University of São Paulo
"Luiz de Queiroz" College of Agriculture

Redefining the hormonal control of tomato (*Solanum lycopersicum* cv. Micro-Tom) fruit development

Ignacio Achon Forno

Dissertation presented to obtain the degree of Master in Science. Area: Plant Physiology and Biochemistry

Piracicaba
2017

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RESUMO

Redefinindo o controle hormonal do desenvolvimento do fruto do tomateiro (*Solanum lycopersicum* cv. Micro-Tom)

O controle hormonal do desenvolvimento do fruto do tomateiro (*Solanum lycopersicum* cv. Micro-Tom) já foi extensamente estudado, principalmente no amadurecimento. No entanto, ainda existem consideráveis lacunas em nosso conhecimento da função e distribuição espaço-temporal dos hormônios durante o desenvolvimento do fruto. Foram realizados ensaios histoquímicos com cinco linhas transgênicas de tomate *cv.* Micro-Tom que carregavam o gene repórter GUS fusionado a cinco diferentes promotores responsivos aos hormônios, auxina, citocinina (CKs), giberelina (GAs), ácido abscísico (ABA) e etileno, com o fim de redefinir a distribuição espaço-temporal deles ao longo do desenvolvimento do fruto, desde estágios iniciais pre-anteses até o estágio red ripe (RR). Os níveis de CKs foram altos na fase pre-anteses, inicialmente na placenta e posteriormente nos óvulos, indicando um papel importante deste hormônio no crescimento do ovário, pre- fertilização. Nos estágios inicias, post-fertilização, o conteúdo de CKs foi alto nas sementes e depois nas camadas epidérmicas e subepidérmicas internas e externas do pericarpo. Alto conteúdo de auxina foi observado, durante todos o desenvolvimento do fruto, no pedicelo, sugerindo o transporte basípeto de auxina para a planta mãe. Os níveis de etileno aumentaram durante o crescimento do ovário antes da anteses. Interessantemente, o nível de etileno não diminuiu imediatamente post-fertilização. Os conteúdos de GA e ABA foram baixos prévios a anteses e nos estágios inicias pre-fertilização, especificamente na placenta e pericarpo. Inversamente, o conteúdo desses hormônios aumentou na fase de expansão celular do crescimento do fruto. Os grãos de pólen que chegaram ao stigma, post deiscência das anteras, apresentaram altos níveis de ABA e auxina. Além disso, reportamos um antagonismo entre conteúdo de etileno e giberelina durante o desenvolvimento do fruto, onde o nível de etileno começou a decrescer durante a fase de expansão celular do crescimento do fruto, momento em que o nível de giberelina começou a aumentar na placenta e pericarpo.

Palavras-chave: Tomate; Desenvolvimento do fruto; Hormônios; Gene reporter GUS L

ABSTRACT

Redefining the hormonal control of tomato (*Solanum lycopersicum* cv. Micro-Tom) fruit development

The hormonal control of tomato (*Solanum lycopersicum*) fruit development have been extensively studied, mainly during ripening. Notwithstanding, considerable gaps still exist in our understanding of the function and spatial-temporal distribution of hormones during fruit development. Here, we performed histochemical analysis of tomato *cv.* Micro-Tom transgenic lines harboring the gene reporter *GUS* fused to five different promoter responsive to the hormones, auxin, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA) and ethylene, in order to redefine their spatial-temporal distribution during tomato fruit development, from early pre-anthesis stages to red ripe (RR) stage. CKs levels were high during pre-anthesis, initially in placental tissues and subsequently in ovule, indicating a major function of this hormone during pre-fertilized ovary growth. At early post-fertilization stages, CKs contents were high in seeds, and afterward in the outer and inner epidermical and subepidermical layer of pericarp. High auxin content was observed, during all pre-anthesis and fruit development stages, in pedicel, suggesting a basipetal transport to the mother plant. Ethylene contents increased during pre-anthesis ovary growth. Interestingly, ethylene contents did not decrease, immediately, post-fertilization. The content of GAs and ABA was low at pre-anthesis and early post-fertilization stages, specific in placental tissues and pericarp. Conversely, these hormones accumulated mainly during cell expansion phase of fruit growth. Pollen grains that reached the stigma, post anther dehiscence, showed high content of auxin and ABA. In addition, we reported an antagonism between ethylene and GAs contents during tomato fruit development, where the level of ethylene started to decrease during the cell expansion phase of the fruit growth, moment when the level of GAs started to increase in placental tissues and pericarp.

Keywords: Tomato; Fruit development; Hormones; Gene reporter GUS L

1. INTRODUCTION

In angiosperms, embryonic development occurs concomitantly with the development of the ovary into a specialized organ, the fruit (Gillaspy et al., 1993). In tomato, a multicarpelar berry that is considered as a model system for fleshy climacteric fruit development (Azzi et al., 2015), fruit develops from the ovary, which consist in two or more carpels forming the locules, separated by a septum that fuses with each of the carpels, and ovules are attached to the central septum via placental tissue (Gasser and Robinson-Beer, 1993; Pattison et al., 2015).

Tomato fruit development is usually divided in four phases; fruit set, growth, maturation and ripening. The fruit set, defined as the transition of a quiescent ovary to a rapidly growing young fruit, depends on the successful completion of pollination and double fertilization, when one sperm fuses with the eggs cell to form the embryo and the other sperm fuses with the two haploid polar nuclei in the central cell to give rise to the endosperm (Raghavan, 2003). The fruit growth is divided in two stages: a very active period of cell division within the pericarp, mainly in the outer layer, which last about 7 to 10 days after successful fertilization, whereas after cell division phase during the following days until the fruit reach it final size, the fruit growth depend mainly on cell expansion. Fruit growth is followed by the fruit maturation, where it acquire the prerequisite competence to enter into the final development stage, ripening. Fleshy fruit such as tomato undergo a ripening process in which the biochemistry, physiology, and structure of the organ are developmentally altered to influence appearance, texture, flavor and aroma (Gillaspy et al., 1993; Giovannoni, 2004; de Jong et al., 2009a; McAtee et al, 2013; Kumar et al, 2014; Liu et al, 2015). It is worth noting that the four phases described above are well accepted by most author, although they do not take into account the initial ovary development before anthesis, where important events, such as the definition of the number of carpels, and thus the number of locules and the final size of the fruit, take place (Xu et al., 2015).

New evidences suggest that functions of plant hormones are not limited to a restricted phase in fruit development, and that there exists a complex crosstalk between them during the whole fruit development (Kumar et al., 2014; McAtee et al., 2013; Pattison et al., 2015). Increase of auxin contents upon pollination, mostly synthesized in the embryo and exported to pericarp, plays a major role in the regulation of fruit set and growth (Gillaspy et al., 1993; Serrani et al., 2008, 2007; Vriezen et al., 2008; de Jong et al., 2009a; Mariotti et al., 2011; Pattison and Catala, 2012; McAtee et al., 2013;). This induction of fruit set and growth by increased auxin post-fertilization is, at least partially, due to an increase in gibberellins (GAs) biosynthesis (Serrani et al., 2008). Exogenous applications of either auxin or GAs induce parthenocarpic fruits that present, respectively, increased cell layers or greater cell expansion (Serrani et al., 2007), suggesting that auxin contributes to cell division in fruit, while gibberellin to cell expansion.

Application of synthetic cytokinins (CKs) induces parthenocarpic fruit through promotion of cell division (Ding et al., 2013). Additionally, the cytokinin transduction pathway is active in cell division phase of fruit development, but the first peak of cytokinin accumulation occurs during anthesis (Matsuo et al., 2012). These evidences suggest that cytokinin might be involved in the growth of ovaries until pollination and afterwards in the cell division period post-pollination.

Acid abscisic (ABA) and ethylene biosynthesis genes expression is attenuated post-pollination, which suggest an antagonist role of these two plant hormones in fruit set (Vriezen et al., 2008; Shinozaki et al., 2015). In climacteric fruit, such as tomato, ethylene production reaches its maximum at the onset of ripening, which indicates a main role of this hormone at this phase of fruit development (Liu et al., 2015). On the other hand, the facts that the expression of ethylene biosynthesis genes (*SlACS2*, *SlACS4* and *SlACO1*) is induced by exogenous ABA, and that

ABA maximum precedes ethylene release, reveals an ABA/ethylene interaction acting at the level of ethylene biosynthesis (Liu et al., 2015). These evidence imply that ABA may function as a trigger for the initiation of fruit ripening by inducing ethylene-mediated pathway and other ethylene-independent processes (Zhang et al., 2009; Mou et al., 2016). Moreover, Jones et al. (2002) and Trainotti et al. (2007) suggests a crosstalk between ethylene and auxin during onset of ripening.

Although the importance of hormones in fruit development is well known (Zhang et al., 2016), considerable gaps in our knowledge about hormonal networks and crosstalk between different hormones still exist (Kumar et al., 2014). Moreover, the spatial-temporal distribution of hormones and expression of genes related to hormonal metabolism and signaling during the entire fruit development, from pre-anthesis to red ripe stages, is poorly understood (Zhang et al., 2016). The aim of this work was to redefines the hormonal control of fruit development by using transgenic plant harboring the gene reporter GUS fused to five different promoter responsive to auxin, CKs, GAs, ABA and ethylene. Through histochemical analysis, we demonstrates the spatial-temporal distribution of the five classics plant hormones from 12 days before anthesis, referred to here as -12 days post anthesis (-12 DPA), to the red ripe (RR) stage in tomato *cv.* Micro-Tom fruit. According to our results, GAs and ABA have its main functions in the cell expansion phase of fruit development, reaching its maximum production at mature green stage and cell expansion phase, respectively, and presenting apparently no activity during fruit set and early fruit growth. Surprisingly, an increase in the ethylene contents was observed, during the ovary growth, at pre-anthesis stages without diminishing during early post-fertilization stages. Furthermore, CKs show a major role during ovary growth at pre-anthesis stages in placenta and ovules. We additionally, proposes an interplay between ethylene and GAs during the transition from cell division to cell expansion phase of fruit growth.

2. BIBLIOGRAPHIC REVIEW

2.1. An overview of fruit anatomy and development

Fruits, often defined as structures derived from a mature ovary containing seeds (Seymour et al., 2013), are vital plant structures that support their development and dispersal, and play an important role in human diet (Liu and Franks, 2015). Fruit types can be divided using the combination of the following characteristics: dehiscence or indehiscence, dry or fleshy, and free (apocarpous) or fused (syncarpous) carpels (Seymour et al., 2013). According to these, tomato present an indehiscence fleshy syncarpous fruit.

The tomato has been putting forward as a model system to study fleshy fruit development (Azzi et al., 2015). Its fruit, a bi- or multilocular berry, develops from ovary after successful pollination, when the ovary wall transformed into pericarp, which encloses the locular cavity containing the seeds attached to a central paranchymatous axis or columella (Gillaspy et al., 1993). The peripheral part of the columella, or placental tissue, develops into a gel-like substance, consisting of large, thin-walled cells that are highly vacuolated and surrounds the seeds filling the locular cavity (Lemaire-Chamley et al., 2005; de Jong et al., 2009a). The pericarp consists in three layers: exocarp, mesocarp and endocarp. The exocarp, the outer layer, consists of a cuticle layer that thickens as the fruit ages, and the skin, which includes an epidermal cell layer and three to four layers of a collenchymatous tissue, that include mitotically active and enlarging cells, where starch accumulates and few plastids are retained. The intermediate layer, the mesocarp, is a parenchymatous tissue formed by big cells with large vacuoules. The endocarp, the innermost structure, limits the pericarp and is adjacent to the locular region (Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008; Pesareri et al., 2014).

Fertilization normally initiates the development of the tomato fruit, de-repressing cell division and fruit growth in a synchronized manner (Gillaspy et al., 1993). Fleshy fruit undergo a progression of specific stages. The first stage is divided in two phases, the first one is characterized by an intensive cell division phase. This high cell division activity occurs first in the outer and inner pericarp, columellar and placental tissue and peripheral integument layers of the developing seeds. Later mitoses activity is confined to the vascular tissues, outer epidermical and subepidermical pericarp cell layers and developing embryo (Gillaspy et al., 1993; Cheniclet et al., 2005; McAtee et al., 2013). During the active cell division phases a progressive increase in pericarp cell number occurs. The end of this phase, around 8 to 12 days after anthesis, is marked by a sharp fall in the rate of cell division (Pesareri et al., 2014). The second phase of the fruit growth stage is characterized by cell expansion and leads to a significant increase in weight. This expansion phase is accompanied by endoreduplication; that is, a multiplication of the genome without mitosis, leading to an increase in DNA content per cell (Bergervoet et al., 1996).

By the end of the cell expansion phase, the fruit reaches the mature green (MG) stage, when it attain its final size, which is both genetically and environmentally determined (Chevalier et al., 2007). In addition, even though the final fruit size is determined by cell division and expansion, which are tightly controlled by factors that drive the core cell cycle (Czerednik et al., 2012), cell expansion makes the greatest contribution to this trait. At this stage, the fruit achieves the competence to ripen (McAtee et al., 2013). Ripening involves profound changes in metabolism of the tissue surrounding the seeds to aid their dispersal, including drastic alterations in color, texture, and sugar content (Seymour et al., 2013). Two main phases can be distinguished in tomato ripening: breaker (BK) and red ripe (RR) stages. Accumulation of lycopene, typical carotenoid pigment of tomato fruit, occurs during the ripening process, in chloroplasts, which differentiate into chromoplasts, as the thylakoid membranes break down and it becomes a

carotenoid accumulating structure, while chlorophyll is degraded (Egea et al., 2011). This conversion of chloroplasts into chromoplasts causes the changes in color from green to yellow-orange at BK. Further, tomato ripening is also accompanied by the expression of genes involved in cell wall modification, such as polygalacturonase and expansin, volatile production, and accumulation of monomeric sugar, fructose and glucose, and organic acids in vacuoles (Karlova et al., 2014).

2.2. Micro-Tom as a model system to study fleshy fruit development

Tomato presents many characteristics of a biological model: it is an autogamous diploid species with a small genome (930 Mb) distributed in 12 chromosomes; it has a saturated genetic linkage map with traits of great economic and biological importance, and highly efficiency transformation protocols are already developed. In addition, several mutants affected in fruit size, shape, development and ripening have been isolated (Campos et al., 2010). The fact that tomato fruit is an edible climacteric fleshy fruit, unlike *Arabidopsis*, makes it a better candidate to study fruit development in climacteric species.

The miniature tomato *cv*. Micro-Tom (MT) harbours some distinctive mutation. The most well-known mutant alleles are: *dwarf* (*d*), a brassinosteroid related mutation responsible for the small plant size, and *self-prunning* (*sp*), responsible for its determinate growth habit. These two mutant alleles give Micro-Tom desirable characteristic like; compact plant size (~15 cm) and short life cycle (~10 weeks). Additionally, giving that one of the major problems on plant hormone studies in tomato is that many hormonal mutations are present in diverse cultivars, the information/comparison between them is poorly exchangeable. Our research group developed a collection of hormonal mutations in *cv*. MT (http://www.esalq.usp.br/tomato), which makes it an excellent model to analyze hormonal mutation in the same genetic background taking advantage of the characteristics mentioned above (Campos et al., 2010).

2.3. Hormones biosynthesis and signaling

As sessile organisms, plants require the abilities to sense environmental signals regarding current and probable future conditions, and to respond in adaptive manners that permit their growth, development, and reproduction. Therefore, the integration of various external signals with endogenous development programs is essential for their survival. Key environmental cues mainly include changes in water, soil and light variables, while major intrinsic regulators of many developmental processes include the plant hormones (Zdarska et al., 2015).

Due to their sessile lifestyle, plants need to adjust to numerous external stimuli and coordinate their growth and development accordingly. The plant hormones are a structurally unrelated collection of small molecules derived from various essential metabolic pathways. These compounds are important regulators of plant growth and mediate responses to both biotic and abiotic stresses. The classical phytohormones, identified during the first half of the twentieth century, are auxin, abscisic acid (ABA), cytokinins (CKs), gibberellins (GAs) and ethylene. More recently, several additional compounds have been recognized as hormones, including brassinosteroids, jasmonate, salicylic acid, nitric oxide and strigolactones (Santner et al., 2009; Santner and Estelle, 2009).

Auxin is crucial in regulating plant growth and development from embryogenesis through maturity, controlling nearly every aspect of a plant´s life. In plants, a significant amount of indole-3-acetic acid (IAA), the

predominant biologically active form of auxin, is synthesized via a simple two-step route where indole-3-pyruvic acid (IPyA) produced from L-tryptophan by tryptophan aminotransferases (*TAA1/TAR*) is converted to IAA by the YUC family of Flavin monooxygenases (Brumos et al., 2014).

The TIR1/AFB family of F-box proteins are auxin receptors. TIR1 is a component of the Skp 1/Cullin/F-box (SCF) ubiquitin ligases (E3). The auxin transcriptional response is controlled by two large families of transcription factors. One of these families are the Auxin Response Factor (ARF) proteins, which bind DNA directly and either activate or repress transcription depending on the ARF type, and the other are the Aux/IAA proteins, which exert their effects by binding to the ARF proteins through a conserved dimerization domain. At least in the case of the activating ARFs, the effect of Aux/IAA binding is to repress transcription. When auxin levels increases, it is perceived by the nuclear auxin receptor TIR1 and the closely related AFB F-box proteins, which recruit the Aux/IAA proteins to the $SCF^{TIR1/AFB}$ complex for ubiquitination and proteasome-mediated degradation, leading to derepression of ARFs that activate auxin-induced gene expression (Dharmasiri et al., 2005; Santner and Estelle, 2009; Kong et al., 2016). The genes derepressed by ARFs tend to harbor auxin response elements (AREs) in their promoter regions. Such AREs can be used to build up synthetic promoters (*e.g. DR5*, Ulmasov et al., 1997), which can be fused to reporter genes (*e.g.* GUS) and used as markers of endogenous auxin status.

CKs are known to be key regulator implicated in a broad range of developmental processes including germination, root and shoot meristem function and leaf senescence (To and Keiber, 2008). In general, the first step in CKs biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (IPT), producing isopentenyladenine (iP) nucleotides as CK precursor. The cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, convert iP nucleotides into *trans-zeatin* (tZ) nucleotides. To become biologically active, CK nucleotides produced by IPTs and CYP735As must be converted to free-base form. A CKs-activating enzyme (LOG), which directly converts CK nucleotides to the active nucleobases, was recently identified in rice and *Arabidopsis* (Matsuo et al., 2012).

CKs signaling is remarkably similar to the two-component signaling systems that are commonplace in bacterial species. In two-component signaling system, a sensor histidine (His) kinase perceives a stimulus and autophosphorylates on a conserved His residue in the kinase domain. The signal is transduced via phosphoryl transfer to a conserved aspartic acid (Asp) residue on the receiver domain of a response regulator, which activates downstream response. CKs is perceived by a membrane-associated hybrid kinase that transfers a phosphate to *Arabidopsis His Phosphotransfer* (AHP) proteins. The AHPs are translocated into the nucleus where they phosphosylate *Arabidopsis Response Regulator* (ARR) proteins. The ARRs can be either negative (type A ARRs) or positive (type B ARRs) effectors of cytokinin signaling (Santner et al., 2009). The type B ARRs are the ones that are phosphorylated (and activated) by AHP proteins. Upon activation, type B ARRs induce type A ARRS transcription. Therefore, transcript accumulation of type A ARRs (*e.g. ARR5*) or the activity of heterologous genes (*e.g. GUS*) fused to their promoters are markers of the endogenous CK status (D'Agostino et al., 2000).

Bioactive gibberellins (GAs) are diterpene plant hormones that are biosynthesized through complex pathways and control diverse aspects of growth and development. GAs are biosynthesized from geranylgeranyldiphosphate (GGDP), a common C_{20} precursor for diterpenoids. The *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), are involved in the conversion of GGDP to the tetracyclic hydrocarbon intermediate *ent*-kaurene, the both later enzymes are localized in plastids. Then, *ent*-kaurene is converted to GA_{12} by two P450s, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). These monooxygenases are localized in the endoplasmic reticulum. Finally, dioxygenases acting in the cytosol convert GA_{12} into GA_4, a bioactive form, through oxidation on C-20 and C-3 by GA 20-oxidase and GA 3-oxidase. GA_{12} is also a substrate for GA 13-oxidase

for the production of GA_{53}, which is a precursor for GA_1, another GA bioactive form, in the 13-hydroxylated pathway (Yamaguchi et al., 2008). Besides GAs biosynthesis, some dioxygenases, such as GA 2-oxidases, can inactivate GAs. Such enzymes are transcriptionally induced by the GA transduction pathways, as a negative feedback controlling GA endogenous levels. Since the promoter region of GA 2-oxidase respond to GA, it can be fused to reporter genes (*e.g GUS*) to monitor the endogenous GA status (Dayan et al., 2012). GAs response are negative regulated by DELLA proteins, which are considered repressor of growth. The DELLAs are named after the conserved N-terminal DELLA domain and also contain a C-terminal GRAS domain. GAs promote degradation of DELLA proteins, thus inducing the GAs transcriptional response. The Gibberellin Insensitive Dwarf1 (GID1), a protein localized in the nucleus, acts as GAs receptor and binds biologically active GAs. GID1s interact with DELLA proteins in a GA-dependent manner, and promote its degradation via enhancing the DELLA/$SCF^{SLY/GID2}$ interaction (Santner and Estelle, 2009).

The phytohormone ABA serves as an endogenous messenger in biotic and abiotic stress response in plants. ABA biosynthesis is divided in two pathways. The first, which includes the synthesis of the C_{40} carotenoid precursor (β-carotene), and a second specific ABA biosynthetic pathway. The β-carotene hydroxylase enzyme catalyzes the zeaxanthin production, which is subsequently converted to violaxanthin by zeaxanthin epoxidase (ZEP) and further to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED) in plastid. In cytosol, xanthoxin is converted to ABA via the intermediate ABA aldehyde by xanthoxin oxidase/ short-chain dehydrogenase/ reductase and aldehyde oxidase (AO) (Weng et al., 2015).

This hormone binds to the high affinity ABA binding proteins RCARs/PYR1/PYLs, a family with the START domain. These ABA binding protein form a complex with Phosphatases type 2C (PP2C), this heteromeric complex form the ABA receptor. The phosphatase activity of the PP2C inhibits the action of the protein kinases OST1 and related SnRKs. In the presence of ABA, the phosphatase activity of the receptor is blocked. As, a consequence, the protein kinases are released from inhibition and directly phosphorylate and regulate key target of ABA signaling pathway. Key transcriptional regulators of ABA-dependent gene expression are ABFs/AREBs (ABA-responsive Element Binding Factor/Protein). In nucleus, OST1 and the related SnRK2.2/SnRK2D and SnRK2.3/SnRK2I directly target ABF/AREBs, and phosphosrylated ABFs binds as dimer to the ABA-responsive cis-element (ABRE), and provide the ABA-responsive transcription (Raghavendra et al., 2016). Genes whose transcription is induced by ABA, such as *RD29B*, can have their promoter regions fused to *GUS* in order to monitor the endogenous ABA status in tissues and organs (Christmann et al., 2005).

