Reductions of endogenous gibberellin content impact fruit development and modify tomato fruit metabolism during ripening

Auxiliadora O. Martins

Universidade Federal de Vicosa

Welder A. Silva

Universidade Federal de Vicosa

Rebeca P. Omena-Garcia

Universidade Federal de Vicosa

Franciele S. Oliveira

Universidade Federal de Vicosa

José G. Vallarino

Universidad de Málaga: Universidad de Malaga

Sonia Osorio

Universidad de Málaga: Universidad de Malaga

Alisdair R. Fernie

Max-Planck-Gesellschaft

Adriano Nunes-Nesi

Universidade Federal de Vicosa

Dimas Mendes Ribeiro

Universidade Federal de Vicosa

Wagner Araujo (✉ wlaraujo@ufv.br)

Universidade Federal de Vicosa <https://orcid.org/0000-0002-4796-2616>

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Abstract

Gibberellins (GAs) play a crucial role in modulating developmental processes throughout the plant life cycle. Among the processes in which GAs are involved, they are of significance during the transition and maintenance of the reproductive meristem, as well as in allowing the floral organs development. GAs are also able to regulate, alongside with auxin and cytokinin, the initial processes of fruit development, most likely because they are responsible for both division and cell expansion. It is unknown whether fluctuations in the endogenous content of GAs impact fruit development and metabolism during ripening. To investigate these questions, tomato mutant plants deficient in GAs biosynthesis (*gib3*, moderately deficient; *gib2*, intermediate deficiency and *gib1*, extremely deficient in GA) were used. Notably, *gib2* and *gib1* mutants were characterized by a complete interruption of their reproductive development at the floral bud level. Although *gib3* plants displayed a slightly delay in fruit development, at the end of fruit ripening both WT and *gib3* fruits were highly similar. Little differences were found between WT and *gib3* mutant plants during floral development and total fruit yield. We demonstrated that reduced GAs in *gib3* mutant did not promote morphological modifications in fruits and relatively few metabolic changes were observed between genotypes during fruit ripening. Overall, typical metabolic changes, including increments in amino acids and soluble sugars coupled with reductions in starch, were observed during ripening. Collectively, we demonstrate that GAs plays a significant role on change vegetative to reproductive stage, as well as on the timing of set fruit.

Key Message

Tomato plants with reduced endogenous content of gibberellins show interruption or delay of the reproductive stage.

Introduction

The transition from vegetative to reproductive development is of great importance throughout the life cycle of plants (Galvão et al. 2015). In the case of angiosperm, reproductive success and the establishment of the germ line occurs only after this transition, i.e, the flowering process (Huang et al. 2017). A simplistic explanation of the flowering process is that a signal from the leaves moves to the shoot apex and induces flowering involving the integration of innumerable environmental signals (Bernier and Périlleux, 2005; Bouché et al. 2017). It has been also described that endogenous signals such as age and hormone levels are able to impact flowering time (Aya et al. 2009; Andrés et al. 2014; Zhang et al. 2016). Accordingly, the fruit set is characterized by the rapid development of the flower's ovary following the occurrence of pollination and consequent fertilization (Gillaspy et al. 1993; Serrani et al. 2007b; Shinozaki et al. 2016). This highly regulated sequence of events entails not only a strong imbalance of phytohormones but also a complex activation of programs for the ovule development (Obroucheva, 2014).

The fruit, specific organ from angiosperm, can be classified in fresh and dry fruit and its development presents different phases (Knapp, 2002; Seymour and Granel, 2014). Recent studies have proposed four characteristic physiological stages occurring during fruit development process (McAtee et al. 2013; Obroucheva, 2014) rather than the three initially proposed (Gillaspy et al. 1993). Although both models are characterized by specific characteristics, a similar feature is the transition between vegetative-reproductive processes that can occur gradually and with some overlap. The first stage is the fruit set that is mainly characterized by intense hormonal changes and the initial development of the embryo. During growth, which covers a long period, an intense division and cellular expansion occurs, thus determining the final fruit size. The maturation or breaker phase is a key point between final fruit growth and the ripening/senescence phase. In this stage, the fruit has reached the final size and all competence to ripen, although it has yet to start the ripening process (Ireland et al. 2013). The later stage is usually associated with significant metabolic and physiological changes (Klee and Giovannoni, 2011). Throughout fruit developmental process there is an exquisite crosstalk between different hormones. In addition to being stage-specific, the hormones involved with ripening/senescence in fruits may act both synergistically and antagonistically (Hanke et al. 2008; Huang et al. 2017; Batista-Silva et al. 2018). Gibberellins (GAs) are associated with the promotion of the vegetative to reproductive phase transition in plants (Pharis and King, 1985; Huang et al. 2017). Accordingly, GAs act in the establishment and maintenance of floral meristem (Olszewski et al. 2002; Gomez-Mena, 2005; Andrés et al. 2014) and at the development of floral organs such as petals (Hirano et al. 2008), anthers and pollen (Chhun et al. 2007; Zhang et al. 2010; Kwon et al. 2015) and carpet cells (Plackett et al. 2011). Together with auxin and cytokinin, GAs are required during early stages of fruit development (Gillaspy et al. 1993; Serrani et al. 2007a; McAtee et al. 2013). Thus, the content of GAs increase rapidly after fertilization of the ovary acting as a positive signal during the initial fruit development (Mapelli et al. 1978; Dorsey et al. 2009; Shinozaki et al. 2015). High concentrations of GAs are also found during the growth phase of the fruit. This is due to the fact that GAs play also a crucial role during the division process (Achard et al. 2009; Claeys et al. 2012; Sablowski and Carnier Dornelas, 2014) and cellular expansion (Bouquin, 2003; Locascio et al. 2013). In general, fruit set is result of successful ovule fertilization (Gillaspy et al. 1993; Olimpieri et al. 2007). However, ovary development can occur independently of this process, which leads to the formation of seedless, or parthenocarpic, fruit. Parthenocarpy may also occur in the nature for example with dead pollen or originating from incompatible species, as well as fertilization followed by embryo abortion. Accordingly, the parthenocarpy can also be artificially induced via increase in hormone content brought about genetic manipulation and/or exogenous application, more precisely involving auxin and GA. This process was further verified in plants with increased content and/or GAs signaling (Rieu et al. 2008; Livne et al. 2015) as well as in non-pollinated ovaries treated with exogenous Gas (Fos et al. 2001; Serrani et al. 2007a).

