AUXILIADORA OLIVEIRA MARTINS

THE FUNCTIONS OF GIBBERELLINS IN DIFFERENT TISSUES OF THE TOMATO PLANT (Solanum lycopersicum L.)

Thesis presented to the Universidade Federal de Viçosa, as part of the requirement of the Plant Physiology Graduate Program for the obtention of the degree of Doctor Scientiae.

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To my parents: Nadir and Expedito,
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these years, teaching me mainly the importance of the
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"I can not give you everything you want or need.
So fly and get it for yourself. "
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CONTENTS

ABSTRACT

MARTINS, Auxiliadora Oliveira, D.Sc., Universidade Federal de Viçosa, July, 2017. **The functions of gibberellin in different tissues of the tomato plant (Solanum lycopersicum L.)**. Adviser: Wagner Luiz Araújo.

This thesis is largely focused on improving the current understanding about the role played by gibberellins (GAs) levels in different tissues and in the fruit development process. Two well-defined goals were established: (I) to gain more insights into how and to which extent manipulation of GA levels might differently affect plant growth in general; (II) to generate experimental evidences for the role GAs play during the transition from vegetative to reproductive phase. For this, we firstly reviewed the current evidence of the connection between central energy and GAs metabolism, via the tricarboxylic acid (TCA) cycle. The key point at this process is the 2-oxoglutarate, a TCA cycle intermediate, which has been suggested to play a role in the control of GA biosynthesis. This control is due to the fact that the enzymes responsible, ultimately for the maintenance of the pool of bioactive GAs, are dependent on 2-oxoglutarate as cofactor. Following, different but complementary experiments were carried out using wild type (WT) and mutant tomato (Solanum lycopersicum L.) plants in the GAs biosynthesis (gib3: moderately deficient, gib2: moderately deficient and gib1: extremely deficient). In the first experiment WT plants and the mutants were used to investigate, in details, the metabolic and physiological impacts of the variation of GA levels in shoot and root tissues. The results showed that, in general, depletion in the endogenous GAs levels promoted greater impacts on leaf tissues when compared to root tissues. In both tissues, gib3 mutant plants were very similar to WT. On the other hand, gib2 and gib1 mutant plants presented drastic and gradual reductions in carbohydrate contents in the foliar tissues, with relatively fewer alterations in roots. Similar behavior was also observed for malate and fumarate. It should be noted that these reductions largely follow the pattern of the reduction

of the endogenous GAs content. We also observed that, although the total amino acid pool was not strongly impacted, the individual profile of amino acids was significantly altered with the highest variations occurring in the shoots of gib2 and gib1 mutant plants. These results suggest a differential fine-tuning of metabolism as a function of GA content fluctuations in shoot and roots. The second experiment was conducted with the same genotype as described previously, and the impact of GA reduction on flowering and fruit development was evaluated. Reductions in GA levels affected the reproductive process in gib2 and gib1 mutant plants, thus compromising the natural perpetuation of the genotypes, since floral development was restricted at the bud level. Few variations were observed between WT and gib3 mutant plants in relation to the flowering process, production and final fruit morphology. When evaluating fruit development in three different phases during ripening, we observed that both morphology and metabolism were altered in the early stages, with generally lower values in fruits of gib3. However, at the final stage of the ripening the phenotype is completely recovered. Thus, our results indicate that the changes occurred in the transition from semi autotrophic to completely heterotrophic metabolism and that the reduced GAs content verified in gib3 mutant promotes only a delay in fruit development in good agreement with the action of this hormone being only reported in the early stages of fruit development. The effect of impaired GA biosynthesis appears to be fairly specific, particularly on floral establishment and metabolic reprogramming during fruit development.

RESUMO

MARTINS, Auxiliadora Oliveira, D.Sc., Universidade Federal de Viçosa, julho de 2017. **Funções das giberelinas em diferentes tecidos de tomateiro (Solanum lycopersicum L.).** Orientador: Wagner Luiz Araújo.

A presente tese teve como foco ampliar os conhecimentos acerca do papel desempenhado por diferentes níveis de giberelinas em diferentes tecidos e no processo de desenvolvimento de frutos. Dois objetivos bem característicos foram estabelecidos: (I) obtenção de maiores informações sobre como e, em que medida a manipulação dos níveis de GA pode afetar diferencialmente o crescimento das plantas em geral; (II) gerar evidências experimentais do papel fundamental desempenhando pela GAs durante a transição estadio vegetativo-reprodutivo. Para tal, foram realizados diferentes experimentos utilizando plantas tipo selvagem (WT) e mutantes na biossíntese de giberelinas (gib3: moderadamente deficiente, gib2: medianamente deficiente e gib1: extremamente deficiente), juntamente com a elaboração de um review. Neste review foram apresentadas evidências da conecção do metabolismo central, via ciclo dos ácidos tricarboxílicos (TCA), ao metabolismo da GAs. O ponto chave desse processo é o 2-oxoglutarato, um intermediário do ciclo TCA, e que foi sugerido de apresentar um papel no controle da biossíntese de GAs. Esse controle se deve pelo fato de que as enzimas responsáveis, em última instância, pela manutenção do pool de GAs biotivas são dependentes do 2-oxoglutarato como cofator. No primeiro experimento foram utilizadas plantas WT e os mutantes gib3, gib2, e gib1, nas quais foram avaliados os impactos metabólico e fisiológico da variação dos níveis de GAs em tecidos foliares e radiculares. Os resultados mostraram que, de modo geral, a depleção nos níveis endógenos de GAs promoveu maiores impactos nos tecidos foliares quando comparados a tecidos radiculares. Em ambos os tecidos o mutante gib3 foi bem similar ao WT para a maioria das análises. Os mutantes gib2 e gib1 apresentaram drástica e gradativa reduções nos conteúdos de

carboidratos nos tecidos foliares, com poucas alterações nas raízes. Semelhante comportamento foi verificado para malato e fumarato. Vale lembrar que essas reduções seguem o padrão da redução do conteúdo endógeno de GAs. Também verificou-se que, embora o pool de aminoácidos tenha apresentado algumas variações, o perfil individual dos aminoácidos mostrou-se bastante alterado, tendo as maiores variações nos tecidos foliares dos mutantes gib2 e gib1. Esses resultados sugerem um ajuste fino do metabolismo em função das flutuações do conteúdo de GAs. O segundo experimento foi conduzido com os mesmos genótipos descritos anteriormente, avaliando-se o efeito da redução das GAs no processo de florescimento e desenvolvimento de frutos. Reduções dos níveis de GAs afetaram drasticamente o processo reprodutivo em plantas gib2 e gib1, comprometendo assim a perpetuação natural dos genótipos, visto que o desenvolvimento floral ficou restrito a nível de botão. Poucas variações foram encontradas entre plantas WT e gib3 no que se refere ao processo de florescimento, produção e morfologia final dos frutos. Quando avaliou-se o desenvolvimento dos frutos em três fases distintas do desenvolvimento, observamos que tanto a morfologia quanto o metabolismo foi alterado nas fases inicias, com valores geralmente menores em frutos gib3. No entanto, durante a fase de amadurecimento o fenótipo é completamente recuperado. Assim, os resultados obtidos indicam que as modificações ocorridas na transição do metabolismo semi autotrófico a completamente heterotrófico é independente das GAs.

CHAPTER I

GENERAL INTRODUCTION

Higher plants are sessile and therefore cannot escape from adverse environmental conditions that are a constant throughout the plant life cycle. The immobile nature of plants thus needs more protection and enabled them to develop unique mechanisms that allow plants to adapt and survival to different environmental conditions (Golldack et al., 2013; Martinez-Bello et al., 2015; Tyler et al., 2004). To cope with these stressful conditions a highly regulated alterations in their metabolic pathways is required to adjust to changed environments. In general these adjustments occur through the integration of intrinsic (hormones) and extrinsic signals (environmental stimuli) (Hou et al., 2013; Li and He, 2013). Phytohormones are highly bioactive natural products of low molecular weight that are often present in very low concentrations acting as chemical messengers, triggering and controlling physiological processes during plant growth and development as well as in response to plant stresses (Chiwocha et al., 2003; Novak et al., 2017).

It is important to mention that hormones do not act only in a linear path and as such, the pathways of hormonal synthesis and degradation are interconnected by a complex network of feedback loops and interactions that ultimately determine the final output of individual hormonal actions. Hormonal networks exhibit strong flexibility and allow for multiple transcriptional and post-transcriptional interactions at the level of metabolism, transport, signaling, and downstream responses to integrate many internal and external stimuli that affect different development processes throughout the plant's life cycle (Vanstraelen and Benkova, 2012). To further complicate this exquisite network there is an extensive feedback in these pathways coupled with significant cross-regulation (Lilley et al., 2013; Vanstraelen and Benkova, 2012). To date, at

least eight classes of hormones have been identified and characterized in plants including auxins, gibberellins (GAs), abscisic acid (ABA), cytokinins (CKs), ethylene, brassinosteroids (BRs), jasmonates (Tg) and strigolactones (Hedden and Thomas, 2012; Li and He, 2013; Sanchez-Rodriguez et al., 2010). Among these hormones, GAs and BRs are the main growth promoters (Chiwocha et al., 2003; Hedden and Thomas, 2012), whereas vegetative growth patterns are controlled largely by the combined actions of auxins, CKs, strigolactones, GAs, and BRs (Davies, 1995; Wolters and Jurgens, 2009). This thesis is mainly focused on enhancing our understanding of the role of GAs in governing plant growth and impacting plant metabolism in general. This is mainly due to the fact that the GA signaling pathway is a critical control point through which diverse environmental and hormonal factors are integrated into growth responses. Thus, in the following sections below I briefly review the current knowledge regarding the GA synthesis and signalling pathways and briefly examine how they participate in the regulation of growth, and flowering in higher plants.

On the metabolism and the functions of GAs

GAs are a complex family of structurally related tetracyclic diterpenoid compounds with a regulatory function in various developmental processes in plants, including seed germination, stem elongation, leaf expansion, trichome differentiation, floral initiation, pollen maturation and fruit development (Achard et al., 2006; Chen et al., 2014; Daviere and Achard, 2013; Gao et al., 2011; Li and He, 2013; Ragni et al., 2011; Ueguchi-Tanaka et al., 2005). Over than 130 molecules have been already identified, but only four of them are biologically active, namely GA_1, GA_3, GA_4 and GA_7 (Camara et al., 2015).

The action of GAs has been extensively associated with stem elongation and germination and in some plants it promotes flowering and fruit development. Remarkably, genetic or chemical manipulations of GA levels are usual agricultural practices to optimize plant growth and yields. The development of semidwarf grain varieties presenting an attenuated stem elongation was the single most significant achievement in 20th century agricultural practices that culminated with a dramatically increased global crop yields (Hedden, 2003; Phillips, 2004).

The action of GAs occurs indirectly regulating plant growth by promoting the inhibition of growth repressor factors. This can be compared to a car parked on a hill which has its movement prevented by the action of the brakes. When there is brake release, or in other words the inhibition of the inhibitor, other forces such as gravity, can act promoting the movement of the car. Similarly, GA does not directly cause growth, but rather interferes with the action of growth inhibitors (TTPB9LectureNote, 2014).

Several factors involved with the signal transduction pathway have been identified through GA response mutants (Achard et al., 2006; Daviere and Achard, 2013; Richards et al., 2001). The biosynthesis of GAs is highly complex being regulated by both intrinsic and extrinsic factors, which in turn, modulate the homeostasis of this hormone.

Although GAs are reported to be synthesized at the site of action (Kaneko et al., 2003), there are certain organ or tissue that cannot produce enough GAs by itself and are as such dependent on the import of this hormone from other parts of the plant (Lenton et al., 1994; Pimenta Lange and Lange, 2016; Yamaguchi, 2008). The precise mechanisms involved at the GAs transport remains relatively unclear; however, it is well known that the transport of GAs over long distances occurs in several species of plants. Thus, GAs synthesized in the leaves have been implicated in floral initiation at the apical meristem (King, 2012), as well as in organ

growth (Chen et al., 2016; Dayan et al., 2012; Hedden and Sponsel, 2015; Ragni et al., 2011). Furthermore, application of stable deuterium-labeled gibberellins, namely GA_9, GA_{12}, GA_{19}, GA_{20} and GA_1, as well as grafting of GAs mutants with WT has shown that the transport of this hormone can occur in both long and small distances mediated by the xylem and/or phloem. Interestingly, it was also verified that inactive form are predominant over the active ones (Pimenta Lange and Lange, 2016; Proebsting et al., 1992; Regnault et al., 2015). By using grafting experiments it was expertly demonstrated that both roots and shoots produce sufficient precursors and/or GAs to compensate the deficient biosynthesis in mutants used as scions and stocks (Ragni et al., 2011).

Once we considered that the shoot is self-sufficient in the production of GAs, the import of root GAs does not appear to be physiologically relevant (Hedden and Sponsel, 2015). Another intriguing feature is that root tissues are usually characterized by a higher sensitivity to GAs when compared to shoot tissues (Barboza-Barquero et al., 2015). This sensitivity is extremely high, varying from concentrations of nM in roots to μM concentrations in shoots (Tanimoto, 1987; Tanimoto, 2012). This differential response between shoots and root system was also observed following the application of biosynthetic inhibitor of GAs in Arabidopsis thaliana plants (Bidadi et al., 2010). These authors verified that under the presence of uniconazole there were increases in root length whereas an inhibition of shoot growth was observed which was, most likely, due to an increased expression of GA20ox and GA3ox at the roots. Hybrids of populus with reduced sensitivity and biosynthesis of GAs also presented a higher allocation of biomass within the roots (Elias et al., 2012).

Several studies have clearly demonstrated that levels and signaling of GA also affect plant response to drought, salinity and other environmental stresses (Achard et al., 2006;

Magome et al., 2004; Shan et al., 2014). In this context, it has been shown that Arabidopsis plants deficient or insensitive to GAs present higher tolerance to salinity (Magome et al., 2004). This higher tolerance to different stresses can also be partly due to the differential response presented by the root system in detriment to the shoot under these conditions (Sharp, 2002; Sharp and Davies, 1989). It seems reasonable to suggest that signs originating from the roots are likely transported to shoot, which result in the reduction and/or inhibition of plant growth/development under unfavorable conditions (Arraes et al., 2015; Chu et al., 2014; Golldack et al., 2013; Sharp, 2002; Valluru et al., 2016; Xiong et al., 2006; Zhang et al., 2007).

GAs also play a key role in the transition from vegetative to reproductive stage. This transitional process is called flowering and has fundamental significance for plant development. Thus, an adequate flowering time ensures the success of the next generation and consequently the species continuity (Romera-Branchat et al., 2014; Song et al., 2015; Takeno, 2016; Turnbull, 2011). The involvement of GAs at the flowering has been reported in Arabidopsis plants deficient in the GAs biosynthesis and/or defects in signal transduction (ga1-6 and gai, respectively) showing that this mutation is associated with a delay in flowering (Koornneef and van der Veen, 1980; Peng and Harberd, 1993). On the other hand, plants with high GAs signaling (rgl2 and spy) were characterized by an early flowering when compared to WT (Cheng et al., 2004; Jacobsen and Olszewski, 1991).

The mechanisms by which GAs act at the flowering process are still not very clear (Olszewski et al., 2002; Pimenta Lange and Lange, 2016); however, it is known that GAs act at the differentiation and the maintenance of the floral meristem (Andrés et al., 2014; Fleet and Sun, 2005). Once there is differentiation of the reproductive meristem, the floral organs formation is initiated, culminating in the production of fruits, as long as there are favorable

environmental conditions. It is important to mention that fruit development goes through different phases, from fertilization process to maturation/senescence. Initially it was thought that fruit development occurred as a function of the hormones synthesis during seeds development (Gustafson, 1939; Nitsch, 1952). However early abortion of ovary fertilized in peas prevented fruits development (Eeuwens and Schwabe, 1975). Another evidence that set fruit may be independent of fertilization and seed development was the demonstration that the exogenous application of GAs resulted in the formation of parthenocarpy fruits (Dorcey et al., 2009). Although several hormones have been reported to be involved at the promotion of parthenocarpy fruits, such as auxins (Balbi and Lomax, 2003; Pandolfini et al., 2007), brassinosteroids (Fu et al., 2008), CKs (Li et al., 2003; Yu et al., 2001) and GAs (Fos et al., 2001; Olimpieri et al., 2007), only unpollinated ovaries treated with GA_3 resulted in the development of fruits similar to those of pollinated ovaries (McAtee et al., 2013). The presence of GAs, together with other hormones, during the phases that embrace from set fruit to total fruits growth is likely associated with the action of GAs in both cell division and cell expansion processes (Bouquin et al., 2002; Claeys et al., 2012; Locascio et al., 2013; Ubeda-Tomas et al., 2008). This facts apart, it is important to stress that GAs have not been reported to date as able to act at the maturation and ripening/senescence phases (Fos et al., 2000; Shinozaki et al., 2016). During the maturation stage, it seems that fruits present all the necessary skills for ripening, although it has not yet started (Cara and Giovannoni, 2008; McAtee et al., 2013). During the ripening process, all fruit types face up to both physiological and metabolic modification, which lead to changes in the color and cell wall composition. Unique characteristic in the fleshy fruits is the breaking of carbohydrates stored in sugars and the reduction of acidity. There is also an increase in flavor and volatile aroma (Klee and Giovannoni, 2011). In tomato fruits the most important metabolites

are sugars and organic acids (Roessner-Tunali, 2003; Zanor et al., 2009; Zhao et al., 2017). A close relationship between these metabolites was observed in fruits of tomato plants with lower expression of the mitochondrial malate dehydrogenase (mtMDH) and fumarase (Centeno et al., 2011). Fruits with high content of malate had reductions at the content of sugars, whereas fruits with reduced content of this organic acid presented increases in the carbohydrates levels. Further characterization of plant hormone metabolism and signaling is important to precisely clarify the regulatory networks that operate during ripening.

Layout and aims of the chapters

This thesis is largely focused on the role of GA levels in different tissues and during fruit development. Thus, the main objectives of this work were: (i) to gain more insights into how and to which extent manipulation of GA levels might differently affect plant growth in general; (ii) to generate experimental evidences for the role GAs might play during the transition to juvenile to adult phase. To reach these goals several different but complementary experimental approaches were undertaken and therefore this thesis is organized as a compilation of three independent stand-alone chapters. In each chapter an introduction and discussion as well as details of the methods used are included. At the end of this thesis, one chapter entitled “Concluding Remarks” synthesizes the main findings of this work and a brief discussion about the challenges and perspectives in understanding GA function during plant development is additionally presented.

Chapter 2. 2-Oxoglutarate: linking TCA cycle function with amino acid, glucosinolates, flavonoids, alkaloids and gibberellin biosynthesis

Although the tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (2-OG) is an obligatory substrate in a range of oxidative reactions, it was only recently demonstrated that the antisense inhibition of the TCA cycle enzyme 2-OG dehydrogenase culminate with an modified fruit ripening associated with changes in the levels of GA. In this chapter, recent advances in the biochemistry and metabolism of 2-OG are revisited and its connection with GA in higher plants are presented suggesting that GA biosynthesis is, at least partially, controlled by the levels of 2-OG in plants.

Chapter 3. Differential responses between roots and shoot in tomato plants with different gibberellin levels

An adequate interconnection root-shoot is necessary for the adaptation, survival and optimal plant development in response to different environmental conditions. We have previously observed a decoupling between carbon and nitrogen metabolism in leaf tissues of plants with alterations in the endogenous content of GAs. This was associated with a favoring of the root growth to the detriment of the shoot. In an attempt to better understand how and to which extent this connection occurs and the importance of GAs in different organs, metabolic and physiological analyzes were performed on those different tissues of WT plants and mutants on the GAs biosynthesis (gib3, gib2 and gib1) growing under normal optimal environmental conditions. Our results demonstrated that the greatest perturbations caused by GAs depletion were observed in shoot of mutant plants gib2 and gib1 and that the profile of organic acids and

amino acids were significantly affected suggesting an exquisite metabolic reprogramming following fluctuation in the levels of GAs.

Chapter 4. Both transition from vegetative-reproductive stage and fruits development are mediated by GA in tomato

GAs are also able to regulate, alongside with other hormones, the initial processes of fruit development most likely because GAs are responsible for both division and cell expansion process. Here, we investigate whether fluctuations in the endogenous content of GAs impact fruit development and metabolism during ripening. By using the same mutant plants deficient in the biosynthesis of GAs (gib3, moderately deficient; gib2, intermediate deficiency and gib1, extremely deficient in GA) described above we demonstrated that gib2 and gib1 mutants were characterized by a complete interruption of their reproductive development at the floral bud level. We further demonstrated that the reduction in the content of GAs in gib3 mutant did not promote morphological fruit modifications. Collectively, our results demonstrate that the modification of a semi autotrophic metabolism to a completely heterotrophic metabolism takes place independently of GA content.

Chapter 5. Concluding remarks

Altogether, the results of this work clearly shown that GAs are responsible for acting on various processes of plant development. Moreover, our results indicate that this action is highly dependent on tissue, concentration and/or development stage. Reductions in GA content caused a significant impact on growth and primary metabolism of shoots, with little if any alterations in roots. In this brief section I will discuss and integrate the results obtained in the previous

chapters discussing potential regulatory modes of this intricate metabolic network. Finally, possible avenues for future research on this topic are provided.

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2-Oxoglutarate: linking TCA cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis

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The tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (2-OG) is used as an obligatory substrate in a range of oxidative reactions catalyzed by 2-OG-dependent dioxygenases. These enzymes are widespread in nature being involved in several important biochemical processes. We have recently demonstrated that tomato plants in which the TCA cycle enzyme 2-OG dehydrogenase (2-ODD) was antisense inhibited were characterized by early senescence and modified fruit ripening associated with differences in the levels of bioactive gibberellin (GA). Accordingly, there is now compelling evidence that the TCA cycle plays an important role in modulating the rate of flux from 2-OG to amino acid metabolism. Here we discuss recent advances in the biochemistry and molecular biology of 2-OG metabolism occurring in different biological systems indicating the importance of 2-OG and 2-OG dependent dioxygenases not only in glucosinolate, flavonoid and alkaloid metabolism but also in GA and amino acid metabolism. We additionally summarize recent findings regarding the impact of modification of 2-OG metabolism on biosynthetic pathways involving 2-ODDs.

Keywords: dioxygenases, 2-oxoglutarate dependent dioxygenases, gibberellin, TCA cycle, flavonoid, alkaloid

INTRODUCTION

2-Oxoglutarate (2-OG), a key organic acid of the tricarboxylic acid (TCA) cycle (Lancien et al., 2000; Scheible et al., 2000), is also an obligatory substrate for 2-OG-dependent dioxygenases (2-ODDs), as depicted in Reaction 1. Briefly, dioxygenases can be defined as enzymes catalyzing reactions in which both atoms of molecular oxygen are incorporated into substrates (**Figure 1**). In the hydroxylation reaction catalyzed by dioxygenases, one atom of molecular oxygen is incorporated into the substrate, while the other atom of oxygen is incorporated into 2-OG resulting in the subsequent formation of succinate and the release of carbon dioxide.

The 2-ODDs are considered the largest known family of non-heme oxidizing enzymes (Prescott and John, 1996; Ozer and Bruick, 2007; Kawai et al., 2014). Members of this family are found throughout biology catalyzing a number of oxidation reactions and have been identified in many organisms ranging from prokaryotes to eukaryotes. Furthermore, oxidative reactions catalyzed by 2-ODD are involved in biosynthetic processes leading to materials of medicinal or agrochemical importance including collagen or other modified polypeptides and amino acids, plant secondary metabolites, phytohormones such as ethylene and gibberellins (GAs) as well as β-lactam antibiotics, i.e., penicillins and cephalosporins (Vaillancourt et al., 2006; Loenarz and Schofield, 2008; Martens et al., 2010 and references therein). Altogether this indicates that 2-ODD and its substrate, 2-OG, are highly important in plant metabolism as a whole and thus a massive impact of their genetic modification on plant metabolism in different plant tissues is expected. It is worth mentioning that a difficulty impeding metabolic engineering within this enzyme family is the fact that multiple pathways within the metabolic network could be affected, linking the enzymes to the specific target metabolites.

Here we discuss recent advances in the biochemistry and molecular biology of 2-OG metabolism occurring in different biological systems indicating the importance of 2-OG and 2-ODDs not only in glucosinolate, flavonoid, and alkaloid metabolism but also in GA and amino acid metabolism.

