of the interactions of qualitative and quantitative genetic factors on fruit yield and quality in tomato (*Solanum lycopersicum*)

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Doctor Scientiae

VIÇOSA - MINAS GERAIS
2024

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Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Agustin Zsogon

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2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

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B238i
2024

Barbosa, Guilherme Mateus Dias, 1988-
Influence of the interactions of qualitative and quantitative genetic factors on fruit yield and quality in tomato (*Solanum lycopersicum*) / Guilherme Mateus Dias Barbosa. – Viçosa, MG, 2024.
1 tese eletrônica (113 f.): il. (algumas color.).

Texto em inglês.
Orientador: Agustin Zsögön.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Vegetal, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2025.155
Modo de acesso: World Wide Web.

1. Tomate - Melhoramento genético. 2. Marcadores genéticos. 3. Heterozigosidade. 4. Alelos. 5. Epitase. I. Zsögön, Agustin, 1980-. II. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-Graduação em Fisiologia Vegetal. III. Título.

CDD 22. ed. 635.6422

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

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APPROVED: August 30, 2024.

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I offer to my family,

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

I first thank God for granting me health, strength, and wisdom to live.

I extend my gratitude to the Federal University of Viçosa, especially to the Graduate Program in Plant Physiology, for providing access to its facilities during the development of this work, and for the knowledge gained at this institution since my undergraduate studies.

Thanks also to CAPES and CNPq for providing a scholarship during part of my course, and to Embrapa Hortaliças for offering facilities to carry out some of the analyses in this thesis.

I am grateful to my advisor, Dr. Agustín Zsögön, for his support throughout the development of this research project, and to my co-advisor, Dr. Leonardo Silva Boiteux.

I am grateful to Dr. Maria Esther de Noronha Fonseca, for her guidance in the transcriptomic analysis at Embrapa Hortaliças.

I also deeply appreciate my examining committee members, Dr. Kaio Olímpio das Graças Dias, Dr. Dimas Mendes Ribeiro, Dr. Lázaro Eustáquio Pereira Peres, and Dr. Samuel Cordeiro Vitor Martins, for agreeing to evaluate my thesis and providing all the necessary feedback to improve this work.

Thanks to my colleagues in the Molecular Plant Physiology Laboratory at UFV and Embrapa Hortaliças for their help and support during the various stages of my experiments.

I am very grateful to my beloved wife, Vanessa Maria Gonçalves, and my son, Joaquim Barbosa Gonçalves, who are my greatest motivation. To my parents, Sebastião Ferreira Barbosa and Nilda Dias Barbosa, and my siblings, Samuel and Nayara, for all their love, support, patience, and understanding throughout all stages of my life.

To the Brazilian people for funding this work, my sincere thanks, and may this work contribute to the development of our nation.

“Science is the key to our future, and if you don’t believe in science, you’re holding us back”
(Bill Nye)

ABSTRACT

BARBOSA, Guilherme Mateus Dias, D.Sc., Universidade Federal de Viçosa, August, 2024. **Influence of the interactions of qualitative and quantitative genetic factors on fruit yield and quality in tomato (*Solanum lycopersicum*)**. Adviser: Agustin Zsogon. Co-advisers: Leonardo Silva Boiteux and Samuel Cordeiro Vitor Martins.

The study of gene relationships at the level of a single locus (allelic relationships) as well as at the level of multiple loci (epistasis), is crucial for crop genetic improvement. In the case of single loci, understanding overdominance can enable the selection of heterozygotes that maximize hybrid vigor. For multiple loci, understanding epistasis allows for the combination of desirable traits. In this research, certain tools become important, such as the evaluation of yield in field trials to verify whether the observed effect is verified in real crop environments, where factors like climate, soil, and agricultural practices influence plant performance and the identification of differentially expressed genes (DEGs) to pinpoint which candidate genes are potentially involved in traits of interest. In this context, the present study aimed to evaluate a set of tomato (*Solanum lycopersicum* L.) F1 hybrids involving the *epinastic* (*epi*) mutation to estimate potential heterotic effects associated with the presence of this mutation in different tomato genetic backgrounds. Mutations in pleiotropic genes potentially involved in the synthesis of plant hormones, such as *epi*, can result in broad changes in gene expression patterns and alter numerous metabolic pathways that may affect tomato growth and production. Here, transcriptomic analysis via RNA-seq was employed as a tool to identify a set of differentially expressed genes (DEGs) potentially modulated by the *epi* mutation in contrasting genetic backgrounds. A chromosome landing strategy on the candidate *epi* gene(s) was carried out involving a combination of genomic mapping and information derived from the transcriptome of two contrasting near-isogenic lines (NILs). The results suggest that a heterozygous mutation can alter both the level of allelic interaction and several epistatic interactions, particularly in relation to hormonal pathways, which culminates in phenotypes that may be of interest from a breeding perspective.

Keywords: Heterosis ; ethylene; mutation ; Solanaceae; transcriptome; overdominance

RESUMO

BARBOSA, Guilherme Mateus Dias, D.Sc., Universidade Federal de Viçosa, agosto de 2024. **Influência das interações de fatores genéticos qualitativos e quantitativos no rendimento e qualidade de frutos em tomate (*Solanum lycopersicum*)**. Orientador: Agustin Zsogon. Coorientadores: Leonardo Silva Boiteux e Samuel Cordeiro Vitor Martins.

O estudo das relações gênicas a nível de um lócus único (relações alélicas), quanto a nível de múltiplos loci (epistasia), é crucial para o melhoramento genético. No caso de lócus único, entender a sobredominância pode permitir a seleção de heterozigotos que maximizem o vigor híbrido. Para múltiplos loci, a compreensão das interações gênicas permite a combinação de características desejáveis. Para essa pesquisa algumas ferramentas se tornam importantes como, avaliação da produtividade em ensaios de campo para avaliar verificar se o efeito observado é confirmado em ambientes reais de cultivo, onde fatores como clima, solo, e práticas agrícolas influenciam o desempenho das plantas e a identificação de genes diferencialmente expressos (DEGs) para apontar quais genes candidatos estão potencialmente envolvidos em características de interesse, Neste contexto, o presente trabalho propôs a avaliar um conjunto de híbridos F1 envolvendo o gene *epinastic* (*epi*) para estimar potenciais efeitos heteróticos associados à presença desta mutação em diferentes origens backgrounds do tomateiro. Mutações em genes pleiotrópicos potencialmente envolvidos na síntese de hormônios, como o *epi*, podem resultar em amplas mudanças no padrão de expressão gênica e alterar numerosas vias metabólicas que podem afetar o crescimento e a produção do tomate. Análise transcritômica via RNA-seq foi empregada como uma ferramenta para investigar o painel de genes diferencialmente expressos (DEGs) potencialmente modulados pela mutação *epi* em plântulas de diferentes fundos genéticos. Uma estratégia de “aterrissagem cromossômica” foi implementada visando identificar genes candidato(s) para a mutação *epi*. Esta estratégia envolveu uma combinação de mapeamento genômico e informações derivadas do transcritoma de duas linhagens quase isogênicas contrastantes (NILs). Os resultados sugerem que uma mutação em heterozigose pode alterar tanto o nível de interação alélicas, como várias interações epistáticas, principalmente em relação as vias hormonais, o que culmina em fenótipos que podem ser interessantes para o melhoramento genético.

Palavras-chave: Heterose; etileno ; mutação ; Solanaceae ; transcritoma ;

sobredominância

CONTENTS

Chapter 1

General Introduction

GENERAL INTRODUCTION

Agricultural productivity, as well as product quality, are characteristics influenced by a complex interaction of genetic and environmental factors (RAWAL et al. 2016, AFIFAH et al. 2021). Among the genetic components, allelic relationships (at the single locus level) and gene interactions (epistasis) play a crucial role (KRIEGER et al. 2010; FERREIRA et al. 2018). Understanding the mechanisms that control gene interactions can lead to significant advancements in plant breeding, aiming for higher yields, the development of disease-resistant plants (VIGNESH et al. 2009), and products that better meet consumer needs.

Allelic Relationships (Single Locus)

Allelic reactions refer to interactions at a locus between different forms of a single gene (alleles). These relationships may include complete dominance, codominance, incomplete dominance, and overdominance (SAPIR et al. 2008; KRIEGER et al. 2010). In complete dominance, one allele imposes its full phenotypic expression over the other. For example, in maize (*Zea mays*), plant height is controlled by a single gene with complete dominance, where the allele for tall plants is dominant over the allele for dwarf plants (GRIFFITHS et al. 2005). In tomato (*Solanum lycopersicum*), various genes exhibit complete dominance expression patterns, including fruit coloration, hormonal mutants, morphological mutant, and pathogen resistance (for an extensive list of these mutants, see https://tgrc.ucdavis.edu/monogenic). In codominance, both alleles simultaneously express their characteristics, as observed in various allozyme systems (enzyme variants encoded by different alleles of the same locus) in tomatoes (RICK & YODER, 1988). The theory of incomplete dominance seeks to explain the incomplete expression of deleterious alleles as a basis for heterosis. In incomplete dominance, the heterozygote phenotype is intermediate between the phenotypes of the two homozygotes, as observed in snapdragon flowers (*Antirrhinum majus*) (RIEGER et al. 2001), stem length, panicle length, and panicle number in rice (*Oryza sativa*) (JUN et al. 1985), and the erect leaf phenotype in tomato (GONZÁLES-ARCOS et al. 2019). In overdominance, the heterozygote exhibits a phenotype superior to both homozygotes, as seen in some cases of hybrid vigor in tomatoes, particularly in heterozygous mutants (BIRCHLER et al., 2010, KRIEGER et al. 2010; VICENTE et al. 2015).

Epistasis (Multiple Loci)

Interactions among multiple loci are fundamental to the expression of complex and quantitative traits, such as productivity and some of its components. The combination of genes from different loci can result in phenotypes that are not predictable by the sum of the individual loci's effects. For example, wheat yield is influenced by the complex interaction of multiple QTLs (Quantitative Trait Loci) (KEARSEY & FARQUHAR, 1998). A specific case that occurs in the interaction between alleles is the phenomenon of epistasis, in which one locus can mask or modify the expression of different genes as well as alleles at another locus. An example of an epistatic gene in tomatoes is the *rin* (ripening inhibitor) mutant, which alters components of fruit flavor, color, aroma, texture, and size (MASAKI et al. 2013; WANG et al. 2020).

Impact on Yield

Studies have demonstrated that both allelic interactions at a single locus and gene interactions for multiple loci can significantly influence agricultural crop productivity. Examples of research in plant breeding aimed at understanding allelic interactions as well as gene interactions and their impact on crop productivity and quality include studies on heterosis and the localization of QTLs within the genome (FERREIRA et al. 2018; JUST et al. 2007; ZSÖGÖN 2011; NAVES et al. 2021).

Heterosis or Hybrid Vigor

The term "heterosis" was coined by SHULL in the 1910s to illustrate a plant genetic phenomenon whereby hybrids exhibit superior phenotypic traits compared to the parental materials. Thus, heterosis is characterized by an increase in vigor of a heterozygous progeny relative to the average of genetically divergent parents (FU et al. 2014). Although this phenomenon has been observed since the early days of modern agriculture, associated with increased fertility, height, and total weight in some cross-pollinated plant species (HOCHHOLDINGER et al. 2018), the use of the first hybrids began in the 1920s, with the first commercial maize hybrids in 1924. In the state of Iowa (USA), the use of hybrid maize increased from 10% in 1935 to over 90% in the following four years. From then on, there was a growing transition from open-pollinated cultivars to hybrids (CRABB 1947; CROW 1998). By 1950, most maize fields in the United States were using hybrid seeds (CROW 1998).

Following the commercial success of hybrid maize, the use of hybrid seeds extended to other crops such as beetroot (*Beta vulgaris*), sorghum (*Sorghum bicolor*), onion (*Allium cepa*), eggplant (*Solanum melongena*), peppers and chili peppers (*Capsicum* spp.), rice *(Oryza sativa*), cotton (*Gossypium hirsutum*), sunflower (*Helianthus annuus*), rapeseed (*Brassica napus*), and also tomato (*Solanum lycopersicum* L.) (MELCHINGER & GUMBER 1998; TAMTA & SINGH 2017; HOCHHOLDINGER & BALDAUF 2018; KUMAR et al. 2020).

Genetic Models to Explain the Phenomenon of Heterosis

To elucidate the mechanisms behind heterosis, several classic models have been proposed, such as dominance, overdominance, pseudo-overdominance, and epistasis. Below is a brief description of each model.

The *dominance* model hypothesis argues that heterosis occurs due to the complementation of recessive alleles (usually deleterious) present in contrasting inbred parents (SCHNABLE et al. 2013). Inbred parents have homozygous alleles with deleterious effects (inbreeding depression), which are masked or suppressed in the hybrid combination through the complementation of inferior alleles by superior alleles. Complementation occurs at multiple loci, where deleterious effects are not expressed, resulting in a superior F_1 hybrid. In this model, heterozygosity is not a requirement for heterosis; rather, the main contribution is the increase in the number of superior loci (YAO et al. 2013). However, the uncertainty of whether all complementation of recessive alleles would result in an additive effect on the final phenotype remains the main gap in the dominance model (ZELIANG et al. 2013).

Alternatively to the dominance model, another model proposed by classical genetics is *overdominance*, which suggests that heterosis results from the superiority of heterozygotes over each of the homozygotes (BIRCHLER et al. 2010). This model supports that a heterotic response is already caused by heterozygosity in small genomic regions, which potentially includes heterozygosis in single loci. Interactions between various alleles that do not occur in any homozygous states (dominant/recessive) and occur in the heterozygous state give rise to superior progeny performance.

In turn, the *pseudo-overdominance* model results from the imbalance between the dominance and overdominance models. This model is based on the fact that some small genomic regions in hybrids may have repulsion variations between two or more different

genes (BIRCHLER et al. 2006). In this model, dominant homozygous alleles (favorable) are linked to recessive alleles (unfavorable) in parental lines. This model demonstrates that dominant homozygous alleles (favorable) are linked to recessive alleles (unfavorable) in parental lines, but after hybridization, they reach a heterozygous state and behave as a "overdominant" locus. Thus, determining the possible roles of multiple genes in the superior performance of hybrids appears to be the greatest challenge.

In addition to these models described so far to explain allelic interactions, another model considered to explain heterosis is the *epistasis* model, which explains how the interaction of genes from at least two loci determines a phenotypic expression. A classic study suggested that both intra-allelic and inter-allelic interactions, as well as the crosstalk between genes and the environment, can influence the phenomenon of heterosis (POWERS 1944). Liang (2015), in his work on the genetic basis of heterosis in upland cotton (*Gossypium hirsutum* L.), showed a significant increase in productivity for the number of bolls per plant, directly contributing to fiber yield, as a result of an epistatic interaction. A metabolic profile was performed on two Arabidopsis populations (369 RILs, and their test-cross descendants, and 41 introgression lines (ILs) and their test crosses) (LISEC et al. 2009). This study revealed 147 QTLs for absolute intermediate heterosis of metabolites (aMPH), as well as 153 and 83 QTLs for additive and dominant effects. Thus, it can be concluded that epistasis significantly contributes to metabolite heterosis in Arabidopsis.

Evidence of Heterosis in Tomato

Heterosis and hybrid vigor have also been observed in tomatoes (BHATT et al., 2001; BAI & LINDHOUT, 2007). HEDRICK & BOOTH (1907) were the first to observe increased yield and a greater number of fruits in hybrid tomato plants compared to parental materials. WELLINGTON (1912) pointed out the commercial potential of producing F_1 hybrids in tomatoes.

Commercial use of heterosis in tomatoes has been pursued through F_1 combinations derived from crosses between inbred parents, where the interaction between multiple loci in heterozygosity generates the hybrid vigor effect. However, there are reports that the effect of overdominance due to heterozygosity at a single locus is sufficient to produce significant heterotic effects (KRIEGER et al. 2010; GUO et al. 2013; REHMAN et al. 2021).

A notable study on heterosis manifestations determined by mutations in major genes, as well as their potential for overerdominance effects, was conducted by KRIEGER et al. (2010). Some genes, particularly those with pleiotropic effects of agronomic interest, fit more appropriately into this category. In the study conducted by KRIEGER et al. (2010), heterotic effects were conditioned by a mutation in the *single flower truss* (*sft*) locus, which encodes the *SFT* (*SINGLE FLOWER TRUSS*) protein, responsible for encoding the main component of florigen. The recessive mutation responsible for loss of function (*sft*) results in late flowering and altered sympodial growth. Phenotypic analyses led to the hypothesis that the balance between the sft and *SELF-PRUNING* (*SP*) genes is primarily responsible for regulating the transition from the vegetative to the reproductive phase in tomato, both for the initial meristem and the sympodial meristems (LIFSCHITZ & ESHED, 2006).

VICENTE et al. (2015) also found similar results for tomato productivity when studying the vegetative-reproductive balance in a semi-determinate mutant hybrid controlled by a single locus. Determinate growth (*sp/sp*) causes nearly simultaneous fruit ripening, which, along with the compact growth habit, allows for large-scale mechanical harvesting (STEVENS & RICK, 1986). However, the downside is that determinate growth produces fruits with lower total soluble solids content (EMERY & MUNGER, 1970; ROUSSEAX et al. 2005). On the other hand, plants with a semi-determinate growth habit tend to exhibit a combination of favorable attributes, including increases in both production and total soluble solids content in the fruits (FRIEDMAN et al. 2002; CARMEL-GOREN et al. 2003; KRIEGER et al. 2010). This is possible because the semi-determinate growth habit balances the excessive vegetative growth of indeterminate plants and the excessive reproductive growth of determinate plants.

The results indicate a notable example of superdominance controlled by a single gene for tomato production with pleiotropic effects, demonstrating that superdominance can be achieved through subtle changes in gene dosage rather than complex genic interactions. As mentioned by KRIEGER et al. (2010), the use of potentially heterotic major genes is a new methodology for identifying mutant genes for potential hybrid use in agriculture.

The *epinastic* (*epi*) Mutation

One mutation with potential for use as a source of single-locus heterosis is the *epinastic* (*epi*) mutation, which is related to the balance of the ethylene hormone (BARRY, 2001; BASTOS, 2017). The *epi* mutant has been mapped to a 1 Mb region on chromosome *4* (BARRY, 2001). Its phenotype exhibits characteristics indicative of altered cell expansion, similar to wild-type plants when treated with ethylene. For example, stems and petioles have larger diameters than those of wild-type plants, and petioles display epinastic curvature. Leaves also exhibit a characteristic epinastic morphology and have ethylene production at higher levels than wild-type plants (FUJINO et al., 1988; BARRY, 2001; ZSOGON et al., 2008; BASTOS, 2017).

BASTOS (2017) demonstrated that the *epi* mutant in heterozygous form (+/*epi*) exhibits hybrid vigor (heterosis) through the effect of overerdominance for a set of vegetative and reproductive traits. However, this hypothesis has not yet been thoroughly evaluated in different varietal backgrounds, including accessions with indeterminate, semi-determinate, and determinate growth habits. For agronomic purposes, it is important to evaluate the effects of the mutation in heterozygous form across various genetic backgrounds to study whether there is a gene interaction between the mutant locus in heterozygous form and the geneses of the cultivar background of interest.

Mutations in genes with potentially pleiotropic effects can lead to alterations in the expression pattern of several other genes and, consequently, affect numerous metabolic pathways. In this context, transcriptome analysis can indicate which genes are differentially expressed or modulated (DUAN et al., 2019). Although the *epi* mutation is mapped to a region on chromosome *4*, the responsible gene locus has not yet been characterized. Therefore, gene characterization would be very important to validate the candidate gene and expand knowledge about this mutation (BASTOS, 2017; LI et al., 2022).

Analysis of Gene Expression Associated with Hormonal Mutants

Mutations in pleiotropic genes potentially involved in plant hormone synthesis, such as *epi*, can lead to broad alterations in gene expression patterns and affect numerous metabolic pathways (McCOURT, 1999). Recent microarray and transcriptome analysis (RNA-Seq) studies involving hormonal mutants in tomato and Arabidopsis have allowed for the study of effects on gene expression patterns and modulation of various hormonal signaling pathways and crosstalk between hormones in these species.

ALBA et al. (2005) identified 869 differentially expressed genes in the development of the tomato pericarp by analyzing gene expression profiles via cDNA microarray. Parallel phenotypic and targeted metabolite comparisons were used to support the expression analysis. It was observed that transcript accumulation in tomato is often ethylene-dependent and extensively coordinated. The mutation of an ethylene receptor *Never-ripe* (*Nr*) altered the expression of 37% of these 869 genes, reducing sensitivity to the hormone and inhibiting fruit ripening. Fruit morphology, seed number, ascorbate accumulation, carotenoid biosynthesis, ethylene evolution, and the expression of a wide range of genes were influenced by the *Nr* mutant during fruit ripening, indicating that multiple physiological aspects before and during fruit ripening are governed by ethylene.

DUAN et al. (2019) investigated differentially expressed genes (DEGs) and gene expression profiles in the cold-resistant tomato mutant (*M*) and wild-type tomato (*CK*) in response to low-temperature stress. Results indicated that 1,007 genes were positively and significantly regulated and 502 genes were negatively regulated in 1,509 DEGs from CKUC/CKC, and 751 genes were positively regulated and 647 genes were negatively regulated in 1,398 DEGs from MUC/MC. A total of 726 DEGs were tracked and annotated in contrasts between M and CK. Analysis of the unitary transcripts indicated that nine and 16 gene ontology terms were significantly enriched in CKUC/CKC and MUC/MC, respectively. Subsequent KEGG pathway analysis for different genes found that flavonoid and phenylpropanoid biosynthesis were significantly enriched in MUC/MC, highlighting their importance in the biosynthesis pathway. Interestingly, 24% of the 920 DEGs detected by ROBINSON & PARKIN (2008) in response to cold in *A. thaliana* overlapped with the results of DUAN et al. (2019).

