REBECA PATRICIA OMENA GARCIA

EFFECTS OF GIBBERELLIN DEFICIENCY IN THE ROOT-SHOOT ADJUSTMENTS DURING WATER DEFICIT IN TOMATO PLANTS

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.

VIÇOSA
MINAS GERAIS – BRASIL
2017

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
G216e
2017

Garcia, Rebeca Patricia Omena, 1989-
Effects of gibberellin deficiency in the root-shoot adjustments during water deficit in tomato plants / Rebeca Patricia Omena Garcia. – Viçosa, MG, 2017.
vii, 144f. : il. (algumas color.) ; 29 cm.

Inclui apêndices.
Orientador: Adriano Nunes Nesi.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Plantas - Crescimento. 2. Hormônios vegetais. 3. Giberelinas. 4. Análise foliar. 5. Raízes. 6. Deficiência hídrica. I. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-graduação em Fisiologia Vegetal. II. Título.

CDD 22 ed. 571.2

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APPROVED: July 31st, 2017.

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ACKNOWLEDGMENTS

First, I would like to thank God, for being present in my life and guiding my ways.

I want to thank the Universidade Federal de Viçosa (UFV), mainly to the Plant Physiology Graduate Program, for the opportunity to take the PhD course and for the support and structure offered for the development of my research. To the CAPES (Coordination for the Improvement of Higher Level Personnel) for granting the scholarship, I thank.

I sincerely thank my advisor Prof. Adriano Nunes Nesi, for guidance, knowledge conveyed, understanding and patience. Thank you for these five and a half years of teachings.

I am grateful tor Prof. Wagner Araújo for his co-supervision, for the disposition and indispensable contribution in this work.

I want to thank to other professors and technicians of Plant Physiology Program for contribution in my training and professional growth. To Prof. Dimas Ribeiro, I am grateful for the support and help during those years of hormones research.

I am also grateful to all Plant Growth Unit (UCP) team, mainly to Acácio, Alice, Danielle, Elias, Franciele, Franklin, João Henrique, Jorge, Luiz Valente, Marcelo, Paula, Rinamara and Willian for the good living, help in experiments and moments of relaxation at laboratory. I want think to Dora for help and partnership mainly at the end of this PhD. I want also think to Lucas Drumond and especially to Pedro Brandão for making our work more fun, for the animation in the development of experiments and analyzes, and for advice in difficult moments.

To my class friends since the master course, Adinan Alves, Amanda Ávila and Fernanda Vidal for support, group studies and company in disciplines and lunches in the university restaurant. I am very grateful to David Medeiros, also a laboratory fellow for his friendship, advice and contributions in this work.

To my long-time friends Flávia Schimpl, Haroldo and Laís Queiroz, for the friendship of almost 10 years and for knowing that I can always count, I thank.

I also express my gratitude to my “Viçosense family”, Christiane Martins, Lidiane Silva and Thais Correa for the great moments shared, laughter, advice and friendship for all life.

I also want to thank to my newest “Coimbrense family” Didi, Mileide and Miló for great conversations and advice. Special thanks to Jamil for the companionship and support since I met him and especially in these last months. You were very important to the conclusion of this PhD.

I would like to thank to my family, brothers, parents, “father-uncle”, “father-step-father”, grandparents, uncles and cousins who even so far helped to overcome my difficulty moments and the days of missing. To Deyse Maria, Evellyn Priscilla, Hermelindo Freire and Maria Evelyn for including me in your prayers and for never failing to believe in my potential.

Anyway, I thank all the people who directly or indirectly participated and contributed to the completion of this stage.

BIOGRAPHY

Rebeca Patricia Omena Garcia, daughter of João Chaves Garcia Filho and Deyse Maria Martins Omena, was born in Manaus, Amazonas state, Brazil, on July 18th, 1989. In 2007, she started the undergraduate course in Agronomy on Center of Agrarian Sciences at Federal University of Amazonas, Manaus, Amazonas state, Brazil and achieved bachelor degree in February 2012. In February 2012, she started Magister course at the Federal University of Viçosa (UFV), Viçosa, Minas Geris state, Brazil, under the supervision of Prof. Adriano Nunes Nesi, achieving the Master degree in Plant Physiology in Ferbruary 2014. In the same period, she started her doctoral studies at the UFV and her submitting to thesis defense in July 2017 under the supervision of the same adviser.

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ABSTRACT

OMENA-GARCIA, Rebeca Patricia, D.Sc., Universidade Federal de Viçosa, July, 2017. **Effects of gibberellin deficiency in the root-shoot adjustments during water deficit in tomato plants**. Advisor: Adriano Nunes Nesi.

Gibberellins (GAs) belong to a class of plant hormones with multiple functions in the regulation of physiological processes associated with growth and development. It has been suggested that GAs also play an important role for plant tolerance to a range of adverse conditions. Especially on water deficit, little is known about the physiological and metabolic implications of changes in the endogenous GAs levels in plants. GAs are synthesized in young and actively growing organs in both leaves and roots being able to act locally or be transported to other tissues or organs. For a better understanding of the GAs effects on growth and tolerance to water deficit distinct experimental approaches were adopted. The first approach aimed to identify the physiological and metabolic effects of reduced GAs levels in tomato mutant plants, defective in GA biosynthesis, exposed to water deficit. The second approach aimed to understand the role of GA in root growth, morphology and primary metabolism, while the third approach aimed at understanding the regulation of organ growth through long-distance traffic of signaling molecules by conducting vessels in GA-deficient plants. The results presented in this thesis provided new information for the current knowledge involving GAs such as: (i) GA content promotes acclimatization and tolerance to water deficiency by altering the metabolism of proline and directing the biomass partitioning to the roots, maintaining leaf turgor; (ii) GAs alter the metabolism of amino acids and organic acids promoting greater root growth compared to shoot suggesting that root growth and primary metabolism is decoupled from shoot in GA deficient plants; (iii) shoot and root growth is reversed in scions and rootstocks mutant probably due to long-distance signals transport between these organs, and the GAs mobility may be strongly involved. Thus, the results presented here demonstrate that GAs are involved not only in regulating plant growth as a whole but also in stress responses.

RESUMO

OMENA-GARCIA, Rebeca Patrícia, D.Sc., Universidade Federal de Viçosa, julho de 2017. **Efeitos da deficiência de giberelinas no ajustamento raiz-parte aérea durante deficiência hídrica em plantas de tomate**. Orientador: Adriano Nunes Nesi.

As giberelinas (GAs) pertencem a uma classe de hormônios vegetais com múltiplas funções na regulação de processos fisiológicos associados ao crescimento e desenvolvimento. Sugere-se que as GAs podem desempenhar um papel importante na tolerância de planta a uma série de condições adversas. Especialmente sobre deficiência hídrica, pouco se sabe sobre as implicações fisiológicas e metabólicas das mudanças nos níveis endógenos de GAs nas plantas. As GAs podem ser sintetizadas em órgãos jovens e ativamente crescentes, tanto em folhas quanto em raízes, podendo agir localmente ou ser transportadas para outros tecidos ou órgãos. Diante do exposto, para um melhor entendimento dos efeitos da GAs no crescimento e tolerância á deficiência hídrica diferentes abordagens experimentais foram adotadas. A primeira buscou identificar os efeitos fisiológicos e metabólicos do reduzido nível de GAs em plantas de tomate mutantes na biossíntese de GAs quando expostas á deficiência hídrica. A segunda abordagem teve o objetivo de compreender o papel das GA no crescimento, morfologia e metabolismo primário de raízes enquanto a terceira abordagem visou o entendimento da regulação do crescimento de órgãos através do tráfego a longas distâncias de moléculas sinalizadoras pelos vasos condutores em plantas deficientes em GAs. Os resultados apresentados nesta tese forneceram novas informações para o conhecimento atual que envolve as GAs: (i) o conteúdo de GA promove aclimatação e tolerância a deficiência hídrica por alterar o metabolismo da prolina e direcionar a alocação de biomassa para as raízes mantendo o turgor foliar; (ii) as GAs alteram o metabolismo de aminoácidos e ácidos orgânicos promovendo maior crescimento radicular em comparação com a parte aérea sugerindo que o crescimento e o metabolismo primário de raízes é desacoplado da parte aérea em plantas deficientes em GA e; (iii) o crescimento da parte aérea e radicular é revertido em enxertos e porta-enxertos mutantes provavelmente devido ao transporte a longas distancias de sinais entre esses órgãos, podendo fortemente ser GAs móveis. Portanto, os resultados apresentados demonstram que as GAs estão envolvidas na regulação do crescimento da planta como um todo e nas respostas a estresses.

General introduction

General introduction

Plant hormones are low molecular weight natural products that act at micromolar (or very low) concentrations to regulate all physiological and developmental processes during the plant life cycle (Chiwocha et al., 2003). Classes of phytohormones have been identified and characterized in plants. Among them, eight are very well known: gibberellins (GAs), auxins, abscisic acids, cytokinins, ethylene, brassinosteroids, jasmonates and strigolactones (Chiwocha et al., 2003; Vanstraelen and Benková, 2012; Li and He, 2013).

GAs belong to a class of plant hormones with multiple functions in the regulation of physiological processes associated by growth and development (Hedden and Kamiya, 1997; Olszewski et al., 2002; Sakamoto et al., 2004; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011). Physiologically, the main effect of GAs on superior plants is stimulating organ growth through cell stretching and, in some cases, cell division. In addition, GAs promote phase transition during development, such as between seed dormancy and germination, juvenile and adult growth phases, and vegetative and reproductive development (Hedden and Thomas, 2012). GAs also play important role in fertility and act in response to development (intrinsic) and in responses to environmental stimuli (extrinsic), which can regulate their biosynthesis, inactivation, perception or signal transduction, acting at various pathway points (Hedden and Thomas, 2012).

In shoot and roots, GAs are synthesized in young and actively growing organs, such as developing leaves and expanding internodes (Smith et al., 1992), which are also sites of action of these GAs (Hedden, 2012). GAs are synthesized from carotenoid precursors through various GAs biosynthesis pathways that differ in hydroxylation pattern and have been identified in a different plant species (Hedden and Thomas, 2012). Levels of GAs are limited by the final stages of their biosynthesis and the GAs responses are regulated by negative feedback in which high concentrations of GAs downregulate their own biosynthesis path and positive feedback acting on their catabolism or inactivation (Hedden e Kamiya, 1997, Yamaguchi e Kamiya, 2000). Bioactive GAs are produced by the action of GA20-oxidases and 3β-hydroxylases. GA20-oxidases convert GA_{12} to GA_9 from the non-hydroxylating pathway and GA_{53} to GA_{20} from the C-13 hydroxylation pathway. Subsequently, GA_9 and GA_{20} are converted into bioactive GAs, GA_4 and GA_1, respectively, by the action of 3β-hydroxylases

(Hedden e Phillips, 2000). Many different GAs structures have been identified in plants but a few of these act as endogenous growth regulators because they are precursors of the biosynthetic pathway or are in their inactive form (Cowling et al., 1998; Yamaguchil and Kamiya, 2000; Richards et al., 2001).

Relatively few GAs have intrinsic biological activity in higher plants (angiosperms), the most common active forms being GA_1, GA_3, GA_4 and GA_7. The universal occurrence of GA_1 and GA_4 in plants suggests that these are the functionally active forms for promoting growth. Giberelin A_3 (GA_3), known as gibberellic acid, is the main product of the fungus Gibberella fujikuroi (now reclassified as Fusarium fujikuroi) which is produced for commercial application but is also endogenous in some evolved plant species (Hedden and Thomas, 2012). GA_3 and GA_7, in contrast to GA_1 and GA_4, contain a double bond between C-1 and C-2 and also have intrinsic biological activity, but with exception of developing seeds of certain species, and are usually present in plants in a low concentrations when compared to their saturated analogs (Hedden, 2012). The intrinsic biological activity and, therefore, the hormonal function of these molecules has been verified from analysis of mutants GAs deficient (Hedden and Phillips, 2000).

The GA-biosynthetic pathway can be divided into three stages according to subcellular compartmentalization and the nature of the enzymes involved (Figure 1). The first stage occurs in plastids and results in the formation of the hydrocarbon ent-kaurene from geranylgeranyl diphosphate by separate enzymes, ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS). In the second stage that happen in the endoplasmic reticulum membrane by two membrane-associated cytochrome P450 monooxygenases (P450s) to formation GA_{12}. This multifunctional P450s are ent-kaurene oxidase (KO) converts ent-kaurene through ent-kaurenol and ent-kaurenal to ent-kaurenoic acid, which is converted by ent-kaurenoic acid oxidase (KAO) to GA_{12} via ent-7α-hydroxykaurenoic acid and GA_{12}-aldehyde. The GA_{12} lies at a branch point in the biosynthetic pathway: 13-hydroxylation to GA_{53} initiates the formation of 13-hydroxylated GAs, whereas 20-oxidation results in the production of non-13-hydroxyGAs. The conversion of GA_{12} and GA_{53} by GA 13-oxidase in parallel pathways to the active end-products of the pathway, GA_4 and GA_1, that is the final stage. The formation of the active hormones happen by several stapes of oxidation through the action of soluble 2-oxoglutarate-dependent dioxygenases (2-ODDs), named GA 20-

oxidase and GA 3-oxidase. In the reaction sequence catalysed by GA 20-oxidase, C-20 is oxidised to the alcohol and then aldehyde, from which it is removed to form the C_{19}-GA product GA_9 or its 13-hydroxylated analogue GA_{20}. Finally, the active products of the pathway, GA_1 and GA_4, are formed in a single step from GA_{20} and GA_9, respectively, by the action of GA 3-oxidase. In addition, the GA 2-oxidase can to convert active GAs, GA_1 and GA_4, in inactive forms, GA_8 and GA_{34}, respectively. Reactions that result in GA deactivation or prevent the formation of biologically active hormones are catalysed by P450s and 2-ODDs (Hedden, 2012; Hedden and Thomas, 2012; Hedden and Sponsel, 2015).

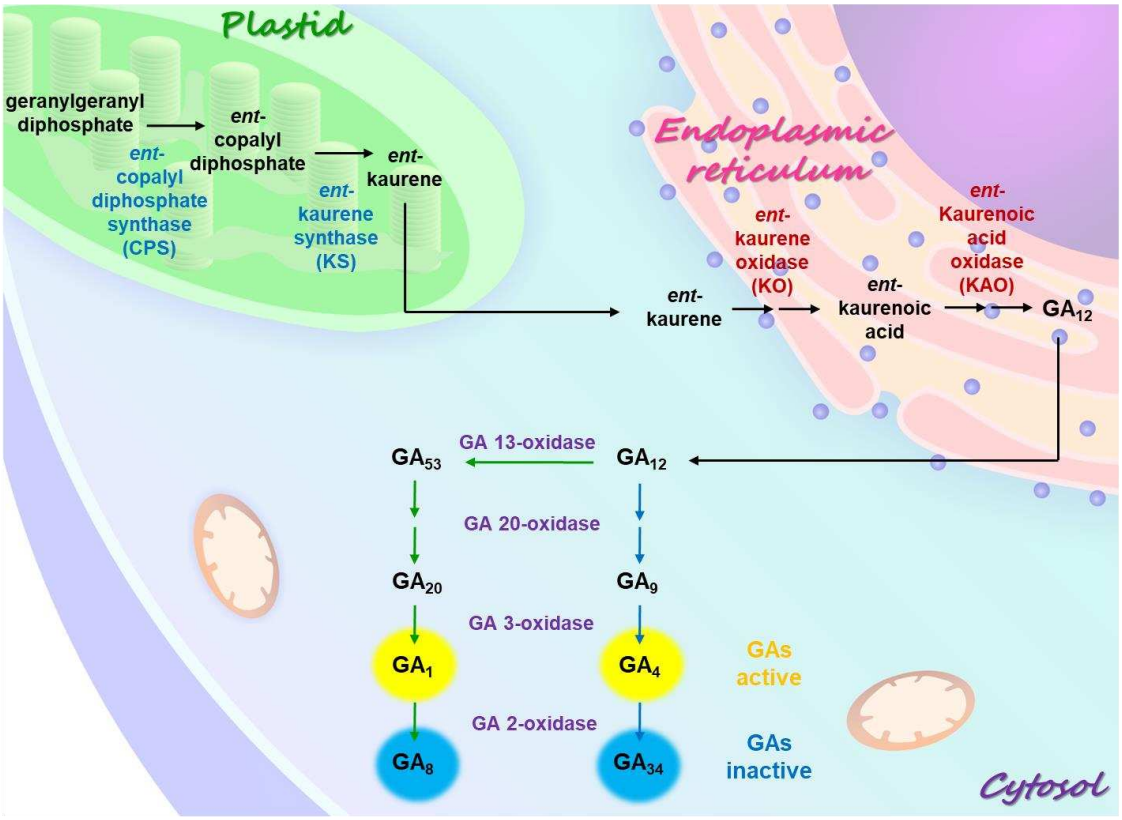


Figure 1. Gibberellin-biosynthetic pathway from geranylgeranyl diphosphate to the biologically active (GA_1 and GA_4) and inactive products (GA_8 and GA_{34}).

It has been demonstrated that hormones are important players for the plant responses to water deprivation. It is verified that high concentrations of ABA cause stomatal closure and induce the expression of genes related to water stress (Seki et al., 2007), such as genes responsible for the synthesis of osmotically active compounds (Antoni et al., 2011). Furthermore, other growth promoting hormones such as cytokinins and gibberellins (GAs) may also being involved in stress responses (Magome

et al., 2004; Achard et al., 2006; Rivero et al., 2007; Albacete et al., 2008; Ha et al., 2012). It is believed that the levels of these hormones in the plant affect their performance under stress conditions and consequently their tolerance. Additionally, besides cytokinin and GA, auxins and brassinosteroids are prominent, whereas, ABA, ethylene, and jasmonic acid are commonly associated with stress responses (Wolters and Juergens, 2009).

The relationship of GA levels and tolerance to abiotic stresses has been verified in different plant species (Achard et al., 2006; Maggio et al., 2010; Dubois et al., 2013; Ho et al., 2013; Nir et al., 2013; Nir et al., 2014; Yang et al., 2014); (Zawaski and Busov, 2014). In tomato plants overexpressing of genes associated with GA catabolism (AtGAMT1) presented a dwarf phenotype and water deficit tolerance. Further, these plants showed lower transpiration rate under water limitation, which was attributed to reduced stomatal conductance due to the smaller stomata with reduction of stomatal pores presented (Nir et al., 2013; Nir et al., 2014). Other author suggest that paclobutrazol (GA biosynthesis inhibitor) application could significantly improve tolerance in tomato plants under limited water availability through selective changes in morpho-physiology and induction of stress-related molecular processes (Pal et al., 2016). The GA content is recognized as important for processes related to plant growth and development (Olszewski et al., 2002; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011; Ribeiro et al., 2012), but little is known about the role of the GAs metabolism in plants exposed to water deficit. Therefore, more studies in plants with altered levels of endogenous GAs are still needed to better understand the mechanisms responsible for responses presented by these plants since GAs are very likely to be variable and not maintained under different extreme conditions.

Studies involving GAs and regulatory processes of shoot growth and development are more common. GAs regulation in root development and elongation mechanisms have not yet been fully elucidated and few studies have been found (Tanimoto, 1987, 1994, 2012). It is worth mentioning that the role of GAs in root/shoot ratio of several species is well known since the "green revolution" which introduction of semi-annual varieties were introduced and enormous improvements in productivity (Hedden, 2003). Understanding the role of GAs in roots came decades later using severely GAs deficient plants, either genetically mutated in biosynthesis genes or using GA inhibitors (Tanimoto, 2012). These studies provided valuable new information on a

roots GAs responses. For example, when using mutant plants in GAs biosynthesis, plant active GA production is regulated locally, indicating that shoot regulation is different from root regulation. Furthermore, roots elongation and thickening these plants can be regulated by a lowest GA concentration and/or by a greater roots sensitivity to GA than to stem giving advantage when GA production is limited (Tanimoto, 2012). In this sense, it is still important studies that aim to understand the role of GAs in root growth, morphology and metabolism to complement the findings so far.