Regardless of the type of fruit development, the content of bioactive GAs decreases at the end of the growth phase being undetectable during the maturation and ripening phases (McAtee et al. 2013; Batista-Silva et al. 2018; Hu et al. 2019). During these last two stages fruits undergo highly regulated metabolic and physiological modification that affect the consistency, colour, flavour, and ultimately, taste of the fruit (Obroucheva, 2014; Zhang et al. 2015; Qin et al. 2016). In tomato fruits, this dramatic metabolic change is

characterized by transition from the partially photosynthetic to the true heterotrophic metabolism, although the net photosynthesis seems not be important to fruits metabolism, but plays a significant role on seeds development (Lytovchenko et al. 2011). During this process, there is conversion from the chloroplast to the chromoplast with dominance of carotenoids and lycopenes in ripening (Carrari and Fernie, 2006). These authors also verified an extensive alteration in the contents of carbohydrates, organic acids and amino acids during fruit development reviewed in Batista-Silva et al. (2018). Wild type (WT) and *gib3* mutant tomato plants showed a decoupling between the carbon and nitrogen metabolism in leaves, most likely due to the manipulation of the endogenous levels of Gas (Ribeiro et al. 2012b; Martins et al. 2019). Furthermore, immature tomato WT fruit are characterized by high levels of GA, whereas low GA levels were found in fruits of *gib3* mutant plants with no significant GA levels in *gib2* and *gib1* fruits (Koornneef et al. 1990). Moreover, *gib* mutants are typical GA-responsive mutants, since reduced plant height, leaf size and flower morphology can be easily reversed to WT by GA application (Koornneef et al. 1990; Asahina et al. 2002). Here, we assess distinct aspects of the influence of GA levels on growth and primary metabolism of fruits through morphological, physiological and biochemical analysis of the GA tomato mutants during fruit development.

Our results demonstrate that impaired GA biosynthesis observed in *gib1* and *gib2* mutant plants (extremely and intermediate deficient in GA levels, respectively) strongly impacts juvenile-adult phase transition and the reproductive organs development. By contrast, the moderate deficiency in GAs observed in *gib3* mutant plants culminated with morphological variations at the early stages of fruit development but was fully recovered following full fruit development. The effects of impaired GA biosynthesis appear to be fairly specific, particularly on floral establishment and metabolic reprogramming during fruit development.

Material And Methods

Plant material and growth conditions

The present work was conducted in Viçosa (20° 45 'S, 42° 15 'W, 650 m altitude), Minas Gerais, Brazil. Wild-type tomato plants (*Solanum lycopersicum* L. cv Moneymaker) (WT) and mutants deficient in biosynthesis of gibberellin (*gib3*, moderately deficient in GAs; *gib2*, intermediate deficiency in GAs and *gib1*, extremely deficient in GAs) were kindly provided by Dr. M. Koornneef (Max Planck Institute for Plant Breeding Research, Cologne, Germany). Mutations in the initial enzymes of GA synthesis, namely *ent*-copalyl diphosphate synthase and *ent*-caurene synthase, are found, respectively, in *gib1* and *gib3* mutants (**Supplementary Fig. 1**; Bensen and Zeevaart, (1990)). Both enzymes are located in plastids and only 6% and 7% of the total WT expression of *ent*-copalyl diphosphate synthase and *ent*-caurene synthase are observed in *gib1* and *gib3* mutant plants, respectively (Bensen and Zeevaart, 1990). In addition, a mutation in the gene encoding the *ent*-caurenic acid oxidase, an endoplasmic reticulum located enzyme, is found in *gib2* mutant plants (Bensen and Zeevaart. 1990; **Supplementary Fig. 1**). It has been previously demonstrated that a very low GA like activity is observed in *gib3* whereas it is negligible in *gib2* and *gib1* extracts (Koornneef et al. 1990). Taken together, these results provide

compelling evidence that strongly reduced GAs levels are found in the mutant plants used here. The GA pathway highlighting the enzyme affected in each mutant and the vegetative phenotype of the mutants is additionally provide in **Supplementary Fig. 1**.

Germination was carried out in a growing chamber (Forma Scientific, Inc, Ohio, USA) under a photoperiod of 12/12 h (day/night), temperature 25/16 °C (day/night), relative humidity $65 \pm 5\%$ and light intensity of $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. After seven days, seedlings were transferred to plastic containers (10 L) containing commercial substrate and soil (50/50 v/v) supplemented, initially, with 14 g of NPK (4:14:8) per pot which means 0.56 g of N, 0.856 g of P (P_2O_5) and 0.93 g of K (K_2O) and fertilization throughout the experiment, as recommended for tomato cultivation (Nowaki et al. 2017). After transfer to pots, the plants were grown in a greenhouse under semi-controlled conditions (maximum photosynthetically active radiation (PAR) of c. $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, with a mean temperature of 30 ± 2 °C). Plants were watered regularly and throughout the entire growth period the plants were maintained under naturally fluctuating conditions of light intensity, temperature and relative air humidity. The plants were irrigated daily as needed and no restriction of root development was observed at the end of the. The plants were maintained under these conditions for five months until the collection of vegetal material for the biochemical analyses, a posteriori. Two experiments were conducted in parallel, one for the evaluation of total fruits production and the second to analyse the reproductive process and fruit development.

Evaluation of fruit development and total fruit production

Wild type and mutants plants were grown side by side under greenhouse in the conditions described above and their development was closely observed until the production of fruits. Half part of plants was used to measure total fruit production and another half part was used to measure fruit development. Each single day, new flowers were tagged and the flowering and fruiting dates recorded on the tag. Index of floral fixation or percentage of flower abortion was determined by the difference between the number of flowers tagged and the total number of fully developed fruits. The fruits were harvested when they presented the reddish coloration characteristic of mature fruits. The fruit mean weight, total number and weight of the fruits per plant were measured as well as the fruit fresh and dry weights.