Ethylene, a simplest olefin, exist in the gaseous state under ambient conditions. It regulates many aspects of the plant cycle, including seed germination, root initiation, root hair development, flower development, fruit ripening and response to abiotic and biotic stresses (Lin et al., 2009). In higher plants, ethylene biosynthesis originates from *S*-adenosyl-methionine (SAM) and comprises two steps catalyzed by 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) and 1-amino-clyclopropane-1-carboxylic oxidase (ACO), the later converting ACC into ethylene (Liu et al., 2015).

Ethylene is perceived by the receptor protein ETR1, located in the endoplasmic reticulum. The protein Response to Antagonist1 (RAN1) play important roles at the receptor levels, delivering the copper ion, essential for ethylene binding activity. Directly downstream of the ethylene receptors acts the Constitutive Triple-Response1 (CTR1), a mitogene-activated protein kinase kinase kinase (MAPKKK). In the absence of ethylene, the receptors activate CTR1, which suppresses the ethylene response via inactivation of Ethylene Insensitive2 (EIN2). Ethylene regulates ripening-related genes through a transcriptional cascade that comprises primary EIN3/Ethylene

Insensitive3-Like1 (EIL) and secondary Ethylene Response Factors (ERFs). There are 77 ERFs genes in the tomato genome, but recent work pointed out that *ERF.E1*, *ERF.E2* and *ERF.E4*, are the ones most involved in the control of fruit ripening (Liu et al., 2016). Two Ethylene Insensitive3-binding F-box (EBF1 and EBF2) have been shown to regulate ethylene signaling and fruit ripening through mediating the degradation of EIN3/EIL proteins. Finally, when ethylene binds the receptor, it induces their inactivation, and by consequence, switches off CTR1 phosphorylation activity. Active EIN2 stabilize EIL transcription factors, which can activate the expression of target genes, including those encoding the ERF transcription factor via binding to primary ethylene response elements (Santner and Estelle, 2009, Liu et al., 2015). Genes whose transcription is induced by ethylene, such as *EIN3*, can have their promoter regions fused to *GUS* in order to monitor the endogenous ethylene status in tissues and organs (Steponova et al., 2007).

2.4. Hormonal control of fruit set and fruit growth

Fruit development and ripening are well-coordinated, temporally and spatially tightly regulated complex processes involving the interplay of a number of biotic and abiotic factors. Plant hormones have long been known to regulate the development and the ripening of fruits (Srivastava et al., 2005). As can be seen in Figure 1., the five classical hormones modulate growth and development at various stages of the developing fruit (Gillaspy et al., 1993).

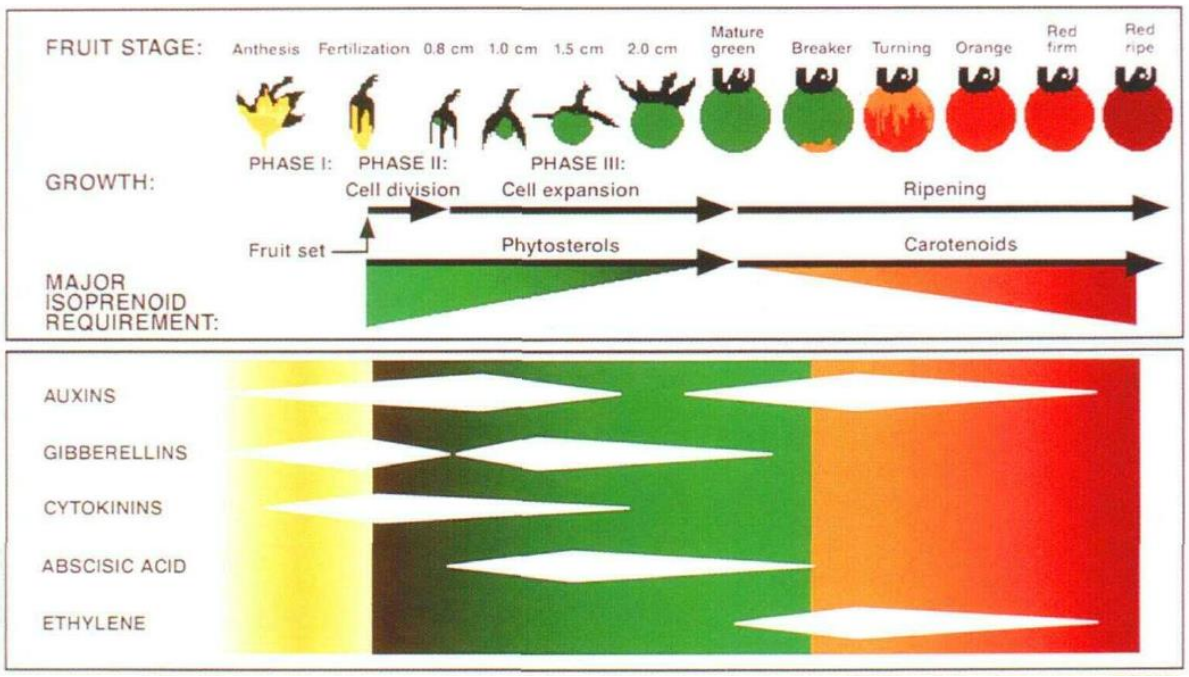

Figure 1. Hormonal changes during fruit development (Gillaspy et al., 1993).

Fruit set, the changeover from the static condition of the flower ovary to the rapidly growing condition of the young fruit (Gillaspy et al., 1993), is the first step in fruit development; it is established during and soon after fertilization. Seed bearing plants have a unique double fertilization event with two pollen nuclei fertilizing the embryo and the endosperm (Raghavan, 2003). The fertilization event leads to the development of the seed that de-represses cell division and fruit growth in a synchronized manner. Fruit set has traditionally been attributed to the action of

three hormones, auxin, GAs and CKs. Hence, the application of these hormones alone can trigger fruit development to a certain extent and, in many plant species, application in combination will induce normal fruit growth even in the absence of fertilization (Gillaspy et al., 1993; McAtee et al., 2013).

In particular, auxin plays a major role in the regulation of fruit set and growth, since studies in tomato and *Arabidopsis* have revealed that the auxin-signaling pathway is involved in controlling the early events of fruit initiation. Two components of the auxin-signaling pathway, ARF8 from Arabidopsis and IAA9 from tomato, have been implicated, respectively, in stimulating and in repressing fruit initiation in the absence of the fertilization cue (Wang et al., 2005; Goetz et al., 2006). *Aux/IAA* genes encodes short-lived nuclear proteins, which can dimerize with ARFs while these are bound to the auxin response elements in the promoters of early auxin response genes (Ulmasov et al., 1999), The ARF-Aux/IAA heterodimers restrain the transcription of the early auxin response genes. After pollination and fertilization, auxin causes the degradation of the Aux/IAA by 26S proteasome and the ARF is released, leading to the activation of the auxin reponse genes (Dharmasiri et al., 2005). In turn, some of these auxin response genes encode for Aux/IAA, such *IAA2* and *IAA14*, whose transcription is induced by auxin treatment of the unpollinated ovary. The mRNAs levels of IAA2 and IAA14 were also found to increase in the pollinated ovary, specifically in the placenta and ovular tissues (Vriezen et al., 2008). The transcriptional activation of some Aux/IAA genes, either after pollination or auxin application, suggests that a minimum level of Aux/IAA is required in order to create a negative feedback loop in the auxin signal transduction pathway, which enables the plant to fine-tune the strength of the auxin response (de Jong et al., 2009a).

On the other hand, gain-of-function mutations on *SlARF8* uncouple fruit initiation from fertilization, resulting in the formation of seedless and parthenocarpic fruit. Goetz et al. (2007) proposed a model where ARF8 and Aux/IAA9 orthologs interact to form a regulatory complex. This complex can bind to the promoters of a range of primary auxin responsive genes that play an essential role in repressing fruit initiation in the absence of fertilization. The presence of an aberrant ARF8 could partially destabilize this complex, leading to parthenocarpic fruits. Additionally, *SlARF7* was found to be expressed at high level in unpollinated mature ovaries and transgenic plants with decreased *SlARF7* mRNA levels formed seedless fruits. Thus, in unpollinated ovaries, *Sl*ARF7 moderates the auxin response by activating the transcription of auxin response-attenuating genes, e.g. members of the *Aux/IAA* gene family, which, in turn, block the auxin response and thus fruit set and development. Upon pollination, the auxin content within the ovary increases, resulting in an auxin signaling cascade that initiates fruit set and development. At the same time, auxin inhibits expression of *SlARF7* (de Jong et al., 2009b).

Several evidences suggest auxin being a central regulator of tomato fruit development, and show that the embryos are the major site of this hormone biosynthesis. The expression of three genes; *TAR2*, and the *YUCCA* genes *to*FZY2 and *to*FZY6, encoding TAR and YUCCA enzymes in the major auxin biosynthetic pathway (Brumos et al., 2014), increased in 4 DPA fruit compared to ovaries at anthesis. The transcript of the mentionated genes accumulated primarily in seed tissues and the funiculus, with *TAR2* and *to*FZY6 showing peak expression in the embryo (Pattison et al., 2015). It has been observed that auxin levels increase in the seed during its development, concomitant with fruit growth stages where cell division is followed by a cell-expansion phase (Kumar et al., 2014).

The increased levels of auxin in the seeds (Gustafson, 1939) suggest that the seeds are the predominant source of auxin, which moves to other tissues to promote cell division and expansion (Ozga et al., 2002). However, after fruit set, precise spatial and temporal synthesis, transport and action of auxin are required for fruit development (Gillaspy et al., 1993). A major mechanism that regulates auxin distribution is polar auxin transport mediated by PIN and AUX/LAX proteins, which control cellular auxin efflux and influx respectively (Vanneste and Friml, 2009).

Pattison and Catalá (2012) detected the expression of eight *SlPIN* in fruit, and divided them in three classes based on preferential expression in seeds/locular tissue (*SlPIN5*), placenta (*SlPIN1*, *SlPIN4*, *SlPIN7*, *SlPIN8*) or pericarp (*SlPIN6*), relative to the other fruit tissues. *SlPIN*s genes expressed in placenta showed higher expression during early stages of fruit development and lower expression during ripening. *SlPIN5* expression peaked in seeds/locular tissue at 14DPA and declined gradually to almost undetectable levels during ripening.

The auxin transport, monitored by *DR5*::GUS, suggests that auxin is transported from the seeds, via funiculus and vasculature of the placenta to the fruit pedicel and mother plant, generating an internal-to-external IAA gradient, during cell expansion stage (Pattison and Catalá, 2012). The high expression of the auxin plasma membrane targeted efflux carriers *SlPIN1*, *SlPIN3*, *SlPIN4*, *SlPIN7* and *SlPIN9*, in either funiculus or placenta at 4 DPA, coincides with the auxin internal-to-external gradient in tomato fruit (Pattison et al., 2015). The basipetal polar auxin transport from the fruit to the mother plant via the pedicel was previously shown (Serrani et al., 2010). Auxin activity in the funiculus and pedicel, which are site of abscission, suggests that auxin may prevent premature seed, flower bud or fruit abscission (Pattison and Catalá, 2012).

Auxin promotes fruit set and growth, at least partly, by controlling the GAs levels. The increase of GAs content in the ovary upon pollination is associated with the upregulation of *SlGA20ox1*, *SlGA20ox2* and *SlGA20ox3* genes, which encode the GA 20-oxidase biosynthetic enzymes, but not of the genes encoding *SlGA3ox*, and is it not associated with the downregulation of genes encoding SlGA2ox, which are GAs inactivating enzymes (Serrani et al., 2007). The application of auxin and the overexpression of genes involved in IAA biosynthesis (Pandolfini et al., 2002) induce fruit set and growth in tomato, generally more efficiently than the application of GAs. Furthermore, application of GA biosynthesis inhibitors to pollinated or 2,4-D treated ovaries, totally inhibited tomato fruit set. These evidences indicates that auxin alters GA metabolism and increases active GA_1 levels in unpollinated ovaries through the upregulation of genes encoding enzymes of GAs biosynthesis (*CPS*, *GA20ox* and *GA3ox*) and down-regulation of genes encoding GA2ox enzyme (Serrani et al., 2008).

According to Matsou et al. (2012) the first peak of CKs is reported at anthesis and it is not linked to pollination. Transcript level of the CK biosynthetic genes *SlIPT3*, *SlIPT4*, *SlLOG6*, and *SlLOG8* are high at anthesis, and the levels of tZR (trans-zeatin riboside), iPR (isopentenyladenosine), DZR (dihydrozeatin riboside), and iP (isopentenyladenine) are increased in ovaries. It was suggested that, prior to fertilization, factors produced by the sporophytic tissue surrounding the developing ovary are required to trigger and maintain cell division in the fruit primordia, until the ovary reaches mature size. Therefore, high CK levels might be necessary for the growth and/or maintenance of unpollinated ovaries until successful pollination (Gillaspy et al., 1993; Matsuo et al., 2012). A second peak in CK concentrations in developing tomato fruit occurs at 5 DPA. A peak in the tZ (trans-zeatin) content corresponded with the cell division phase of fruit growth, suggests that tZ is involved in cell division in ovaries after pollination. Such increase in CK levels post-pollination is due to the high transcript level of *SlIPT1*, *SlIPT2*, *SlCYP735A1*, *SlCYP735A2*, and *SlLOG2* genes (Matsuo et al., 2012).

While auxin, GA and CK levels are increasing at fruit set, ABA and ethylene levels decreases. Thus, it is suggested that ABA and ethylene play an antagonistic role to that of auxin and GA in fruit set, in order to keep the ovary in a temporally protected and dormant state; either to protect the ovary tissue or to prevent fruit development before pollination and fertilization occur (Vriezen et al., 2008; Kumar et al., 2014). The transcripts levels of *SlACS6*, *SlACO2* and *SlACO4* increased between -7 and -2 DPA, remained high at anthesis, and started to decrease after fertilization, suggesting that the progression of ovary expansion coincides with the down-regulation of ethylene biosynthesis and signaling genes (Shinozaki et al., 2015). ABA levels are relatively high in mature ovaries and

decrease directly after pollination. ABA content is regulated by the balance between its biosynthesis and catabolism. It is generally accepted that the cleavage reaction catalyzed by NCED is a major rate-limiting step and point of regulation in ABA biosynthesis. *SlNCED1* mRNA levels decreased, after pollination, in ovules, placenta tissue, and the pericarp. On the other hand, ABA is mainly inactivated by 8´-hydroxilation, which is catalyzed by cytochrome P450 mono-oxygenases (*SlCYP707A1*). mRNA concentration of *SlCYP707A1* strongly increased after pollination in whole ovary (Nitsch et al., 2009).

While auxin mostly controls cell division during fruit set, it is thought to play an important role during the growth phase by influencing cell enlargement together with GA. As was mentioned earlier, after the cell division phase of fruit growth starts the cell expansion phase, when GA presents a major role. Some genes belonging to the expansins, endo-xyloglucan transferase and pectate lyases families have been shown to be regulated by either auxin, GA, or both in tomato (de Jong et al., 2011). ABA has also been associated with the expansion phase in tomato (Gillaspy et al., 1993) and ABA-deficient mutants have a small fruit size (Nitsch et al., 2012).

2.5. Hormonal control of fruit ripening

Fruit ripening involves well-orchestrated coordination of several regulatory steps, which brings about changes to the fruit metabolic and physiological traits. With its progression, the color of fruits decreases with the accumulation of sugars, the flavor and aroma compounds accumulate, and cell wall dynamics change, leading the softening of the pericarp. This process involves initiation of multiple genetic and biochemical pathways, where ethylene and ABA are suggested to be the major contributors, whereas other growth regulators are required for fine tuning the process (Klee and Giovannoni, 2011).

Climacteric fruits, such as tomato, are characterized by a ripening-related increase in respiration and elevated ethylene synthesis to rapidly coordinate and synchronize ripening. Tomato has proven a highly useful model system for fruit development and is the system in which the role of ethylene during fruit ripening has been most thoroughly studied. In tomato plants, nine genes encoding ACS have been described to date, and three are differentially expressed during fruit ripening: *SlACS2*, *SlACS1A* and *SlACS4*, suggesting its potential contribution to the climacteric ethylene production (Barry et al., 2000). Noteworthy, *SlACO1* and *SlACO2* display the most striking ripening-regulated pattern of expression peaking at the breaker stage, whereas *SlACO4* undergoes a steady but slight increase throughout ripening (Liu et al., 2015). Two systems of ethylene biosynthesis have been proposed in climacteric fruits. System 1 is responsible for producing basal ethylene levels that are detected in all tissues, including those of nonclimacteric fruit. System 1 is known to be ethylene autoinhibitory and is reported to function during fruit growth, whereas system 2 operates during the climacteric ripening and is autocatalytic. System 1 relies on *SlACS1A* and *SlACS6*, both being negatively regulated by ethylene, whereas the up-regulation of *SlACS2* and *SlACS4* through a positive feedback by ethylene is responsible for the activation of system 2 (Barry et al., 2000; Cara and Giovanonni, 2008; Liu et al., 2015).

In tomato, ripening is regulated by a number of transcription factors in conjunction with the plant hormone ethylene. Ripening-associated transcription factors (TF) have been found to regulate the biosynthesis of ethylene. Example of such TF are, the MADS-domain protein RIPENING INHIBITOR (RIN), the SBP transcription factor COLORLESS NON-RIPENING (CNR) and the NAC domain family transcription factor NON-RIPENING (*NOR*). The *rin* and *Cnr* mutations effectively block the ripening process and result in fruits that fail both to produce elevated ethylene and to respond to exogenous application of the gas. These data suggested that

both genes lie upstream of ethylene production and have functions that are ethylene dependent and independent (Karlova et al., 2014).

Examples of well characterized ethylene regulated fruit genes include the *ACS* and *ACO*, which are components of the ethylene biosynthesis; the fruit specific polygalacturonase (*PG*), which is involved in depolymerization of cell wall pectin during ripening; the pectin methylesterase (*PME*), which provides accessibility to pectin by PG; and the fruit-specific phytoene synthase (*PSY1*), which catalyzes the rate limiting and highly regulated reaction from GGDP to phytoene in the carotenoid biosynthesis pathway responsible for the pigmentation of fruit (Cara and Giovanonni, 2008). To date, the ripening mechanism of climacteric fruits, especially with respect to the effects of ethylene, is well studied.

ABA is considered another factor controlling ripening for the following reasons: i) a sharp increase in ABA accumulation during the onset of fruit ripening is reported, ii) the ABA accumulation precedes ethylene release in climacteric fruits, iii) the application of exogenous ABA enhances the production of several metabolites involved in fruit ripening, thereby promoting fruit ripening, and in ABA-deficient tomato mutants, the fruit do not show the normal growth pattern observed in the wild type (Leng et al., 2014).

Sun et al. (2012) reported that ABA directly participate in the cell wall catabolism to promote the softening of tomato fruit, which was via the regulation of a series of relevant enzymes and genes expression. Furthermore, the application of exogenous ABA could facilitate tomato fruit ripening by enhancing carotenoids biosynthesis as well as chlorophyll degradation. Ji et al. (2014) reported that *SlNCED1* and *SlCYP707A2* are key genes in the regulation of ABA synthesis and catabolism, and are involved in fruit ripening as positive and negative regulators, respectively. In transgenic plants with a significant reduction in NCED activity was reported a downregulation in the transcription of genes encoding major cell wall-degrading enzymes, specifically *PG*, *PME* and expansin (*Exp*), which led to a significant extension of shelf life (Ji et al., 2014).

Numerous studies have observed that ABA accumulates preceding ethylene release in climacteric fruits, implying ABA may function as an upstream regulator of ethylene biosynthesis and response (Mou et al., 2016). ABA may act as a trigger for the initiation of fruit ripening by inducing ethylene-mediated pathway and other ethylene-independent processes (Zhang et al., 2009). A noticeable reduction of ethylene was detected in the fruits treated with the ABA-inhibitor NDGA. Additionally, the ABA induced tomato ripening was not found in the fruits with subsequent 1-MCP treatment, indicating ABA´s stimulation of ripening progress was at least partially dependent on ethylene (Mou et al., 2016).

In climacteric fruits, several lines of evidence suggest that both ethylene and IAA are involved in crosstalk with each other during ripening (Trainotti et al., 2007; Kumar et al., 2014).

3. GENERAL OBJECTIVE

The aim of this work was to characterize the hormone status of five classical hormones during tomato (*Solanum lycopersicum* L. *cv*. Micro-Tom) fruit development.

4. SPECIFICS OBJECTIVES

- To define, through histochemical analyses by using transgenic plants harboring the gene reporter GUS fused to different promoter responsive to the five classical hormones, the spatial-temporal distribution of these hormones during the tomato fruit development.

- To perform histological analyses of ovaries after GUS staining.
- To determine the number of cell layers in the pericarp from -12 DPA to 10 DPA.
- To study the role of ethylene during early fruit development using the applications of AVG (ethylene inhibitor) on WT and the ethylene-insensitive *Never Ripe (Nr)* ovaries at -3 DPA and measuring their diameter 7 days post application.
- To study the crosstalk between ethylene and GA using *Nr* (ethylene insensitive) and *35S::GA20Ox (GA overproducer)* plants expressing *GA2Ox*::GUS.
- To define, through histochemical analyses, the GAs concentration changes during fruit development in *Nr* and *35S::GA20Ox*.
- To perform time-course experiments measuring the ovary diameter of certain hormonal mutant, from anthesis to red ripe stage.
- To quantify IAA and ABA in tomato fruits from 20 DPA to red ripe stage.

5. MATERIALS AND METHODS

5.1. Plant materials and growth conditions

The experiments were conducted at the Laboratory of Hormonal Control of Plant Development, localized in the Biological Science Department, ESALQ/USP. Plants were kept, in a greenhouse, under automatic irrigation system (4 times per day), an average mean temperature of 28°C, photoperiod of 11h/13h (winter/summer), and 250-350 μmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) by natural radiation reduction with a reflecting mesh (Aluminet-Polysack Industrias Ltda, Leme, SP, Brazil).

Plants were sowed in pots of 350 ml (40 seeds per pot) and, after the emergence of the first true leaf, were transplanted to 250 ml pots, leading one plant per pot. The plastic pots containing a 1:1 mixture of commercial substrate Basaplant® and expanded vermiculite, supplemented with 1 g of NPK 10:10:10 L^{-1} substrate and 4 g of dolomite limestone ($MgCO_3$+$CaCO_3$) L^{-1} substrate. Two weeks after transplant, a foliar application of Peters® fertilizer was performed. At the flowering stage and the start of fruit set, plants were supplemented with NPK (0.2 g per 150 ml pot).