A revolution in transcriptome analysis occurred with the introduction of next-generation RNA sequencing (RNA-seq) technology. With this technology, there is no need to use a set of probes to detect all expressed genes (GUPTA et al., 2013). In species like tomato, RNA sequencing (RNA-Seq) has proven to be a simpler and more powerful approach to quantify expression at the transcriptome level. Regarding RNA-seq analysis of DEGs involved in hormonal signaling or crosstalk between hormones, an interesting study was conducted by GUPTA et al. (2013). Analysis of tomato genome expression profiles revealed crosstalk between auxin and cytokinin in the root system, where new genes induced and repressed by cytokinin and auxin were identified as DEGs, and

changes in the expression levels of several of these genes were confirmed by qPCR. Many of these regulated genes in tomato represent orthologous genes regulated by cytokinin or auxin identified in other species, including CKX, ARRs, Aux/IAA, and ARFs. More recently, Jin et al. (2022) identified a spontaneous mutant of premature senescence MT318 controlled by a single recessive nuclear gene. Maximum photochemical efficiency (Fv/Fm), superoxide dismutase (SOD) content, and chlorophyll content in leaves of the MT318 mutant decreased gradually, while malondialdehyde (MDA) content increased significantly. Therefore, in this context, transcriptome analysis via RNA-seq represents a powerful tool to investigate DEGs associated with the *epi* mutation and to indicate which metabolic pathways are being altered by this allelic variant.

Mapping of QTLs

The identification of QTLs (Quantitative Trait Loci) in tomato has been crucial for understanding the genetic basis of complex quantitative traits such as yield, disease resistance, and fruit quality. Among the tomato characteristics, fruit size and shape, due to their economic importance, were some of the first traits to be mapped. Adhikari et al. (2020) identified new QTLs on chromosome 10 controlling the elongated fruit shape, accounting for up to 24% of the phenotypic variation. These genetic discoveries explained the large proportions of diversity in fruit morphology and enabled breeding programs to manipulate the morphological traits of tomato fruits to produce fruits demanded by specific market segments (RODRIGUEZ et al. 2011).

Another important trait for fruit quality is soluble sugar. Tang et al. (2021), in studying which genes might be controlling the soluble sugar trait in mature tomato fruits, identified 47 genes in two chromosomal regions through bulk segregant analysis (BSA) sequencing, which may affect the soluble sugar content in tomato fruits.

Consumers also often associate fruit color and uniformity with freshness and quality. Yellow shoulder disorder (YSD), characterized by yellow or greenish discoloration on the fruit skin, is a major concern for producers as it decreases the fruit's market value. Therefore, YSD has been studied for some time. Topcu (2024) identified three QTLs that explain approximately 30% of the phenotypic variation. Additionally, candidate genes in each QTL interval were identified based on their expression profiles and genetic polymorphisms, such as single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) in gene/intergenic regions.

Fruit color and color uniformity are of the utmost importance for fresh consumption and the tomato processing industry. This is mainly because consumers generally associate vibrant and uniform colors with freshness and quality. Yellow shoulder disorder (YSD), characterized by yellow or greenish discoloration on the fruit skin, is one of the main concerns for tomato producers worldwide, as YSD symptoms negatively affect market value and consumer appeal. Therefore, YSD has been studied for a long time, but the genetic aspect of the problem has not been a major focus and thus has remained largely unknown. After sequencing the complete genomes of the bulks, three Quantitative Trait Loci (QTLs) were identified, namely YSD1.1 on chromosome 1, YSD3.1 on chromosome 3, and YSD11.1 on chromosome 11, using QTL-seq and traditional linkage mapping. These three QTLs explained approximately 20% of the phenotypic variation in the F_2 mapping population. Additionally, candidate genes in each QTL interval were identified based on their expression profiles and genetic polymorphisms, such as single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) in gene/intergenic regions. These genes are involved in fruit ripening, cell wall modification, lipid metabolism, and starch degradation. This study reports the first QTLs associated with YSD resistance and 17 KASP markers that can be used in marker-assisted selection for YSD in tomato.

Objectives

This work aimed to elucidate the behavior of allelic and gene interactions in tomato and how they influence fruit yield and quality with the following objectives: **(1)** To study the potential expression of overdominance effects associated with the *epimastic* mutation in different genetic backgrounds (varietal groups), **(2)** to estimate the agronomic potential of this gene, **(3)** to catalog a subset of genes that exhibited altered gene expression both in contrasting isolines (with and without the *epi* gene) and in the hybrid combination, and **(4)** to identify potential candidate genes.

To achieve this, the work is divided into the following chapters: first (**Chapter 2**), contrasting near-isogenic lines (NILs - with and without the *epi* gene) were produced through backcrosses and later used as parents for hybrids from different tomato varietal groups. Comparative studies with hybrids derived from contrasting NILs allow for a refined analysis of the impact of the *epi* gene on expression across a set of traits potentially affected by ethylene balance in tomatoes. In the following chapter (**Chapter 3**), we

analyzed the transcriptome of the *epi* mutant and listed the main genes that were differentially expressed due to the *epi* mutation. Finally, in the last chapter (**Chapter 4**), we integrated transcriptome data and identified potential candidate genes for the *epi* mutation.

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Chapter 2

Investigating potential heterotic effects in tomato hybrids from distinct genetic backgrounds employing contrasting near-isogenic lines for the hormonal mutant *epinastic* (*epi*).

Investigating potential heterotic effects in tomato hybrids from distinct genetic backgrounds employing contrasting near-isogenic lines for the hormonal mutant *epinastic*.

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Abstract – Heterosis or hybrid vigor is a genetic phenomenon in which the expression of a trait in the progeny is superior to that of the parental inbred lines. Hybrid vigor has also been observed in tomato (*Solanum lycopersicum* L.). In commercial tomato breeding, heterosis has been sought in F_1 combinations from crossings of highly inbred parents, where the interaction between several loci in heterozygosity results in hybrid vigor. However, there is experimental evidence that, due to the overdominance and pleiotropic effects, the heterozygosity of a single locus is sufficient to achieve heterosis. Previous trials supported the hypothesis that the single locus *epinastic* (*epi*) mutation, when in heterozygous condition, can express an overdominance effect, resulting in significant increases in yield and fruit quality. In this context, the objective of the present study was to evaluate a set of F_1 hybrids involving the *epi* gene to estimate potential heterotic effects associated with the presence of this mutation across distinct tomato genetic backgrounds. Thus, hybrid seeds were generated by crossing five commercial cultivars with indeterminate growth habit (heterozygous for the self-pruning gene *Sp*/*sp*) and five cultivars with determinate growth habit (*sp*/*sp*) with two contrasting near-isogenic lines ('VFN8 *epi*' and 'VFN8' without the *epi* mutation), totaling 20 F_1 hybrid combinations (ten mutants and ten non-mutants). The heterosis was detected in only two of these 20 contrasting hybrids, one involving a determinate inbred line, and the other involving an indeterminate inbred line. Hence, the *epi* mutant gene in heterozygosis in the F_1 generation can, in specific genetic backgrounds, express heterosis for yield regardless of the growth habit. In our experimental conditions, the heterotic effects may have been caused by a maternal effect, since these two heterotic hybrids were the result of crosses using the mutant as a maternal line (pollen recipient). Thus, our results indicate *epi* as a novel gene with potential to be explored in plant breeding, since it confers heterosis in reproductive traits. However, the effect of the *epi* mutation needs to be better evaluated in reciprocal crosses and different genetic backgrounds due to the high likelihood of the involvement of epistatic interactions with the expression of heterosis observed herein.

Keywords: *Solanum lycopersicum***,** recessive mutation, yield, fruit quality, breeding.

INTRODUCTION

The global production of tomato (*Solanum lycopersicum* L.) in 2021 has been around 189.1 million metric tonnes (MT) (https://www.tomatonews.com/en/worldwide-total-fresh-tomato-production-in-2021_2_1911.html). In Brazil, 59.7 thousand hectares of tomatoes were planted in 2018, producing 4.1 million tons of fruit (https://www.ibge.gov.br/). The high tomato yields is largely due to the development of superior cultivars via classical breeding strategies (GEORGIEV et al. 1991; ARIIZUMI et al., 2013). In addition to yield, other characteristics of interest must be present in novel varieties, which may vary according to the varietal segment, including earliness, fruit shape and size, attractive fruit color, and the ability to withstand the biotic and abiotic stresses (BORÉM et al. 2017). In this scenario, the development of hybrid cultivars must be highlighted, due to their expression of heterosis or hybrid vigor (FERNIE et al. 2006; SOLIEMAN et al. 2013; KUMAR et al. 2015; TAMTA & SINGH, 2017).

Heterosis or hybrid vigor is a genetic phenomenon in which the performance or expression of a characteristic in the progeny is superior to that of the parents, such as vigor, growth rate, and biomass yield (HUYEN, 2016). The time frame for the creation of hybrids is considered to be around 1920 (CROW, 1998). Since its discovery, some hypotheses have been raised in order to explain the heterosis. The first was the dominance hypothesis, which is based on the argument that heterosis occurs due to the complementation and/or suppression of the deleterious effects of recessive alleles present in the inbred parents involved in a given cross (SCHNABLE et al. 2013). The inbred parents have alleles with deleterious effects in homozygosity (that may lead to inbreeding depression), which will be masked/abolished in the hybrid combination, through the complementation of the inferior alleles by the superior alleles. Complementation occurs at multiple loci, where there will be no expression of deleterious effects, resulting in a better-performing F_1 hybrid (BIRCHLER et al. 2010; TIAN et al. 2019, KUMAR et al. 2020). The second hypothesis to explain heterosis is that of overdominance, which argues that individual loci in heterozygosity lead to phenotypically superior performance compared to a homozygous condition (BIRCHLER et al. 2010; TIAN et al. 2019, KUMAR et al. 2020). The last hypothesis is that of epistasis, which argues that the expression of hybrid vigor would result from interactions between genes at different loci (= epistatic), which can generate transgressive segregants in relation to the contrasting parents (YU et al. 1997; TIAN et al. 2019). Dominance and overdominance do not

definitively explain the phenomenon of heterosis. However, these hypotheses do not exclude the potential cooperative involvement of epistatic interactions between different genes that probably also contribute to heterosis (HOCHHOLDINGER & BALDAUF, 2018).

Heterosis and hybrid vigor have also been observed in tomato (BHATT et al., 2001; BAI & LINDHOUT, 2007). HEDRICK & BOOTH (1907) were the first to observe higher yield and a greater number of fruits in tomato hybrid plants when compared to parental materials. WELLINGTON (1912) pointed out the commercial potential of producing F1 tomato hybrids. Since then, this phenomenon has been widely studied aiming to elucidate its relationship with fruit yield and its phenotypic components (AMIN et al. 2001; BHATT et al. 2001; KURIAN et al. 2001; BAI & LINDHOUT, 2007; GARG et al. 2013; SHALABY, 2013; SOLIEMAN et al. 2013; KUMAR et al. 2015; TAMTA & SINGH, 2017). Heterosis in commercial tomato breeding has been sought in F_1 combinations originated through the crossing of highly inbred parents, where the interaction between several loci in heterozygosity results in hybrid vigor. However, there are some experimental indications that due to the overdominance effect, the heterozygosity of a single locus is sufficient to achieve heterosis. Given this possibility, studies have identified that simple mutations have enormous potential to be overdominant, and thus there is a renewed interest in characterizing and using this class of mutants (KRIEGER et al. 2010). The tomato *epinastic* (*epi*) mutant gene is related to ethylene balance and, when heterozygous, has shown promising results in relation to higher yield and higher fruit mass (weight) and sugar content (°Brix) (BASTOS, 2017). The *epi* mutant was mapped to a 1 Mb region on chromosome 4 (BARRY, 2001; ZSOGON et al. 2008; BASTOS, 2017). Its phenotype exhibits characteristics indicative of altered cell expansion, similar to wild-type plants treated with ethylene (BASTOS, 2017). For example, stems and petioles have larger diameters than in wild-type plants and petioles exhibit a peculiar epinastic curvature. The leaves also present an epinastic and twisted morphology and ethylene production reaches levels above those observed in wild-type plants (FUJINO et al. 1988a; 1988b; BARRY et al., 2001). This set of characteristics indicates that the mutant *epi* as having potential for use in applied plant breeding. However, it is necessary to demonstrate the capacity of this mutation to promote heterosis in different genetic backgrounds and environmental contexts, which requires more extensive studies (KRIEGER et al. 2010). Therefore, it is necessary to evaluate the

incorporation of this mutation in different commercial cultivars to estimate its potential heterotic impact on different varietal tomato segments (TAMTA & SINGH, 2017). Based on the previous results (BASTOS, 2017), a working hypothesis was raised that the single-locus *epi* mutation, when in heterozygous condition, may express hybrid vigor (heterosis) due to overdominance, resulting in significant increases in yield. The objective of the present study was to evaluate different hybrids involving the *epi* gene across distinct varietal backgrounds to estimate potential heterotic effects on yield and fruit parameters.

MATERIAL AND METHODS

Plant material and generation of hybrids – Field assays were carried out in order to understand whether the single-locus heterozygous mutant (*epi*) affects the commercial characteristics of tomato plants. With this aim, experimental hybrid seeds were generated by crossing five commercial cultivars/elite inbred lines with indeterminate growth habit (heterozygous for the *self-pruning* gene *Sp*/*sp*) and five cultivars/elite inbred lines with determinate growth habit (*sp*/*sp*) with two contrasting near-isogenic lines ('VFN8 *epi*' and 'VFN8' without the *epi* mutation), totaling 20 F_1 hybrid combinations (ten mutants and ten non-mutants). These hybrids were generated through controlled pollinations carried out at the Federal University of Viçosa (UFV). The commercial cultivars/inbred lines with determinate growth habit used in the crosses were: 'Heinz 1706', 'UC82-B', 'T-5', 'VFNT' and 'LAM 186'. The commercial cultivars/inbred lines with indeterminate growth habit were: 'LAM 147' (= 'Santa Clara *Sw*5'), 'Ailsa Craig', 'LAM 374', 'LAM 375' and 'Moneymaker'. The crossings were done by emasculating the flowers in the pre-anthesis period, and pollination was performed using a fine brush to apply pollen to the stigma of the emasculated flower. The cultivars and the direction of the crossings are shown in **Table 1**. After the fruits ripened, they were harvested for seed extraction in the Plant Molecular Physiology Lab of UFV.

Field trials – Tomato seeds of mutant F_1 hybrids, non-mutant F_1 hybrids and parental cultivars/inbred lines were sown and germinated in polyethylene trays, and upon reaching four true leaves, the seedlings were transplanted and grown in the experimental fields of the National Center for Vegetable Research (CNPH, EMBRAPA), Brasília, DF. Cultural practices, including soil management, fertilization, irrigation, and pest and disease control, will be consistent with the standard agricultural practices and with the tomato

cultivar/inbred line to be used. The experiment had 20 F_1 hybrid genotypes (five *epi* F_1 hybrids with determinate growth habit (*sp*/*sp*), five *epi* F_1 hybrids with indeterminate growth habit (*Sp*/*sp*), five determinate non-mutant F_1 hybrids, five indeterminate non-mutant F_1 hybrids), ten parental lines, the 'VFN8 *epi*' background, and the 'VFN8' background. This test was carried out with four replications, eight plants per replication, totaling 64 experimental units per treatment.

Fruit yield and fruit quality analyses – The fruits were harvested in three periods during crop development. Ripe and unripe fruits as well as fallen fruits and the fruits still attached to the plants were harvested. All fruits were counted in order to estimate yield, with the quantity of fruits and their mass (weight) being counted. The following parameters were evaluated: fruit weight (gram) per plant, individual fruit weight (gram), quantity of fruits per plant, and yield (expressed as ton ha^{-1}). To evaluate fruit quality, eight (8) fruits were randomly selected for each treatment. Fruit quality was evaluated for total soluble solids, which were determined as ºBrix using a digital refractometer.

Statistical analysis – For each contrast group, the corresponding F1 combination involving the *epi* mutant and the F_1 combination involving the non-mutant line were used. Statistical analyses were carried out using R software (SNK 5%).

RESULTS

Expression of yield heterosis by overdominance in experimental tomato hybrids with determinate growth habit – The potential expression of heterosis by overdominance for the *epi* locus in heterozygosity was estimated in cultivars/inbred lines of different genetic backgrounds displaying determinate growth habit as a common characteristic. The varieties with determinate growth habit have the recessive *self-pruning* allele in homozygosis (*sp*/*sp*) (KRIEGER et al., 2010). In the present set of trials, five determinate growth lines were used ('UC-82', 'VFNT', 'Heinz 1706', 'T5' and 'LAM 186'). The contrasts involving the cultivars 'UC-82' and 'T5' did not show significant results. For the contrast involving the non-mutant hybrid (3.802 ton.ha^{-1}) and the hybrid involving the *epi* mutant (4.413 ton.ha^{-1}) showed significant results with productivity. In the contrast 'Heinz 1706', only the parental had a significant result with productivity (3.403 tons.ha^{-1}). The F_1 hybrid with the *epi* mutation was superior in productivity (4.372

tons.ha^{-1}) and fruit mass (weight) per plant (282.348 g) for the contrasts involving the cultivar 'LAM 186' (**Table 2**). In the present study, it was observed that the *epi* mutation did not alter the amount of total soluble solids in the crosses involving 'LAM 186' (**Figure 1**). In experiments carried out with different lines with a determined growth habit used as genetic background in the treatments, the results did not demonstrate statistical significance for the productivity of the epinastic hybrid in relation to the other treatments (Table 4).

Expression of yield heterosis by overdominance in experimental tomato hybrids with indeterminate growth habit – Previous studies have detected overdominance for single-locus hybrids with a *sp*/*sp* genetic configuration, which controls determinate growth habit. In the present study, we evaluated whether the overdominance effect would be repeated in a group of tomato hybrids with indeterminate growth habit and heterozygous for the self-pruning gene (*Sp*/*sp*). Herein, five cultivars/inbred lines with indeterminate growth habit were used (viz. 'Ailsa Craig', 'Santa Clara *Sw*-5', 'Money Maker', 'LAM 374', and 'LAM 375'). No significant results were observed for yield in the contrasts involving the cultivars 'Ailsa Craig' and 'Money Maker'. For the contrast involving 'LAM 374', only a mutant and a non-mutant hybrids showed significant results for yield (5.043 ton.ha^{-1}) and (4.476 ton.ha^{-1}), respectively. In the contrast involving the cultivar 'Santa Clara *Sw*-5', the parental and the non-mutant hybrid showed significant results, with yields of 3.963 ton.ha^{-1} and 3.539 ton.ha^{-1}, respectively. The F_1 hybrid with the *epi* mutation was superior in yield (5.839 ton ha-1) and fruit weight per plant (422.121 g) for the contrasts involving the cultivar 'LAM 375' (**Table 3**). Similar to the results for the determinate cultivar, the increase in productivity in the hybrid involving 'LAM 375' did not imply a reduction in the total soluble solids content (**Figure 2**). In experiments carried out with different lines with an indeterminate growth habit used as genetic background in the treatments, the results did not demonstrate statistical significance for the productivity of the *epinastic* hybrid in relation to the other treatments, although for the contrasts involving indeterminate lines there was a greater expression of heterosis in general. (Table 5).

Pleiotropic effects of the *epi* mutation in heterozygosity – Single-locus hybrids can present a pleiotropic action, in which a single locus can alter a broad set of characteristics in the plant. In the present study, the recessive *epi* mutation was responsible for exhibiting

heterosis in two hybrids with distinct growth habits: determinate (parental inbred line ‘LAM 186’) and indeterminate (parental inbred line ‘LAM 375’). Therefore, we decided to carry out a comparative analysis of which phenotypic characteristics could be directly linked to higher yield in these two contrasting hybrids. The *epi* mutation in heterozygosity for the mutant hybrid [‘VFN8’ *epi* × ‘LAM 186’ F_1] showed greater individual fruit mass (weight) compared to the non-mutant hybrid [‘VFN8’ × ‘LAM 186’ F_1] (**Figure 4**). For the mutant hybrid [‘VFN8’ *epi* × ‘LAM 375’ F_1], the *epi* mutation in heterozygosity was responsible for a greater quantity of fruits per plant in relation to the non-mutant hybrid ([‘VFN8’ × ‘LAM 375’ F_1]) (**Figure 3**). Therefore, two characteristics of a quantitative nature were altered by the *epi* mutation in heterozygous condition, which was reflected in an increase in yield (ton.ha^{-1}) in these two contrast groups, demonstrating that in each group a different phenotypic characteristic was altered, suggesting that the mutation in heterozygosity for a single locus has potential pleiotropic effects.

Evaluation of genetic interaction between the loci *epinastic* and *self-pruning* – The F_1 hybrids with the *epi* mutation showed superior yield and fruit weight per plant for the contrasts involving the cultivar ‘LAM 186’ with determinate (*sp*/*sp*) growth habit and ‘LAM 375’ with indeterminate (*Sp*/*sp*) growth habit. However, for the other contrast groups, both for determinate and indeterminate growth habit, the mutation did not present significant results.

DISCUSSION

In tomato, the expression of heterosis has demonstrated for a wide array of traits including more vigorous growth, faster fruit development, earliness, increased yield, and increased resistance to biotic and abiotic effects (YORDANOV, 1983). The commercial exploitation of hybrids in tomato breeding has become important due to their advantages over open-pollinated varieties in relation to increased yield but also due their higher levels of homeostasis, resulting in phenotypically more stable genotypes (CHRISTAKIS & FASOULAS, 2002). Despite the relatively high price of seeds, modern tomato growers prefer hybrids when the major interest is to maximize profits (SOLIEMAN et al. 2013).

Heterotic effects were observed in crosses involving a determinate inbred line ‘LAM 186’. Thus, the *epi* mutation in heterozygosis can express yield heterosis by overdominance in a subgroup of cultivars with determinate growth habit. Similar results

were found by KRIEGER et al. (2010) and VICENTE et al. (2015), who detected overdominance effects in hybrids involving the *single-flower truss* (*sft*) mutant in a determinate background (*sp*/*sp*). It is interesting to highlight that the *epi* mutation did not alter the amount of total soluble solids, demonstrating good potential for agronomic use, since heterosis usually increases yield but usually with negatively impacts the content of total soluble solids (TANKSLEY et al. 1996; FULTON et al. 1997; GRANDILLO et al. 1999; RONGA et al. 2019).