THE IMPORTANCE OF 2-OXOGLUTARATE METABOLISM IN HIGHER PLANTS

2-Oxoglutarate participates in a range of reactions in distinct plant cell compartments (Weber and Flügge, 2002; Foyer et al., 2003), also being a key metabolite at the crossroads of carbon/nitrogen metabolism as it is required for ammonia assimilation (Hodges, 2002). Despite this fact, it still remains rather unclear where the major site of production of 2-OG resides. This organic acid can be produced from either sugar respiration or amino acid transamination following the concerted action of isocitrate dehydrogenases, aminotransaminases, and glutamate dehydrogenases (Lancien et al., 2000). We have previously demonstrated that the mitochondrial enzyme 2-OG dehydrogenase (OGDH) has an important role in 2-OG production and metabolism, controlling the levels of this important organic acid in plant cells (Araújo et al., 2008, 2012a,b). These results in conjunction with others in which similar reduction in the TCA cycle activity was also demonstrated (Sienkiewicz-Porzucek et al., 2008, 2010) indicates

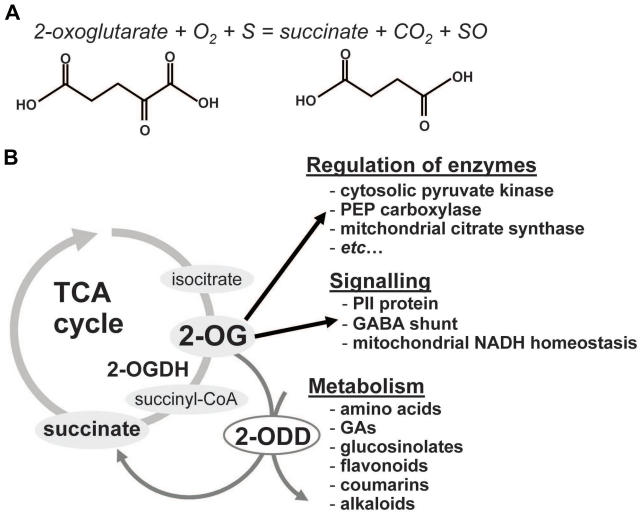

FIGURE 1 | The general reaction mechanism catalyzed by a 2-oxoglutarate-dependent dioxygenase (2-ODD; A) and a schematic overview of reaction and function of 2-ODD in plant (B). (S) is a substrate and (SO) is the hydroxylated product. 2-OG, 2-oxoglutarate; OGDH, 2-oxoglutarate dehydrogenase, GA, gibberellins; 2-ODD, 2-oxoglutarate-dependent dioxygenase.

that mitochondrial TCA cycle enzymes contribute considerably to the regulation of nitrogen assimilation in leaves and that a substantial portion of 2-OG production occurs in the mitochondria itself. It is worth mentioning that the exact balance between the use of cytosolic versus mitochondrial routes for synthesis of 2-OG remains rather unknown. Thus the discovery that the carbon for nitrogen assimilation in leaves comes from carbon stored from the previous night (most likely organic acids such as citrate stored in the vacuole and released to the cytosol; Gauthier et al., 2010) raises the possibility that 2-OG for nitrogen assimilation is generated predominantly in the cytosol and therefore mitochondrial enzymes make only a quantitatively minor contribution. It is important to note that 2-OG is also a direct regulator of several enzymes (e.g., cytosolic pyruvate kinase and PEP carboxylase, mitochondrial citrate synthase, and alternative oxidase) associated with sugar and/or organic acid flux and redox control between cytosol and mitochondria (for a review see Hodges, 2002).

In addition to this, 2-OG has itself been suggested to play a role as a signal metabolite in plants (Lancien et al., 2000; Ferrario-Mery et al., 2001; Feria Bourrellier et al., 2009). This role is, however, largely based on analogy to the important role it plays in conjuncture with the plastidial PII protein in plants (Uhrig et al., 2009). Reports to date suggest that whilst PII may regulates a small number of enzyme systems in plants including *N*-acetyl-glutamate kinase (Ferrario-Mery et al., 2006; Feria Bourrellier et al., 2009) and plastidial acetyl-CoA carboxylase (Feria Bourrellier et al., 2010) its role is unlikely to be as pivotal in plants as in non-plant systems(Araujo et al., 2012). That said it is clear that the production of 2-OG in the mitochondria or cytosol is an important determinant of some plastidial activities.

ON THE CONNECTIONS BETWEEN 2-OXOGLUTARATE METABOLISM AND GIBBERELLIN IN HIGHER PLANTS

Much effort has been expended on elucidating the physiological functions of the various genes regulated by GA (Yamaguchi, 2008). However, studies concerning the associated effects of GA on energy metabolism and growth are rare. This fact notwithstanding, characterization of the pyruvate dehydrogenase kinase 1 (PDK1) has demonstrated that GA modulates the activity of the mitochondrial pyruvate dehydrogenase by regulating *PDK1* expression and controlling growth in rice (Yazaki et al., 2003; Jan et al., 2006). Collectively it also indicates that GA might modify primary metabolism at the entry point of TCA cycle. In addition it has been demonstrated by the overexpression of genes associated with GA biosynthesis or catabolism that GA levels play key roles on transcriptional programs influencing plant growth (Biemelt et al., 2004; Dayan et al., 2010). Furthermore, reduction of TCA cycle enzymatic activity has led to reduction of GA levels in tomato roots (van der Merwe et al., 2009).

In this vein tomato plants with reduced levels of the TCA cycle enzyme 2-OGDH were recently characterized by early leaf senescence and a modified fruit ripening most likely due to differences in the levels of bioactive GAs (Araújo et al., 2012b). Given that the reduction in the activity of this enzyme was associated to a higher impact on respiration rates than observed previously in other TCA enzyme it seems reasonable to suggest that 2-OG might be of critical importance in the regulation of respiration rates in higher plants. It should be mentioned that both the chemical (Araújo et al., 2008) and molecular (Araújo et al., 2012b) inhibition of 2-OGDH was characterized by significant alterations in both sugars and TCA cycle intermediates. Notably, a compensatory augmentation in the flux of the GABA shunt was clearly observed most likely in an attempt to restore the TCA cycle. In addition, the changes in the GABA shunt suggest that its up regulation is needed to maintain succinate supply to the mitochondrial electron transport chain. This finding highlights the metabolic importance of the GABA shunt in plants (Fait et al., 2008) and is similar to findings observed following neuronal OGDH inhibition (Sá Santos et al., 2006; Shi et al., 2009). Remarkably this compensatory up regulation of the GABA shunt was coupled with significant shifts in cellular pools of both nitrate and amino acids in general. Specifically, metabolite profiling of the OGDH antisense lines demonstrated that steady state levels of photorespiratory intermediates, namely glycerate, and glycine, were reduced, coupled with a significant reduction in the label redistribution to glycine and serine (Araújo et al., 2012b). These results suggest that the down-regulation of the TCA cycle activity was integrated with an up-regulation in the flux through the photorespiration pathway as part of a reprogramming to maintain either mitochondrial NADH homeostasis and/or the glutamate pool size. NADH and NADPH levels play an important role in mitochondrial respiratory metabolism and it can explain the maintenance of the levels of both NAD and NADPH in OGDH antisense plants (Araújo et al., 2012b). Altogether the results of this work coupled with others described above have clearly demonstrated that the alteration of the mitochondrial 2-OG metabolism has greater impacts in plant respiration and its connections than previously expected. It is important

to mention that the precise nature of this interaction between 2-OG metabolism and hormone-mediated control of growth and senescence by GA remains an exciting topic for future research. For the purposes of this review perhaps most important is to outline the key role of 2-OG and by extension 2-ODD in GA metabolism.

It also presents metabolic evidence for a key role of 2-OG and 2-ODD in both GA metabolism. The synthetic enzymes involved in GAs biosynthesis have been well-characterized (Graebe, 1987). In GA biosynthesis, several types of oxidation enzymes are involved in GA biosynthesis, such as *ent*-kaurene oxidase (AtKO, CYP701A1) and *ent*-kaurenoic acid oxidase (AtKAO, CYP88A3, and CYP88A4) types, GA-β-hydroxylase and GA20-oxidase (2-ODD) types (Yamaguchi, 2008; Kawai et al., 2014). By great efforts of former works in GA metabolism, several 2-ODDs involved in GA biosynthesis have been found in several plant species (Prescott, 1993; **Figure 2A**); for example, 2β-hydoxylase(s) from *Phaseolus vulgaris* (Griggs et al., 1991) and from *Pisum sativum* (Smith and Macmillan, 1986), 2β-hydoxylase(s) from *P. vulgaris* (Smith et al., 1990), GA20-hydroxylase from *P. sativum* (Lange and Graebe, 1989) and from *Cucurbita maxima* (Lange and Graebe, 1989). Half of the reactions in GA biosynthesis are mainly converted by 2-ODDs (Hedden et al., 1982; Prescott, 1993). The identification and characterization of the 2-ODDs involved in

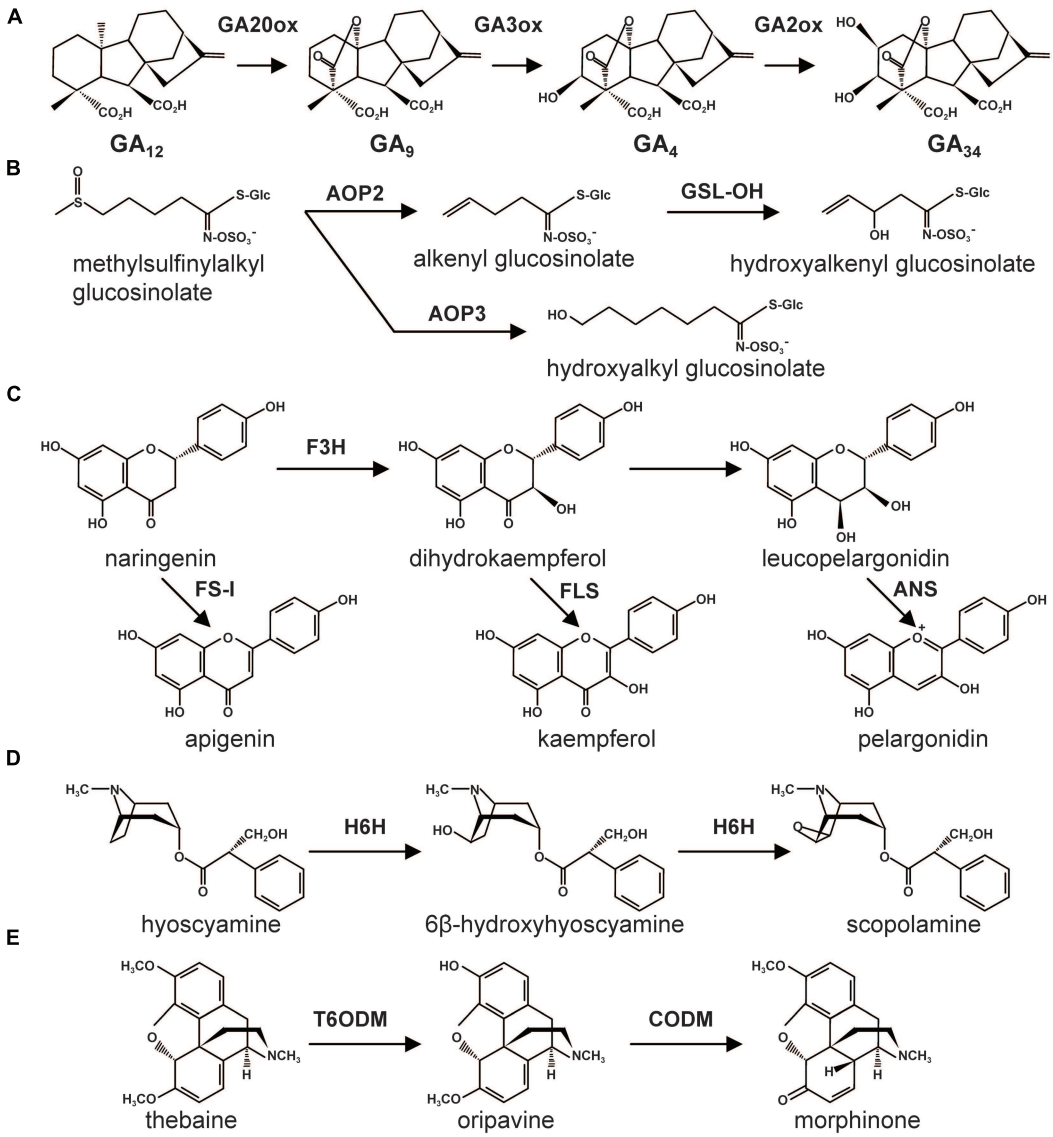

FIGURE 2 | Major enzymatic reactions of 2-ODD involved in GA and plant secondary metabolism. 2-ODDs involved in **(A)** GA, **(B)** glucosinolate, **(C)** flavonoid, **(D)** tropan alkaloid, **(E)** isoquinorine biosynthesis are described. GA20ox, GA20-oxidase; GA3ox, GA3β-hydroxylase; GA2ox, GA 2-oxidase; AOP, alkenyl/hydroxy (OH) alkyl producing enzymes; GSL-OH, glucosinolate 2-oxoacid-dependant dioxygenase gene; FS-I, flavone synthase I; FLS, flavonol synthase, F3H, flavanone 3-hydroxylase, ANS, anthocyanin synthase; H6H, *hyoscyamine 6*-hydroxylase; T6ODM, 6-demethylase; CODM, codeine *O*-demehtylase.

GA biosynthesis has also led to major advances in the elucidation of the pathway including understanding of chemical diversity of GAs. Furthermore, it seems reasonable to consider that a part of GA biosynthesis is controlled by 2-OG content in plant.

THE METABOLIC IMPORTANCE OF 2-ODD IN THE REGULATION OF SECONDARY METABOLISM

The oxygenase which leads to the incorporation of oxygen atoms from molecular oxygen is one of the most important enzymes in terms of conferring variation within plant secondary metabolism. Two functionally different classes of oxygenases, namely cytochrome P450 enzymes and 2-ODD, are involved in several pathways of plant secondary metabolism namely hydroxycinnamates, flavonoids and alkaloids (Tohge et al., 2013). As for GA biosynthesis, almost half of the oxidation reactions which are involved in late steps of their biosynthesis are catalyzed by 2-ODD (Tohge et al., 2013). In the biosynthesis of glucosinolates which are nitrogen/sulfur-containing secondary metabolites mostly found in *Brassica* species, three 2-ODDs, namely alkenyl /hydroxy (OH) alkyl producing enzymes (AOP1, AOP2, AOP3; Kliebenstein et al., 2001) and glucosinolate 2-oxoacid-dependent dioxygenase gene (GSL-OH; Hansen et al., 2008) have been found in *Arabidopsis* (**Figure 2B**). Since GSL-OH is evolutionarily highly distant from AOP2, it has been suggested that GSL-OH represents an independent recruitment from a different 2-ODD clade to the same metabolism (Kawai et al., 2014).

In flavonoid biosynthesis, four types of 2-ODDs; flavonol synthase (FLS), flavanone 3-hydroxylase (F3H), anthocyanin synthase (ANS also known as LDOX), and flavone synthase I (FS-I) are characterized as key enzymes in late steps of flavonoid aglycone formations resulting to in species specific flavonoid profiles (**Figure 2C**; Martens et al., 2001; Turnbull et al., 2004; Tohge et al., 2013). Flavonols are catalyzed from flavanone by hydroxylation of carbon-3 and 2,3-dehydration by F3H and FLS, respectively. On the other hand, ANS which catalyses the formation of dihydroflavonols from leucoanthocyanidins is known to be one of as one of the key enzymes involved in red pigmentation via anthocyanin and proanthocyanidin biosynthesis. FS-I which is found mainly in monocot and Apiaceae species uses flavanone as the substrate for flavone backbone formation. Given that F3H provides the precursor of substrates for FLS and ANS, it has been suggested that the evolution of FLS and ANS occurred after the emergence of F3H during 2-ODD evolution in seed plants (Stafford, 1991). Phylogenetic tree analysis using gene family of FLS, F3H, and ANS from several plant species revealed clear separation between FLS, F3H, and ANS as well as monocots, dicots and leguminous species as subclade of FLS, F3H, and ANS. This fact may suggest that FLS, F3H, and ANS have evolved in early stages of evolution (Tohge et al., 2013). In the other branches of phenolic secondary metabolism such as coumarin and 2,4-di-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) biosynthesis, several 2-ODDs have been found as key enzymes in their biosynthesis namely, *p*-coumaroyl-CoA 2′-hydroxylase (C2′H; Vialart et al., 2012), feruloyl-CoA 6′-hydroxylase (F6′H; Kai et al., 2008), and DIBOA-7-hydroxylase (BX6; Frey et al., 2003).

Several 2-ODDs are also involved in the synthesis of different alkaloids, which is the second largest class of plant secondary metabolism, have been characterized in several plant species. In the biosynthesis of tropane alkaloid, hyoscyamine 6-hydroxylase (H6H) in scoporamine biosynthesis of *Hyoscyamus niger* (**Figure 2D**; Matsuda et al., 1991), *Anisodus tanguticus* (Liu et al., 2005), and *Atropa belladonna* (Suzuki et al., 1999), 2′-deoxymugineic-acid 2′-dioxygenase (IDS3) in mugineic acid biosynthesis of *Hordeum vulgare* (Nakanishi et al., 2000) have been characterized. In addition, two 2-ODDs involved the biosynthesis of morphine namely thebaine 6-demethylase (T6ODM) and codeine *O*-demethylase (CODM) have been found form *Papaver somniferum* (**Figure 2E**; Hagel and Facchini, 2010).

SUMMARY

In summary, 2-OG is not only a TCA cycle intermediate but also a co-factor for a diverse range of enzymes involved in amino acid, glucosinolate, flavonoid, alkaloid, and GA metabolism. Specifically in the case of GA and amino acid, recent evidence indicates that 2-OG levels control the rate of their biosynthesis. Further work is, however, needed to establish it also for glucosinolate, flavonoid, and alkaloid biosynthesis. Moreover, whilst preliminary studies have been attempted to understand evolutionary origins of the 2-ODD, more comprehensive analysis of this will be required to deepen our understanding of this important gene family.

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CHAPTER III

Differential responses between roots and shoot in tomato plants with different gibberellin levels

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ABSTRACT

The ability to adapt to the environment is one of the many characteristics required for plant organisms. A refined communication capable of integrating the endogenous and exogenous signals and sharing them among the most different parts of the plant is what confers such adaptability. Once they growth in highly distinct environments, toot and shoot have been reported to present different responses to a given environmental condition. Accordingly, a higher sensibility of roots to gibberellins (GAs), has allowed better adjustments in the plant growth/development and possibly triggering mechanism of tolerance stress conditions. Thus, here we investigated the differential responses between roots and shoot following reductions of the endogenous GAs content. Wild type (WT) and plants deficient in the GAs biosynthesis (gib3, moderately deficient, gib2, intermediate deficiency and gib1, extremely deficient in GAs) were used. It has been observed that depletion of GAs affects shoot in a greater extension in detriment of the root growth in all the mutants. It was also verified that the lower the GAs content (gib2 and gib1), the greater the extension at the metabolic disturbances. Low carbohydrate content in leaves was verified in plants with higher root growth, suggesting an enhancement of the flow of skeletons to support root growth. Large variations were observed in the pool of amino acids profile, either in roots or shoot, especially for mutants with drastic GAs reductions. This increase of some amino acids content was followed by reductions in the TCA components, a process that is directly linked to the nitrogen metabolism.

INTRODUCTION

Due to their sessile nature plants need to constantly adapt to the most different conditions imposed by the environment (Golldack et al., 2013). Many of these distinct environmental conditions are from a stressful nature either biotic or abiotic (Eremina et al., 2016). Such complex conditions have driven the plasticity through the adversities that allowed the establishment of current plant species (Dangl and Jones, 2001; Pinto et al., 2011). This fact apart, the perception of the exogenous signals governing plant responses can occur differently between the different parts of the plant. Such responses need to be shared with other part of the plant in order to maximize the answers whether it will occurs at whole plant or specific tissue level. This highly regulated communication occurs through the signaling networks that act integrating the environmental signals with endogenous programs (Goossens et al., 2016; Thatcher et al., 2009). Finally, this integration will lead to the alteration of morphological and physiological traits within both roots and shoot, that ultimately depends on the availability of environmental resources (Wang et al., 2009).

The development of root is of pivotal significance for plants growth, which is extremely dependent on the correct balance between absorption and investment of resources (Araya et al., 2016). Coordinated activities of the shoot-root system, also called interdependence relationship (Chu et al., 2014; Yang et al., 2004), is responsible for the development and productivity of plants. Accordingly, not only the photosynthetic process is affected by both the size and the activity of the roots, but also the growth and maintenance of the root system are dependent on the photoassimilates imported from the leaves (Araya et al., 2016; Manschadi et al., 2007; Osaki et al., 1997; Yang et al., 2004;

Zhang et al., 2009). It is also known that the architecture of the root system is crucial not only for its own function but also to the shoot proper function (Wang et al., 2009). External and/or internal clues are important to the development of the roots (Zhang et al., 2009).

Although an integral part of the plant organs, research with roots have been less frequent, when compared to the shoot (Huang et al., 2012). Relatively few studies have been done with root system most likely because this belowground organ is extremely laborious to evaluate (Yang et al., 2008; Zhang et al., 2009). Furthermore, it has been only recently that investigation with roots has been performed without focusing solely on growth and carbon relationships (Bassirirad, 2000; Giri et al., 2017; Huang et al., 2012). Reductions in root growth and, consequently in the shoot, were observed when ryegrass plants were submitted to different types of stresses such as: drought (Lawlor and Cornic, 2002), high temperature (Huang et al., 2012) and nitrogen starvation (Grassi et al., 2003). Alterations in the source-drain relationship between the root-shoot as a consequence of the restriction of water and mineral supplementation were observed. It also affected the production of hormones synthesized in the root and transported to the shoot (Hao et al., 2011). Similarly to metabolites and minerals, hormones can be also transported throughout the body of the plant to regions of greatest demand. In general, the synthesis of gibberellin (GAs) occurs at its site of action, although there are certain tissues and/or organs that are dependent on an external source of this hormone. Example to this are the cereal aleurone layer that receives GA from the scutellum of the embryo (Lenton et al., 1994), as well as the anthers which exports GAs from other floral organs (Yamaguchi, 2008). In addition, different tissues may vary their response to GAs in function to the GA

concentration (Tanimoto, 1987; Tanimoto, 2012) and the tissue sensitivity (Stowe and Yamaki, 1959).

Arabidopsis plants treated with exogenous GAs and/or inhibitors of GAs biosynthesis showed very different behaviors between shoot and root system. Increments of the endogenous content of GAs, by exogenous application, promoted enhanced leaf expansion and biomass gain of the shoot, however, this higher content of GAs inhibited root growth (Bidadi et al., 2010). By contrast, the presence of inhibitors of the GA biosynthesis inhibited shoot growth whereas increased root growth, leading to changes in root to shoot ratio (Bidadi et al., 2010). This behavior is associate to the higher sensitivity of roots to low concentrations of GAs (Barboza-Barquero et al., 2015). Thus, under low concentrations of GAs (nM), there is inhibition of shoot growth and increase of root growth where the growth of lateral roots is more evident. However, at higher concentrations (μM) the inverse is usually observed (Tanimoto, 1987; Tanimoto, 2012). Thus, although the action of GAs is essential for root elongation, at high concentrations GAs are inhibitory. Remarkably, roots usually present levels of GAs next to saturation (Hedden and Sponsel, 2015). It is clear that an adequate interconnection root-shoot is necessary for the adaptation, survival and optimal plant development to different environmental conditions. A decoupling between the carbon and nitrogen metabolism was observed in leaf tissues of plants with alterations in the endogenous content of GAs, as well as a favoring of the root growth to the detriment of the shoot. In an attempt to better understand how and to which extension this connection occurs and the importance of GAs in different organs, metabolic and physiological analyzes were performed on those different tissues of WT plants and mutants on the GAs biosynthesis (gib3, gib2 and

gib1) growing under normal optimal environmental conditions. Our results demonstrated that the greatest perturbations caused by GAs depletion were observed in shoot of mutant plants gib2 and gib1 and that the profile of organic acids and amino acids were significantly affected suggesting an exquisite metabolic reprogramming following fluctuation in the levels of GAs.

MATERIAL AND METHODS

Plant material and growth conditions

The experiment was conducted in Viçosa (20º 45 'S, 42º 15 'W, 650 m altitude), Minas Gerais state, south-eastern 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).