Furthermore, the GAs are synthesized in leaves and roots young and actively growing (Smith et al., 1992) which are also sites of action of these GAs (Hedden, 2012). It has been previously reported that GAs in Arabidopsis roots are mainly accumulated in the endoderm cells of stretching zone and the presence of an GA active transport mechanism has also been strongly suggested (Shani et al., 2013). However, GAs production in maturing leaves occurs in proplastids associated with the foliar vascular system (Silverstone et al., 1997), providing the GAs export to other parts of the plant by phloem (Eriksson et al., 2006). Currently, it is known that GAs can be transported cell-cell (or between tissues) through membrane transporters (Tal et al., 2016) or from one organ to another through xylem and phloem conducting vessels (Regnault et al., 2015; Regnault et al., 2016). Therefore, GAs can act in the places where they are synthesized or can be transported to other cells, tissues or organs promoting responses at short- and long-distances.

The optimal levels of active GAs are controlled by regulatory mechanisms that involved GA biosynthesis genes. Moreover, little is known about the regulation of GA transport from biosynthesis places to tissues and organs that require GAs for growth. The grafting and micrografting technique is an excellent approach to study the long-range GAs signaling in plants (Bidadi et al., 2014; Notaguchi and Okamoto, 2015; Spiegelman et al., 2015; Regnault et al., 2016). Studies of identification and characterization of the long-range signaling molecules by conducting vessels in plats, such as hormones, RNAs and proteins, are still important (Giavalisco et al., 2006; Lough and Lucas, 2006; Omid et al., 2007; Bidadi et al., 2014; Spiegelman et al., 2015).

The role of GAs in growth is much better known than in plant metabolism. When we refer to extreme abiotic conditions, this knowledge is even smaller, especially under water deficiency. In addition, responses on shoot and root growth, architecture

and morphology may be differentiated depending on the concentration and GAs sensitivity in each organ of the plant. Moreover, growth can be regulated by molecules that can be transported over long-distances by xylem and phloem, such as hormones, RNAs, peptides, and so on. Therefore, studies evidencing the GAs participation in growth and morphology responses of shoot and roots under optimal and extreme conditions are still important.

1.1 Layout and aims of the chapters

This thesis is largely focused on effects of gibberellins deficiency in the root-shoot adjustments during water deficit in tomato plants. That said, the main aims of this work were: (i) identify the effects of endogenous alteration of GA levels in tomato plants exposed to water deficit; (ii) obtain a comprehensive role of GA to root growth, morphology and primary metabolism and; (iii) understand the regulation of shoot and root growth by long-range traffic of signaling molecules by conducting vessels in GA-deficient tomato plants. In order to reach these goals several experiments were performed and therefore this thesis is organized in three independent stand-alone chapters. Each chapter is composed of an introduction, results and discussion, as well as the methods used. It was also included at the end of this thesis the main findings of this work and the perspectives to understanding of the role of GA biosynthesis.

Chapter 1. Changes in biomass partitioning and proline accumulation promote water deficit tolerance in gibberellin-deficient tomato plants

Although endogenous GA content is recognized as important for processes related to plant growth and development, little is known about the role of the GAs metabolism in plants exposed to water deficit. It has recently been suggested that the metabolism of GA plays an important role in the resistance and acclimatization of plants to conditions of water deficit. Therefore, more studies in plants with altered levels of endogenous GAs are still needed to better understand the mechanisms responsible for responses presented by these plants since GAs are very likely to be variable and not maintained under different extreme conditions. In this chapter, I used tomato mutants with reduced levels of GAs, gib1, gib2 and gib3 (highly, intermediate and moderately

deficient in GAs levels, respectively) submitted to water deficit and a detailed physiological and metabolic characterization was performed for in order to verify whether the endogenous content of GAs is involved in the tolerance responses to water deficit. The data suggest that under water deficit, the GAs content promote acclimation or tolerance responses mainly by increasing the proline levels and altering the size of root system in order to maintain the leaf turgor for longer.

Chapter 2. Root morphological and metabolic characterization in mutant tomato plants for gibberellin biosynthesis

The growth and development of plants are known to be regulated by endogenous GA content. Many studies have investigated the role of GA in shoot growth processes and little importance has been given to the roots. However, GAs regulation in root development and root elongation mechanisms have not yet been fully elucidated. In this chapter, I investigated of the impact of reduced GAs levels on root metabolism, growth and morphology in the same tomato plants (Solanum lycopersicum cv. Moneymaker). I found that changes in GA levels lead to alterations on the amino acids and organic acids metabolism promoting greater root growth compared to shoot, and these roots are composed mainly of fine roots, suggesting that root growth and primary metabolism are decoupled from the shoot in GA-deficient plants.

Chapter 3. Reciprocal grafting promote shoot and root growth in GA-deficient tomato plants

The long-range traffic of signaling molecules by conducting vessels such as hormones, RNAs and proteins have been identified and characterized in plants. In chapter 3, was through reciprocal grafting of GAs mutants and control plants. We found reversal of the shoot and root phenotype of GA-deficient tomato mutants probably due to the transport of molecules between these organs, especially the translocation of GAs by phloem.

Literature cited

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of Plant Responses to Environmentally Activated Phytohormonal Signals. Science **311:** 91-94

Albacete A, Ghanem ME, Martínez-Andújar C, Acosta M, Sánchez-Bravo J, Martínez V, Lutts S, Dodd IC, Pérez-Alfocea F (2008) Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (Solanum lycopersicum L.) plants. J Exp Bot **59:** 4119-4131

Antoni R, Rodriguez L, Gonzalez-Guzman M, Pizzio GA, Rodriguez PL (2011) News on ABA transport, protein degradation, and ABFs/WRKYs in ABA signaling. Current Opinion in Plant Biology **14:** 547-553

Bidadi H, Matsuoka K, Sage-Ono K, Fukushima J, Pitaksaringkarn W, Asahina M, Yamaguchi S, Sawa S, Fukuda H, Matsubayashi Y, Ono M, Satoh S (2014) CLE6 expression recovers gibberellin deficiency to promote shoot growth in Arabidopsis. The Plant Journal **78:** 241-252

Chiwocha SD, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross AR, Kermode AR (2003) A method for profiling classes of plant hormones and their metabolites using liquid chromatography- electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (Lactuca sativa L.) seeds. The Plant Journal **35:** 405-417

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin Dose-Response Regulation of GA4 Gene Transcript Levels in Arabidopsis. Plant Physiol **117:** 1195-1203

Dubois M, Skirycz A, Claeys H, Maleux K, Dhondt S, De Bodt S, Vanden Bossche R, De Milde L, Yoshizumi T, Matsui M, Inzé D (2013) ETHYLENE RESPONSE FACTOR6 Acts as a Central Regulator of Leaf Growth under Water-Limiting Conditions in Arabidopsis. Plant Physiol **162:** 319-332

Eriksson S, Böhlenius H, Moritz T, Nilsson O (2006) GA4 is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. The Plant Cell **18:** 2172-2181

Gao X-H, Xiao S-L, Yao Q-F, Wang Y-J, Fu X-D (2011) An Updated GA Signaling 'Relief of Repression' Regulatory Model. Mol Plant **4:** 601-606

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Towards the proteome of Brassica napus phloem sap. PROTEOMICS **6:** 896-909

Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran L-SP (2012) Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci **17:** 172-179

Hedden P (2003) The genes of the Green Revolution. Trends in Genetics **19:** 5-9

Hedden P (2012) Gibberellin Biosynthesis. In eLS. John Wiley & Sons, Ltd

Hedden P, Kamiya Y (1997) Gibberellin Biosynthesis: Enzymes, Genes and Their Regulation. Plant Physiol **48:** 431-460

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci **5:** 523-530

Hedden P, Sponsel V (2015) A century of gibberellin research. Journal of plant growth regulation **34:** 740-760

Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. Biochemical Journal **444:** 11-25

Ho S-L, Huang L-F, Lu C-A, He S-L, Wang C-C, Yu S-P, Chen J, Yu S-M (2013) Sugar starvation- and GA-inducible calcium-dependent protein kinase 1

feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. Plant Mol Biol **81:** 347-361

Li Q-F, He J-X (2013) Mechanisms of signaling crosstalk between brassinosteroids and gibberellins. Plant signaling & behavior **8:** e24686

Lough TJ, Lucas WJ (2006) INTEGRATIVE PLANT BIOLOGY: Role of Phloem Long-Distance Macromolecular Trafficking. Annual Review of Plant Biology **57:** 203-232

Maggio A, Barbieri G, Raimondi G, De Pascale S (2010) Contrasting Effects of GA3 Treatments on Tomato Plants Exposed to Increasing Salinity. Journal of Plant Growth Regulation **29:** 63-72

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2004) dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. Plant J **37:** 720-729

Nir I, Moshelion M, Weiss D (2014) The Arabidopsis GIBBERELLIN METHYL TRANSFERASE 1 suppresses gibberellin activity, reduces whole- plant transpiration and promotes drought tolerance in transgenic tomato. Plant, cell & environment **37:** 113-123

Nir IDO, Moshelion M, Weiss D (2013) The Arabidopsis Gibberellin Methyl Transferase 1 suppresses gibberellin activity, reduces whole-plant transpiration and promotes drought tolerance in transgenic tomato. Plant Cell Environ**:** n/a-n/a

Notaguchi M, Okamoto S (2015) Dynamics of long-distance signaling via plant vascular tissues. Frontiers in Plant Science **6:** 161

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin Signaling: Biosynthesis, Catabolism, and Response Pathways. Plant Cell **14:** S61-S80

Omid A, Keilin T, Glass A, Leshkowitz D, Wolf S (2007) Characterization of phloem-sap transcription profile in melon plants. Journal of Experimental Botany **58:** 3645-3656

Pal S, Zhao J, Khan A, Yadav NS, Batushansky A, Barak S, Rewald B, Fait A, Lazarovitch N, Rachmilevitch S (2016) Paclobutrazol induces tolerance in tomato to deficit irrigation through diversified effects on plant morphology, physiology and metabolism. Scientific reports **6:** 39321

Regnault T, Davière J-M, Achard P (2016) Long-distance transport of endogenous gibberellins in Arabidopsis. Plant Signaling & Behavior **11:** e1110661

Regnault T, Davière J-M, Wild M, Sakvarelidze-Achard L, Heintz D, Carrera Bergua E, Lopez Diaz I, Gong F, Hedden P, Achard P (2015) The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. Nature Plants **1:** 15073

Ribeiro DM, Araújo WL, Fernie AR, Schippers JHM, Mueller-Roeber B (2012) Translatome and metabolome effects triggered by gibberellins during rosette growth in Arabidopsis. J Exp Bot **63:** 2769-2786

Richards DE, King KE, Ait-ali T, Harberd NP (2001) How Gibberellin Regulates Plant Growth and Development: A Molecular Genetic Analysis of Gibberellin Signaling. Annu Rev Plant Phys **52:** 67-88

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. P Natl Acad Sci **104:** 19631-19636

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, Miyao A, Hirochika H, Kitano H, Ashikari M, Matsuoka M (2004) An Overview of Gibberellin

Metabolism Enzyme Genes and Their Related Mutants in Rice. Plant Physiol **134:** 1642-1653

Seki M, Umezawa T, Urano K, Shinozaki K (2007) Regulatory metabolic networks in drought stress responses. Curr Opin Plant Biol **10:** 296-302

Shani E, Weinstain R, Zhang Y, Castillejo C, Kaiserli E, Chory J, Tsien RY, Estelle M (2013) Gibberellins accumulate in the elongating endodermal cells of Arabidopsis root. Proceedings of the National Academy of Sciences **110:** 4834-4839

Silverstone AL, Chang Cw, Krol E, Sun Tp (1997) Developmental regulation of the gibberellin biosynthetic gene GA1 in Arabidopsis thaliana. The Plant Journal **12:** 9-19

Smith VA, Knatt CJ, Gaskin P, Reid JB (1992) The distribution of gibberellins in vegetative tissues of Pisum sativum LI Biological and biochemical consequences of the le mutation. Plant physiology **99:** 368-371

Spiegelman Z, Ham B-K, Zhang Z, Toal TW, Brady SM, Zheng Y, Fei Z, Lucas WJ, Wolf S (2015) A tomato phloem-mobile protein regulates the shoot-to-root ratio by mediating the auxin response in distant organs. The Plant Journal **83:** 853-863

Sun T-p, Gubler F (2004) Molecular Mechanism of Gibberellin Signaling in Plants. Annu Rev Plant Biol **55:** 197-223

Tal I, Zhang Y, Jørgensen ME, Pisanty O, Barbosa IC, Zourelidou M, Regnault T, Crocoll C, Olsen CE, Weinstain R (2016) The Arabidopsis NPF3 protein is a GA transporter. Nature communications **7**

Tanimoto E (1987) Gibberellin-dependent root elongation in Lactuca sativa: recovery from growth retardant-suppressed elongation with thickening by low concentration of GA3. Plant and cell physiology **28:** 963-973

Tanimoto E (1994) Interaction of gibberellin A3 and ancymidol in the growth and cell-wall extensibility of dwarf pea roots. Plant and Cell Physiology **35:** 1019-1028

Tanimoto E (2012) Tall or short? Slender or thick? A plant strategy for regulating elongation growth of roots by low concentrations of gibberellin. Annals of Botany **110:** 373-381

Vanstraelen M, Benková E (2012) Hormonal interactions in the regulation of plant development. Annual review of cell and developmental biology **28:** 463-487

Wolters H, Juergens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet **10:** 305-317

Yamaguchil S, Kamiya Y (2000) Gibberellin Biosynthesis: Its Regulation by Endogenous and Environmental Signals. Plant Cell Physiol **41:** 251 - 257

Yang R, Yang T, Zhang H, Qi Y, Xing Y, Zhang N, Li R, Weeda S, Ren S, Ouyang B, Guo Y-D (2014) Hormone profiling and transcription analysis reveal a major role of ABA in tomato salt tolerance. Plant Physiology and Biochemistry **77:** 23-34

Zawaski C, Busov VB (2014) Roles of Gibberellin Catabolism and Signaling in Growth and Physiological Response to Drought and Short-Day Photoperiods in Populus Trees. PLoS ONE **9:** e86217

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) Elongates Uppermost Internode Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. Plant Cell **18:** 442-456

Chapter 1

Changes in biomass partitioning and proline accumulation promote water deficit tolerance in gibberellin-deficient tomato plants

Abstract

Water deficit is recognized as one of the major factors affecting the metabolism, growth, development and survival of higher plants. To overcome the negative impacts of the water deprivation, plants have developed several mechanisms in response to this stress condition. It has been suggested that gibberellins (GAs) can play an important role for plant tolerance to a range of adverse conditions. However, little is known about the physiological and metabolic implications of changes in the endogenous GAs levels in plants when submitted to water deficit. To better understand the contribution of GAs in plants responses to water deficit conditions, it was evaluated here the physiological and metabolic impacts of altered levels of endogenous GAs in tomato plants deficient in the GAs biosynthesis, named gib1, gib2 and gib3, under water deficient conditions and re-watering. Interestingly, the mutant plants kept the turgor pressure in the leaf for longer time and recovered photosynthesis faster than wild-type (WT) plants. gib2 and gib1 plants showed no apparent wilt even when they reached low water potential values (around -1.3 MPa). These responses were related to the dwarfism, shading and leaf thickness observed in these mutants. Curiously, the mutant plants allocated more biomass in the roots than in the shoots compared to WT plants. Furthermore, the maintenance of leaf turgor and increased water use efficiency observed in the mutant plants were attributed to the osmotic adjustment of leaf and root cells, mainly due to higher proline accumulation in these plants. Altogether, these results show that under water deficit, plants with reduced GAs content are able to cope the water deprivation by increasing the proline levels and roots volume in order to maintain the leaf turgor for longer, suggesting a role of GAs for acclimation or tolerance responses to water deficit.

Introduction

Among the abiotic factors to which plants can be affected, water deficit is one of the more important. It occurs when the loss of water by the plant exceeds the capacity of water absorption by the roots from the soil for long enough to cause irreversible damage to the plant (Jaleel et al., 2007). Water deficit is one of the stresses that most affect plant physiology, survival and yield (Charlton et al., 2008; Ramírez et al., 2009; Urano et al., 2009; Ning et al., 2010; Begcy et al., 2012; Loyola et al., 2012). However, the plants have developed adaptive mechanisms to overcome the negative impacts of the water restriction. It has been shown that under this condition plants close the stomata, reducing the photosynthetic activity, alter the elasticity of the cell wall and produce toxic compounds that can lead to plant death (Ahuja et al., 2010).

Under water deficit conditions changes at the transcript, protein and metabolite levels occurs suggesting a complex reprogramming of the plant metabolism (Bokhari et al., 2007; Shulaev et al., 2008; Chae et al., 2009; Ahuja et al., 2010). Consequently, changes in growth are often observed as a result of water deficit. While the shoots are strongly reduced in several species (Loggini et al., 1999; Ravindra et al., 1991; Lerna e Mauromicale, 2006; Shao et al., 2008; Tahi et al., 2008; Sánchez-Rodríguez et al., 2010), the root system is increased under water deficit in order to increase the absorbing root area to keep adequate water supply to the plant (Sharp et al., 1989; Sharp and Davis, 1989; Sharp, 2002; Fu and Harberd, 2003).

It has been demonstrated that hormones are important players for the plant responses to water deprivation. It is verified that plants cultivated under water deficit have their growth regulated mainly by abscisic acid (ABA) (Munns, 2005). It has been observed that high concentrations of ABA cause stomatal closure and induce the expression of genes related to water stress (Seki et al., 2007), such as genes responsible for the synthesis of osmotically active compounds (Antoni et al., 2011). Furthermore, other growth promoting hormones such as cytokinins and gibberellins (GAs) may also being involved in stress responses (Magome et al., 2004; Achard et al., 2006; Rivero et al., 2007; Albacete et al., 2008; Ha et al., 2012). It is believed that the levels of these hormones in the plant affect their performance under stress conditions and consequently their tolerance. Additionally, besides cytokinin and GA, auxins and brassinosteroids are

prominent, whereas, ABA, ethylene, and jasmonic acid are commonly associated with stress responses (Wolters and Juergens, 2009).

The GAs belong to a large family of tetracyclic diterpenoids that promote various processes related to development of organs and tissues (Hedden and Kamiya, 1997; Olszewski et al., 2002; Sakamoto et al., 2004; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011), which are usually suppressed by osmotic stress (Hu et al., 2007). Moreover, modification in the flow through GAs biosynthetic pathway has been observed in response to environmental stimuli (Hedden and Kamiya, 1997; Hedden and Kamiya., 1997). Therefore, the regulation of GA biosynthesis is of fundamental importance for plant development and adaptation (Hedden and Kamiya, 1997; Hedden and Kamiya., 1997). In addition, the content of bioactive GAs is a key factor controlling their homeostasis by negative and positive feedbacks. Negative feedback regulation occurs when the bioactive GAs levels inhibit the enzymes GA_3 oxidase and GA_{20} oxidase, responsible for the final steps of GAs activation. The regulation for positive feedback occurs on GA2ox, which performs the inactivation of these GAs (Hedden and Kamiya, 1997; Hedden and Proebsting, 1999). Many different GAs structures have already been identified in plants but only few of them act as endogenous growth regulators, being precursors of the biosynthetic pathways or are in their inactive form (Cowling et al., 1998; Yamaguchil and Kamiya, 2000; Richards et al., 2001).