Flowering evaluation

The day when the plants start and end to flower was recorded for each plant. The following parameters were measured: the beginning of the flowering (emergence of the first inflorescence); inflorescence per plant; total number of flowers per inflorescence, as well as the average time of floral anthesis per inflorescence. Every single day, new flowers were tagged and the flowering and fruiting dates recorded on their respective tag, we also determined the percentage of floral abortion, calculated by the difference between the total number of flowers and the number of fruits produced per plant. The number of petals per flower and the viability of the pollen were also measured. Pollen grains were stained with aceto-carmine and photographed using a digital camera (Axiocam MRc) coupled to stereo microscope (Zeiss

model AX10). Pollen grains that presented abnormal size and transparent colouring were considered unfeasible.

Fruit morphology

Fruits morphology was determined in tagged fruits at 20 days after anthesis (DAA; mature green), 40 DAA (breaker stage) and 60 DAA (ripe, but non-overripen) by determining their fresh and dry weight, diameter, height, volume and number of seeds. The fruits were oven-dried to constant dry weight at 72 °C for 72 h. All these morphological analyses were carried out in at least 10 fruits/plants on each evaluated age, and we used at least five plants/genotype. Additionally, diameter and height measurements were performed in at least three different positions in each fruit to obtain an average measurement.

Determination of fruit metabolite profiling

Fruit mesocarp samples were taken from fruits with 20, 40 and 60 DAA and immediately frozen and powdered in liquid nitrogen, and then stored at -80 °C until further analysis. All analysis (graphics) are expressed in dry weight.

Chlorophyll content (a and b) and total carotenoids were determined from 25 mg fresh matter in 85% acetone extract at 4 °C, as described previously (Wellburn, 1994). The total chlorophyll contents (*a+b*) as well as the chlorophyll a/b ratio was calculated. Approximately 5 mg of dry matter (roots and leaves) were subjected to hot ethanol extraction by incubation at 70 °C for 30 min. In the ethanol-soluble fraction, the concentration of glucose, fructose and sucrose were measured as described previously (Fernie et al. 2001); total amino acids (Cross et al. 2006); malate and fumarate (Nunes-Nesi et al. 2007). In the ethanol insoluble fraction, it was determined starch contents (Fernie et al. 2001) and total soluble proteins contents by the Bradford technique (Bradford et al. 1976).

NAD(H)s and NADP(H)s were determined as described previously by Gibon and Larher, (1997). Briefly, approximately 30 mg of fresh matter were used and thus pyridine nucleotides were assayed using the phenazine methosulfate-catalysed reduction of dichlorophenolindophenol in the presence of ethanol and alcohol dehydrogenase (for NAD⁺ and NADH) or glucose 6-phosphate (G6P) and G6P dehydrogenase (for NADP⁺ and NADPH). The equation used to determine the concentration of nucleotides was obtained by linear regression from a standard curve and subsequent normalization for the dry mass.

Metabolite profiling

Fruit pericarp samples ($\pm$ 50 mg, fresh matter) were used to quantify the relative metabolite levels following an established gas chromatography mass spectrometry (GC-MS) based metabolite profiling method. The extraction, derivatization, standard addition, and sample injection were performed exactly as previously described Lisec et al. (2006). The mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al. 2005). This analysis allowed the determination of approximately 50 different compounds, representing the main classes of compounds (amino acids, organic acids, sugars and others).

Gibberellin measurement

Sample extraction and purification were carried out as described by Chiwocha et al. (2003) with minor modifications. In brief, approximately 600 µg of leaf tissue were extracted by 24 h incubation in 30 ml of 99:1 isopropanol:glacial acetic acid (v/v) containing 15 ng of each deuterium-labeled standards (d2-GA1, d2-GA3, and d2-GA4 whereas GA7 and 9 as external standards) at 4 °C in the dark (OlChemIm Ltd, Czech Republic). Then, supernatants were collected after centrifugation for 15 min at 300 *g*. Extractions were repeated with 10 mL of 99:1 isopropanol:glacial acetic acid (v/v). Both supernatants were mixed and passed through 80% methanol pre-equilibrated SPE-C18 columns (Strata-X 33u Polymeric Reversed Phase, Phenomenex) and filtrates were collected on ice. Extracts were concentrated to complete dryness using Speed-Vac concentrator. The residues were dissolved in 300 µL methanol, sonicated for 5 min, centrifugated at 14000 *g* for 8 min and the supernatants were passed through 0.2 µm syringe filters prior UHPLC-MS/MS analysis. GAs analyses were performed using UHPLC-MS/MS system (UHPLC; Vanquish; Thermo Fisher Scientific) system coupled to a Q-Exactive-Orbitrap mass spectrometer (Thermo-Fisher). In total, 10 µL of each sample were injected onto a Hypersil Gold C18 column (3 µm, 100 x 2.1 mm, Thermo Fisher Scientific). Samples were separated by a gradient elution of mobile phase A (water/methanol/acetic acid; 89.5/10/0.5) against an increasing percentage of mobile phase B (methanol/acetic acid; 99.5/0.5). Quality control samples, internal standard and solvent blanks were also prepared and analyzed along with each batch of tissue samples.

Statistical analyses

Unless otherwise stated, data were obtained from 10 individual plants per genotype. The experiment was designed in a completely randomized distribution. The effect of genotypes was determined by analysis of variance ($P < 0.05$). The means were submitted to the Scott-Knott test. All statistical analyses were carried out using Statistical Analysis System (SAS; North Carolina State University, EUA).