Tomato seeds were germinated in Petri dishes with two layers of filter paper (Whatman nº 1) soaked in GA solution (Duchefa, Haarlem, The Netherlands; GA_3 50 µM). 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 ± 5 % and light intensity of 150 µmol.m^{-2}.s^{-1} during the light period. After seven days, seedlings were transferred to 1.4 L pots containing commercial substrate supplemented with 14 g of NPK per pot. After transfer to pots, the plants were grown in a greenhouse with semi-controlled conditions (maximum photosynthetically active radiation (PAR) of c. 1500 µmol photons m^{-2} s^{-1}, with a mean temperature of 30 ±

2 °C). The plants were irrigated daily as needed and no restriction of root development was observed at the end of the experiment. All the samplings and measurements were performed on 5-week-old plant. For biochemical analysis, both root and leaf samples were harvested in the middle of the photoperiod and immediately flash frozen in liquid nitrogen and then stored at -80 °C until further analysis.

Metabolite analysis

Metabolite extraction was performed by grinding the lyophilized material with liquid nitrogen and immediate addition of the appropriate extraction buffers. Approximately 5 mg of dry matter (roots and leaves) were subjected to ethanolic extraction by incubation at 70 °C for 30 min. After centrifugation (16,200 x g, 5 min) the chlorophyll contents (a and b) was determined as previously described (Porra et al., 2006). The total chlorophyll contents (a+b) as well as the chlorophyll a/b ratio were calculated. The concentrations of glucose, fructose and sucrose were determined as previously described (Fernie et al., 2001); total amino acids (Cross et al., 2006); malate and fumarate (Nunes-Nesi et al., 2007). In the ethanol insoluble fraction, starch contents (Fernie et al., 2001) and total soluble proteins contents by the Bradford technique (Bradford, 1976).

Pyridine nucleotides

NAD(H)s and NADP(H)s were determined as described previously (Gibon and Larher, 1997). Briefly, approximately 5 mg of dry matter were used and thus pyridine nucleotides were assayed using the phenazine methosulfate-catalyzed 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 profile

To obtain a broad overview of the major pathways of central metabolism an established gas chromatography mass spectrometry (GC-MS)-based metabolite profiling method was used to quantify the relative metabolite levels in both leaf and root samples (~50 mg fresh weight). The extraction, derivatization, standard addition, and sample injection were performed exactly as previously described (Lisec et al., 2006). This analysis allowed the determination of approximately 50 different compounds, representing the main classes of compounds (amino acids, organic acids, sugars and others).

Statistical analyses

Unless otherwise stated, data were obtained from seven plants in individual pots per genotype. The experiment was designed in a completely randomized distribution. The effect of genotype (mutation) was determined by analysis of variance ($P < 0.05$). The means were submitted to the Dunnett test. All statistical analyses were carried out using Statistical Analysis System (SAS; North Carolina State University, EUA). To evaluate the relations between the different genotypes and part of the plants, we also used Pearson

correlations coefficients (GraphPad Prism software) and principal component analysis (Minitab 17 software) based on the correlation matrix for the adjusted means.

RESULTS

To investigate the functional role of GA levels we here used the previously characterized tomato mutant plants gib3, gib2 and gib1, which present moderate, intermediate and extreme deficiency in the biosynthesis of GAs, respectively. Both gib2 and gib1 mutants showed significantly reduced content of chlorophyll a and b (dry mass base) when compared to their WT control whereas gib3 plants showed similar content to WT plants (Figure 1). It is worth to mention, however, that this reduction (gib2 and gib1) is compatible with the reduction of the endogenous content of GAs, which drastically impacts the whole plant development. The changes observed in all the genotypes were similar among genotypes, since chlorophyll a/b ratio did not differ (Figure 1D).

Given that a differential growth response in roots and shoots was previously observed (Martins, 2013), we decided to next investigate whether this response also extend to metabolism in general. Although in completely different amounts between the two systems, shoot and roots, glucose and fructose contents presented similar behavior (Figure 2A and 2B). gib3, gib2 and gib1 plants were characterized by significant reductions of these metabolites in the shoot, and this reduction was observed to be higher in gib2 and gib1 plants (to 10 to 15 % of levels observed in the WT plants, respectively). Accordingly, in gib3 this reduction was around 50 % (Figure 2A and 2B). Lower content of the glucose and fructose also were observed in the roots of gib2 mutant, which one differed in both metabolites, whereas roots of gib1 mutant differed only to glucose. The content of sucrose, in quantitative terms, was similar between roots and shoots, and it was

significant reduced only in leaves of gib1 plants (Figure 2C). Similar to the situation observed for fructose and glucose, leaf starch levels was significantly lower in the mutants gib3, gib2 and gib1, respectively, although with different intensity (Figure 2D). By contrast, the accumulation of starch in roots was similar for all genotypes. Notably, the reduction of the endogenous levels of GAs did not affect the metabolism of sucrose and starch in the roots. Similar results were observed in Arabidopsis plants submitted to low GA regimens (Ribeiro et al., 2012b). This behavior can, at least partially, explain the maintenance and / or increase of roots growth observed in these same plants previously (Martins, 2013). Malate (Figure 2E) and fumarate (Figure 2F) content were also evaluated and, similarly, root metabolism was less susceptible to the endogenous variation of GAs concentration and, as such, only the extremely deficient mutant in the biosynthesis of GAs (gib1) showed alteration, with increment of the content of both malate and fumarate. On the other hand, fumarate content in shoots was reduced in all mutant genotypes, whereas malate was significantly reduced only in the gib2 and gib1 mutant genotypes (Figure 2F and 2G). Regarding the quantification of nitrogen compounds, that is amino acids and proteins, the root system of these plants was, in general, more affected than the shoot (Figure 2G and 2H). Thus, the amino acids contents in roots were gradually increased for gib2 and gib1 mutant plants, respectively, whereas in shoot this increase was verified for gib3 and gib2 mutants. For proteins gib2 and gib1 mutant plants presented similar behavior as amino acids in roots, whereas shoot tissues were characterized by reductions in gib1 mutant plants.

To better understand the variations occurring between the genotypes and mainly the differences between the parts of the plant, a more detailed investigation of the

primary metabolism of these plants was carried out through the metabolic profile in GCMS. Over 70 metabolites were successfully quantified in this analysis, representing different groups of compounds, such as amino acids, sugar, organic acids, fatty acids among others (Figure 3). The main compounds belonging to the glycolytic pathway and the tricarboxylic cycle (TCA), as well as its derivatives and branching points are schematically represented in Figure 4. The full dataset is additionally available with their respective relative values and standard error in the Supplementary Table 2.

The metabolite profile revealed, similar to that observed above, that both gib2 and gib1 plants presented greatest differences in relation to their WT control plants. As expected, the greatest variations occurred in leaf tissues in comparison to the changes observed in roots. Notably, the highest variation was observed with the several amino acids identified. Thus, the levels of valine, methionine, tyrosine, lysine, isoleucine and asparagine were largely increased in gib1 mutant plants. Similar behavior was also observed for the gib2 mutant plants, although such increase occurred in lower intensities. Interestingly, asparagine increased in gib1 mutant more than 10 times in relation to the WT levels. By contrast, glutamine, glutamate, glycine, homoserine, ornithine and tryptophan were characterized by reductions in their content and, in general, this reduction was similar forgib2 and gib1 mutant plants.

Regarding the gib3 mutant it was observed that, among the few amino acids that significantly changed, glycine content was reduced, as it was in gib2 and gib1 plants, while glutamine and tyrosine varied in the opposite way to the other mutants showing increments in gib3 whereas in gib2 and gib1 plants it was reduced. Similar to that observed in the shoot, tyrosine, methionine, isoleucine and histidine significantly increase

their root contents, as well as citrulline. The root contents of proline and hydroxyproline were reduced in all mutant genotypes. It is also worth to notice that gib3 mutant plants were characterized by, unlike the shoot, increases in tyrosine, ornithine and cysteine. When comparing the individual behavior of the amino acids analyzed by GCMS (Figure 3) with the total amino acid contents (Figure 2G), no direct correlation was observed. It is probably because for total amino acids significant differences were only found in roots of gib2 and gib1 mutant plants, whereas the total amino acids content for shoot was different in gib3 and gib2 mutant plants. Genotypes with drastic reductions at the GAs content, gib2 and gib1, presented significant reductions for all organic acids quantified in leaf tissues. These reductions are possibly explained by the increase in amino acids occurring in these genotypes, since some of these compounds are deviation points for amino acid synthesis (Figure 4). In roots, the effect was the opposite, since theses organics acids were observed to increase for gib3 and gib2, except for 2-oxoglutarate, which showed a higher increase in gib3 and reductions in gib2 and gib1 mutant plant.

In both leaf and roots, the levels of sugars were significantly reduced especially in the gib2 and gib1, following the same behavior observed for organic acids. These results are compatible with a higher demand of these compounds for energy generation and consequently maintenance of roots growth of these mutant genotypes in detriment to shoots when compared to WT control plants (Martins, 2013). In general, most of the compounds grouped without specific classification (Figure 3) showing significant reductions in both leaves and roots. Remarkably, dehydroascorbate and ascorbic acid display a gradual decrease in leaves following the trend of reducing the endogenous content of GAs (gib3, gib2 and gib1). Few metabolites showed significant increases and

as such only nicotinic acid was found in leaves of all mutant genotypes, whereas in the roots system adenosine and glycerate were found only in gib3 mutant plants. The levels of uracil showed gradual increase in gib3, gib2 and gib1 mutant plants.

We decided to next evaluate the redox status of these plants by determining the pyridine nucleotides (NAD(P)(H)s) (Figure 5). NAD^+ content presented significant increases only in root tissues of gib2 and gib1 mutant plants (Figure 5A).

By contrast to the situation observed for NAD^+, leaf $NADP^+$ contents were characterized by gradual reductions in the gib3, gib2 and gib1 mutant plants, respectively. In roots however only gib1 mutant plants differed from the WT control, presenting enhanced levels as occurred for NAD^+ (Figure 5B). Regarding the reduced nucleotides, NADH (Figure 5C) and NADPH (Figure 5D), no significant variations were observed in roots. A similar result was also observed for NADPH in leaf samples. Leaf NADH content was significantly reduced in all mutants, similarly to that previously found for $NADP^+$. Interestingly, neither the NAD/NADH (Figure 5E) nor the NADP/NADPH (Figure 5F) ratio was altered in roots of all the mutants in comparison to WT values, thus demonstrating the maintenance of a redox balance in this tissue. Although the NAD/NADH ratio showed a trend of gradual increments in the shoots of the mutant, this was only statistically significant for the gib1. Interestingly, an opposite behavior was observed for the NADP/NADPH showing significant reductions for gib2 and gib1 mutant plants.

To provide a broad evaluation of the behavior of the metabolites here analyzed, the dataset was submitted to correlation analysis using the Pearson coefficient in an attempt to determine which changes were most closely associated with the phenotypes

observed (Supplemental Figure S1). When evaluating the strength of these correlation and their significance it becomes apparent that positive correlations were presented mainly among chlorophylls; chlorophylls and nucleotides; chlorophylls and some acids such as: isocitric, octadecanoic, nanoic, hexadecenoic, glucuronic and galactonic; chlorophylls and sugars such as trehalose; sucrose, ribose, gentibiose, myo inositol and squalene (Supplementary Figure S1). There was also a positive correlation between some sugars. Additionally, most sugars were negatively correlated with chlorophylls, proteins, and some acids, more specifically those with positive correlation with chlorophyll and within own the group. It is noteworthy that there were the compounds which had little or no correlation within the group including the amino acids GABA, cysteine, β-alanine, alanine, asparagine, aspartate, pyroglutamate, valine and tyrosine; the sugar glucose, glucose-6P, the glycolic acid and glycerol.

In order to explore in more details the effect of reducing the endogen levels of GAs on plant metabolism, the metabolite dataset was analyzed using principal component analysis (PCA) using the first two components, which cover most of the variance of the dataset. Shoot and roots (Figure 6A and 6B, respectively) were first evaluated individually and the whole data as further analyzed (Figure 6C). This fingerprint analysis revealed that the first two axes explain 88 %, 83.1 % and 78.8 % of the dataset variance in leaves, roots and the whole plant, respectively.

As observed in our previous analyses, larger variations were found in leaf tissues where the separation of genotypes along axis 1 (PC1 - 72.1 %) was mainly due to the contribution of total amino acids in gib2 and gib1, whereas acids, in general, for gib3 and WT plants (Figure 6A). The separation along axis 2 (PC2 - 15.6 %) was more specifically

due to: asparagine and phenylalanine in gib1 plants; tyramine and octadecanoic acid in gib2 plants; malate and fumarate in gib3, succinate, isocitrate and tryptophan plants in WT plants. Differently from the situation observed in shoot, the gib2 and gib1 mutants were grouped very closely both in PC1 (58.7 %) and PC2 (24.4 %) when analyzing the root metabolites (Figure 6B). Once more, amino acids were responsible for this separation of gib2 and gib1 in PC1 in relation to the gib3 and WT, whereas the later genotypes had greater contributions from sugars and acids in general. In PC2, gib3 mutant plants, as a function of pyruvate, 2-oxoglutarate and cysteine, were completely separated from WT, which had greater contributions from tryptophan, sucrose, fructose and citric acid (Figure 6B). When we evaluate these variables at the whole plant level (Figure 6C) we found a more consistent relationship between all the results described so far. Clearly we observed the formation of three highly characteristic groups: group A being formed by the metabolite composition of roots of all genotypes, group B formed by the metabolite composition of shoots of the gib2 and gib1 mutant plants and, a third group (C) formed by the metabolite composition of shoots of the mutant gib3 and the WT. Notably, the separation between the groups in regarding to metabolite composition of shoots and roots occurred in component 1 (58.4 %) and it was due to some amino acids and sugars as main contributors to the roots, whereas chlorophylls, proteins, amino acids, nucleotides and acids contributed to the separation observed for the metabolite composition of leaves.

DISCUSSION

Plant growth and development are tightly regulated by multiple environmental cues and nutritional supplements (Fernie et al., 2001). Among these factors, plant hormones are known to play an essential role in the regulatory events associated with growth and development (Ljung et al., 2015). GAs has been extensively shown to be a growth promoter, an effect more specifically observed in the shoot (Busov et al., 2006; Hooley, 1994). However, as regards to the root system, the precise manner by which GAs regulate the development of this organ remains poorly understood (Fu and Harberd, 2003; Tanimoto, 2012). It has been previously observed an uncoupling between growth and carbon metabolism in both Arabidopsis and tomato plants submitted to distinct gibberellin regime by exogenous application of paclobutrazol (PBZ) and/or GAs (Martins, 2013; Ribeiro et al., 2012b). In both studies the addition of PBZ, an inhibitor of GA biosynthesis, was associated with a negative effect in the growth of these plants. Arabidopsis plants did not differ in the root-shoot ratio, whereas tomato plants showed a significant increase in this parameter. Interestingly, tomato plants deficient in GAs biosynthesis (gib3, gib2 and gib1) also did not show significant variations in root-to-shoot ratio (Martins, 2013). In addition, it is important to note that both organisms/plants and different parts of the same plant may respond differently to GAs (Tanimoto, 1987; Tanimoto, 1994) or exhibit different sensitivities (Barboza-Barquero et al., 2015; Elias et al., 2012; Stowe and Yamaki, 1959). Different root-shoot sensitivity was also verified to the perception of exogenous signals. Despite a severe reduction on shoot development without any effect on root growth tomato plants overexpressing AtGAMT1 showed also a higher drought tolerance within the shoots (Nir et al., 2014). In the same context, both

bean (Hungria and Kaschuk, 2014) and ryegrass (Huang et al., 2012; Xu and Huang, 2000) were characterized by enhanced sensitivity at roots under high temperatures, that also affected the carbon nitrogen balance between root-shoot. Here, we provide further evidence of the importance of the GA levels in governing an exquisite but differential metabolic reprogramming between root and shoot of tomato plants.

We found that moderate and extreme GAs biosynthetic deficiency culminate with significant reductions in the chlorophylls content when compared to their respective WT (Figure 1). On the other hand, carrot plants (Gopi et al., 2007) and Arabidopsis plant (Ribeiro et al., 2012a; Ribeiro et al., 2012b) treated with triazoles, inhibitory molecules of GAs biosynthesis, as well as mutant plants with higher rates of GAs inactivation (Nir et al., 2014) were characterized by increases in these compounds. This reduction was followed by the absence of changes in both amino acid and protein contents in shoots (Figure 2). We also observed increases in the contents of amino acids and proteins in the root system of these plants (Figure 2G and 2H), thus characterizing a metabolic context in which nitrogen is accumulated in roots in detriment of the shoot content allowing for the maintenance and/or growth increments of this organ (Hua et al., 2014; Martins, 2013; Sharp, 2002). It is estimated that in plants, in general, about 75 % of the amino acids are derived from the proteins degradation, and the remaining 25 % are synthesized from carbon skeletons (Davies, 1979; Huang et al., 2012). However the origin of amino acids pool may significantly vary in tissue and development stage. Protein degradation is the major amino acids source in mature, stressed and senescent cells, as well as in seed in germinating process. In contrast, in developing cells amino acid biosynthesis is seemingly more important (Galili et al., 2014; Hildebrandt et al., 2015; Watanabe et al.,

2013). This may explain, at least partially, the higher reduction of the carbohydrates glucose, fructose and starch found in the shoot (Figure 2A, 2B and 2D). Collectively, this indicates reallocation of these assimilates for the maintenance of respiration and supporting energy generation in the roots.

It is also known that nitrogen assimilation and growth are energetically expensive processes and that shoots are responsible for the supplementation of all the carbon skeleton to root (Huang et al., 2012; Lambers et al., 1998). Our results provide compelling evidence for the translocation of the photoassimilates allowing an enhancement of the nitrogen metabolism and root growth. This is further explained by the similar content of sucrose between root-shoot (Figure 2). This behavior characterizes the unidirectional sugar transport from shoot to root and did not allowed the accumulation of carbohydrates in shoots as observed by the reduced, content of starch. It is noteworthy that sucrose is one the main sugar transported in plants (Rolland et al., 2006). Altogether these data indicate that GAs are able to regulate not only plant growth, but also to reprogram carbon metabolism with associated impacts on nitrogen metabolism, ultimately leading to changes in the flow of these metabolisms between roots and shoot.

The significant, and gradual, decrease in the starch content of starch in tomato leaves (Figure 2D), following the reduction in the endogenous levels of GAs, are consistent with the results found in Arabidopsis mutant plants defective in the metabolism of starch (Paparelli et al., 2013). Due to the poor accumulation and/or degradation of the starch, these mutants were sugar starved, affecting the biosynthesis of GAs and consequently leading to growth inhibition. This was evidenced by the direct relationship between the lower sugar content in the leaf at night (Caspar et al., 1991;

Zeeman and AP Rees, 1999) and the reduced expression of the enzyme ent-kauren synthase (KS, a biosynthetic pathway enzyme) in the next day (Paparelli et al., 2013). The interdependence between carbon-nitrogen metabolisms has been well characterized in Arabidopsis plants submitted to either partial or full nitrogen limitation (Ikram et al., 2012). In both conditions, plants presented higher accumulation of starch in the leaves in detriment of starch levels in roots, whereas amino acid content presented an inverse pattern. Significant reductions, for shoot tissues, in malate (gib2 and gib1)and fumarate contents (gib3, gib2 and gib1) of mutant plants (Figure 2E and 2F) coupled with increases of these organic acids in roots of the gib1 mutant are suggestive of higher activity of the tricarboxylic acid cycle (TCA). Furthermore, these organics acids can be used as an alternative and flexible source of carbon skeletons in Arabidopsis plants (Chia et al., 2000; Zell et al., 2010). It is tempting to suggest that this additional function of organic acids might occur in response to limitations in GA content in an attempt to sustain growth, particularly in roots. Due to its physic-chemical characteristics malate acts also as a carrier of reducing power between the organelles chloroplast, peroxisome and mitochondria (Niessen et al., 2012; Pracharoenwattana et al., 2007). The point of entry and/or exit of malate from mitochondrial metabolism occur through the TCA cycle. In addition, TCA cycle is one of the main components of mitochondrial respiration, connecting the glycolytic pathway and extra-mitochondrial malate to the electron transport chain (Araujo et al., 2012; Fernie et al., 2004; Millar et al., 2004; Sweetlove et al., 2010). The components of the TCA cycle have been extensively characterized during the last years (Araujo et al., 2012; Araújo et al., 2012; Nunes-Nesi et al., 2011; Sweetlove et al., 2010; Sweetlove et al., 2007). Thus, the TCA cycle is not only the key point in

cellular energy metabolism, but it also plays an integrating role between carbon-nitrogen metabolisms (Hodges, 2002), which seems to be also influenced by endogenous manipulation of GA contents, as previously demonstrated (Martins, 2013) and by the results presented here (Figure 3 and 4). Approximately 70 % of the TCA intermediaries were quantified among over than 70 compounds identified in our metabolic profile (Figure 3 and 4). Different classes of compounds were represented including sugars, organic acids, fatty acids, and amino acids among others. As expected, the largest significant variations were found in leaf tissues rather than in roots. Accordingly, both gib2 and gib1 mutant plants were also the most affected. Together with amino acids, the organic acids, which are intermediates or precursor of the TCA cycle, were the most affected. It is noteworthy that the vast majority of amino acids are produced directly or indirectly from intermediates of the glycolytic pathway or the TCA cycle (Hodges, 2002). Considering organics acids and amino acids quantified, only the levels of aspartate did not significantly changed. Two important points to note about TCA cycle are 2-oxoglutarate and oxaloacetate: these metabolites are diverted to the synthesis of the amino acids glutamate and aspartate, respectively (Figure 4). Remarkably, from these two compounds the most of the amino acids are derived. 2-oxoglutarate together with glycine and serine, which are metabolites directly related to the photorespiratory process (Igarashi et al., 2003), although they presented some significant differences, did not present a characteristic pattern that explain changes in this process. Photorespiration is a process that occurs naturally in C3 plants (Dellero et al., 2016; Hagemann and Bauwe, 2016).

In general for shoot and root tissues the branched-chain amino acids valine, leucine and isoleucine together with lysine and tyrosine, an aromatic amino acid, showed significant increases mainly in gib2 and gib1 mutants., It is known that this set of amino acids may be involved in alternative respiratory routes (Araújo et al., 2011; Kleessen et al., 2012). However, the increase in the content of these amino acids, as well as some others amino acids, and the absence of greater perturbations at the TCA are likely associated with a normal protein metabolism, which had increases in gib2 and gib1 roots. Glutamate, glutamine and asparagine, which are known to be amino acid of nitrogen storage and transport in cell metabolism (Miflin and Habash, 2002), as well as the others, were significant reduced/increased in gib2 and gib1 plants. In spite of the function conferred to them, they do not seem to contribute to the increase of total amino acids in the roots, where they were not detected. Besides to being essentially the building blocks of proteins, amino acids act directly on cytosolic and mitochondrial energy metabolism, either by feeding the glycolysis and the TCA cycle with carbon skeleton or by donating electrons directly to the electron transport chain (Araujo et al., 2010; Araújo et al., 2011; Barros et al., 2017). Amino acids also are reported to act on the resistance against stress, signaling, plant growth, among others (Hildebrandt et al., 2015; Shi and Chan, 2013; Szabados and Savoure, 2010). In general, the content of sugar, more specifically glucose, fructose and trehalose, that are known to feed the glycolytic pathway showed gradual and significant reductions in a similar context to the reductions in GA contents. This behavior was observed for both roots and shoot, except for trehalose that was detected only in the shoot. Sucrose content was similar between genotypes as well as between different parts of the plant. The absence of changes in sucrose, which is one the major sugar transported

in plant, is consistent with the low accumulation of starch in leaves of mutant plants in response to the reduction of GAs. Accordingly, starch is a transient carbon skeleton reserve that accumulates during the day and is remobilized at night allowing for the maintenance of growth and metabolism (Smith and Stitt, 2007; Stitt and Zeeman, 2012; Sulpice et al., 2009).

Collectively, our results present evidence of a highly exquisite metabolic adjustment in response to fluctuations of GA levels and this adjustment is different between roots and shoots. We have furthermore demonstrated the effect of the endogenous manipulation of levels GAs in altering plant development most likely associated with modifications in both carbon and nitrogen metabolism. These variations resulted in a clear separation of the genotypes based in their metabolite composition (Figure 6) as observed by the PCA analysis. That said, the exact mechanistic relationship between energetic metabolism, metabolic reprogramming, and GAs itself governing plant growth differently in shoots and roots remains to be examined in detail in future studies. It is important to mention, however that future studies are clearly necessary to unravel the mechanisms through which the GA orchestrates all these modifications.