The relationship of GA levels and tolerance to abiotic stresses has been verified in different plant species (Achard et al., 2006; Maggio et al., 2010; Dubois et al., 2013; Ho et al., 2013; Nir et al., 2014; Yang et al., 2014; Zawaski and Busov, 2014). In tomato plants overexpressing the GA-methyl transferase 1 gene from Arabidopsis thaliana (AtGAMT1), which encodes an enzyme that catalyzes the methylation of active GAs generating inactive GAs, low levels of bioactive GAs were observed (Nir et al., 2014). These transgenic plants also presented a dwarf phenotype and water deficit tolerance. However, when exogenous GA was applied, normal growth and water deficit sensitivity were restored (Nir et al., 2014). Further, these plants showed lower transpiration rate under water limitation, which was attributed to reduced stomatal conductance due to the smaller stomata with reduction of stomatal pores presented by the AtGAMT1 overexpression lines (Nir et al., 2014).

Although endogenous GA content is recognized as important for processes related to plant growth and development (Olszewski et al., 2002; Sun and Gubler, 2004;

Zhu et al., 2006; Gao et al., 2011; Ribeiro et al., 2012), little is known about the role of the GAs metabolism in plants exposed to water deficit. It has recently been suggested that the metabolism of GA plays an important role in the resistance and acclimatization of plants to conditions of water deficit (Dubois et al., 2013; Ho et al., 2013; Nir et al., 2014; Zawaski and Busov, 2014). Therefore, more studies in plants with altered levels of endogenous GAs are still needed to better understand the mechanisms responsible for responses presented by these plants since GAs are very likely to be variable and not maintained under different extreme conditions. Here, we used tomato mutants with reduced levels of GAs, gib1, gib2 and gib3 (highly, intermediate and moderately deficient in GAs levels, respectively) submitted to water deficit in order to verify whether the endogenous content of GAs is involved in the tolerance responses to water deficit. The results obtained in this study suggest that under water deficit, plants with reduced GAs content are able to cope with the water deprivation mainly by increasing the proline levels and roots volume in order to maintain the leaf turgor for longer, suggesting a role of GAs for acclimation or tolerance responses to water deficit.

Material and methods

Description of genotypes used

The mutant tomato plants (Solanum lycopersicum L. cv. Moneymaker) deficient in GAs biosynthesis used in this study were produced and characterized genotypically and phenotypically previously by (Koornneef et al., 1990). The mutants were obtained by immersion of seeds of commercial cultivar Money-maker in solution of ethyl methane sulfonate mutagenic agent (EMS). The genotypic analysis performed initially revealed that the mutations generated by EMS occurred at different loci, located on different chromosomes (Koornneef et al., 1990). The mutants named gib3, gib2 and gib1 have mutations in chromosomes seven, one and six, respectively (Koornneef et al. 1990).

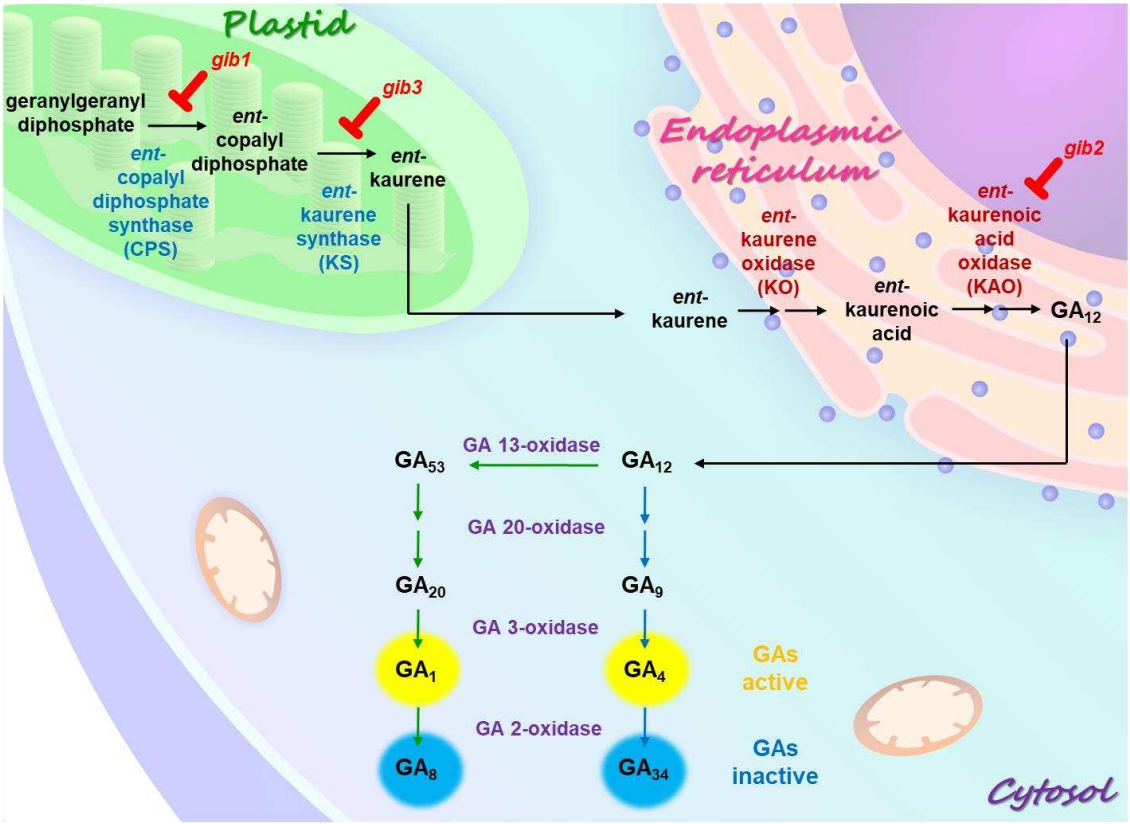


Figure 1. Gibberellin-biosynthetic pathway scheme showing the enzyme and cell place in which each plant, gib3, gib2 and gib1, was mutated.

The characterization of gib1 and gib3 mutants revealed that these genotypes have reduced ent-copalyl diphosphate synthase enzyme activities, with a reduction of 94% in relation to wild type (WT) and ent-kaurene synthase, with a reduction of 93%

compared to WT, respectively (Figure 1). These enzymes participate of first reactions of GAs synthesis located in the plastids. For gib2, although the level of activity reduction was not known, it must have affected the expression of the ent-kaurenoic acid oxidase enzyme located in the endoplasmic reticulum (Bensen and Zeevaart, 1990); Zeevaart 1986). Together, low gene expression and low activity of these three enzymes resulted in mutant plants with reduced GAs levels (Koornneef et al., 1990).

Experimental conditions

The experiment was performed at Viçosa (20º45'S, 650 m altitude), Minas Gerais, in green house, of Department of Plant Biology of Universidade Federal de Viçosa.

Wild type tomato plants and mutants deficient in the biosynthesis of GAs, gib1, gib2 and gib3 (highly, intermediate and moderately deficient) were used, kindly provided by Dr. Koornneef (Max Planck Institute for Plant Breeding Research, Cologne, Germany).

The seeds were germinated in Petri dish (15 cm of diameter) containing two layers of germtest paper towels moistened with 10 μM GA_{4+7} solution. Subsequently, the plates were housed in a growth chamber BOD type (Forma Scientific, Inc, Ohio, EUA), under photoperiod of 12/12 hours (day/night), temperature 25/16 ºC (day/night), relative humidity 65±5% and luminous intensity of 150 μmol photons m^{-2} s^{-1}, for a period of seven days. Each day, germinated seeds with a radicle emission of one centimeter in length were transferred to vase of 0.08 dm^3 containing Tropstrato HT® commercial substrate for vegetables and remained in growing chambers until cotyledonary leaves expansion. The pots were kept in a greenhouse and, after the appearance of three completely expanded leaves, the seedlings were transplanted to vessels of 1.4 dm^3 Containing substrate for vegetables supplemented with the 4:14:8 NPK granulated (equivalent at 4% of (NH_4^+) (NO_3^-), 14% of P_2O_5, 8% of K_2O) in the proportion of 0.5 kg of NPK to 10.0 kg of substrate. Plants were cultivated for six weeks in a greenhouse.

After six weeks, the treatments consisted of periodically watered plants (control, C1), plants under water deficit (WD) where the water was totally suppressed and plants that underwent a period of water deficit but received watering until the total recovery of the photosynthetic parameters, such as photosynthesis and stomatal conductance (R). In addition, in order to compare the stress condition with the optimum condition to the

crop, the treatments WD and R presented their control, C1 and C2, respectively. Control treatment plants were watered daily in order to keep the substrate moisture of pots close to the field capacity until the end of experiment. Physiological assessments and leaf and root collections for biochemical analyzes were initiated when the water potential of at least 50% of the plants of each genotype reached -1.3 MPa. The determination of water potential was carried out in the morning with the aid of a pressure pump Scholander type. For the treatment that consisted of rewatered plants, parameter used to start the collect was the photosynthesis and stomatal conductance measured with an Open System Infrared Gas Analyzer (IRGA). The average of plants values of R treatment being identical to average of values of fully watered plants (control, C2) indicating recovery after stress.

The evaluations were carried out on completely expanded leaves after analyzes of water potential and gas exchange for plants under water deficit and under recovery accompanied by their controls, C1 and C2 respectively.

Growth analysis and sample harvesting

Growth in shoot height was determined during the experiment. Plant growth was monitored every three days. For this, height of plants was monitored with help of a millimeter rule from the lap height to the apical bud throughout the experiment. The final height, shoot dry weight (leaves and stem), root dry weight, total leaf area and specific leaf area were determined at the end. Subsequently, relative growth rate in height (RGR-h) was calculated by Evans (1972) and Hunt (1982).

Eight leaf discs (0,519 cm of diameter) were dried at 65 ºC until constant dry weight for determination of specific leaf area. The total leaf area was estimated from the specific leaf area using the dry weight of all leaves. The determination of both specific leaf area and total leaf area was performed following protocol described by (Mielke et al., 1995). The specific leaf area was calculated using equation:

$$\text{Specific leaf area } (m^2\ g^{-1}) = \frac{\text{leaf area of discs } (m^2)}{\text{dry weight of discs (g)}}.$$

Leaf samples were collected, immediately frozen in liquid nitrogen and stored at -80 ºC until analysis. Leaves, stems and roots were separated and immediately taken to the oven with forced ventilation at 65 ºC to obtain the dry weight (DW) after constant

weight. Values of dry weight, biomass partitioning of leaves, stems and roots and shoot-root ratio were calculated by Benincasa (2003).

Gas exchange and chlorophyll a fluorescence analysis

The photosynthetic rate (A_N), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and leaf transpiration rate (E) were measured simultaneously to chlorophyll a fluorescence parameters in penultimate leaflet of the third leaf, counted from the apex. For this, used an Open System Infrared Gas Analyzer (IRGA), with a chamber of sample area of 2 cm^2, model LI 6400XT (LI-COR, Lincoln, NE, EUA), and equipped with coupled fluorometer (LI-6400-40, LI-COR Inc.). Instantaneous gas exchanges were measured after two hours illumination during the light period, under 1000 μmol of photons m^{-2} s^{-1} (light saturation) of photosynthetically active photon flux density (PPFD), determined by A/PPFD curves - net photosynthesis (A_N) in response to PPFD curves, and standard condition of 400 ppm of CO_2. The ratio stomatal was 0.5 (amphistomatic leaves) and the leaf-to-air vapor pressure deficit (VPD) was kept at 1.2 to 2.0 kPa. An amount of blue light applied at 10% of the density of the photosynthetic stream to maximize a stomatal aperture. Greenhouse temperature during the evaluation was 23.7 ± 2.5 °C. Respiratory rates in the dark (R_d) were also determined using the same equipment during the dark period, after two hours of the end of the light period, using the same leaflet to determine the other clear parameters.

The determination of the chlorophyll a fluorescence occurred in leaves adapted to the dark after the measurement of the parameters with IRGA. The leaves were initially exposed to a faint red-distant pulse of light (1-2 μmol of photons m^{-2} s^{-1}), for determination of initial fluorescence (F_0). Then, a pulse of saturating light with irradiance of 6000 μmol of photons m^{-2} s^{-1} and time of one second, was applied to estimate the maximum emitted fluorescence (F_m). The minimum fluorescence of light adapted leaves (F_0') and maximum chlorophyll fluorescence (F_m') were determined using the same equipment concomitantly collecting the gas exchange data. The maximum photochemical efficiency of PSII ($F_v/F_m=[(F_m-F_0)/F_m)]$), of excitation energy capture efficiency by PSII open reaction centers ($F_v'/F_m'=[(F_{m'}-F_s)/F_{m'})]$), of photochemical extinction coefficients (q_L) and non-photochemical (NPQ) and of electron transport rate (ETR), was also estimated like DaMatta e Rena (2002).

The results of A_N, R_d and E were expressed per mass unit (kg) while g_S and ETR were expressed per area unit (m^2).

Determination of plant water status

Water status of the plant was determined during the experiment by means of a pressure chamber Scholander type (model 1000, PMS Instruments, Albany, NY, USA) in before tomorrow. The penultimate leaflet of the fourth leaf from apex to base was cut at insertion of petiole and immediately placed in the chamber for measuring the water potential (Ψ_w) (Scholander et al., 1964). The results were expressed in MPa.

In addition, water status was also determined by the relative water content (RWC), according to the protocol described by Cairo (1995). After nondestructive evaluations, nine leaf discs (0.519 cm of diameter) were collected from leaves located at base, middle and apex of plant at the end of experiment. Immediately the discs were weighed to determine fresh weight (FW) and placed in Petri dishes containing distilled water. After a period of four hours, in which the disks remained immersed in water, the disks were carefully dried between adsorbent paper under the same pressure on them, so that there was loss of external moisture without extravasation of water from the fabrics, and again heavy to obtain the turgid weight (TW). Afterwards, the disks were placed in paper bags and forced into a greenhouse with forced ventilation at 65 ^{O}C to obtain the dry weight (DW) up to constant weight. With fresh, turgid and dry mass, the RWC was calculated according to the equation below and the results were expressed as percentage:

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} * 100$$

Leaf water loss measurements

For water loss measurements, the penultimate leaflet of fully expanded fifth leaf was detached and immediately had its mass determined. Then the leaflet was maintained with the abaxial side facing up under the same growing conditions of the plants. The leaf weight was determined over a period of two hours at 10 min intervals. Subsequently, water loss was calculated as the percentage of initial fresh weight loss (Araújo et al., 2011).

For leaf water loss measurements after submersion in ABA solution, leaflets were maintained for four hours in deionized water, 10 µM and 50 µM ABA solution shortly after excision. Leaflet was maintained with the abaxial side facing down, in

contact to solution. After this period, the leaflets were weighed for two hours at 10 min intervals.

Dynamics of stomatal closure following leaf excision and ABA

The dynamics of stomatal closure following leaf excision and ABA was performed according with (Brodribb and McAdam, 2011). To record the dynamics of stomatal closure following leaf excision, one leaf of each plant, from four individuals were used. Leaves were collected and excised in the morning either, cut underwater directly. Portable infrared gas analysers (Li-6400XT, Lincoln, NE, USA) were used to measure stomatal conductance (g_S) (mol m^{-2} s^{-1}) following leaf excision. Ambient conditions within the leaf chamber were maintained constant during the experiment (leaf temperature was maintained at 25 ºC, PPFD at 1000 μmol photons m^{-2} s^{-1}, leaf chamber flow rate at 300 μmol s^{-1}, CO_2 concentration at 400 μmol mol^{-1} and VPD manually regulated between 1.2 and 2.0 kPa). During measurements g_S, A_N and other leaf environmental traits were logged every 60 seconds. Intact leaves or leafy shoots were allowed to equilibrate in the chamber for 10 minutes while supplied with water. Gas exchange data was recorded on the excised leaf until g_S had reached a minimum and stabilized.

To assess the response of g_S to ABA in mutants plants in GAs biosynthesis, leaves of each plant were excised and immediately cut into a Petri dish containing deionized water (MilliQ, Millipore, Billerica, MA, USA). Leaf tissue was included in the cuvette of a IRGA under the conditions described above and leaf traits were logged every 60 seconds. After g_S read during 10 minutes, an aliquot of a stock solution of unlabeled ABA was added to the leaf water supply increasing the concentration of ABA in the water to 1185 ng ml^{-1} (concentration that reduced g_S plant at an intermediate rate when compared to the other concentrations, selected according to the ABA concentration test in WT plants). After the addition of ABA to the foliar water supply, g_S continued to be recorded every 60 s for at least 90 minutes.

Determination of pigments, sugars, starch, proteins, amino acids, malate, fumarate and proline

For the determination of the metabolites, leaf and root samples were collected in the middle of the light period and immediately frozen in liquid nitrogen and stored at -80ºC until extraction. Aliquots of approximately 30 mg of fresh mass of both tissues

were used for biochemical analysis. Leaf and root samples were submitted to hot ethanol extraction and photosynthetic pigments (Porra et al., 1989), glucose, fructose, sucrose (Fernie et al., 2001), amino acids (Gibon et al., 2004), malate and fumarate contents (Nunes-Nesi et al., 2007) were determined in the soluble fraction in ethanol. In the insoluble fraction, the starch (Fernie et al., 2001) and protein content (Gibon et al., 2004) was measured. Leaf data were normalized and presented by dry weight.

The proline concentration was determined in both leaves and roots as previously described (Bates et al., 1973). For this, 30 mg of leaf and 50 mg of root was lyophilized and used for extraction and subsequent analytical march. The absorbance was read in a spectrophotometer at 520 nm and proline concentration was calculated by standard curve.

Determination of the metabolic profile

Leaf and root samples were previously collected in the middle of the light period and immediately frozen in liquid nitrogen and stored at -80 °C until extraction. The samples were extracted in methanol, water and chloroform as previously described (Lisec et al., 2006), and used for the determination of the metabolic profile by gas chromatography associated with mass spectrometry (GC-MS) (Roessner et al., 2001). Peaks were manually annotated, and ion intensity was determined by the aid of TagFinder software (Luedemann et al., 2012), using a reference library from the Golm Metabolome Database (Kopka et al., 2005) and following the recommended reporting format (Fernie et al., 2011).

Experimental design

The experimental design was a randomized complete block design, in a 4x4 factorial scheme, corresponding to four water regimes: (i) watered plants (C1), control of the treatment under water deficit; (ii) plants under water deficit (WD); Watered plants, control of re-watering treatment (C2) and; (iv) re-watered plants, recovery after drought (R). Four genotypes (WT, gib3, gib2 and gib1) were also used, with six replicates for each treatment, considering each sample as a repeat. The results were submitted to analysis of variance (ANOVA) and, to verify statistical difference between genotypes in each of the treatments, the means were compared by Student t test at 5% of probability ($P<0.05$), always comparing the mutant lines to WT, with the aid of the algorithms present in the program Microsoft Excel® (Microsoft, Seattle). Furthermore,

a graphical representation of data set of the metabolic profile was represented as heatmap using Multiple Experiment Viewer Software (MeV) version 4.5, for this, values was normalized to the mean response calculated for WT in treatment C1. For the multivariate analyzes of principal component (PCA) were performed using the MeV program.

Results

GAs-deficient plants present delay in the reduction of the water status

Wild type (WT) and mutant plants in GA biosynthesis (gib3, gib2 and gib1) were submitted to water deficit by interruption of the irrigation until reaching the water potential (Ψw) of -1.3 MPa. Afterwards, the plants were re-watering. During and at the end of these two steps, physiological and growth parameters were evaluated as well as leaves and roots were harvested to further biochemical analyses. It was observed that under control condition (C) the growth in WT and gib3 plants was similar, whereas gib2 and gib1 plants presented lower growth phenotype (Figure 1A). In addition, it was observed that leaves of the mutant plants, gib2 and gib1, presented dark green color when compared to WT. Although, this intense green color of gib2 and gib1 leaves did not correlate with total chlorophyll content (Chl) expressed per dry weight (Supplementary Figure S4 A and B). This fact was attributed to leaf morphology and anatomy, characterized by leaf shrinkage and thickness (Figure 1A).