The available transgenic plants harboring the GUS reporter genes are described in Table 1. MT-*DR5*::GUS was donated by Dr. Jose Luis Garcia-Martines (Universidad Politécnica de Valencia, Spain). MT-*ARR5*::GUS was derived from genetic transformation (Pino et al., 2010) using a construct donated by Dr. JJ.Kieber (University of North Carolina, USA). MT-*GA2Ox*::GUS was derived from genetic transformation using the vector donated by Dr. Jonathan Dayan and Dr, Tai-Ping Sun (Duke University, USA). MT-*EBS*::GUS was derived from genetic transformation using a vector generated at Joe Ecker Lab (Salk Institute, USA) and donated by Dr. Anna N. Stepanova and Dr. Jose M. Alonso (North Carolina State University, USA). MT-*RD29B*::GUS was produced in this work through introgression, using the methodology described in Carvalho et al., (2011), from a transgenic plants produced in cv. Moneymaker by Lisette Nitsch at Dr. Ivo Rieu's Lab (Radboud University, The Netherlands)from the vector donated by Dr. Alexander Christmann (Technische Universitat Munchen, Germany). The mutants *procera* (*pro*), *Never ripe* (*Nr*) and *entire* (*e*) in the MT back ground were described previously (Carvalho et al., 2011). The *35S::GA20Ox* transgene in the MT background was donated by Dr. Esther Carrera (Universidad Politécnica de Valencia, Spain).

Closed flower buds were collected from 12 days before anthesis, referred here as -12 days post anthesis (-12 DPA), to anthesis (0 DPA). The measurement of tomato flower buds length at preanthesis stages were used to define how many days before anthesis they were at the moment of sampling (Faria, 2014). We also marked the flowers at anthesis, to know, how many days after anthesis the fruits were collected.

Table 1. Tomato (*Solanum lycopersicum* cv. MicroTom) transgenic plants harboring the gene reporter GUS fused to promoting region induced by the five classical hormones.

Genotype	Description	Reference
MT-*pDR5*::GUS	Gene reporter GUS fused to a promoter induced by auxin.	Ulmasov et al., 1997.
MT-*pARR5*::GUS	Gene reporter GUS fused to a promoter induced by cytokinins.	D'Agostino et al., 2000.
MT-*pGA2Ox*::GUS	Gene reporter GUS fused to a promoter induced by gibberellins.	Dayan et al., 2012.
MT-*pRD29B*::GUS	Gene reporter GUS fused to a promoter induced by ABA.	Christmann et al., 2005.
MT-*pEBS*::GUS	Gene reporter GUS fused to a promoter induced by ethylene.	Stepanova et al., 2007.

5.2. Histochemical analysis

Histochemical *GUS* (β-glucoronidase) was performed in the transgenic plants mentioned in Table 1. Flower buds and fruits, from -12 DPA to red ripe stage, were cut longitudinally and incubated, in the dark, at 37°C in GUS staining solution [80mM sodium phosphate buffer, pH 7.0; 8mM EDTA; 0.4 mM potassium ferrocyanide; 0.05% Triton X-100; 0.8 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-Gluc); 20% methanol]. Fruits and flower buds were vacuum-infiltrated for 15 minutes previous the incubation at 37°C. Following GUS staining, the reaction was stopped with 70% ethanol. Images capture of the flower buds after GUS staining was performed using the Leica Application Suite-LAS program, fruit from 10 DPA ahead were photographed with a camera.

5.3. Histological analysis

Samples of flower buds and ovaries, after GUS staining, stored in 70% alcohol were rehydrate and fixed in Karnovsky solution (Karnovsky, 1965). Later, dehydrated in an increasing ethanol series (10-100%), and subsequently infiltrated into synthetic resin using a HistoResin embedding kit (Leica, www.leica-microsystems.com), according to the manufacturer's instructions. Tissue sections were obtained using a rotatory microtome (Leica). Ovaries samples used for counting of pericarp cell layers were stained with toluidine blue 0.05% (Sakai, 1973).

5.4. AVG application

For experiments in which AVG was applied, WT, *Never ripe* mutant (*Nr*) and *GA2Ox*::GUS plants were used. At -2 DPA, emasculated flowers were hand-pollinated and 100 μM of AVG were applied. Ovary diameter was measured 7 days after pollination. For mock control (0 μM AVG) milli-q water without AVG was applied in ovaries.

Noteworthy, 20 µl of 100 µM AVG solution were applied to 10 ovaries from 10 different MT, *Nr* and *GA2Ox*::GUS plants.

5.5. Generation of *Nr* x *EBS*::GUS, *Nr* x *GA2Ox*::GUS, *35S*::*GA20Ox* x *EBS*::GUS and *35S*::*GA20Ox* x *GA20Ox*::GUS

Pollen from transgenic plants *EBS*::GUS and *GA20Ox*::GUS were used to pollinate ovaries of both *Nr* and *35S*::*GA20Ox* plants. Additionally, WT plants were also used as pollen receptor, in crosses with *EBS*::GUS and *GA20Ox*::GUS, generating a hemizygous plants for the GUS gene reporter. The F1 plants of all crosses have the same gene dosage, since all of them are hemizygous for the GUS genes. The fruits from the F1 plants were used in the analysis.

5.5.1. Ovary diameter

To measure the ovary diameter from anthesis (0 DPA) to red ripe stage, 10 plants of WT and the mutants *entire* (*e*), *procera* (*pro*), *Nr* were used. Only 5 fruits were left per plant and a digital pachymeter was used to determinate the ovary diameter. **Table 2.** describes the mutants and others transgenic plants used in this work.

Table 2. Description of hormonal mutants and transgenic plants.

Genotype	Description	Reference
entire (*e*)	*entire* codes for AUX/IAA9, a transcriptional repressor of auxin signaling. The mutated allele increases auxin response.	Wilkinson et al. (1995).
Never Ripe (*Nr*)	*Never ripe* codes for ethylene receptor (*SlETR3*). Plants harboring the mutated allele have low ethylene sensitivity.	Wang et al. (2005).
procera (*pro*)	*pro* shows a constitutive GA response due to a point mutiation in the VHV(I/V)D motif, witch is thought to be important for DELLA action..	Bassel et al. (2008).
MT-*35S::GA20Ox*	Citrus GA biosynthetic gene *CcGA20Ox1*, is overexpressed. The plants have altered GA profile containing higher concentrations of GA_4.	Garcia-Hurtado et al. (2012).

5.6. Indole acetic acid (IAA) and ABA quantification

Extraction of free IAA and ABA were performed according to Ludwig-Muller et al. (2008), using frozen samples of tomato fruits ground in a mortar, in liquid N_2. An isopropanol:acetic acid (95:5) solution was added to the tissue powder (4:1 v/w) together with 0.5μg of each labelled ABA standard ([$^2H_6$]ABA) and IAA standard ([$^{13}C_6$]IAA). The mixture was kept under shaking at 4°C for 2 h and centrifuged at 13 000 *g*. The supernatant was collected and concentrated to 50μL under an N_2 gas flow. Sample were then fractionated with 0.5 Ml of ethyl acetate and the organic phase was transferred to another tube and dried under N_2 gas flow. The material was methylated with diazomethane, dried under N_2 flow and re-suspended in ethyl acetate. The analysis was performed on a Hewlett-Packard (Wilmington, DE, USA) gas chromatograp model 6890 coupled to a mass spectrometer model 5973 in selective ion monitoring mode (GC-MS-SIM).

The chromatograph was equipped with an HP-1701 column (30 m, ID 0.25, 0,50 μm thick internal film) using helium as the carrier gas at a flow rate of 4 mL min^{-1}: 3 min at 150°C, followed a ramp by 5°C min^{-1} to 210°C and 15°C min^{-1} to 260 °C. Ions with a mass ratio/charge (m/z) of 130 and 189 (corresponding to endogenous IAA) and 136 and 195 (corresponding to [$^{13}C_6$]IAA), and also ions with (m/z) 134, 162 and 190 (corresponding to endogenous ABA) and 138, 166 and 194 (corresponding to [$^2H_6$]ABA) were monitored. Concentrations were calculated based on extracted chromatograms at m/z 130 and 136 for IAA and 162 and 266 for ABA. The extractions were performed in triplicate.

6. RESULTS

6.1. Hormonal regulation of tomato flower development at pre-anthesis stages.

The GUS activity was evaluated, from flower buds (Figure 2) to red ripe fruits, in five different transgenic plants of *Solanum lycopersicum cv*. Micro-Tom harboring the gene reporter GUS fused to a responsive promoter to auxin (*p*DR5), CKs (*p*ARR5), GAs (*p*GA2Ox), ABA (*p*RD29B) and ethylene (*p*EBS). The time course evaluation started at -12 DPA. In this stage, the carpels that form the ovary are already fused, locular cavities are formed, style is emerged and ovule are clearly visible on the placental tissue (Brukhin et al., 2003; Xiao et al., 2009).

At -12 DPA, *DR5*::GUS staining was observed in the megasporocyte (Figure S1). Interestingly, at the subsequent pre-anthesis stages when megasporocyte undergoes meiosis to produce four haploid nucleus and later mitotic divisions to give rise to mature embryo sac (Brukhin et al., 2003; Reiser and Fischer, 1993; Xiao et al., 2009), no *DR5*::GUS staining was observed. Further, no expression of *DR5*::GUS was perceived in ovary tissue during tomato flower development (-12 to 0 DPA), while, a high *DR5*::GUS activity was noticed in the peduncle at the same period. Remarkably, *DR5*::GUS activity was limited to the pith, while no GUS staining was observed in vascular bundles (Figure 3A; Figure S1). At -6 and -3 DPA, a strong GUS staining was observed in the stamen due to a *DR5*::GUS expression on pollen grain and anther, specifically in epidermis (Figure S1). Before -6 DPA, no *DR5*::GUS staining was observed in stamen (Figure 3A; Figure S1). In addition, from -6 to -3 DPA, *DR5*::GUS activity was observed in the junction of ovary and style (Figure 3A; Figure S2).

High cytokinin signaling was showed in placental tissue at -12 DPA (Figure S3). Notably, at later pre-anthesis stages, the expression of *ARR5*::GUS in placental tissue decreased and started to increase in ovules, specifically in outer and inner integument (Figure 3B; Figure S3). Furthermore, *ARR5*::GUS activity was observed in the carpel wall at – 9 DPA. However, this GUS activity, decreased at subsequent stages until the anthesis (Figure S4). No *ARR5*::GUS staining was showed in pollen grain from -12 DPA to -3 DPA, but a faint *ARR5*::GUS staining was observed in anthers at -12 DPA (Figure S3). A high increase of stigma width was presented from -6 to 0 DPA (anthesis) (Figure S4), coinciding with the appearance of *ARR5*::GUS activity in stigma at that period (Figure 3B; Figure S5).

No *GA2Ox*::GUS and *RD29B*::GUS activity was showed in the ovary at pre-anthesis stages, whereas, stamen showed high *GA2Ox*::GUS and *RD29B*::GUS staining at -6 and 0 DPA (Figure 3C and 3D). Interestingly, stamen of *GA2Ox*::GUS plant showed GUS activity in both anther and pollen grain, while, *RD29B*::GUS stamen showed GUS activity just in pollen grain (Figure S6).

Ethylene contents increase in ovary during tomato flower development. The *EBS*::GUS expression which was not observed at -12 DPA, increased from -9 to -3 DPA, specifically in columella (Figure 2E; Figure S7). Interestingly, no *EBS*::GUS expression was noticed in ovules during the whole flower development. At the end of pre-anthesis stages, *EBS*::GUS staining was observed in stamen, coinciding with the anther dehiscence (Figure 2E). Intriguingly, *EBS*::GUS activity was showed in peduncle, specifically in vascular bundles, from -9 DPA to 0 DPA, while no *EBS*::GUS activity was noticed in pith (Figure S7).

As shown in Figure 2, a prominent and constant ovary growth occurs during pre-anthesis, since the ovary diameter almost duplicates within this period (from -12 DPA to 0 DPA). The increase was mainly dependent on cell division, as evidenced by the number of the cell layers in the carpel wall, which increased from 7 to 11 (Figure S8). This pre-anthesis ovary growth coincides with an increase in the content of ethylene, suggesting that this hormone

may be involved in the cell division before anthesis. Additionally, a high expression of *ARR5*::GUS, responsive to cytokinin, was observed in placental tissue and later in ovules, indicating that cytokinin is also involved in the promotion of the cell division at this stage of floral development. Apparently, GAs and ABA do not have a major role in ovary growth at pre-anthesis since no expression of *GA2Ox*::GUS and *RD29B*::GUS was observed in ovary tissue. Interestingly, although no *DR5*::GUS expression was showed in ovary tissues from -9 to 0 DPA, the strong GUS staining observed in the peduncle, in that stages, was probably due to the basipetal transport of auxin, synthesized in ovule, to the mother plant (Serrani et al., 2010).

The stamen length present a faster growth compared to both style and ovary length. Hence, at -9 DPA, stamen reached 40% of its final length while style reached just 27%. Furthermore, the fact that the length of the stamen was higher in all pre-anthesis stages than the sum of the style and ovary length, suggest that this can be caused by the high auxin content of the stamen, which prevent heterostyly, *i.e.* the protrusion of the style beyond the staminal cone (Figure S4). No GUS activity was observed for any hormone class in styles from -12 DPA to 3 DPA (Figure 3 and 4).

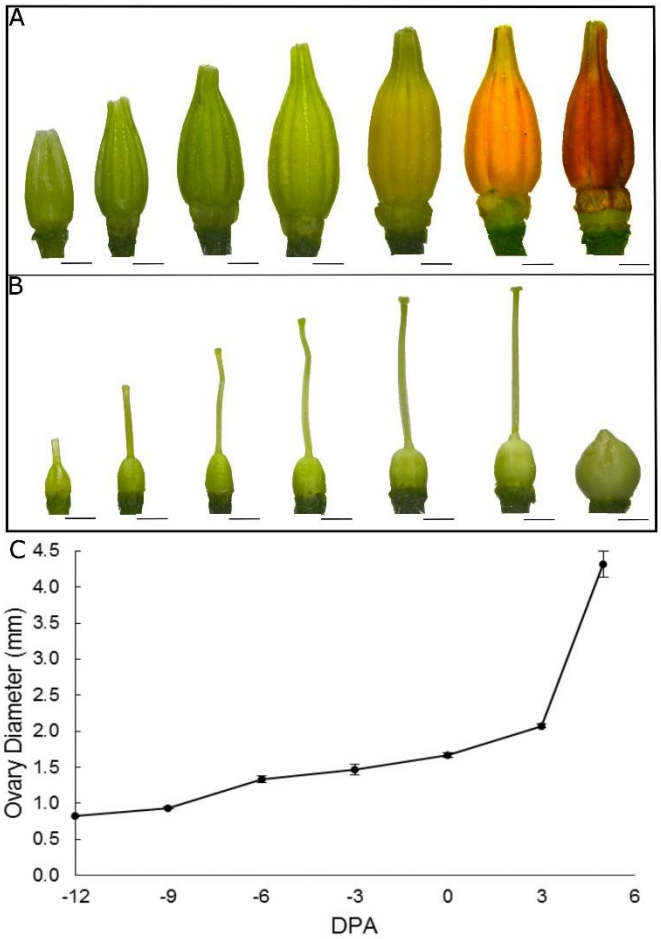

Figure 2. Time course of tomato flower from early pre-anthesis stages to early post-fertilization stages (A-B). At B the flowers are emasculated. Progression of ovary diameter during tomato flower development and initial cell division phase of fruit development (C). Data are ± SEs (n= 10 ovaries). DPA, days post anthesis. Scale bars= 1 mm.

6.2. Hormonal control of the "cell division phase" of early fruit development

Auxin presence remained in stamen and peduncle at anthesis (Figure 4A; Figure S1). In the post-dehiscence of anther, the pollen grains that reached the stigma, at 3 DPA, presented a strong *DR5*::GUS staining, which is consistent with the idea that the auxin content of the pollen grain may have a stimulates role in fruit set. Further, no *DR5*::GUS staining was observed in stigma tissue at 3 DPA (Figure S2). A remarkable increase in ovary diameter occur from 3 to 5 DPA (Figure 2), a period probably coinciding with ovule fertilization. At the same period (3 to 5 DPA) a strong increase of *DR5*::GUS activity was observed in ovary, and the GUS activity was similar in pericarp and internal tissue. Therefore, the fertilization promotes auxin biosynthesis (Figure 4A). As shown in Figure S8 the fertilization triggered a high cell division activity, giving that the number of pericarp cell layers increased from 11 to 20 in the period from 0 to 5 DPA.

A strong *ARR5*::GUS staining was observed in stigma at anthesis, however at 3 DPA the GUS staining decrease (Figure 4B, Figure S5). At 0 DPA, *ARR5*:GUS activity was observed in placental tissue, which started to decrease from 3 to 5 DPA (Figure S3). However, *ARR5*::GUS expression remained high in ovule at 0 and 3 DPA, mainly in the integument, and at 5 DPA, mostly in embryo (Figure S3). These evidences suggest that cytokinin plays a major role in pre-anthesis ovary growth and in early seed development.

No *GA2Ox*::GUS and *RD29B*::GUS activity was observed in ovary from anthesis to 5 DPA, whereas, at anthesis, anther still showed *GA2Ox*::GUS expression and pollen grain showed *GA2OX*::GUS and *RD29B*::GUS expression (Figure 4C and 4D; Figure S6). Figure S6 shows that pollen grains that reached stigma, during pollination, presented high ABA signal, which may be linked to the known role of this hormone in the desiccation and protection of pollen grain (Gonzalez and Iusem, 2014). Further, *RD29B*::GUS staining was showed in stigma at 3 DPA, but no *RD29B*::GUS staining was observed at anthesis and pre-anthesis stages (Figure 3D; Figure 4D; Figure S6), indicating that ABA may also have participates in style senescence post-pollination and fertilization.

A high ethylene content was observed at anthesis and 3 DPA, specifically in the columella, placental tissue and vascular bundle, in the peduncle (Figure 4E; Figure S7). Interestingly, no decrease in the *EBS*::GUS expression was observed post-fertilization. Conversely, at 5 DPA, *EBS*::GUS activity remained in pericarp and internal tissues, excepting in the seeds, where no *EBS*::GUS activity was observed (Figure 4E; Figure S7). Ovary diameter increases dramatically from 3 to 5 DPA (Figure 2), which is attributed to the known high cell division, mainly in pericarp, at this stage. The fact that *EBS*::GUS activity remains high at this phase suggests that ethylene may be involved in the early fruit growth. Furthermore, at anthesis, the anther showed *EBS*::GUS staining, indicating the role of ethylene in anther dehiscence, and a weak *EBS*::GUS staining was observed in some pollen grains. Additionally, a high *EBS*::GUS activity was observed in stigma at 3 DPA (Figure 4E; Figure S7).

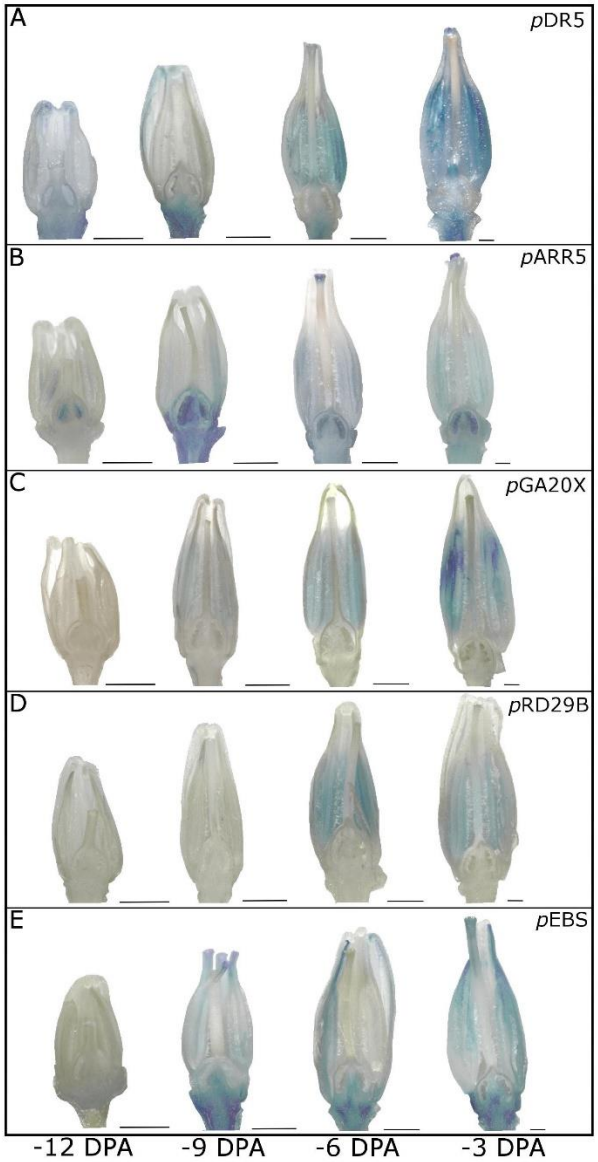

Figure 3. *DR5*::GUS (A), *ARR5*::GUS (B), *GA2Ox*::GUS (C), *RD29B*::GUS (D) and *EBS*::GUS (E) expression of tomato flower buds during pre-anthesis stages. Blue color corresponds to GUS activity. DPA, days post anthesis. Scale bars= 1 mm.

6.3. Interplay between hormones during the transition from cell division to cell expansion phase of tomato fruit development

At 10 DPA, *DR5*::GUS expression was observed in funiculus and peduncle, whereas no auxin content was presented in pericarp and placenta (Figure 5A). Nevertheless, from 15 to 25 DPA, the auxin content in placental tissues increased and then declined, since almost no *DR5*::GUS expression was observed in pericarp and placenta at 30 DPA. This evidence suggests that auxin levels in pericarp decrease during cell expansion phase, reaching its minimum at the end of this phase (Figure 5A; Figure 6A). Seeds showed a strong *DR5*::GUS staining from 15 to 30 DPA, which is in agreement with the idea that seeds are the auxin source in fruit, from where it is transported to the pericarp and placenta promoting growth (Pattison and Catalá, 2012). During that period, a strong *DR5*::GUS staining

was observed in peduncle, evidencing the occurrence of an auxin basipetal transport from the fruit (seeds) to the mother plant.

ARR5:: GUS activity was observed in the outer and inner epidermal and subepidermical layers of pericarp from 10 to 30 DPA (Figure 5B), whereas a strong *ARR5*::GUS activity was observed in fruit shoulder, especially at 15 DPA. Seeds also showed high CKs content, mainly at 10 DPA and then decreased (Figure 5B).