Results

The flowering of tomato plants is strongly affected by reduction in the endogenous ga levels

Through quantitative analysis, via UHPLC-MS/MS, no differences in the content of the bioactive gibberellins GA1 and GA3 in any of the mutant plants was observed (**Supplementary Fig. 2A-B**). However, gradual reductions were observed in the content of the bioactive gibberellin GA4 in *gib2* and *gib1* mutant plants (**Supplementary Fig. 2C**), as was verified for the content of GA7, which was also reduced for the mutant *gib3* (**Supplementary Fig. 2C**). In good agreement with the phenotype observed previously (Martins et al. 2019), *gib1* mutant plants also displayed reductions in the content of GA9 (**Supplementary Fig. 2E**), which is the precursor of the bioactive GA4.

From the simple visual inspection of WT plants when compared to *gib3*, *gib2* and *gib1* mutants, we observed a clear developmental reproductive pattern that was clearly different and characteristic among the tested genotypes. Similar to what was observed during vegetative stage in these mutants (Martins et al. 2019), reductions in the endogenous content of bioactive GAs also impacted the reproductive stage of these plants (**Supplementary Fig. 1**). Through visual and photographic monitoring for ten weeks, it was observed that the endogenous reduction of GA content strongly affects the reproductive development of these plants (Fig. 1). Wild type plants exhibited the first inflorescence three weeks after the transplant (35 days after sowing) whereas the appearance of inflorescences in plants with lower GA content was delayed by one week in relation to WT plants. Although *gib3* mutant plants showed a vegetative development highly similar to WT, a delay in the appearance of the first inflorescence as well as in fruit emergence as long as one week was observed in relation to WT plants. Notably, in both *gib2* and *gib1* plants the inflorescence development was limited to the appearance of buds, which did not develop further in flowers (Fig. 1). By further analysing the development of the inflorescences in a more detailed manner, it was verified that only *gib1* mutant plants produced a small number of inflorescences per plant in comparison to WT plants (Fig. 2A). Furthermore, the total number of flowers per inflorescence was higher in *gib3* plants whereas it was significantly reduced in *gib2* and *gib1* plants in comparison to WT (Fig. 2B).

The anthesis period, i.e. the total time required (in days) for all flowers present in an inflorescence to fully open, was greater in approximately three days in *gib3* in relation to WT plants (Fig. 2C). As previously demonstrated (Fig. 1), *gib2* and *gib1* mutant plants have no anthesis period since the buds are not able to develop in flowers, most likely due to the drastic reduction in the content of GAs. In addition, we observed that the number of unfeasible pollen grains was similar between WT and *gib3* plants (Fig. 2D). Notably, this analysis was carried out solely in WT and *gib3* plants, since in *gib2* and *gib1* the floral development is stalled before this step and can only be concluded with the addition of exogenous GA (Nester and Zeevaart, 1988; Jacobsen and Olszewski, 1991).

Depletion in the ga level promotes delayed development of morphological characters during early stages of fruit development

Given that both *gib2* and *gib1* mutant plants had their reproductive development fully impaired by the reduction of the endogenous content of GAs, all following analyses were performed only on fruits of WT and *gib3* plants (**Supplementary Fig. 3**). There were no significant differences in total number of fruits per plant (**Supplementary Fig. 3A**), percentage of floral abortion (**Supplementary Fig. 3B**), the fresh and dry weight per fruit (**Supplementary Fig. 3C-D**), fresh and dry weight fruit/plant (**Supplementary Fig. 3E-F**), the dry weight/fresh weight ratio (**Supplementary Fig. 3G**) and the number of seeds per fruit (**Supplementary Fig. 3H**). The relatively small reduction of the GA content in *gib3* plants seems to not impact the full development of tomato fruits, since these analyses were carried out when the fruits were at the ripening stage. Thus, although the reduction of GAs in *gib3* plants promoted a delay in the appearance and development of the inflorescences, both the production and morphology of the fully developed fruits were not altered in these plants.

To further investigate to which extent the responses are similar during fruit development, we next decided to analyse fruits at different ages (Fig. 3). By analysing fruits harvested with 20, 40 and 60 days after floral anthesis (DAA) and comparing the genotypes effect on the fruit development, it was observed that height (Fig. 3A) and diameter (Fig. 3B) of 20 DAA fruits from *gib3* plants were reduced in relation to the WT fruits. Despite of that, the total fresh weight (Fig. 3C), total dry weight (Fig. 3D), dry weight to fresh weight ratio (Fig. 3E) and volume (Fig. 3F) were not affected by the reduction in GA levels. We further observed that 40 DAA fruits of *gib3* mutant were characterized by a significant reduction in their development in all evaluated traits, except for the percentage of DW that did not differ. Surprisingly, at 60 DAA the fruits of *gib3* mutant plants were similar to WT fruits for most of the characters, except for the reduced values of dry weight and consequently affecting percentage of DW. This somehow growth recovery at 60 DAA indicated that the reduction of GAs in *gib3* plants promotes morphological changes only at the temporal level, simultaneously with a slight delay in fruit development. The differences in time effect between genotypes were also evaluated and, as expected, there were significant differences among the three developmental stages with progressive increments in the traits evaluated here. The percentage of fruit DW values did not differ at 20 and 40 DAA, but was significantly reduced at 60 DAA (Fig. 3E).

Metabolic changes in response to variation of endogenous ga levels in tomato fruits

Given the above-mentioned results and the visual aspects presented by the fruits in the different stages of development (**Supplementary Fig. 4**), we next decided to further perform an extensive metabolic analysis during fruit development. Changes in nitrogen metabolism were observed by the higher levels of chlorophyll *a* (Fig. 4A) in *gib3* fruits at 20 DAA; however, this increase was not maintained in fruits of 40 DAA. Despite of these changes in chlorophyll *a*, the levels of chlorophyll *b* (Fig. 4B) and chlorophyll *a/b* ratio (Fig. 4C) were invariant between genotypes at 20 and 40 DAA. Since the fruits were already ripening at 60 DAA, the reduction of pigments was substantial, and they were not even detected at 60 DAA. Consistent with the chlorophyll results, total carotenoid contents (Fig. 4D) were gradually and significantly higher in the *gib3* mutant than in WT at 40 and 60 DAA, although when we analyse each genotype over time, at 60 DAA presented a significant effect within the genotypes. The values of total amino acid (Fig. 4E) and soluble protein (Fig. 4F) exhibited a similar behaviour during fruit development. Total amino acid content was similar between WT and *gib3* fruits at the different time points evaluated and significantly increased at 60 DAA in both genotypes (Fig. 4E). As for amino acids, protein content did not present significant differences between the genotypes at 20 and 40 DAA (Fig. 4F). The fruits of 60 DAA from *gib3* mutant exhibited a reduction of 25% in the protein content in comparison with WT fruits. Noteworthy, proteins levels were significant and progressively increased throughout fruit development, and as such WT fruits presented three times higher content at 60 DAA when compared with 20 DAA.