Regarding lines with indeterminate growth habit, the results reported here are in contrast with a subset of results obtained by KRIEGER et al. (2010) and VICENTE et al. (2015). In these studies, an overdominance effect was observed only for parents with determinate growth habit. In the present study, it was demonstrated that the F_1 hybrid with the *epi* mutation was superior in yield and fruit mass (weight) per plant for the contrasts involving the 'LAM 375' inbred line. These results indicate that the single-locus *epi* mutation, when in heterozygosity, can positively impact the expression of overdominance heterosis for yield also in cultivars with indeterminate growth habit. It is also important to highlight that, similarly to the results for the determinate cultivar, the yield increase in the mutant hybrid involving the 'LAM 375' did not imply a reduction in the total soluble solids content. Tomato breeding has sought higher yield with higher ^{o}Brix, although there is a strong and inverse correlation between fruit production and total solids content (TANKSLEY et al. 1996; FULTON et al. 1997; GRANDILLO et al. 1999; SCHAUER et al. 2006 RONGA et al. 2019). In this regard, our results are similar to those obtained by KRIEGER et al. (2010), in which substantial increases in yield were observed without decreasing sugar content. Furthermore, KRIEGER et al. (2010) found that the most likely heterotic component in *sft*/+ plants was the increase in inflorescence production. In the case of the *epi* mutant, the candidate phenotype fulfilling the role of heterotic component is cell proliferation since yield was accompanied by increased fruit size and also the plants phenotypically presented the most developed vegetative part in relation to the non-mutant hybrid.

In four contrasts of lines used as background for determinate growth habit and in four indeterminate lines, there were no significant results for heterosis in productivity for the *epinastic* mutant, but general heterosis (comparing mutant and non-mutant hybrid together) at a higher level of contrasts for indeterminate lineages suggests that heterosis has to do with the flowering pathway, as suggested by KRIEGER et al. 2010.

BASTOS (2017) was the first to observed significant growth differences between reciprocal hybrids for the *epi* gene, and these data are consistent with reports that different parental combinations can produce F_1 combinations with distinct levels of heterosis for a specific set of traits (CHEN, 2013; GROSZMAN, 2014). Herein, some contrasts did not show expression of heterosis, suggesting that there could be suppressed by some maternal effect, since these hybrids were precisely the contrasts in which the mutant was used as the female parent. Hybrid seeds obtained from the cross with +/+ inbred line as the female parent and *epi*/*epi* as the pollen donor are easier, since the set of flowers is larger in the WT than in the *epi* mutant (BASTOS, 2017). Coincidentally, in these specific contrasts with 'LAM 186' and 'LAM 375', we chose to use the *epi* mutant ('VFN8 *epi*') as the female (pollen receptor) parent and not as the pollen donor (see **Table 1**). In a cross in which *epi* is the pollen donor, any changes either in the expression or in the interaction of nuclear genes of mitochondrial and chloroplast genes are evaluated in these the F_1 combination. In future studies, *epi*/*epi* inbred lines could be used preferentially as the female parent to confirm the hypothesis of the modulation of heterosis expression conditioned by maternal effect.

Leaves play important roles in plant metabolism, and, in this sense, the anatomical characteristics of the leaves are fundamental to imply greater photosynthesis and subsequent adaptation to the environment (KALVE et al., 2014; TIAN et al., 2016). BASTOS (2017) found a greater thickness of the palisade parenchyma in the +/*epi* hybrid using the background of the cultivar 'Micro-Tom'. Many studies have correlated a more developed mesophyll with a higher rate of photosynthesis, and greater photosynthetic capacity is associated with characteristics that favor gas exchange (PEGUERO-PINA et al., 2017). Palisade and spongy mesophylls are tissues that, when they have altered characteristics, directly influence the photosynthetic rates of the plant. Palisade parenchyma is associated with a greater quantity of chloroplasts and spongy parenchyma is associated with gas exchange with the environment (TIAN et al., 2016).

The regulation of organ size is yet poorly understood involving a complex spatial and temporal coordination of cell division and expansion (GONZALEZ et al., 2010; KALVE et al., 2014; KALVE et al., 2014). Larger leaf size associated with higher cell number is a common heterotic phenotype in hybrids as suggested by several studies (BLUM et al., 2013; GOFF, 2011; GONZALEZ et al. 2012; GROSZMANN et al., 2015; HE et al., 2010). In leaf development, it is known that cell division has a great influence

on leaf size, because when cell division ceases, the cell continues to expand until the final size of the leaf (KALVE et al., 2014). This transition from cell division to cell expansion is caused by changes in the cell cycle and prolongation of the cell proliferation period in hybrids and non-hybrids, resulting in larger leaf size due to the increase in the number of cells, mainly auxin (GONZALEZ et al., 2010; KALVE et al., 2014).

In tomato, three cell types with different responses to auxin and ethylene have been identified, and early studies with the *epi* mutant (URSIN & BRADFORD, 1989). This study demonstrated that *epi* has different cell types when comparison to WT, because 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., 1989; URSIN & BRAFORD, 1989). The DR5::GUS assays seem to confirm the idea that individual cell types respond differently to hormones in an *epi*-mediated mechanism (BASTOS, 2017).

BASTOS (2017) also found that stomatal conductance was increased in heterozygous (+/*epi*) in comparison to WT homozygous (+/+) plants. The effects observed in gas exchange without changes in stomatal index and stomatal density indicate that the CO_2 fixation capacity in +/*epi* is mainly controlled by a physical limitation, especially in stomatal opening. One of the factors that strongly regulates stomatal closure is the presence of ABA and it was demonstrated in *Arabidopsis* that ABA-induced closure is impaired by ethylene (TANAKA et al., 2006), thus, the overproduction of ethylene in +/*epi* compared to +/+ plants could explain the greater stomatal conductance.

Although the results for both contrasts were promising (including one with a determined background and another with an indeterminate background), our results continued to suggest the occurrence of epistatic interactions between the mutant locus in heterozygosity and the genetic background of the other contrasts, since eight contrasts did not show significant differences, for the same experimental conditions. These results with epistatic interaction were found by KRIEGER et al. (2010) always with the indeterminate background (*Sp*/+).

The heterotic response can vary widely depending on the environment and genetic background (SCHNABLE & SPRINGER, 2013). The F_1 hybrid with the *epi* mutation was superior in yield and fruit weight per plant for the contrasts involving the cultivar ‘LAM 186’ (with determinate growth habit) and ‘LAM 375’ (with indeterminate growth habit). However, for the other groups of contrasts (both for determinate and indeterminate growth habit) the mutation did not show significant impact, thus indicating that there may

be an effect of gene interaction and/or epistasis between *epi* mutation, WT as well as other loci of each specific genetic background of these two lines, since the experiment was carried out under the same experimental conditions for all groups. Although cultivars with indeterminate growth habits are intended for greenhouse cultivation, here the test was carried out entirely in the field, to estimate a more severe environmental impact. In this sense, we can initially exclude the potential presence of relevant interactions between the *epi* and *sp* loci.

CONCLUSIONS

Although heterosis was not significantly detected in most of the contrasts evaluated, we conclude that the *epi* mutant gene in heterozygosis in the F_1 combinations can, in specific genetic backgrounds, exhibit heterosis for yield regardless of the growth habit. Although heterosis occurred for hybrid combinations with determinate and indeterminate growth habits, this phenomenon was coincidently observed in two hybrids that the mutant near isogenic line was used as female (pollen receptor) parent. Even though further studies are needed to prove this hypothesis, we suggest that modulation of the heterosis level may result from overdominance in a single gene due to the maternal effect. In conclusion, the present work provides evidence that the *epi* mutation could be a potential gene to be explored in plant breeding, since it drives heterosis in traits of horticultural relevance. From the physiological point of view, based on other studies involving *epi* mutants, we suggest that the increase in yield is more likely associated with a greater number of fruits per plant or greater fruit size, probably associated with greater cell proliferation due to hormonal crosstalk between ethylene and other hormones (BASTOS, 2017).

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Table 1 – Characteristics of the parental materials (cultivars/elite inbred lines), contrasting near-isogenic inbred lines ('VFN8' and 'VFN8 *epi*') and experimental hybrids that were generated and used in the crosses. Parental materials are in bold.

Growth habit	Parental cultivars/inbred lines and experimental hybrids		Experimental objective
	Female (pollen donor)	**Male (pollen donor)**	
Determinate	**Heinz 1706**		**Heterosis**
	UC82-B	VFN8 *epi*	
	T-5	VFN8 *epi*	
	VFNT	VFN8 *epi*	
	VFN8 *epi*	**LAM 186**	
Indeterminate	**Santa Clara *Sw*5**	VFN8 *epi*	
	Ailsa Craig	VFN8 *epi*	
	LAM374 (*bif*/*bif*)	VFN8 *epi*	
	VFN8 *epi*	**LAM375**	
	Money Maker	VFN8 *epi*	
Determinate	**Heinz 1706**	VFN8	**Controls**
	UC82-B	VFN8	
	T-5	VFN8	
	VFNT	VFN8	
	VFN8	**LAM 186**	
Indeterminate	**Santa Clara *Sw*5**	VFN8	
	Ailsa Craig	VFN8	
	LAM374 (*bif/bif*)	VFN8	
	VFN8	**LAM375**	
	Money Maker	VFN8	

Table 2 – Average yield and fruit mass (weight) per plant among parental lines, contrasting near-isogenic inbred lines ('VFN8' and 'VFN8 *epi*'), F_1 hybrids for the contrast group involving the 'LAM 186' inbred line (with determinate growth habit).

Parental lines and crosses	**Yield (ton ha^{-1})***	**Fruit weight per plant (g)***
[LAM 186]	1.343 b	97.068 b
[VFN8]	1.123 b	81.188 b
[VFN 8 *epi*]	1.364 b	98.611 b
[VFN 8 × LAM 186 F_1]	2.225 b	160.87 b
[VFN 8 *epi* × LAM 186 F_1]	**4.372 a**	**282.348 a**
CV (%)	**37.19**	**38.27**

*Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test.

Table 3 – Average yield and fruit mass (weight) per plant among parental lines, contrasting near-isogenic inbred lines ('VFN8' and 'VFN8 *epi*'), and F_1 hybrids for the contrast group involving the inbred line 'LAM 374' (indeterminate growth habit).

Parental lines and crosses	**Yield (ton.ha^{-1})***	**Fruit weight per plant (g)***
[LAM 375]	2.295 bc	165.923 bc
[VFN8]	1.123 c	81.188 c
[VFN8 *epi*]	1.364 bc	98.611 bc
[VFN8 × LAM 375 F_1]	2.756 b	199.222 b
[VFN8 *epi* × LAM 375 F_1]	**5.839 a**	**422.121 a**
CV (%)	**22.99**	**22.98**

*Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test

Table 4 – Average yield and fruit mass (weight) per plant among parental lines, contrasting near-isogenic inbred lines ('VFN8' and 'VFN8 *epi*'), F_1 hybrids for the contrast group of determinate growth habit.

Parental lines and crosses	**Yield (ton.ha^{-1})***	**Fruit weight per plant (g)***
[UC82B]	2.828 a	204.418 a
[VFN8]	1.123 a	81.188 a
[VFN8 *epi*]	1.364 a	98.611 a
[UC82B × VFN8 F_1]	2.522 a	182.305 a
[UC82B × VFN8 *epi* F_1]	2.222 a	160.626 a
CV (%)	**44.45**	**44.44**
[VFNT]	2.249 ab	162.581 ab
[VFN8]	1.123 b	81.188 b
[VFN8 *epi*]	1.364 b	98.611 b
[VFNT × VFN8 F_1]	3.802 a	274.867 a
[VFNT × VFN8 *epi* F_1]	4.413 a	319.046 a
CV (%)	**40.91**	**40.9**
[Heinz 1706]	3.403 a	246.039 a
[VFN8]	1.123 b	81.188 b
[VFN8 *epi*]	1.364 ab	98.611 ab
[Heinz 1706 × VFN8 F_1]	3.018 ab	218.153 ab
[Heinz 1706 × VFN8 *epi* F_1]	2.621 ab	189.453 ab
CV (%)	**44.29**	**44.28**
[T5]	1.367 a	98.819 a
[VFN8]	1.123 a	81.188 a
[VFN8 *epi*]	1.364 a	98.611 a
[T5 × VFN8 F_1]	1.416 a	102.332 a
[T5 × VFN8 *epi* F_1]	2.031 a	146.842 a
CV (%)	**40.57**	**40.56**

*Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test

Table 5 – Average yield and fruit mass (weight) per plant among parental lines, contrasting near-isogenic inbred lines ('VFN8' and 'VFN8 epi'), F1 hybrids for the contrast group of indeterminate growth habit.

Parental lines and crosses	**Yield (ton.ha^{-1})***	**Fruit weight per plant (g)***
[Ailsa Craig]	-	-
[VFN8]	1.123 a	81.188 a
[VFN8 *epi*]	1.364 a	98.611 a
[Ailsa Craig × VFN8 F1]	2.470 a	178.522 a
[Ailsa Craig × VFN8 *epi* F1]	2.265 a	163.713 a
CV (%)	**54.83**	**54.82**
[Santa Clara *Sw*-5]	3.963 a	286.523 a
[VFN8]	1.123 b	81.188 b
[VFN8 *epi*]	1.364 b	98.611 b
[Santa Clara *Sw*-5 × VFN8 F1]	3.539 a	255.857 a
[Santa Clara *Sw*-5 × VFN8 *epi* F1]	3.140 ab	227.025 ab
CV (%)	**37.67**	**37.66**
[Money Maker]	2.622 a	189.779 a
[VFN8]	1.123 a	81.188 a
[VFN8 *epi*]	1.364 a	98.611 a
[Money Maker × VFN8 F1]	3.157 a	228.197 a
[Money Maker × VFN8 *epi* F1]	3.080 a	222.645 a
CV (%)	**55.86**	**55.86**
[LAM 374]	1.320 b	95.440 b
[VFN8]	1.123 b	81.188 b
[VFN8 *epi*]	1.364 b	98.611 b
[LAM 374 × VFN8 F1]	5.043 a	364.576 a
[LAM 374 × VFN8 *epi* F1]	4.476 a	323.615 a
CV (%)	**30.86**	**30.85**

*Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test

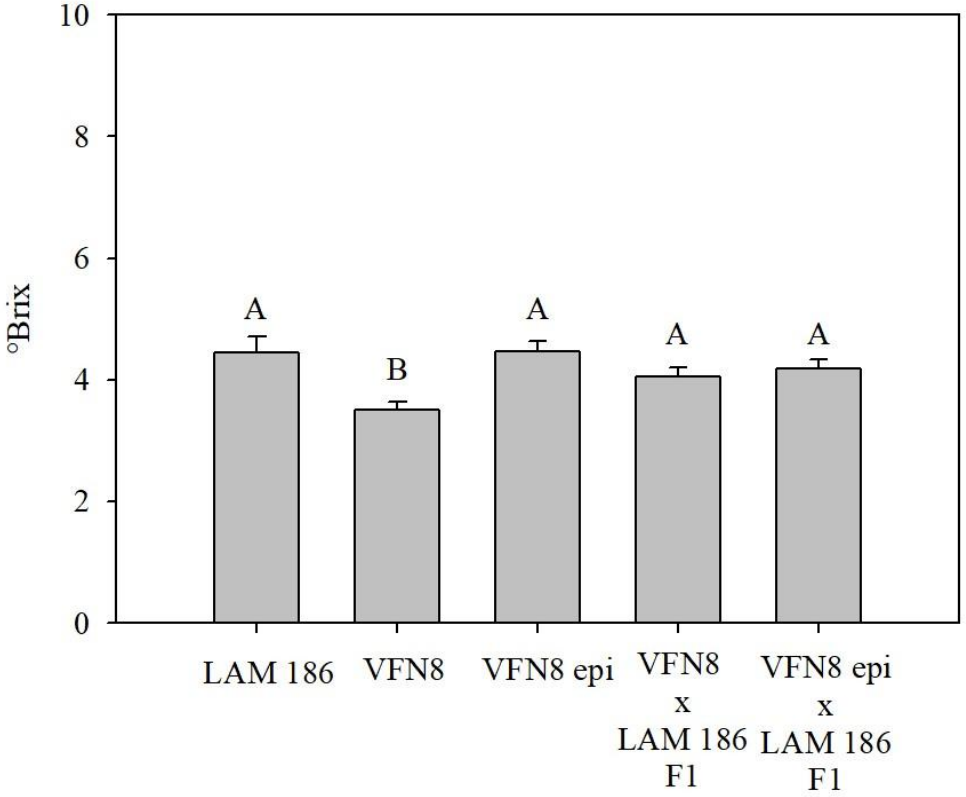

Figure 1. Averages of total soluble solids (°Brix) for the contrast involving 'LAM 186', 'VFN8', 'VFN8 *epi'*, ['VFN8' × 'LAM 186' F_1], ['VFN8 *epi'* × 'LAM 186' F_1]. Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test.

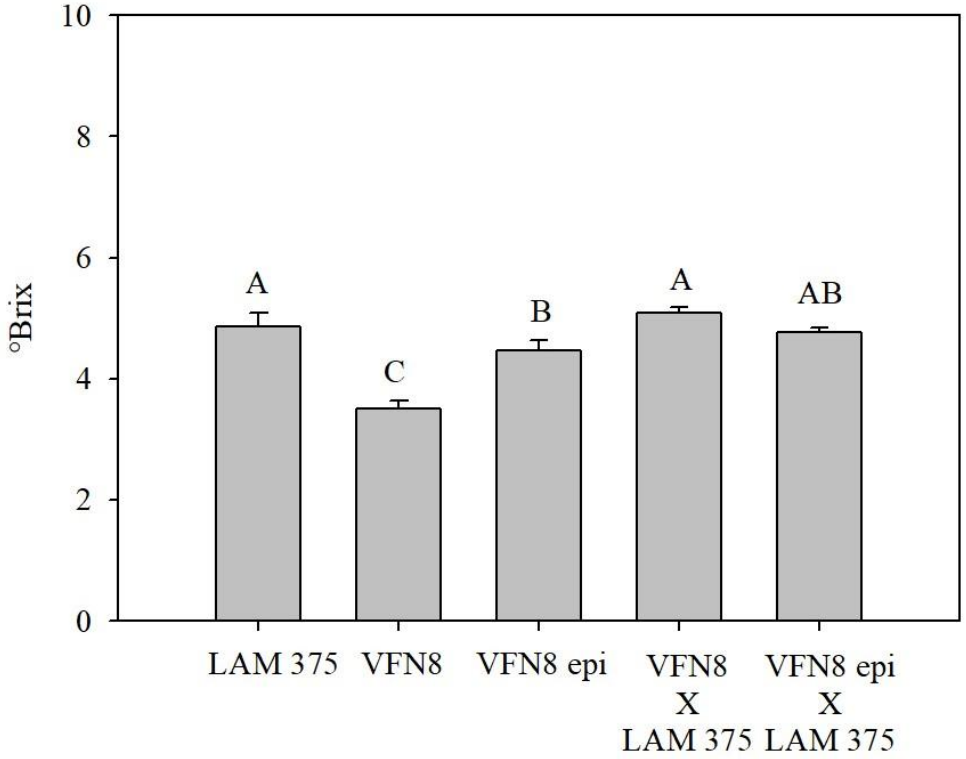

Figure 2. Averages of total soluble solids (°Brix) for the contrast involving 'LAM 375', 'VFN8', 'VFN8 *epi'*, ['VFN8' × 'LAM 375' F_1], ['VFN8 *epi'* × 'LAM 375' F_1]. Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test.

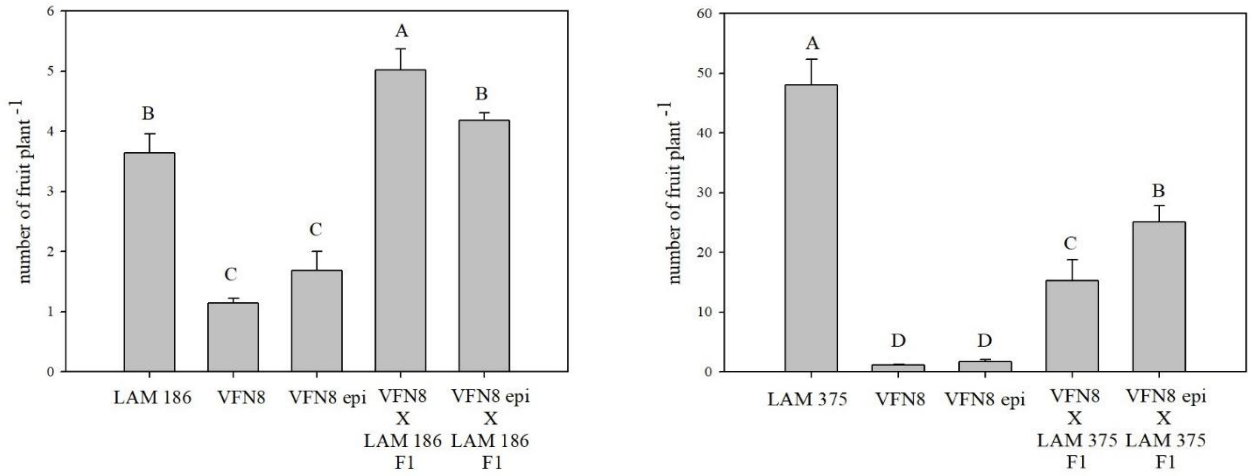

Figure 3. Average number (n^o) of fruits per plant. **(Left)** Contrast involving 'LAM 186', 'VFN8', 'VFN8 *epi*', ['VFN8' × 'LAM 186' F_1], ['VFN8 *epi*' × 'LAM 186' F_1]. (**Right**) Contrast involving 'LAM 375', 'VFN8', 'VFN8 *epi*', ['VFN8' × 'LAM 375' F_1], ['VFN8 *epi*' × 'LAM 375' F_1]. Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test.