The water status of WT and mutant plants was determined daily during 23 days after application of the treatments. When submitted to water deficit (WD) condition for eight days, WT and gib3 plants were found to have visible wilt symptoms (Figure 1A), while gib2 and gib1 plants showed no symptoms. The plants from these two genotypes required 15 days and more than 20 days to show apparent wilt signs (Figure 1A). It is important to note that the plants from distinct genotypes reached Ψ_w of -1.3 MPa at different days after stopping the irrigation and recovered Ψ_w at the day after re-watering (Figure 1B and D). Plants WT and gib3 reached Ψ_w close to -1.3 MPa after eight days of irrigation suspension, while gib2 and gib1 reached the same Ψw only after 15 and 20 days, respectively.

Under control conditions (C1 and C2) and after re-watering, RWC values were greater than 80% in the WT and mutants plants. However, genotypes gib2 and gib1 presented significantly higher RWC values when compared to WT (Figure 1C). Under water deficit, reductions in RWC were observed, reaching values around 61% for WT and gib3, whereas for gib2 and gib1 the values were equal to 68% and 70%, respectively.

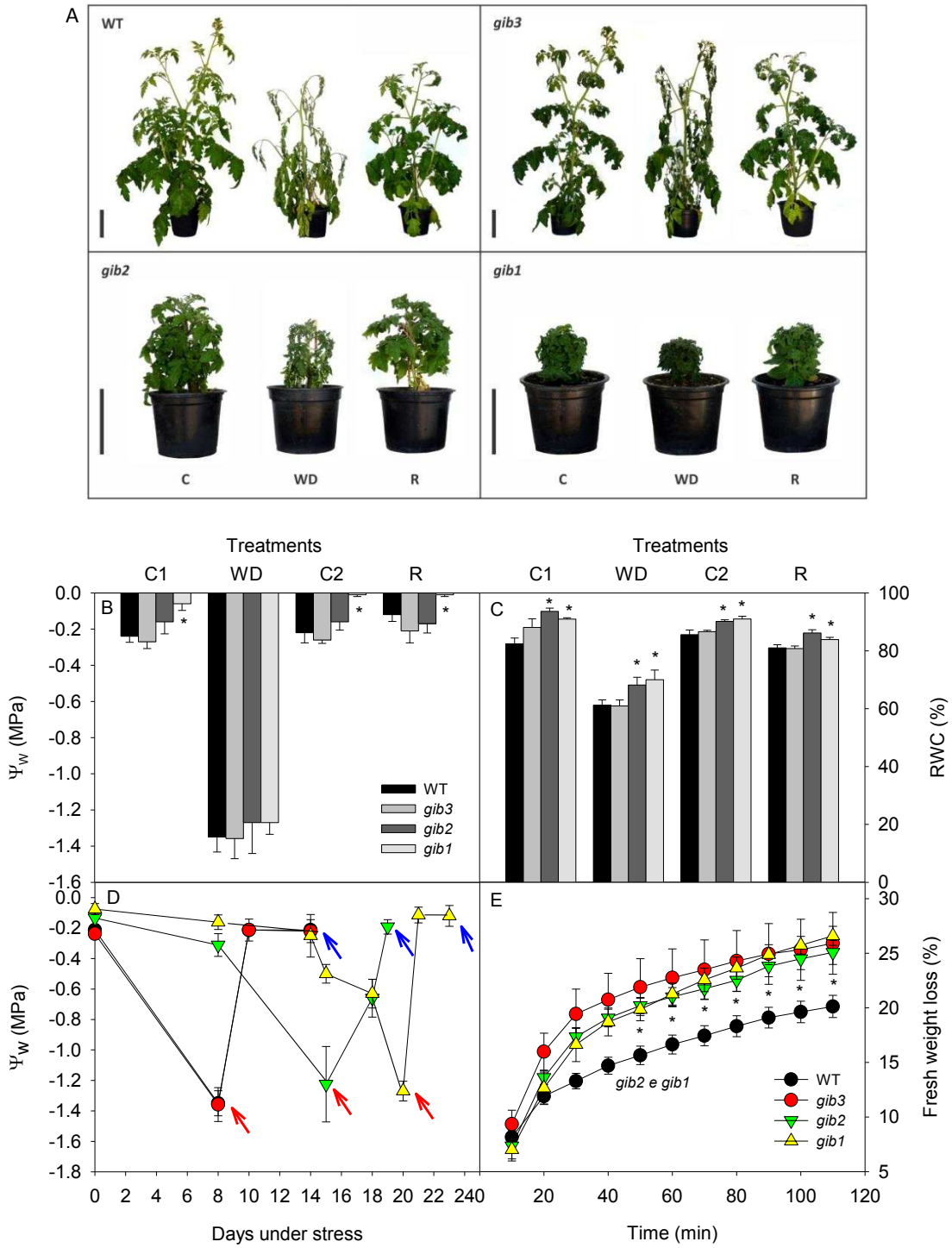


Figure 1. Visual aspect and water status of leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with low levels of endogenous GAs, gib3, gib2 and gib1, exposed to water deficit and after recovery. (A) WT and mutant plants in GA biosynthesis at the end of eight, 15 and 20 days under water deficit, respectively. C represents control treatment which plants were irrigated throughout the experiment; WD represents treatment of water deficit which plants remained without irrigation for a period of eight, 15 and 20 days for gib3, gib2 and gib1, respectively; And R, recovery treatment which plants went through a period of water deficit (variable for each genotype) and later were reirrigated until recovery of gas exchange parameters. Representative vertical bars equal to 11.5 cm. (B) Water potential (Ψ_w) of leaves of plants with different GAs endogenous levels at the end of each treatment; (C) relative water content (RWC) of plants at the end of each treatment exposure; (D) Ψ_w of leaves of plants with

GAs reduced endogenous levels during the days of exposure to water stress; (E) Leaf water loss from mutant plants in GA biosynthesis with eight weeks of culture for two hours. Values are presented as means ± standard error of at least six plants. Red arrows indicate collection points for treatment with plants under water deficit (WD) and their respective control (C1). Blue arrows indicate collection points of treatment samples with plants that have gone through a period of drought and recovered the physiological parameters (R) and their respective control (C2). Asterisks in figure E indicates the moment (in minutes) which values of gib2 and gib1 mutants show significantly different ($P<0.05$) by Student's t test in relation to WT. Asterisks of Figures B and C indicate significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in same treatment, always comparing to WT.

Mutant plants with reduced endogenous GA levels reached Ψ_w -1.3 MPa later. Therefore, we decided to evaluate the water loss in detached leaves (Figure 1E). Surprisingly, the mutants showed fresh weight loss rates similar to the WT only in the first 50 min. Thus, after 50 minutes gib3, gib2 and gib1 presented 22%, 20% and 19% of water loss, respectively, whereas in WT plants it was on average 16%. Later, greater fresh mass loss in the mutants compared to the WT were observed until the end of the analysis. Although the mutant plants showed greater water losses, only gib2 and gib1 differed significantly from WT at 50 min at 120 min of analysis.

It has been observed that high ABA concentrations in leaves cause stomatal closure and consequently reduce stomatal conductance (g_S) (Seki et al., 2007). Thus, it is expected that during the water loss in the leaves previously incubated in ABA solution, will be reduced due to stomatal closure. That said, we verified the sensitivity of mutant genotypes and WT to ABA (Figure 2). After excision, the leaves were incubated for four hours in distilled water (control) and in ABA solution at two concentrations, 10 μM and 50 μM (Figure 2A, B and C). Similar behavior of the genotypes was verified in terms of water loss, with higher water losses in the mutant leaves (Figure 2A). The same pattern of response was verified for the two other ABA concentrations (Figure 2B and C). However, the presence of ABA at 10 μM concentration reduced the water loss in leaves of all tested genotypes when compared to the control (water) condition (Figure 2B). The water loss was even lower when the ABA concentration was increased to 50 μM (Figure 2C).

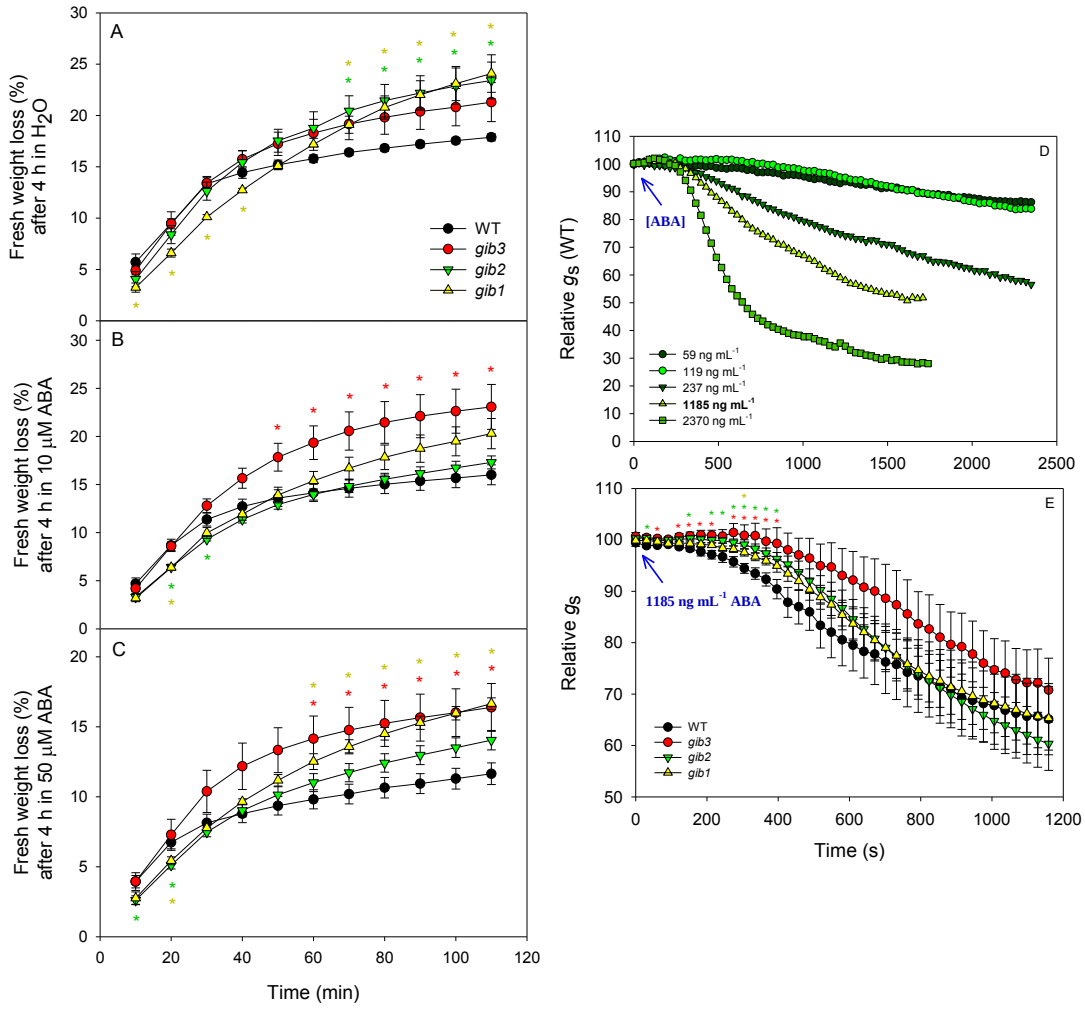


Figure 2. Detached leaf fresh mass loss for two hours after submerging in ABA solution and ABA response curves of leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis with eight weeks of cultivation under growth optimal conditions. (A) Detached leaf fresh mass loss after submersion in distilled water for 4 h. (B) Detached leaf fresh mass loss after submersion in solution containing 10 µM ABA for 4 h. (C) Detached leaf fresh mass loss after submersion in solution containing 50 µM ABA for 4 h. (D) Stomatal conductance (g_S) as time function of WT plants under different ABA concentrations for a period of approximately 40 min. (E) Stomatal conductance (g_S) as time function of WT plants and mutants in GAs biosynthesis under 1185 ng mL^{-1} of ABA in the solution over a period of 20 min. Values are presented as means ± standard error of at least six plants. Asterisks indicates the moment (in minutes or seconds) which mutants values show significantly different ($P<0.05$) by Student's t test in relation to WT. Red, green and yellow asterisks represent significant differences of gib3, gib2 and gib1 mutants, respectively. In figure D, show individual values of WT plants under different concentrations of ABA.

The ABA influence on the decrease of g_S was further verified by performing ABA response curves by following the g_S after application of ABA (Figure 2D and E). Initially, a test using only WT plants was performed to select the ABA concentration to be used (Figure 2D). The ABA concentration selected was 1185 ng mL^{-1}, which was closer to that found in angiosperm xylem sap, of 1500 ng mL^{-1} (Brodribb and McAdam,

2011). Thereafter, all genotypes were assayed and a delay in g_S decay could be observed with significant differences between mutants and WT approximately 100 to 400 seconds after ABA application at the final concentration in the previously established medium (Figure 2E). However, from these two analyzes (water loss in detached leaves and ABA-gs response curve) it was observed that the plants have different levels of sensitivity to ABA, with mutant genotypes being lesser sensitive to ABA.

GAs drives growth and biomass partitioning to roots, especially under water deficit

The mutant plants presented reductions in total and specific leaf area, leaf dry weight, stem dry weight, root dry weight, total root/shoot ratio, and consequently biomass partitioning (Figure 3).The total leaf area was lower in plants from all mutant lines under both tested conditions (Figure 3A). Plants exposure to water deficit (WD), even in those in which irrigation was restored (R), resulted in reduction of total leaf area in both mutant and WT plants, with strong reductions in plants with the highest GA levels. In the case of specific leaf area, the results were similar to those found for total leaf area in which mutant plants presented a smaller specific leaf area in all tested conditions, with exception of C2 (Figure 3B). Such results of specific leaf area in C1 indicate that plants with reduction in GA presented increases in the leaf thickness and the characteristics of the mutation of these plants remain even under WD.

The dry weight of leaves (Figure 3C), stems (Figure 3D), roots (Figure 3E), and total (Figure 3F) were reduced in mutant plants when compared to WT in all treatments, with strong reductions in gib1 and gib2. When the plants were submitted to WD treatment, there was a greater reduction in leaf dry weight, stems dry weight and total dry weight when compared to treatment C1, while root dry weight was reduced dramatically only in WT and gib3 plants. The dry weight of the plants in R treatment also showed reductions when compared to the treatment C2.

The higher root/shoot ratio values in gib2 and gib1 plants in all applied treatments indicates a higher biomass partitioning in roots than in shoot, which may explain the longer time required for wilting in these plants when compared to WT and gib3 (Figure 3G). The absolute values of the root/shoot ratio increased in WD treatment and in R one.

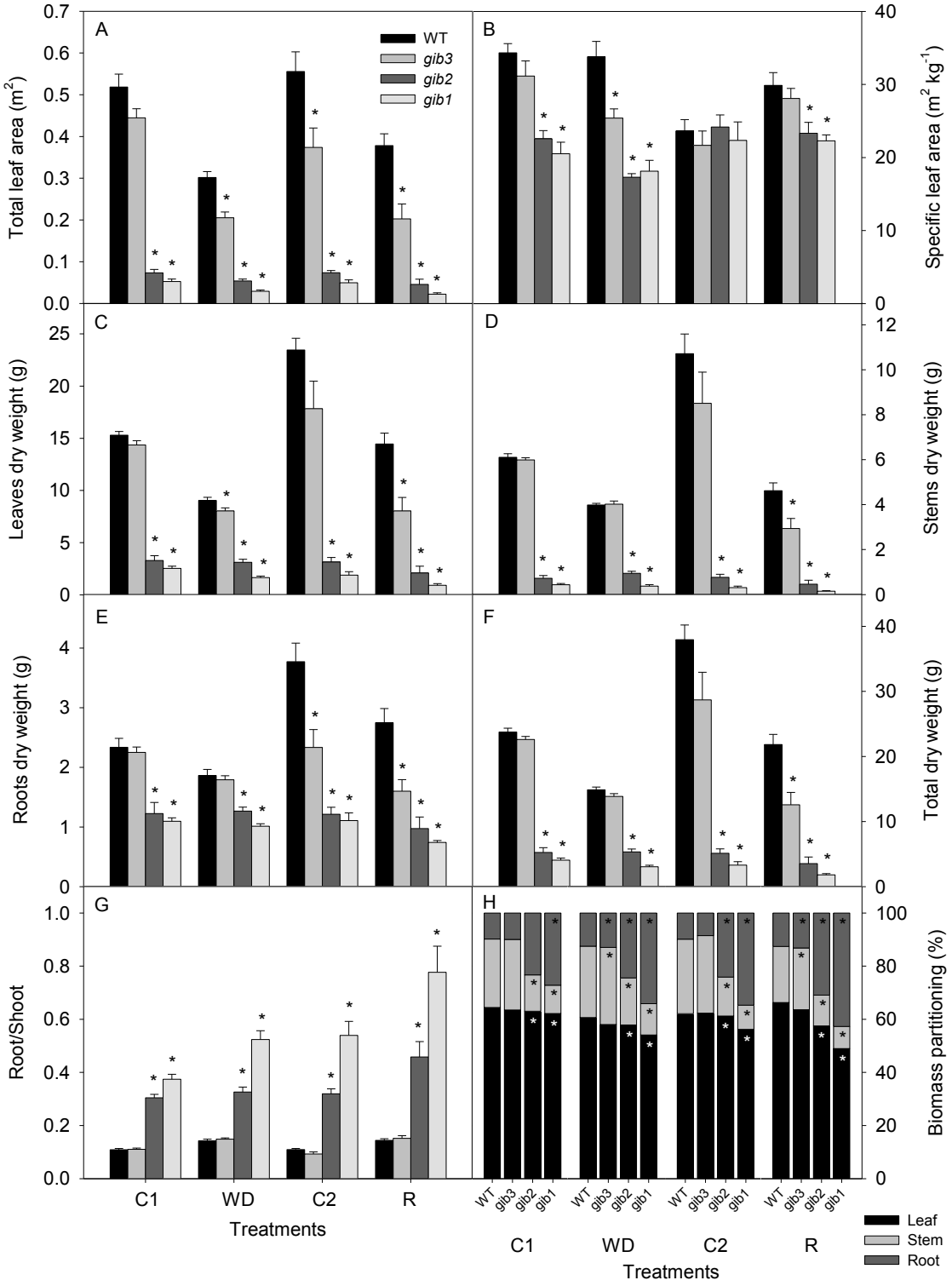


Figure 3. Variation of biometric parameters in response to water deficit in tomato plants (Solanum lycopersicum cv. Moneymaker) with different levels of endogenous GAs. (A) Total leaf area; (B) specific leaf area; (C) leaf dry weight; (D) stem dry weight; (E) root dry weight (F) total dry weight of plant; (G) ratio between root and shoot; (H) biomass partitioning which bars represent the percentage of the total dry weight of each organ. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 1.

At the end of the experiment, the height of WT and mutants plants were determined (Supplementary Figure S1A). gib2 and gib1 plants, presented lower height when compared to WT. Interestingly, the gib3 mutant presented higher plants than WT, differing significantly in the WD treatment and its control (C1). In WD and R treatment, a slight reduction in the final height of WT and gib3 plants could be observed when compared to their respective controls.