CONCLUSIONS

We found that the GA depletion promoted a more drastic effect on the shoot metabolism in detriment to the root metabolism. More generally, mutant plants gib3 (moderately deficient in GAs) showed to be relatively similar to WT plants in both tissues, root and shoots. Unlike gib3, the mutants gib2 and gib1 (moderately and extremely deficient in GAs, respectively) were found to be significantly discrepant from WT plants. For most metabolites, gib2 and gib1 plants had gradually increase or decreases, following the pattern observed for biometric data (Martins 2013). Accordingly, this result is more compatible with the reduction in the GAs content, which is more pronounced in gib1. The carbohydrate content, except for sucrose, and organic acids, had gradual reductions in the mutants in the shoot, and interestingly the reductions were strongly associated with the increment observed in root growth, thus showing that the contribution from shoot in the maintenance at the roots growth occurs even when the shoot growth is inhibited. The total amino acid pool had little alterations when we compared to the profile of specific amino acids. Thus, gib2 and gib1 were highly affected in both roots and shoot, highlighting the changes observed for valine, tyrosine, lysine, isoleucine that had their content increased up to two times in leaves of gib1 plants in comparison to WT levels. Amino acid uptake were coupled to reductions of the organic acids of the TCA, possibly by a mechanism associated with deviation of compounds allowing the maintenance of the pool of amino acids.

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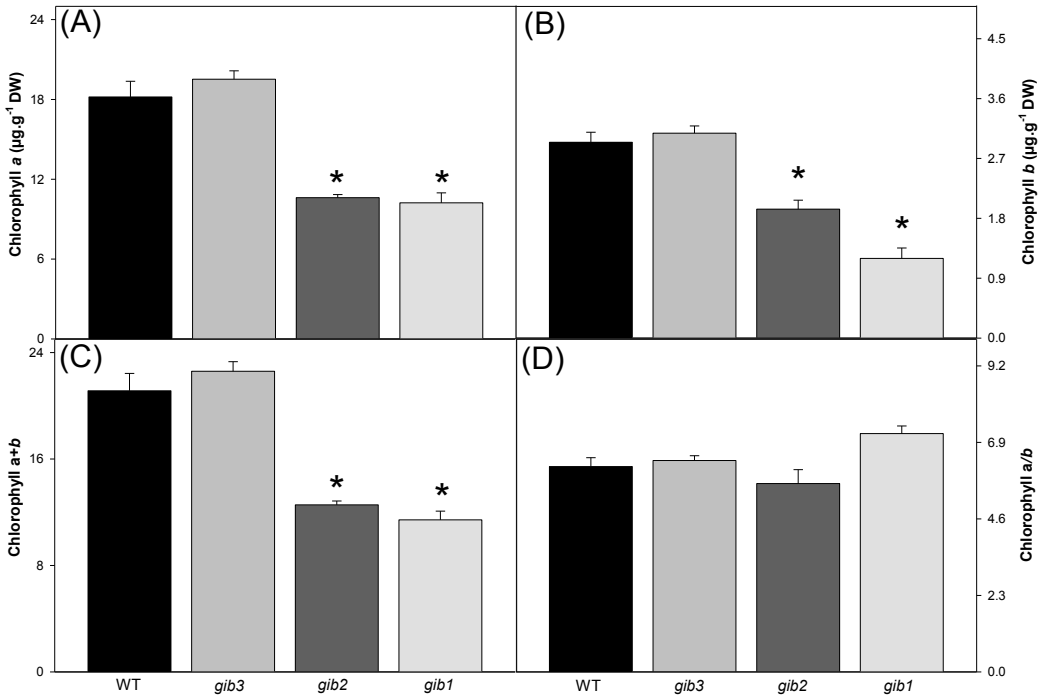

Figure 1. Variation in chlorophyll concentration in response to endogenous reduction in GAs levels in shoots of plants WT and mutants plants in biosynthesis of gibberellin gib3, gib2 and gib1 (as described in Material and Methods). (A) Chlorophyll a. (B) Chlorophyll b. (C) Chlorophyll a+b. (D) Chlorophyll a/b. Asterisks indicate values determined by the Dunnett-test to be significantly different from control ($P < 0.05$). Values are presented as means of seven replicates ±SE.

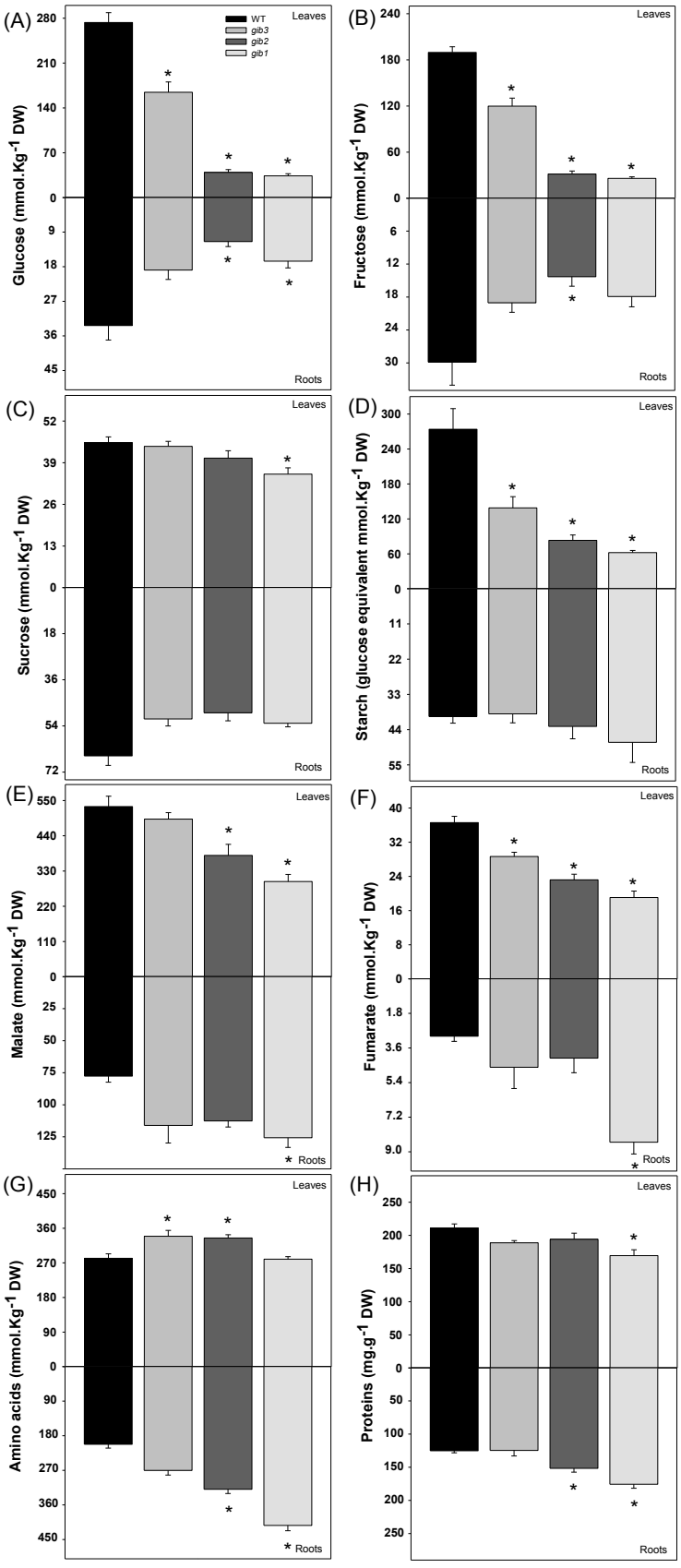

Figure 2: Comparison of the metabolite levels in shoots and roots of plants WT and mutants plants in biosynthesis of gibberellin gib3, gib2 and gib1 (as described in material and methods). (A) Glucose. (B) Fructose. (C) Sucrose. (D) Starch. (E) Malate. (F) Fumarate. (G) Total amino acids. (H) Total soluble proteins. Asterisks indicate values determined by the Dunnett-test to be significantly different from control ($P < 0.05$). Values are presented as means of seven replicates ±SE.

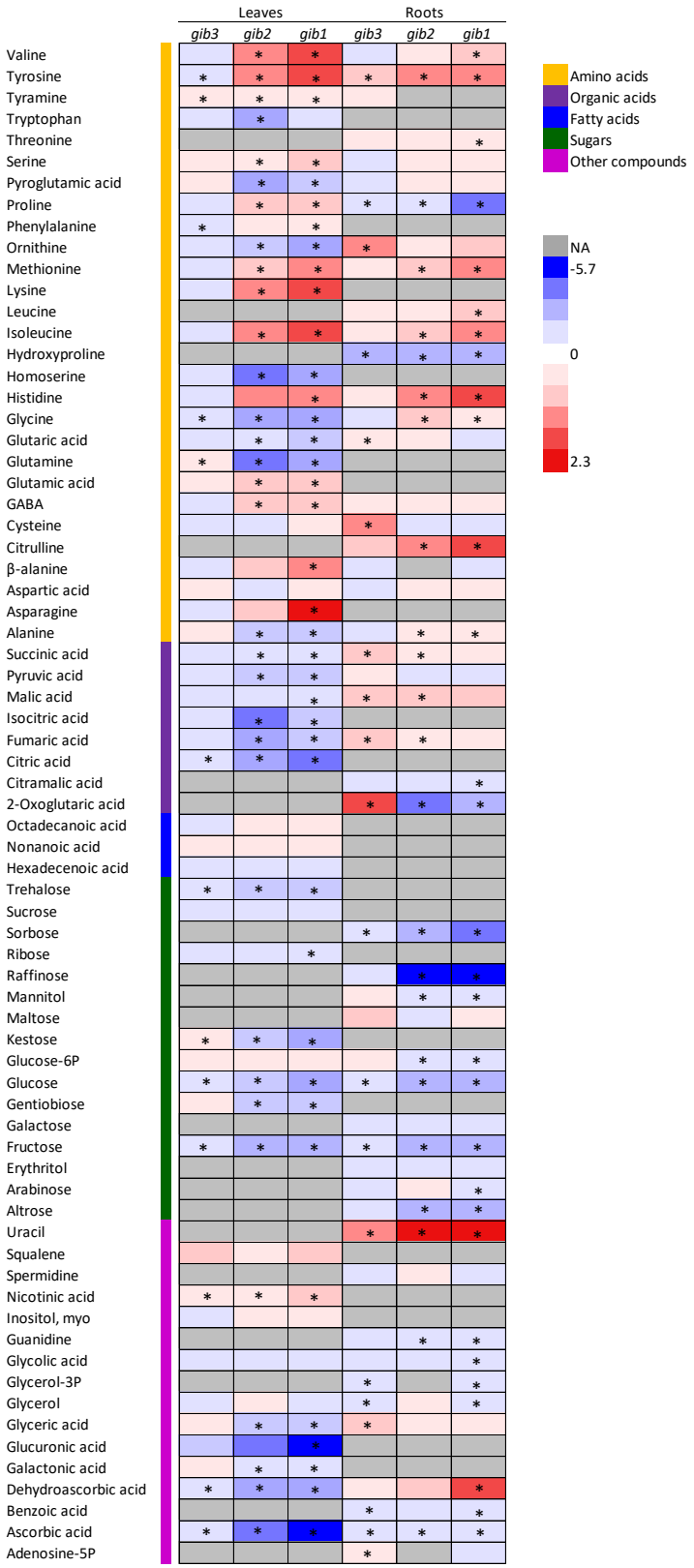

Figure 3: Heat map representing changes in relative metabolite contents. Metabolites were analyzed in shoots and roots of plants WT and mutant plants in biosynthesis of gibberellin gib3, gib2 and gib1 (as described in material and methods). Different shades of red and blue express the extent of the change according to the color bar scale provided (log2 ratio of control); grey color indicate not measured. Asterisks indicate values determined by the Dunnett-test to be significantly different from WT plants ($P < 0.05$). Values are presented as means of six replicates.

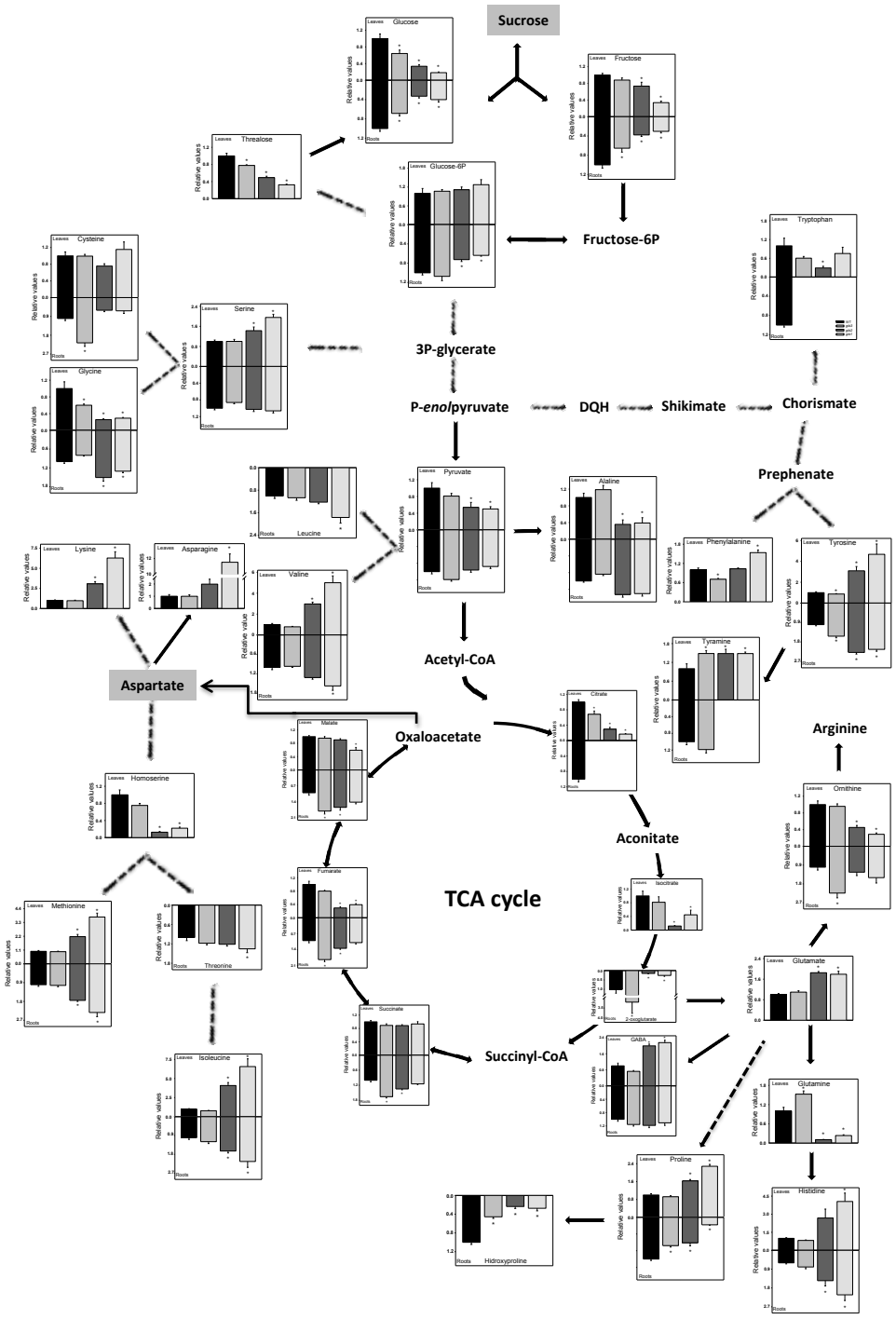

Figure 4: Changes in metabolite profiles in shoots and roots of plants WT and mutant plants in the biosynthesis of gibberellin (gib3, gib2 and gib1). Metabolites without a significant difference between genotypes are indicated by a grey square. Metabolites outside grey squares indicate that they were not measured. Continuous arrows indicate a one-step reaction, and broken arrows indicate a series of biochemical reactions. Values are presented as means of six replicates. Asterisks indicate values determined by the Dunnett-test to be significantly different from control ($P < 0.05$).

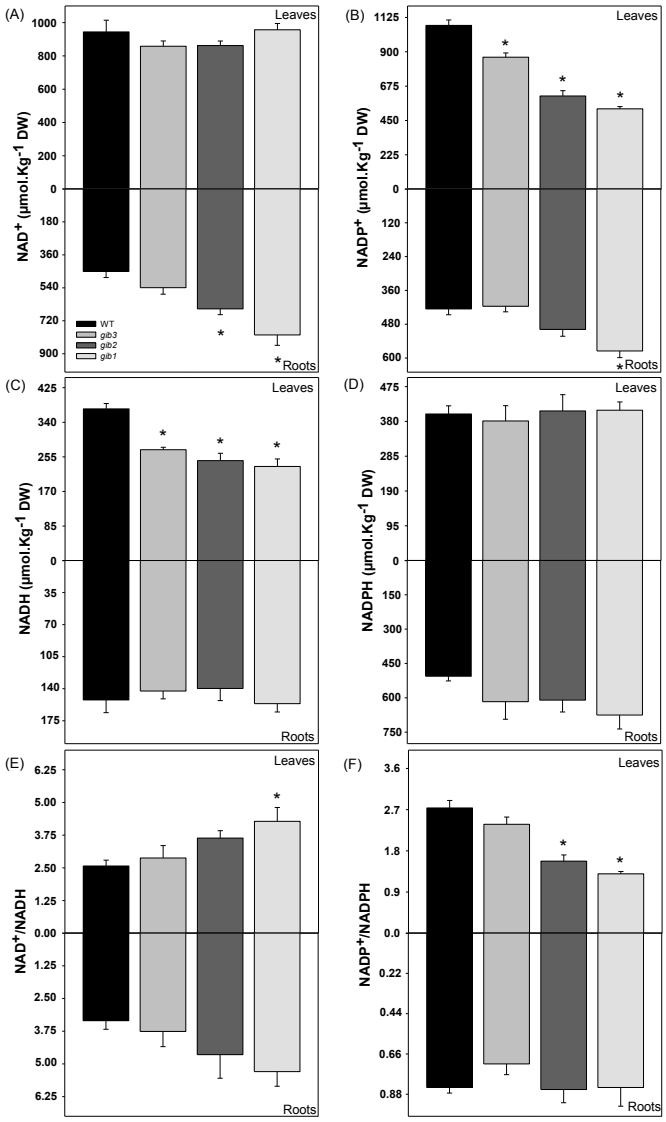

Figure 5: Comparison of the pyridine nucleotide levels and ratios in shoots and roots. WT and mutant plants in biosynthesis of gibberellin gib3, gib2 and gib1 (as described in material and methods) were used. (A) NAD+. (B) NADP+. (C) NADH. (D) NADPH. Asterisks indicate values determined by the Dunnett-test to be significantly different from control ($P < 0.05$). Values are presented as means of seven replicates ±SE.

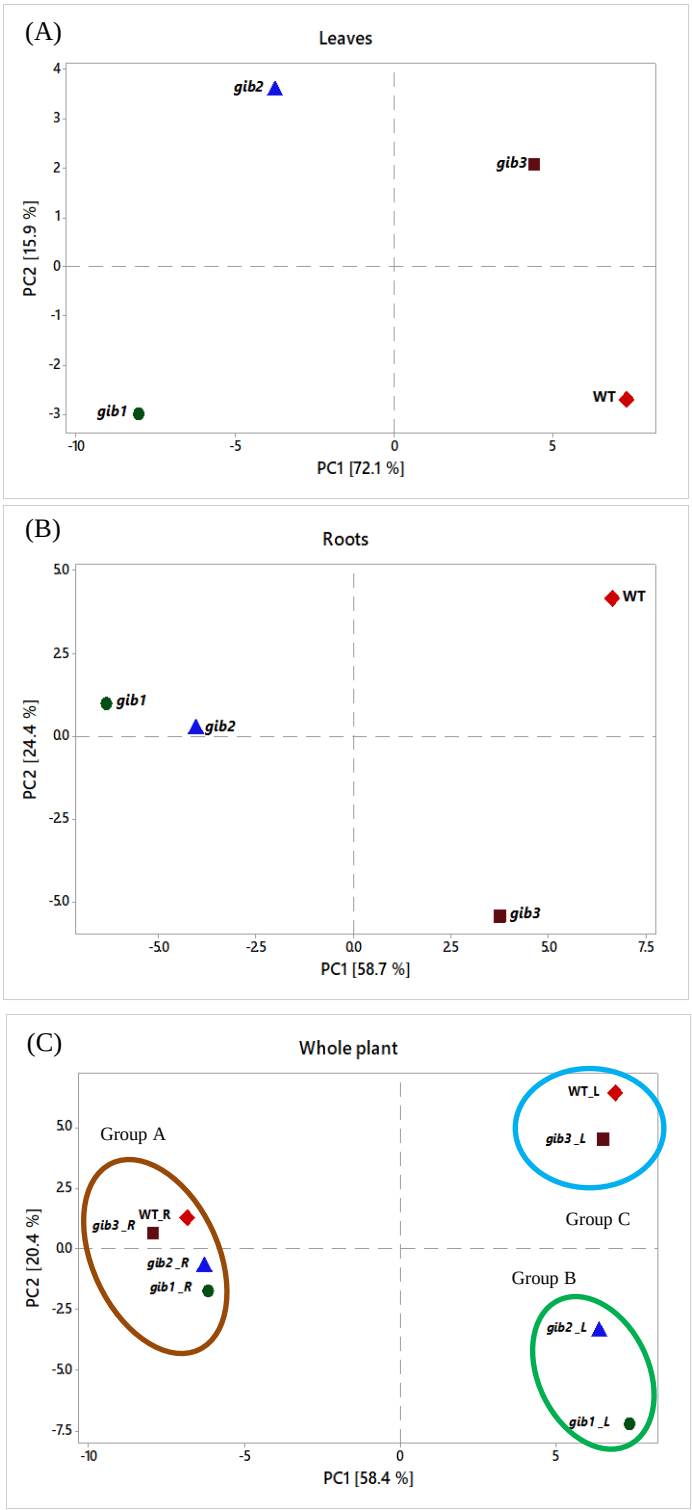

Figure 6: Principal component plot (PC1 vs. PC2 plots) of the metabolite dataset obtained in tomato plants WT, gib3, gib2 and gib1. (A) Leaves. (B) Roots. (C) Whole plants. Brown circle indicates group of roots; green circle indicates group of leaves from gib2 and gib1; blue circle indicates group of leaves from gib3 and WT. The contributions of each metabolite to the separation of the genotypes in the two components (PC1 and PC2) are found in Supplementary Table 1. Metabolites were determined as described in the Materials and Methods section

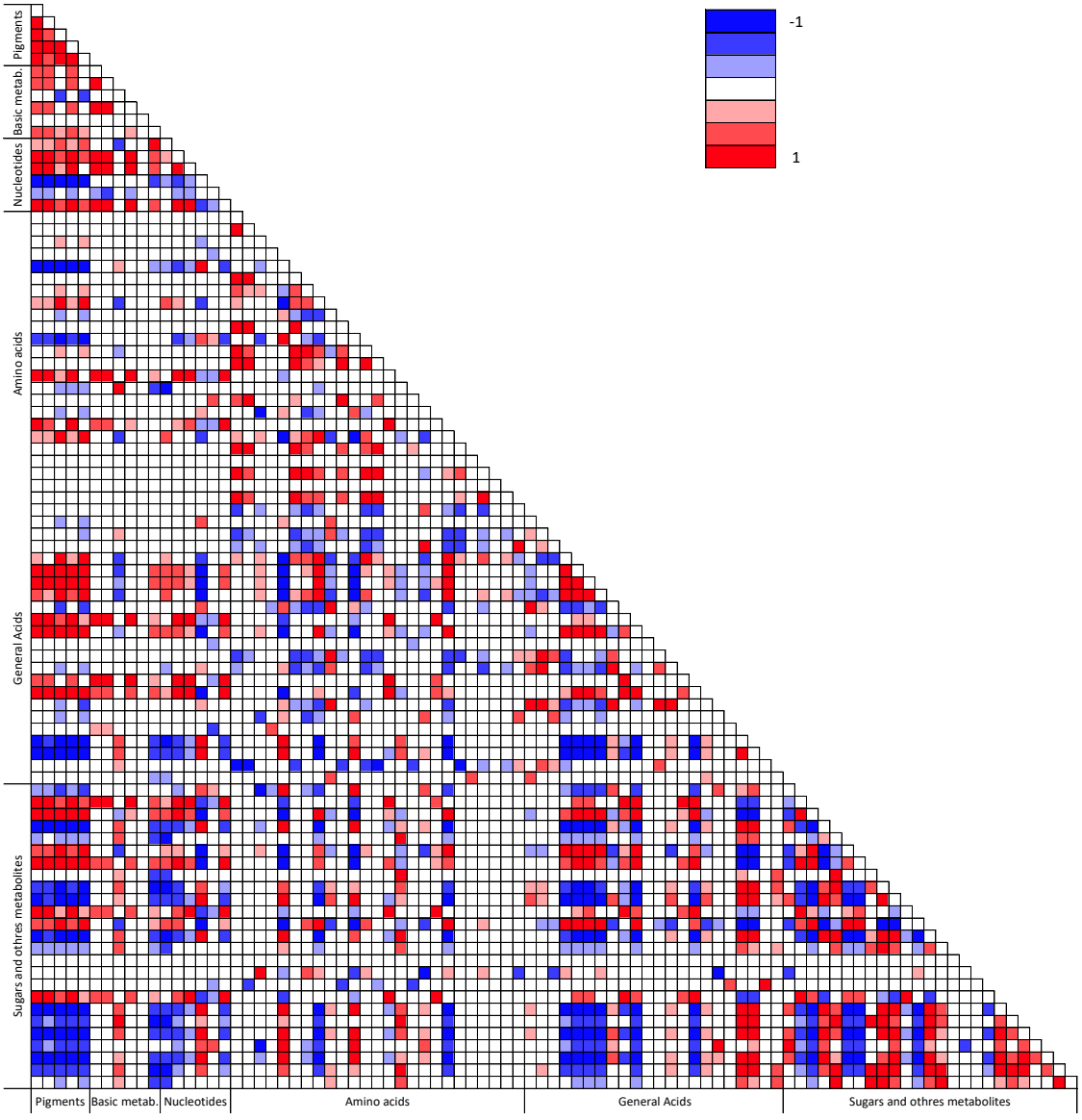

Figure Supplementary 1. Visualization of metabolite-metabolite correlations. Heatmap of metabolite-metabolite correlations for root and shoot of tomato plants WT and mutants in GAS biosynthesis (gib3, gib2 and gib1). Metabolites were grouped by compound class, and each square represents the correlation between the metabolite heading the column with the metabolite heading the row. Correlation coefficients and significances (two tailed) were calculated by applying Pearson algorithm using GraphPad Prism software. Each square indicates a given r value resulting from a Spearman correlation analysis. Red and blue colors indicate positive and negative correlation, respectively, to ($P<0.05$).