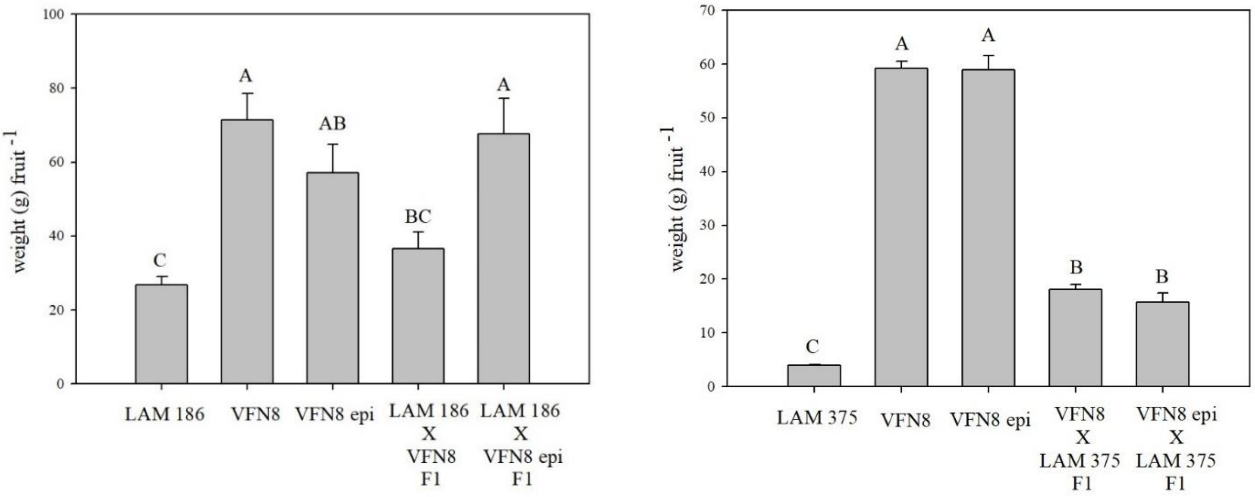

Figure 4. Average mass (weight in grams) of individual fruit (grams). (**Left**) Contrast involving 'LAM 186', 'VFN8', 'VFN8 *epi*', ['VFN8' × 'LAM 186' F_1], ['VFN8 *epi*' × 'LAM 186' F_1]. (**Right**) Contrast involving 'LAM 375', 'VFN8', 'VFN8 *epi'*, ['VFN8' × 'LAM 375' F_1], ['VFN8 *epi*' × 'LAM 375' F_1]. Means followed by the same letter in the column within each contrast group do not differ by the 5% SNK test.

Chapter 3

RNA-seq analysis reveals massive changes in the transcriptional landscape of near-isogenic lines contrasting for the *epinastic* mutation in tomato.

RNA-seq analysis reveals massive changes in the transcriptional landscape of near-isogenic lines contrasting for the *epinastic* mutation in tomato.

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ABSTRACT

The spontaneous mutation *epinastic* (*epi*) was first observed in tomato (*Solanum lycopersicum*) cultivar 'VFN8', being characterized by foliar epinasty, dark green leaves, erect growth, branched root system, thickened stem and petioles, reduced anthocyanin content, and overproduction of ethylene in the stem apices. Homozygous *epi* plants also displayed a constitutive ethylene response and a more intense transport of 3-indoleacetic acid (AIA) in both roots and hypocotyls. Contrasting near isogenic lines (NILs) for the *epi* mutation were developed in distinct backgrounds. Mutations in pleiotropic genes potentially involved in the synthesis of plant hormones, such as *epi*, can result in broad changes in the pattern of gene expression and alter numerous metabolic pathways that might affect tomato growth and yield. However, the effects of this mutation at the whole-transcriptional landscape were not investigated thus far. Herein, we employed transcriptomic analysis via RNA-seq as a tool to investigate the panel of differentially expressed genes (DEGs) potentially modulated by the *epi* mutation in seedlings of contrasting NILs. The 29,548 mapped genes were filtered according to the criterion of $p<0.05$ and two-fold differential expression. A total of 797 DEGs was detected after analysis using this set of parameters, which were subdivided according to the three major gene ontology (GO) terms: 'biological process' (41%); 'cellular component' (38%); and 'molecular function' (41%). DEG of five GO subgroups potentially related to the phenotypic syndrome associated with the *epi* mutation were further analyzed, including 'hormone metabolic process', (four DEGs) 'regulation of gene expression' (29 DEGs), 'RNA processing' (two DEGs) and 'developmental process' + 'growth' (23 DEGs). As somewhat expected, the majority of the DEGs were related to the crosstalk of hormonal pathways and their regulation. Previous functional analysis of a wide array of these DEGs indicated their association with one or more phenotypic traits of the *epi*-related syndrome. In conclusion, further analysis of these *epi*-associated DEGs will provide the basis for the establishment of conventional and biotech breeding strategies aiming to fine-tuning the expression of most suitable combinations of yield-enhancing genetic factors.

Keywords: *Solanum lycopersicum***,** recessive mutation, yield, breeding.

INTRODUCTION

The spontaneous recessive mutation *epinastic* (*epi*) was first observed in tomato (*Solanum lycopersicum*) cv. 'VFN8', being characterized by strong foliar epinasty, dark green leaves, erect growth, branched root system, reduced anthocyanin content, and overproduction of ethylene in the apex of the plants (FUJINO et al., 1988, 1989; URSIN & BRADFORD, 1989). Roots and hypocotyls of *epi* plants are shorter and thickened due to a reorientation of cell expansion, and a distinct curvature of the apical hook is observed. The phenotypic syndrome associated with *epi* mutant is similar to the triple response even in the absence of ethylene (BARRY et al., 2001). A constitutive ethylene response (BARRY et al., 2001) and a more intense transport of 3-indoleacetic acid (AIA) in both roots and hypocotyls were also observed in homozygous *epi* plants compared to wild-type (WT), suggesting that ethylene stimulates localized auxin biosynthesis and/or changes in auxin transport, leading to auxin accumulation in the tissues (NEGI et al., 2010).

Mutations in pleiotropic genes potentially involved in the synthesis of plant hormones, such as *epi*, can result in broad changes in the patterns of gene expression and alter numerous metabolic pathways (McCOURT, 1999). Recent technological strategies have made possible to study the effects on global gene expression patterns and the modulation of various hormonal signaling pathways and crosstalk between hormones in tomato fruit (ALBA et al. 2005) and in foliar (SHI et al. 2013) tissues. A revolution in transcriptomics analysis in tomato occurred with the introduction of next-generation high-throughput RNA sequencing (RNA-seq) technology (ROTHAN et al. 2019). One major goal of transcriptomic studies is to connect gene expression with phenotype (ZHOU et al., 2019). In tomato, RNA-seq has proven to be a simpler and more powerful approach to quantify expression at the transcriptome level. Analysis of expression profiles revealed crosstalk between auxin and cytokinin in the tomato root system, in which new genes induced and repressed by cytokinin and auxin were identified as DEGs and changes in the expression levels of several of these genes were confirmed by qPCR (GUPTA et al. 2013). Several of these tomato-regulated genes represent cytokinin- or auxin-regulated orthologous genes identified in other species, including CKX, RR type A, Aux/IAA, and ARF. More recently, a premature senescence phenotype (MT318) was characterized in a spontaneous mutant associated with a single recessive nuclear gene (JIN et al., 2022). The maximum photochemical efficiency (*Fv*/*Fm*), superoxide dismutase (SOD) and

chlorophyll content in the leaves of the MT318 mutant gradually decreased, while the malondialdehyde (MDA) content increased significantly. The molecular basis of the regulatory network of auxin-ethylene interaction was also investigated during tomato fruit ripening (LI et al., 2017). Global transcriptome profiling analysis showed that ethylene modulated auxin transport, metabolism, and signaling processes by affecting the expression patterns of genes encoding auxin carrier proteins, aldehyde dehydrogenase, and primary auxin-responsive proteins. Potential hormonal interaction points were also identified, which can be employed to predict the candidate of the crosstalk regulators of these two hormones during the fruit ripening process (LI et al., 2017). However, transcriptomic information about the ethylene-auxin interplay in foliar tissues are not yet available.

Plant growth and yield includes coordination of many regulatory pathways (CARRARI & FERNIE, 2006; ARIIZUMI et al. 2013; QUINET et al. 2019). Mutations in pleiotropic genes potentially involved in the synthesis of plant hormones, such as *epi*, can result in broad changes in the pattern of gene expression and alter numerous metabolic pathways that might affect tomato growth and yield. However, the effects of this mutation at the whole- transcriptional landscape were not investigated thus far. In this context, we employed transcriptomic analysis via RNA-seq as a tool to investigate the panel of differentially expressed genes (DEGs) potentially modulated by the *epi* mutation in two contrasting NILs.

MATERIAL AND METHODS

Plant cultivation – Seedlings of two contrasting NILs for the locus *epi* ('VFN8 *epi*' and 'VFN8 WT') were grown under greenhouse conditions with supplemental LED lighting running in a regime for 12 hours of light and 12 hours of dark with day temperature of 26 ºC and night temperature of 20 ºC. Seedings were grown in soil up to the development of their first true leaves (approximately 8–10 cm tall). Afterwards, the above ground tissues of the seedings (without roots) were harvested and immediately put into liquid nitrogen and subsequently kept into deep freezer (-80 ºC) up to RNA extraction.

RNA extraction and construction of transcript libraries – The tissue samples (50 mg) were removed from the freezer and RNA was purified using the Reliaprep kit (Qiagen).

A final aliquot of 10 μL was used to measure the quality and quantity of the extracted DNA in a NanoDrop® spectrophotometer (Thermo Fisher Scientific, USA) and TapeStation (Agilent) Electrophoresis. Samples with RIN > 8.0 were employed in subsequent experimental steps. Aliquots of total RNA extracted from seedlings of the hybrid and its corresponding NILs were sent for library synthesis for a commercial company under recommended shipment conditions. All steps related to sample preparation for the library acquisition and sequencing stages were performed by the commercial company. The sequencing platform used was the Illumina HiSeq 2500, paired ends, choosing to map 20 million reads in each biological replicate.

Bioinformatics analysis of RNAseq data – The FASTq sequences from the three replicates of experiments named as S3 ('VFN8 WT', Wild Type) and S4 ('VFN8 *epi*') were aligned to the *Solanum lycopersicum* reference genome (SolGenomics; NCBI) using SeqManNGen v.17 (DNAStar, Madison, WI USA). In the SeqMan NGene software, the sequence quality analysis procedures, adapter trimming used by the sequencing platform, and alignment with the reference genome (cultivar 'Heinz 1706') were performed automatically, following the default settings of the program. SNPs/InDels were also mapped. The mapped sequences (reads) were used to evaluate differential gene expression, using the ArrayStar software (DNAStar, Madison, WI USA). The reads were normalized using DEseq2 statistical analysis (LOVE et al., 2014). Genes with differential expression were identified by performing t-tests, which included FDR (False Discovery Rates) control in order to adjust the significance levels (BENJAMINI & HOCHBERG, 1995). Genes considered differentially expressed were those that presented an expression variation (fold change) greater than 2.0 between samples, with a significance value (*p*-value) equal to or less than 0.05%. Gene annotations were performed using the SeqMan Pro software, using standard program algorithms (Gnomom). Subsequently, functional analysis of the genes was performed manually using the UniProt database, belonging to the UniProt consortium (https://www.uniprot.org).

Gene Ontology (GO) analyses – GO analyses were performed using the DAVID platform (https://david.ncifcrf.gov) (SHERMAN et al., 2021; HUANG et al., 2009). DEGs for each comparison were submitted to the DAVID platform using the *S. lycopersicum* as the reference genome. GO terms were defined when the significance level was < 0.05 (Statistical test method: Fisher, Multi_test adjustment method: Hocheberg FDR). Minimum number of mapping entries = 5. Genes with reads ≥ 50 at

least in one sample were considered expressed genes and used as the background gene list. Significant GO term was defined when significant level < 0.05 (Statistical test method: Fisher, Multi_test adjustment method: Hocheberg FDR). Minimum number of mapping entries = 5. After the initial analysis, isoforms were checked to verify whether their presence masked the expression results. GO annotations were transferred to ArrayStar to perform cluster analysis and to design the heat maps.

RESULTS

The 29,548 mapped genes were filtered according to the criterion of $p<0.05$ and two-fold differential expression. A total of 797 DEGs was detected after analysis using this set of parameters (**Supplementary Table 1**). DEGs were subdivided according to the three major gene ontology (GO) terms (**Figure 1**): 'biological process' (41%); 'cellular component' (38%); and 'molecular function' (41%). Of these DEG groups, we focused the analyses of GOs potentially related to the phenotypic syndrome associated with the *epi* mutation, including the following **five GO subgroups**: 'hormone metabolic process', 'regulation of gene expression', 'RNA processing', 'developmental process' +'growth'.

Within the GO subgroup 'hormone metabolic process', four DEGs were identified (**Figure 2**) for the alleles of the genes *ARF3* and *LOG8* (down-regulated in NIL *epi*) and *AO1* and *det2* (up-regulated in this NIL). The GO subgroup encompassing 'regulation of gene expression' displayed the largest number of DEGs. In total, 29 DEGs were identified for this GO subgroup. The following alleles were down-regulated in NIL *epi*: *TCP11*, *ARF2*, *ARF19*, *ARF4*, *ARF3*, *hb-1*, *MYC2*, *CIB4*, *EIL1*, *TIP*, *FIE*, *HY5*, *ARF13*, *DREB2*, *MYC1*, *GRAS2*, *ASR1*, *NAC1*, *SHINE3*, and *TCP5* (**Figure 3**). On the other hand, the alleles of *TCP24*, *MBF1a*, *HSFA3*, *ARF1*, *ERF4*, *TCP17*, *PAP1*, *TCP3*, and *PCL1* were up regulated in the *epi* NIL (**Figure 3**). Only two up-regulated DEGs DEGs were detected in the *epi* NIL in the analysis of the GO subgroup encompassing 'RNA processing' term: *FEY* and *CCR2* (**Figure 4**). Twenty-three (23) DEGs were detected in the GO subgroups corresponding to the terms 'developmental process' + 'growth' (**Figure 5**). The following genes were down-regulated in *epi* NIL in comparison with the WT NIL: *ARS1*, *HY5*, *ARF2*, *gts1*, *hsc70-1* (allele from the chromosome 6), *hsc70-2* (chromosome 10), *BAM1*, *hb-1*, *Coi1*, *SGT1*, *FEY*, *MAPK6*, *CIP4*, *PIN3*, and *pip1*. The following genes were up regulated in the *epi* NIL: *FIE*, *hxk1*, *TCP24*, *UBC1*, *profilin*, *TCP17*, *AGO10*, and *TCP3*.

The DEGs corresponding to BAM1, *hb-1*, *MAPK6*, *profilin*, *gts1*, and *PIN3* were identified in both 'developmental process' and 'growth' subgroups (**Figure 5**).

DISCUSSION

The phenotypic syndrome associated with the *epi* mutation is characterized by structural and metabolic changes including foliar epinasty, leaves with dark green pigmentation, erect growth, branched root system, reduced anthocyanin content, and overproduction of ethylene in the apex of the plants (FUJINO et al., 1988, 1989; URSIN & BRADFORD, 1989). Roots and hypocotyls of *epi* plants are shorter and thickened and a constitutive ethylene response and a more intense transport and accumulation auxin in the tissues roots and hypocotyls in comparison with to WT plants (BARRY et al., 2001). Stems and petioles have larger diameters than WT plants and petioles exhibit a peculiar epinastic curvature. The leaves also present an epinastic and twisted morphology and ethylene production reaches levels above those observed in WT plants (FUJINO et al. 1988a; 1988b; BARRY et al., 2001). The plants with one dosage of the *epi* mutation displayed higher yield and higher fruit mass (weight) and sugar content (°Brix) (BASTOS, 2017). This set of characteristics indicates that the mutant *epi* as having potential for use in applied plant breeding. Based upon this set of phenotypic traits associated with the *epi* mutation, we focused our investigation in DEGs with potential involvement in this syndrome, including **five major GO subgroups**: 'hormone metabolic process', 'regulation of gene expression', 'RNA processing', 'developmental process' + 'growth'.

HORMONE METABOLIC PROCESS

The four DEGs identified within the GO subgroup '**hormone metabolic process'**, are the alleles of the genes *ARF3* and *LOG8* (down-regulated) and *AO1* and *det2* (up-regulated in NIL *epi*). The ***AO1* gene** is a member of the ***ALDEHYDE OXIDASE FAMILY*** known to be the catalysts of the last step in the biosynthesis of abscisic acid (ABA). *AO1* is expressed mainly in shoots and roots in tomato being associated with overproduction of auxin in the *superroot*1 mutant in *Arabidopsis thaliana* (SEO et al., 1998). The ***ARF3 (AUXIN RESPONSE FACTOR 3)* gene** is associated with the expression of wiry leaf syndrome in tomato (YIFHAR et al., 2012) and it will be discussed further in the the GO subgroup encompassing 'regulation of gene expression'. The tomato

det2 (STEROID 5 ALPHA REDUCTASE 2) gene is involved in the biosynthesis of α-tomatine (AKIYAMA et al., 2019). Alpha-tomatine and dehydrotomatine are steroidal glycoalkaloids (SGAs) that accumulate in the mature green fruits, leaves, and flowers of tomatoes and function as defensive compounds against a wide range of pathogens and predators. The cloning and functional characterization of *LeDET2* provided the evidence of the presence of two isoenzymes in the tomato genome (ROSATI et al., 2005). The full-length cDNA (*LeDET2*) encoding a 257 amino acid protein homolog of the *Arabidopsis DET2* (*AtDET2*), displaying 76% similarity with *AtDET2* and structural characteristics conserved among plant and mammalian steroid 5 alpha reductase genes. On the other hand, the ***LOG8*** **gene** encodes a cytokinin riboside 5'-monophosphate phosphoribohydrolase, which was found to be modulated under salt and oxidative stress in tomato (KESHISHIAN et al., 2018). However, the function of *AO1* and *det2* in the *epi*-associated phenotype remains unclear.

REGULATION OF GENE EXPRESSION

The GO subgroup encompassing **'regulation of gene expression'** displayed the largest number of DEGs. In total, **29 DEGs** were identified for this GO subgroup. The following alleles were down-regulated in NIL *epi*: *TCP11*, *ARF2*, *ARF19*, *ARF4*, *ARF3*, *hb-1*, *MYC2*, *CIB4*, *EIL1*, *TIP*, *FIE*, *HY5*, *ARF13*, *DREB2*, *MYC1*, *GRAS2*, *ASR1*, *NAC1*, *SHINE3*, and *TCP5* (**Figure 3**). On the other hand, the alleles of *TCP24*, *MBF1a*, *HSFA3*, *ARF1*, *ERF4*, *TCP17*, *PAP1*, *TCP3*, and *PCL1* were up regulated in the *epi* NIL (**Figure 3**). Bellow we provide the information available about these genes in tomato and other model plants.

The DEG ***HSFA3*** (***HEAT STRESS TRANSCRIPTION FACTOR A3***) gene was detected in a transcriptomic study in association with Brassinolide regulation in tomato fruits during cold injury (BAI et al., 2021). HSFA3 was one of the DEGs involved in the cold stress response of plants (BAI et al., 2021). HSFA3 plays an important role in cold tolerance mechanisms in tomato, especially in mitigating injuries caused by cold exposure in fruits (RÉ et al., 2016). On the other hand, it was also demonstrated that this DEG is involved in the response to thermal heat stress, where temperatures around 45° induced its gene expression (TOKIĆ et al., 2023). Studies demonstrate that HSF interacts with

several other genes such as *bZIP28*, *multiprotein bridging factor* 1c (*MBF1c*), and *calmodulin-binding protein kinase* 3 (*CBK3*) in response to heat stress (LI et al. 2017).

***AUXIN RESPONSE FACTORS* (*ARFS*)** are an important family of plant-specific transcription factors that bind directly to auxin response elements in the promoters of auxin-responsive genes (LI et al., 2023; LI et al., 2024). Herein, we detected a wide array of DEGs from this family of transcription factors. These genes are involved in auxin signal transduction in plants, playing crucial roles in growth and developmental regulation of roots, stems, leaves, flowers, fruits, and seeds as well as responses to biotic and abiotic stresses, and phytohormone crosstalk (KUO et al., 2022; LI et al., 2024). Using whole-genome sequencing, 22 ARF genes have been identified in tomato with some of them have already been functionally characterized (LI et al., 2023).

The **DEG of the *ARF2A* (*AUXIN RESPONSE FACTOR 2A***), a recognized auxin signaling component also detected in our transcriptomic analysis. This was found to confer enhanced tolerance to salt and drought stresses (EL MAMOUN et al., 2023). In addition, the functional characterization of *SlARF2* knockdown tomato mutants revealed that the downregulation of this gene enhanced primary root length and root branching and reduced plant wilting (EL MAMOUN et al., 2023). The involvement of ethylene in fruit ripening is result of crosstalk between ethylene and other hormones in ripening. *ARF2A* expression is ripening regulated and reduced in the *rin*, *nor* and *nr* ripening mutants. Over-expressing *ARF2A* in tomato resulted in blotchy ripening in which certain fruit regions turn red and possess accelerated ripening and parthenocarpy. *ARF2A* over-expressing fruit displayed early ethylene emission and ethylene signaling inhibition delayed their ripening phenotype, suggesting ethylene dependency. Comprehensive hormone profiling revealed that altered *ARF2A* expression in fruit significantly modified abscisates, cytokinins and salicylic acid while gibberellic acid and auxin metabolites were unaffected (BREITEL et al, 2016). *ARF2A* homodimerizes and interacts with the ABA *STRESS RIPENING* (*ASR1*) protein, suggesting that *ASR1* might be linking ABA and ethylene-dependent ripening. These results revealed that *ARF2A* interconnects signals of ethylene and additional hormones to co-ordinate the capacity of fruit tissue to initiate the complex ripening process. *ARF2* is also responsive to exogenous application of ethylene, auxin and abscisic acid (ABA).