The gib2 and gib1 plants were found to have reduced RGR-h under normal growth conditions (Supplementary Figure S1B). During water stress, the RGR-h of the gib2 and gib1 plants was higher than WT (Supplementary Figure S1 C). However, under the same condition, WT and gib3 plants considerably decreased their growth compared to the same genotypes in the control (C1) treatment.

Plants with low GAs levels present higher photosynthetic capacity

During the plants cultivation under the different tested conditions, the CO_2 assimilation rate (A_N) was monitored from the application of the water deficit treatment and after the re-watering, until the recovery of the plants (R) (Figure 4). Thus, when the plants reached Ψ_w -1.3 MPa (Figure 1D), gas exchange analyze was performed. These time points corresponded to 8, 15 and 20 after the irrigation suspension for WT and gib3, gib2, and gib1, respectively (Figure 4A). When plants reached -1.3 MPa the A_N was dramatically reduced for all the genotypes. After re-watering, although Ψ_w of the plants reached values close to zero already in the next day, the plants needed a longer time to recover their gas exchanges parameters, represented here by the A_N. Thus, WT and gib3, which present phenotypic similarities to each other, presented A_N similar to the control condition after five days of re-watering, while gib2 and gib1 recovered A_N after three and two days, respectively.

The mutant plants exhibited a tendency of higher values in the gas exchanges parameters expressed per mass unit, A_N, g_S and WUE (Figure 4). The A_N of WT and mutant plants presented a variable pattern among the genotypes within each treatment (Figure 4B). In C1 there was a reduction in A_N in gib2 while the other genotypes did not differ from WT. Under WD, all genotypes show drastic reduction in A_N mainly gib3 plants while gib1 and gib2 presented the highest values. In the treatment R there was an increase in the photosynthetic rates in the mutant genotypes when compared to WT, a

pattern similar to that found in the C2 treatment. The g_S showed the same trend observed for A_N, which plants with low GAs levels presented higher g_S (Figure 4C). Interestingly, in all tested conditions, mutant plants had higher g_S values, up to five times higher than WT plants, except for WD treatment where g_S was only slightly higher. Under optimal growth conditions (C1 and C2), the mutant plants showed significant increase in g_S, except for gib3 in the C1 treatment. When plants were exposed to water deficit, there was a reduction of g_S in all genotypes when compared to the control condition, with the most drastic reductions in WT plants. After re-watering, the g_S was reestablished and the mutant genotypes showed even greater g_S values relative to WT. Similar to g_S, the mutants presented higher transpiration rates (E) under all conditions (Supplementary Figure S3 A).

It was verified that mutant plants presented higher C_i in the treatments C1, C2 and R when compared to the values observed in WT (Supplementary Figure S3 B). Under WD treatment, higher values of C_i for gib3 and lower for gib2 and gib1 were observed. However, these reductions in C_i were not drastic enough to cause photosynthesis limitation due to CO_2 lack at the Rubisco carboxylation centers (biochemical limitation). Probably, the reductions in A_N observed were due to diffusive limitations since g_S drastically reduced under water deficiency (Figure 4B and C).

In general, gib2 and gib1 were found to have higher values of instantaneous water use efficiency (WUE) compared to WT under water deficit (Figure 4D). However, similar trends were observed in the control treatments (C1 and C2) and R, whose all mutant genotypes presented reduced WUE. Dark respiration (R_d) did not differ dramatically among genotypes in the controls (Figure 4E). However, when the plants were submitted to WD, the genotypes gib2 and gib1 showed a strongly reduction in R_d and, when they were re-watered, these genotypes recovered the respiration rates. When comparing the genotypes in each condition, C1 and WD, we observed that mutant plants gib2 and gib1 exhibited greater isotopic discrimination of ^{13}C in both control (C1) and WD conditions (Figure 4F). However, in these genotypes the ^{13}C isotope discrimination was lower under stress conditions compared to C1.

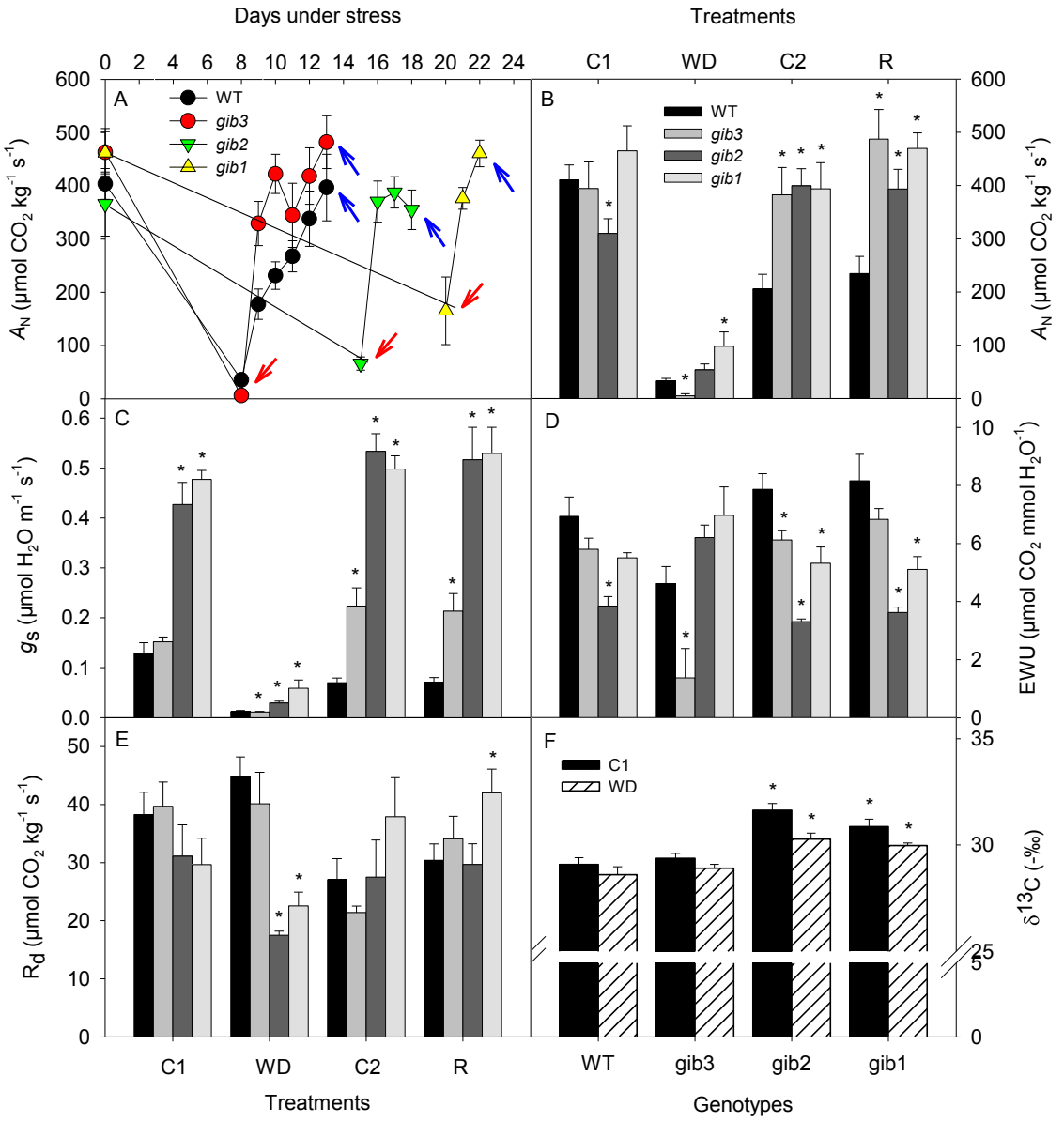


Figure 4. Alteration in gas exchange parameters and ^{13}C isotope discrimination ($\delta^{13}C$) of leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with low GAs levels in response to water deficit. (A) Net CO_2 net assimilation rate (A_N) during all period of water deficit and recovery period; (B) Net CO_2 assimilation rate (A_N) at the end of the experiment; (C) Stomatal conductance (g_S); (D) dark respiration rate (Rd) and; (E) Instantaneous water use efficiency (WUE) and; (F) ^{13}C isotopic discrimination ($\delta^{13}C$) of leaves under water deficit. Values are presented as means ± standard error of at least six plants. Red arrows indicate sample harvesting points for treatment with plants under water deficit (WD) and their respective control (C1). Blue arrows indicate sample harvesting points for plants that have gone through a period of drought and recovered the physiological parameters (R) and their respective control (C2). Statistical analysis as described in Figure 1.

The maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) was higher than 0.80 in all genotypes indicating that even under water deficits, it did not cause severe damage to the photochemical apparatus and PSII (Supplementary Figure S2 A). As expected the values for F_v'/F_m' were lower compared to F_v/F_m in all mutants

and treatments (Supplementary Figure S2 B). The photochemical extinction coefficient (q_L) and electron transport rate (ETR) under water deficit conditions caused reductions in WT and gib3, not drastic in gib2 and gib1 differing significantly from WT indicating that the energy was being directed to the photochemical step of photosynthesis (Supplementary FigureS2 C and E). Under WD, all plants presented increase in the non-photochemical quenching in the heat form when compared to C1 (non-photochemical extinction coefficient - NPQ) (Supplementary FigureS2 D). In R, the plants recovered q_L, NPQ and ETR values from both WT and mutant genotypes.

Water restriction alters carbohydrates and organic acids accumulation in leaves in gib2 and gib1 plants

In order to understand the effects of endogenous GAs low levels on carbon and nitrogen metabolism under stress conditions, a biochemical characterization of leaves and roots of the genotypes was performed. Several changes were observed in the levels of compounds associated with carbon metabolism (Figure 5 and 6). In the leaves, glucose and fructose contents were similar in C1 and WD (Figure 5A and B). However, plant exposure to water deficit, accumulate more glucose and fructose in all genotypes comparing to C1, mainly gib2 and gib1 compared to the control condition. After re-watering, the levels of these sugars were reduced in the mutants when compared to WT, but similar to the values presented by the respective control condition. Sucrose did not accumulated significantly in all genotypes under different tested conditions (Figure 5C). In the WD and R treatments no significant differences were observed in the sucrose content between genotypes. Starch concentration at the middle of the day was reduced in the mutant plants under control treatments (C1 and C2) and R (Figure 5D). However, under water deficit, starch levels decreased only in WT and gib3. When irrigation was restored, the behavior of WT and mutants were similar to plants kept under optimum conditions (C2).

We further measured the levels of malate and fumarate (Figure 5E and F). Water deficit led to increases in the malate levels in gib2 and gib1 and reductions in gib3 (Figure 5E). After re-watering, significant reductions in malate levels were observed in gib2 and gib1. The fumarate levels significantly increased in gib2 and gib1 plants under C1 condition (Figure 5F). However, gib2 and gib1 plants had significantly reduced

fumarate levels under water deficit. During the recovery, no significant differences were observed in the fumarate contents among the genotypes.

In the roots, the sugar accumulation pattern was similar to the leaves in terms of the highest glucose and fructose concentrations and lower sucrose levels in the tested genotypes (Figure 6A, B and C). The genotypes showed increases in glucose content in the WD treatment, being higher in the mutant genotypes when compared to the C1 condition (Figure 6A). However, the levels of fructose in WD were reduced compared to C1 (Figure 6B). After re-watering, glucose levels were significantly reduced only in the gib1 plants (Figure 6A). Fructose presented similar values in the genotypes under R and C2 with the exception of gib2 that showed reduction after re-watering (Figure 6B). The genotype effect under sucrose accumulation was evidenced in mutant roots when grown under optimum conditions (C1 and C2) and after re-watering (Figure 6C). During water deficit, sucrose levels were dramatically reduced and minor reductions were observed for gib2 and gib1, whose gib1 was significantly higher than WT. When the plants were re-watered, increment in sucrose levels was observed maintaining the same pattern of the condition C2. The starch content in the roots with water deficit was not significantly changed due to the applied treatments for all the genotypes (Figure 6D).

Under optimal conditions there was a clear trend of reduction in total chlorophyll (Chl) levels of leaves in gib2 and gib1 grown under optimal conditions and significant reduction in the same genotypes when such plants were submitted to water deficit conditions (Supplementary figure S4 A). Reduction of total Chl content in WT and gib3 plants was also observed, with the lowest reductions in older gib2 and gib1 plants, cultivated under continuous irrigation (C2). After re-watering, no differences were observed between the genotypes. Plants with the lowest GA levels, gib2 and gib1, presented the lowest Chl a/b ratio in all tested conditions (Supplementary Figure S4 B). Plants gib2 and gib1 have less Chl a over Chl b, whose greatest differences have been observed in C1 and WD.

Unlike in leaves, in roots both malate and fumarate contents were increased under water deficit, with mutant genotypes showing higher values in comparison to WT (Figure 6E and F). After re-watering, malate content was reduced in all genotypes without significant differences between mutants and WT (Figure 6E). Concerning the

fumarate content, there were reductions in WT and gib3 plants, and in gib2 and gib1 the values remained higher after adequate water supply (Figure 6F).

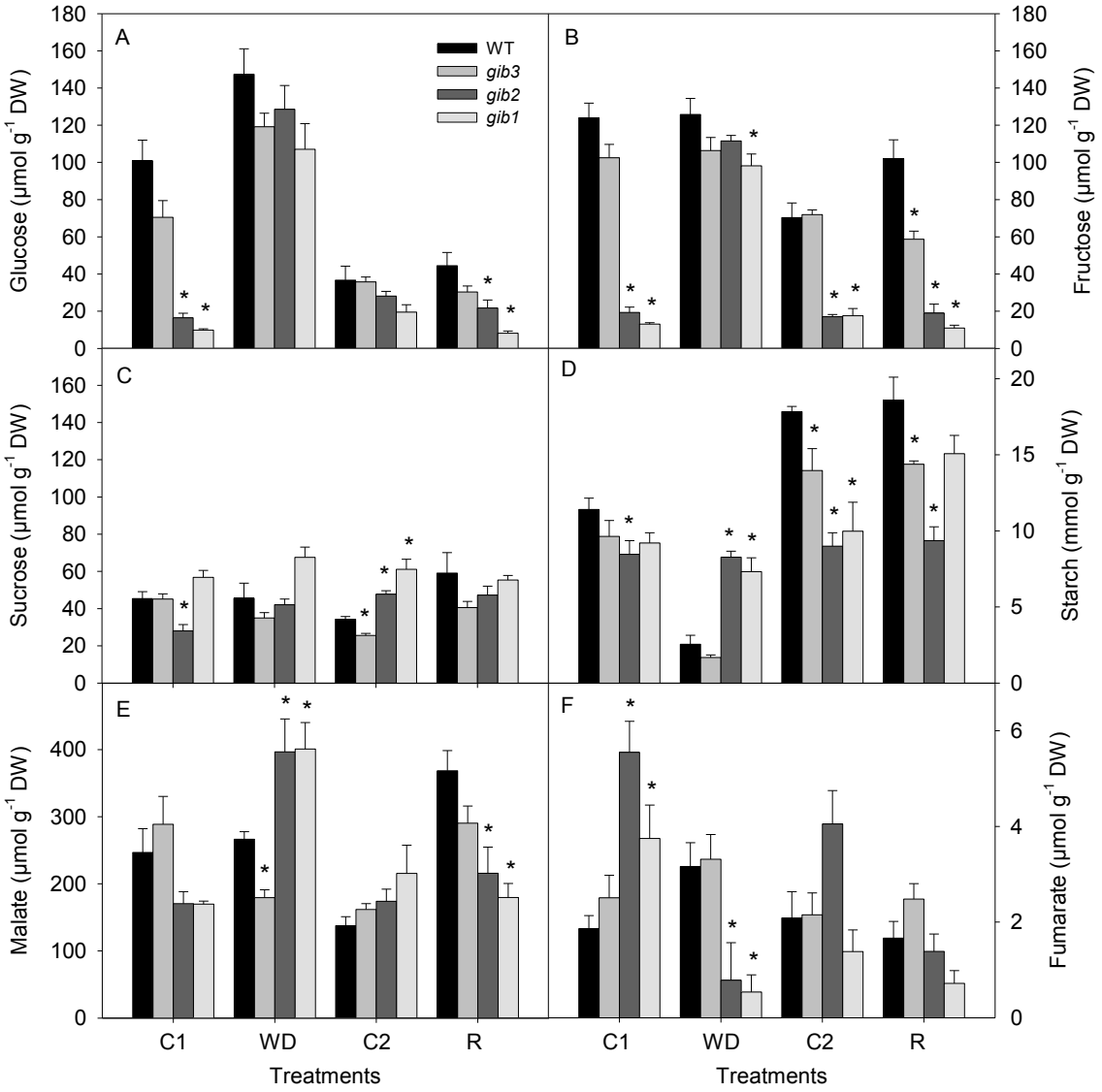


Figure 5. Changes in metabolite contents involved in carbon metabolism in leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with different endogenous levels of GAs under water deficit and after recovery period. (A) Glucose; (B) Fructose; (C) Sucrose; (D) Starch; (E) Malate and; (F) Fumarate. Values are presented as means ± standard error of six plants. Statistical analysis as described in Figure 1.

Also in leaves, mutant plants presented accumulation in the total amino acids concentration under water deficit, but with no significant difference in relation to WT (Supplementary Figure S4 C). After re-watering, the amino acid contents of the genotypes were reduced, resembling the initial values of daily irrigated plants (C2). After suspension of irrigation (WD), protein levels were reduced, however, the highest values were found in the mutant genotypes, with gib1 presented statistical difference when compared to WT (Supplementary Figure S4D). The nitrate content in the leaves

of the four genotypes used showed a drastic increase when the plants were undergoing water stress (Supplementary Figure S4 E). There was a slight increase in nitrate levels in the mutants relative to WT. After recovery, the genotypes slowly returned to nitrate levels as in C2.