Variables	PC1	PC2
Chl *b*	0.122	0.101
Chl *a/b*	0.136	-0.005
Chl *a+b*	0.126	0.089
Fructose	0.081	0.178
Sucrose	-0.108	0.081
Starch	0.087	0.154
Ptnas	0.111	0.045
NAD+	0.118	-0.027
NADH	0.118	0.100
NADP+	0.099	0.152
NADPH	-0.128	-0.029
NAD+/H	-0.068	-0.158
NADP+/H	0.112	0.132
Valine	0.065	-0.198
Tyrosine	0.042	-0.215
Threonine	-0.135	-0.012
Serine	0.078	-0.183
Proline	0.095	-0.121
Phenylalanine	0.129	-0.061
Ornithine	-0.103	0.083
Methionine	0.039	-0.208
Leucine	-0.127	-0.020
Lysine	0.095	-0.154
Isoleucine	0.062	-0.203
Homoserine	0.097	0.153
Hydroxyproline	-0.105	0.022
Histidine	0.011	-0.205
Glycine	-0.096	0.082
Glutamine	0.089	0.149
Glutamic acid	0.128	-0.072
GABA	0.053	-0.198
β-alanine	0.081	-0.151
Asparagine	0.076	-0.153
Alanine	-0.071	0.133
Succinic acid	-0.102	0.009
Pyruvic acid	-0.095	0.150
Pyroglutamic acid	-0.082	0.128
Phosphoric acid	0.125	-0.088
Octadecanoic acid	0.137	0.000

Variables	PC1	PC2
Nonanoic acid	0.135	0.011
Nicotinic acid	0.131	-0.060
Malic acid	-0.110	0.046
Isocitric acid	0.107	0.122
Hexadecenoic acid	0.136	0.015
Glycolic acid	0.013	0.127
Glyceric acid	-0.088	0.139
Glutaric acid	-0.103	0.110
Glucuronic acid	0.089	0.161
Galactonic acid	0.127	0.083
Fumaric acid	-0.106	0.093
Citric acid	0.047	0.151
Citramalic acid	-0.134	0.004
Benzoic acid	-0.135	-0.001
Ascorbic acid	-0.056	0.204
2-Oxoglutaric acid	-0.840	0.020
Uracil	-0.110	-0.029
Trehalose	0.118	0.115
Sucrose	0.136	0.019
Spermidine	-0.134	-0.004
Sorbose	-0.107	0.025
Squalene	0.129	-0.013
Ribose	0.131	0.058
Mannitol	-0.127	0.013
Maltose	-0.136	-0.002
Kestose	0.102	0.142
Inositol, myo	0.135	-0.032
Guanidine	-0.133	0.005
Glycerol-3P	-0.108	0.013
Glucose	-0.019	0.191
Gentiobiose	0.108	0.130
Galactose	-0.137	-0.002
Fructose	-0.117	0.020
Erythritol	-0.137	-0.003
Citrulline	-0.110	-0.031
Arabinose	-0.136	-0.007
Altrose	-0.122	0.017
Adenosine-5P	-0.104	0.019

Supplementary table 1: individual contribution over than 77 variables used in the principal components analysis (Figure 6). PC1: main components on axis 1. PC2: main components on axis 2.

	Leaves				Roots			
	WT	*gib3*	*gib2*	*gib1*	WT	*gib3*	*gib2*	*gib1*
Valine	1 ± 0.1053	0.7875 ± 0.0411	2.9730 ± 0.1734	5.0017 ± 0.6542	1 ± 0.0713	0.9674 ± 0.0332	1.3137 ± 0.0520	1.5724 ± 0.1366
Tyrosine	1 ± 0.0856	0.8715 ± 0.0383	3.0955 ± 0.4108	4.6745 ± 0.9907	1 ± 0.0526	1.5357 ± 0.0756	2.2882 ± 0.0754	2.1463 ± 0.0883
Tyramine	1 ± 0.1543	1.4824 ± 0.0954	1.4814 ± 0.1378	1.4812 ± 0.0486	1 ± 0.0709	1.1873 ± 0.0808	-	-
Tryptophan	1 ± 0.2443	0.6048 ± 0.0547	0.2916 ± 0.0536	0.7541 ± 0.1994	1 ± 0.0404	-	-	-
Threonine	-	-	-	-	1 ± 0.1005	1.1809 ± 0.0493	1.2126 ± 0.0564	1.3544 ± 0.1178
Serine	1 ± 0.0550	1.0016 ± 0.0795	1.4292 ± 0.1465	1.9560 ± 0.1258	1 ± 0.0377	0.8621 ± 0.0311	1.0265 ± 0.0519	1.0619 ± 0.0510
Pyroglutamic acid	1 ± 0.0363	1.0473 ± 0.0416	0.2618 ± 0.0228	0.4342 ± 0.0315	1 ± 0.0513	0.9186 ± 0.0564	1.2343 ± 0.1026	1.2822 ± 0.1268
Proline	1 ± 0.0575	0.9219 ± 0.0459	1.6306 ± 0.0620	2.2847 ± 0.0836	1 ± 0.0342	0.6784 ± 0.0345	0.6109 ± 0.0743	0.1801 ± 0.0089
Phenylalanine	1 ± 0.0565	0.7041 ± 0.0269	1.0311 ± 0.0270	1.5370 ± 0.0829	-	-	-	-
Ornithine	1 ± 0.0863	0.9569 ± 0.0596	0.4519 ± 0.0474	0.2887 ± 0.0320	1 ± 0.1379	2.2399 ± 0.2269	1.2400 ± 0.1618	1.5011 ± 0.2568
Methionine	1 ± 0.0661	0.9733 ± 0.0405	2.1727 ± 0.1753	3.7392 ± 0.3059	1 ± 0.0771	1.0338 ± 0.0631	1.7507 ± 0.0498	2.3301 ± 0.1991
Lysine	1 ± 0.0678	0.9714 ± 0.0662	3.0671 ± 0.2783	6.2801 ± 0.7782	-	-	-	-
Leucine	-	-	-	-	1 ± 0.0904	1.0670 ± 0.0903	1.2242 ± 0.0553	1.7666 ± 0.2030
Isoleucine	1 ± 0.0581	0.7865 ± 0.0391	4.0872 ± 0.3892	6.5650 ± 1.0142	1 ± 0.0856	1.1954 ± 0.0883	1.6421 ± 0.0923	2.1425 ± 0.2813
Hydroxyproline	-	-	-	-	1 ± 0.0454	0.4559 ± 0.0345	0.2363 ± 0.0416	0.2743 ± 0.0546
Homoserine	1 ± 0.1147	0.7512 ± 0.0476	0.1261 ± 0.0161	0.2197 ± 0.0253	-	-	-	-
Histidine	1 ± 0.0544	0.8257 ± 0.0399	2.6828 ± 0.7436	4.0562 ± 0.6966	1 ± 0.0824	1.3276 ± 0.1418	2.4206 ± 0.4199	3.5481 ± 0.4556
Glycine	1 ± 0.1617	0.6007 ± 0.0293	0.2550 ± 0.0204	0.2907 ± 0.0168	1 ± 0.0539	0.7993 ± 0.0237	1.5069 ± 0.1185	1.3057 ± 0.0500
Glutaric acid	1 ± 0.0792	0.7906 ± 0.0470	0.6935 ± 0.0238	0.5329 ± 0.0640	1 ± 0.1090	1.3132 ± 0.0408	1.2044 ± 0.0533	0.8973 ± 0.0288
Glutamine	1 ± 0.1157	1.5265 ± 0.1011	0.1032 ± 0.0088	0.2336 ± 0.0241	-	-	-	-
Glutamic acid	1 ± 0.0408	1.0945 ± 0.0545	1.8504 ± 0.0484	1.7916 ± 0.1171	-	-	-	-
GABA	1 ± 0.1373	0.7267 ± 0.0424	1.9968 ± 0.0909	2.1597 ± 0.1213	1 ± 0.0593	1.1554 ± 0.0487	1.1830 ± 0.0676	1.1052 ± 0.0788
Cysteine	1 ± 0.0947	0.9932 ± 0.0422	0.7554 ± 0.0553	1.1531 ± 0.1799	1 ± 0.0987	2.1666 ± 0.1683	0.6067 ± 0.0668	0.6346 ± 0.1240
Citrulline	-	-	-	-	1 ± 0.1032	1.4369 ± 0.0730	2.3154 ± 0.2239	3.5447 ± 0.2415
β-alanine	1 ± 0.1954	0.9476 ± 0.0754	1.7680 ± 0.2123	3.8052 ± 0.3070	1 ± 0.0411	0.8224 ± 0.0352	-	0.8526 ± 0.0734
Aspartic acid	1 ± 0.1553	1.2503 ± 0.0365	0.8776 ± 0.1429	1.2008 ± 0.1222	1 ± 0.0284	0.8630 ± 0.0803	1.1868 ± 0.0989	1.2289 ± 0.1094
Asparagine	1 ± 0.1341	0.9951 ± 0.1206	1.9890 ± 0.4044	11.5272 ± 1.0608	-	-	-	-
Alanine	1 ± 0.0993	1.1865 ± 0.1023	0.3558 ± 0.1068	0.3887 ± 0.1270	1 ± 0.0290	0.8371 ± 0.0336	1.3224 ± 0.0647	1.3013 ± 0.0604
Succinic acid	1 ± 0.0399	0.8921 ± 0.0475	0.8867 ± 0.0276	0.9362 ± 0.0694	1 ± 0.0665	1.6544 ± 0.0526	1.3464 ± 0.0347	1.1450 ± 0.0306
Pyruvic acid	1 ± 0.1306	0.8084 ± 0.0652	0.5381 ± 0.1202	0.5014 ± 0.0546	1 ± 0.0540	1.1888 ± 0.0403	0.9625 ± 0.0531	0.8767 ± 0.0490
Malic acid	1 ± 0.0305	0.9542 ± 0.0489	0.9022 ± 0.0359	0.5930 ± 0.0831	1 ± 0.1066	1.8034 ± 0.1288	1.6397 ± 0.1250	1.4116 ± 0.0749
Isocitric acid	1 ± 0.1410	0.8138 ± 0.1604	0.1187 ± 0.0143	0.4466 ± 0.1375	-	-	-	-
Fumaric acid	1 ± 0.0954	0.8008 ± 0.0233	0.2969 ± 0.0369	0.3943 ± 0.0337	1 ± 0.0957	1.8330 ± 0.0918	1.3382 ± 0.0454	1.0883 ± 0.0605
Citric acid	1 ± 0.0630	0.6841 ± 0.0847	0.2950 ± 0.0496	0.1696 ± 0.0139	1 ± 0.0701	-	-	-
Citramalic acid	-	-	-	-	1 ± 0.0424	0.8011 ± 0.0432	0.7927 ± 0.0906	0.6219 ± 0.0235
2-Oxoglutaric acid	-	-	-	-	1 ± 0.2059	3.2091 ± 0.5515	0.1522 ± 0.0217	0.2577 ± 0.0499
Octadecanoic acid	1 ± 0.0282	0.9920 ± 0.0226	1.0633 ± 0.0431	1.0208 ± 0.0109	-	-	-	-
Nonanoic acid	1 ± 0.0596	1.2843 ± 0.0569	1.1538 ± 0.0868	1.0481 ± 0.1067	-	-	-	-
Hexadecenoic acid	1 ± 0.0230	0.9818 ± 0.0259	0.9827 ± 0.0407	0.9170 ± 0.0107	-	-	-	-
Trehalose	1 ± 0.0627	0.7770 ± 0.0159	0.4917 ± 0.0332	0.3251 ± 0.0132	-	-	-	-
Sucrose	1 ± 0.0938	0.8498 ± 0.0476	0.9370 ± 0.0172	0.8291 ± 0.0811	-	-	-	-
Sorbose	-	-	-	-	1 ± 0.0773	0.6706 ± 0.0891	0.2561 ± 0.0174	0.2018 ± 0.0140
Ribose	1 ± 0.1016	0.7289 ± 0.0684	0.6856 ± 0.0828	0.5971 ± 0.0686	-	-	-	-
Raffinose	-	-	-	-	1 ± 0.1514	0.8874 ± 0.0725	0.0200 ± 0.0023	0.0219 ± 0.0020
Mannitol	-	-	-	-	1 ± 0.0937	1.0619 ± 0.1676	0.4992 ± 0.0407	0.5233 ± 0.0451
Maltose	-	-	-	-	1 ± 0.1144	1.3992 ± 0.2119	0.9961 ± 0.0453	1.0179 ± 0.0908
Kestose	1 ± 0.0303	1.2880 ± 0.0887	0.3267 ± 0.0323	0.2426 ± 0.0221	1 ± 0.0446	1.0732 ± 0.0892	0.7260 ± 0.0399	0.6383 ± 0.0170
Glucose-6P	1 ± 0.1544	1.0665 ± 0.0523	1.1133 ± 0.0889	1.2754 ± 0.1609	1 ± 0.0575	0.6864 ± 0.0510	0.3297 ± 0.0359	0.4005 ± 0.0515
Glucose	1 ± 0.1107	0.6430 ± 0.0817	0.3372 ± 0.0394	0.1848 ± 0.0169	-	-	-	-
Gentiobiose	1 ± 0.1105	1.2835 ± 0.2560	0.4105 ± 0.0480	0.3260 ± 0.0435	-	-	-	-
Galactose	-	-	-	-	1 ± 0.0646	0.9169 ± 0.0437	0.8729 ± 0.0206	0.8494 ± 0.0471
Fructose	1 ± 0.0397	0.8792 ± 0.0516	0.7353 ± 0.0917	0.3387 ± 0.0363	1 ± 0.0686	0.6541 ± 0.0811	0.3780 ± 0.0326	0.3040 ± 0.0332
Erythritol	-	-	-	-	1 ± 0.0312	0.9567 ± 0.0366	0.9685 ± 0.0712	0.8582 ± 0.0600
Arabinose	-	-	-	-	1 ± 0.0241	0.9436 ± 0.0176	1.1222 ± 0.0119	0.9978 ± 0.0217
Altrose	1 ± 0.0407	0.9944 ± 0.0493	1.2117 ± 0.3340	0.8008 ± 0.0595	1 ± 0.0947	0.9713 ± 0.1438	0.3988 ± 0.0298	0.4201 ± 0.0288
Uracil	-	-	-	-	1 ± 0.1491	2.5275 ± 0.2176	4.9054 ± 0.1804	4.3275 ± 0.1306
Squalene	1 ± 0.0960	2.0413 ± 0.3910	1.3609 ± 0.1736	1.7548 ± 0.5436	-	-	-	-
Spermidine	-	-	-	-	1 ± 0.0602	0.8435 ± 0.0330	1.1083 ± 0.1237	0.8205 ± 0.0274
Nicotinic acid	1 ± 0.0677	1.3685 ± 0.0381	1.6153 ± 0.0702	1.8895 ± 0.1292	-	-	-	-
Inositol, myo	1 ± 0.0907	0.8547 ± 0.0978	1.0861 ± 0.0865	1.2603 ± 0.0930	-	-	-	-
Guanidine	-	-	-	-	1 ± 0.0598	0.8359 ± 0.0161	0.6136 ± 0.0339	0.6922 ± 0.0557
Glycolic acid	1 ± 0.0869	0.9339 ± 0.0959	0.9481 ± 0.0560	0.7772 ± 0.0270	1 ± 0.0339	0.8770 ± 0.0203	0.9948 ± 0.0455	0.6805 ± 0.0267
Glycerol-3P	-	-	-	-	1 ± 0.0551	0.9269 ± 0.0427	-	0.6877 ± 0.0240
Glycerol	-	-	-	-	1 ± 0.0244	0.8886 ± 0.0144	1.0412 ± 0.0303	0.8565 ± 0.0323
Glyceric acid	1 ± 0.0750	1.1278 ± 0.0588	0.4380 ± 0.0504	0.5174 ± 0.0453	1 ± 0.1231	1.4752 ± 0.0692	1.1012 ± 0.0547	1.0488 ± 0.0522
Glucuronic acid	1 ± 0.4343	0.4975 ± 0.1019	0.1696 ± 0.0276	0.0873 ± 0.0171	-	-	-	-
Galactonic acid	1 ± 0.0598	1.0684 ± 0.0497	0.5655 ± 0.0275	0.6005 ± 0.0612	-	-	-	-
Dehydroascorbic acid	1 ± 0.0375	0.8703 ± 0.0466	0.2989 ± 0.0206	0.1872 ± 0.0068	1 ± 0.1071	1.1805 ± 0.0865	1.5904 ± 0.1724	2.8113 ± 0.3297
Benzoic acid	-	-	-	-	1 ± 0.0551	0.8213 ± 0.0368	0.9099 ± 0.0430	0.7578 ± 0.0261
Ascorbic acid	1 ± 0.0618	0.8188 ± 0.0624	0.1407 ± 0.0142	0.0554 ± 0.0051	1 ± 0.0157	0.8305 ± 0.0233	0.5862 ± 0.0232	0.6433 ± 0.0445
Adenosine-5P	-	-	-	-	1 ± 0.1608	1.1484 ± 0.2506	-	0.5177 ± 0.0453

Supplementary table 2: Table with relatives values ± standart error for metabolite contents. Metabolites were analyzed in shoots and roots of plants WT and mutant plants in biosynthesis of gibberellin gib3, gib2 and gib1 (as described in material and methods). Values are presented as means of six replicates.

CHAPTER IV

Both transition from vegetative-reproductive stage and fruits development are mediated by GA in tomato

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ABSTRACT

Gibberellins, a large group of plant hormones produced by the dipertenes pathway, play a crucial role in modulating several developmental processes throughout the plant life cycle. Among the many processes in which GAs are involved, they are of critical importance during the transition and maintenance of the reproductive meristem, as well as to allow the development of floral organs. GAs are also able to regulate, alongside with other hormones, the initial processes of fruit development, most likely because GAs are responsible for both division and cell expansion process. Here, we investigate whether fluctuations in the endogenous content of GAs impact fruit development and metabolism during ripening. To this mutant plants deficient in the biosynthesis of GAs (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. Little, if any, differences were found between WT and gib3 mutant plants at floral development and total fruit yield. We further demonstrated that the reduction in the content of GAs in gib3 mutant did not promote morphological modifications in fruits. Relatively few metabolic changes were observed between genotypes during fruit ripening. Overall, typical metabolic changes of fruit ripening including increments in amino acids and soluble sugars coupled with reductions in starch were observed during ripening. Collectively, our results demonstrate that the modification of a semi autotrophic metabolism to a completely heterotrophic metabolism takes place independently of GA content.

INTRODUCTION

The transition from vegetative to reproductive development is of great importance throughout the life cycle of plants (Galvao et al., 2015). In the case of angiosperm, reproductive success and the establishment of the germ line occurs only after this transition, that is, 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 Perilleux, 2005; Bouche et al., 2017). It has been also described that endogenous signs such as age and hormone levels are able to impact flowering time (Andrés et al., 2014; Aya et al., 2009; Zhang et al., 2016). Accordingly, the fruit set is characterized by the rapid development of the flower's ovary after 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, is classified in fresh and dry fruit and its development presents different phases (Knapp, 2002). Recent studies have proposed four characteristic physiological stages occurring during fruit development process (McAtee et al., 2013; Obroucheva, 2014) against the three initially proposed previously (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 characterized mainly 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 has yet to start the ripening process (Ireland et al., 2013). The later stage is usually associated with significant metabolic and physiological changes within the fruit (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., 2007; Huang et al., 2017). Gibberellins (GAs) are associated with the promotion of the juvenile-adult phase transition in plants (Huang et al., 2017; Pharis, 1985). Accordingly, GAs act in the establishment and maintenance of floral meristem (Andrés et al., 2014; Gomez-Mena et al., 2005; Olszewski et al., 2002) and at the development of floral organs such as petals (Hirano et al., 2008), anthers and pollen (Chhun et al., 2007; Kwon et al., 2015; Zhang et al., 2010) 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; McAtee et al., 2013; Serrani et al., 2007a). Thus, the content of GAs increase rapidly after fertilization of the ovary acting as a positive signal during the initial fruit development (Dorcey et al., 2009; Mapelli et al., 1978; 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 et al., 2002; Locascio et al., 2013). In general, fruit set is result of successful ovule fertilization (Gillaspy et al., 1993; Olimpieri et al., 2007). However, the ovary development can be independently of this process, which leads to the formation of seedless fruit called parthenocarpic. Parthenocarpy may also occur in the nature as a result of fertilization with dead grains pollen or originating from

incompatible species, as well as fertilization followed by embryo abortion. Accordingly, the parthenocarpy can be also artificially induced via increase in hormones content through 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 (Garcia-Hurtado et al., 2012; Rieu et al., 2008), 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, parthenocarpic or not, the content of bioactive GAs decreases at the end of the growth phase being undetected during the maturation and ripening phases (McAtee et al., 2013). During these last two stages fruits undergo highly regulated metabolic and physiological changes that affect the consistency, color, flavor and ultimately taste of the fruit (Obroucheva, 2014; Qin et al., 2016; Zhang et al., 2015). In tomato fruits, this drastic metabolic change is characterized by transition from the partially photosynthetic to the true heterotrophic metabolism. 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. Tomato plants WT and gib3 mutant plants showed a decoupling between the carbon and nitrogen metabolism in leaves. This decoupling was due to the manipulation of the endogenous levels of GAs (Martins, 2013). 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 tomato mutant 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 impact juvenile-adult phase transition and the reproductive organs development. By contrast, the

moderate deficiency in GAs observed in gib3 mutant plants culminated with morphological variation at the early stages of fruit development but was fully recovered following full fruit development. The effect of impaired GA biosynthesis appears to be fairly specific, particularly on floral establishment and metabolic reprogramming during fruit development. We compare and contrast our results with those previously published on the impact of the GA on tomato fruit and leaves and further discuss them within the context of current models of GA biosynthesis and signaling.