DEGs of *ARF3* (also detected in the 'HORMONE METABOLIC PROCESS') **and *ARF1*** were identified. These genes are associated wiry mutants, representing a class of phenotype that mimics viral infection by inducing a set of morphological modifications in the foliar tissue (YIFHAR et al., 2012).

The ***ARF4* DEG** was also detected in our transcriptomic analysis. The *ARF4* gene in tomato associated with modification in a set of anatomical and morphological traits of the foliar tissue of the tomato mutant *obscuravenosa* (*obv*), including physiological changes in leaf hydraulics and photosynthetic rate (MOREIRA et al., 2022).

The ***ARF13*** (together with *ARF24*, *ARF13-1*, and *SlARF24*) gene was studied in the *entire* (*e*) leaf mutant in tomato (WU et al., 2018). ***ARF24 = SlARF24*** (together with *SlARF6A*) was able to bind to the promoter region of the auxin efflux carrier *PIN-FORMED1* (*SlPIN1*) which mediates local auxin accumulation. Therefore, *SlARF24* may act as transcriptional activator regulating expression of genes involved in leaflet initiation, suggesting its role in regulating in leaf shape development via direct binding to auxin-responsive genes (WU et al., 2018).

The ***ARF19* gene** is involved post-transcriptional regulation in tomato (ZOUINE et al., 2014). Five SlARFs (viz. ***SlARF5, SlARF8A, SlARF8B, SlARF19A,* and *SlARF19B*)** were evaluated by two-hybrid (Y2H) assays and showed that all of them interacted with *SlDELLA* and *SlIAA9* (HU et al., 2018). The interaction between *SlARFs/SlIAA9* and *SlDELLA* plays an important role in regulating fruit initiation.

The ***CIP4* (*CONSTANS INTERACTING PROTEIN 4*; *CIP4*)** gene belongs to a family of proteins involved cytokinesis (FORERO & CVRČKOVÁ, 2019), which is a phenotype intensified in the *epi* mutants (BASTOS, 2017).

The *DREB2* (*DEHYDRATION-RESPONSIVE ELEMENT-BINDING 2 = ERF-H14 – ETHYLENE RESPONSE FACTOR H.14*) gene codes for a transcription factor that mediates salt stress tolerance in tomato (HICHRI et al., 2016). This *DREB2*-type regulator involved in salinity response, being part of the repertoire of environmental adaptive mechanisms requiring regulation of downstream genes.

The ***MYC1* and *MYC2* genes** code for transcription factors with basic helix-loop-helix transcription factors that have a dual role in the regulation of constitutive and stress-inducible specialized metabolism in tomato (SWINNEN et al., 2022). The tomato HAIRY

ROOT is conditioned by the double (*myc*1 and *myc*2) loss-of-function mutation. This phenotype is result of the suppression of the constitutive expression of *SGA* biosynthesis genes, and severely reduced levels of the main tomato *SGAs* alpha-tomatine and dehydrotomatine (SWINNEN et al., 2022). In general, ***MYC2*** plays a central role in the jasmonic acid (JA) signaling in plants, participating in multiple signaling pathway-networks that integrate light signaling, hormone signaling, natural product synthesis, and the complex processes of plant growth and development (LUO et al., 2023).

Members of the ***GRAS* (*GIBBERELLIC ACID INSENSITIVE – GAI, REPRESSOR OF GA1 – RGA*, and *SCARECROW – SCR*)** family encode transcriptional regulators that play roles in diverse fundamental processes gene family named after the three founding members of plant growth and development such as gibberellin signal transduction, root radial patterning, axillary meristem formation, phytochrome-A signal transduction, and gametogenesis (HUANG et al., 2015). Genomic analysis identified 53 putative *SlGRAS* genes in tomato (HUANG et al., 2015). Knockout involving the *SlGRAS9* genes resulted in marked increase in chlorophyll content, reprogrammed chloroplast biogenesis and enhanced accumulation of starch and soluble sugars in tomato fruits (SHI et al., 2024). Here, we identified a ***GRAS2* (= *SCL*)** DEG, which is a gene involved in multiple biological processes in plants, playing a vital role in regulating fruit weight in tomato (LI et al., 2018).

The ***FIE* (*FERTILIZATION-INDEPENDENT ENDOSPERM* = *SlWDR171***) was detected here. These proteins are members of Polycomb Repressive Complex 2 (*PRC2*) that plays important roles in the developmental regulation of plants by controlling *epi* genetic modification of the genome. It has been well documented that *FIE* plays important regulatory roles in diverse developmental processes in model plant *Arabidopsis thaliana*. Mutation of these genes results in autonomous endosperm, developing without fertilization. In addition, a gene of this class is indispensable for the transition from the syncytium to cellularized cells in Arabidopsis (for review see CHENG et al., 2020). A *FIE* gene from *Malus hupehensis* var. *pingyiensis* was introduced into tomato. The hemizygous transgenic tomato lines produced curly leaves and decreased in seed germination. In addition, the co-suppression of the transgenic *MhFIE* and endogenous (*SlFIE*) genes occurred in homozygous transgenic tomatoes. As a result, *FIE* silencing brought about abnormal phenotypes during reproductive development in tomato, such as increased sepal and petal numbers in flower, a fused ovule and pistil and parthenocarpic

fruit formation (LIU et al. 2012). The ectopic expression of a *FIE* gene and *SlFIE* co-suppression notably influenced the expression of genes associated with leaf, flower, and fruit development.

The gene ***LeTIP* (= *TONOPLAST INTRINSIC PROTEIN – TIP*),** is a member of the aquaporin family (for review see KUROWSKA et al., 2020). The genes code for transmembrane channels located mostly at the tonoplast of plant cells. The *TIPs* are known to transport water and many other small solutes such as ammonia, urea, hydrogen peroxide, and glycerol (SUDHAKARAN et al., 2021). The gene expression analysis indicated that the expression of *TIP* genes varies during different developmental stages and also during stressed conditions (REGON et al., 2014). However, *TIPs* also play an important role in growth and developmental processes like radicle protrusion, anther dehiscence, seed germination, cell elongation, and expansion (SUDHAKARAN et al., 2021). Some of these developmental are clearly affected in the *epi*-related syndrome.

TCP* proteins [*Maize TEOSINTE BRANCHED (TB)* 1, *Antirrhinum majus CYCLOIDEA* (*CYC*)**, and rice ***PROLIFERATING CELL FACTORS* (*PCFs*)]** are plant-specific transcription factors characterized by the TCP domain, a motif encompassing a non-canonical basic-helix-loop-helix (*bHLH*) structure (PARAPUNOVA et al. 2014), related to DNA-binding and protein to protein interaction (ZHOU et al., 2022). Members of *TCP* gene family are involved in biological processes such as senescence, circadian rhythm, hormone signaling, leaf development, axillary shoots formation, Lateral branches formation, fruit development, floral induction, flower development, and ripening (PARAPUNOVA et al. 2014; SILVA et al. 2019; WEI et al., 2021; EDRIS et al., 2023). Interestingly, gibberellin and the microRNA319-regulated *TCP* transcription factors promoting flowering in *Arabidopsis* (a facultative long-day species) but suppressing it in tomato (a day-neutral species) (SILVA et al. 2019). Whole genomic analysis in tomato identified 30 *SlTCP* genes that were phylogenetically subdivided into class I and class II (PARAPUNOVA et al. 2014). Within class II, two further lineages are found in angiosperms, *Antirrhinum CINCINNATA* (*CIN*) and *TCP* genes (NICOLAS & CUBAS, 2016). Class *IIb* or *CIN-TCPs* is a group of eight *TCP* proteins in Arabidopsis involved in leaf growth regulation (*AtTCP−2*, *AtTCP−3*, *AtTCP −4*, *AtTCP −5*, *AtTCP −10*, *AtTCP −13*, *AtTCP −17*, and *AtTCP −24*). Tomato has 11 CIN-like *TCP* proteins in this homology group, among which the earlier identified *LANCEOLATE*, ***SlTCP1* to *SlTCP6,

SlTCP10, ***SlTCP−24***, *SlTCP−28*, *SlTCP−29*, and *SlTCP−30* (PARAPUNOVA et al. 2014). Herein, we detected four DEGs from this family of transcription factors (***TCP3, TCP6***, and ***TCP24*)**.

The ***TCP24* (= *SlTCP24*)** is a tomato homolog from one Arabipdosis genes implicated in regulating leaf morphogenesis that regulate the activities of *ASYMMETRIC LEAVES*1 mutant and auxin response during foliar differentiation of Arabidopsis (KOYAMA et al., 2010; EDRIS et al., 2023). In *Arabidopsis*, the *TCP3*, *TCP17*, and *TCP24* genes are targeted by different microRNAs, but they display partially redundant roles (LI, 2015; NICOLAS & CUBAS, 2016). Single and multiple mutants of these genes are associated with a wide range of phenotypic manifestations in the foliar tissue ranging from slightly enlarged leaves to serrated crinkly leaves (NICOLAS & CUBAS, 2016). The DEG ***TCP17***) was detected in the OGs ('**DEVELOPMENT PROCESS'** and **'GROWTH'**) may regulate the shade-induced hypocotyl elongation rate in *A. thaliana.* (ZHOU et al., 2018). Functional analysis in tomato revealed the involvement of ***SlTCP24***, which synergistically regulate compound leaf development with *SlTCP29* by targeting genes involved in cytokinin metabolic pathway (HU et al., 2023). However, no functional study is available with ***TCP3, TCP6,* and *TCP17*** homologs in tomato and other Solanaceae (SI et al., 2023). ***TCP11*** was involved in early tomato fruit development (EDRIS et al., 2023). Morphological changes in fruit phenotype in association with the *epi* mutation are not typical, even though increase in fruit mass in hybrid combination has been observed (BASTOS, 2017).

The *SHINE3* (*ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR*) is a transcription factor that regulates biosynthesis of cutin, controlling proper tomato fruit formation and epidermal pattern (SHI et al., 2013). The silencing of the *SHINE3* gene in Arabidopsis caused a decrease in cutin load and to changes in cell wall structure (SHI et al., 2011). Interestingly, *SHIN3* displayed a non-significant expression in the transcriptional regulatory network associated with systemic defense, plant growth, and flowering in tomato during priming with *Trichoderma harzianum T34* (AAMIR et al., 2023).

The ***ZINC-FINGER HOMEODOMAIN* (*ZF-HD*) *TRANSCRIPTION FACTORS*** play a key role in the control of plant growth and development and being also involved in plant

responses to stress. Twenty-two (22) *ZF-HD* genes in the tomato genome and classified them into seven groups located on six chromosomes (Hu et al., 2018).

Herein, the ***LeHB-1*** (= *hb*-1 = *hd*1; AOKI et al., 2010) was also identified as a DEG. ***LeHB-1* gene** was found to be a major transcription factor in the ethylene biosynthesis pathway, being involved in the control of ripening and in floral organogenesis (LIN et al., 2008). Fruit ripening involves a complex interplay between ethylene and ripening-associated transcriptional regulators. ***ETHYLENE RESPONSE FACTORS* (*ERF*s)** are downstream components of ethylene signaling, known to regulate the expression of ethylene-responsive genes (LIU et al., 2014). Experimental data suggest the existence of a complex network enabling interconnection between *ERF* genes and the expression of ripening regulators, such as *HB-1* which may account for the pleiotropic alterations in fruit maturation and ripening (LIU et al., 2014).

The DEG ***ELONGATED HYPOCOTYL 5* (*SlHY5*)** (**= *HY5,*** *THY5*, *LeHY5*, *thy5*) is a basic Leucine zipper transcription factor that directly bound to and activated the transcription of genes encoding a gibberellin-inactivation enzyme, (*GIBBERELLIN2-OXIDASE4*), and an abscisic acid biosynthetic enzyme (*9-CIS-EPOXYCAROTENOID DIOXYGENASE6 – SlNCED6*). Thus, phytochrome A-dependent *SlHY5* accumulation resulted in an increased abscisic acid/gibberellin ratio, which was accompanied by growth cessation and induction of cold response (WANG et al., 2018). In fruits, *SlBBX20* (*Solyc12g089240*), is transcriptionally induced by the master transcription factor *RIPENING INHIBITOR* (*SlRIN*) and together with *SlHY5* up-regulates flavonoid biosynthetic genes (SHIOSE et al., 2024). *HY5* could activate the starch degradation-related genes such as *PWD*, *BAM1* (one of the detected DEGs in our assay), *BAM3*, *BAM8*, *MEX1*, and *DPE1* by directly binding to their promoters.

The DEG ***MBF1a*** (***MULTIPROTEIN BRIDGING FACTOR 1A GENE EXPRESSION***) is a member of proteins associated with stress responses. *MBF1* proteins are transcription co-factors that form a bridge between transcription factors (TF) and the *TATA BOX BINDING PROTEIN* (*TBP*), which is part of the basal transcription machinery (SANCHEZ-BALLESTA et al., 2007). The first transcription factors that were shown to interact with *MBF1* belong to the *bZIP* family, although interactions with other families have been reported (JAIMES-MIRANDA & CHAVEZ MONTES, 2020). In tomato, *ER24* (an ethylene responsive group *II MBF1* protein) loss-of-function results in

altered seed germination phenotype (SANCHEZ-BALLESTA et al., 2007; HOMMEL et al., 2008).

The DEG ***PAP1* (*PURPLE ACID PHOSPHATASE* 2**) is a member of a family of metallo-phosphoesterase enzymes, are involved in phosphorus nutrition in plants (SUEN et al., 2015). The tomato genome encodes 25 *PAP* members (SRIVASTAVA et al., 2020). Transcript abundance analysis showed that most of these *PAPs* are activated under Pi deficiency in tomato. Physio-biochemical analyses revealed relatively lower total root-associated acid phosphatase activity in the seedlings of *S. pimpinellifolium* than their cultivated tomato seedlings under Pi deficiency (SRIVASTAVA et al., 2020). However, the correlation of this DEG with the *epi*-related syndrome is elusive.

The *NAC* (*NAM, ATAF1/2*, and *CUC2*) is one of the largest transcription factor families in plants, playing roles in response pathways to various abiotic and abiotic stresses and such as drought, salinity, low temperature, and pathogen infection (OU et al., 2024; ZHU et al., 2024). *NACs* are plant-specific transcription factors that also play essential roles in plant development, being involved in cell enlargement (CHEN et al., 2021). *NAC* proteins comprise a highly conserved N-terminal *NAC* structural domain and a multifunctional C-terminal regulatory structural domain. The *NAC* structural domain has the ability to recognize specific *cis*-acting elements and bind to both DNA and proteins (OU et al., 2024). Herein, we detected a DEG for ***NAC1* (*TRANSCRIPTION FACTOR SLNAC1*).** This gene in tomato was found to be an important factor in abiotic stress response and is fine-tuned at both transcriptional and posttranslational levels. The ***SlNAC1* gene** is strongly induced by multiple abiotic stresses and the *SlNAC1* protein is subjected to ubiquitin proteasome-mediated degradation. Transgenic tomato plants expressing the $Pro_{StNAC1}::SlNAC1^{\Delta 191\text{-}270}$ transgene did not display any undesired traits and exhibited enhanced tolerance to cold, drought and salt stresses (NIU et al., 2022). *NAC1* gene is down regulated in *epi*, indicating that this mutation might result in inferior levels of tolerance to abiotic and abiotic stresses.

RNA PROCESSING

Down-regulated DEGs of the ***FEY* (*RETINOL DEHYDROGENASE 12 = FOREVER YOUNG OXIDOREDUCTASE***) and ***CCR2* (*CINNAMOYL-COA REDUCTASE***) were

detected in this OG. They are two genes coding for a Rossmann-fold NAD(P)-binding domain-containing proteins (NCBI Conserved domains). The NADPH domain is found in numerous dehydrogenases of metabolic pathways such as glycolysis, and many other redox enzymes (NCBI Conserved domains). NADPH oxidase can induce reactive oxygen species (ROS) production and microtubule orientation (SANDALIO et al., 2016). The balance between ROS production and elimination by antioxidants defines the role of these compounds as intra- and inter-cellular signals or toxic compounds according to their concentrations (SANDALIO et al., 2016). ROS regulate *TRANSPORT INHIBITION RESPONSE* (*TIR1*) activation, which is a receptor of *ARFs*. ROS accumulation induced by auxin triggers epinasty by changing cell wall biosynthesis, cell expansion, and vascular tissue proliferation (SANDALIO et al., 2016). ROS promote the post-translational modification of tubulin and actin, giving rise to changes in cytoskeleton organization and cell size. All these factors promote curly leaf phenotypes, resulting in epinasty or hyponasty (SANDALIO et al., 2016). Interestingly, epinasty is the most typical phenotype defining the *epi* mutation.

DEVELOPMENTAL PROCESS AND GROWTH

Twenty-three (23) DEGs were detected in the GO subgroups corresponding to the terms **'developmental process'** and **'growth'** (**Figure 5**). The following genes were down-regulated in *epi* NIL in comparison with the WT NIL: *ARS1*, *HY5*, *ARF2*, *gts1*, *hsc70-1* (chromosome 6), *hsc70-2* (chromosome 10), *BAM1*, *hb-1*, *Coi1*, *SGT1*, *FEY*, *MAPK6*, *CIP4*, *PIN3*, and *pip1*. The following genes were up regulated in the *epi* NIL: *FIE*, *hxk1*, *TCP24*, *UBC1*, *profilin*, *TCP17*, *AGO10*, and *TCP3*. The DEGs corresponding to *BAM1*, *hb-1*, *MAPK6*, *profilin*, *gts1*, and *PIN3* were identified in both 'developmental process' and 'growth' subgroups (**Figure 5**).

A down-regulated DEG of ***ARS1* (*ALTERED RESPONSE TO SALT STRESS 1*)** was detected here. This protein codes a R1-type *MYB* transcription factor which is involved in stomatal closure under salt acclimation (CAMPOS et al., 2016). A screening under salt stress conditions of a t-DNA mutant collection of tomato led to the identification of this mutant, which showed a salt-sensitive phenotype (CAMPOS et al., 2016).

A novel strongly down-regulated DEG in the OG was related to ***pip1* (*APOPLASTIC PHYTOPHTHORA-INHIBITED PROTEASE 1*).** The gene codes for a ***PAPAIN-LIKE***

IMMUNE PROTEASE* (*PLCP*).** These genes are promising engineering targets for crop protection, given their significant roles in plant immunity (SCHUSTER et al., 2024). The *Avr2* gene of *Cladosporium fulvum* encodes a small secreted protein that inhibits ***PIP1 in the apoplast of tomato leaves (ILYAS et al., 2015). *PIP1* is also inhibited by *Epic1* and *Epic2B*, which are secreted cystatin-like proteins produced by *Phytophthora infestans* during infection, indicating a protease-inhibitor arms race in this pathosystem. Additional studies demonstrated that the depletion of pip1 induces hyper-susceptibility to bacterial, fungal and oomycete tomato pathogens in tomato (ILYAS et al., 2015). The association of *epi* with the downregulation of pip1 is yet elusive. However, we observed that *epi* plants were highly susceptible to powdery mildew in contrast with the WT line (**manuscript in preparation**), indicating that this mutation could increase disease susceptibility.

The spatiotemporal localization of the plant hormone auxin acts as a positional cue for proper patterning in many developmental processes, including early root, leaf, and flower organogenesis (ZHANG & SHANG, 2024). Auxin guides the organization of these processes by inducing changes in transcriptional responses and by affecting cell wall properties (MARTINEZ et al., 2016). Unlike other known plant hormones, auxin is actively transported in a directional fashion, allowing the creation of spatio-temporally regulated auxin concentrations. The largest contributors of directional transport in the *PAT* system are the ***PIN-FORMED* (*PIN*) auxin transporters**. Most *PIN*s (*PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7*) accomplish directional transport by localizing asymmetrically on the plasm membrane of a cell, transporting auxin out of the cell in the direction of *PIN* localization. Auxin, as a weak acid, is freely taken up into a cell; therefore transport of auxin out of the cell by *PIN* proteins is the determining factor for directional auxin movement. Herein, we identified a DEG of the tomato ***PIN3* (*AUXIN EFFLUX CARRIER COMPONENT 3* = *SLPIN3*)**. In *Arabidopsis*, lateral roots originate from pericycle cells deep within the primary root. New lateral root primordia (LRP) have to emerge through several overlaying tissues. A *PIN3* ortholog is involved in the formation and emergence of the lateral root primordium (LRP) When the LRP penetrates the endodermis, cortex, and epidermis, auxin is directed to flow to cortex and epidermis cells, mediated by *PIN3*. A study investigated the positive effects of Melatonin on adventitious root formation of de-rooted tomato seedlings indicated a significant increase in the r**elative expression of *PIN3* in response to melatonin treatment** in both plant apex and hypocotyl (WEN et

al., 2016), suggesting the involvement of this gene in root system formation. *PIN* genes are also involved in the development of epinasty by interfering with microtubules, acting, cell wall synthesis, cell expansion, and vascular tissue proliferation (SANDALIO et al., 2016), which are events associated with the *epi* mutation.

An upregulated DEG of a tomato ***PROFILIN* (*Solyc08g066110*)** was also detected in our transcriptomic analysis. Profilins regulate actin filament polymerization by interacting with actin monomers and play crucial roles in actin dynamics in cells (SAARIKANGAS et al, 2010). Remodeling of the cytoskeleton coordinates growth in plant cells, including trafficking and exocytosis of membrane and wall components during cell expansion, and regulation of hypocotyl elongation in response to light (LIAN et al., 2021).