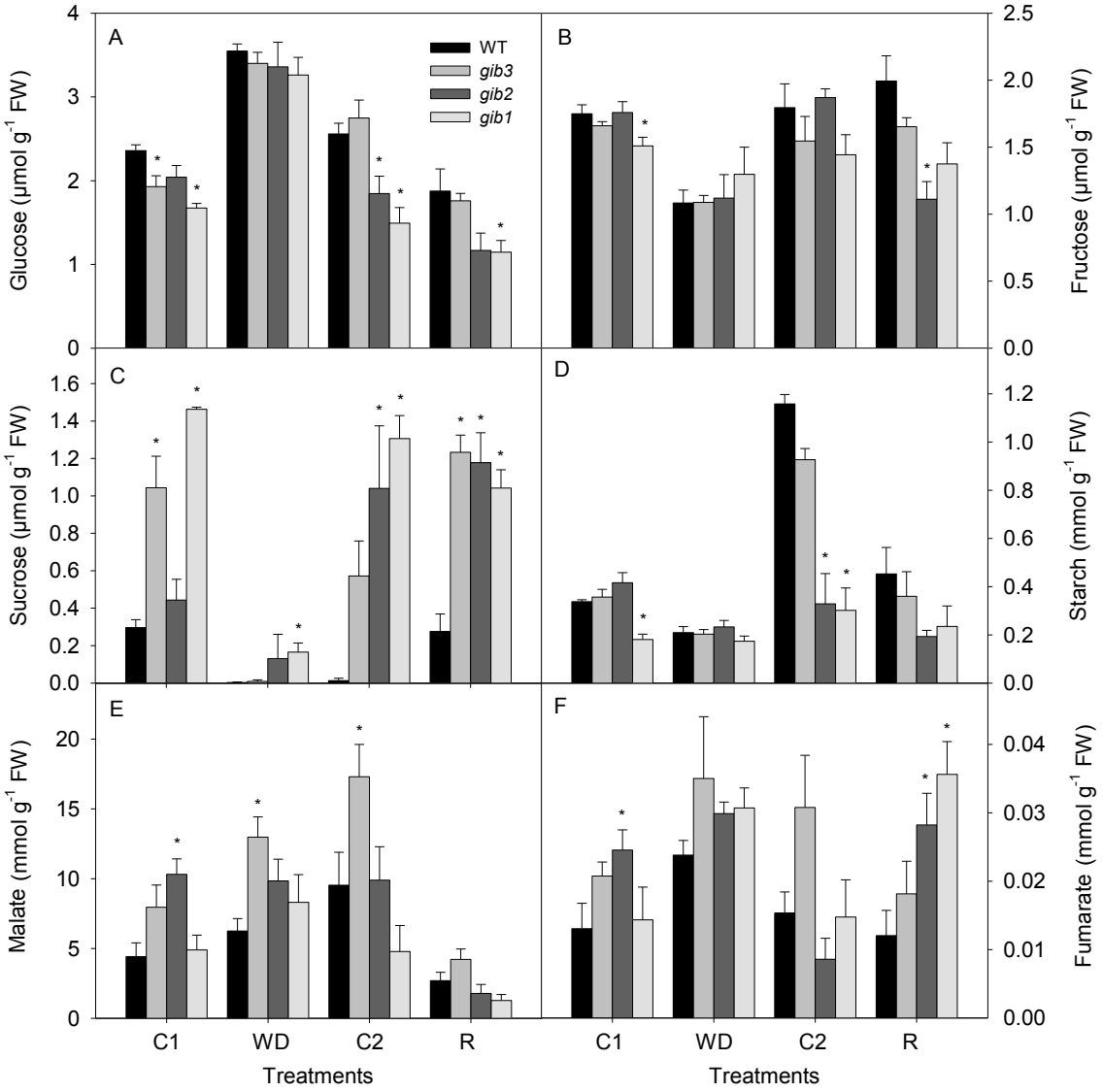


Figure 6. Changes in metabolite content involved in metabolism of carbon in roots of tomato plants (Solanum lycopersicum cv. Moneymaker) with different levels of endogenous GAs exposed to water deficit and after recovery. (A) Glucose; (B) Fructose; (C) Sucrose; (D) Starch; (E) Malate and; (F) Fumarate. Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing to WT.

Through of the nitrate and amino acids ratios, nitrate and proteins and nitrate and total chlorophylls, we can verify the direction of the assimilated nitrate that was absorbed and transported to the leaves (Supplementary figure S4 F, G and H). In general, the assimilation of nitrate in amino acids of leaf mutants showed increments

after recovery from stress. It was observed that the nitrate in leaves of mutant plants is mainly assimilated into chlorophylls under water deficit and after recovery.

It was observed that mutants in GA biosynthesis showed high total amino acid contents in roots in all applied treatments, with gib1 differed significantly from WT in C1 and WD treatment (Supplementary Figure S5 A). Mutant plants and WT increased the amino acid content in roots exposed to the water limitation condition. The total proteins accumulation in roots was directly proportional to the GA content of mutant plants submitted to water deficit (Supplementary Figure S5 B). The gib2 and gib1 mutants had less protein than WT in WD.

The roots nitrate content of the plants submitted to water deficit was increased, mainly in gib2 and gib1 (Supplementary Figure S5 C). While they were re-watering, nitrates levels reduced to values close to the initial ones (in C1), however the mutant genotypes presented larger values and significant when compared to the WT. In roots, nitrate was assimilated into proteins and amino acids mainly in the gib2 and gib1 genotypes in the water absence (Supplementary Figure S5 D and E). After re-watering, nitrate assimilation into proteins and amino acids remained high in WT and mutant genotypes when compared to C2 treatment.

The metabolites content are altered according to the genotype and the treatments

The metabolic profile of WT and mutants plants with reduced GAs levels in leaves presented a different pattern when compared to roots (Figure 11). In a large part of the analyzed metabolites, the behavior pattern of WT and gib3 plants was similar, while the genotypes gib2 and gib1 presented a more similar behavior regardless of the treatment applied and the analyzed organ (Figure 11).

The amino acids content in leaves varied in plants under water deficit and after recovery of the photosynthetic parameters in relation to control conditions (Figure 7A). Under water deficit, reduction in the levels of the amino acids serine, threonine and glutamate were observed, whereas increases in alanine, asparagine, glutamine, glycine, hydroxyproline, isoleucine, phenylalanine, lysine, proline, tyramine, tryptophan, valine and β-alanine. After re-watering, there was an increase in asparagine, glycine, hydroxyproline, proline and tyramine and reduction in the arginine, glutamate, phenylalanine, and methionine content. In leaves, in all treatments, 2-oxoglutarate was

reduced in the mutants, being the most drastic reduction in C1 and R and less drastic in WD in the gib2 and gib1 plants, differing statistically to WT. Dehydroascorbate also showed the same reduction pattern, whose tree mutants were significantly reduced in comparison to WT in all treatments. Aspartate also decreased in mutants in WD and R. In mutants exposed to WD, maltose, raminose and turanose increased, while fucose, glycoheptose and glucose-6-phosphate present reductions in mutants. In R, glycoheptose, glucose-6-phosphate, lixose/xylose, trehalose and turanose reduced in the three mutants, while gentioiose had a slight and significant increase when compared to WT. In addition, other metabolites were highlighted with increases in WD, such as caffeic acid, galactinol, glycerol, glycerol-3-phosphate and phosphoric acid. The glyceric acid content was reduced in all genotype in response to the water deficit.

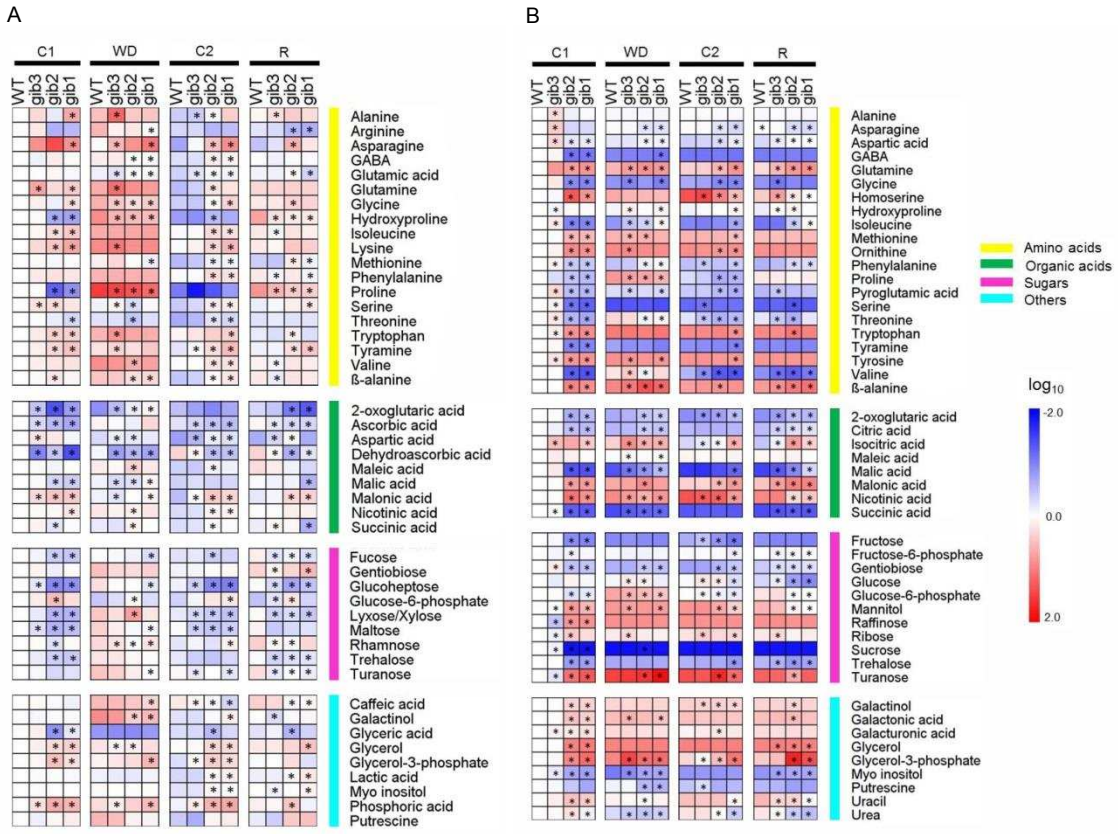


Figure 7. Heat map based on the metabolic profile of leaves (A) and roots (B) tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis, gib3, gib2 and gib1, under water deficit and after re-watering. Red indicates relative increase, and blue reduction, of metabolite content compared to WT of each treatment. Values are plotted as log on base 10 of six plants. Statistical analysis as described in Figure 1. Proline was quantified by a spectrophotometric methodology and the data were calculated by dry weight for leaves and roots. The other metabolites were determined by GC-MS and the data were calculated by dry weight for leaves and by fresh weight for roots.

In roots, the levels of glutamine, homoserine, methionine, ornithine, tryptophan, tyrosine and β-alanine were increased in the mutant genotypes and in the four treatments, except proline that increased in WD and R and valine which increased in WD (Figure 7B). Amino acids such as asparagine, aspartate, GABA, glycine, isoleucine, phenylalanine, serine and tyramine have reductions,. Threonine and valine increased in the mutants without WD and reduced in R. GABA, glycine, isoleucine and serine showed the stronger reductions, even though they were not significant in some treatments. Under water deficit and after recovery the organic acids isocitrate and nicotinate showed significant increases in the mutants plants, whereas 2-oxoglutarate, citrate, and succinate were significantly reduced. In gib2 and gib1 plants exposed to WD treatment, the gentiobiose and sucrose were reduced in comparison to WT. The mutants presented significant reductions in the levels of these same sugars in the R treatment, which fructose-6-phosphate, glucose and trehalose were also decreased in this treatment. Conversely, the sugars which showed increments in WD of the mutants were mannitol and turanose. In R, the increase pattern was also maintained for the mutants in that cited sugars. The genotypes gib3, gib2 and gib1, had an increase in galactonate and glycerol-3-phosphate, and a reduction in myo-inositol, putrescine and urea contents, due to water deficit and after recovery from stress.

Multivariate analysis

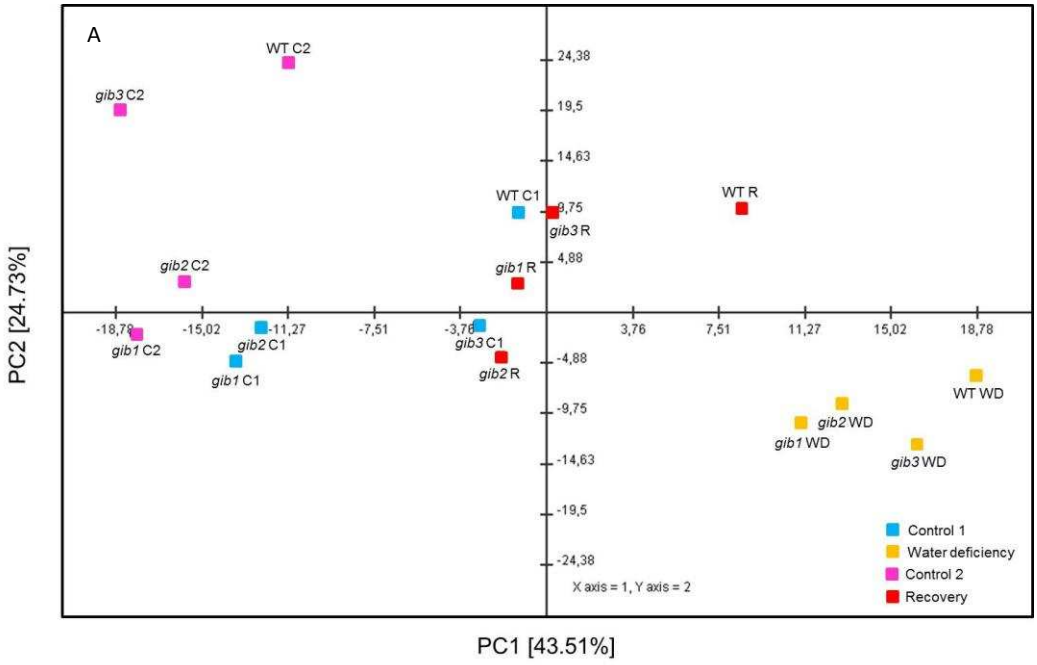
Principal component analysis (PCA) showed a separation of the genotypes submitted to water deficit and subsequent re-watering, based on the metabolic profile of leaves and roots (Figure 8). It is possible to observe a grouping of all genotypes when submitted to each treatment, mainly when submitted to water deficit in both leaves and roots (Figure 8A and C). In addition, in the C1, C2, and R treatments, the separation was influenced by both the genotype and the treatment applied in both organs. In leaves and roots, genotypes WT and gib3 present similarities in the quantified metabolites levels since they presented clusters in each one of the treatments. Similarly, gib2 and gib1 were also grouped in C1, C2 and R treatment in leaves and roots, presenting greater similarities in metabolite levels when compared to WT and gib3 together.

When analyzing the PCA with the four genotypes under water deficit only, a clear separation between WT and gib3 plants of gib2 and gib1 plants was observed in leaves and roots (Figure 8B and D). Such separation was not possible to be observed when PCA was constituted of the other treatments worked.

The PCA of leaves made with all treatments, the component one (PC1) explained 43.51% of the genotype separation according to the treatment applied (Figure 8A). Analyzing the second principal component (PC2), which explained 24.73% of the variation, the separation was clearer for plants of all genotypes exposed to water deficit. The individual contribution of the variables was similar, with some metabolites and their eigenvectors that contributed to the separation of each major component in descending order: proline (5.1861), hydroxyproline (2,8237), glycoheptose (1.7829), lixose/xylose (1.4925), arginine (1.2178), glutamine (1.1235), maltose (1.0702) and ascorbate (1.0534) for PC1. In addition, asparagine (-3.5510), dehydroascorbate (2.6612), proline (-2.2803), glutamine (-2.0313), alanine (-1.8858), lysine (-1.7667), putrescine (-1.6048), tryptophan (-1.4075), were the ones that contributed the most to separation and clustering in PC2.

In PCA of leaves showing the genotypes grouping in WD treatment, PC1 explained 54.50% of the separation and PC2 explained 20.33% (Figure 8B). The individual contribution of each variable was: asparagine (3.3349), 2-oxoglutaric acid (3,1463), dehydroascorbic acid (-1.5086), arginine (-1.4187), putrescine (-1.3510), galactinol (-1.2551), turanose (-1.2524), maltose (-1.2466) for PC1. Alanine (-3.8463), dehydroascorbic acid (2.5208), glutamine (-2.1092), arginine (1.5034), methionine (-1.3944), lyxose/xylose (1.2391), tryptophan (-1.1670), phosphoric acid (1.1118) were the ones that contributed most to PC2 separation.

When analyzing PCA of roots with all treatments, PC1 explained 60.13% of the separation, and WT, gib3, gib2 and gib1 under water deficit and gib2 and gib1 after re-watering showed similarities in the quantified metabolites content (Figure 8C). The PC2 explained only 17.31% of the separation, with the WT and gib3 genotypes in the control condition being less similar to the other genotypes in the other treatments in relation to the metabolites analyzed. It is possible to highlight some metabolites and their eigenvectors that contributed individually to PC1. They are: sucrose (-2.3957), turanose (1.9112), malic acid (-1.8146), succinic acid (-1.7138), serine (-1.6433), raffinose (1.4592), GABA (-1.4254), glycine (-1.3198). Those who contributed individually to PC2 were: glycerol-3-phosphate (-2.7819), proline (-2.3764), homoserine (2.0635), isoleucine (-2.0453), isocitric acid (-1.9952), valine (-1.889), malic acid (-1.8438), urea (1.6973).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3,76
7,51
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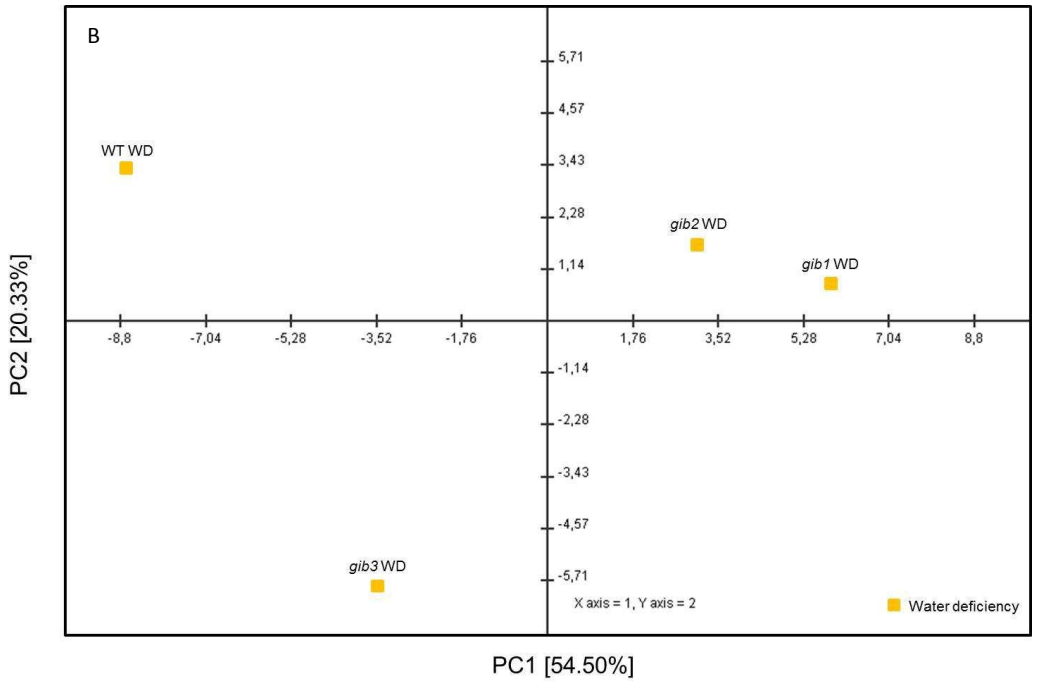
Control 1
Water deficiency
Control 2
Recovery
PC2 [24.73%]
PC1 [43.51%]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7,04
8,8
X axis = 1, Y axis = 2
Water deficiency
PC2 [20.33%]
PC1 [54.50%]

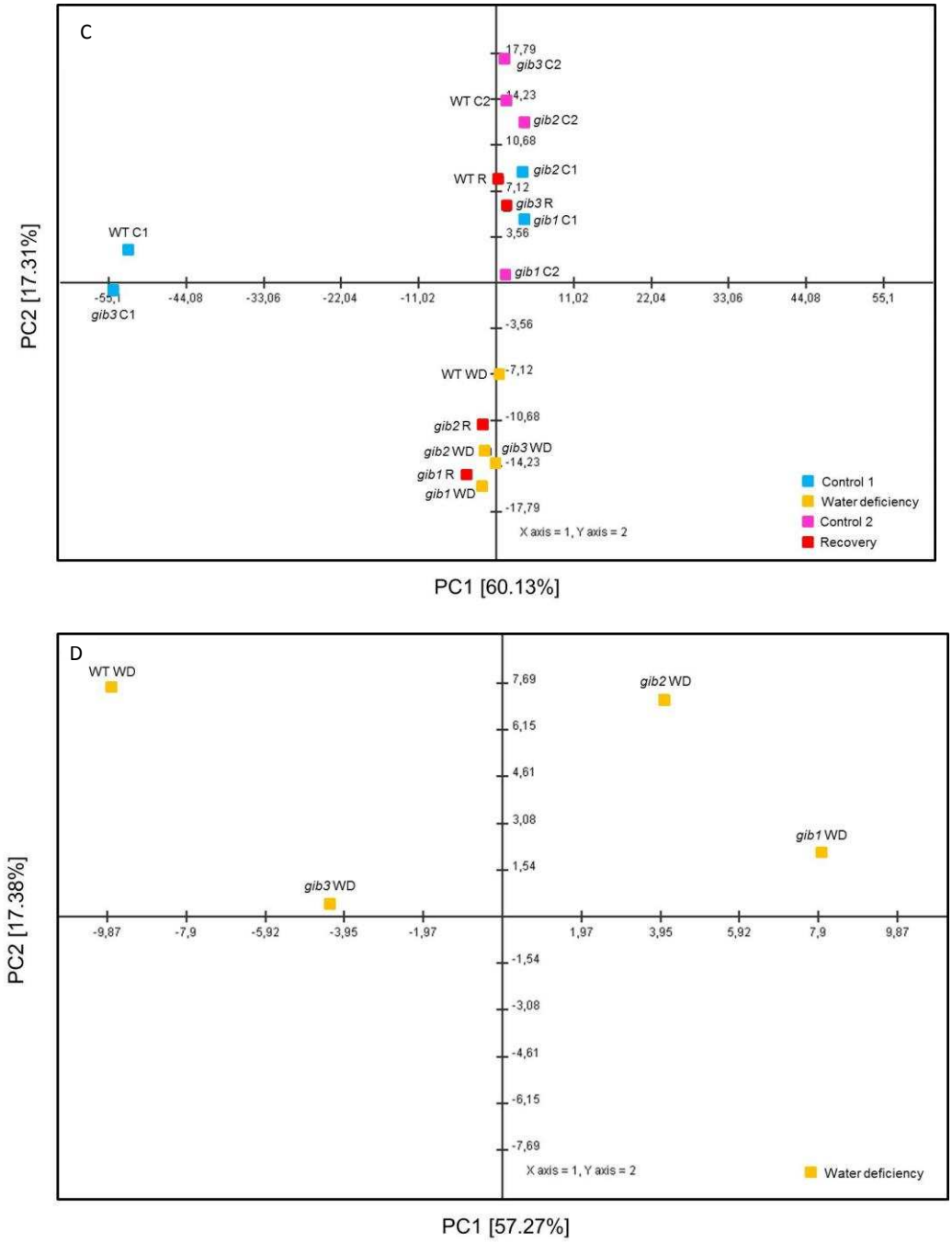


Figure 8. Principal components analyses (PCA) of leaves and roots from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis, gib3, gib2 and gib1, under water deficit and after re-watering, based on the metabolic profile. Leaves PCA under all treatments (A) and only water deficit (B); roots PCA under all treatments (C) and only water deficit (D).