The cell division phase of fruit growth in the cv. Micro-Tom ends between 5 and 10 DPA, given that the number of pericarp cell layer almost did not change from 5 to 10 DPA (Figure S8). Thus, the predominant cell expansion phase probably begins at 10 DPA and goes up to 32-36 DPA, when fruit reaches its final size at the mature green (MG) stage. Therefore, the GAs and ABA contents in pericarp during cell division phase are low, since *GA2Ox*::GUS and *RD29B*::GUS expression was observed only in seeds, at 10 and 15 DPA. Notwithstanding, at 20 DPA onwards strong *GA2Ox*::GUS and *RD29B*::GUS staining was showed in pericarp, placenta and seeds (Figure 5C and 5D). This data suggest that these two hormone may have a major role in cell expansion phase of tomato fruit growth.

No *EBS*::GUS expression was observed in seeds from 10 to 30 DPA, whereas *EBS*::GUS expression was noticed at 10 DPA, in placenta and pericarp, and then decreased to a basal level from 15 to 30 DPA (Figure 5E). This evidence shows a high content of ethylene during the end of cell division (10 DPA) and a decrease at the cell expansion phase (15 DPA). This suggests that ethylene may be involved in the transition from cell division to cell expansion phase. An inverse pattern of GA and ethylene expression seems also to occur, as evidenced by the concomitant high *EBS*::GUS and low *GA20Ox*::GUS staining, from anthesis to 10 DPA in pericarp and placenta (Figure 4 and 5). Conversely, when *EBS*::GUS activity began to decline (15 DPA), *GA2Ox*::GUS and *RD29B*::GUS activity increased in pericarp and placenta (Figure 5).

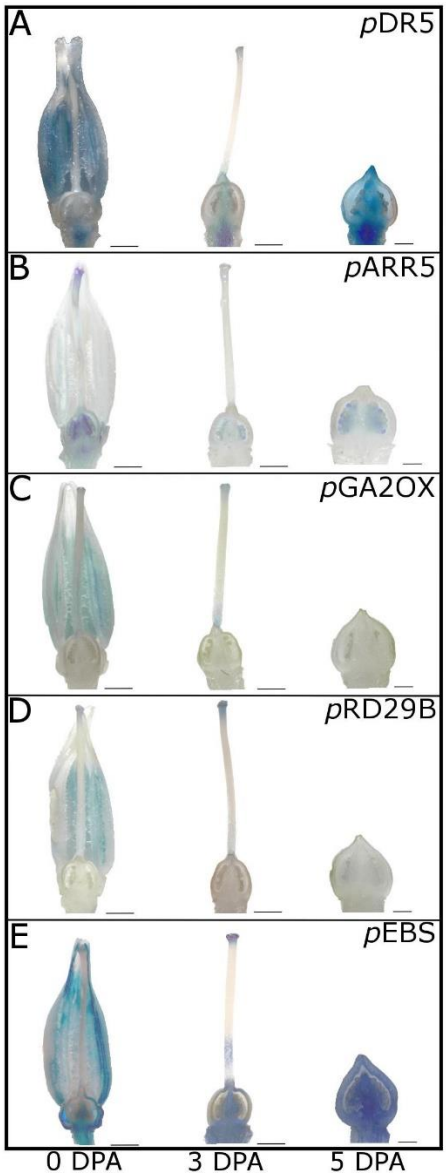

Figure 4. *DR5*::GUS (A), *ARR5*::GUS (B), *GA2Ox*::GUS (C), *RD29B*::GUS (D) and *EBS*::GUS (E) expression of representative tomato ovary at anthesis and early post-anthesis stages. Blue color corresponds to GUS activity. DPA, days post anthesis. Scale bars= 1 mm.

6.4. Hormonal balance at mature green, breaker and red ripe stages

At mature green and breaker, no *DR5*::GUS staining was observed in pericarp, whereas strong *DR5*::GUS staining was observed in seeds (Figure 6A). This suggests that auxin contents in pericarp needs to decrease, allowing the commence of the ripening process. At red ripe, no *DR5*::GUS expression was observed in seeds and pericarp, though *DR5*::GUS expression was showed in peduncle, as it occur during the whole fruit development, from -12 DPA to red ripe (Figure 3A to 6A).

ARR5::GUS staining was observed in the inner and outer epidermical and subepidermical layers in pericarp, and in seeds at mature green. This *ARR5*::GUS staining decreased at breaker stage in seeds, whereas a weak

ARR5::GUS staining was observed in inner subepidermical and epidermical layers in pericarp. No *ARR5*::GUS activity was observed at red ripe (Figure 6B).

Both *GA2Ox*::GUS and *RD29B*::GUS expression reached their maxima at mature green and decreased at breaker stage, and both pericarp and seeds showed GUS expression. No *GA2Ox*::GUS and *RD29B*::GUS expression was observed at red ripe (Figure 6C and 6D). Regarding ethylene, a weak *EBS*::GUS expression was observed in pericarp at mature green. Further, a strong *EBS*::GUS staining was observed in pericarp and placenta at breaker stage, during the onset of ripening, though no *EBS*::GUS staining was observed in seed at mature green and breaker stages. No *EBS*::GUS expression, whatsoever, was observed at red ripe (Figure 6E).

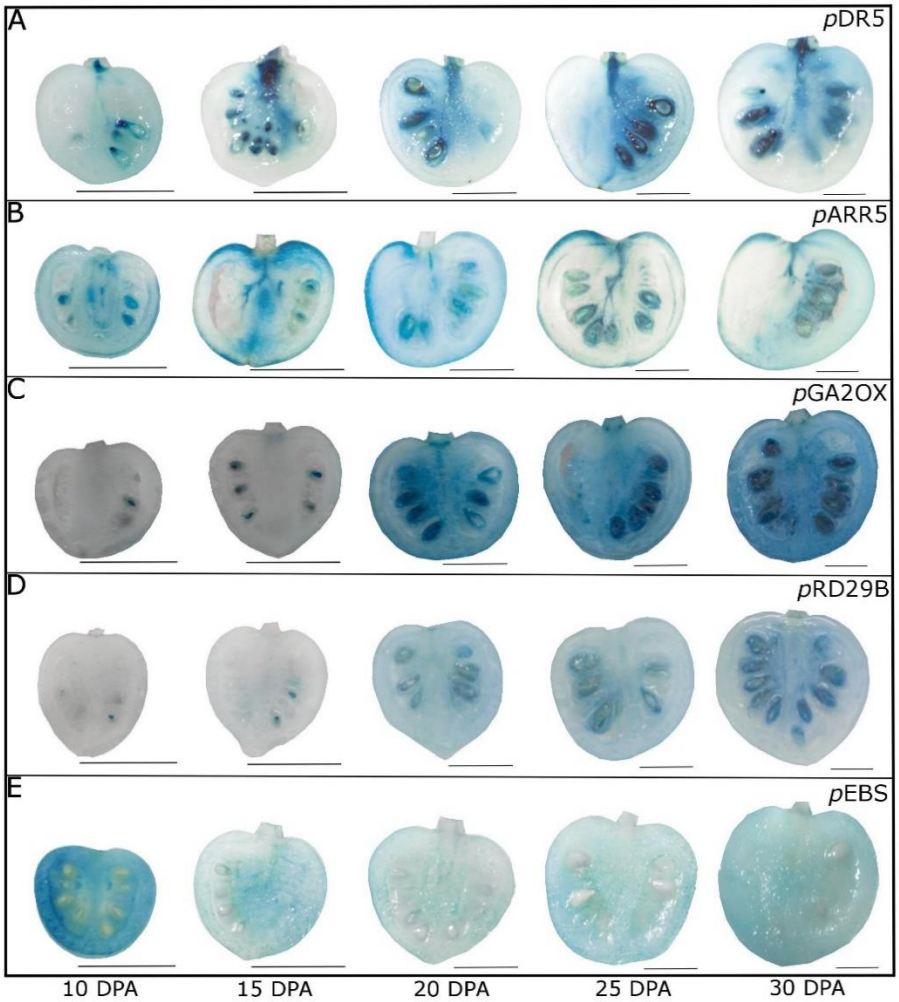

Figure 5. *DR5*::GUS (A), *ARR5*::GUS (B), *GA2Ox*::GUS (C), *RD29B*::GUS (D) and *EBS*::GUS (E) expression of representative tomato fruits during development. Blue color corresponds to GUS activity. DPA, days post anthesis. Scale bars= 1 cm.

6.5. *GA2Ox*::GUS staining was higher, at 15 DPA, in the placenta and the pericarp of the ethylene insensitive *Never ripe* (*Nr*) mutant

Since an antagonism in the spatial and temporal expression of *EBS*::GUS and *GA2Ox*::GUS was reported here during tomato fruit development (Figure 3 to 6), the ethylene insensitive *Never ripe* (*Nr*) mutant expressing the *GA20Ox*::GUS in the hemizygous form was used to analyze if whether the GAs content is modified due to alteration in ethylene sensitivity. The GA-overproducer *35S*::*GA20Ox* transgene, also expressing the GA20Ox::GUS in the hemizygous form, was used as a positive control. Interestingly, at 10 DPA, *GA2Ox*::GUS expression was higher in *Nr* seeds, compared to the positive control *35S*::*GA20Ox* and the hemizygous *GA20Ox*::GUS control (WT) not harboring the *Nr* mutation and the *35S*::*GA20Ox* transgene. However, at 15 DPA, *Nr* and *35S*::*GA20Ox* apparently showed similar intensity of *GA2Ox*::GUS staining in seeds. In addition, a weak *GA2Ox*::GUS staining was observed in the placenta at 15 DPA in *Nr* and *35S*::*GA20Ox*, while no GUS activity was seen in the placenta of WT (Figure 7).

6.6. Application of AVG, an ethylene biosynthesis inhibitor, decreased the diameter of hand-pollinated ovaries

Considering that the *EBS*::GUS activity was observed in placenta and pericarp during early post-fertilization cell division phase, an ethylene inhibitor was applied in order to check out if ethylene has a role regulating fruit cell division by itself or in crosstalk with other hormone. The application of 100 μM AVG in hand pollinated ovaries at -3 DPA, dramatically decreased the ovary diameter at 7 days post application (Figure 8A). To find out if the reduction in ovary diameter was related to cell number or size, we conducted histological analysis. As shown in Figures 8C and 8D, apparently the reduction in ovary diameter post AVG application was mainly due to a reduction in the number of cell layer across the pericarp. Additionally, AVG was also applied to *Nr* ovaries to figure out if the reduction in ovary diameter was related to ethylene. Interestingly, the *Nr* ovaries diameter also decreased post AVG application, although the reduction was slightly attenuated in the *Nr* (Figure 8B).

Aiming to check if the inhibition of ethylene biosynthesis by AVG application alter the GAs metabolism, we realized histochemical analysis of *GA2Ox*::GUS ovaries, 7 days after AVG application. The *GA2Ox*::GUS expression did not alter due to AVG application as no GUS staining was observed in ovaries at that moment.

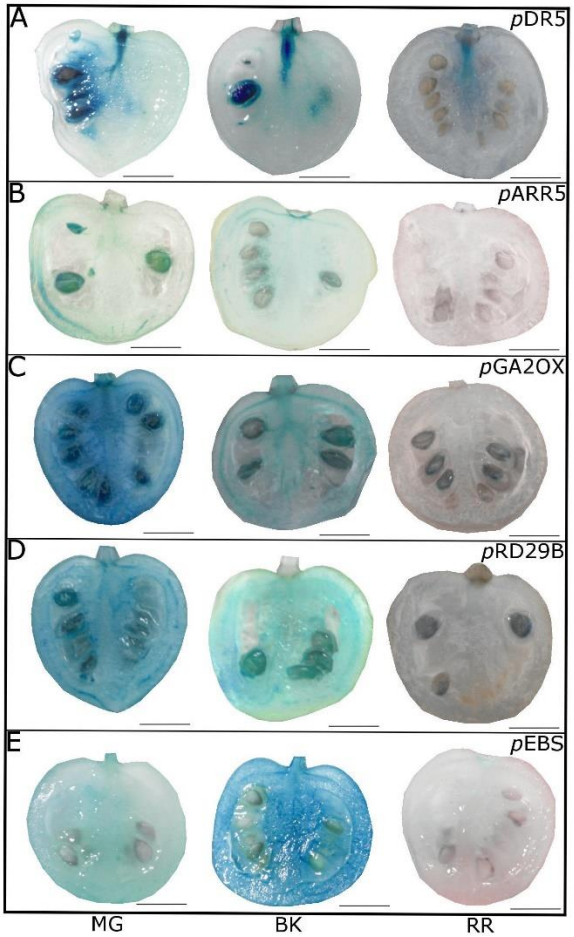

Figure 6. *DR5*::GUS (A), *ARR5*::GUS (B), *GA2Ox*::GUS (C), *RD29B*::GUS (D) and *EBS*::GUS (E) expression of representative fruits at mature green (MG), breaker (BK) and red ripe (RR) stages. Blue corresponds to GUS activity. Scale bars= 1 cm.

6.7. Time course of ovary diameter in tomato hormonal mutant's affecting auxin, gibberellin and ethylene sensitivity shows the contribution of each hormones class during cell division and expansion phases

At anthesis and at the end of cell division phase (10 DPA), the auxin-consititutive signaling *entire* (*e*) mutant presented the highest ovary diameter (Figure 9). At red ripe stage (final fruit size), *e* presented the highest ovary diameter and the GA-constitutive signaling *pro* mutant showed the smallest fruits. According to Figure 9, the cell expansion phase of fruit growth is the major contributor of final fruit size, since WT reached just 33% of the final fruit diameter at the end of cell division phase (10 DPA). Noteworthy, *Nr* and *pro* reached only 29% and 30% of the final fruit diameter at the end of cell division phase, respectively. On the other hand, *e* reached 39% of the final fruit diameter at 10 DPA.

7. DISCUSSION

7.1. Hormones playing a major role in ovary growth at pre-anthesis development

The strong *ARR5*::GUS staining observed in the placenta and the ovule during the pre-anthesis stages (Figure 3B; Figure S3), is corroborated by Matsuo et al. (2012), which reported that tomato genes involved in cytokinin biosynthesis and activation were highly expressed before anthesis (*SlIPT3*; *SlIPT4*) or at anthesis (*SlLOG6*; *SlLOG8*), and that the level of *trans*-zeatin riboside (tZR), *trans*-zeatin (ZR), isopentenyladenosine (iPR), dihydrozeatin riboside (DZR) and isopentenyladenine (iP) were high in ovaries at anthesis. These evidences suggests that CKs synthesized in ovary, prior to fertilization, is required to trigger and maintain cell division until ovary reaches its final size (Gillaspy et al., 1993). Noteworthy, ovary diameter increased almost twofold from -12 to 0 DPA, mainly due to cell division (Figure 2; Figure S8). Bohner and Bangerth (1988), and Tanksley (2004) reported that this cell division before anthesis is critical to determinates final fruit size in tomato. Hence, the allele *fw2.2*, which accounts for 20-30% of fruit enlargement during tomato domestication (Frary et al., 2000), acts during the phase of cell proliferation in the ovary, extending this period (Tanksley, 2004). It remains to be determined, however, whether or not *fw2.2* and cytokinin interplay during cell proliferation and ovary growth.

High *ARR5*::GUS activity was observed in stigma from -6 DPA to 0 DPA (Figure 3B; Figure 4B; Figure S5), matching with an increase of stigma width higher than that from -12 to -9 DPA (Figure S4). The differentiation of the upper style into stigmatic tissue is a process that involves cell proliferation, which was previos proposed to be linked to the action of cytokinin promoting cell division (Gasser and Robinson-Beers, 1993; Inze e De Veylder, 2006).

The strong *DR5*::GUS staining in pedicel from -12 DPA to -3 DPA (Figure 3A) may represent the basipetal auxin transport from the ovary to the mother plant. Serrani et al. (2010) showed that auxin is transported basipetally from ovaries and apical shoots, indicating that auxin biosynthesis is taking place in unpollinated ovaries, prior anthesis. It is well known that application of auxin transport inhibitors in tomato pedicels causes the development of parthenocarpic fruit (Serrani et al., 2010). This auxin transport may prevent premature abscission of flower buds, as pedicel is a site of abscission, and may plays a crucial role in auxin homeostasis (Pattison and Catalá, 2012). In addition, the strong *DR5*::GUS activity indicates that auxin content is high in peduncle compared to ovaries, at pre-anthesis stages (Nishio et al., 2010).

DR5::GUS expression in ovule was observed at -12 DPA (2 mm flower bud) in megasporocyte (Figure S1), while *DR5*::GUS staining was not seen in ovary at subsequent pre-anthesis stages, when megasporocyte underwent two meiotic divisions to create haploid megaspores. Afterward, one megaspore underwent mitotic division to gives rise to the embryo sac, which displaced the nucellus at micropylar and chalazal ends during its growth (Yadegari et al., 2004; Brukhin et al., 2007; Xiao et al., 2009) (Figure S1). The absence of *DR5*::GUS expression in ovary is likely to be due to the basipetal auxin transport previously mentioned, generating a higher auxin content in pedicel. The auxin efflux transporters PIN proteins, regulates auxin homeostasis in ovaries (Nishio et al., 2010; Pattison and Catalá, 2012). Mounet et al. (2012) reported that *SlPIN4*, was the *SlPIN* gene highly expressed in tomato ovary, and its expression increased during flower development. In addition, the silencing of *SlPIN4* leads to the development of parthenocarpic fruits. On the other hand, there are reports that *SlPIN1* and *SlPIN2* are highly expressed in ovaries, reaching their peaks near to anthesis. Additionally, *SlPIN2*::GUS is expressed in ovules at anthesis (Nishio et al., 2010). These evidences suggest that basipetal auxin transport is critical to maintain

an adequate level of this hormone in ovaries, at pre-anthesis stages, to prevent parthenocarpic fruit development or abscission of flower buds.

The *DR5*::GUS activity in pollen grain and anther from -6 to -3 DPA indicates a role of auxin in the development of these organs. These GUS activity are probably mainly due to local auxin synthesis, since auxin biosynthesis genes (*YUC2* and *YUC6*) are indeed transcribed in anthers (Cheng et al., 2006). Tomato genes encoding a YUCCA-like flavin monooxygenases, enzyme that catalyze a rate-limiting step in some auxin biosynthesis pathways, named *toFZY*, are expressed during flower development, having two maxima. The first peaks in 2 mm flower buds, due to a high expression of *toFZY4*, and the second peaks just prior to anthesis, due to high *toFZY2* expression (Expósito-Rodriguez et al., 2011). In *Arabidopsis*, no *DR5*::GUS staining was detectable in stamen during microsporogenesis, while it became intense at the end of meiosis, in tapetum, middle layer and endothecium, and microspore. Cecchetti et al. (2008) suggest that this hormone coordinates the timing of anther dehiscence and pollen maturation. As shown in Figure 3A, *DR5*::GUS activity started, in stamen at -6 DPA, and according to Brukhin et al. (2007) at that stage of 6 mm flower bud, meiosis was already ended and microspore start to differentiate into pollen grain. Thus, in tomato, *DR5*::GUS activity in anther and pollen grains probably also started after meiosis and continued during pollen grains maturation (Figure S1).

During the pre-anthesis, the stamen presented an early elongation compared to the style. Hence, at -9 DPA the stamen already reached 38% of its final length, while the style, only reached the 24% (Figure S4). Therefore, the high auxin level in stamen may present a regulatory role. In *Arabidposis*, the stamen removal promotes an early petal elongation, indicating that the high auxin level in stamen may retard the growth of other floral organs (Aloni et al., 2006). This auxin function may be important to prevent the existence of staminal cones with protruding style (heterostyly), ensuring that stamen cones remain higher than stigma, leading to self-pollination in Micro-Tom. Alternatively, the absence of a functional allele of *Style2.1*, a gene necessary for style elongation (Cheng et al., 2007), in cultivated tomato varieties, including MT (Carrera et al., 2012), may prevent that the style responds to the auxin stimulus in order to parallel the stamen growth. A possible interplay between *Style2.1* and auxin during tomato *Style2.1* growth remains to be determined.

GAs are essential for normal tomato flower development. Hence, tomato GAs mutants, such as *gib-1*, which has a reduced ability to synthesize copalyl pyrophosphate from geranylgeranyl pyro-phosphate, presents infertile flower with anther and pollen grain development arrested (van den Heuvel et al., 2001). The transcripts of three *GA 20-oxidase* genes were detected in tomato flower buds, with *SlGA20ox-1* transcript level increased to a high level from 7mm flower bud to open flower. Besides, transcript corresponding to *GA 20-oxidase* were abundant in anther and low in ovary. Additionally, *in situ* hybridization demonstrates a *SlGA20ox-1* signal in placenta (Rebers et al., 1999). Nonetheless, no *GA2Ox*::GUS expression was observed in ovary tissues from -12 DPA to -3 DPA. However, strong *GA2Ox*::GUS activity was observed in stamen from -6 to 0 DPA, due to GUS staining in anther and pollen grain (Figure 3C; Figure S6). The anther, generally presents the higher GAs content among floral organs (Pharis and King, 1985), indicating that stamen tissues act like floral GAs sources (Hirano et al., 2008). In *Arabidopsis*, various GAs biosynthesis genes (*AtGA3ox2, AtGA3ox3* and *AtGA3ox4*) are expressed in most anther tissues, reaching their peaks in the tapetum, prior to its degeneration, and in mature anther walls at anthesis (Hu et al., 2008). In rice, *OsCPS* expression were found in pollen at bicellular microspore stage (Chhun et al., 2007). These evidences indicate a major role of GAs in tapetum functions and in regulating important checkpoints during pollen grain development (Plackett et al., 2011).

No *RD29B*::GUS activity was observed, in ovary, from -12 to -3 DPA (Figure 3D). These results contradict Nitsch et al. (2009), which reported the expression of *SlNCED1* in ovary wall, placenta and ovule in unpollinated tomato ovary. Nevertheless, a strong *RD29B*::GUS staining was observed in stamen, specifically pollen grains, from -6 to 0 DPA (Figure 3D, Figure S6). The ASR (ABA STRESS AND RIPENING) proteins play a major role in pollen grain development, mainly during the drying stage, where protection against drying conditions is crucial for grain maturation (González e Iusem, 2014). Giving that the expression of these *ASR* genes is controlled by ABA (Rossi et al., 1998; Carrari et al., 2004) at pre-anthesis stages, the high level of ABA in pollen grain could be precisely to induce the expression of these genes and to guarantee pollen protection.

Interestingly, *EBS*::GUS activity was observed in vascular bundle from -9 to -3 DPA (Figure S5). It is known that ethylene, via *ERFs* expression, is necessary for normal vascular cell division in *Arabidposis* and poplar. Hence, the *ethylene overproducer1* (*eto1*) mutant, which has an elevated ethylene biosynthesis (Guzman and Ecker, 1990), presents an increase in vascular cell division due to an increased expression of some *ERF*s that promotes cell division (Etchells et al., 2012).