MATERIAL AND METHODS

Plant material and growth conditions

The present work was conducted in Viçosa (20º 45'S, 42º 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). Tomato seeds were germinated in Petri dishes with two layers of filter paper (Whatman nº 1) soaked in GA solution (Duchefa, Haarlem, The Netherlands; GA_3 50 μM). 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 ± 5 % and light intensity of 150 $\mu mol.m^{-2}.s^{-1}$. 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 per pot and fertilization throughout the experiment, as recommended for the crop. After transfer to pots, the plants were grown in a greenhouse under semi-controlled conditions

(maximum photosynthetically active radiation (PAR) of c. 1500 µmol photons m^{-2} 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 analysis, a posteriori. Two experiments were conducted in parallel, one for the evaluation of total fruits production and the second to analyze the reproductive process and fruit development.

Experiment I: Total fruit production

WT 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. Each single day, new flowers were tagged and the flowering and fruiting dates recorded on the tag. Index of floral fixation or percentage of flowers 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.

Experiment II: fruit development

WT and mutants plants were grown side by side under greenhouse in the same conditions as described above and their development was closely observed until the production of fruits.

Flowering date

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; 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. 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 coloring were considered infeasible.

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) by determining their fresh weight, diameter, height, volume and number of seeds. The fruits were oven-dried to constant dry weight at 72 ^{o}C for 72 h.

Biochemistry of fruits

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 ^{o}C until further analysis.

Pigments determination

Chlorophyll content (a and b) and total carotenoids were determined from 25 mg fresh matter in in 85 % acetone extract at 4 ^{o}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, 1976).

Pyridine nucleotides

NAD(H)s and NADP(H)s were determined as described previously (Gibon and Larher, 1997). Briefly, approximately 5 mg of dry matter were used and thus pyridine nucleotides were assayed using the phenazine methosulfate-catalyzed 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 profile

Fruit pericarp samples (~50 mg, fresh weight) 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). This analysis

allowed the determination of approximately 50 different compounds, representing the main classes of compounds (amino acids, organic acids, sugars and others).

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

Visual inspection of Inflorescence formation and development

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 very different and characteristic among the tested genotypes. Through visual and also photographic monitoring during ten weeks, it was observed that the endogenous reduction of GA content strongly affects the reproductive development of these plants (Fig. 1). WT showed the first inflorescence three weeks after the transplant 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 were characterized by a vegetative development very similar to WT, it was observed a delay in the appearance of the first inflorescence as well as in fruit emergence as long as one week in relation to WT plants. Notably, in 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 analyzing the

development of the inflorescences in a more detailed manner, it was verified that only gib1 mutant plants presented a significantly smaller quantity of inflorescence per plant in comparison to WT plants (Fig. 2A). 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 inflorescences to fully open, was greater in approximately three days in gib3 plants in relation to WT (Fig. 2C). As previously demonstrated (Fig. 1), gib2 and gib1 mutant plants have no anthesis period since the buds are not able to grow in flowers, possibly due to the drastic reduction in the content of GAs. We also quantified the viability of the pollen grains in WTs and gib3 plants showing that the number of infeasible pollen grains was similar (and relatively low) between WT and gib3 plants (Fig 2D). This analysis was carried out solely in WT and gib3 plants, since in gib2 and gib1 the floral development is paralyzed before this step and is only concluded with the addition of exogenous GA.

Morphological evaluation of fruits

Considering that both gib2 and gib1 mutant plants had their reproductive development fully impaired by the reduction of the endogenous content of GAs, we performed all the following analyzes only on WT and gib3 plants (Fig. 3). There were no significant differences in total number of fruits per plant (Fig. 3A), percentage of floral abortion, calculated by the difference between the total number of flowers and the number of fruits produced per plant (Fig. 3C), the dry weight per fruit (Fig. 3D), the dry weight/fresh weight ratio (Fig. 3E) and the number of seeds per fruit (Fig. 3F). The relatively small reduction of the GA content in gib3 plants seems to not impact the full development of tomato fruits, since these analyzes were

carried out when the fruits were in the ripening stage. 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 demonstrate to which extension the genotype responses are similar during fruit development, we next decided to analyze fruits at different ages (Fig. 4). By analyzing 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 the 20 DAA fruits from gib3 presented significant reductions in relation to the WT fruits in terms of fruit height (Fig. 4A) and fruit diameter (Fig. 4B). However, total fresh weight (Fig. 4C), total dry weight (Fig. 4D), DW/FW ratio (Fig. 4E) and volume (Fig. 4F) was not affected by the reduction in GA levels. We observed that at 40 DAA the fruits of gib3 mutant had their development significantly reduced in all evaluated traits, except for the DW/FW ratio that did not differ. Surprisingly, at 60 DAA the fruits of mutant plants were similar to WT fruits for most of the characters, presenting only reduced values in dry weight and consequently affecting DW/FW. This somehow growth recovery at 60 DAA demonstrates that the reduction of GAs in gib3 promotes differences only at the temporal level, causing a slight delay in fruit development. The effect of time within the genotypes was also evaluated and, as expected, there were significant differences among the three stages of development with progressive increments in the traits evaluated here. The DW/FW ratio of fruits presented similar values at 20 and 40 DAA, these values being significantly reduced at 60 DAA.

Metabolic changes in response to endogenous variation of GA levels in fruits

Given the above mentioned results and the visual aspects presented by the fruits in the different stages of development (Fig. 5), we 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. 6A) in gib3 fruits at 20 DAA; however, this increase is not maintained in fruits of 40 DAA. The levels of chlorophyll b (Fig. 6B) and chlorophyll a/b (Fig. 6C) were invariant between genotypes at 20 and 40 DAA times. Fruit development between the 20DAA and 40 DAA culminated with relatively small variations in the content of these pigments in both genotypes. Due to the fact that the fruits were already at an advanced ripening stage between 40 and 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. 6D) were gradually and significantly higher in the gib3 mutant than in WT at all time points here evaluated, although only at 60 DAA a significant effect of the time points occur within the genotypes. Total amino acid content (Fig. 6E) and total soluble protein (Fig. 6F) showed a very similar behavior 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. 6E). As for amino acids, protein content did not present significant differences between the genotypes at 20 and 40 DAA (Fig. 6F). At 60 DAA fruits of the gib3 mutant presented a 25 % lower protein content than 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 we compared with 20 DAA.

The contents of soluble sugars (glucose, fructose and sucrose), starch and the organic acids malate and fumarate were also evaluated (Fig. 7). Briefly, glucose (Fig. 7A), fructose (Fig.

7B) and sucrose (Fig. 7C) increased gradually throughout fruit development. In general, glucose and fructose increased significantly 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 and again only in gib3. Surprisingly, the fruits from gib3 presented values significantly elevated in relation to the WT for all sugars evaluated at 60 DAA. Unlike soluble sugars, starch content in both genotypes presented significant differences throughout the fruit development. Between 20 and 40 DAA significant increases in starch content were observed reaching the highest contents observed. Between 40 and 60 DAA reductions in starch content were observed. The reduction in WT fruits was less pronounced, since we observed similar values between 20 and 60 DAA, however in gib3 fruits the levels of starch was lower in 60 DAA 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. 7E) and fumarate (Fig. 7F) was not affected by the reduction of the endogenous level of GAs, except for fumarate at 60 DAA. Both metabolites presented 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 reduction of GA biosynthesis on primary metabolism during fruit development we decided to extend the analysis by studying the major primary metabolic pathways, by using an established GCMS protocol. 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. 8 and Supplementary Figure 1). 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 Figure 8 are either directly derived or feed the glycolytic pathway and the tricarboxylic acid cycle (TCA), both processes that are involved with cell energetic metabolism. Perhaps surprisingly, the metabolite profiles of the mutant lines followed a remarkably similar pattern to those of the wild type. Thus, the levels of sucrose, mannose, glucose-6P and fructose-6P did not present large variations either between genotypes or along the time, and the values were very similar to those of the WT fruit at 20 DAA. It is noteworthy that some of these sugars were not even detected in some samples such as mannose at 60 DAA and fructose-6 at 40 DAA in WT. In gib3 fruit mannose was not detected at 60 DAA and fructose-6P at 40 and 60 DAA. Similar behavior 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 detecte lysine and methionine. Although asparagine, homoserine, aspartate and threonine did not differ between genotypes at 20 DAA, at this time point reductions in isoleucine were observed in gib3 fruits. 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 presented differences between the genotypes at 20 DAA, significant reductions in histidine and glutamine were observed in gib3 fruits at 40 DAA. Fruits from the gib3 mutant plant were characterized by reductions in proline, hydroxyproline and ornithine during ripening, whereas GABA reduced only at 60 DAA. By analyzing these compounds during ripening for each genotype, it is possible to verify that the great majority presented gradual and significant increments as the fruit developed. It is worth mentioning that these compounds reflected well the behavior found in 2-oxoglutarate. Similar behavior to the compounds derived from 2-oxoglutarate was also verified for cysteine, serine and glycine, which are all derived from 3P-glycerate. The amino acids valine, leucine and alanine, that are synthesized from pyruvate, as well as phenylalanine and tyrosine presented the lower contents during ripening in both genotypes, with significant variation just for leucine between WT and gib3 fruits. Although during the growing stage (between20 and 40 DAA) we did not found difference between genotypes but only for phenylalanine at 40 DAA. 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 genotype at 40 and 60 DAA whereas at 20 DAA we were unable to identify this metabolite for gib3 fruits. As well as tryptophan, tyramine increased its content at 60 DAA.

Although relatively few morphological or metabolic disturbances have been observed during fruit development, the changes observed in 2-oxoglutarate, the substrate of the 2-oxoglutarate that also generates the reduced coenzyme NADH, it seems reasonable to suggest that these changes may affect the redox balance. Therefore, we decided to assay the levels of

pyridine dinucleotides during fruit ripening in wild-type 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 (Figure 9).

Principal component analysis (PCA) was also performed to explore more deeply the contribution of changes in the endogenous levels of GAs in the metabolite composition across fruit developmental stages (Fig. 10). This analyzes showed that indeed the dominant source of variation in the combined dataset is the differential contribution of the metabolite composition across fruits ripening in both genotypes. We first analyzed the variation observed in WT and gib3 fruits individually (Fig. 10A and 10B, respectively) and the whole data as further analyzed (Figure 10C). This analyzes revealed that the first two components explained, together 100 % of the data variability observed in WT fruit (Fig.10A; PC1 covers 69.2 % of the total variance and PC2 30.8 %). By analyzing the PC1 it was clearly observed that fruits at 20 DAA and 40 DAA were similar to each other whereas very distinct from fruits at 60 DAA. It is however important to mention that in PC 2 all fruits were distinct from each other. By contrast to the situation observed for WT fruits, fruits at 20 DAA from gib3 mutant plants were very distant from the others by PC1, whereas gib3 fruits at 40 and 60 DAA were similar (Fig. 10B; PC1 covers 55.4 % of the total variance and PC2 44.7 %). When we evaluated the PC2 all fruits presented distinct behavior. Next, we further evaluated the metabolite composition of fruits from both genotypes simultaneously and observed that there was a reduction of percentages that explain the variation along the axes and the formation of three groups (Fig. 10C; PC1 covers 43.2% and PC2 28.9). Interestingly, WT fruits at 20 and 40 DAA and fruits gib3 at 20 DAA were grouped closely either by PC1 or PC2 whereas fruits from gib3 at 40 DAA remained separated from the remaining fruits in both components. The metabolite composition of fruits from WT and gib3

fruits at 60 DAA were well separated in relation to PC1, however, in PC2 they showed a very similar behavior. The metabolic events occurring in WT fruits across the developmental stages are best explained, among others, from acids in general for the time 20 DAA, malate and starch for 40 DAA and several amino acids for 60 DAA (the parameters with high a main impact on the variance of the dataset are additionally presented in Supplementary Table S1). In fruits at 20 DAA from gib3 mutant plants, the chlorophyll content had a main impact on the total variance, as were the amino acids for fruits at 40 DAA and the nucleotides for fruits of 60 DAA of this genotype. A strong impact of chlorophylls, isocitrate and sucrose accounted for the whole variance within fruits (Fig. 10C), allowing that no segregation between WT fruits at 20 and 40 DAA as well as 20 DAA from gib3 were observed. Fruits at 40 DAA from gib3 received great influence of isomaltose and putrescina whereas WT fruits at 60 DAA were heavily influenced by mannose and fruits at 60 DAA from gib3 were influenced mainly by nucleotides, the same way when they were evaluated separately.

DISCUSSION

The effects of the manipulation on the endogenous levels of GAs on plant growth have been described (Strader et al., 2004; Wuddineh et al., 2015; Zhang et al., 2016) but, surprisingly, no attempt has been made to elucidate the morphological and biochemical mechanisms underlying the plant response to GAs fluctuations during fruit development to date. In this study, we decided to detailed explore in tomatoes, a species of agronomic interest, the influence of changes in the endogenous levels of GAs during reproductive development. 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 (Andrés et al., 2014; Gao et al., 2012; Olszewski et al., 2002). 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 GA-related requirements. Thus, gib2 and gib1 mutants had their development restricted to the flower bud stage as previously verified (Jacobsen and Olszewski, 1991; Nester and Zeevaart, 1988). Similar changes were also observed previously in rice (Oriza sativa L. cv Nipponhare) plants 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 concentrations of GAs for the complete flowers 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 over than 50 ng per bud (Nester and Zeevaart, 1988; Shinozaki et al., 2015). Although gib3 mutant plants were characterized by some morphological differences, at inflorescence level, in relation to its respective WT (Fig. 2A and B), the endogenous reduction of GAs was not able to affect both the final phenotype and fruit production (Fig. 3 and 4). Likewise, these results are in good agreement with those previously observed for the same genotypes when only the vegetative stage was evaluated (Martins, 2013; Omena-Garcia, 2014). 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 with a reduced size (Carrera et al., 2012; Olszewski et al., 2002). It seems reasonable to assume that this smaller fruit size was triggered by the difference 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).

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; Obroucheva, 2014; Shinozaki et al., 2015; Srivastava and Handa, 2005). It is known that GAs are involved in cell division (Lammens et al., 2008; Sablowski and Carnier Dornelas, 2014), and cell expansion process (Band et al., 2012; Bouquin et al., 2002) and as such analyzes 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. 4A-D and 4F); however, at 60 DAA these differences were not found anymore suggesting, thus, that the reduction of GAs only promoted a delay in the growth and development of the mutant fruits. These results corroborate with the model previously suggested by Srivastava & Handa (2005), in which they describe that GA peaks during fruit formation mainly between 7 to 15 DAA and 3 to 5 weeks after anthesis. Remarkably, this time coincide exactly with division and cellular expansion phases.

The impacts of GAs on primary metabolism has been previously demonstrated (Araujo et al., 2014; Araújo et al., 2012b; Ribeiro et al., 2012a; Ribeiro et al., 2012b), although this relationship is still not well established. To further investigate the effect of the endogenous reduction of this hormone on fruit metabolism, metabolic analyzes were performed

concomitantly with morphological ones. 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 (Fig. 5). This fact is represented by the reduction of chlorophyll contents (Fig. 6A and B) and consequent carotenoids accumulation (Fig. 6C) 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 6E), proteins (Fig. 6F) and the organic acids malate (Fig. 7E) and fumarate (Fig. 7F), probably reflect the low 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 (Araujo et al., 2012b; Carrari et al., 2006), that seems to not be affected by relatively small reductions in the levels of GAs. By analyzing 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. 7A, 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 sugar content largely justifies the starch levels (Fig. 7D), that increased until 40 DAA and decreased in fruits at 60 DAA. The general picture observed is, however, similar to that found in tomato fruits at 65 DAA of plants with low expression of the mitochondrial enzyme fumarase (Centeno et al., 2011) and control plants at 55 DAA, except for starch (Araujo et al., 2012b). Perhaps more importantly, this inversion in the content of carbohydrate reserves as well as other metabolites during the fruit ripening process culminates in aesthetic and desirable quality characteristics such as texture, color, taste and flavor (Qin et al., 2016; Srivastava and Handa, 2005; Zhang et al., 2015). 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 changes observed when GA levels are strongly reduced, relatively minor changes in GA levels 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 modifications 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. Therefore it was decided to follow the repertoire of metabolic changes that occur during fruit development by using an established GC-MS approach. Several compounds were detected and, among them, large changes in the levels of amino acids between genotypes and throughout fruit development, especially at 40 and 60 DAA (Figure S1). This behavior is somehow similar to what was previously observed in leaf and root tissues of these same plants (Martins et al, 2017, data not yet published). Largest increases were observed in amino acids derived from oxaloacetate, 3β-glycerate and 2-oxoglutarate, and importantly these results are consistent with the results described previously (Araujo et al., 2012a; Araujo et al., 2012b; Carrari and Fernie, 2006). Besides direct involvement in amino acid metabolism, 2-oxoglutarate is also involved in the metabolism of several metabolites including flavonoids, glucosinolate, alkaloids, coumarins and GAs (Araujo 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 recently demonstrated in tomato

plants with reductions in OGDH activity. Theses plant presented 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., 2012). Our results aid further evidence in the connection of primary metabolism, mediated by changes in 2-oxoglutarate, and GAs metabolism supporting the growing body of evidence associated with a significant network behavior 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 et al., 1985), since it is a direct precursor of the biosynthetic pathway of chlorophyll (Carrari and Fernie, 2006; Szafranska et al., 2017). Accordingly, chlorophylls are usually present in 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 color transitions (Bartley and Scolnik, 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; Kuhn and Grof, 2010). It is worth to notice that this process follows different patterns/mechanism of unloading according to the species (Biais et al., 2014; Kuhn and Grof, 2010; Lalonde et al., 2003; Ruan and Patrick, 1995; 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 tomato starch. Thirdly, dilution which may change the concentration without, however, altering the amount of the metabolite and is dependent on the environmental conditions (Dai et al., 2016; Genard et al., 2014). 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 sealing has been oriented, through processes of improvement, to sugar content higher than 60 % of the dry weight (Carrari and Fernie, 2006; Yelle et al., 1991). 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 remains an exciting topic for future research.

CONCLUSION

In the present study we investigated the role GAs play during the transition process from juvenile-adult phase and during the reproductive organs development. The function of GAs in the maintenance of the flowering process was demonstrated especially in gib2 and gib1 mutant plants. The drastic reductions in the endogenous content of GAs observed in those 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 fruits from WT plants. Surprisingly, the morphological variations that were observed in young fruits were not sustained during fruit development with the phenotype being fully recovered at the ripe fruits. This result indicates that the reduced GAs content verified in gib3 mutant promotes only a delay in fruit development in good agreement with the action of this hormone being only reported in the early stages of fruit development. Thus, the metabolic changes observed between genotypes were mainly starting 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 while 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.

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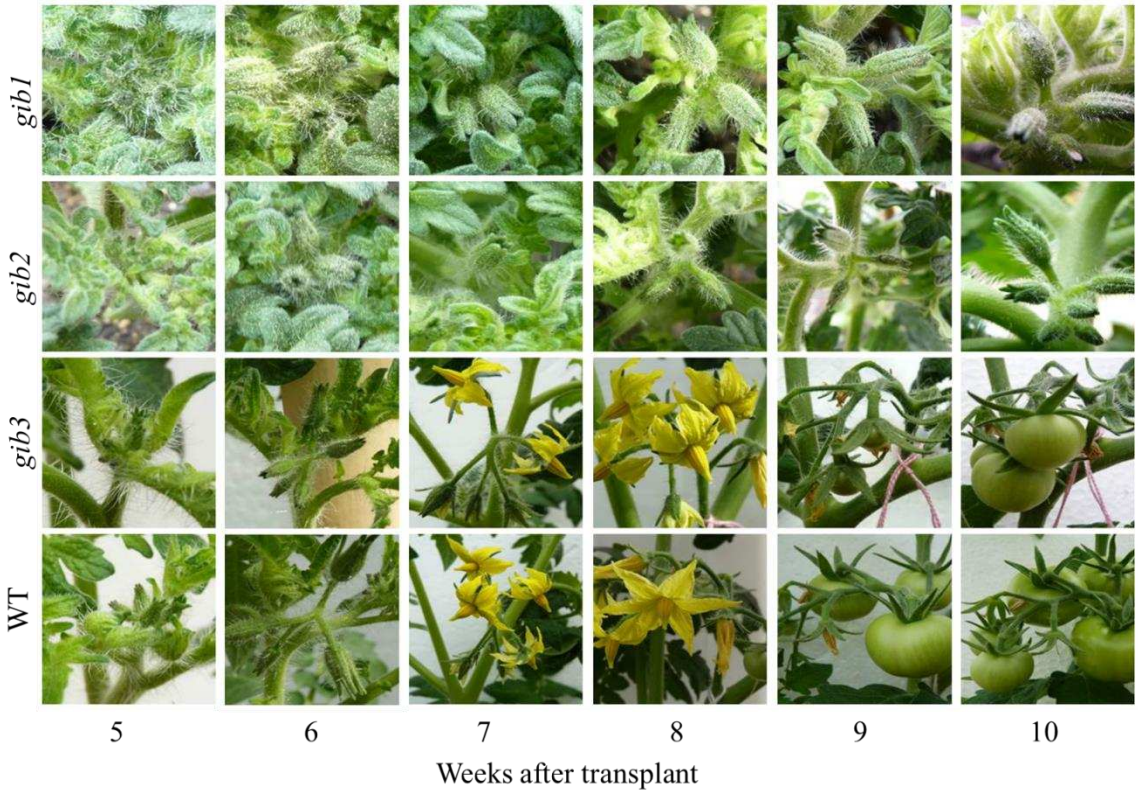


Figure 1: Photographic record of the monitoring of inflorescence development in plants WT (wild type) and mutants: gib3 (moderately deficient in GAs), gib2 (intermediate deficiency in GAs) and gib1 (extremely deficient in GAs).

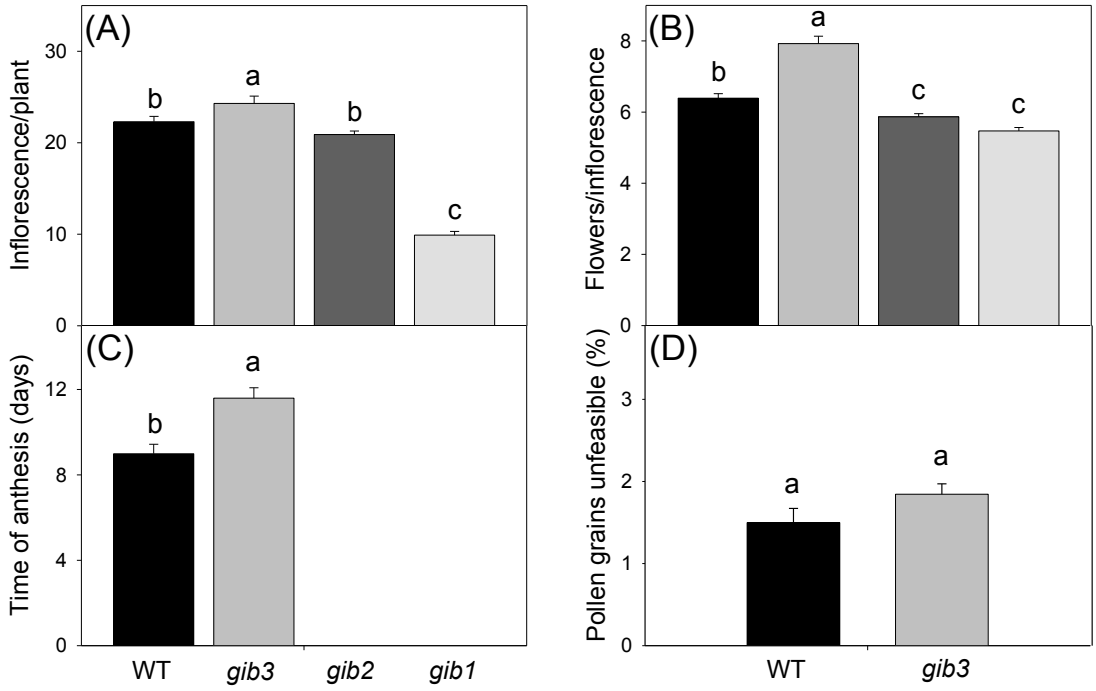


Figure 2: Variation in tomato fruits development parameters in response to endogenous reduction in GA levels. The following were analyzed: Number of inflorescences per plant (A); Total flowers per inflorescence (B); Floral anthesis period (C); Number of non-viable pollen grains (D). Values represent the mean ± SD of at least ten independent plants. Means followed by the same lowercase did not differ from the control in ($P < 0.05$), according to the Scott-Knott test. Abbreviations as described in figure1.