In PCA of roots showing the genotypes grouping in WD treatment, PC1 explained 57.27% of the separation and PC2 explained 17.38% (Figure 8D). The individual contribution of each variable was: isoleucine (3.2804), malic acid (3.1749), putrescine (-2.3601), phenylalanine (2.0058), myo inositol (1.6032), threonine (-1.444), β-alanine (1.4341), turanose (1.4032) for PC1. Putrescine (-4.1516), valine (-3.5000),

threonine (-2.7295), malic acid (-2.0972), isoleucine (-1.8261), myo inositol (-1.7848), phenylalanine (-1.6614), hydroxyproline (-1,6064) were the major contributors to PC2 separation.

Oxidative and cellular damage indicators and redox state of leaves

In leaves, the mutant plants naturally present less activity of the catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD), which act as a front line in the ROS removal, and these responses were even more contrasting under WD in gib2 and gib1 (Supplementary Figure S5 A, B and C). The lipid peroxidation, caused by reactive oxygen species (ROS), during stress can be estimated by the malonaldehyde (MDA) concentration and for this purpose this metabolite was quantified in leaves (Supplementary Figure S5 D). The MDA concentration among the genotypes was similar under optimum conditions for cultivation (C1 and C2) and after re-watering, indicating that there is no damage to the cell membrane. However, when the plants were submitted to water deficit, genotypes WT and gib3 presented high MDA concentrations whereas in gib2 and gib1 the lowest concentrations were observed.

Leaf and root redox status was determined by quantification of NAD (P) (H) levels (Supplementary Figure S6). The NAD+/NADH ratios were estimated, there was little variation among the genotypes under the control conditions (Supplementary Figure S6 C). Mutant plants that passed through a water deficit period and even those that were re-watering later, presented a small ratio differing significantly from WT, except for gib3 under water deficit. In the case of phosphate nucleotides, the $NADP^+$/NADPH ratio was altered and, in general, mutant plants showed a lower ratio indicating that the reduced form prevailed under oxidized form when compared to WT (Supplementary Figure S6 F).

Discussion

Mutants with reduced GA content presented higher turgidity under drought even with high g_s and loss of leaf water

In this study, plants with low endogenous GAs levels (mainly gib2 and gib1) under water deficit presented a higher water retention capacity than wild (WT). It was found that gib2 and gib1 required a greater number of days to reach Ψ_w of -1.3 MPa under water deficit (Figure 1D). Moreover, it was found that despite the Ψ_w reduction in these plants affected the A_N. It was also observed that gib3, gib2 and gib1 plants presented high photosynthetic rates after the re-watering, whose plants presented a reduced time for the total recovery of A_N (Figure 4A). Further, they showed to be able of maintaining water in the leaf longer than WT plants when soil water is scarce. This fact was favored by the drastic reductions in g_S and E observed in the mutant plants under water deficit (Figure 4C and Supplementary Figure S3 A). Similar results were also observed in experiments with tomato plants overexpressing GAMT1 from Arabidopsis, which encodes the GA inactivation enzyme, named GA 2-oxidase (Nir et al., 2014). This work suggests that the low GAs levels induce a greater tolerance to water deficit due to the ability to keep the leaf tissues hydrated for longer, having lower E and thus using the soil available water slower (Nir et al., 2014).

Although all plants had low Ψ_w (-1.3 MPa), wilt symptoms were not observed in gib1 and less pronounced in gib2 (Figure 1A). Interestingly, these two genotypes showed the lowest reductions in g_s and E compared to WT under water deficit conditions, which together with the lower A_N reduction led to a higher water use efficiency (WUE; Figure 4C and Supplementary Figure S3 A and B). In addition, our data on water loss in detached leaves surprisingly showed increased water losses in the mutant plants. It should be remembered that unlike the observed by Nir et al. (2013), leaves of genotypes gib2 and gib1 present higher stomatal density and stomatal pore opening (Martins, 2013). The high stomata number found, as well as the greater stomatal pores opening, can explain the observed results of high g_s, E and leaf water loss in plants with lower endogenous GA levels contributing to lower WUE (Figure 4). It should be noted that other studies with tomato plants have also demonstrated effects of low GA levels on stomatal density. Tomato plants overexpressing the gene encoding the dehydration response binding protein (SlDREB), have been found to have lower GA levels due to inhibition of the ent-cauren synthase (CPS) and GA 20-oxidase (GA20ox).

These plants presented lower stomatal density, greater drought resistance, together with less leaf expansion and internodes elongation (Li et al., 2012).

To explain the fact that gib2 and gib1 plants reached Ψw of -1.3 MPa later than WT and gib3 plants we evaluate the water loss in detached leaves after incubation in ABA solution (Figure 2). We suggest that this greater water loss of the mutant plants would be related to the loss ABA signaling after the removal of leaf from plant for analysis of water loss in detached leaves. The high ABA concentrations in leaves causes the stomatal closure and consequently reduce the stomatal conductance (g_S) (Seki et al., 2007). However, the mutant plants had the highest water losses compared to WT at low and high ABA concentrations, 10 and 50 μM, respectively. To complement the above analysis and to exclude the ABA influence on the reduction of expected fresh mass loss for the mutant genotypes, a further experiment was conducted to verify the plants sensitivity to ABA (Brodribb e McAdam, 2011). In this case, the influence of ABA on the decrease of g_S was verified by performing ABA response curves (Figure 2). A stomatal closure delay of the mutant plants at the test start was found. Therefore, with the analyzes of water loss and ABA response curve, it can be concluded that mutant plants probably have a lower ABA sensitivity than WT, explaining in part the higher water loss in detached leaves, the higher values of gs and E, but without explaining the greater turgidity.

It was found in gib2 and gib1, greater isotopic discrimination of ^{13}C (value of $\delta^{13}C$ plus negative) in both control (C1) and WD conditions (Figure 4F). Less negative values of $\delta^{13}C$ may arise due to reductions in g_S or high CO_2 assimilation, leading to high long term water use efficiency (WUE) (Farquhar et al., 1989). In a situation of stress and low CO_2 input by stomata, plants fix the isotope ^{12}C and also the ^{13}C as a way to compensate for the lower CO_2 input. This lower discrimination of mutants under stress may also explain the lower reductions in A_N observed here (Figure 4B) and in previously (Meinzer et al., 1990; Pinheiro et al., 2005).

We suggest that the greater turgidity and greater days number to reach Ψ_w -1.3 MPa in the gib2 e gib1 genotypes may be related to plant architecture and leaf morphology, which have been characterized by dwarfism, shrivelling and thickening of leaves (Koornneef et al., 1990) which may hinder water loss and wilt development. Additionally, our specific leaf area results corroborate this hypothesis since the gib2 and gib1 plants have smaller leaves and larger thicknesses compared to WT.

Another possible explanation for this wilt phenotype would be that gib2 and gib1 plants because they have high active osmolytes levels are able to maintain leaf turgescence for a longer time period, as has been observed in other species (Medeiros et al., 2012); Medeiros et al., 2013). Proline and glycine betaine have an important role as compatible osmolytes (Noctor et al., 2011) and are the main amino acids found in high concentrations in plants exposed to water deficit (Nakamura et al., 2001; Ashraf and Foolad, 2007; Arivalagan and Somasundaram, 2016). Glycine-batatin was found in trace concentrations in leaves and roots without distinction between treatments (Data not shown). This is because tomato plants naturally do not accumulate glycine-betaine as well as potato, rice and Arabidopsis plants (Wyn Jones and Storey, 1981; Park et al., 2004).

In drought conditions, plants need to re-establish their cellular turgor and one of the most efficient strategies is to synthesize osmotically active organic compounds that accumulate as a result of reprogrammed metabolism (Soudry et al., 2005). One of the best known osmoprotectants is proline, which can play several roles in stress tolerance. In addition to maintaining stable osmotic balance, it can protect membrane enzymes and lipids from degradation, act as a free radical scavenger in the antioxidative defense process, and provide a energy source for post-stress recovery mechanisms (Hare et al., 1999; Hong et al., 2000; Ashraf and Foolad, 2007). Proline was highly accumulated in roots and mainly in leaves of mutant plants in the GAs biosynthesis submitted to water deficit (Figure 7). Our results indicate that mutant plants reprogrammed their metabolism by promoting the osmotic adjustment of the cells as a way of tolerating the imposed stress. In many plants, proline accumulation can be considered an indicator of stress tolerance (Hajlaoui et al., 2010) and can still be positively correlated with drought tolerance (Reddy et al., 2004).

Phytohormones regulate proline production and also stress tolerance. It is, thus, expected that there is a close relationship between phytohormones and proline metabolism (Per et al., 2017). In several instances GA has been found to exhibit positive relationship with proline accumulation. Foliar application of GA_3 on maize was found to counteract the adverse effects of salt stress through increased proline and macro and micronutrients accumulation, and also in maintaining the cell membrane permeability (Tuna et al., 2008). There are few studies that relate the GA content to proline accumulation in plants under water deficit. Here, we suggest that lower concentrations

of GAs may also favor the proline accumulation in plants and promote tolerance to water stress.

In addition, amino acids and nitrate were also highly accumulated in leaves and roots of mutants plants (Supplementary Figure S4 and S5). We suggest that the nitrate absorbed by roots or the one transported to leaves were assimilated mainly for the production of proteins and these were converted to amino acids later, among them the proline was present in high concentrations, to promote the osmotic adjustment and to tolerate the drought. Transgenic Arabidopsis plants overexpressing the SlDREB1 gene exhibited increases in proline and soluble sugars and markedly decreased MDA content in response to water stress (Jiang et al., 2017). These authors have suggested that gene overexpression leads to favorable physiological changes involving osmotic adjustment promoting tolerance (Jiang et al., 2017). Transgenic rice lines GA deficient due to ectopic expression of GA2ox6 had a high accumulation of proline under water deficit, being one of the factors responsible for the greater tolerance of these plants (Lo et al., 2016). It should be noted that proline accumulation may also explain the lower contents of MDA found, the maintenance of turgidity and non-visualization of wilt of gib2 and gib1 plants. In addition, proline levels did not return to basal levels after plant recovery in both leaves and roots. This suggests that such plants may be more tolerant when subjected to a second cycle of severe water stress.

Mutant plants in GA biosynthesis suffer less from water stress

Many studies have reported the effects of drought on the expression and activity of antioxidant system enzymes such as CAT, APX and SOD (Gill and Tuteja, 2010; Noctor et al., 2014). CAT and APX were the main enzymes involved in the hydrogen peroxide (H_2O_2) removal (Noctor et al., 2014) and SOD operating in the conversion of superoxide ($O_2^{\bullet-}$) to H_2O_2 (Gill and Tuteja, 2010). The lower activities of the CAT, APX and SOD enzymes found in mutant plants may indicate that they are suffering less from applied stress than WT plants, producing fewer ROS, especially under water deficit (Supplementary Figure S5 A, B and C). A evidence for these mechanism was the lower lipid peroxidation due to the lower MDA content observed in the gib2 and gib1 mutants during the water deficit period (Supplementary Figure S6 D) suggesting that oxidative damages did not occur or occurred at low levels due to low endogenous GA content. In many cases, MDA is the most abundant product of lipid aldehyde breakdown (Esterbauer and Cheeseman, 1990) and participates in the deterioration of several

functions by attacking biomolecules such as proteins and nucleic acids (Yamauchi et al., 2008).

Studies show that DELLA proteins were not degraded in adverse environments, resulting in lower GA levels and, therefore, decreased growth and increased plant survival (Achard et al., 2006; Achard et al., 2008). GA deficit or inhibition of GA signaling in Arabidopsis suppresses the accumulation of ROS under salinity or mannitol-induced stresses delaying cell death and increasing plant tolerance to stress (Achard et al., 2008). In our work, probably DELLA proteins are not responsible for the smallest CAT, APX and SOD activities found in the genotypes gib2 and gib1 (Supplementary Figure S6). Conversely, maize plants treated with exogenous GAs, with high GAs levels, reduced the activity of SOD, peroxidases and polyphenol oxidase contributing to tolerance to osmotic stress (Tuna et al., 2008).

The F_v/F_m ratio is used as an indicator of photosynthetic capacity in plants, and has become an important physiological feature in studies related to various types of stress (Krause and Weis, 1991). If the plant is in optimum conditions, in most species, the value of this variable can vary from 0.75 to 0.85. The values lower than one indicate compromises in the maximum quantum efficiency of PSII and, consequently, in the photosynthetic potential of the plant (Maxwell and Johnson, 2000). When plants under go stress as drought (Ögren and Öquist, 1985), the decline in F_v/F_m is a good indicator of damage in the photochemical apparatus of plants. Therefore, it is believed that these WT and mutant plants in the GAs biosynthesis were not suffering drastically with applied stress, indicating efficiency in the use of radiation without damage to the photosynthetic biochemical apparatus, specifically to PSII.

Altered biomass partitioning is important to the higher tolerance to water deficit in gib2 and gib1 plants

The dwarf phenotype (Figure 1) observed in gib2 and gib1 has also been observed in genotypes of other species (Fujioka et al., 1988; Magome et al., 2004; Sakamoto et al., 2004; Nir et al., 2013) and may be advantageous in adverse environments (Magome et al., 2004; Li et al., 2012). Some studies have suggested that the reduction of active GA levels promotes water stress tolerance indirectly through suppression of growth (Magome et al., 2004; Achard et al., 2006; Shan et al., 2007; Wang et al., 2012). When plants are submitted to water stress, the gas exchange

between the leaf and the atmosphere usually decreases due to reduction in g_S and hydraulic conductance causing less assimilation of atmospheric CO_2 (Silva et al., 2013). It is believed that the lower growth may lead to the redirection of growth promotion resources to support the mechanisms that promote survival (Achard et al., 2006). Thus, plants with lower growth may be less vulnerable to stress (Achard et al., 2006). Although the nature of these mechanisms remains unknown, it is believed that the GAs levels may be related to growth control as a function of the environmental condition, thus being related to survival in adverse conditions (Achard et al., 2006).

Water deficit alters the root/shoot ratio due to rapid inhibition of shoot growth and maintenance of root growth (Sharp, 2002). On the other hand, water stress may not only contribute to the maintenance of root growth but may also increase root growth (Leach et al., 2011; Saucedo et al., 2012). The highest values of root/shoot ratio in gib2 and gib1 plants in all applied treatments indicate higher biomass partitioning in roots, followed by leaves and then stems (Figure 3G and H), a fact that may explain the longer time required for observation of wilt in these plants when compared to WT and gib3. Thus, these results suggest that the mutants have a larger root area to absorb soil water and a smaller leaf area, thus reducing water loss through transpiration. Drought tolerance has been associated with slower use of water by plants due to reduced leaf area and E (Lawlor, 2013). The absolute values of the root/shoot ratio increased especially in the WD treatment and also in the R when compared to the control condition of each one (Figure 3G). Corroborating with the results found in the literature, this shows that the plants have a greater investment in roots under water scarcity (Zawaski and Busov, 2014) with the purpose of increasing the area of water absorption and also the roots elongation in search of water in deeper soils.

In a study with mutants GA deficient it was found that some genes encoding GA2ox are expressed in different parts of the plant under drought conditions or short days, with the GA2ox3 gene expressed in the apical meristem (SAM) and GA2ox7 gene in roots, inducing the production of lateral roots (Zawaski and Busov, 2014). GA deficient mutants Populus diverted resources from the larger A_N to a greater investment in roots (Zawaski and Busov, 2014). The authors also report that the resistance mechanisms to drought were altered due to the rapid perception of the water deficit. In the present work, a higher biomass partitioning was observed in roots of the gib2 and gib1 genotypes (Figure 3 and 4), with an increase in A_N rates (Figure 4A and B) and

absence of damage in the same photosystems under water deficit conditions (Supplementary Figure S2). In addition, it was observed high rates in A_N and E in gib2 and gib1 plants, which may explain the higher root growth than shoot. It is also suggested that the greater accumulation of dry roots mass of gib3, gib2 and gib1, indicating a greater growth of the root system, can allow a greater volume of soil to be exploited and thus sustaining higher rates of transpiration and higher efficiency in water use (Figure 4D) (Elias et al., 2012). In addition, the carbon from A_N may be used to improve tolerance mechanisms protecting against cell damage due production of compatible solutes and elimination of ROS or for root growth, which would be advantageous for increased water uptake (Zawaski and Busov, 2014).

GAs modulate global changes in the primary metabolism of plants under stress

In this work, was observed a relationship between carbon metabolism and growth when mutant plants submitted to water deficit. In water deficit conditions, a higher RGR, starch and malate accumulation was observed in gib2 and gib1, and sucrose and fumarate levels were reduced in leaves, while sucrose, malate and mainly fumarate increased in roots (Figure 5C, D, E and F). We suggest that plants with reduced GAs levels, gib2 and gib1, did not show a drastic reduction in RGR-h also because they were suffering less with applied stress. Consequently, glucose, fructose and sucrose, and even malate, may have been used by mutants to promote height growth since there were reductions in leaf sugars when compared to WT under water deficit, even if not significant. Root growth of the mutants in WD probably was due to consumption of starch and sucrose (Figure 6). The accumulation of non-structural sugars, such as sucrose, is important in the response to water and salt stress acting in the growth maintenance and as osmoprotective molecules allowed the turgescence maintenance, cell membranes stability and preventing the proteins degradation (Juan et al., 2005; Arbona et al., 2013; Pérez-López et al., 2013; Wu et al., 2013; Almeida et al., 2014; Arbona et al., 2017).