An increase in ethylene content was observed from -9 to -3 DPA in ovaries (Figure 3E, Figure S5). Shinozaki et al (2015) mention an increase in the expression of some ethylene biosynthetic genes, like *SlACS6*, *SlACO2* and *SlACO4*, from -7 to -2 DPA. These evidences suggest an increase in ethylene biosynthesis during pre-anthesis stages, reaching its peak at anthesis (Pattison et al., 2015; Vriezen et al., 2008). Noteworthy, the rise of *EBS*::GUS expression coincided with pre-anthesis ovary growth, where the cell layers of ovary wall increased from 7 to 11 (Figure S8). These results suggest that ethylene may have a role in cell division prior fertilization. Vriezen et al. (2008), McAtee et al. (2013), Kumar et al. (2014) and Pattison et al. (2015) explain that the high level of ABA and ethylene content in ovaries at anthesis, generate a temporally dormant stage before pollination. Nevertheless, no *RD29B*::GUS activity was observed at that stage (Figure 3D and 4D) and, as was mention above, the raise of *EBS*::GUS staining matched with an increase in the number of cell layer in the ovary wall until it reaches its final size prior fertilization (Figure 2; Figure S8).

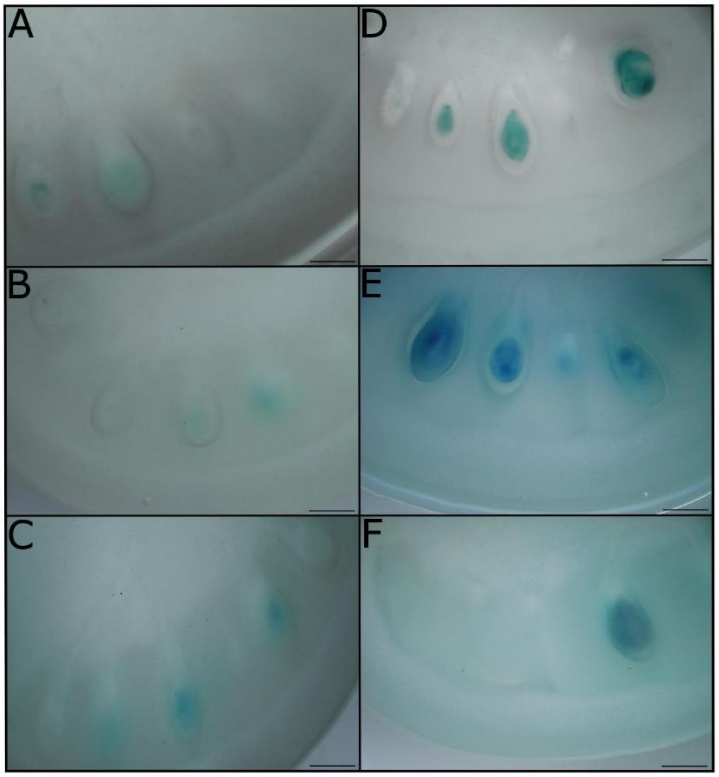

Figure 7. *GA2Ox*::GUS expression in WT x *GA2Ox*::GUS (A-D), *35S::GA20Ox* x *GA2Ox*::GUS (B-E) and *Nr* x *GA2Ox*::GUS (C-F) at 10 DPA (A-C) and 15 DPA (D-F). Scale bars= 1 mm.
At 10 DPA a higher *GA2Ox*::GUS activity was observed in *Nr* seeds (C). A *GA2Ox*::GUS staining was seen in placenta and pericarp in *35S::GA20Ox* x *GA2Ox*::GUS (E) and *Nr* x *GA2Ox*::GUS (F) at 15 DPA. WT x *GA2Ox*::GUS showed *GA2Ox*::GUS expression just in seeds at 15 DPA (D). DPA, days post anthesis.

7.2. Hormonal status during early cell division phase post fertilization

Strong *DR5*::GUS activity was observed in stamen at 0 DPA (Figure 4A). This contrasts with *Arabidopsis*, where no *DR5*::GUS staining was observed in both pollen and anther, when the later become bilocular, the first step of anther dehiscence (Cecchetti et al., 2008; Wilson et al., 2011). Interestingly, in tomato, *DR5*::GUS staining was observed at 3 DPA in pollen grain that reached stigma (Figure S2), suggesting that the high auxin content present in pollen grain may have a stimulating role to initiate fruit set. Gustafson (1936) demonstrated that the application of synthetic auxin to stigma causes the development of parthenocarpic fruits, suggesting that growth hormones present in pollen grain are released to the ovary via pollen tube to trigger fruit growth (de Jong et al., 2009a). Figure 2 shows a dramatic increase in ovary diameter from 3 DPA (2,108mm) to 5 DPA (4,320mm), indicating that in tomato, *cv.* Micro-Tom, fertilization probably occur between 3 and 4 DPA, matching with an increase in *DR5*::GUS activity (Figure 4A).

Fertilization induces the auxin biosynthesis, suggesting that this hormone is the first signal to trigger fruit set (Serrani et al., 2008). Our results also indicate that fertilization triggers auxin biosynthesis, since *DR5*::GUS activity increased after pollination (Figure 4A). At 4 DPA, genes encoding enzymes of the major auxin biosynthetic pathway (*TAR2*, *toFZY2* and *toFZY6*) increase its expression relative to 0 DPA and accumulate primarily in seed tissues and funiculus, with a peak expression in the embryo. Additionally, *SlPIN1*, *SlPIN3*, *SlPIN4*, *SlPIN7* and

SlPIN9 shows high expression in funiculus and placenta at 4 DPA (Pattison et al., 2015). This indicates that seeds are the auxin source of fruits (de Jong et al., 2009a) and from there it is transported via funiculus and vascular tissues of placenta to the fruit pedicel (Pattison and Catalá, 2012).

At 5 DPA, *DR5*::GUS staining had similar intensity in placenta, pericarp and seeds (Figure 4A), which corroborate Pattison and Catalá (2012) results, in which IAA levels were similar in the pericarp and internal tissues (seed and placenta) at 5 DPA. Fertilization have significant effects on the expression of *Aux/IAAs* and AUXIN RESPONSE FACTORs (*ARF*) through the increase of auxin biosynthesis. Some *ARFs,* like *SlARF7*, decrease mRNA levels after pollination, suggesting that it may act as a negative regulator of fruit set (de Jong et al., 2009b and de Jong et al., 2011). Similarly, *IAA9*, a member of tomato *Aux/IAA* gene family also acts as a repressor of fruit set, since the reduction of its transcripts levels trigger fruit development prior fertilization (Wang et al., 2005).

From 0 DPA to 5 DPA, a strong *ARR5*::GUS activity was observed in ovule, suggesting a role of cytokinins in early seed development (Figure 4B; Figure S3). Matsuo et al. (2012) reported that the expression of *SlIPT1*, *SlIPT2*, *SlCYP735A1*, *SlCYP735A2* and *SlLOG2* increased after fertilization. Further, a rise in tZ (trans-zeatin) content occurs at 5 DPA, coinciding with the cell division phase of fruit growth, which indicate a role of cytokinin on cell division in tomato fruit (Matsuo et al., 2012). The high and localized *ARR5*::GUS activity, in seeds, (Figure 4B; Figure S3) indicates a main role of this hormone in seeds development (Sun et al., 2010).

No *GA2Ox*::GUS expression was observed in ovary from 0 to 5 DPA (Figure 4C), suggesting that the main role of GAs is not during early cell division phase of fruit development, but during the cell expansion phase of fruit growth, where *GA2Ox*::GUS activity increased (Figure 5C). Previous studies reported an increase in GAs content and *SlGA-20-Oxs* expression after pollination, specifically *SlGA20Ox1* and *SlGA20Ox3*. Additionally, pollination seems to have no effect on GAs inactivation as no decrease of *SlGA2Oxs* expression is observed in pollinated ovaries after anthesis. Conversely, the expression of *SlGA2Oxs* decreased after application of 2,4-D (Serrani et al., 2007, 2008; de Jong et al., 2011). *DR5*::GUS expression started earlier in both seeds and pericarp compared to *GA2Ox*::GUS expression (Figure 4 to 6). Serrani et al. (2008) suggest that auxin acts prior to GAs in fruit set, and that it role in fruit growth is mediated by GAs, enhancing *SlGA20Ox1* and *SlGA20Ox3* expression and decreasing *SlGA2Ox2* expression.

Expression of ABA and ethylene biosynthesis genes decreases after pollination (Vriezen et al., 2008; Pattison et al., 2015; Shinozaki et al., 2015). Genes encoding neoxantin synthase (*SlNSYs*) and 9-cis-epoxycarotenoid dioxygenase (*SlNCEDs*) are expressed in lower levels after fertilization, and a gene encoding ABA 8'hydroxylase (*CYP707A4*) was highly induced after fruit set, indicating a decrease in biosynthesis and an increase in degradation of ABA (Vriezen et al., 2008). Interestingly, no *RD29B*::GUS staining was observed in ovaries from 0 to 5 DPA (Figure 4D), but only in pollen grains. Thus, we cannot suggest even a high ABA content in ovary at anthesis nor a strong decrease on ABA levels after fruit set, based on *RD29B*::GUS expression. Shinozaki et al. (2015) reported that the expression of *SlACS6*, *SlACS2* and *SlACO4* also increased from -7 to 0 DPA, and its transcripts levels decrease after fertilization (Shinozaki et al., 2015). Curiously, *EBS*::GUS activity, that was already high from -9 to 0 DPA (Figure 4E), did not decreased post fertilization and remained high from 3 to 5 DPA (Figure 4E). This suggests that ethylene may have a function on cell division phase of fruit growth, since the application of AVG, at -3 DPA in hand pollinated ovaries caused a reduction of ovary diameter 7 days later (Figure 8). Ethylene promotes stem cell division in the quiescent center (QC) of *Arabidopsis* root. Hence, the ethylene mutant *eto1* and wild-type plants growing in the presence of ethylene present a higher division in the QC, while the application of AVG eliminates the extra QC cell division in *eto1* (Ortega-Martinez et al., 2007).

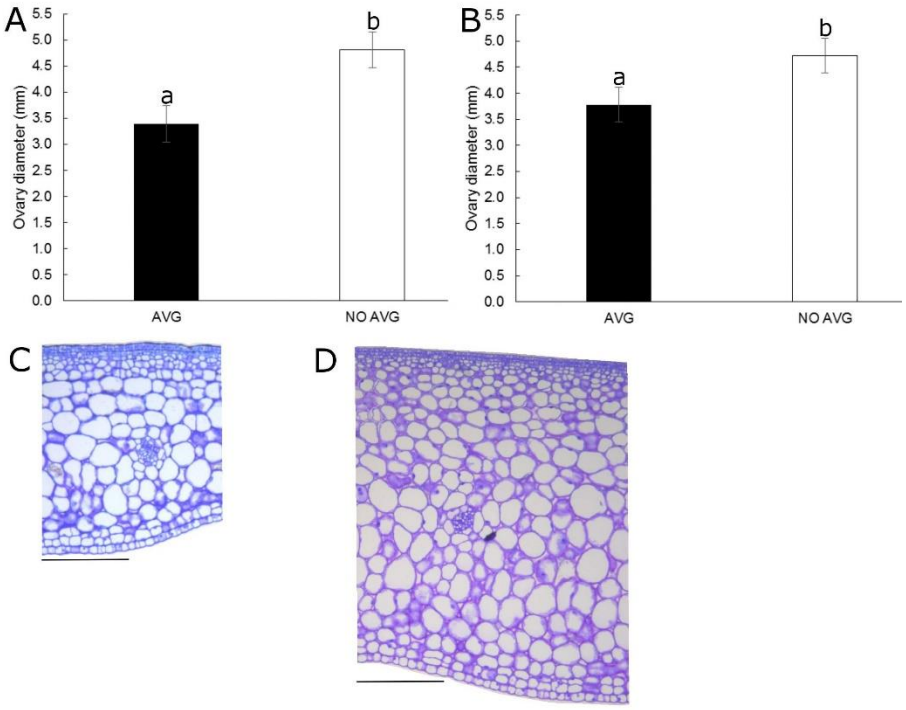

Figure 8. Effects of AVG in ovary diameter 8 days post application in WT (A) and *Nr* (B). Cross section of ovary wall/pericarp in WT 7 days post AVG application (C) and control (D). Scale bars= 200 μm.
A reduction in ovary diameter was observed post AVG application in *WT* and *Nr*, the later at a lower rate (A-B). The apparent cell layers number in pericarp was reduced after AVG application (C-D). Data are ± SEs (n=10 ovaries). Letters indicate significant differences (P< 0,05; Student's test).

7.3. GAs and ABA levels increase at the beginning of cell expansion phase while ethylene levels decrease

A t 10 and 15 DPA a strong *DR5*::GUS activity was observed in funiculus and peduncle and *DR5*::GUS activity was almost undetectable in pericarp and placenta (Figure 5A). Pattison et al. (2015), as was mentioned above, reported a strong expression of auxin biosynthesis and transport genes in the funiculus. Interestingly, *DR5*::GUS staining was observed in placenta, at 20 and 25 DPA, and decreased at the following stages, remained in seeds. This coincides with IAA dosages by GC-MS where its concentration in pericarp decrease from 20 DPA onwards, being almost null at red ripe stage (Figure S9). In seeds and placenta the peak of IAA concentration was observed at 26 DPA, matching with the *DR5*::GUS expression seen in placenta at that stage. Contrarily, Pattison and Catalá (2012) reported IAA peaks in seeds and the placenta at 34 DPA in *cv.* Ailsa Craig. Additionally, previous studies, as mentioned above, indicated that auxin is mainly synthesized in seeds and transported to pericarp. Nevertheless, Zhang et al. (2016) found that *ToFZY1* and *ToFZY5* were expressed in pericarp during early fruit development in *Solanum pimpinellifolium*, suggesting that auxin may be also synthesized in tomato pericarp.

ARR5::GUS activity was observed in outer and inner epidermal and subepidermal cell layer from 10 to 30 DPA (Figure 5B). Mitoses occurred at high rate in these cells, after pollination (Cheniclet et al., 2005). Thus, the increase on cell layer number in the pericarp is mainly due to cell division that occurr in the in the two outer and inner epidermal and subepidermal cells (Cheniclet et al., 2005). Cytokinin is widely known to promote cell division

(Sakakibara, 2006). Further, *ARR5*::GUS expression was seen in fruit shoulder (Figure 5B), suggesting that this region of tomato fruit may present a high cell division rate.

Noteworthy, no *GA2Ox*::GUS and *RD29B*::GUS activity was observed in pericarp and placenta from 10 to 15 DPA. Notwithstanding, strong *GA2Ox*::GUS and *RD29B*::GUS activity was observed in seeds (Figure 5C and 5D). According to the data from ABA measurement in GC-MS, apparently the seeds and placenta are the mainly source of this hormones in the tomato fruit. The ABA content in seeds reached its peak at 20 DPA, whereas the peak in pericarp, at lower levels, was noticed at Mature Green (MG) (Figure S8). Weng et al. (2015a, 2015b) reported that ABA biosynthetic genes (*NOT*, *SIT*, *FLC*) were expressed in funiculus and placental area at 5 DPA. Interestingly, previous studies, mainly based on the expression of ABA biosynthetic genes, mention a first ABA peak in tomato ovary at anthesis (Vriezen et al., 2008; Nitsch et al., 2009; Pattison et al., 2015). Our results, based on *RD29B*::GUS expression (Figure 4D), did not confirm this ABA accumulation in the ovary at anthesis.

In *Arabidopsis*, auxin and GAs act in a hierarchical scheme, in which, after fertilization auxin mediates GAs biosynthesis activation, specifically in the fertilized ovules, from where it is transported to promotes GAs signaling in valves (Dorcey et al., 2009). This evidence supports our results, given that *GA2Ox*::GUS expression was seen initially in seeds, from 10 to 15 DPA (Figure 5C). Pattison et al. (2015) reported an increase of *SlGA20oxs* transcript, particularly in the funiculus, after pollination.

According to Azzi et al. (2015) and de Jong et al. (2011), the cell division phase of tomato fruit growth goes until 8 to 12 DPA, and afterward fruit grows due to cell expansion phase until reaches its final size. Nevertheless, in cv. Micro-Tom, as Figure S8 shows, apparently the cell expansion phase starts between 5 to 10 DPA, concomitantly with an already attenuated cell division activity. Based on this data, the high *EBS*::GUS staining remained during the whole cell division phase, and started to decrease during cell expansion phase (Figure 5E). One possibility is that ethylene production by system 1 (Liu et al., 2015) decrease from anthesis to 10 DPA, reaching its basal level while cell division phase ends. Figures 5E and 6E show that *EBS*::GUS activity decreases from 10 to 15 DPA, and is maintained at a basal level from 15 DPA to mature green (MG), when fruit grows mainly due to cell expansion.

Interestingly, *GA2Ox*::GUS and *RD29B*::GUS expression in placenta and pericarp started at 20 DPA, matching with the decrease of *EBS*::GUS. Evidences indicate that ethylene act during fruit set suppressing GAs metabolism. Shinozaki et al. (2015) showed that ethylene insensitive mutant *Solanum lycopersicum Ethylene receptor 1-1* (*Sletr1-1*) and *Solanum lycopersicum Ethylene receptor 1-1* (*Sletr1-2*), present parthenocarpic fruits, and unpollinated *Sletr1-1* showed higher expression of *SlGA2ox2*, *SlGA2ox3*, *SlGA20ox4* and *SlGA3ox1* compared to unpollinated WT, at 8 DPA. In addition, the pollination-independent ovary growth was due to an increase in cellular expansion of the mesocarp cells, a typical feature of GAs induced fruit. These evidences suggest the existence of an antagonistic role between these two hormones during fruit development, matching with our results of the *EBS*::GUS and *GA2Ox*::GUS expressions (Figure 2 to 5), in which during the cell expansion phase, ethylene decrease to a basal level and GAs levels increase. Several studies reported an increase of GAs biosynthetic genes during cell expansion phase (de Jong et al., 2014; Kumar etl al., 2014; Azzi et al., 2015). In addition, tomato ethylene mutant *Nr* presents elongated fruits and a thicker pericarp, indicating that ethylene action is not only limited to climacteric ripening, but also influences multiple aspects of fruit development prior ripening (Alba et al., 2005).

The peak of ABA concentration in seeds and placenta was at 20 DPA, during the cell expansion phase of fruit growth (Figure S9). Previous studies mention a role for ABA in cell expansion phase of tomato fruit growth (Nitsch et al., 2012; Weng et al., 2015a, 2015b). The ABA deficient mutants *not* and *flc*, and the double mutant *not/flc*

show small fruit, which are even smaller in the duoble *not/flc*, indicating a concentration-dependent effect of ABA. No differences in cell number was detected in pericarp of these ABA mutants, compared to WT. Nonetheless, in *not/flc* a significant reduction of cell size in pericarp indicates that ABA promotes cell expansion during fruit development (Nitsch et al., 2012). In fact, this evidence coincides with the *RD29B*::GUS expression and ABA concentration dynamics during fruit development, reinforcing the hypothesis of a main ABA role in the cell expansion phase.

7.4. ABA and GAs maxima precede the ethylene climacteric burst in tomato fruit

From mature green (MG) to red ripe (RR), *DR5*::GUS expression was not seen in pericarp, matching with the IAA concentration measured by GC-MS (Figure S9), which decrease at that period to levels almost undetectable at red ripe (RR). In addition, no *DR5*::GUS staining was observed in seeds at RR stage and there was a decrease in IAA concentration in seeds from MG to RR (Figure S9), suggesting a reduction in the auxin synthesis. Further, the *DR5*::GUS staining was observed in peduncle, which indicates that the basipetal auxin transport continues, preventing fruit abscission (Serrani et al., 2010; Pattison and Catalá, 2012).It is known that ripening associated *GH3* genes, which convert the free IAA to a conjugated form (Wang et al., 2008), are upregulated in pericarps, which could also account for the decreasing of AIA levels (Kumar et al., 2014). As for cytokinins, it was observed an *ARR5*::GUS activity in the inner and outer epidermical and subepidermical cells at MG, and a weaker activity at BK, indicating that cell division continues, probably at a lower rate compared to early stages (Figure 6B).

Apparently, GAs content are high until MG stage, starting afterward to decrease becoming undetectable at RR stage (Figure 5). These evidences indicate high GAs level in placenta and pericarp from 20 DPA to MG phase (Figure 5C and 6C), in which fruit grows mainly due to cell expansion (Gillaspy et al., 1993; Azzi et al., 2015;). ABA plays an important role in tomato ripening (Zhang et al., 2009). Additionally, the *RD29B*::GUS peak activity precedes *EBS*::GUS maxima, and Zhang et al. (2009) mention that ABA may trigger the fruit ripening by inducing ethylene-mediated pathway and other ethylene independent processes. Mou et al. (2016) reported that ABA maxima precedes the climateric ethylene production, and it has been already shown that exogenous ABA application promotes ethylene biosynthesis through up-regulation of *SlACS2*, a crucial speed-restricting enzyme of ethylene synthesis in tomato fruit (Mou et al., 2016). Furthermore, *RD29B*::GUS expression was high from 20 DPA to MG and then started to decrease (Figure 6D and 6D). This pattern was similar to that of *GA2Ox*::GUS, suggesting an ABA role in cell expansion phase of fruit growth, as it is known for GA.

Several studies mention an increase in ethylene production during ripening in climacteric tomato fruit (Cara and Giovannoni, 2008; Osorio et al., 2013; McAtee et al., 2013; Liu et al., 2015). The *EBS*::GUS expression matches with that peak on ethylene production during the onset of ripening (Figure 6E). In addition, *EBS*::GUS expression was weak from 15 DPA to MG and dramatically increased at BK (Figure 5E and 6E). Ethylene production evolution during fruit development is characterized by an auto-inhibitory pre-climateric ethylene synthesis (System 1) and an autocatalytic climacteric synthesis (System 2) during ripening. These two ethylene system production are controlled via differential regulation of different ACC synthase and ACC oxidase genes (Barry et al., 2000).

7.5. Nr showed a higher activity of *GA2Ox*::GUS

Interestingly, at 10 DPA, a stronger *GA2Ox*::GUS activity was observed in seeds of *Nr* mutant compared to *35S*::*GA20Ox* and WT (Figure 7). This evidence suggests, as was mentioned by Shinozaki et al. (2015), that a low ethylene sensitivity may increase the GAs content. The fact that, at 15 DPA, in *Nr* and *35S*::*GA20Ox* a weak *GA2Ox*::GUS staining was observed in placenta and pericarp, and no GUS staining was observed in WT, indicates that these two genotype has a higher GAs content (Figure 7).