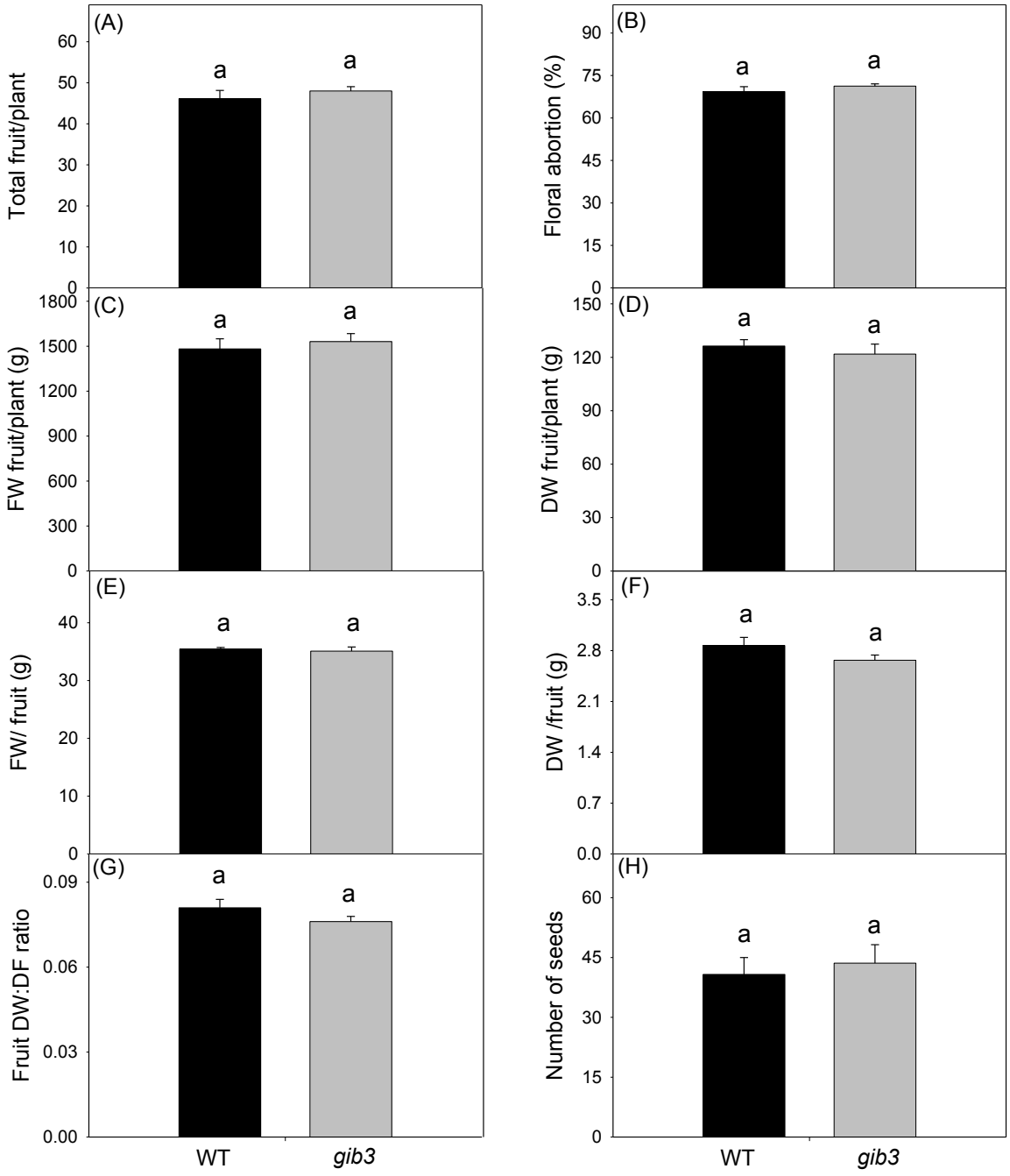


Figure 3: Variation in the morphological development parameters of tomato fruits in response to endogenous reduction in GA levels. Were analyzed: Total fruit production per plant (A); Floral abortion (B); Fresh fruit of weight - FW (C); Dry weight of fruit - DW (D); Ratio DW:FW (E); Number of seeds per fruit (F). Values represent the mean ± SD of at least ten independent plants and ten fruits/plants. Abbreviations as described in figure 1. Statistical analysis as described in Figure 2.

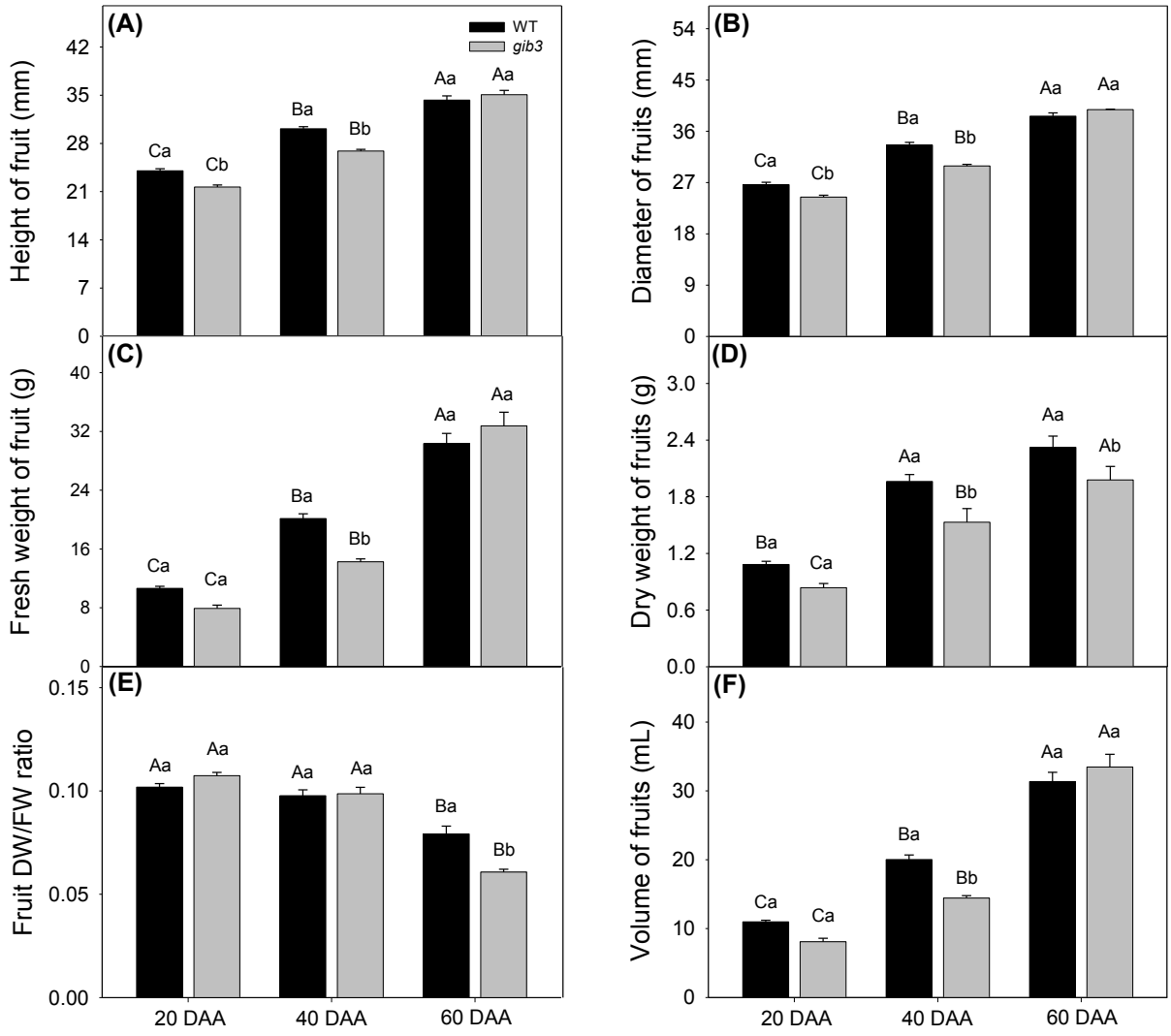


Figure 4: Changes in the morphological development parameters of tomato fruits in response to endogenous reduction in GA levels. The fruits were evaluated in three different ages: 20 DAA, 40 DAA and 60 DAA. Were analyzed: Height of fruit (A); Diameter of fruit (B); Fresh fruit of weight - FW (C); Dry weight of fruit - DW (D); Ratio DW/FW (E), volume of fruit (F). Values represent the mean ± SD of at least ten independent fruits/plants. 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

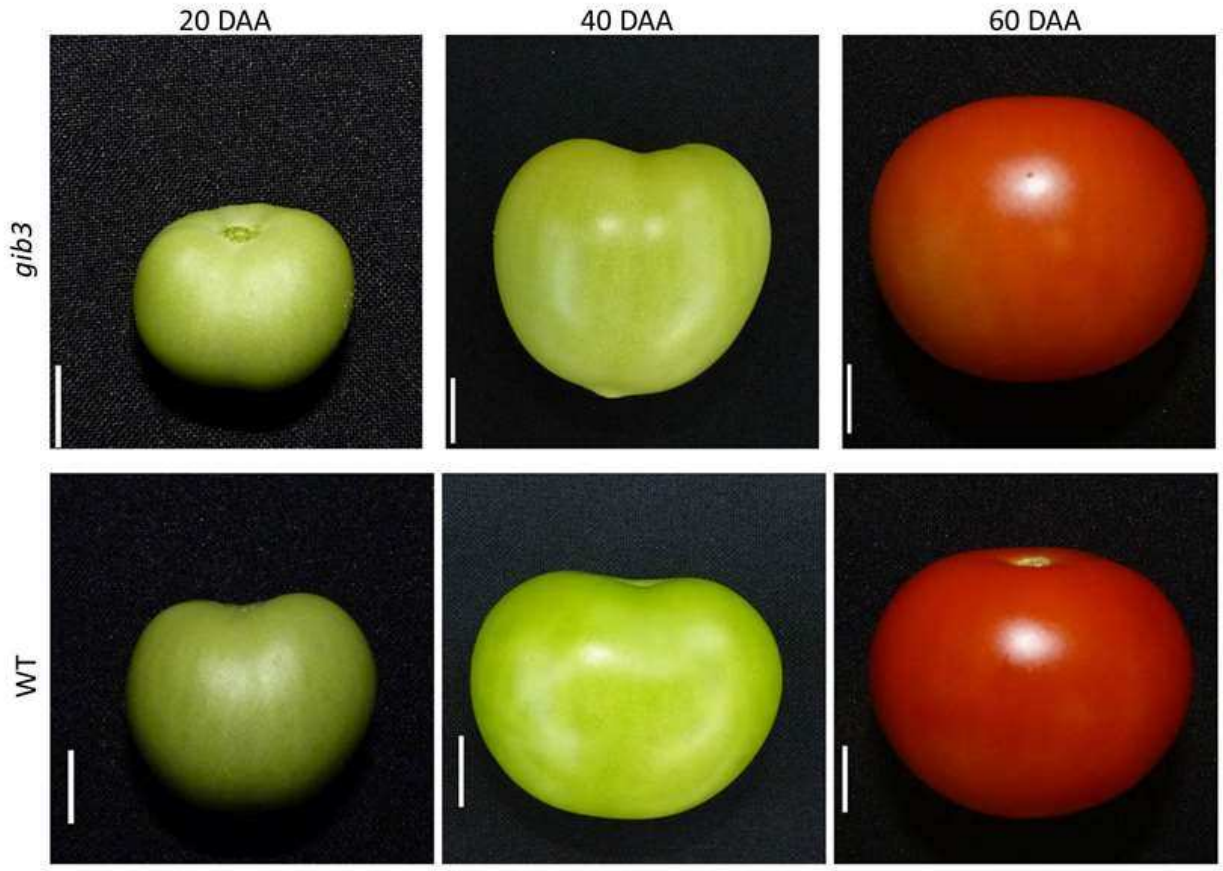


Figure 5: Representative photography of wild plant (WT) and gib3 mutant fruits at three distinct developmental stages: 20 DAA; 40 DAA; 60 DAA, respectively. Scale: bars =1 cm.

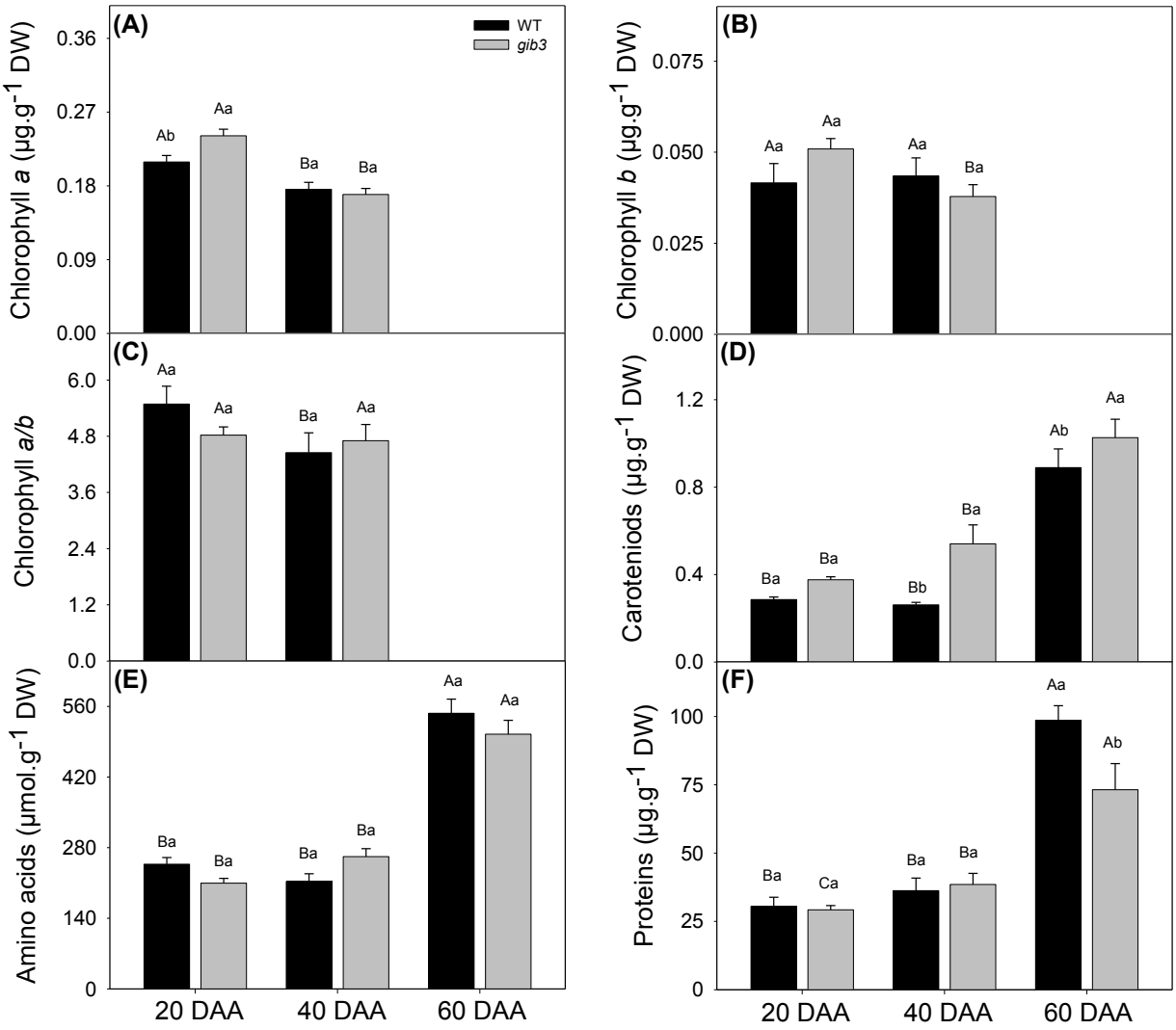


Figure 6: Variation on nitrogen tomato fruits metabolism in response to endogenous reduction in GA levels in three different ages. Were analyzed: Chlorophyll a (A); Chlorophyll b (B); Chlorophyll a/b (C); Carotenoids (D); Amino acids (E); Total soluble proteins (F). Values represent the mean ± SD of at least ten independent fruits. Abbreviations as described in Figure 1. Statistical analysis and ages as described in Figure 4.

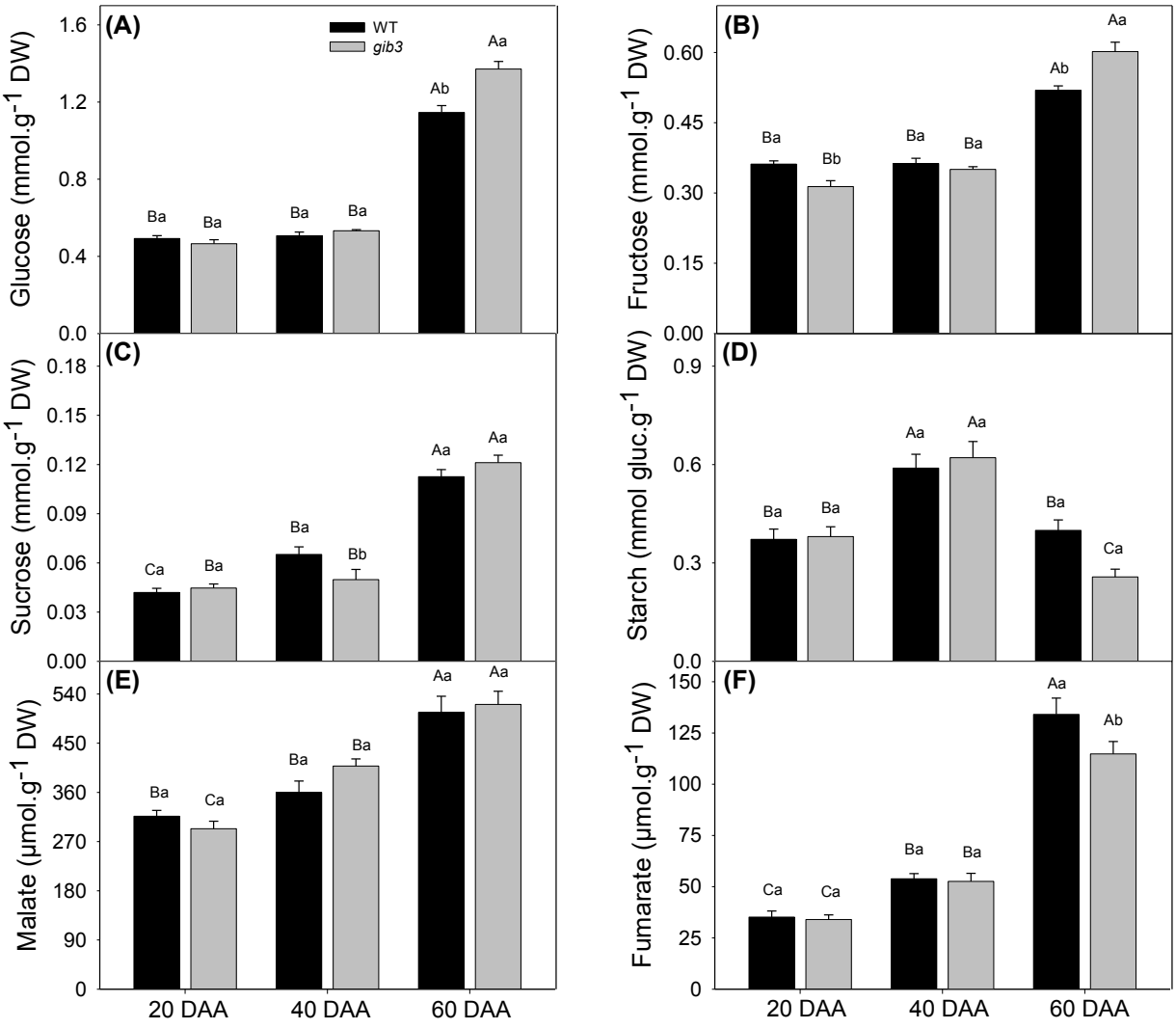


Figure 7: Metabolic variation in the carbohydrates and organic acids in tomato fruits metabolism in response to endogenous reduction in GA levels in three different ages. Glucose (A); Fructose (B); Sucrose (C); Starch (D); Malate (F) and Fumarate (G). Values represent the mean ± SD of at least ten independent fruits. Abbreviations as described in Figure 1. Statistical analysis and ages as described in Figure 4.

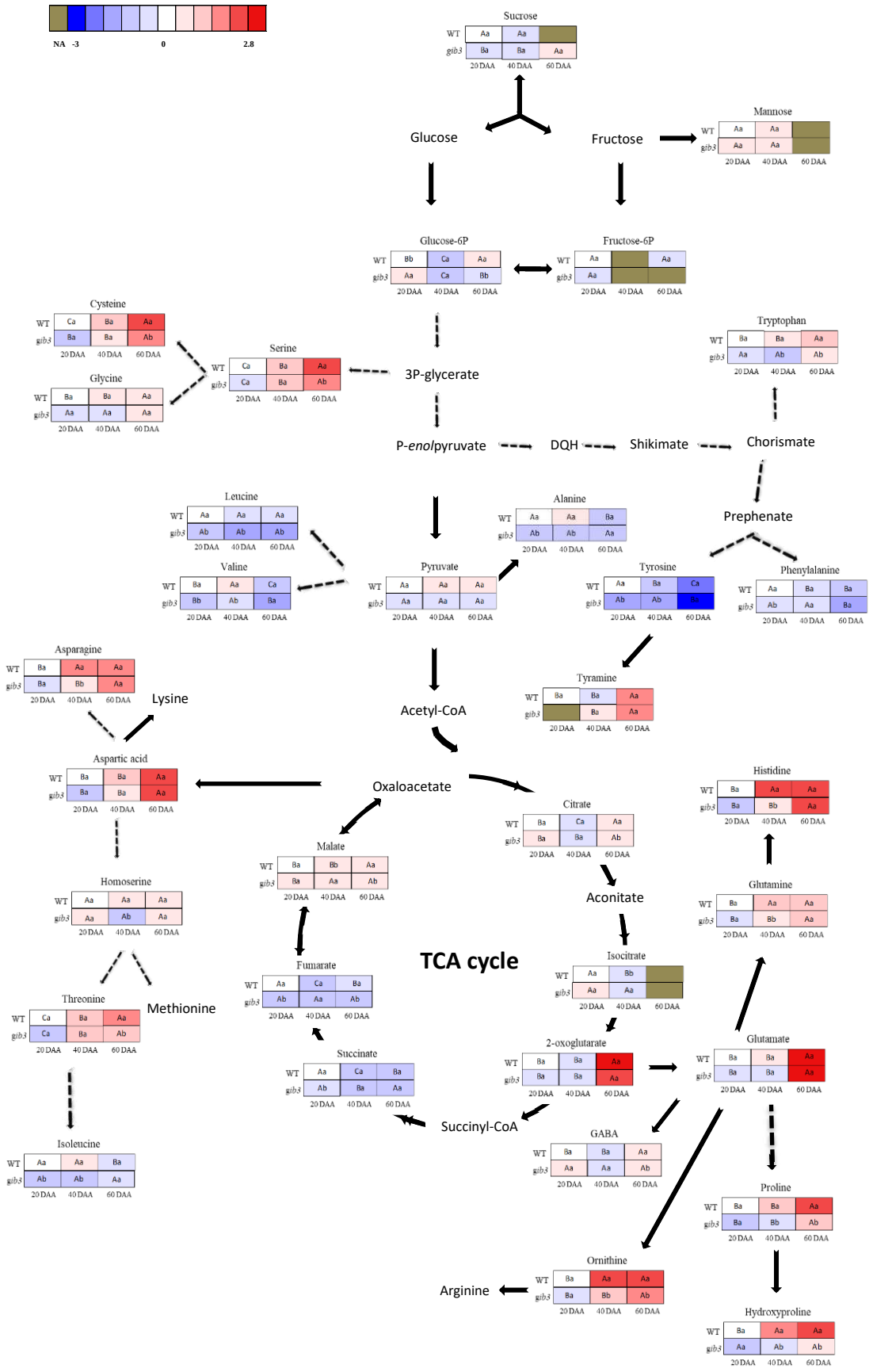


Figure 8: Changes in metabolite profiles in fruits of tomato plants. Metabolites outside squares indicate that they were not measured. Continuous arrows indicate a one-step reaction, and broken arrows indicate a series of biochemical reactions. Values represent the mean ± SD of at least six independent fruits. Abbreviations as described in Figure 1. Statistical analysis and ages as described in Figure 4.