The covalent attachment of the protein ubiquitin to other cellular proteins has been implicated in a number of important physiological processes including selective protein degradation, DNA repair, cell cycle control, and organelle biosynthesis (FEUSSNER et al., 1997). The ubiquitination is a complex process, which involves three enzymes namely, *E1* ubiquitin-activating enzyme, *E2* ubiquitin-conjugating enzyme, and *E3* ubiquitin ligase (SHARMA & BHATT, 2017). An up-regulated DEG corresponding to ***LeUBC1* (an *E2 UBIQUITIN-CONJUGATING ENZYME – UBC*; Gene ID: 101250809 *LeUBC1 = UBC27*)** from tomato (FEUSSNER et al., 1997) was detected here. *LeUBC1* was identified as a member of a small *E2* subfamily and transcripts of the gene displayed strong accumulation after exposure to heavy metal (cadmium) stress (FEUSSNER et al., 1997). *In-silico* methods allowed the identification of 59 genes with *UBC* family domains in the tomato genome. The gene expression analysis of RNA sequencing data of these genes revealed expression profile of tomato *E2* genes in seedling, root, leaf, seed, fruit, and flower tissues (SHARMA & BHATT, 2017). The *E2* enzymes are reported to involve in both biotic and abiotic stress, including positive regulation of plant immunity, osmotic stress tolerance, drought tolerance, and salt tolerance (for review see SHARMA & BHATT, 2017). However, it is the first report of the upregulation of *LeUBC1* associated with a morphological mutant such as *epi*, which contribution to the phenotype is yet elusive.

A DEG of ***MAPK6* (*MITOGEN-ACTIVATED PROTEIN KINASE 6* Gene ID: 100736474 = JF791807 = *SlMAPK6*)** correspond to a member of the mitogen-activated

protein kinases (*MAPKs*), a family of Ser/Thr protein kinases, that play an essential role in mediating biotic and abiotic stress responses in plants. The *MAPK* cascade signaling system has been relatively conserved throughout the evolution of eukaryotes and is involved in the regulation of growth and development and metabolism. KONG et al. (2012) investigated 16 putative *SlMAPK* genes from tomato genome and compared them with those from Arabidopsis. Tissue-specific expression of *SlMAPK6* was measured in WT plants by quantitative RT-PCR. The results showed that *SlMAPK6* was highly expressed in the tissues of the stems, leaves, and flowers but was expressed at low levels in the tissues of the roots, sepals, and fruits. *SlMAPK6*-knockout lines were obtained and compared with WT. The mutant lines CRISPR-3 and CRISPR-7 showed increased numbers of axillary buds and true leaves, thickened stems, and longer leaflets. In addition, *SlMAPK6* was found to positively regulate genes of the strigolactone (*SlCCD7* and *SlCCD8*) and the gibberellin synthesis (GA20ox3 and GA3ox1) and negatively regulates the axillary bud development-related genes *Ls*, *BL* and *BRC1b/TCP8* and the GA synthesis inhibitory gene GAI. Therefore, *SlMAPK6* appears to regulate the synthesis of strigolactone and GA to induce the growth and development of tomato axillary buds (LI et al., 2021), which is in agreement with a subset of traits associated with the *epi* mutation.

PHYTOSULFOKINE* (*PSK*)** is a plant pentapeptide hormone that fulfills a wide range of functions. *PSK* acts to promote plant growth at the expense of the pathogen defense against (hemi)biotrophic pathogens *PSK* has frequently been reported to function in the inverse regulation of growth and defense in response to (hemi)biotrophic pathogens (DING et al., 2023). *PSK*-induced phosphorylation of glutamine synthetase GS2 at two serine residues regulates this balance The *PSK*-induced phosphorylation of glutamine synthetase GS2 at two serine residues regulates the balance between plant growth at the expense of the pathogen defense against (hemi)biotrophic pathogens. Interestingly, the DEG of ***GTS1 – GLUTAMINE SYNTHETASE CYTOSOLIC ISOZYME 1-1 (Gene ID: 543756; Solyc04g014510.2) down regulated in *epi* is 80% identical to *GS2* (NP_001310598.1) and contains a *GS2* phosphorylation site at *Ser334*, which specifically regulates plant defense, and *Ser360*, which regulates growth.Glutamine synthetase cytosolic isozyme 1-1 (***GTS1***) is 80% identical to *GS2* (NP_001310598.1) and contains a *GS2* phosphorylation at *Ser334* specifically regulates plant defense, whereas *Ser360* regulates growth. However, the participation of ***GTS1*** in the *epi*-associated phenotype remains unclear.

Plant **OXYLIPINS** constitute a group of bioactive fatty acid derivatives that perform several important roles in growth and development, which includes jasmonic acid (JA) and methyl jasmonate (MeJA). These signaling compounds play important roles in regulating defense responses against herbivores and pathogens (LI et al., 2004). JA is a signaling molecule that regulates a broad range of plant defense responses against herbivores and some microbial pathogens. Molecular genetic studies in Arabidopsis have established that JA also performs a critical role in anther and pollen development. A loss of function mutation in the F-box protein of one DEG detected here (***CORONATINE-INSENSITIVE1 – COI1 = SlCOI1*****)** gene in tomato exhibited a wide array of defected in JA-signaled processes, including reduced pollen viability, the inability to express JA-responsive genes, severely compromised resistance to two-spotted spider mites, and abnormal development of glandular trichomes (LI et al., 2004). More recently, the role of phytohormone JA signaling in ROS scavenging was investigated employing *SlCOI1* as a target for silencing and for overexpression in tomato transgenic hairy roots (HR) under the constitutive promoter. All observation confirmed the regulatory role of *COI1*-mediated JA signaling in regulation of enzymatic components involved in ROS scavenging (KADAM & BARVKAR, 2024). As previously discussed, ROS balance is associated with epinastic leaves (SANDALIO et al., 2016), which is the key phenotype of the *epi* mutant.

Eukaryotic microRNAs (miRNAs) loaded into *ARGONAUTE* (*AGO*) proteins recognize complementary sequences on target mRNAs and silence their expression. These properties of the *AGO* proteins allow their participation in plant developmental processes and virus defense as core elements of transcriptional regulator or/and post-transcriptional regulator in RNA induced silencing complex (*RISC*), which is guided by small RNAs to repress target genes expression. Fifteen 15 putative *AGO* genes were identified in tomato genome. Highly conserved in plants, miR168 is a key feedback regulator of the expression of the central miRNA effector, *AGO1*, enabling proper functioning of the entire miRNA pathway. Here, we identified a DEG of the ***AGO10* (*PROTEIN ARGONAUTE 10*)**, which is the closest paralog of *AGO1*. The 22-nt *miR168-AGO10* complex antagonizes *AGO1* accumulation in part via “transitive RNAi”, a silencing-amplification process, to maintain appropriate *AGO1* cellular homeostasis. Interesting, the tombusviral P19 silencing-suppressor protein displays markedly weaker affinity for the 22-nt form among its isomiR168 cargoes, thereby promoting *AGO10-directed suppression* of *AGO1*-

mediated antiviral silencing. In addition, patterning of the root xylem into protoxylem (PX) and metaxylem is regulated by auxin-cytokinin signaling and microRNA *miR165a/166b*-mediated suppression of genes encoding Class III *HOMEODOMAIN LEU-ZIPPER* (*HD-ZIPIII*) proteins (BLOCH et al., 2019). In tomato, abscisic acid (ABA) signaling enhanced PX differentiation longitudinally and radially, indicating an evolutionarily conserved mechanism. ABA increased expression of *miR165a/166b* and reduced expression of the *ARG10* (also known as ZWILLE – ZLL), which is the repressor of *miR165a/166b*, resulting in reduced levels of all five HD-ZIPIII RNAs. Thus, this gene plays a role in ABA-mediated xylem patterning and maturation as well as in lateral root initiation (BLOCH et al., 2019).

The **DEG of *BAM1* (*BETA AMYLASE 1* = *Solyc09g091030.3.1*)** is a homolog of Arabidopsis *BAM1* gene. *BAM* enzymes are usually associated with starch breakdown, which are localized in the nucleus rather than targeted to the chloroplast. These proteins possess a set of *BRASSINAZOLE RESISTANT1* (*BZR1*)-type DNA binding domains—also found in transcription factors mediating brassinosteroid (BR) responses. The *BZR1-BAM* proteins activate gene expression in Arabidopsis. Deregulation of *BZR1-BAMs* (the bam7/bam8 double mutant and *BAM8*-overexpressing plants) causes altered leaf growth and development (REINHOLD et al., 2011), which could have a role in the *epi*-mediated syndrome.

Sterols accumulate in different forms, as free sterols (*FSs*) with a free β-hydroxyl group at C-3 position on the backbone, conjugated esters, sterol glycosides (*SGs*), and acyl *SGs*. *SGs* are produced by UDP-glucose: sterol glycosyltransferases (*SGTs*), which catalyze the transfer of a glucose residue from UDP-glucose to the free hydroxyl group at position C-3 of *FSs* (RAMIREZ-ESTRADA et al., 2017). Sterols are important for regulating plant growth and development, as changes in cellular sterol composition affect a variety of cellular processes, such as vascular and stomatal patterning, cell division, expansion, and polarity, cell-to-cell connectivity, and hormonal control, among others (RAMIREZ-ESTRADA et al., 2017). Here we found a DEG of the ***STEROL GLYCOSYLTRANSFERASE 1* (*SGT1*) *SGT1*** gene silencing caused moderate plant dwarfism and reduced fruit size (Chávez et al., 2023), which could be related to the *epi* phenotype.

A DEG for the ***HEXOKINASE 1*** **(*hxk1*= *SlHXK1*)** was also observed here**.** Tomato lines with silent ***SlHXK1*** (SlHXK1-RNAi lines) displayed advanced leaf senescence and stunted plant growth. Physiological features including plant height, leaf length, thickness and size (LI et al., 2020), which are some features shared with the *epi* mutation. The SlHXK1-RNAi lines also displayed reduced contents of chlorophyll and starch. Therefore, *SlHXK1* display a significant involvement in leaf senescence and plant growth and development in tomato through affecting starch turnover (LI et al., 2020).

CONCLUSIONS

DEG of five GO subgroups potentially related to the phenotypic syndrome associated with the *epi* mutation were analyzed, including 'hormone metabolic process', (four DEGs) 'regulation of gene expression' (29 DEGs), 'RNA processing' (two DEGs) and 'developmental process' + 'growth' (23 DEGs). As somewhat expected, the majority of the DEGs were related to the crosstalk of hormonal pathways and their regulation. Previous functional analysis of a wide array of these DEGs indicated their association with one or more phenotypic traits of the *epi*-related syndrome. In conclusion, further analysis of these *epi*-associated DEGs will provide the basis for the establishment of conventional and biotech breeding strategies aiming to fine-tunning the expression of most suitable combination of yield-enhancing genetic factors.

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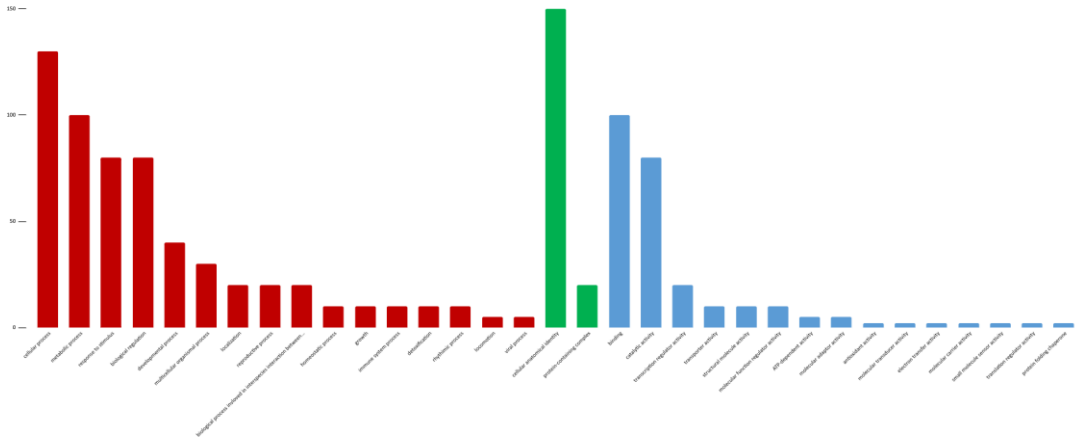

Figure 1 – General gene ontology (GO) of the 29,548 mapped genes in the transcriptomic analysis of contrasting near isogenic lines (NILs) for the epinastic (*epi*) mutation in tomato. Red bar corresponding to the group of 'Biological process'; green bar corresponding to genes of the 'cellular component', and blue bar corresponding to the group 'molecular function'.

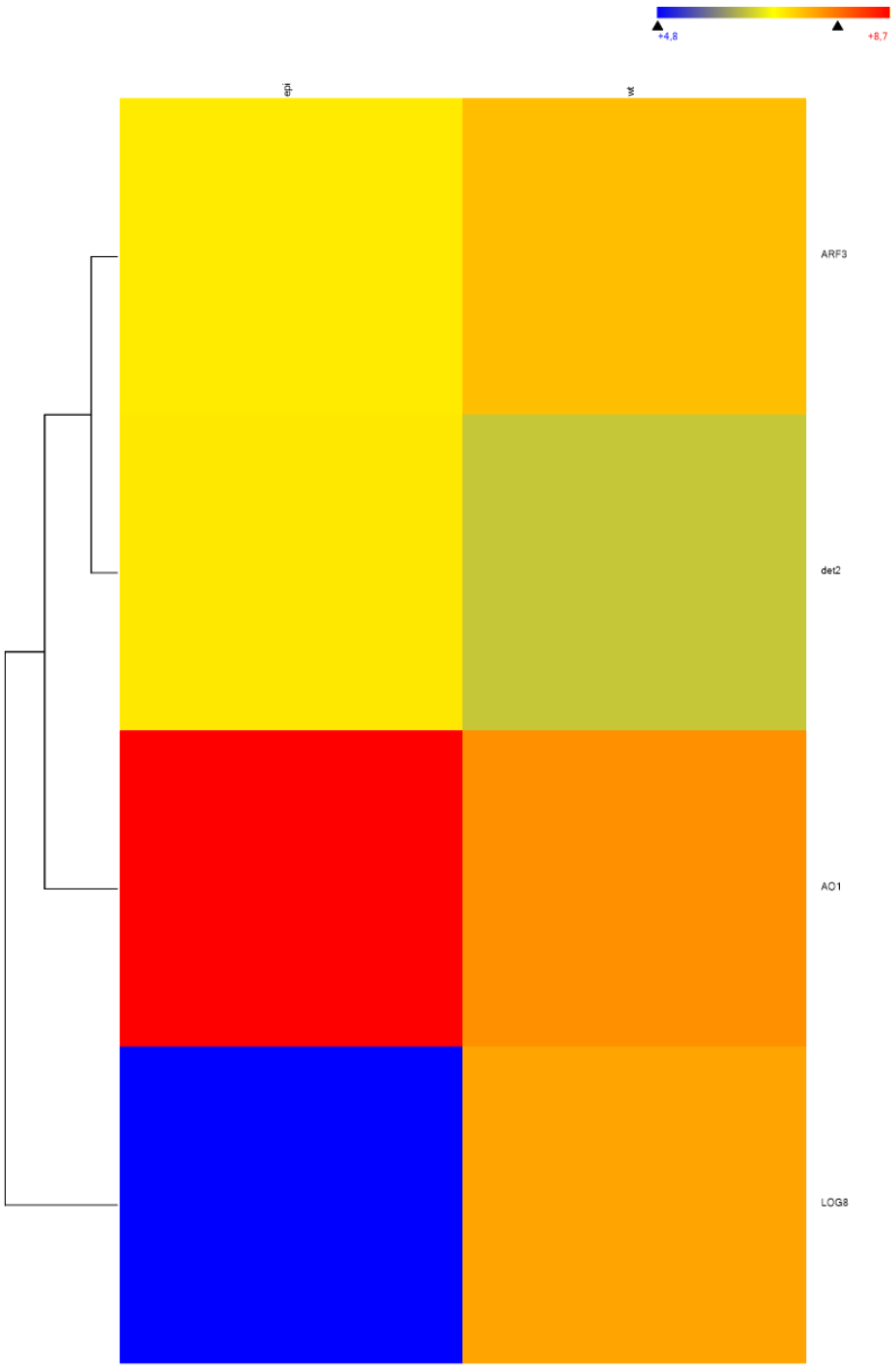

Figure 2 – Heat map of four differentially expressed gene (DEGs) of the ontology gene class **'hormone metabolic process'** detected in the transcriptomic analyses of contrasting near isogenic lines (NILs) for the epinastic (*epi*) mutation in tomato. Red color indicates up-regulated genes, and blue indicates down-regulated genes (see color rule at the upper right corner). Hierarchical cluster using Euclidean distance metric and Centroid Linkage Method in the ArrayStar program (DNAstar).

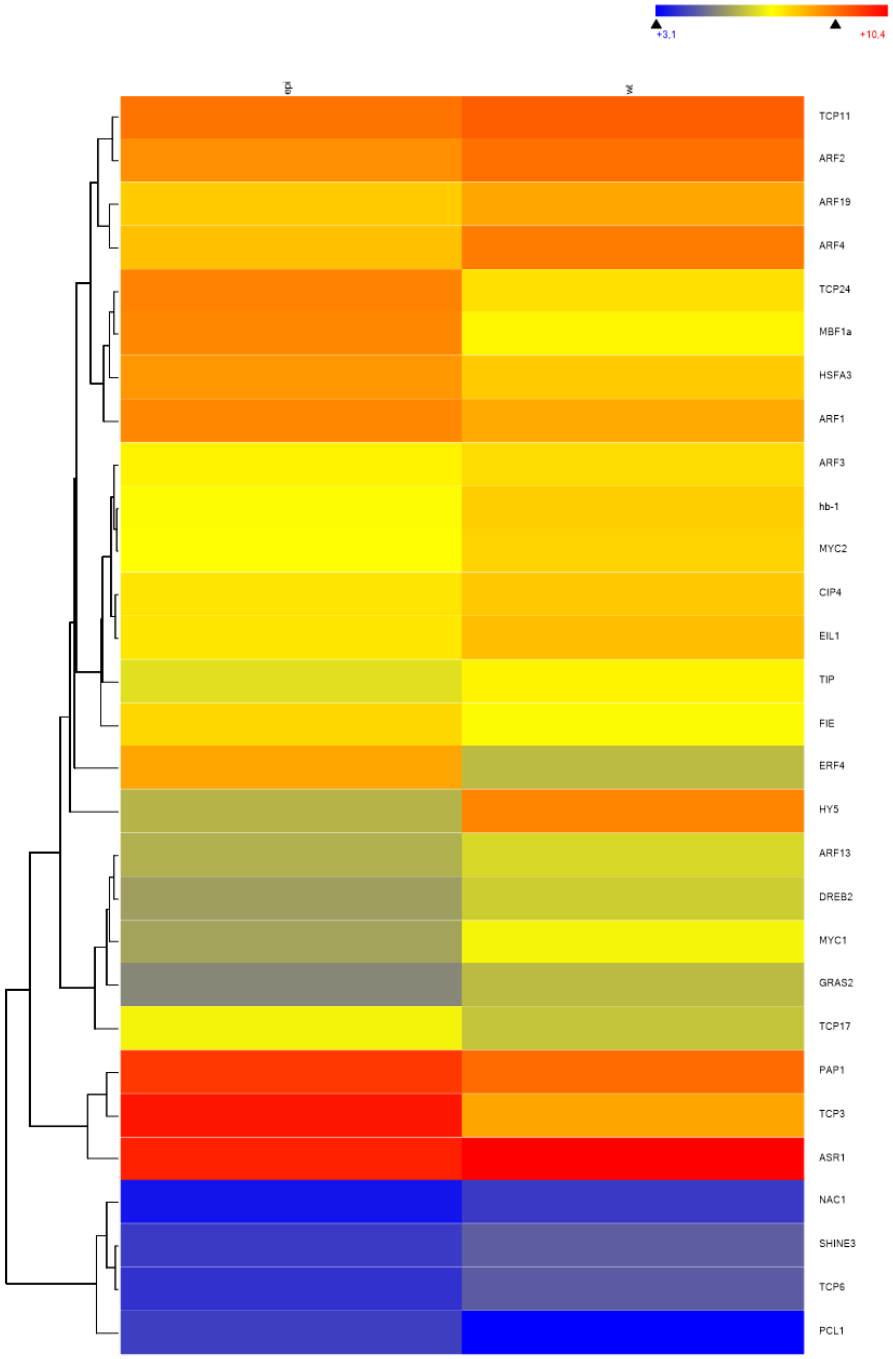

Figure 3 – Heat map of 29 differentially expressed gene (DEGs) of the ontology gene class **'regulation of gene expression'** detected in the transcriptomic analyses of contrasting near isogenic lines (NILs) for the epinastic (*epi*) mutation in tomato. Red color indicates up-regulated genes, and blue indicates down-regulated genes (see color rule at the upper right corner). Hierarchical cluster using Euclidean distance metric and Centroid Linkage Method in the ArrayStar program (DNAstar).