In terms of metabolites directly related with carbon metabolism, we evaluated the contents of soluble sugars (glucose, fructose and sucrose), starch and the organic acids (malate and fumarate) (Fig. 5). Briefly, the levels of glucose (Fig. 5A), fructose (Fig. 5B) and sucrose (Fig. 5C) increased gradually throughout fruit development. In general, glucose and fructose increased within each genotype only at 60 DAA, while sucrose increased significantly at all time points. At 20 DAA glucose and sucrose were

virtually invariant between genotypes, whereas fructose was reduced in *gib3*. At 40 DAA we observed reductions only in sucrose in fruits from *gib3* plants. Surprisingly, fruits from the same genotype displayed values significantly higher in relation to the WT for most sugars evaluated at 60 DAA. Unlike soluble sugars, the levels of starch were altered in the genotypes throughout the fruit development (Fig. 5D). Between 20 and 40 DAA significant increases in starch content were observed reaching the highest observed values during fruit development. After the 40 DAA, the starch levels reduced in both WT and *gib3* fruits. At 60 DAA the WT fruits presented starch levels similar to at 20 DAA, while in the *gib3* fruits these levels were lower than 20 DAA. The pattern observed for starch is compatible with those found in sugar, characterizing the conversion of the carbohydrate reserve, starch, into sugar as the ripening occurs. No differences were observed for starch when comparing the genotypes at each time points. The content of the organic acids malate (Fig. 5E) and fumarate (Fig. 5F) was not affected by the reduction of the endogenous level of GAs, except for fumarate at 60 DAA. Both metabolites displayed gradual increments of their content throughout fruit development. Fumarate differed significantly between all periods, while malate differed only between the last two stages of the development.

To further explore the consequences of the reduced GA biosynthesis on primary metabolism during fruit development we extended the analyses by studying the major primary metabolic pathways, by using an established gas chromatography mass spectrometry (GCMS) approach. These analyses revealed that among over 50 successfully annotated compounds related to primary metabolism, considerable changes occurred in the levels of a wide range of amino acids, organic acids, and sugars (Fig. 6 and **Supplementary Fig. 5**). In general, higher variations in amino acid levels between genotypes or throughout fruit development within each genotype were verified at 40 and 60 DAA. In other groups of metabolites, the variations occurred mainly at 60 DAA. For sake of simplicity, the metabolites highlighted in Fig. 6 are either directly derived or feed the glycolytic pathway and the tricarboxylic acid (TCA) cycle, both processes involved with cell energetic metabolism. Surprisingly, the metabolite profiles of the *gib3* mutant line followed a similar pattern to those of the WT. Thus, the levels of sucrose, glucose-6P and fructose-6P did not present large variations either between genotypes or along the fruit development.

Similar behaviour was observed for organic acids and as such significant increases were verified during fruit ripening for malate and especially for 2-oxoglutarate. Malate is further converted to oxaloacetate, which, together with 2-oxoglutarate, are points of diversion/deviation of TCA cycle intermediates for the biosynthetic pathway of various amino acids (Lancien et al. 2000). Regarding the oxaloacetate-derived amino acids, we were not able to detect lysine and methionine. Although asparagine, homoserine, aspartate and threonine did not differ between genotypes at 20 DAA, reductions in isoleucine were observed in *gib3* fruits at this time point. The levels of isoleucine, homoserine and asparagine reduced at 40 DAA whereas at 60 DAA only threonine reduced in *gib3* fruits. During ripening aspartate, asparagine and threonine showed, in general, significant increases in both genotypes, while homoserine and isoleucine were virtually invariant. From the 2-oxoglutarate, another point of diversion of the TCA cycle six amino acids were detected including histidine, glutamine, glutamate, proline, ornithine, hydroxyproline, and GABA. Although none of them displayed differences between the genotypes at 20 DAA, significant reductions in histidine and glutamine were observed in *gib3* fruits at 40 DAA. Fruits from *gib3* mutant

plants were characterized by reductions in proline, hydroxyproline and ornithine during ripening, whereas GABA reduced only at 60 DAA. By analysing these compounds during ripening for each genotype, it is possible to verify that the great majority exhibited gradual and significant increments during fruit developed. It is worth mentioning that these compounds reflected well the behaviour found in 2-oxoglutarate. Similar behaviour to the compounds derived from 2-oxoglutarate was also verified for cysteine, serine and glycine, which are all derived from 3P-glycerate. Not only the amino acids valine, leucine and alanine, which are synthesized from pyruvate, but also phenylalanine and tyrosine, presented lower contents during all development in both genotypes, with significant reduction in the three stages just for leucine. The levels of tryptophan were distinct between WT and *gib3* fruits at 40 and 60 DAA, and a gradual increment was observed from 40 to 60 DAA in WT fruits. For tyramine there was similar content for both genotypes at 40 and 60 DAA whereas at 20 DAA we were unable to identify this metabolite for *gib3* fruits. Similarly, to tryptophan, an increased content of tyramine was observed at 60 DAA.