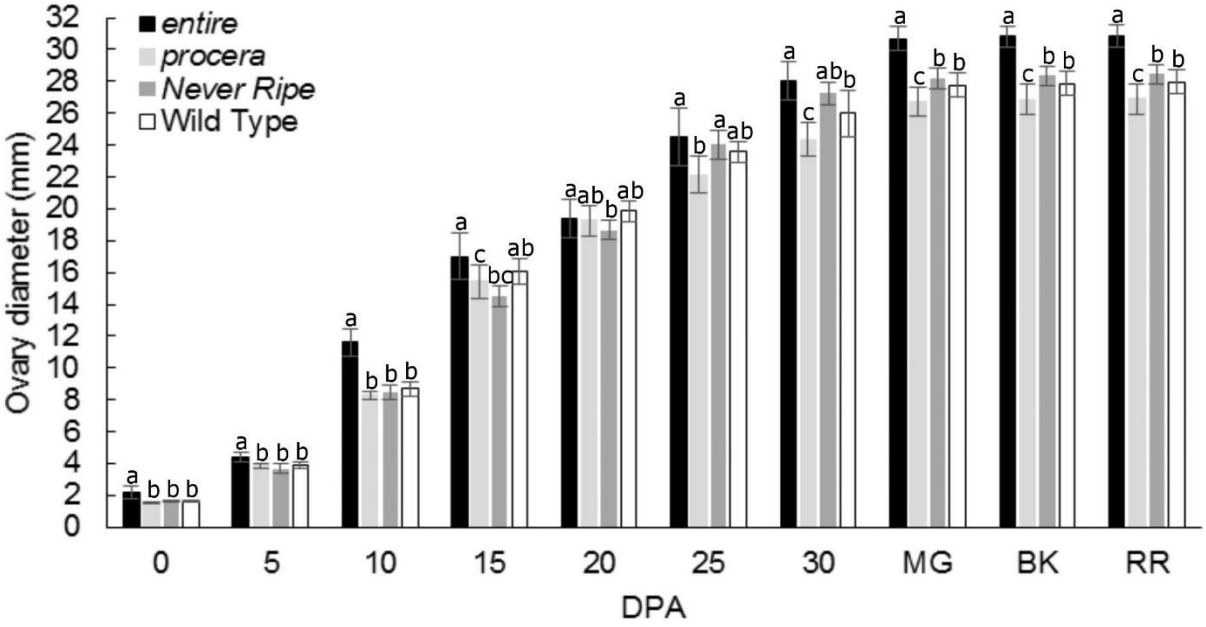

Figure 9. Time course of ovary diameter from anthesis (0 DPA) to red ripe (RR) stage of several hormones mutant in MT background. Data are ± SEs (n= 10 ovaries). Letters indicate significant differences (P< 0,05; Tukey's test). DPA, days post anthesis, MG, mature green; BK, breaker; RR, red ripe.

7.6. Reduction of ovary diameter due to AVG application reinforces the role of ethylene in cell division phase

The reduction of ovary diameter, mainly due to a decrease in the number of cell layer across pericarp, post AVG application (Figure 8), and the high *EBS*::GUS staining from -9 DPA to 10 DPA (Figure 3-5),suggest that ethylene may has positive role in the cell division phase of fruit growth. Moreover, high ethylene content coincides with a period when ovary grows mainly due to cell division, both in pre-anthesis and early post fertilization stages (Figure 3-5; Figure S8).

7.7. The cell division phase of fruit development contributes less the final fruit size in the *Nr* and *pro* mutant

Auxin acts prior to GAs as the early post fertilization signal (Serrani et al., 2008). This coincides with the temporal expression of *GA2Ox*::GUS and *DR5*::GUS (Figure 5). The fact that at anthesis and at the end of cell division (10 DPA), *e* showed the largest ovary diameter and that *pro* showed a smaller ovary diameter than *e* suggest that auxin has a major role in ovary growth during pre-anthesis stages and cell division phase compared to GAs.

These data suggest that the major function of GAs during fruit growth may occurs in the cell expansion phase. There are several evidences that these two hormones (IAA and GAs) has different effects on cell division and cell expansion (de Jong et al., 2009a). In auxin-induced parthenocarpic fruits, cell division takes places at a higher rate, resulting in a fast initial increase in pericarp volume, similar to the observed here in *e*, while the pericarp of GAs-induced parthenocarpic fruits presents a thickness comparable to that of seeded fruit, nonetheless, with fewer larger cells (Serrani et al., 2007). Additionally, the fact that in *Nr* the fruit growth during cell expansion phase contributed to 71% of the final size, more than in WT (67%), suggests that a reduction in ethylene sensitivity may promotes cell expansion, probably due to an increase in GAs biosynthesis or a reduction in the GAs inactivation (Shinozaki et al., 2015).

7.8. Redefining the hormonal balance during tomato fruit development

Taken together, the results presented here redefined the hormonal balance during tomato fruit development (summarized in Figure 10). In this working hypothesis, auxin and cytokinin are the main hormones in stimulating ovary/fruit development during pre-anthesis and early post-fertilization cell division phase, whereas GAs and ABA are responsible for fruit growth during cell expansion phase (Figure 10 and S10). On the other hand, ethylene may presents a role in cell division phase, suppressing GAs metabolism, giving that an antagonist temporal and spatial distribution of *EBS*::GUS and *GA2OX*::GUS was noticed, and that *Nr* fruits showed a higher *GA2Ox::GUS* expression in placenta and pericarp compared to WT (Figure 6C, 6E and 7). The decrease of ethylene content during cell expansion phase may release ethylene repressed GAs metabolism, which in turns, promotes cell expansion. In seeds (ovules), the hormones balance, in general, parallels the pericarp, with the notable exception of ethylene, which seems not to accumulate in seeds, and the more persistent accumulation of auxin, probably reflecting their further role in germination.

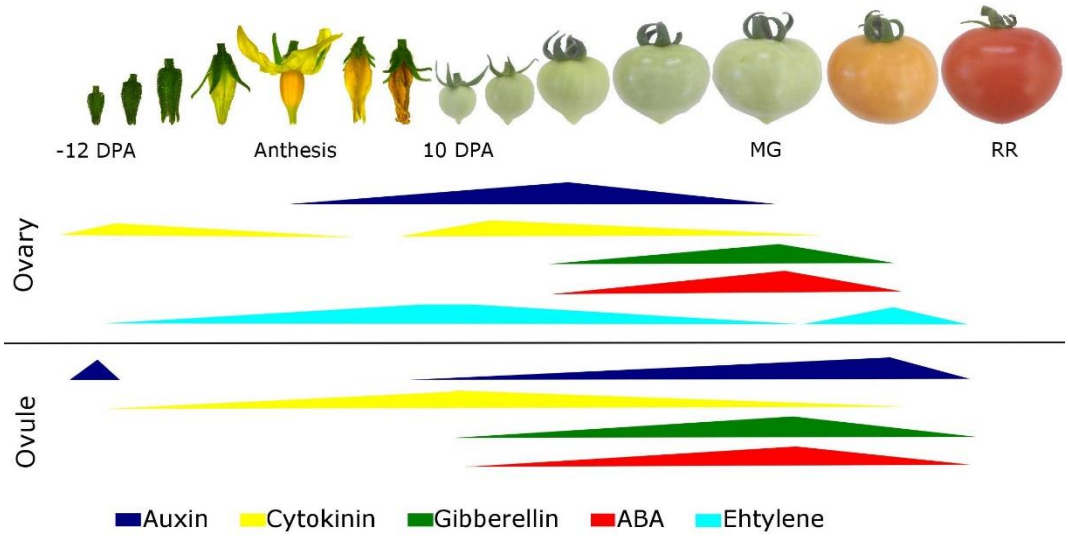

Figure 9. Schematic diagrams showing the gradual changes in the concentrations of auxin, CKs, GAs, ABA and ethylene from early pre-anthesis stages (-12 DPA) to red ripe stage. DPA, days post anthesis; MG, mature green; RR, red ripe.

BIBLIOGRAPHIC REFERENCE

ALBA, R.;PAYTON, P.; FEI, Z.; MCQUINN, R.; DEBBIE, P.; MARTIN, G.B.; TANKSLEY, S.D.; GIOVANNONI, J.J. Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. **The Plant Cell**, Baltimore, v. 17, p. 2954-2965, Nov. 2005.

ALONI, R.; ALONI, E.; LANGHANS, M.; ULLRICH, C.I. Role of auxin in regulating Arabidopsis flower development. **Planta**, New York, v. 223, p. 315-328, Oct. 2006.

AZZI, L.; DELUCHE, C.; GÉVAUDANT, F.; FRANGNE, N.; DELMAS, F.; HERNOULD, M.; CHEVALIER, C. Fruit growth-related genes in tomato. **Journal of Experimental Botany**, Oxford, v. 66, p. 1075-1086, Jan. 2015.

BASSEL, G. W.; MULLEN, R. T.; BEWLEY, J. D. *procera* is a putative DELLA mutant in tomato (*Solanum lycopersicum*): effects on the seed and vegetative plant. **Journal of Experimental Botany**, Oxford, v. 59, p. 585-593, Feb. 2008.

BARRY, C. S.; LLOP-TOUS, M. I.; GRIERSON, D. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. **Plant Physiology**, Minneapolis, v. 123, p. 979-986, Jul. 2000.

BERGERVOET, J. H.; BERHOEVEN, H. A.; GILISSEN, L. J.; BINO, R. J. High amounts of nuclear DNA in tomato (Lycopersicon esculentum Mill.) pericarp. **Plant Science**,Limerick, v. 116, p. 141-145, May. 1996.

BLEECKER, A. B.; KENDE, H. Ethylene: a gaseous signal molecule in plants. **Annual Review of Cell and Developmental Biology**, Palo Alto, v. 16, p. 1-18, Jan. 2000.

BOHNER, J.; BANGERTH, F. Effects of fruit set sequence and defoliation on cell number, cell size and hormone levels of tomato fruits (*Lycopersicon esculentum* Mill.) within a truss. **Plant Growth Regulation**, Boston, v. 7, p. 141-155, Mar. 1988.

BRUKHIN, V.; HERNOULD, M.; GONZALEZ, N.; CHEVALIER, C; MOURAS, A. Flower development schedule in tomato *Lycopersicon esculentum* cv. sweet cherry. **Sexual Plant Reproduction**, Berlin, v. 15, p. 311-320, Mar. 2003.

BRUMOS, J.; ALONSO, J. M.; STEPANOVA, A. N. Genetic aspects of auxin biosynthesis and its regulation. **Physiologia Plantarum**, Lund, v. 151, p. 3-12, May. 2014.

CAMPOS, M. L.; CARVALHO, R. F.; BENEDITO, V. A; PERES, L. E. P. Small and remarkable: the Micro-Tom model system as a tool to discover novel hormonal functions and interactions. **Plant signaling & Behavior**, Georgetown, v. 5, p. 267-270, Mar. 2010.

CARA, B.; GIOVANNONI, J. J. Molecular biology of ethylene during tomato fruit development and maturation. **Plant Science**, Limerick, v. 175, p. 106-113, Apr. 2008.

CARVALHO, R. F.; CAMPOS, M. L.; PINO L. E.; CRESTANA, S. L.; ZSOGON, A.; LIMA, J. E.; BENEDITO,V. A.; PERES, L. E. P. Convergence of developmental mutants into a single tomato model system: Micro-Tom as an effective toolkit for plant development research. **Plant Methods**, London, v. 7, p. 1-14, Jun. 2011.

CARRARI, F.; FERNIE, A .R.; IUSEM, N. D. Heard it through the grapevine? ABA and sugar cross-talk: the ASR story. **Trends in Plant Science**, Oxford, v. 9, p. 57-59, Feb. 2004.

CARRERA, E.; RUIZ-RIVERO, O.; PERES L.E.P; ATARES, A.; GARCIA MARTINEZ, J. L. Characterization of the *procera* tomato mutant shows novel functions of the SlDELLA protein in the control of flower morphology, cell division and expansion, and the auxin-signaling pathway during fruit set and development. **Plant Physiology**, Baltimore, v. 160, p. 1581-1596, Nov. 2012.

CECCHETTI, V.; ALTAMURA, M. M.; FALASCA, G.; COSTANTINO, P.; CARDARELLI, M. Auxin regulates Arabidopsis anther dehiscence, pollen maturation, and filament elongation. **The Plant Cell**, Baltimore, v. 20, p. 1760-1774, Jul. 2008.

CHEN, K. Y.; CONG, B.; VREBALOV, J.; TANKSLEY, S. D. Changes in regulation of a transcription factor lead to autogamy in cultivated tomatoes. **Science**, Washington, v. 318, v. 643-645, Oct. 2007.

CHANDLER, J. The hormonal regulation of flower development. **Journal of Plant Growth Regulation**, New York, v. 30, p. 242-254, Oct. 2011.

CHENG, Y.; DAI, X.; ZHAO, Y. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. **Genes & Development**, New York v. 20, p. 1790-1799, Apr. 2006.

CHENICLET, C.; RONG, W.Y.; CAUSSE, M.; FRANGNE, N.; OLLING, L.; CARDE, J. P.; RENAUDIN, J. P. Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. **Plant Physiology**, Minneapolis, v. 139, p. 1984-1994, Dec. 2005.

CHHUN, T.; AYA, K.; ASANO, K.; YAMAMOTO, E.; MORINAKA, Y.; WATANABE, M.; KITANO, H.; ASHIKARI, M.; MATSUOKA, M.; UEGUCHI-TANAKA, M. Gibberellin regulates pollen viability and pollen tube growth in rice. **The Plant Cell**, Baltimore, v. 19, p. 3876-3888, Dec. 2007.

CHRISTMANN, A.; HOFFMANN, T.; TEPLOVA, I.; GRILL, E.; MÜLLER, A. Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed Arabidopsis. **Plant Physiology**, Minneapolis, v. 137, p. 209-219, Jan. 2005.

CUCINOTTA, M.; COLOMBO, L.; ROIG-VILLANOVA, I. Ovule development, a new model for lateral organ formation. **Frontier in Plant Science**, Lausanne, v. 5, p. 117, Mar. 2014.

CZEREDNIK, A.; BUSSCHER, M.; BIELEN, B. A.; WOLTERS-ARTS, M.; DE MAAGD, R.A.; ANGENENT, G.C. Regulation of tomato fruit pericarp development by an interplay between CDKB and CDKA1 cell cycle genes. **Journal of Experimental Botany**, Oxford, v. 63, p. 2605-2617, Jan, 2012.

D'AGOSTINO, I. B.; DERUERE, J.; KIEBER, J. J. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. **Plant Physiology**, Minneapolis, v. 124, p. 1706-1717, Dec. 2000.

DAYAN, J.; VORONIN, N.; GONG, F.; SUN, T. P.; HEDDEN, P.; FROMM, H.; ALONI, R. Leaf induced gibberellin signaling is essential for internode elongation, cambial activity, and fiber differentiation in tobacco stems. **The Plant Cell**, Baltimore, v. 24, p. 66-79, Jan. 2012.

DE JONG, M.; MARIANI, C.; VRIEZEN, W. H. The role of auxin and gibberellin in tomato fruit set. **Journal of Experimental Botany**, Oxford, v. 60 (5), p. 1523-1532, Mar. 2009a.

DE JONG, M., M. WOLTERS-ARTS, R. FERON, C. MARIANI, AND W. H. VRIEZEN, W. H. The Solanum lycopersicum auxin response factor 7 (*SlARF7*) regulates auxin signaling during tomato fruit set and development. **The Plant Journal**, Oxford, v. 57, p. 160-170, Jan. 2009b.

DE JONG, M.; WOLTERS-ARTS, M.; GARCÍA-MARTÍNEZ, J.L.; MARIANI, C.; VRIEZEN, W. H.; The *Solanum lycopersicum* AUXIN RESPONSE FACTOR 7 (*Sl*ARF7) mediates cross-talk between auxin and gibberellin signalling during tomato fruit set and development. **Journal of Experimental Botany**,Oxford, v. 62, p. 617-626, Feb. 2011.

DHARMASIRI, N.; DHARMASIRI, S.; ESTELLE, M. The F-box protein TIR1 is an auxin receptor. **Nature**, London, v. 435, p. 441-445, May. 2005.

DORCEY, E.; URBEZ, C.; BLÁZQUEZ, M.A.; CARBONELL, J.; PEREZ-AMADOR, M.A. Fertilization dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in Arabidopsis. **The Plant Journal**, Oxford, v. 58, p. 318-332, Jan. 2009.

EGEA, I., W. BIAN, C. BARSAN, A. JAUNEAU, J.-C. PECH, A. LATCHÉ, Z. LI, AND C. CHERVIN, 2011, Chloroplast to chromoplast transition in tomato fruit: spectral confocal microscopy analyses of carotenoids and chlorophylls in isolated plastids and time-lapse recording on intact live tissue. **Annals of Botany**, London, v. 108 p. 291-297, Apr. 2011.

EXPÓSITO-RODRÍGUEZ, M.; BORGES, A. A.; BORGES-PÉREZ, A.; PÉREZ, J. A. Gene structure and spatiotemporal expression profile of tomato genes encoding YUCCA-like flavin monooxygenases: the ToFZY gene family. **Plant Physiology and Biochemistry**, Paris, v. 49, p. 782-791, Mar. 2011.

FRARY, A.; NESBITT, T. C.; GRANDILO, S.; KNAAP, E: CONG, B.; LIU, J.; ELBER, R.; ALPERT, K. B.; TANKSLEY, S. S. fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. **Science**, Washington. v. 289, p. 85-88, Jul. 2000.

GARCIA-HURTADO, N.; CARRERA, E.; RUIZ-RIVERO, O.; LOPEZ-GRESA, M. P.; HEDDEN, P.; GONG, F.; GARCIA-MARTINEZ, J. L. The characterization of trangenic tomato overexpressing gibberellin 20-oxidase reveals induction of parthenocarpic fruit growth, higher yield, and alteration of gibberellin biosynthetic pathway. **Journal of Experimental Botany**, Oxford, v. 66, p. 5803-5813, Set. 2012.

GASSER, C. S.; ROBINSON-BEERS, K. Pistil development. **The Plant Cell**, Baltimore, v. 5, p. 1231-1239, Oct. 1993.

GILLASPY, G.; BEN-DAVID, H.; GRUISSEM, W. Fruits: a developmental perspective. **The Plant Cell**, Baltimore, v. 5, p. 1439-1451, Oct. 1993.

GOETZ, M.; HOOPER, L. C. JOHNSON, S. D.; RODRIGUES, J. C. M.; VIVIAN-SMITH, A.; KOLTUNOW, A.M. Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in Arabidopsis and tomato. **Plant Physiology**, Minneapolis, v. 145, p. 351-366, Oct. 2007.

GONZÁLEZ, R. M.; IUSEM, N. D. Twenty years of research on Asr (ABA-stress-ripening) genes and proteins. **Planta**, New York, v. 239, p. 941-949, Feb. 2014.

GUSTAFSON, F. G. Inducement of fruit development by growth-promoting chemicals. **Proceedings of the National Academy of Sciences**, Washigton, v. 22, p. 628-636, Oct. 1936.

GUZMAN, P.; ECKER, J. R. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. **The Plant Cell**, Baltimore, v. 2, p. 513-523, Jun. 1990.

HIRANO, K.; AYA, K.; HOBO, T.; SAKAKIBARA, H.; KOJIMA, M.; SHIM, R. A.; HASEGAWA, Y.; UEGUCHI-TANAKA, M.; MATSUOKA, M. Comprehensive transcriptome analysis of phytohormone biosynthesis and signaling genes in microspore/pollen and tapetum of rice. **Plant and Cell Physiology**, Oxford, v. 49, p. 1429-1450, Aug. 2008.

HU, J.; MITCHUM, M. G.; BARNABY, N.; AYELE, B. T.; OGAWA, M.; NAM, E.; LAI, W. C.; HANADA, A.; ALONSO, J. M.; ECKER, J. R. Potential sites of bioactive gibberellin production during reproductive growth in Arabidopsis. **The Plant Cell**, Baltimore, v. 20, p. 320-336, Feb. 2008.

INZÉ, D.; DE VEYLDER, L. Cell cycle regulation in plant development. **Annual Review of Genetics**, Palo Alto, v. 40, p. 77-105, Sept. 2006.

JI, K.; KAI, W.; ZHAO, B.; SUN, Y.; YUAN, B.; DAI, S.; LI, Q.; CHEN, P.; WANG, Y.; PEI, Y. SlNCED1 and SlCYP707A2: key genes involved in ABA metabolism during tomato fruit ripening. **Journal of Experimental Botany**, Oxford, v.65, p.5243-5255, Jul. 2014.

KARLOVA, R.; CHAPMAN, N.; DAVID, K.; ANGENENT, G. C.; SEYMOUR, G. B.; DE MAAGD, R. A. Transcriptional control of fleshy fruit development and ripening. **Journal of Experimental Botany**, Oxford, v. 65, p. 4527-4541, Jul. 2014.

KLEE, H. J.; GIOVANNONI, J. J. Genetics and control of tomato fruit ripening and quality attributes. **Annual Review of Genetics**, Palo Alto, v. 45, p. 41-59, May. 2011.

KONG, X.; ZHANG, L.; DING, 2016, Z. 26S Proteasome. Hunter and Prey in Auxin Signaling. **Trends in Plant Science**, Oxford, v. 21 (7), p. 546-548, Jul. 2016.

KUMAR, R.; KHURANA, A.; SHARMA, A. K. Role of plant hormones and their interplay in development and ripening of fleshy fruits. **Journal of Experimental Botany**, Oxford, v. 65, p. 4561-4575, Jul. 2014.

LEMAIRE-CHAMLEY, M.; PETIT, J.; GARCIA, V.; JUST, D.; BALDET, P.; GERMAIN, V.; FAGARD, M.; MOUASSITE, M.; CHENICLET, C.; ROTHAN, C.; Changes in transcriptional profiles are associated with early fruit tissue specialization in tomato. **Plant Physiology**, Minneapolis, v. 139, p. 750-769, Oct. 2005.

LENG, P.; YUAN, B.; GUO, Y.; CHEN, P. The role of abscisic acid in fruit ripening and responses to abiotic stress. **Journal of Experimental Botany**, Oxford, v. 65, p. 4577-4588, Jul. 2014.

LIU, M.; PIRRELLO, J.; CHERVIN, C.; ROUSTAN, J. P.; BOUZAYEN, M. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. **Plant Physiology**, Minneapolis, v. 169, p. 2380-2390, Dec. 2015.

LIU, Z.; FRANKS, R. G. Molecular basis of fruit development. **Frontiers in Plant Science**, Lausanne, v. 6, p. 28-31, Feb. 2015.

LUDWIG-MÜLLER, J.; GEORGIEV, M.; BLEY, T. Metabolite and hormonal status of hairy root cultures of Devil's claw (Harpagophytum procumbens) in flasks and in a bubble column bioreactor. **Process Biochemistry**, London, v. 43, p. 15-23, Oct. 2008.

MATSUO, S.; KIKUCHI, K.; FUKUDA, M.; HONDA, I.; IMANISHI, S. Roles and regulation of cytokinins in tomato fruit development. **Journal of Experimental Botany**, Oxford, v. 63, p. 5569-5579, Aug. 2012.

MCATEE, P.; KARIM, S.; SCHAFFER, R. J.; DAVID, K. A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening. **Frontiers in Plant Science**, Lausanne, v. 4, p. 79, Apr. 2013.