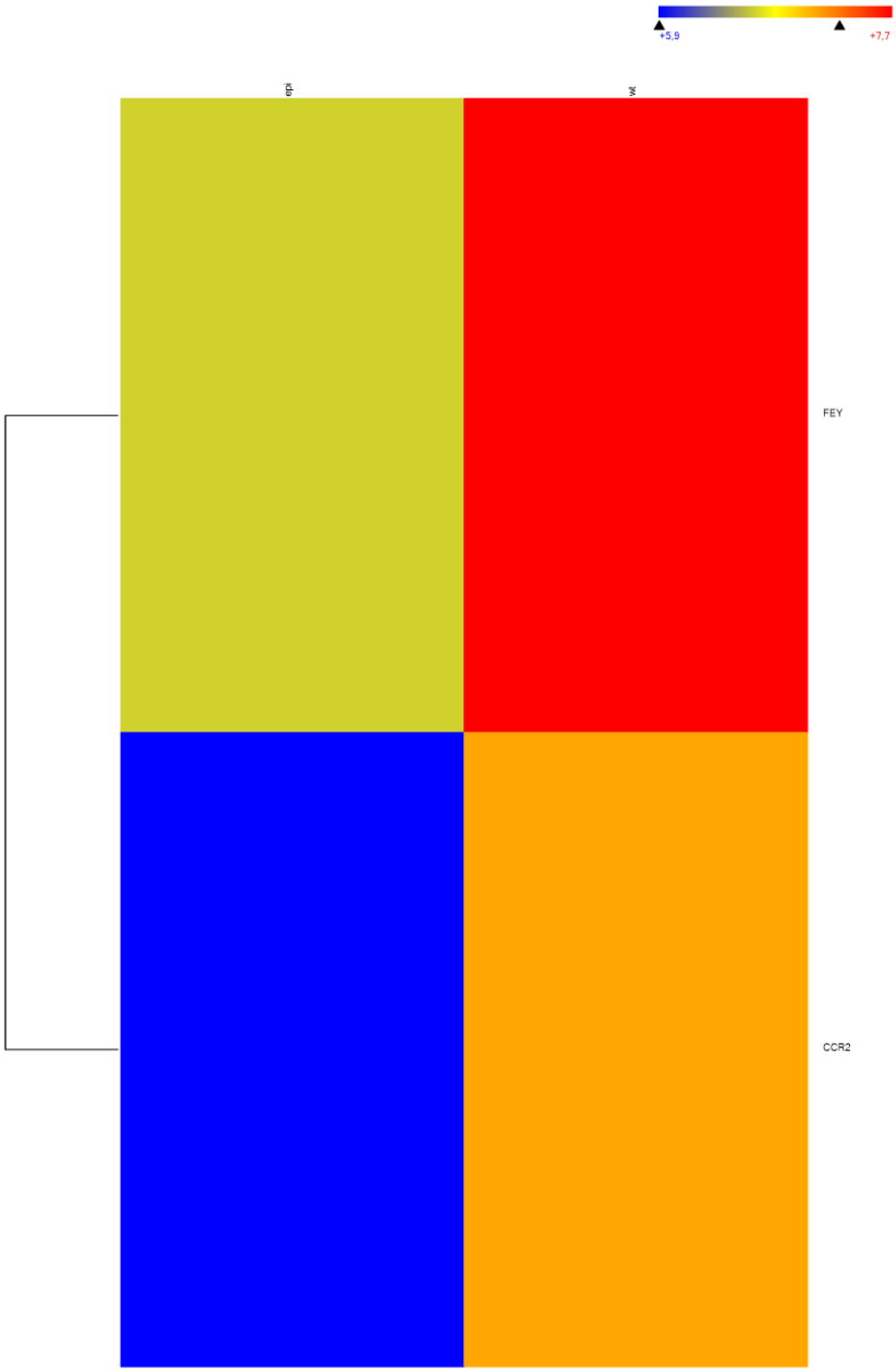

Figure 4 – Heat map of two differentially expressed gene (DEGs) of the ontology gene class **'RNA processing'** detected in the transcriptomic analyses of contrasting near isogenic lines (NILs) for the epinastic (*epi*) mutation in tomato. Red color indicates up-regulated genes, and blue indicates down-regulated genes (see color rule at the upper right corner). Hierarchical cluster using Euclidean distance metric and Centroid Linkage Method in the ArrayStar program (DNAstar).

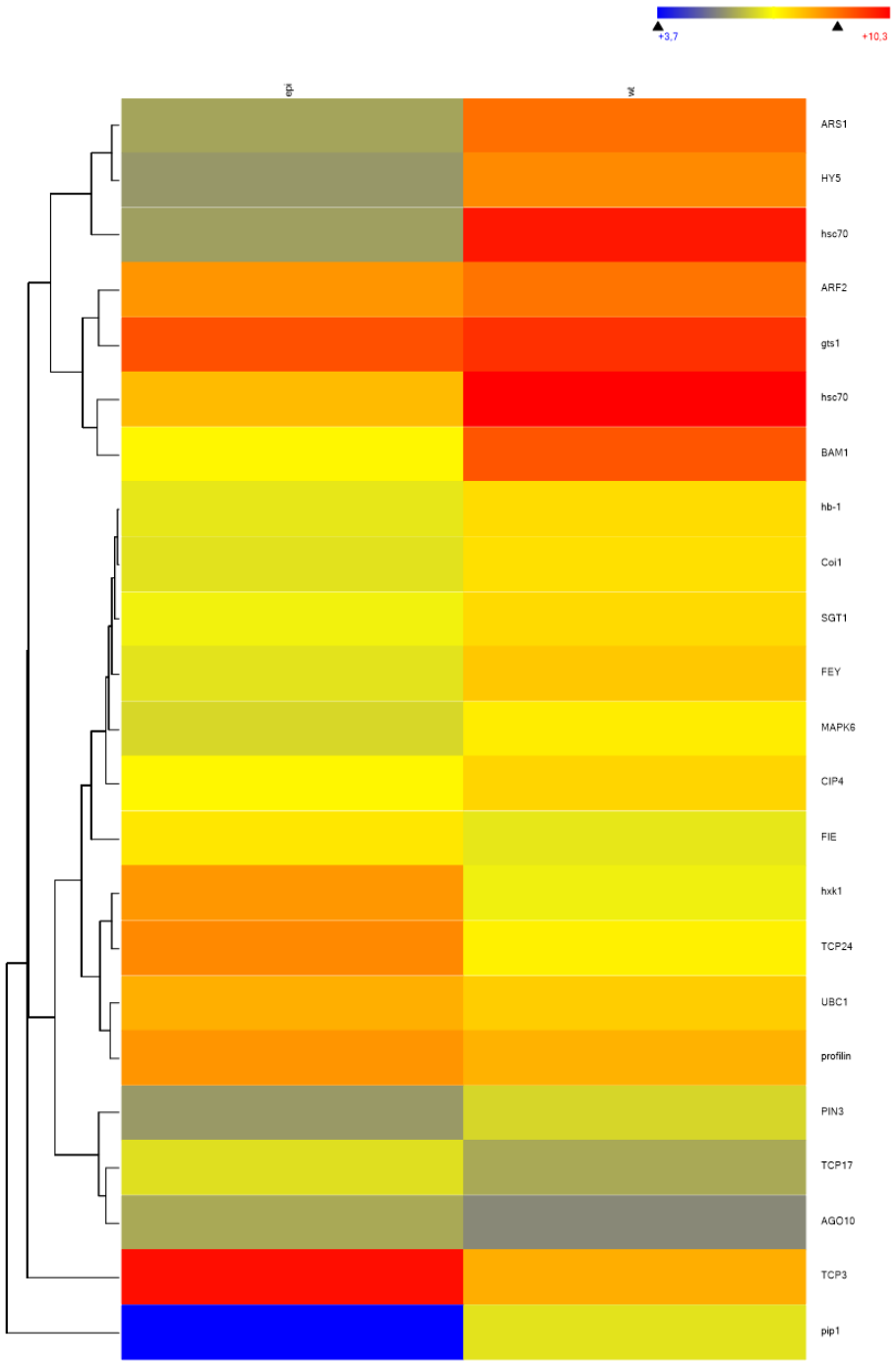

Figure 5 – Heat map of 23 differentially expressed gene (DEGs) of the ontology gene classes **'developmental process' and 'growth'** detected in the transcriptomic analyses of contrasting near isogenic lines (NILs) for the epinastic (*epi*) mutation in tomato. Red color indicates up-regulated genes, and blue indicates down-regulated genes (see color rule at the upper right corner). Hierarchical cluster using Euclidean distance metric and Centroid Linkage Method in the ArrayStar program (DNAstar). Six DEGs also identified of the 'growth' term were **gts1, BAM1, hb-1, MAPK6, profilin, and PIN3.**

CHAPTER 4

Search for candidate *epi* gene(s) via comparative analyses of transcriptomic-derived polymorphic mRNAs in contrasting near-isogenic lines for the mutant locus on the tomato chromosome 4.

Search for candidate *epi* gene(s) via comparative analyses of transcriptomic-derived polymorphic mRNAs in contrasting near-isogenic lines for the mutant locus on the tomato chromosome 4.

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ABSTRACT

The *epinastic* (*epi*) mutation is characterized by inducing striking structural and physiological changes in tomato. The importance of *epi* from the breeding standpoint is its involvement in the expression of heterosis for a relevant set of vegetative and reproductive traits. Therefore, it would be of genetic interest to identify and clone this gene(s). Previous genomic mapping indicated that *epi* locus is positioned on the long arm of the chromosome 4. Herein, we employed a strategy for landing on candidate *epi* gene(s) involving a combination of genomic mapping and transcriptomic-derived information from two contrasting near isogenic lines (NILs). We searched for missense and nonsense (= presence of stop mutation with putative loss-of-function) mRNA polymorphisms within genes encompassing the *epi* locus by analyzing the two independent RNA-seq data (one from the whole above-ground seedlings and one from the meristematic regions) and their corresponding hybrid combination. The employment of contrasting NILs significantly reduces the 'genetic noise', simplifying the process of detecting candidate genes controlling the *epi* associated phenotypic syndrome. Variant calls were detected across the NILs in comparison to the reference genome (cultivar 'Heinz 1706'). The following set of simultaneous 'filtering criteria' were used from both RNA-seq data: **(1)** missense or nonsense homozygous variants occurring exclusively in all the three replicas of one of the NILs; **(2)** variants + wild type genes (in heterozygous configuration) occurring exclusively in the hybrid combination; **(3)** variant positions with a minimum depth of coverage of 10, and **(4)** Q call ≥ 7. Altogether we analyzed 4,086 sequence variants along the entire chromosome 4. Only seven genes were identified fitting the imposed set of 'filtering criteria'. The sequences of these genes were also verified in the variant call files (VCFs) of the original 'VFN8' cultivar (from where the *epi* spontaneous mutation was initially detected) retrieved from the database of 360 tomato genomes from the Sol Genomics network. As somewhat expected, all genes with polymorphic mRNAs were in close linkage on chromosome 4 (from 58,372.069 to 65,529.058). The seven genes were exocyst complex component – *SEC5A* (*Solyc04g071350*); syntaxin-81 (*Solyc04g071730.3.1*); light-harvesting complex-like protein – *OHP2* (*Solyc04g071930.3.1*); xanthoxin dehydrogenase – *XanDH* (*Solyc04g071940.3.1*); uncharacterized protein (*Solyc04g071970.3*); uncharacterized protein with a kinesin-like motif (*Solyc04g072940.3*); and β-tubulin (*Solyc04g081490.3*). Analyses of gene function, structural modification of the protein, type of mutation

(missense or nonsense), and expression patterns across the NILs indicated the allelic variants of *XanDH* (a putative truncated gene) and *β-tubulin* (a deleterious mutation) as the strongest candidates for this trait. The presence of two genes coding for uncharacterized proteins in this linkage group will demand additional reverse genetics analyses to exclude both as candidates. However, both displayed neutral impacts of their amino acid substitutions on the putative protein functions. The information derived from these polymorphic genes can used as molecular markers to fine map *epi* in segregating populations. Also, gene silencing via CRISPR-Cas9 targeting the disruption of these genes can be used to verify the role of one or more of these genes in the *epi*-associated phenotype.

Keywords: *Solanum lycopersicum*, recessive mutation, yield, breeding.

INTRODUCTION

The recessive *epinastic* (*epi*) was initially identified as a spontaneous mutation in tomato (*Solanum lycopersicum* L.) cultivar 'VFN8' (FUJINO et al., 1988a, 1989; URSIN & BRADFORD, 1989; BARRY et al., 2001). The phenotypic syndrome associated with the *epi* mutation is characterized by a wide range of structural and metabolic changes, including foliar epinasty, leaves with dark green pigmentation, erect growth, branched root system, reduced anthocyanin content, and overproduction of ethylene in the apex of the plants (FUJINO et al., 1988; 1989; URSIN & BRADFORD, 1989). Roots and hypocotyls of *epi* plants are shorter and thickened and a constitutive ethylene response and a more intense transport and accumulation auxin in the tissues roots and hypocotyls in comparison with to WT plants (BARRY et al., 2001). Stems and petioles have larger diameters than WT plants and petioles exhibit a peculiar epinastic curvature. The leaves also present an epinastic and twisted morphology and ethylene production reaches levels above those observed in WT plants (FUJINO et al. 1988a; 1988b; BARRY et al., 2001).

Contrasting near isogenic lines (NILs) for the *epi* mutation were developed in distinct backgrounds and the performance of their hybrids have been evaluated (CARVALHO et al., 2011; BASTOS, 2017). The potential of the *epi* is its association with the expression of heterosis for a set of vegetative and reproductive traits in plants heterozygous for this mutation (BASTOS, 2017). The plants with one dosage of the *epi* mutation displayed higher yield and higher fruit mass (weight) and sugar content (°Brix) (BASTOS, 2017). This set of characteristics indicates that the mutant *epi* as having potential for use in applied plant breeding. The locus encompassing the *epi* was mapped on the long arm of the tomato chromosome 4 (BARRY et al., 2001). Therefore, it would be of both breeding and genetic interest to clone this gene(s). In addition, the development of functional molecular markers for the *epi* gene would be useful in assisted selection systems in tomato. However, the *epi* gene(s) was not yet cloned.

Novel large-scale gene expression analytic tools have allowed the investigation of the effects on global expression patterns of candidate genes associated with distinct mutations/traits in tomato (ALBA et al. 2005; SHI et al. 2013). Currently, the high-throughput RNA sequencing (RNA-seq) technology has been widely used in tomato genetics (ROTHAN et al., 2019) since it represents a simpler and more powerful approach to quantify gene expression at genomic level (GUPTA et al. 2013; LI et al., 2017; JIN et al., 2022). One major goal of the transcriptomic analyses is to connect gene expression

with phenotype of interest (ZHOU et al., 2019). Therefore, the availability of information about the genetic location of this mutation in combination with RNA-seq data for contrasting NILs for the *epi* locus may facilitate the chromosome landing (TANKSLEY et al., 1995) on the gene controlling this trait. Therefore, it would be interesting the search for non-synonymous, nonsense, or loss-of-function polymorphisms (SNPs and INDELs) within mRNAs present in this linkage group. These allelic variants linked to this genomic region could be considered as potential candidate genes controlling this trait. The importance in the *epi* from the breeding standpoint, is its involvement in the expression of heterosis for a relevant set of vegetative and reproductive traits. Therefore, it would be of genetic interest to clone this gene(s). Previous genomic mapping indicated that *epi* locus is positioned on the long arm of the chromosome 4. Herein, we employed a strategy for landing on candidate *epi* genes involving a combination of genomic mapping and transcriptomic-derived information from two contrasting near isogenic lines (NILs). We searched for putative missense and nonsense (= stop mutation with putative loss-of-function) mRNA polymorphisms (either SNPs or INDELs) within genes present in the linkage group encompassing the *epi* locus by analyzing the two independent RNA-seq data (one from the whole above-ground seedlings and one from the meristematic regions) and their corresponding hybrid combination. The close genetic relationship between NILs is an important genetic tool since them significantly reduces the 'genetic noise' (i.e. unrelated polymorphisms) originating from other genomic regions and simplifies the process of chromosomal landing on potential candidate genes controlling the *epi*-associated phenotypic syndrome.

MATERIAL AND METHODS

Plant cultivation – Seedlings of the hybrid combination and its corresponding NILs (named as 'VFN8 *epi*' and 'VFN8 WT') were all grown under greenhouse conditions with supplemental LED lighting running in a regime for 12 hours of light and 12 hours of dark with day temperature of 26 ºC and night temperature of 20 ºC. Two subgroups of samples were collected: seedlings and meristematic tips. Seedlings were grown in soil up to the development of their first true leaves (approximately 8–10 cm tall). Afterwards, the above ground tissues of the seedlings (without roots) were harvested from a subgroup of plants and immediately put into liquid nitrogen. The same procedure was carried out for a distinct subgroup of plants from which the meristematic tips were carefully removed. All samples were kept into deep freezer (-80 ºC) up to RNA extraction,

RNA extraction and construction of transcript libraries – The tissue samples (50 mg) were removed from the freezer and RNA was purified using the Reliaprep kit (Qiagen). A final aliquot of 10 μL was used to measure the quality and quantity of the extracted DNA in a NanoDrop® spectrophotometer (Thermo Fisher Scientific, USA) and TapeStation (Agilent) Electrophoresis. Samples with RIN > 8.0 were employed in subsequent experimental steps. Aliquots of total RNA extracted from both tissue samples of the NILs and the hybrid combination were sent for library synthesis in a commercial company under recommended shipment conditions. All steps related to sample preparation for the library acquisition and sequencing stages were performed by the commercial company. The sequencing platform used was the Illumina HiSeq 2500, paired ends, choosing to map 20 million reads in each biological replicate.

Bioinformatics analysis of RNAseq data – The FASTq sequences from the three replicates of the following experiments/libraries: **S3** ('VFN8 WT', Wild Type; seedling), **S4** ('VFN8 *epi*', seedling) and **S5** (hybrid 'VFN8 WT' *x* 'VFN8 *epi*'; seedling); **M3** ('VFN8 WT', Wild Type; meristematic tip), **M4** ('VFN8 *epi*'; meristematic tip), and **M5** (hybrid 'VFN8 WT' *x* 'VFN8 *epi*'; meristematic tip). Sequences were aligned to the latest version of the *Solanum* (*Lycopersicon*) reference genome (Sol Genomics; NCBI) using SeqManNGen v.17 (DNAStar, Madison, WI, USA). In the SeqMan NGene software, the sequence quality analysis procedures, adapter trimming used by the sequencing platform, and alignment with the reference genome (cultivar 'Heinz 1706') were performed automatically, following the default settings of the program. SNPs/InDels were also mapped. The mapped sequences (reads) were used to evaluate differential gene expression, using the ArrayStar software. The reads were normalized using DEseq2 statistical analysis (LOVE et al., 2014). Genes with differential expression were identified by performing t-tests, which included FDR (False Discovery Rates) control to adequately correct significance levels (BENJAMINI & HOCHBERG, 1995). Differentially expressed genes (DEGs) were evaluated across samples, with a significance value (*p*-value) equal to or less than 0.05%. Gene annotations were performed using the SeqMan Pro software, using standard program algorithms (Gnomom). Subsequently, functional analysis of the genes was performed manually using the UniProt database, belonging to the UniProt consortium (https://www.uniprot.org).

Search for single nucleotide polymorphisms associated with genes in close linkage with the *epi* locus ontomato chromosome 4 – The *epi* locus was genetically mapped on

the long arm of chromosome 4 in previous work (BARRY et al., 2001). Herein, we employed a strategy for landing on candidate *epi* gene(s) involving a combination of genomic mapping and transcriptomic-derived information from the two contrasting NILs. We searched for putative missense and nonsense mRNA polymorphisms within genes encompassing the *epi* locus on chromosome 4 by analyzing the two previously described RNA-seq data. We analyzed variant call files (VCFs) of the contrasting NILs (and the corresponding hybrid) with the reference genome of the cultivar 'Heinz 1706' (TOMATO GENOME CONSORTIUM, 2012) which have a standard, WT phenotype. To do so, the following set of simultaneous 'filtering criteria' were used from both RNA-seq data: **(1)** missense or nonsense homozygous variants occurring exclusively in all the three replicas of one of the NILs; **(2)** variants + wild type genes (in heterozygous configuration) occurring exclusively in the hybrid combination; **(3)** variant positions with a minimum depth of coverage of 10, and **(4)** Q call ≥ 7. We double-checked the identified variants with the VCF from the original 'VFN8' (WT cultivar) retrieved from the database of 360 genomes of tomato cultivars and wild species (BAILE et al., 2021). These variants were also manually verified by inspection of the corresponding sequence assemblies to eliminate artifacts arising from assembly differences. Finally, the best candidate genes were chosen considering: the physical/genetic position respect to *epi* as well as the predicted effects of amino acid substitutions on protein function using the PROVEAN tool (CHOI et al. 2012) (http://provean.jcvi.org/index.php) and the expression pattern according to TomExpress RNAseq database (ZOUINE et et., 2017) (http://gbf.toulouse.inra.fr/tomexpress/). Therefore, we took into account the analyses of gene function, structural modification of the protein, type of mutation (missense or nonsense), and expression patterns across the NILs.

RESULTS

We analyzed 4,086 sequence variants along the entire chromosome 4, known to encompass the *epi* mutation (BARRY et al., 2001) in search for potential candidate gene(s) for this trait. However, only seven genes were identified fitting the imposed set of 'filtering criteria' (**Table 1**). As somewhat expected, all genes with polymorphic mRNAs were in close linkage on chromosome 4 (from 58,372.069 to 65,529.058). The WT gene sequences of these genes were also verified in the VCF of the original 'VFN8' cultivar retrieved from the database of 360 tomato genomes (BAILE et al., 2021).

Expression patterns of these seven genes indicated that four of them were DEGs (**Figure 1**). This set of genes was not listed on **Chapter 3** because they do not match the 2-fold change criteria imposed in that work. In addition, many of these genes were not explored on the ontology groups. The genes with polymorphic mRNAs along the chromosome 4 were exocyst complex component – ***SEC5A*** (*Solyc04g071350* = LOC101266933; not a DEG); xanthoxin dehydrogenase – ***XanDH*** (*Solyc04g071940.3.1* = LOC101254297; 1.2-fold down-regulated); ***uncharacterized protein*** (*Solyc04g071970.3* = LOC101254895; 1.2-fold down-regulated); light-harvesting complex-like protein – ***OHP2*** (*Solyc04g071930.3.1* = LOC101253996; 0.7-fold down-regulated); ***syntaxin-81*** (*Solyc04g071730.3.1* = LOC101249911; not a DEG); ***uncharacterized protein*** (*Solyc04g072940.3* = LOC101250397; not a DEG); and ***β-tubulin*** (*Solyc04g081490.3* = TUB; 1.2-fold up-regulated). All genes displayed only one sequence variant within their coding regions, except for the *SEC5A*, which displayed two mutations (**Table 1**).

Predicted deleterious effect(s) of amino acid substitution(s) on function of these proteins was detected only the *β-tubulin* (amino acid change p.L249V) gene, according the PROVEAN tool (**Table 1**). The expression pattern according to TomExpress RNAseq database (http://gbf.toulouse.inra.fr/tomexpress/) indicated that these seven genes are expressed across all plant tissues (**data not shown**).

Coverage depth of the mRNA reads on the genomic regions corresponding to this set of seven polymorphic genes on the tomato chromosome 4 indicated that all mRNAs are completely covered for all genes except for xanthoxin dehydrogenase (*Solyc04g071940.3.1* = LOC101254297), suggesting that the allele in the *epi* NIL codes for a version of a putative truncated protein.