Although relatively few morphological or metabolic disturbances have been observed between genotypes during fruit development, it seems reasonable to suggest that the changes observed in 2-oxoglutarate, the substrate of the 2-oxoglutarate dehydrogenase that also generates the reduced coenzyme NADH, may also affect the redox balance. Therefore, we decided to assay the levels of pyridine dinucleotide during fruit ripening in WT and *gib3* plants. Surprisingly, however, alterations in neither pyridine nucleotide level per se nor in the NADH/NAD and NADPH/NADP ratios were observed between genotypes across fruit ripening (**Supplementary Fig. 6**).

Discussion

The effects of the manipulation on the endogenous levels of GAs on plant growth have been previously described (Strader et al. 2004; Wuddineh et al. 2015; Zhang et al. 2016). To date, the morphological and biochemical mechanisms underlying the plant response to fluctuations in the levels of GAs during fruit development are not yet defined. Here, we decided to explore the influence of changes in the endogenous levels of GAs during reproductive phase in tomato fruits. This approach has revealed novel insights into the links between GA-related fertilization in tomato and yield together with a range of associated metabolic changes. It has been previously shown that GAs are directly involved in plant growth and development including flowering and fruit development (Olszewski et al. 2002; Gao et al. 2012; Andrés et al. 2014). Moreover, GAs regulate floral development and play an important role in male and female fertility (Griffiths et al. 2006; Pimenta Lange et al. 2012; Pimenta Lange and Lange, 2016). Thus, the compromised reproductive development observed in *gib2* and *gib1* mutants (mildly and extremely deficient in the biosynthesis of GAs, respectively), completely differing from the mutant genotype *gib3* (moderately deficient in GA biosynthesis) and WT (Fig. 1), is likely explained by the reduced content of GA4 and GA7 in both genotypes, as well as the reduction of GA9, a precursor of GA4, in *gib1* mutant plants (**Supplementary Fig. 2**), a mutant whose phenotype is more drastic. Thus, *gib2* and *gib1* mutants had their development restricted to the flower bud stage as previously verified (Nester and Zeveaart, 1988; Jacobsen and Olszewski, 1991). Similar changes were also observed previously in rice (*Oryza sativa* L. cv Nipponbare) overexpressing the enzyme GA2ox (Sakamoto et al. 2001), as well as in *Arabidopsis*,

petunia, and tobacco that overexpressed *GAMT1* (Varbanova et al. 2007), in good agreement with the need of adequate GAs levels for the complete flower development (Groot et al. 1987; Van den Heuvel et al. 2002). Despite the fact that the anther development in *gib2* and *gib1* was retained just prior to meiosis, 75 to 90% of this phenotype can be reversed by exogenous application of bioactive GAs at concentrations higher than 50 ng per bud (Nester and Zeevaart, 1988; Shinozaki et al. 2015). Moreover, all *gib* mutants used here are typical GA-responsive mutants since all aspects of the mutants such as reduced plant height, leaf size and flower morphology can be reversed to WT by GA application (Koornneef et al. 1990; Asahina et al. 2002). Although *gib3* mutant plants (moderately deficient in the biosynthesis in GAs) were characterized by minor morphological differences, at inflorescence level, in relation to its WT counterpart (Fig. 2A and B), the endogenous reduction of GAs was not able to affect both the final phenotype and fruit production (**Supplementary Fig. 3** and Fig. 4). Likewise, the results described here are in good agreement with not only with the reduction observed only for the content of GA7 (**Supplementary Fig. 2**) but also with those previously observed for the same genotypes when only the vegetative stage was evaluated (Martins et al. 2019; Omena-Garcia et al. 2019). By contrast, both GA20ox overexpressing plants and the *procera* mutant in tomato showed numerous variations in floral organs coupled with higher number of fruits per plant and a reduced fruit size (Olszewski et al. 2002; Carrera et al. 2012; Nir et al. 2017). It seems reasonable to assume that this smaller fruit size was likely triggered by differences in source-sink strength. It is also worth noting that the final size of a fruit, in addition to the genotype, can be influenced by the position it occupies in the plant stem, climatic conditions and cultural practices to which the plants are subjected (Srivastava and Handa, 2005). It is equally important to mention that a tight fine-tuning between auxin and GA signalling (Hu et al. 2018) as well as a crosstalk between GAs and two unrelated miRNAs (Silva et al. 2019) are seemingly essential for controlling the switch from flower into fruit development in tomato. Moreover, overexpression and loss-of-function of PROCERA, a single tomato DELLA protein, culminated in significant changes in growth, flowering, fruit development, and transpiration (Livne et al. 2015; Nir et al. 2017). Therefore, future genetic studies are clearly required to fully achieve mechanistic understanding of the role of GAS in tomato floral transition and inflorescence development.

Developmental stages of tomato fruits can be divided in time specific steps defined as: (i) floral development and fruit set; (ii) growth characterized by cell division and expansion; (iii) maturation; and (iv) ripening/senescence (Gillaspy et al. 1993; Srivastava and Handa, 2005; Obroucheva, 2014; Shinozaki et al. 2015). It is known that GAs are involved in cell division (Lammens et al. 2008; Sablowski and Carnier Dornelas, 2014), and cell expansion process (Bouquin, 2003; Band et al. 2012) and as such our analyses were carried out at different stages of fruit development. For all morphological parameters (size, diameter, fresh and dry weight, volume) reduced values were observed for *gib3* fruits with 20 and 40 DAA (Fig. 3A-D and 3F); however, at 60 DAA these differences were no longer found suggesting, thus, that the reduction of GAs only promoted a delay in early growth and development of the mutant fruits. These results corroborate with the model previously suggested by (Srivastava and Handa, 2005), in which GA reaches maximum levels during fruit formation mainly between 7 to 15 DAA and 3 to 5 weeks after anthesis. Remarkably, this time match exactly with cell division and cellular expansion phases.