MINTZ-ORON, S.; MANDEL, T.; ROGACHEV, I.; FELDBERG, L.; LOTAN, O.; YATIV, M.; WANG, Z.; JETTER, R.; VENGER, I.; ADATO, A. Gene expression and metabolism in tomato fruit surface tissues. **Plant Physiology**, Minneapolis, v. 147, p. 823-851, Jun. 2008.

MOU, W.; LI, D.; BU, J.; JIANG, Y.; KHAN, Z. U.; LUO, Z.; MAO, L.; YING, T. Comprehensive Analysis of ABA Effects on Ethylene Biosynthesis and Signaling during Tomato Fruit Ripening. **Plos One**, San Francisco, v. 11, p. 1-30, Apr. 2016.

MOUNET, F.; MOING, A.; KOWALCZYK, M.; ROHRMANN, J.; PETIT, J.; GARCIA, V.; MAUCOURT, M.; YANO, K.; DEBORDE, C.; AOKI, K. Down-regulation of a single auxin efflux transport protein in tomato induces precocious fruit development. **Journal of Experimental Botany**, Oxfrod, v. 63, p. 4901-4917, Jul. 2012.

NISHIO, S., MORIGUCHI, R.; IKEDA, H.; TAKAHASHI, H.; TAKAHASHI, H.; FUJII, N.; GUILFOYLE, T. J.; KANAHAMA, K.; KANAYAMA, Y. Expression analysis of the auxin efflux carrier family in tomato fruit development. **Planta**, New York, v. 232, p. 755-764, Jun. 2010.

NITSCH, L.; KOHLEN, W.; OPLAAT, C.; CHARNIKHOVA, T.; CRISTESCU, S.; MICHIELI, P.; WOLTERS-ARTS, M.; BOUWMEESTER, H.; MARIANI, C.; VRIEZEN, W. ABA-deficiency results in reduced plant and fruit size in tomato. **Journal of Plant Physiology**, Oxford, v. 169, p. 878-883, Feb. 2012.

NITSCH, L. M. C.; OPLAAT, C.; FERON, R.; MA, Q.; WOLTERS-ARTS, M.; HEDDEN, P.; MARIANI, C.; VRIEZEN, W. H. Abscisic acid levels in tomato ovaries are regulated by LeNCED1 and SlCYP707A1. **Planta**, New York, v. 229, p. 1335-1346, Mar. 2009.

ORTEGA-MARTÍNEZ, O.; PERNAS, M.; CAROL, R. J.; DOLAN, L. Ethylene modulates stem cell division in the Arabidopsis thaliana root. **Science**, Washington, v. 317, p. 507-510, Jul. 2007.

OSORIO, S.; SCOSSA, F.; FERNIE, A. Molecular regulation of fruit ripening. **Frontiers in Plant Science**, Lausanne, v. 4, p. 198, Jun. 2013.

OZGA, J. A.; VAN HUIZEN, R.; REINECKE, D. M. Hormone and seed-specific regulation of pea fruit growth. **Plant Physiology**, Minneapolis, v. 128, p. 1379-1389, Apr. 2002.

PANDOLFINI, T.; ROTINO, G. L.; CAMERINI, S.; DEFEZ, R.; SPENA, A. Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. **BMC Biotechnology**, London, v. 2, p. 1-11, Jan. 2002.

PATTISON, R. J.; CATALÁ, C. Evaluating auxin distribution in tomato (*Solanum lycopersicum*) through an analysis of the PIN and AUX/LAX gene families. **The Plant Journal**, Oxford, v. 70, p. 585-598, Feb. 2012.

PATTISON, R. J.; CSUKASI, F.; ZHENG, Y.; FEI, Z.; VAN DER KNAAP, E.; CATALÁ, C. Comprehensive Tissue-Specific Transcriptome Analysis Reveals Distinct Regulatory Programs during Early Tomato Fruit Development. **Plant Physiology**, Minneapolis, v. 168, p. 1684-1701, Jun. 2015.

PESARESI, P.; MIZZOTTI, C.; COLOMBO, M.; MASIERO, S. Genetic regulation and structural changes during tomato fruit development and ripening. **Frontiers in Plant Science**, Lausanne, v. 5, p. 124-138, Apr. 2014.

PHARIS, R. P.; KING, R. W. Gibberellins and reproductive development in seed plants. **Annual Review of Plant Physiology**, Palo Alto, v. 36, p. 517-568, Jul. 1985.

PINO, L. E.; CRESTANA, S.; AZEVEDO, M. S.; SCOTTON, D. C.; BORGO, L.; QUECINI, V.; FIGUEIRA, A.; PERER, L. E. P. The Rg1 allele as a valuable tool for genetic transformation of the tomato Micro-Tom model system. **Plant Methods**, London, v. 6, p. 1-11, Oct. 2010.

PLACKETT, A. R.; THOMAS, S. G.; WILSON, Z. A.; HEDDEN, P. Gibberellin control of stamen development: a fertile field. **Trends in Plant Science**, Oxford, v. 16, p. 568-578, Oct. 2011.

RAGHAVAN, V. Some reflections on double fertilization, from its discovery to the present. **New Phytologist**, London, v. 159, p. 565-583, May. 2003.

RAGHAVENDRA, A. S.; GONUGUNTA, V. K.; CHRISTMANN, A.; GRILL, E. ABA perception and signalling. **Trends in Plant Science**, Oxford, v. 15, p. 395-401, May. 2010.

REBERS, M.; KANETA, T.; KAWAIDE, H.; YAMAGUCHI, S.; YANG, Y. Y.; IMAI, R.; SEKIMOTO, H.; KAMIYA, Y. Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. **The Plant Journal**, Oxford, v. 17(3), p. 241-250, Nov. 1999.

ROSSI, M.; CARRARI, F.; CABRERA-PONCE, J.; VÁZQUEZ-ROVERE, C.; HERRERA-ESTRELLA, L.; GUDESBLAT, G.; IUSEM, N. Analysis of an abscisic acid (ABA)-responsive gene promoter belonging to the Asr gene family from tomato in homologous and heterologous systems. **Molecular Genetics and Genomic**, Berlin, v. 258, p. 1-8, Nov. 1998.

RUAN, Y. L.; PATRICK, J. W.; BOUZAYEN, M.; OSORIO, S.; FERNIE, A. R. Molecular regulation of seed and fruit set. **Trends in Plant Science**, Oxford, v. 17, p. 656-665, Nov. 2012.

SAKAKIBARA, H. Cytokinins: activity, biosynthesis, and translocation. **Annual Review of Plant Biology**, v. 57, p. 431-449, Feb. 2006.

SANTNER, A.; CALDERON-VILLALOBOS, L. I. A.; ESTELLE, M. Plant hormones are versatile chemical regulators of plant growth. **Nature Chemical Biology**, New York, v. 5, p. 301-307, May. 2009.

SANTNER, A.; ESTELLE, M. Recent advances and emerging trends in plant hormone signalling. **Nature**, London, v. 459, p. 1071-1078, Jun. 2009.

SERRANI, J. C.; CARRERA, E.; RUIZ-RIVERO, O.; GALLEGO-GIRALDO, L.; PERES, L. E. P.; GARCIA-MARTINEZ, J. L. Inhibition of auxin transport from the ovary or from the apical shoot induces parthenocarpic fruit-set in tomato mediated by gibberellins. **Plant Physiology**, Minneapolis, v. 153, p. 851-862, Jun. 2010.

SERRANI, J. C.; FOS, M.; ATARÉS, A.; GARCÍA-MARTÍNEZ, J. L. Effect of gibberellin and auxin on parthenocarpic fruit growth induction in the cv Micro-Tom of tomato. **Journal of Plant Growth Regulation**, New York, v. 26, p. 211-221, Sept. 2007.

SERRANI, J. C.; RUIZ-RIVERO, O.; FOS, M.; GARCÍA-MARTÍNEZ, J. L. Auxin-induced fruit-set in tomato is mediated in part by gibberellins. **The Plant Journal**, Oxford, v. 56, p. 922-934, Sept. 2008.

SERRANI, J. C.; SANJUÁN, R.; RUIZ-RIVERO, O.; FOS, M.; GARCÍA-MARTÍNEZ, J. L. Gibberellin regulation of fruit set and growth in tomato. **Plant Physiology**, Minneapolis, v. 145, p. 246-257, Sept. 2007.

SEYMOUR, G. B.; ØSTERGAARD, L.; CHAPMAN, N. H.; KNAPP, S.; MARTIN, C. Fruit development and ripening. **Annual Review of Plant Biology**, Palo Alto, v. 64, p. 219-241, Feb. 2013.

SHINOZAKI, Y.; HAO, S.; KOJIMA, M.; SAKAKIBARA, H.; OZEKI-IIDA, Y.; ZHENG, Y.; FEI, Z.; ZHONG, S.; GIOVANNONI, J. J.; ROSE, J. K. Ethylene suppresses tomato fruit set through modification of gibberellin metabolism. **The Plant Journal**, Oxford, v. 83, p. 237-251, May. 2015..

SRIVASTAVA, A.; HANDA, A. K. Hormonal regulation of tomato fruit development: a molecular perspective. **Journal of Plant Growth Regulation**, New York, v. 24, p. 67-82, Sept. 2005.

STEPANOVA, A. N.; YUN, J.; LIKHACHEVA, A. V.; ALONSO, J. M. Multilevel interactions between ethylene and auxin in Arabidopsis roots. **The Plant Cell**, Baltimore, v. 19, p. 2169-2185, Jul. 2007.

SUN, L.; SUN, Y.; ZHANG, M.; WANG, L.; REN, J.; CUI, M.; WANG, Y.; JI, K.; LI, P.; LI, Q. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. **Plant Physiology**, Minneapolis, v. 158, p. 283-298, Jan. 2012.

SUN, X.; SHANTHARAJ, D.; KANG, X.; NI, M. Transcriptional and hormonal signaling control of Arabidopsis seed development. **Current Opinion in Plant Biology**, London, v. 13, p. 611-620, Sept. 2010.

TANKSLEY, S. D. The genetic, developmental, and molecular bases of fruit size and shape variation in tomato. **The Plant Cell**, Baltimore, v. 16, p. 181-189, May. 2004.

TRAINOTTI, L.; TADIELLO, A.; CASADORO, G. The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. **Journal of Experimental Botany**, Oxford, v. 58, p. 3299-3308, Oct. 2007.

ULMASOV, T.; HAGEN, G.; GUILFOYLE, T. J. Dimerization and DNA binding of auxin response factors. **The Plant Journal**, Oxford, v. 19, p. 309-319, Aug. 1999.

ULMASOV, T.; MURFETT, J.; HAGEN, G.; GUILFOYLE, T. J. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. **The Plant Cell**, Baltimore, v. 9, p. 1963-1971, 1997.

VAN DEN HEUVEL, K.; BARENDSE, G.; WULLEMS, G. Effect of gibberellic acid on cell division and cell elongation in anthers of the gibberellin deficient gib-1 mutant of tomato. **Plant Biology**, Stuttgart, v. 3, p. 124-131, 2001.

VAN DOORN, W. G.; KAMDEE, C. Flower opening and closure: an update. **Journal of Experimental Botany**, Oxford, v. 65, p. 5749-5757, Aug, 2014.

VANNESTE, S.; FRIML, J. Auxin: a trigger for change in plant development. **Cell**, Cambridge, v. 136, p. 1005-1016, Mar. 2009.

VRIEZEN, W. H.; FERON, R.; MARETTO, F.; KEIJMAN, J.; MARIANI, C. Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set. **New Phytologist**, London, v. 177, p. 60-76, Aug. 2008.

WANG, H.; JONES, B.; LI, Z.; FRASSE, P.; DELALANDE, C.; REGAD, F.; CHAABOUNI, S.; LATCHE, A.;PECH, J. C.; BOUZAYEN, M. The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. **The Plant Cell**, Baltimore, v. 17, p. 2676-2692, Oct. 2005.

WANG, H.; C.-E. TIAN, C.E.; DUAN, J; WU, K. Research progresses on GH3s, one family of primary auxin-responsive genes. **Plant growth regulation**, New York, v. 56, p. 225-232, Aug, 2008.

WENG, L.; ZHAO, F.; LI, R.; XIAO, H. Cross-talk modulation between ABA and ethylene by transcription factor SlZFP2 during fruit development and ripening in tomato. **Plant signaling & Behavior**, v. 10, p. e1107691. Oct. 2015.

WENG, L.; ZHAO, F.; LI, R.; XU, C.; CHEN, K.; XIAO, H. The zinc finger transcription factor *Sl*ZFP2 negatively regulates abscisic acid biosynthesis and fruit ripening in tomato. **Plant Physiology**, Georgetown, v. 167, p. 931-949, Mar. 2015.

WILKINSON, J. Q.; LANAHAN, M. B.; YEN, H. C.; GIOVANNONI, J. J.; KLEE, H. J. An ethylene-inducible component of signal transduction encoded by *Never ripe*. **Science**, Washington, v. 270, p. 1807-1810. Dec. 1995.

WILSON, Z. A.; SONG, J.; TAYLOR, B.; YANG, C. The final split: the regulation of anther dehiscence. **Journal of Experimental Botany**, Oxford, v. 62, p. 1633-1649, Feb. 2011.

XIAO, H.; RADOVICH, C.; WELTY, N.; HSU, J.; LI, D.; MEULIA, T.; VAN DER KNAAP, E. Integration of tomato reproductive developmental landmarks and expression profiles, and the effect of SUN on fruit shape. **BMC Plant Biology**, London, v. 9, p. 1-21, May. 2009.

XU, C.; LIBERATORE, K. L.; MACALISTER, C. A.; HUANG, Z.; CHU, Y. H.; JIANG, K.; BROOKS, C.; OGAWA-OHNISHI, M.; XIONG, G; PAULY, M.; VAN ECK, J.; MATSUBAYASHI, Y.; KNAAP, E.; LIPPMAN, Z. A cascade of arabinosyltransferases controls shoot meristem size in tomato. **Nature Genetics**, London, v. 47, p. 784-792, May. 2015.

YADEGARI, R.; DREWS, G. N. Female gametophyte development. **The Plant Cell**, Baltimore, v. 16, p. 133-141, Jun. 2004.

YAMAGUCHI, S. Gibberellin metabolism and its regulation. **Annual Review of Plant Biology**, Palo Alto, v. 59, p. 225-251, 2008.

ZDARSKA, M.; DOBISOVÁ, T.; GELOVÁ, Z.; PERNISOVÁ, M.; DABRAVOLSKI, S.; HEJÁTKO, J. Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. **Journal of Experimental Botany**, Oxford, v. 66(16), p. 4913-4931, May. 2015.

ZHANG, M.; YUAN, B.; LENG, P. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. **Journal of Experimental Botany**, Oxford, v. 60, p. 1579-1588, Feb. 2009.

ZHANG, S.; XU, M.; QIU, Z.; WANG, K.; DU, Y.; GU, L.; CUI, X. Spatiotemporal transcriptome provides insights into early fruit development of tomato (*Solanum lycopersicum*). **Scientific Reports**, London, v. 6, p. 23173, Mar. 2016.

Appendix

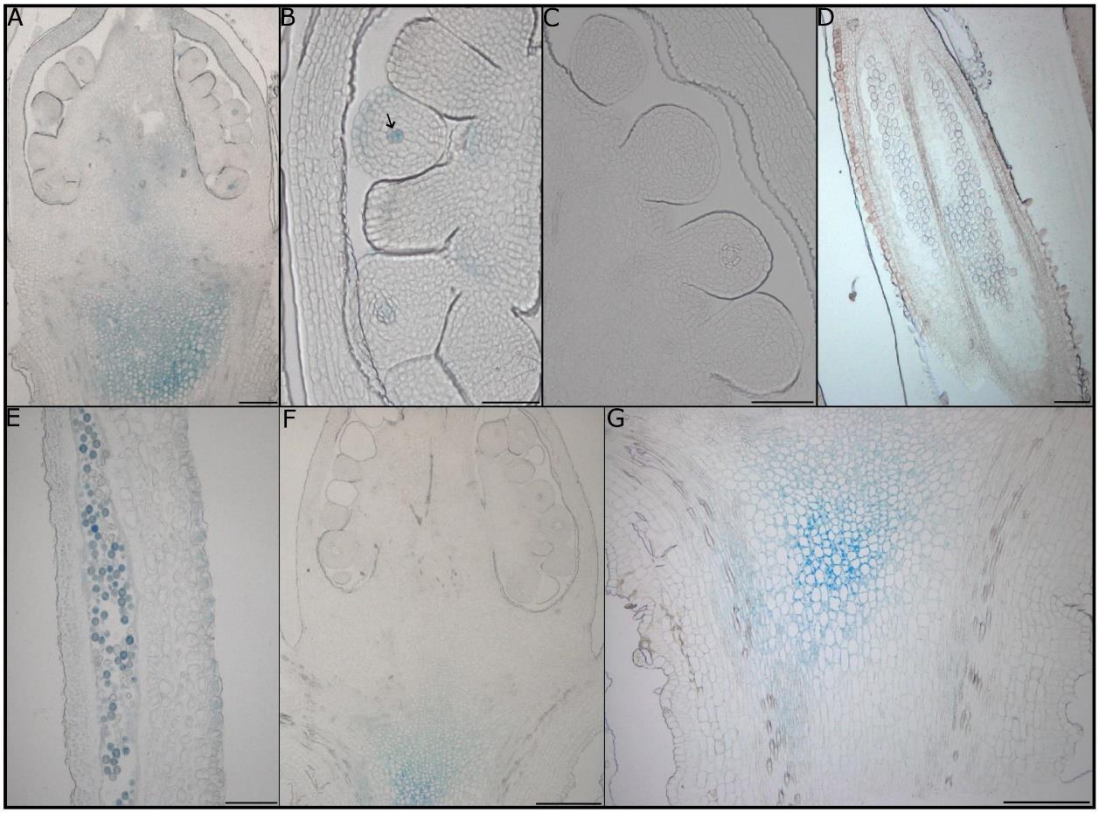

Figure S1. Histochemical analysis of *DR5* expression using the GUS reporter gene in tomato ovary and stamen from early pre-anthesis stages to 5 DPA. DPA, days post anthesis. Scale bars= 200 μm.

At -12 DPA, pedicel and ovule, specifically the megasporocyte, shows GUS activity (A-B). Arrow indicates the megasporocyte (B). At -9 DPA, no GUS staining is observed in ovule (C). Stamen shows no *DR5*::GUS expression in anther and pollen grain at -12 DPA (D). GUS staining is observed in pollen grain and anther at -3 DPA (E). GUS activity in pedicel is observed at antheses, whereas no GUS activity is observed in ovary (F). At 5 DPA, GUS staining is observed in the pith of the peduncle, no DR5::GUS activity is seen in the vascular bundles (G).

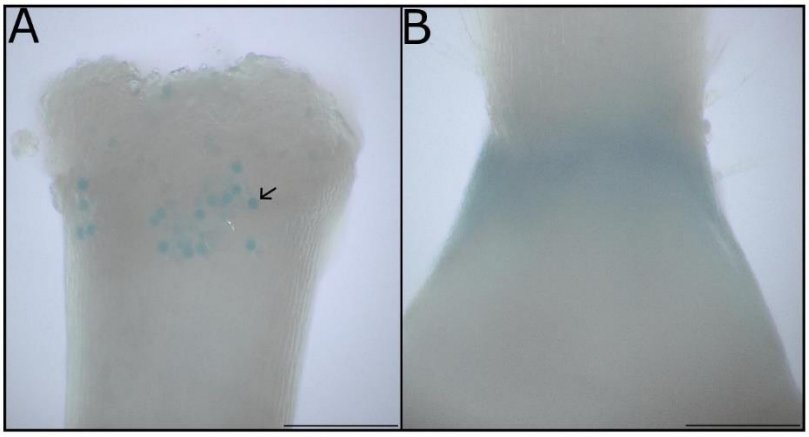

Figure S2. *DR5*::GUS activity in stigma at 3 DPA (A) and in the junction of ovary and style at – 3 DPA (B). Arrow indicates pollen grains. DPA, days post anthesis. Scale bars= 200 μm.

Pollen grains that reached the stigma at 3 DPA showed intense *DR5*::GUS activity (A).The ovary and style junction showed *DR5*::GUS activity at -3 DPA.

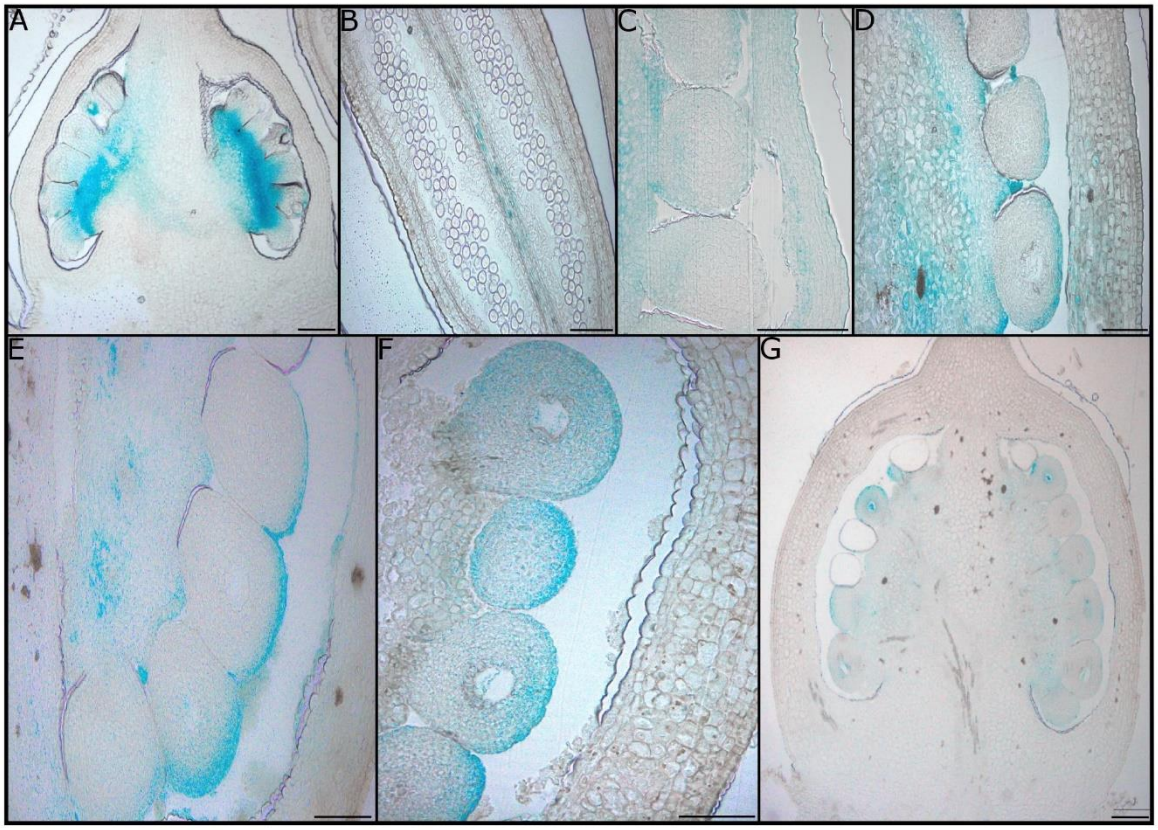

Figure S3. Histochemical analysis of *ARR5* expression using the GUS reporter gene in tomato ovary and stamen from early pre-anthesis stages to 5 DPA. DPA, days post anthesis. Scale bars= 200 μm.