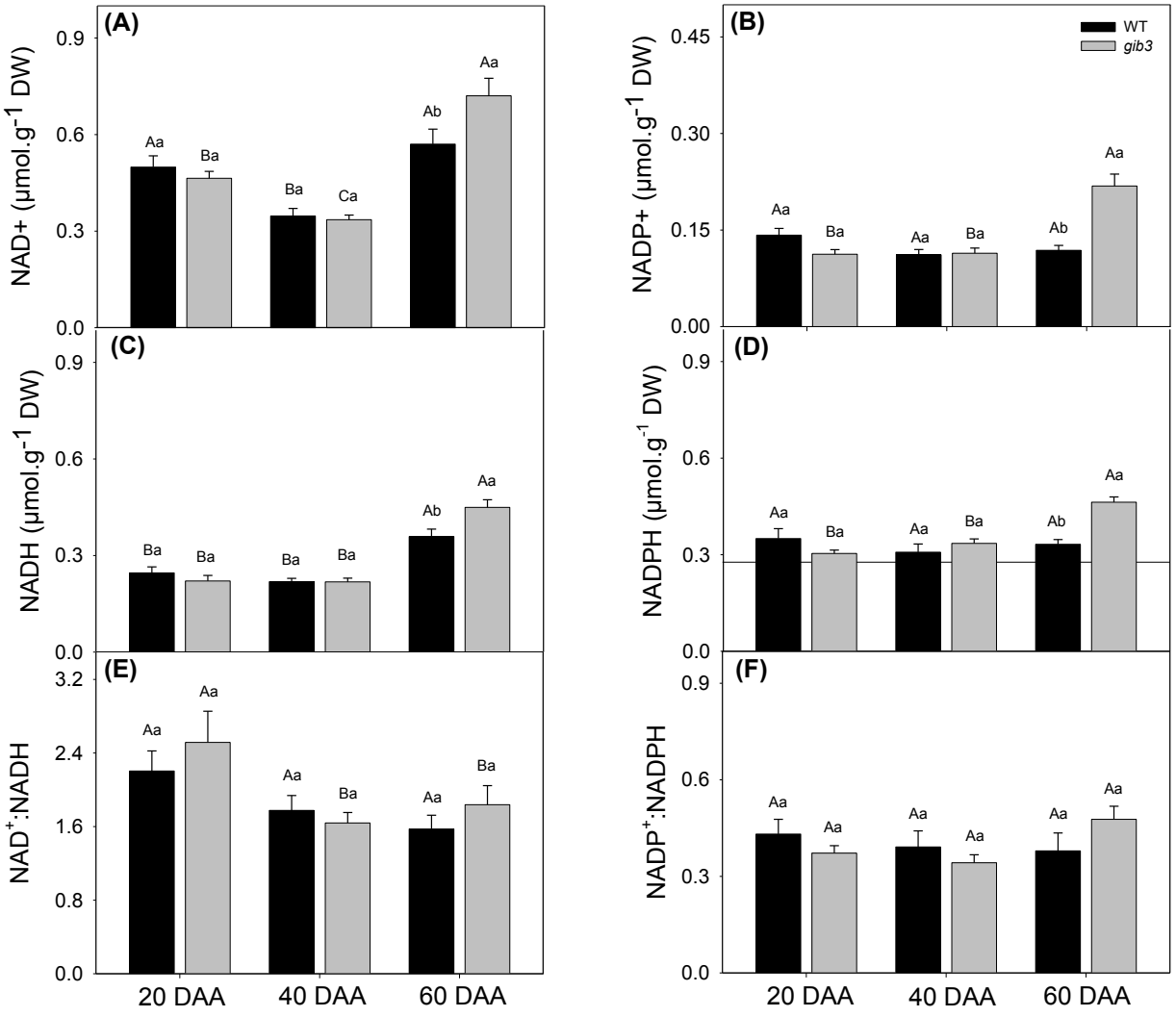


Figure 9: Variations in pyridine nucleotides levels in tomato fruits in response to endogenous reduction in GA levels in three different ages. NAD^+(A). NADH (B). $NADP^+$ (C). NADPH (D). NAD:NADH (E). $NADP^+$/NADPH (F). Values represent the mean ± SD of at least ten independent fruits. Abbreviations as described in Figure 1. Statistical analysis and ages as described in Figure 4.

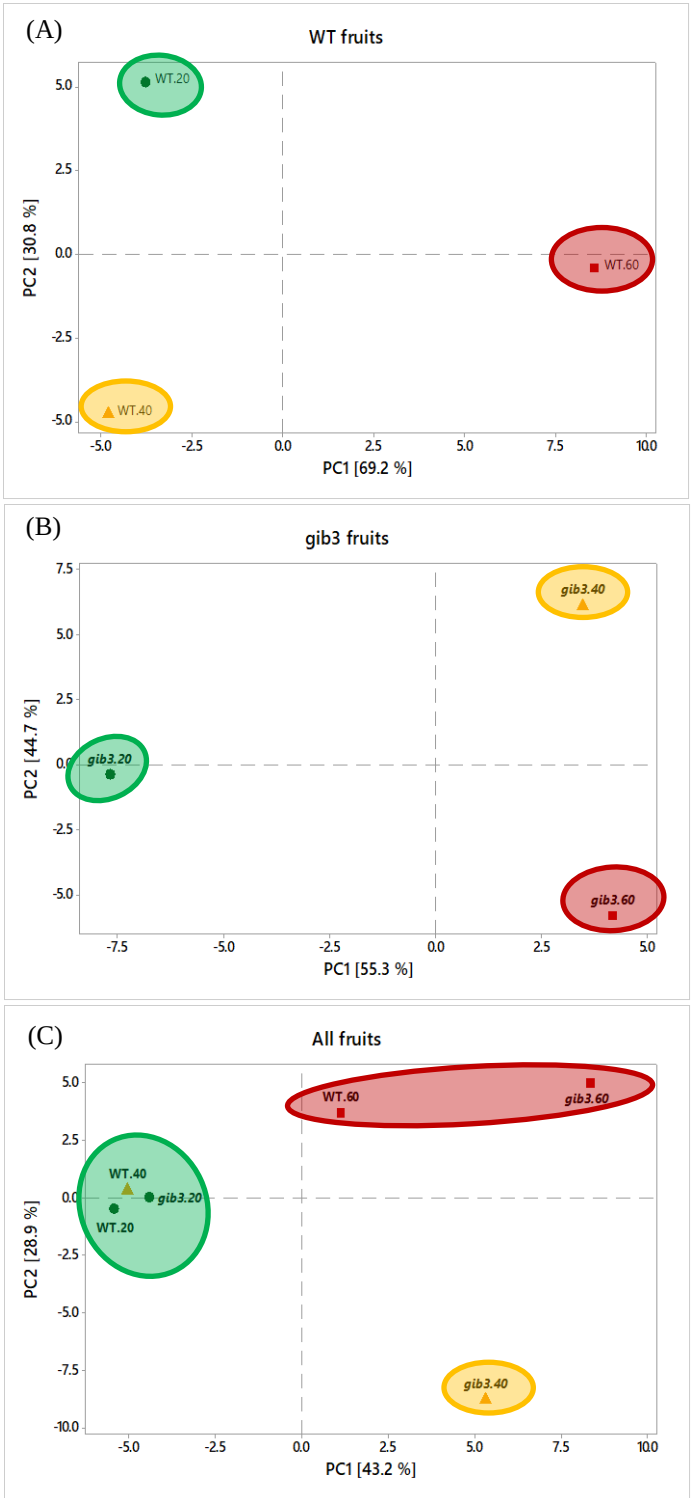
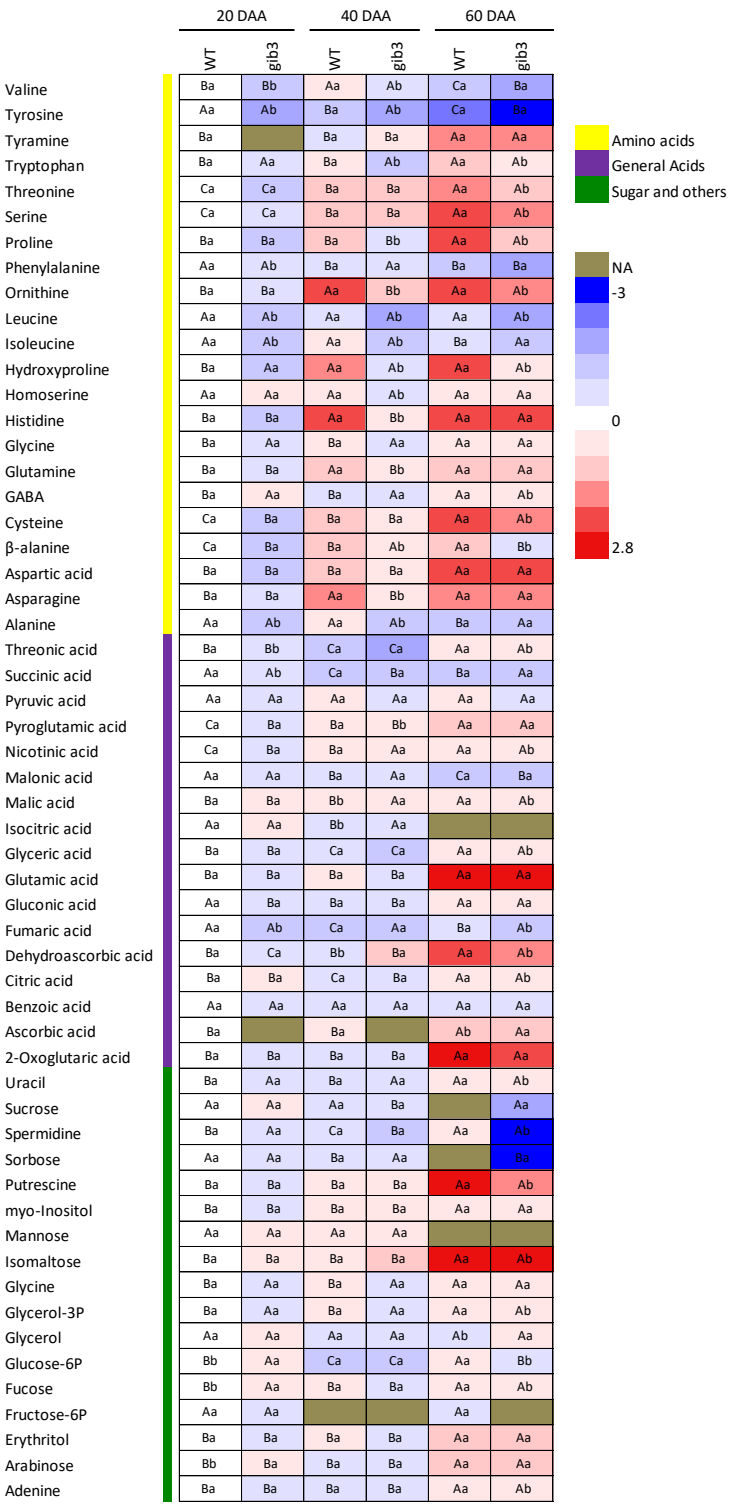


Figure 10: Principal component analyzes (PC1 vs. PC2 plots) of the metabolite data obtained for tomato fruits from wild type (WT) and gib3 across three different ripening stages (20 DAA; 40 DAA and 60 DAA). WT fruits (A). gib3 fruits (B). All fruits (C). The contributions of each metabolite to the separation of the genotypes in the two components (PC1 and PC2) are found in Supplementary Table 1. DAA, days after anthesis.



Suplemmentary figure 1: Heat map representing the changes in relative metabolite contents in tomato fruits of plants WT and mutant plants in biosynthesis of gibberellin gib3 (as described in material and methods). Different shades of red and blue express the extent of the change according to the color bar provided (log2 ratio of control); grey color indicate not measured. Values represent the mean ± SD of at least six independent fruits. Abbreviations as described in Figure 1. Statistical analysis and ages as described in Figure 4.

	WT fruits		gib3 fruits		WT and gib3 fruits	
Variables	PC1	PC2	PC1	PC2	PC1	PC2
Volume	0.12	-0.10	0.11	-0.11	0.11	0.13
Diameter	0.10	-0.13	0.12	-0.10	0.11	0.12
Heigth	0.10	-0.13	0.12	-0.09	0.11	0.11
Fresh weight	0.11	-0.11	0.11	-0.11	0.11	0.13
Dry Weight	0.09	-0.15	0.14	-0.06	0.08	0.08
Proteins	0.13	-0.04	0.11	-0.12	0.11	0.13
Amino acids	0.13	0.01	0.10	-0.12	0.11	0.13
Glucose	0.13	-0.02	0.09	-0.13	0.12	0.14
Fructose	0.12	-0.08	0.09	-0.14	0.11	0.15
Sucrose	0.12	-0.08	0.09	-0.14	0.11	0.15
Starch	-0.06	-0.18	0.02	0.17	-0.03	-0.16
Malate	0.13	-0.06	0.13	-0.08	0.14	0.09
Fumarate	0.13	-0.05	0.11	-0.12	0.11	0.13
Chlorophyll *a*	-0.13	0.05	-0.12	0.11	-0.12	-0.12
Chlorophyll *b*	-0.13	0.01	-0.11	0.11	-0.12	-0.13
Chlorophyll *a+b*	-0.12	-0.10	-0.15	-0.01	-0.16	0.06
Chlorophyll a/ *b*	-0.10	0.14	-0.15	-0.01	-0.16	0.06
Carotenoids	0.13	-0.01	0.11	-0.11	0.14	0.10
NAD+	0.11	0.12	0.04	-0.16	0.08	0.16
NADH	0.13	0.02	0.08	-0.14	0.12	0.15
NADP+	-0.03	0.20	0.08	-0.14	0.10	0.11
NADPH	0.02	0.20	0.10	-0.12	0.12	0.09
NAD+/H	-0.09	0.14	-0.15	-0.04	-0.10	0.01
NADP+/H	-0.08	0.16	0.05	-0.16	0.04	0.14
Valine	0.12	0.09	-0.15	0.01	-0.06	0.07
Tyrosine	-0.05	0.19	-0.13	0.08	-0.11	0.01
Tyramine	0.04	0.19	0.15	0.02	0.15	-0.07
Tryptophan	0.12	0.11	0.12	0.11	0.12	-0.12
Threonine	0.13	0.04	0.12	0.10	0.14	-0.08
Serine	0.13	0.01	0.12	0.10	0.14	-0.10
Proline	0.13	0.05	0.10	0.12	0.12	-0.14
Phenylalanine	-0.04	0.19	-0.11	0.11	-0.14	-0.03
Ornithine	0.13	-0.01	0.11	0.11	0.12	-0.04
Leucine	0.04	0.19	0.08	0.15	-0.04	-0.03
Isoleucine	0.10	0.14	0.06	0.15	-0.02	0.05
Hydroxyproline	0.13	0.01	0.09	0.14	0.09	-0.08
Homoserine	0.13	-0.02	0.15	0.03	0.06	0.04
Histidine	0.13	0.00	0.15	0.05	0.16	-0.04
Glycine	0.13	0.06	0.13	0.08	0.15	-0.11
Glutamine	0.13	0.04	0.15	-0.04	0.16	0.03
GABA	-0.10	-0.13	0.12	0.10	0.11	-0.14
Cysteine	0.13	0.05	0.12	0.10	0.14	-0.11
β-alanine	0.13	0.05	0.03	0.16	0.07	-0.12
Aspartic	0.13	0.05	0.15	0.01	0.16	-0.05
Asparagine	0.13	0.00	0.13	0.08	0.13	-0.02
Alanine	0.09	0.15	-0.15	0.04	-0.08	0.06
Threonic acid	-0.11	0.11	0.14	0.05	0.10	-0.10
Succinic acid	-0.11	0.12	0.15	0.01	-0.07	-0.03
Pyruvic acid	0.10	0.14	0.08	0.14	0.08	-0.08
Pyroglutamic acid	0.13	0.05	0.15	0.02	0.16	-0.05
Nicotinic acid	0.13	0.02	0.02	0.17	0.09	-0.11
Malonic acid	-0.09	0.15	-0.15	0.01	-0.16	0.04
Malic acid	-0.06	-0.18	0.09	0.13	0.10	-0.12
Isocitric acid	-0.13	-0.05	-0.15	-0.01	-0.16	0.06
Glyceric acid	-0.12	0.09	0.14	0.07	0.11	-0.12
Glutamic acid	0.13	0.06	0.15	0.01	0.16	-0.06
Gluconic acid	-0.12	0.10	0.15	-0.02	0.09	-0.07
Fumaric acid	-0.07	0.17	0.09	0.14	-0.04	-0.10
Dehydroascorbic acid	-0.10	0.14	0.14	0.05	0.14	-0.09
Citric acid	-0.13	-0.04	0.13	0.08	0.09	-0.13
Benzoic acid	-0.13	0.07	-0.08	0.14	-0.13	-0.04
Ascorbic acid	0.09	0.15	0.15	-0.04	0.15	0.01
2-Oxoglutaric acid	-0.06	0.18	0.15	0.04	0.15	-0.09
Uracil	-0.11	0.12	0.09	0.14	0.08	-0.17
Sucrose	-0.13	-0.06	-0.14	-0.06	-0.14	0.10
Spermidine	-0.12	0.08	0.02	0.17	-0.04	-0.18
Sorbose	-0.11	0.11	-0.15	-0.04	-0.15	0.08
Putrescine	0.13	-0.01	0.05	0.16	0.06	-0.19
myo-Inositol	0.13	0.00	0.09	-0.14	0.12	0.10
Mannose	0.05	-0.19	-0.06	-0.15	-0.06	0.19
Isomaltose	-0.06	-0.18	0.05	0.16	0.06	-0.19
Glycerol-3P	0.12	0.09	0.13	-0.09	0.15	0.04
Glycerol	-0.12	-0.09	-0.09	0.13	-0.14	-0.08
Glucose-6P	-0.13	-0.05	0.04	0.16	-0.05	-0.16
Fucose	-0.07	-0.18	-0.04	0.16	-0.09	-0.15
Fructose-6P	-0.13	0.03	0.09	0.13	-0.04	-0.13
Erythritol	0.11	0.12	0.02	0.17	0.00	-0.19
Arabinose	-0.11	-0.12	0.00	0.17	-0.05	-0.18
Adenine	0.04	0.19	-0.01	0.17	-0.04	-0.18

Supplementary table 1: individual contribution over than 82 variables used in the principal components analysis (Figure 10). PC1: main components on axis 1. PC2: main components on axis 2.

CHAPTER V

CONCLUDING REMARKS

The main goals of the work presented here were (i) to obtain a comprehensive picture of how and to which extent the manipulation of GA levels might differently affect plant growth in general and (ii) to demonstrate the significance of GAs during the transition to juvenile to adult phase. To this end, different but complementary experimental approaches were used. Although each chapter presented here has an independent discussion focusing on the results described within it, this final chapter discusses this thesis in its integrity in an attempt to detail the main findings in a broader context. Finally, an outlook detailing possible avenues for future research is provided using the work presented here as its foundation.

We have sought to improve our understanding of the role played by GAs during plant growth and development, as well as the associated mechanisms by which GAs play their role. Our results show that a distinct response is observed in different tissues/organs of tomato plants in response to endogenous fluctuations in the levels of GAs. Plants that have a strong depletion in the GAs content (gib2 and gib1) were also characterized by significant reductions in the development of aerial part without, however, affecting the development of the root system. Thus, a normal growth of roots was observed which resulted in a higher root-shoot ratio in those plants. Strong reductions in the carbohydrates and organic acids content in leaves for all mutants (gib3, gib2 and gib1) were observed, being more expressive in gib2 and gib1 mutants. Notably, when analyzing leaf tissues it was noticed that these reductions were gradual and consistent with the expected reductions in GA levels. When considered together, these results coupled with those previously published (Ribeiro et al., 2012) suggest a strong effect of GAs on the carbon and

energetic metabolism of the cell, particularly in leaves. Nitrogen metabolism was also strongly influenced by the drastic reduction in the GAs content, with large variations for certain amino acids. It is important to note that some amino acids (e.g., serine, proline, and leucine) have been associated with signaling functions in plants whereas others are precursors for the synthesis of phytohormones (Szabados and Savoure, 2010; Häusler et al., 2014; Ros et al., 2014). The molecular mechanisms underlying the regulation of amino acid metabolism in plants are largely unknown but can be expected to be very complex (Hildebrandt et al., 2015).

A decoupling between carbon and nitrogen metabolism was previously verified in the same plants with endogenous variations of GA levels and also confirmed following artificial alteration in the levels of GA showing that carbon skeleton are deviated for the maintenance of other metabolic pathways (Martins, 2013). Remarkably, one key point in the integration of carbon-nitrogen metabolism is 2-oxoglutarate, a TCA cycle intermediate. 2-oxoglutarate levels have been reported as capable of influencing several biosynthetic pathways. More recently the influence of this organic acid on the GAs metabolism has been verified, suggesting that it may exert some control in the biosynthetic pathway from this hormone (Araujo et al, 2012).Although the exact point of control mediating the interaction between energy metabolism and hormone-mediated regulation of growth it seems reasonable to suggest that this control would occur mainly in the last steps of either the biosynthesis or degradation of GAs, since the enzymes responsible by maintenance of the bioactive GAs pool are 2-oxoglutarate dependent dioxigenases (Hedden and Phillips, 2000).This fact apart, it clearly remains an exciting topic for future research.

We have also reported here that root tissues in general were not negatively affected by the endogenous depletion of the GA levels. Accordingly, roots have shown a degree of

sensitivity to GAs quite different from that found in shoot tissues (Barboza-Barquero et al., 2015). It is important to highlight that roots are capable of responding at a concentration 1000 times lower than that the concentration necessary to trigger a normal shoot development (Tanimoto, 1987; Tanimoto, 1994). When taken together, these information alongside our results described here it seems highly tempting to suggest that in roots the GAs levels have not been reduced to the minimum amount capable of compromising development. Further analysis of these responses may identify additional components of this cross-section response and clearly indicates that the appropriate elucidation of GA transport mechanism will be essential to elucidate this highly complicate matter.

Further analysis revealed that, similar to the situation observed for shoot development, the depletion of GAs content was determinant in stopping the vegetative-reproductive transitional process in tomato plants. The drastic reductions observed in GA levels in the mutants for GA biosynthesis (gib2 and gib1) culminated with their development being abruptly interrupted in the floral bud stage, making them unable to complete their life cycle by itself. It is important to mention that exogenous application of GAs can rescue the phenotype. Accordingly, adequate GAs concentrations are required for the normal development of floral organs and the obstruction of floral development in these mutants has previously been demonstrated to occur prior the complete formation of the anthers, which acts as a GAs source for other floral organs (Groot et al., 1987). Reversal of the phenotype may occur and depend on the development stage of ovary (Jacobsen and Olszewski, 1993; Nester and Zeevaart, 1988). By contrast to the situation observed for gib2 and gib1 mutant plants, gib3 mutant plants, which are moderately deficient in GAs, had little effect on the flowering process, characterized only by a slight delay. The important role played by GAs has been extensively demonstrated on the fruits establishment and

development, most notably in the fruit set and growing phases (McAtee et al., 2013). This is likely because of GAs action is mostly associated with cell division and expansion. Thus, metabolic and morphological differences in fruits of gib3 mutant plants were verified only at the initial phases, leading to a developmental delay of the fruits in general. This delay is most likely associated with the reduction of the GAs content, which is of fundamental significance in these phases. However, this reduction was not enough to promote greater impacts on the fruits itself, since these phenotypes were fully recovered at the final stage of development. Due to the proven action of GAs been, to date, only demonstrated in the initial stages of the fruit development coupled with the phenotypic recovery of fruits at the ripening it seems reasonable to infer that the metabolic and physiological changes found during ripening are rather natural consequence from changes in the semi autotrophic metabolism to completely heterotrophic metabolism. This change in metabolism is characterized mainly by the conversion of chloroplast to chromoplast, with predominance of non-photosynthetic pigments (Carrari and Fernie, 2006).

When considered together the results of this work clearly demonstrate that the functions of GAs are rather more complex than initially thought. In addition, it provided novel insights into the functions of GAs and highlights the complexity and specificity of plant metabolism in response to fluctuations in the levels of GAs. With the inclusion and knowledge inferred from differential tissue responses to GAs, the work presented here clearly suggest that in order to better understand the function of GAs one need to evaluate not only different tissues but also the metabolic interconnections that are associated with the pathways of biosynthesis and degradation of this highly intriguing hormone. Furthermore, it is likely that many aspects of the regulation of GAs biosynthesis, transport and degradation remain as interesting topics for future studies. The

molecular evidences explaining the movements of GAs within the plants still remain challenging (Gupta & Chakrabarty, 2013) and thus desperately in need of further experimental studies.

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