The impacts of GAs on primary metabolism has been also previously demonstrated (Araújo et al. 2012a; Ribeiro et al. 2012a; Andrés et al. 2014; Nir et al. 2014), although this relationship still remains unclear. Among metabolic changes occurring throughout fruit development the differential accumulation of pigments is visually perceptible (Carrari et al. 2006). The greenish tint of the fruit became reddish across fruit ripening (**Supplementary Fig. 4**). This fact is represented by the reduction of chlorophyll contents (Fig. 4A and B) and consequent carotenoids accumulation (Fig. 4C) that clearly differed between genotypes from 40 DAA being always higher in *gib3* fruits. The presence of little, if any, difference between genotypes when we evaluated the amino acid contents (Fig. 4E), proteins (Fig. 4F) and the organic acids malate (Fig. 5E) and fumarate (Fig. 5F), probably reflect the minor influence of GAs on these metabolites during fruit development. Here, the changes observed occurred mainly at 60 DAA and may be a direct consequence of the natural ripening process (Carrari and Fernie, 2006; Araújo et al. 2012b), that seems to not be affected by relatively small reductions in the levels of GAs. By analysing the contents of soluble sugars little difference was found between WT and *gib3* fruits, although the contents clearly increase in fruits of both genotypes during ripening (Fig. 5A, B and C). They increased in an essentially linear manner reaching maximum values at 60 DAA, which is consistent with results observed for glucose and fructose in tomato fruits previously (Carrari et al. 2006). In addition, the changes observed in soluble sugars content largely justifies the starch levels (Fig. 5D), that increased until 40 DAA and decreased in fruits at 60 DAA, when we observed increases in the soluble sugars levels. This increase on sugars content and decrease on starch between 40 to 60 DAA, however, was similar to that found in tomato fruits at 35 and 65 DAA of plants with low expression of the mitochondrial fumarase (Centeno et al. 2011). Perhaps more importantly, this inversion in the content of carbohydrate reserves as well as other metabolites during the fruit ripening process culminates in organoleptic and desirable quality characteristics such as texture, colour, taste and flavour (Srivastava and Handa, 2005; Zhang et al. 2015; Qin et al. 2016). When considered together, these results coupled with the relatively minor changes in fruit set and fruit growth suggest that GA levels is important per se for normal fruit development and performance. However, it is highly interesting that, despite the dramatic changes in leaf metabolism and plant development observed when GA levels are strongly reduced, relatively minor changes in GA levels, as in *gib3* mutant plants (Bensen and Zeevaart, 1990), do not strongly impact fruit set, yield, and germination rates, suggesting the presence of sophisticated signaling mechanisms by which the plant uses the senescence process to prioritize the supply of nutrients to sustain reproductive tissues.

The alterations observed between fruits of WT and *gib3* mutant plant cannot be directly explained by the influence of GAs in the process of this organ development, at least on late stages, where the alterations look like as common change between semi autotrophic and heterotrophic metabolisms. Therefore, it was decided to follow the repertoire of metabolic changes that occur during fruit development. Large changes in the levels of amino acids were verified between genotypes and throughout fruit development, especially at 40 and 60 DAA (Fig. 6). Largest increases were observed in amino acids derived from oxaloacetate, 3P-glycerate and 2-oxoglutarate, and importantly these results are consistent with previously described results (Carrari and Fernie, 2006; Araújo et al. 2012b). Besides its direct involvement in amino acid metabolism, 2-oxoglutarate is also involved in the metabolism of several metabolites

including flavonoids, glucosinolate, alkaloids, coumarins and Gas (Araújo et al. 2014). Enzymes involved in the final stages of both synthesis and degradation of GAs (GA3ox, GA20ox and GA2ox, respectively) are part of the large 2-oxoglutarate dependent dioxygenases family (Hedden and Phillips, 2000). Notably, the relationship between 2-oxoglutarate and GAs metabolism was previously demonstrated in tomato plants with reduced 2-oxoglutarate dehydrogenase (OGDH) activity. These plants displayed reductions in the transcript levels of the GA2ox4 and GA3ox3 and consequently in the GA3 content, one of the bioactive form of Gas (Araújo et al. 2012b). Our results aid further evidence in the connection between primary metabolism, mediated by changes in 2-oxoglutarate, and GAs metabolism supporting the growing body of evidence associated with a significant network behaviour in the coordination of amino acid metabolism (Galili, 2011; Galili et al. 2014; Hildebrandt et al. 2015).

Increases in the contents of the amino acids histidine, proline, hydroxyproline, ornithine and glutamine are consistent with the observed increase in glutamate, from which they all derive. Increments on the levels of glutamate during fruit ripening is of pivotal significance during this process (Grierson, 1992), since it is a direct precursor of the biosynthetic pathway of chlorophyll (Carrari et al. 2006; Szafranska et al. 2017). Accordingly, chlorophylls are usually present at very low concentration or even absent in mature fruits (Carrari et al. 2006). This reduction occurs due to the degradation of chlorophylls and carotenoids (Ye et al. 2015), characteristic of the conversion of chloroplasts to chromoplasts during fruit ripening which is visually perceived by colour transitions (Bartley et al. 1994; Kahlau and Bock, 2008). Changes in sugar metabolism and its effects on fruit ripening have been previously demonstrated, although the exact mechanism associated with sugar has not yet been fully elucidated (Qin et al. 2016). Carbohydrates are the most abundant compounds on soluble solids of ripe tomato fruits, where the content of glucose, fructose and sucrose are mainly used as indicators of quality (Baxter et al. 2005; Zhang et al. 2015). Increases in soluble sugars were observed in fruits of both genotypes during fruit ripening, ranging from 40 to 70% between 40 and 60 DAA. High sucrose levels were also observed in ripe peach fruits (Vimolmangkang et al. 2016) and berry (Tilbrook and Tyerman, 2009). As such, it is important to mention that different processes can contribute to the increase of soluble sugars in fruits (Dai et al. 2016) including first the import of photoassimilates from leaves via phloem, sucrose being the main sugar transported in higher plants (Braun and Slewinski, 2009; Kühn and Grof, 2010). It is worth to notice that this process follows different patterns and mechanism of unloading according to the species (Ruan and Patrick, 1995; Lalonde et al. 2003; Kühn and Grof, 2010; Biais et al. 2014; Zhang et al. 2015). Secondly, the metabolism of imported compounds into different cell compartments (apoplasm, symplasm and/or vacuole) to synthesize organic acids, cell walls and storage compounds, such as starch. Thirdly, dilution which may change the concentration without, however, altering the amount of the metabolite and is dependent on the environmental conditions (Genard et al. 2014; Dai et al. 2016). Notably, a close relationship was verified here between the increase of the concentration of soluble sugars and the degradation of the starch between the 40 and 60 DAA. It should be noticed that tomato fruit breeding has been largely oriented, through processes of improvement, to sugar content higher than 60% of the dry weight (Yelle et al. 1988; Carrari and Fernie, 2006). Although the precise nature of the interaction between

energy and sugar metabolism coupled with the GA-mediated regulation of fruit growth and development could not be fully resolved in this study, it clearly remains an exciting topic for future research.