At -12 DPA is observed a strong GUS staining in the placenta and a soft GUS staining in ovules (A). Anther shows *ARR5*::GUS expression at -12 DPA (B).At -9 DPA GUS activity is observed in placenta, outer integument and ovary wall (C). At -3 DPA the placenta and outer integument shows GUS staining, the ovary wall shows no GUS staining (D). At anthesis GUS activity is observed in placenta and outer integument (E). At 3 DPA *ARR5*::GUS expression is observed in the integument and nucellus (F). At 5 DPA GUS staining is observed in the embryo, no GUS staining is observed in the ovary wall and placenta at 3 and 5 DPA (F-G).

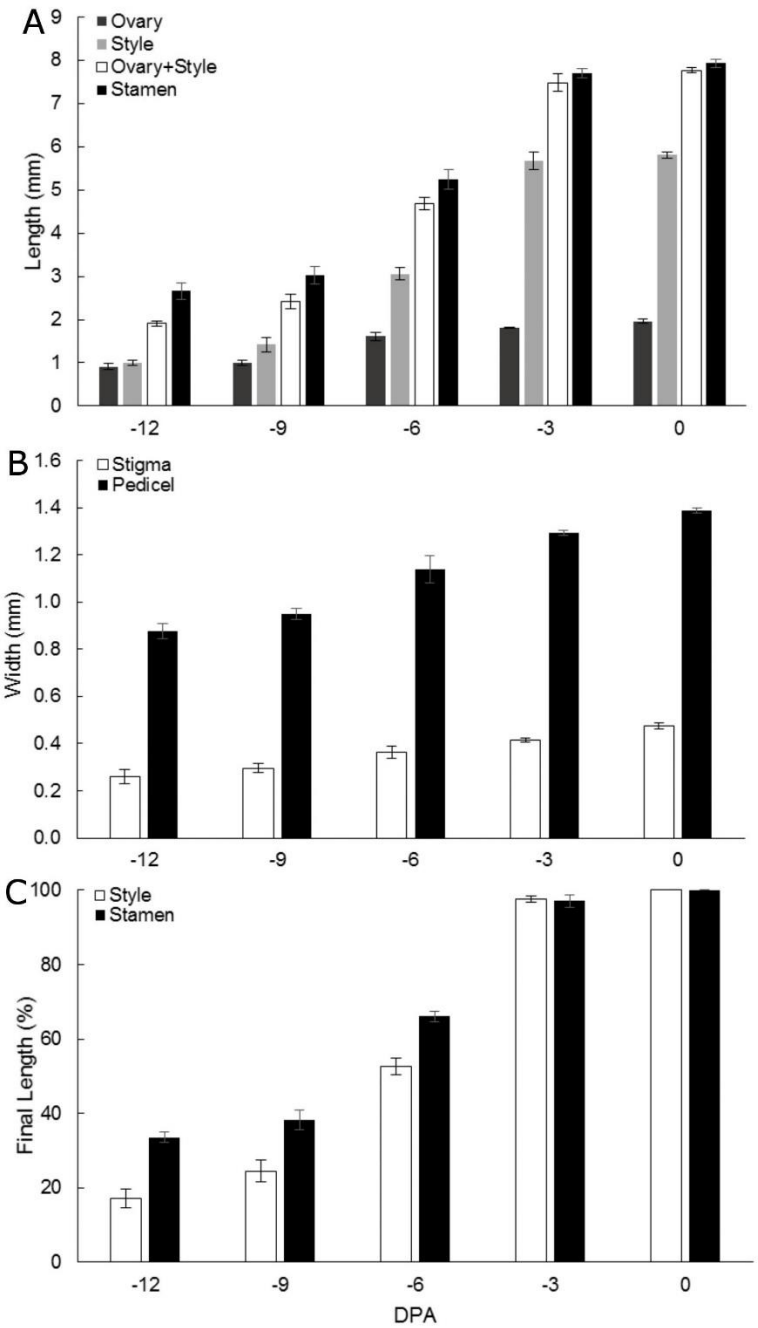

Figure S4. Measurement of different floral organ from -12 DPA to anthesis. Length of style, ovary and stamen of 10 representative tomato flower buds from 10 different plants (A). Progression of stigma and pedicel width of 10 representative tomato flower buds from 10 different plants (B). Comparative growth of stamen and style length during tomato flower development (C). Data are ± SEs (n= 10 flower buds). DPA, days post anthesis.

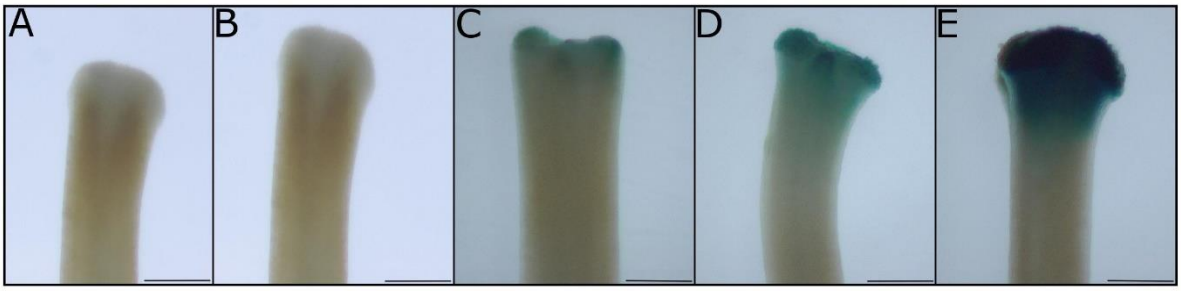

Figure S5. Time course of *ARR5*::GUS expression during stigma differentiation from -12 DPA (A) to 0 DPA (E). DPA, days post anthesis. Scale bars= 250 μm.

At -12 and -9 DPA, no *ARR5*::GUS staining was observed (A-B). An increased ARR5::GUS activity was observed from -6 DPA to 0 DPA (C-E).

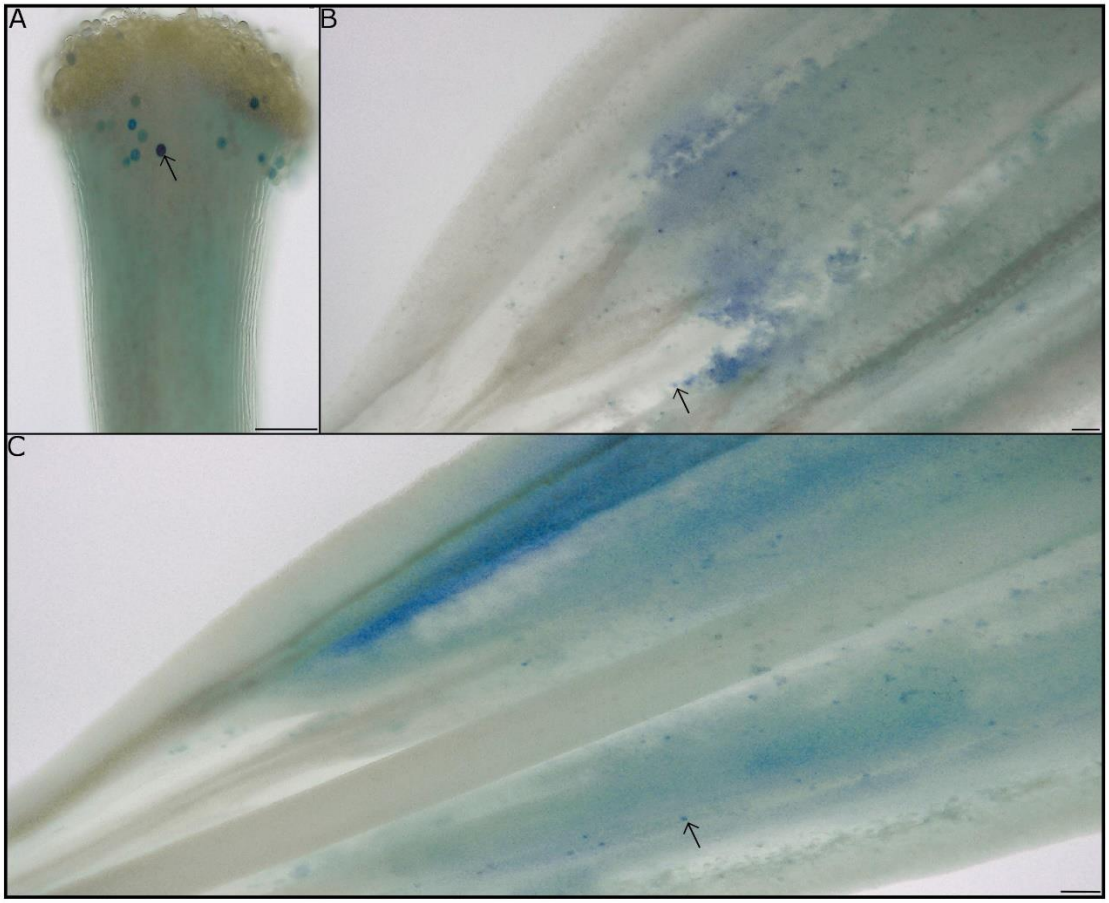

Figure S6. *RD29B*::GUS and *GA2Ox*::GUS expression in tomato stamen and stigma. Scale bars= 200 μm. Pollen grains localized in the stigma of *RD29B*::GUS, at 3 DPA, show GUS staining (A). At 3 DPA, GUS staining is observed in the style (A). Pollen grains of *RD29B*::GUS shows GUS activity at anthesis, no GUS staining is observed in the anthers (B). Stamen of *GA2Ox*::GUS shows GUS staining in the pollen grains and anthers, at anthesis (C). Arrows indicates pollen grains. DPA, days post anthesis.

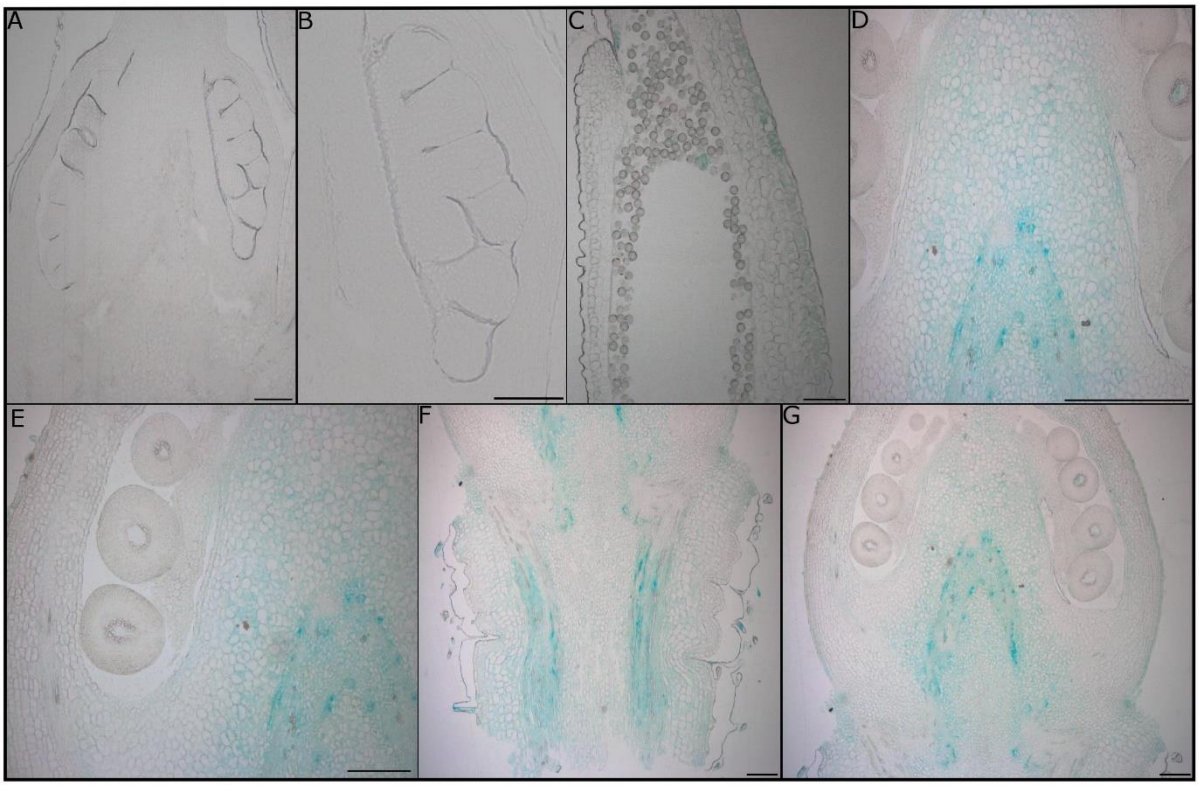

Figure S7. Histochemical analysis of *EBS* expression using the GUS reporter gene in tomato ovary and stamen from early pre-anthesis stages to 5 DPA. DPA, days post anthesis. Scale bars= 200 μm.

No *EBS* expression is observed in ovary at -12 DPA (A-B). GUS staning is observed in anther at anthesis (C). Strong GUS staining is observed in columella and placenta at 5 DPA (G-D). At antheses, GUS activity is observed in columella and placenta. (E). Vascular bundles show strong GUS staining (F).

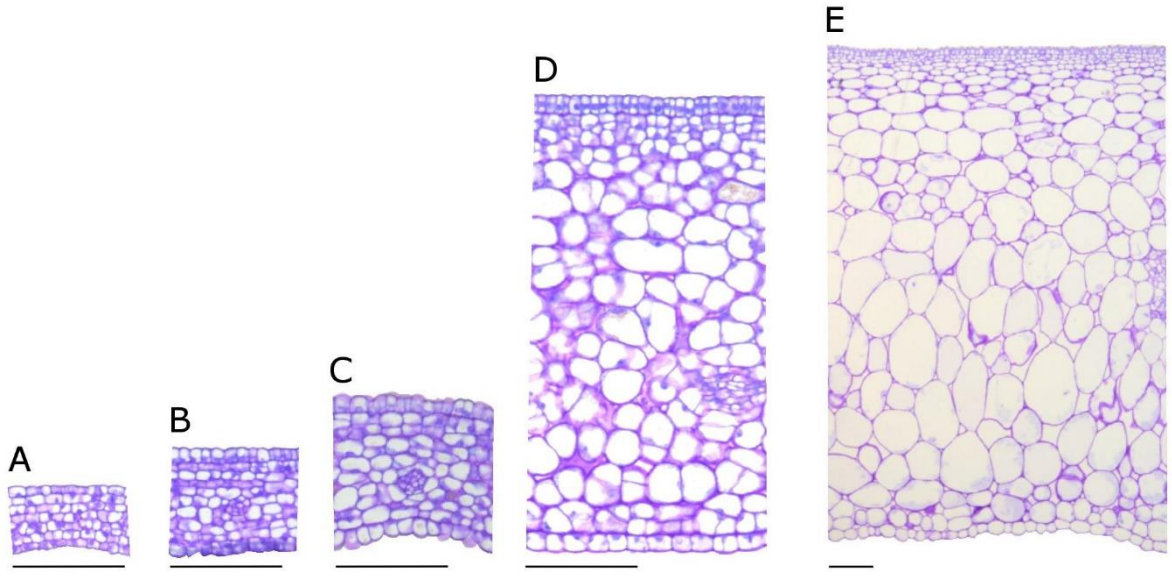

Figure S8. Time course of the number of the pericarp cell layers from -12 DPA to 10 DPA (A-E). Scales bars= 100 µm. At -12 DPA the number of the pericarp cell layers was 7 (A). At -6 DPA, the number of the pericarp cell layer was 10 (B). At anthesis (0 DPA) the number of the pericarp cell layers was 11 (C). At 5 DPA, the number of the pericarp cell layers was 20 (D). At 10 DPA, the number of cell layers was 22 (E).

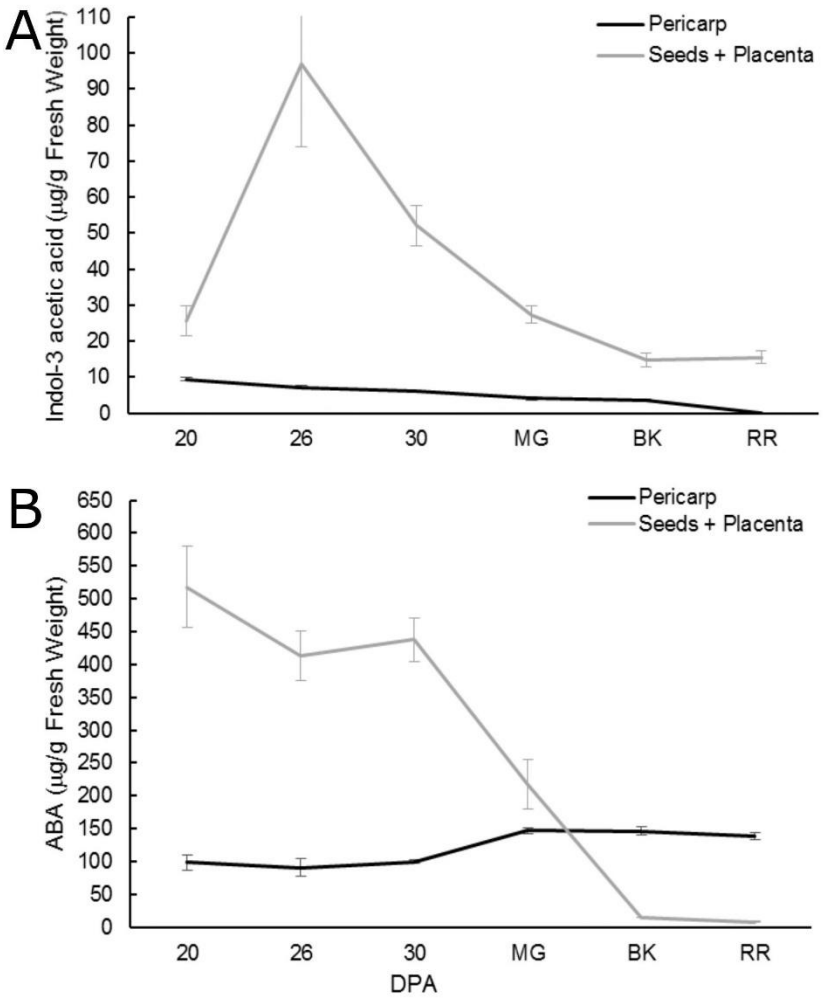

Figure S9. Tissue specific Indol-3 acetic acid (IAA) (A) and ABA (B) levels at different stage of fruit development. Data are means ± SEs (n= 3 fruits). DPA, days post anthesis. IAA and ABA was quantified in seeds and pericarp at 20 DPA, 26 DPA, 30 DPA and MG, BK and RR stages.

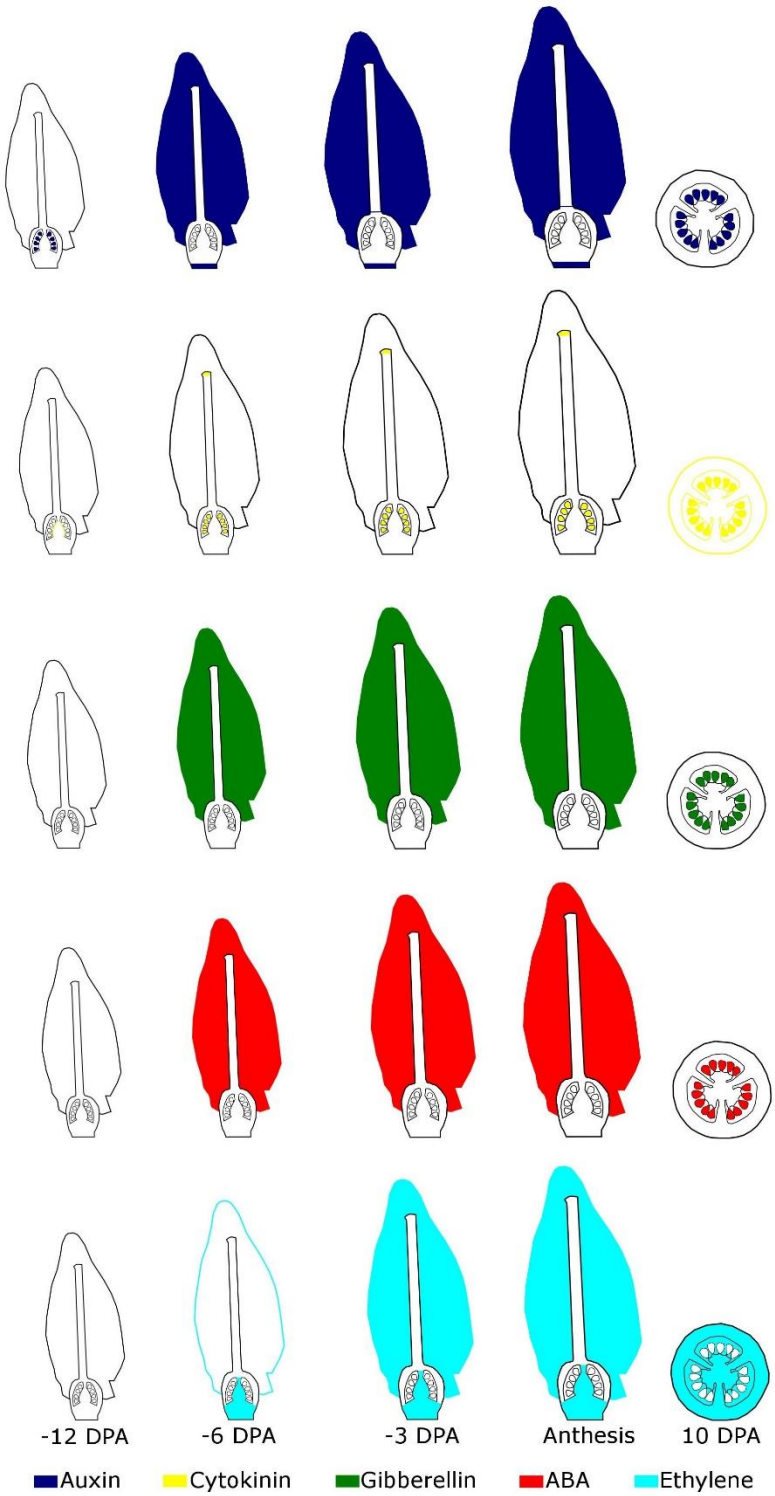

Figure S10. Graphic representation of spatial and temporal hormones content in tomato flower and fruit from -12 DPA to 10 DPA.