DISCUSSION

The phenotypic syndrome associated with the *epi* mutation is characterized by structural and metabolic changes including foliar epinasty, leaves with dark green pigmentation, erect growth, branched root system, reduced anthocyanin content, and overproduction of ethylene in the apex of the plants (FUJINO et al., 1988, 1989; URSIN & BRADFORD, 1989). Roots and hypocotyls of *epi* plants are shorter and thickened and a constitutive ethylene response and a more intense transport and accumulation auxin in the tissues roots and hypocotyls in comparison with to WT plants (BARRY et al., 2001).

The importance in the *epi* from the breeding standpoint is the involvement of this mutation in the expression of heterosis for a relevant set of vegetative and reproductive traits (BASTOS 2017). Therefore, it would be of genetic interest to identify and clone this gene(s).

Previous genetic map information indicated that *epi* locus is located on the long arm of the tomato chromosome 4 (BARRY et al., 2001). Herein, we employed a strategy for landing on the candidate(s) *epi* gene(s), involving a combination of genomic mapping and transcriptomic-derived information. We initially search for the presence of nonsense and missense mRNA polymorphisms of genes present in the linkage group of the chromosome 4 by analyzing the two independent RNA-seq data of NILs contrasting for the *epi* locus. Variant calls were detected across the contrasting NILs and the reference genome of the cultivar 'Heinz 1706' (with the WT phenotype). Altogether we detected 4,086 sequence variants along the entire chromosome 4. However, only seven genes were identified according to the imposed set of 'filtering criteria'. As somewhat expected, all genes with polymorphic mRNAs were in close linkage on chromosome 4 (from 58,372.069 to 65,529.058). For this set of genes, we carried out analyses of gene function, structural modification of the protein, type of mutation (missense or nonsense), and expression patterns across the NILs aiming to identify the strongest candidates for this trait among them.

According to the PROVEAN analysis, the allele of the ***β-tubulin*** (*Solyc04g081490.3*) gene in the *epi* locus displays a deleterious amino acid substitution (change p.L249V). However, this gene was the only one over-expressed DEG in this set of polymorphic alleles in *epi* NIL. In addition, the sequence of the corresponding RNA was found to be complete (non-truncated). Microtubules (MTs) are one of the most conserved cytoskeletal structures of plants, consisting of dimeric proteins with α-tubulin and β-tubulin subunits (BREVIARIO et al., 2013). MTs are essential elements, guiding cell wall cellulose patterns and controlling cell division, size, and shape as well as intracellular trafficking and immune responses (HSIAO & HUANG, 2023). MTs are also important under abiotic stress challenges by adjusting plant growth and plasticity (CHEN et al., 2022). Perturbation of MTs often leads to changes in cell wall composition, which results in modification in the patterns of cell expansion and the overall plant architecture (BAO et al., 2021). In terms of patterns of expression, the ***β-tubulin*** (*Solyc04g081490.3*) fits as a good candidate gene since it is expressed in all organs (TomExpress RNAseq

database http://gbf.toulouse.inra.fr/tomexpress/) where the *epi* mutation is able to induce morphological changes. Nevertheless, epinasty (one of the major traits associated with the *epi* mutation) and other foliar, stem, and root abnormalities due to changes in cytoskeleton organization may arise from post-translational modification of tubulin and actin (SANDALIO et al., 2016). However, there is so far no clear and direct association of the *epi*-associated phenotype with a *β-tubulin* gene mutation. In fact, *β-tubulins* are encoded across plant species by families of multiple genes, which are functionally redundant making most of the mutants phenotypically indistinguishable from the wild type (HASHIMOTO, 2013; RADCHUK, 2008). In the tomato genome, the *Solyc04g081490.3* plus nine other *β-tubulin* genes are present in the genome of the cultivar 'Micro-Tom' (AOKI et al., 2010) and in the TomExpress RNAseq database. However, none of these other *β-tubulins* were DEGs in our transcriptomes (**data not shown**). Therefore, mutation in a single *β-tubulin* coding gene in tomato leading to striking structural and physiological/hormonal regulatory changes (as observed in *epi* plants) will be a very interesting novelty.

Abscisic acid (ABA) biosynthesis is carried out via the carotenoid pathway, initiating from epoxycarotenoid in plastids (CHEN et al., 2020). A rate-limiting step in ABA biosynthesis is the oxidative cleavage of 9-cis-epoxycarotenoid to produce xanthoxin (catalyzed by 9-cis-epoxycarotenoid dioxygenase) also in the plastids (ENDO et al. 2014). In the cytosol, ***xanthoxin dehydrogenase* (*XanDH*)** catalyzes the conversion of xanthoxin to abscisyl aldehyde, which is converted to ABA by an aldehyde oxidase (OA). Herein, we detected a missense (but neutral according to PROVEAN analysis) variant of one ***molybdenum cofactor* (*MoCo*)*-containing XanDH*** (*Solyc04g071940.3.1*) gene in the linkage group. Here, the expression pattern of *Solyc04g071940.3.1* indicated that it is downregulated (1.2-fold). In addition, a putative truncated allelic version of this gene was detected in the *epi* NIL. Distinct mutants displaying reduced capacity to synthesize ABA were reported in tomato, including *flacca*, *notabilis,* and *sit* (SAGI et al., 1999). In the mutant *flacca*, no significant XanDH or AO activities were detected in leaves; however, the mutant exhibited considerable activity in the roots, which contained notable amounts of ABA. The *Solyc04g071940.3.1* is potentially expressed in all tissues according to the TomExpress RNAseq database. The morphological mutant *sitiens* (*sit*) is also defective in ABA biosynthesis (TAYLOR et al., 1988). Fine molecular mapping placed the *sit* mutation on the short arm of chromosome 1 (HARRISON et al., 2011). The

sit gene was found to be a novel ABA aldehyde oxidase apoenzyme-coding gene (76-78% amino acid identity with other AO proteins) (HARRISON et al., 2011). Two allelic *sit* mutants (*sit* and *sit* ^{w}) were identified causing a truncation of exon 2 and the deletion of exon 7, respectively. In the present work, the putative effects of a putative defective version of *Solyc04g071940.3.1* could explain some of the phenotypic effects observed in *epi* plants. However, in the case of *epi* mutation, the levels of ABA across distinct tissues were not yet evaluated.

A missense (but neutral according to PROVEAN analysis) variant of ***chlorophyll-containing light-harvesting complex* (*LHC*)*-like protein* (*one helix protein 2*)** was detected in our analysis. This gene, named as ***OHP2*** (= *Solyc04g071930.3.1*) displayed 0.7-fold down-regulation. The light-harvesting complexes were acquired by primary endosymbiosis and transferred to the host plant genome, allowing the evolution of photosynthetic eukaryotic organisms (LEVIN & SCHUSTER, 2023). In eukaryotic organisms, *OHP1* and *OHP2* genes (or small chlorophyll a/b binding proteins = small-CABs) replace One-Helix LHC-like Proteins (HLIPs) and are similarly induced by intensive high-light. Recent studies suggest that they are crucial for PSII assembly and chlorophyll biosynthesis (HEY & GRIMM, 2018). Mutations of either *OHP1* or *OHP2* genes in Arabidopsis resulted in less pigmentation, a disrupted thylakoid architecture, and severe growth deficits (JAHNS et al. 2017) but not impact in hormone regulation was reported thus far. No information is yet available in tomato about the phenotypic effects of mutation in *Solyc04g071930.3.1* or its influence on plant architecture. However, it the phenotypic effects of *OHP* mutations described in Arabidopsis does not indicate this gene as a good candidate for the *epi* gene.

A missense (but neutral according to PROVEAN analysis) mutation in the ***syntaxin-coding gene SlSYP81.1*** (= *Solyc04g071730*) was also detected in our analyses. *SlSYP81.1* is one of 21 tomato syntaxin-coding genes (BRACUTO et al., 2017) and one of the 63 ***SNARE* (*soluble N-ethylmaleimide-sensitive factor attachment protein receptor*)** genes characterized in the tomato genome (SALINAS-CORNEJO et al., 2019). These gene products play a major role in membrane fusion and vesicle transport during biotic and salt stress conditions (SALINAS-CORNEJO et al., 2023). In potato, the downregulation of *SYNTAXIN-RELATED 1* (*StSYR1*) gene increased resistance *Phytophthora infestans* more likely due to constitutive accumulation of salicylic acid and pathogen-related protein 1 (*PR1*) transcripts. Aberrant callose deposition of *StSYR1*-

RNAi plants after pathogen inoculation coincided with decreased papilla formation at penetration sites. On the other hand, levels of resistance to the necrotrophic fungus *Botrytis cinerea* were not affected (ESCHEN-LIPPOLD et al., 2012). In tomato, RNAi-based silencing of tomato syntaxin *Solyc10g081850.1.1* (named *SlPEN1a*) resulted in compromised resistance to two powdery mildew-inducing fungi: *Oidium neolycopersici* and *Blumeria graminis* f. sp. *hordei*, which is not a natural tomato pathogen (BRACUTO et al., 2017). Likewise, in Arabidopsis the syntaxin *SYP121* (a component of the SNARE complex) mediated a non-host like resistance to the *B. graminis*. However, the phenotypic effects of mutations in *SlSYP81.1* (*Solyc04g071730*) gene have not yet reported in the literature.

Interestingly, a sequence variant was also detected in the ***SEC5A*** (*Solyc04g071350* = LOC101266933) gene ***(a distinct component of exocyst / SNARE complex***) located in top of the chromosome 4. *Solyc04g071350* displayed two missense (but neutral according to PROVEAN analysis) mutations in its sequence. In plants, the highly conserved multiprotein exocyst complex plays an essential role in many biological processes by mediating secretion of post-Golgi-derived vesicles towards the plasma membrane, being implicated in cell morphogenesis (HALA et al., 2008). Emerging evidence shows that the exocyst complex is also a central player in plant–pathogen interactions in many host plants, including Solanaceous species (DU et al., 2018). The *Sec5* gene in Solanaceous plays a role in *P. infestans virulence*, being a target of the RXLR effector of this pathogen and suppressing host defense (OVERDIJK et al., 2021). It is interesting to mention that all these mutational analyses generated changes in host-pathogen interactions but not striking structural modification in the plants. Therefore, there is no strong evidence available that mutations in ***SEC5A*** could be related to the phenotypic syndrome associated with the *epi* mutation, although synergy among exocyst and *SNARE* complex interactions are related to vegetative growth (LARSON et al., 2020). ***SlSYP81.1*** and ***SEC5A*** are expressed in both tissues used in our transcriptomic analyses.

Finally, two uncharacterized proteins were also detected in this genomic region. The ***uncharacterized protein Solyc04g071970.3*** (= LOC101254895) was a DEG (1.2-fold down-regulated), whereas the uncharacterized protein *Solyc04g072940.3* (= LOC101250397) was not a DEG. We carried out additional attempts to characterize these proteins. The *Solyc04g072940.3* codes for protein with a kinesin-like motif, whereas *Solyc04g071970.3* displayed no typical structural/functional motifs. Kinesins are found

in all eukaryotic organisms and are essential cellular components, involved in a wide array of functions such as microtubule dynamics and morphogenesis, chromosome segregation, spindle formation, and elongation and transport of organelles (ALI & YANG, 2020). Kinesins play also essential roles in vesicle trafficking during regulation of plant growth and development (KHOSO et al., 2023). The presence of two genes coding for uncharacterized proteins in this linkage group will demand additional reverse genetics analyses to exclude both as candidates. However, both displayed neutral impact of their amino acid substitutions on the putative protein function.

CONCLUSIONS

Only seven genes were identified fitting the imposed set of 'filtering criteria'. The sequences of these genes were also verified in the VCF of the original 'VFN8' cultivar (from where the *epi* spontaneous mutation was detected) retrieved from the database of 360 tomato genomes from the Sol Genomics network. The seven genes with polymorphic mRNAs were detected in close linkage on chromosome 4 (from 58,372.069 to 65,529.058). These genes were a component of the ***Exocyst complex – SEC5A*** (*Solyc04g071350*); a ***syntaxin-81*** (*Solyc04g071730.3.1*); a ***light-harvesting complex-like protein – OHP2*** (*Solyc04g071930.3.1*); a ***xanthoxin dehydrogenase*** – ***XanDH*** (*Solyc04g071940.3.1*); an ***uncharacterized protein*** (*Solyc04g071970.3*); a distinct ***uncharacterized protein*** (*Solyc04g072940.3*); and a ***β-tubulin*** (*Solyc04g081490.3*).

Analyses of gene function, structural modification of the protein, type of mutation (missense or nonsense), and expression patterns across the NILs indicated the allelic variants of *XanDH* and *β-tubulin* as the strongest candidates for this trait. The presence of two genes coding for uncharacterized proteins will demand additional reverse genetics analyses in order to exclude both as candidates. However, both displayed a putative neutral impact of the amino acid substitutions on protein function.

The information derived from these polymorphic genes can used as molecular markers to fine mapping of the *epi* mutation in segregating populations. Also, gene silencing via CRISPR-Cas9 targeting the disruption of these genes can be used to verify the role of one or more of these genes in the *epi*-associated phenotype.

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Figure 1 – Heat map of seven genes on the tomato chromosome 4 detected in the transcriptomic analyses using seedlings of two near isogenic lines (wild type (WT) versus *epinastic* (*epi*) mutation) and their corresponding hybrid combination. The genes with polymorphic mRNAs along the chromosome 4 were: exocyst complex component – *SEC5A* (*Solyc04g071350* = *LOC101266933*; not a DEG); xanthoxin dehydrogenase (*Solyc04g071940.3.1* = *LOC101254297*; 1.2 fold down-regulated); uncharacterized protein (*Solyc04g071970.3* = *LOC101254895*; 1.2 fold down-regulated); light-harvesting complex-like protein – *OHP2* (*Solyc04g071930.3.1* = *LOC101253996*; 0.7 fold down-regulated); *syntaxin-81* (*Solyc04g071730.3.1* = *LOC101249911*; not a DEG); uncharacterized protein (*Solyc04g072940.3* = *LOC101250397*; not a DEG); and *β-tubulin* (*Solyc04g081490.3* = *TUB*; 1.2 fold up-regulated). Red color indicates up-regulated genes, and blue indicates down-regulated genes (see color rule at the upper right corner). Hierarchical cluster using Euclidean distance metric and Centroid Linkage Method in the ArrayStar program (DNAStar).

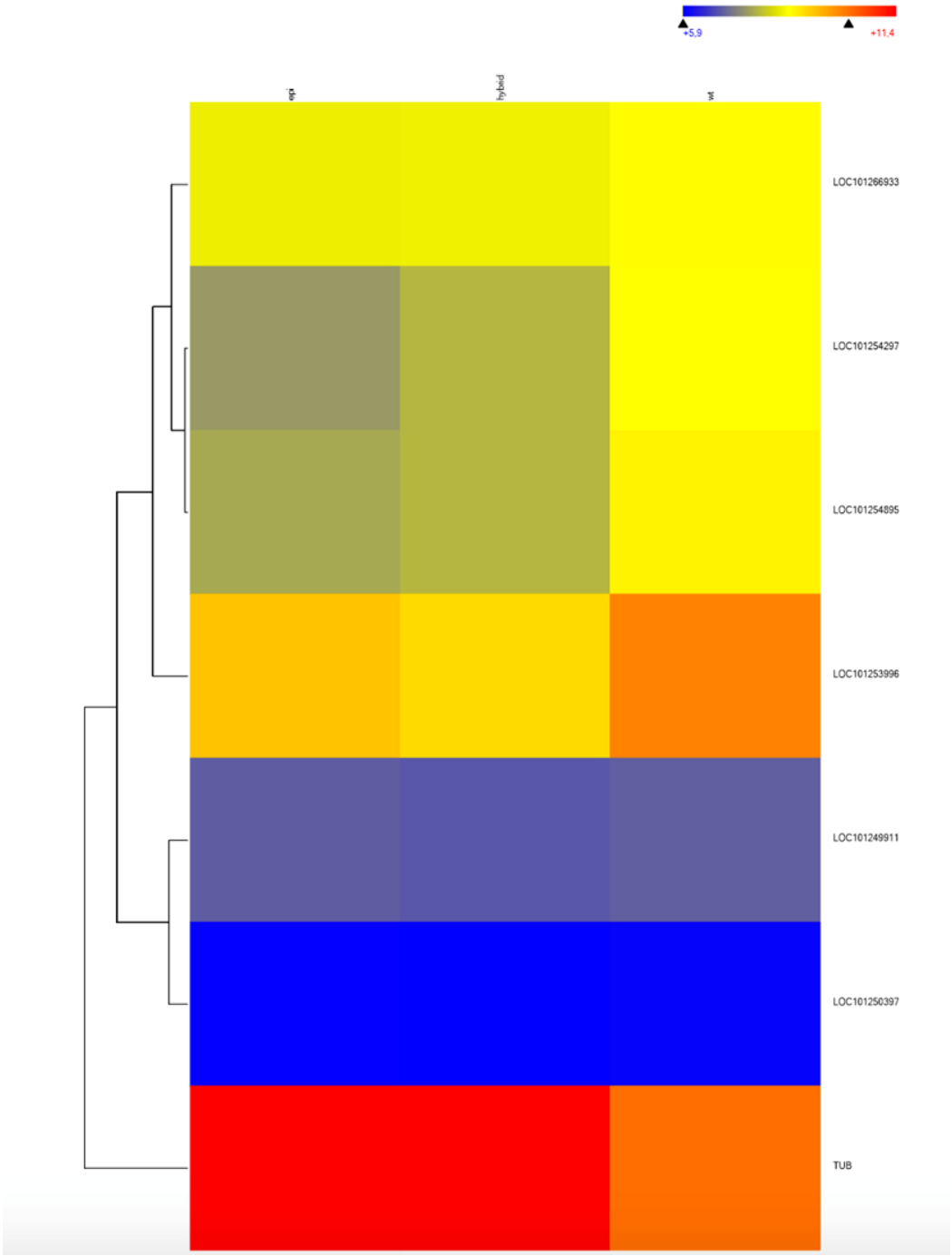

Table 1 – Position of variants present along the chromosome 4, known to encompass the *epi* mutation (BARRY et al., 2001) in search for potential candidate gene(s) for this trait. Percentage of variants found on the total reads in each experiment and replicas and the nucleotide change. The names of the tomato genes correspond to the following GenBank accessions: **exocyst complex component – *SEC5A* (***Solyc04g071350***)**; ***syntaxin-81* (***Solyc04g071730.3.1***)**; **light-harvesting complex-like protein – *OHP2*** (***Solyc04g071930.3.1***); **xanthoxin dehydrogenase (***Solyc04g071940.3.1***)**, **uncharacterized protein (***Solyc04g071970.3***)**; **uncharacterized protein** (***Solyc04g072940.3***), and **β-tubulin (***Solyc04g081490.3***)**.

Reference Name in the tomato genome	**Position**	**Gene and gene product name**	**Aminoacid change**	**Mutation type according to PROVEAN analysis**
Solyc04g071350	58372069	SEC5A (LOC101266933); Mutation #1	p.T467M	Neutral
Solyc04g071350	58384000	SEC5A (LOC101266933); Mutation #2	p.Y1089F	Neutral
Solyc04g071730.3.1	58785789	Syntaxin-81 (LOC101249911)	p.T184I	Neutral
Solyc04g071930.3.1	59032925	OHP2 (LOC101253996)	p.L22S	Neutral
Solyc04g071940.3.1	59034223	Xanthoxin dehydrogenase (LOC101254297)	p.H187R	Neutral
Solyc04g071970.3	59066215	Uncharacterized protein	p.T169I	Neutral
Solyc04g072940.3	60027859	Uncharacterized protein with a kinesin-like motif	p.A23V	Neutral
Solyc04g081490.3	65529058	β-tubulin (NM_001247878.2)	p.L249V	Deleterious

CONCLUSION

Based on the results presented, this thesis significantly contributes to the understanding of heterosis, particularly in the context of overdominance associated with the mutant *epi* gene and its impact on relevant agronomic traits. The data suggest that the modulation of heterosis may be linked to maternal effects in specific hybrid combinations, opening new possibilities for plant breeding. Additionally, the detailed analysis of differentially expressed genes (DEGs) reinforces the complex hormonal interaction that governs the phenotypic traits associated with *epi*, highlighting the crucial role of these DEGs in the hormonal crosstalk that affects productivity. The identification of seven candidate genes, especially *XanDH* and *β-tubulin*, provides a solid foundation for future genetic and biotechnological investigations, with the potential to utilize these variants as molecular markers or targets for genetic editing via CRISPR-Cas9.

In conclusion, this work not only advances knowledge on the genetic and hormonal regulation associated with *epi* but also offers valuable tools for the development of new breeding strategies that can optimize productivity and other agronomic traits of interest.

Given the complexity and variability of heterosis, research on heterosis in +/*epi* should focus on the following main objectives: (1) Confirmation of the role of overdominance and maternal effects in heterosis: Conduct additional studies to confirm and deepen the understanding of how overdominance in a single gene, possibly influenced by maternal effects, modulates heterosis in different hybrid combinations. This may involve the analysis of more genotypes and environments to validate the initial hypothesis; (2) Functional exploration of DEGs associated with *epi*: Further investigate the identified DEGs, especially those involved in hormonal crosstalk, to understand how these interactions affect the phenotypic traits associated with *epi*. Functional studies, such as manipulating the expression of these genes in different contexts, could elucidate the underlying mechanisms and enhance their use in breeding; (3) Validation of the candidate genes *XanDH* and *β-tubulin*: Focus on the functional validation of *XanDH* and *β-tubulin*, identified as strong candidates, to confirm their role in the phenotype associated with *epi*. This can be achieved through genetic silencing experiments using CRISPR-Cas9 or other gene-editing techniques, verifying how the modification of these genes impacts traits of interest; and (4) Characterization of unidentified proteins and development of molecular markers: Perform reverse genetic analyses to characterize the unidentified proteins,

excluding or confirming their role in the expression of the phenotype associated with *epi*. Additionally, develop and apply molecular markers based on the identified polymorphic genes for fine mapping and marker-assisted selection in segregating populations, facilitating the improvement of cultivars.