Conclusions

We investigated the role played by GAs during the transition process from vegetative to reproductive phase and during the reproductive organs development. The function of GAs in the maintenance of the flowering process was very clear in *gib2* and *gib1* mutant plants. Reductions in the endogenous content of bioactive GAs observed in those mutant plants was associated with a significant restriction in their development at floral bud phase. We also found that fruits of *gib3* plants showed little difference from WT fruits. Surprisingly, the morphological variations that were observed in young fruits were not sustained during fruit development with the phenotype being fully recovered in red ripe fruits. Our result indicates that the reduced GAs content verified in *gib3* mutant promotes only a delay in fruit development which is in good agreement with the action of this hormone being solely reported in the early stages of fruit development. The metabolic changes observed between genotypes started mainly at 40 DAA, and this is the phase characterized by the natural fruit ripening and in which there are no reports of the presence and/or action of GAs. Thus, we here present compelling evidence for a significant function of GA in governing early fruit set and development despite minor metabolic changes during later stages of fruit ripening. Although the absence of alterations in fruit yield and seed production despite reductions in the levels of GAs is somewhat surprising it is tempting to speculate that pathways of energy metabolism and hormone metabolism and transport are most likely highly interconnected at the whole-plant level in a manner that allows the plant to prioritize reproductive organs during plant senescence. It will be important to establish the functional significance of this observation in future studies in order to fully understand the molecular regulatory hierarchy regulating GA balance at the whole-plant level.

Declarations

Author contributions:

AOM, AN-N, DMR and WLA designed the research; AOM performed most of the research with the support of WAS, FSO and RPO-G; JGV and SO performed gibberellin analysis; JGV, SO ARF, AN-N, and DMR analysed the data, discussed the results and complemented the writing; AOM, and WLA analysed the data and wrote the article which was later approved by the all the others.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Figures

Figure 1

Photographic record of the monitoring of inflorescence development in response to changes in gibberellins (GAs). The following genotypes were weekly monitored: WT (wild type) and mutants: *gib3* (moderately deficient in GAs), *gib2* (intermediate deficiency in GAs) and *gib1* (extremely deficient in GAs). Scale: bars = 1 cm

Figure 2

Variations on flowering development parameters in tomato plants in response to reduced endogenous of GA levels in WT and mutants plants deficient in the biosynthesis of gibberellins (*gib3*, moderately deficient, *gib2*, intermediate deficiency and *gib1*, extremely deficient). Number of inflorescences per plant (A); Total flowers per inflorescence (B); Floral anthesis period (C); Number of non-viable pollen grains (D). Means followed by the same lowercase did not differ from the control in ($P < 0.05$), according to the Scott-Knott test; and ND means parameter not determined. Values are presented as means of ten biological replicates $\pm$ SE.

Figure 3

Variations on morphological development parameters in tomato fruits in response to reduced endogenous of GA levels in WT and mutants plants deficient in the biosynthesis of gibberellins (*gib3*). The fruits were evaluated in three different ages: 20, 40 and 60 DAA. Height of fruit (A); Diameter of fruits (B); Fresh weight of fruit - FW (C); Dry weight of fruits - DW (D); Ratio DW:FW (E); Volume of fruits (F). Lowercase letters indicate significant differences between genotypes within each time, and capital letters indicate significant differences between periods within each genotype ($P < 0.05$), according to the Scott-Knott test. Abbreviations as described in Figure 1. Values represent the means ± SE of at least ten independent fruits/plants.

Figure 4

Variation on nitrogen metabolism in tomato fruits in response to reduced endogenous levels of GA levels in WT and mutants plants deficient in the biosynthesis of gibberellins (*gib3*). The fruits were evaluated in three different ages: 20, 40 and 60 DAA. Chlorophyll a (A); Chlorophyll b (B); Chlorophyll a/b (C); Carotenoids (D); Amino acids (E); Total soluble proteins (F). Lowercase letters indicate significant differences between genotypes within each time, and capital letters indicate significant differences between periods within each genotype ($P < 0.05$), according to Scott-Knott test; and ND means compounds not determined. Abbreviations as described in Figure 1. Values represent the mean $\pm$ SD of at least ten independent fruits.

Figure 5

Variation on carbon metabolism related metabolites in tomato fruits in response to reduced endogenous levels of GA in WT and mutants plants deficient in the biosynthesis of gibberellins (*gib3*). The fruits were evaluated in three different ages: 20, 40 and 60 DAA. Glucose (A); Fructose (B); Sucrose (C); Starch (D); Malate (E) and Fumarate (F). Lowercase letters indicate significant differences between genotypes within each time, and capital letters indicate significant differences between periods within each genotype (P < 0.05), according to Scott-Knott test. Abbreviations as described in Figure 1. Values represent the mean $\pm$ SE of at least ten independent fruits.

Figure 6

Variation on metabolite profiling in tomato fruits in response to reduced endogenous levels of GA levels in WT and mutants plants deficient in the biosynthesis of gibberellins (*gib3*). Continuous arrows indicate a one-step reaction, and broken arrows indicate a series of biochemical reactions. Lowercase letters indicate significant differences between genotypes within each time point, and capital letters indicate

significant differences between periods within each genotype ($P < 0.05$), according to Scott-Knott test. Abbreviations as described in Figure 1.

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