In the mutants with the lowest GAs levels, the starch and malate accumulation in leaves may have been partially due to the conversion of sucrose and fumarate, respectively. We know that starch is the end product of photosynthesis and the predominant carbohydrate reserve in many species is important for plant development (Gibon et al., 2004; Stitt and Zeeman, 2012). Starch is accumulated during the day and degraded overnight to promote growth in the absence of photosynthesis under optimum

conditions for plant growth (Wiese et al., 2007). Therefore, starch and malate, as carbon sources for the cell, may have been accumulated under water deficit as a survival strategy, in other words, to be used when conditions are again favorable.

The malate accumulation in leaves of gib2 and gib1 in WD treatment, corresponding to an increase of approximately 37% in plants with lower GA content when compared to WT (Figure 5E). Malate may serve as an alternative carbon source and may be metabolized to promote growth (Martinoia and Rentsch, 1994). Otherwise, malate can be transported to the guiding cells contributing to the stomatal opening by decreasing the osmotic and water potential and thus being an osmoregulatory molecule (Nunes-Nesi et al., 2007; Fernie and Martinoia, 2009; Araújo et al., 2011) with probable action also in other cells promoting the osmotic adjustment. Malate accumulated in gib2 and gib1 probably provided the highest g_S observed and served as an osmoregulatory molecule contributing to water status maintenance and as a source of carbon used in plant growth after recovery.

They are important components of nitrogen transport and are responsible for the biosynthesis of several compounds, glutamate and glutamine (Masclaux-Daubresse et al., 2006). The enzyme aspartate aminotransferase converts glutamine and glutamate into aspartate, or asparagine synthase converts them into asparagine. The amino acids glutamine and asparagine have an extra nitrogen atom in the amide group of their side chains and therefore play an important role in cellular metabolism as nitrogen transporters (Urquhart and Joy, 1981). Our results showed accumulation of glutamine and asparagine in leaves and reduction in glutamate in mutant plants submitted to water deficit. At roots, glutamine increased while asparagine decreased in WD (Figure 7A). It is suggested that the nitrate absorbed in roots and found at high concentrations in the mutants was being converted to ammonium and fixed in glutamine under WD (Figure 7B and supplementary figure S5 C). It is likely that the enzyme asparagine synthase was not at high activity since high concentrations of glutamine and low asparagine were found in roots.

The favoritism of nitrogen metabolism and several amino acids accumulation in roots of mutant genotypes under stress and after recovery may have reduced TCA cycle activity, which would explain the reduction in four organic acids (citrate, 2-oxoglutarate, succinate and malate) of the five detected (Figure 7B). Increases in isocitrate in roots of mutant plants in the WD and R treatments may explain the lower values of 2-oxoglutarate, indicating that the mitochondrial isocitrate dehydrogenase

enzyme could be acting at low rates. The water deficit together with the lower GAs concentrations in the mutants may have modulated the flow of 2-oxoglutarate to the synthesis of amino acids, such as glutamine, responsible for nitrogen assimilation, as had been reported (Araújo et al., 2014). Glutamine may be associated with reduced succinate levels, a subsequent intermediate also produced by 2-oxoglutarate in the TCA cycle (Figure 7B). These results indicate changes in respiratory metabolism as a function of proline biosynthesis, produced from glutamate. Therefore, it may be suggested that in roots of mutants under water deficit, energy demand and osmotic adjustment play an important role in the maintenance of root growth.

For the aspartate synthesis is necessary besides the amino acid glutamate, a molecule of the organic acid oxaloacetate. 2-oxuglutarate is required for glutamate synthesis, both compounds reduced significantly in leaves mutants of WD and R. The nitrogen partition of glutamate for glutamine, proline and hydroxyproline for mutants leaves may have contributed to the maintenance of low GABA levels (Figure 7A). GABA is a direct co-substrate for the synthesis of alanine (Rocha et al., 2010) or for synthesis of succinate, and in the succinate case the accumulation can occur under biotic or abiotic stress (Fait et al., 2008). However, significant reductions in succinate in roots of the gib3, gib2 and gib1 under WD and R treatment indicate that 2-oxoglutarate was being diverted from the TCA cycle to glutamate synthesis and was impaired the supply of this metabolite through the conversion of GABA by succinate semialdehyde dehydrogenase. The alanine, glutamate and GABA accumulation in flooded Lotus japonicus roots was important for plant survival (Rocha et al., 2010). These amino acids were increased in Lotus japonicus WT and transgenic plants with low expression of three homologous genes coding for leghemoglobin in nodules under hypoxia (Rocha et al., 2010). Similar results were found in soybean roots, where accumulation of alanine, GABA and succinate under hypoxia was observed. These accumulations were explained by the alanine and GABA shunt metabolism (António et al., 2016). Changes in the alanine, glutamine and GABA levels were also observed in Arabidopsis when respiratory activity was artificially inhibited by rotenone, chemical inhibitor of the electron transport chain I complex (Garmier et al., 2008), or by a genetic approach leading to dysfunction of the I complex (Meyer et al., 2009). Therefore, induced changes in alanine metabolism are characteristic of an adaptive metabolic response to situations in which respiration is inhibited (Rocha et al., 2010). In addition, paclobutrazol-treated (GA biosynthesis inhibitor) Arabidopsis plants had reduced plant

growth and increased alanine levels (Ribeiro et al., 2012). It is possible that alanine may contribute to regulate pyruvate biosynthesis, facilitating the maintenance of carbon/nitrogen balance and plant respiration rate (Ribeiro et al., 2012).

Branched chain amino acids altered in mutant plants under water deficit and after re-watering (Figure 7). Isoleucine, valine and lysine increased in mutants leaves under WD and R, whose the highest values under WD. In roots, valine increased only in WD and isoleucine reduced in WD and R, whose the smallest reductions in gib2 and gib1. According to (Hildebrandt et al., 2015), carbon skeletons from amino acids are converted into precursors or intermediates from TCA cycle to support mitochondrial metabolism and ATP biosynthesis, while the oxidation of certain amino acids (such as leucine, Isoleucine, valine, and lysine) can directly supply electrons to the mitochondrial electron transport chain (Araújo et al., 2010).

Conclusions

In summary this study indicated that reduced endogenous levels of GAs lead to metabolic adjustment due to water deficit that promotes greater tolerance to this condition. Under water deficit, the low GAs content promoted tolerance for turgidity maintenance, lower shoot growth, higher roots biomass partitioning, accumulation of malate, nitrate, amino acids and mainly proline in leaves and roots. Together, these morphological and biochemical changes promoted tolerance to water deficit due the osmotic adjustment which avoided water loss and, together with higher root growth, promoted a better absorption and use of soil water with lower leaf desiccation due to the shoot architecture and leaf morphology due to the reduced endogenous levels of GAs. Studies on morphology and root architecture of these plants are still necessary to better understand the roots influence on tolerance to water deficit. In addition, the understanding of the interaction between shoot and root and the molecules transport at long distances in plants with reduced GAs levels can clarify some issues related to high root growth under stress.

Acknowledgments

This work was supported by funding from the Max Planck Society (to ANN and WLA) and the National Council for Scientific and Technological Development (CNPq-Brazil, Grant 306355/2012-4 to ANN and Grant 483525/2012-0 to WLA) and the FAPEMIG (Foundation for Research Assistance of the Minas Gerais State, Brazil,

Grant APQ-01357-14 to WLA). We also thank the scholarships granted by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES-Brazil) to RPOG. Research fellowships granted by CNPq-Brazil to ANN and WLA are also gratefully acknowledged.

Literature cited

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of Plant Responses to Environmentally Activated Phytohormonal Signals. Science **311:** 91-94

Achard P, Renou J-P, Berthomé R, Harberd NP, Genschik P (2008) Plant DELLAs Restrain Growth and Promote Survival of Adversity by Reducing the Levels of Reactive Oxygen Species. Current Biol **18:** 656-660

Ahuja I, de Vos RCH, Bones AM, Hall RD (2010) Plant molecular stress responses face climate change. Trends Plant Sci **15:** 664-674

Albacete A, Ghanem ME, Martínez-Andújar C, Acosta M, Sánchez-Bravo J, Martínez V, Lutts S, Dodd IC, Pérez-Alfocea F (2008) Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (Solanum lycopersicum L.) plants. J Exp Bot **59:** 4119-4131

Almeida P, Feron R, de Boer G-J, de Boer AH (2014) Role of Na+, K+, Cl−, proline and sucrose concentrations in determining salinity tolerance and their correlation with the expression of multiple genes in tomato. AoB Plants **6:** plu039

Antoni R, Rodriguez L, Gonzalez-Guzman M, Pizzio GA, Rodriguez PL (2011) News on ABA transport, protein degradation, and ABFs/WRKYs in ABA signaling. Current Opinion in Plant Biology **14:** 547-553

António C, Päpke C, Rocha M, Diab H, Limami AM, Obata T, Fernie AR, van Dongen JT (2016) Regulation of Primary Metabolism in Response to Low Oxygen Availability as Revealed by Carbon and Nitrogen Isotope Redistribution. Plant Physiology **170:** 43-56

Araújo WL, Ishizaki K, Nunes-Nesi A, Larson TR, Tohge T, Krahnert I, Witt S, Obata T, Schauer N, Graham IA (2010) Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. The Plant Cell **22:** 1549-1563

Araújo WL, Martins AO, Fernie AR, Tohge T (2014) 2-Oxoglutarate: linking TCA cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis. Frontiers in plant science **5**

Araújo WL, Nunes-Nesi A, Osorio S, Usadel B, Fuentes D, Nagy R, Balbo I, Lehmann M, Studart-Witkowski C, Tohge T, Martinoia E, Jordana X, DaMatta FM, Fernie AR (2011) Antisense Inhibition of the Iron-Sulphur Subunit of Succinate Dehydrogenase Enhances Photosynthesis and Growth in Tomato via an Organic Acid–Mediated Effect on Stomatal Aperture. Plant Cell **23:** 600-627

Arbona V, Manzi M, Ollas Cd, Gómez-Cadenas A (2013) Metabolomics as a tool to investigate abiotic stress tolerance in plants. International journal of molecular sciences **14:** 4885-4911

Arbona V, Manzi M, Zandalinas SI, Vives-Peris V, Pérez-Clemente RM, Gómez-Cadenas A (2017) Physiological, Metabolic, and Molecular Responses of Plants to Abiotic Stress. In M Sarwat, A Ahmad, MZ Abdin, MM Ibrahim, eds, Stress Signaling in Plants: Genomics and Proteomics Perspective, Volume 2. Springer International Publishing, Cham, pp 1-35

Arivalagan M, Somasundaram R (2016) Induction of drought stress tolerance by Propiconazole and Salicylic acid in Sorghum bicolor is mediated by enhanced osmoregulation, compatible solutes and biochemical accumulation. Journal of Applied and Advanced Research **1:** 41-52

Ashraf M, Foolad M (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany **59:** 206-216

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant and Soil **39:** 205-207

Begcy K, Mariano ED, Gentile A, Lembke CG, Zingaretti SM, Souza GM, Menossi M (2012) A Novel Stress-Induced Sugarcane Gene Confers Tolerance to Drought, Salt and Oxidative Stress in Transgenic Tobacco Plants. PLoS One **7:** e44697

Bensen RJ, Zeevaart JAD (1990) Comparison of Ent-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and gib-1, gib-2, and gib-3 tomato plants. J Plant Growth Regul **9:** 237-242

Bokhari SA, Wan X-Y, Yang Y-W, Zhou L, Tang W-L, Liu J-Y (2007) Proteomic Response of Rice Seedling Leaves to Elevated CO2 Levels. J Proteome Res **6:** 4624-4633

Brodribb TJ, McAdam SA (2011) Passive origins of stomatal control in vascular plants. Science **331:** 582-585

Chae L, Sudat S, Dudoit S, Zhu T, Luan S (2009) Diverse Transcriptional Programs Associated with Environmental Stress and Hormones in the Arabidopsis Receptor-Like Kinase Gene Family. Mol Plant **2:** 84-107

Charlton A, Donarski J, Harrison M, Jones S, Godward J, Oehlschlager S, Arques J, Ambrose M, Chinoy C, Mullineaux P, Domoney C (2008) Responses of the pea (Pisum sativum L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy. Metabolomics **4:** 312-327

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin Dose-Response Regulation of GA4 Gene Transcript Levels in Arabidopsis. Plant Physiol **117:** 1195-1203

Dubois M, Skirycz A, Claeys H, Maleux K, Dhondt S, De Bodt S, Vanden Bossche R, De Milde L, Yoshizumi T, Matsui M, Inzé D (2013) ETHYLENE RESPONSE FACTOR6 Acts as a Central Regulator of Leaf Growth under Water-Limiting Conditions in Arabidopsis. Plant Physiol **162:** 319-332

Elias AA, Busov VB, Kosola KR, Ma C, Etherington E, Shevchenko O, Gandhi H, Pearce DW, Rood SB, Strauss SH (2012) Green Revolution Trees: Semidwarfism Transgenes Modify Gibberellins, Promote Root Growth, Enhance Morphological Diversity, and Reduce Competitiveness in Hybrid Poplar. Plant Physiol **160:** 1130-1144

Esterbauer H, Cheeseman KH (1990) [42] Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. Methods in enzymology **186:** 407-421

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2008) Highway or byway: the metabolic role of the GABA shunt in plants. Trends in plant science **13:** 14-19

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annual review of plant biology **40:** 503-537

Fernie AR, Aharoni A, Willmitzer L, Stitt M, Tohge T, Kopka J, Carroll AJ, Saito K, Fraser PD, DeLuca V (2011) Recommendations for reporting metabolite data. The Plant Cell **23:** 2477-2482

Fernie AR, Martinoia E (2009) Malate. Jack of all trades or master of a few? Phytochem **70:** 828-832

Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ (2001) Fructose 2,6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and

increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta **212:** 250-263

Fu X, Harberd NP (2003) Auxin promotes Arabidopsis root growth by modulating gibberellin response. Nature **421:** 740-743

Fujioka S, Yamane H, Spray CR, Gaskin P, Macmillan J, Phinney BO, Takahashi N (1988) Qualitative and Quantitative Analyses of Gibberellins in Vegetative Shoots of Normal, dwarf-1, dwarf-2, dwarf-3, and dwarf-5 Seedlings of Zea mays L. Plant Physiol **88:** 1367-1372

Gao X-H, Xiao S-L, Yao Q-F, Wang Y-J, Fu X-D (2011) An Updated GA Signaling 'Relief of Repression' Regulatory Model. Mol Plant **4:** 601-606

Garmier M, Carroll AJ, Delannoy E, Vallet C, Day DA, Small ID, Millar AH (2008) Complex I dysfunction redirects cellular and mitochondrial metabolism in Arabidopsis. Plant physiology **148:** 1324-1341

Gibon Y, Blaesing OE, Hannemann J, Carillo P, Höhne M, Hendriks JHM, Palacios N, Cross J, Selbig J, Stitt M (2004) A Robot-Based Platform to Measure Multiple Enzyme Activities in Arabidopsis Using a Set of Cycling Assays: Comparison of Changes of Enzyme Activities and Transcript Levels during Diurnal Cycles and in Prolonged Darkness. The Plant Cell **16:** 3304-3325

Gibon Y, Bläsing OE, Palacios- Rojas N, Pankovic D, Hendriks JH, Fisahn J, Höhne M, Günther M, Stitt M (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post- translational activation of ADP- glucose pyrophosphorylase in the following light period. The Plant Journal **39:** 847-862

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48:** 909-930

Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran L-SP (2012) Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci **17:** 172-179

Hajlaoui H, El Ayeb N, Garrec JP, Denden M (2010) Differential effects of salt stress on osmotic adjustment and solutes allocation on the basis of root and leaf tissue senescence of two silage maize (Zea mays L.) varieties. Industrial crops and products **31:** 122-130

Hare P, Cress W, Van Staden J (1999) Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. Journal of experimental botany **50:** 413-434

Hedden P, Kamiya Y (1997) Gibberellin Biosynthesis: Enzymes, Genes and Their Regulation. Plant Physiol **48:** 431-460

Hedden P, Kamiya. Y (1997) GIBBERELLIN BIOSYNTHESIS: Enzymes, Genes and Their Regulation. Plant Physiology **48:** 431 - 460

Hedden P, Proebsting WM (1999) Genetic Analysis of Gibberellin Biosynthesis. Plant Physiol **119:** 365-370

Hildebrandt TM, Nesi AN, Araújo WL, Braun H-P (2015) Amino acid catabolism in plants. Molecular plant **8:** 1563-1579

Ho S-L, Huang L-F, Lu C-A, He S-L, Wang C-C, Yu S-P, Chen J, Yu S-M (2013) Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. Plant Mol Biol **81:** 347-361

Hong Z, Lakkineni K, Zhang Z, Verma DPS (2000) Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline

accumulation and protection of plants from osmotic stress. Plant physiology **122:** 1129-1136

Hu Y, Burucs Z, von Tucher S, Schmidhalter U (2007) Short-term effects of drought and salinity on mineral nutrient distribution along growing leaves of maize seedlings. Environ Exp Bot **60:** 268-275

Jaleel C, Gopi R, Manivannan P, Panneerselvam R (2007) Responses of antioxidant defense system of Catharanthus roseus (L.) G. Don. to paclobutrazol treatment under salinity. Acta Physiol Plant **29:** 205-209

Jiang L, Wang Y, Zhang S, He R, Li W, Han J, Cheng X (2017) Tomato SlDREB1 gene conferred the transcriptional activation of drought-induced gene and an enhanced tolerance of the transgenic Arabidopsis to drought stress. Plant Growth Regulation **81:** 131-145

Juan M, Rivero RM, Romero L, Ruiz JM (2005) Evaluation of some nutritional and biochemical indicators in selecting salt-resistant tomato cultivars. Environmental and Experimental Botany **54:** 193-201

Koornneef M, Bosma TDG, Hanhart CJ, Veen JH, Zeevaart JAD (1990) The isolation and characterization of gibberellin-deficient mutants in tomato. Theor Appl Genet **80:** 852-857

Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B, Bergmüller E, Dörmann P, Weckwerth W, Gibon Y, Stitt M, Willmitzer L, Fernie AR, Steinhauser D (2005) GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics **21:** 1635-1638

Krause G, Weis E (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annual review of plant biology **42:** 313-349

Lawlor DW (2013) Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. Journal of Experimental Botany **64:** 83-108

Leach KA, Hejlek LG, Hearne LB, Nguyen HT, Sharp RE, Davis GL (2011) Primary root elongation rate and abscisic acid levels of maize in response to water stress. Crop science **51:** 157-172

Li J, Sima W, Ouyang B, Wang T, Ziaf K, Luo Z, Liu L, Li H, Chen M, Huang Y, Feng Y, Hao Y, Ye Z (2012) Tomato SlDREB gene restricts leaf expansion and internode elongation by downregulating key genes for gibberellin biosynthesis. J Exp Bot **63:** 6407-6420

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nature Protocols **1:** 387-396

Lo SF, Ho THD, Liu YL, Jiang MJ, Hsieh KT, Chen KT, Yu LC, Lee MH, Chen Cy, Huang TP (2016) Ectopic expression of specific GA2 oxidase mutants promotes yield and stress tolerance in rice. Plant Biotechnology Journal

Loyola J, Verdugo I, González E, Casaretto JA, Ruiz-Lara S (2012) Plastidic isoprenoid biosynthesis in tomato: physiological and molecular analysis in genotypes resistant and sensitive to drought stress. Plant biology (Stuttgart, Germany) **14:** 149-156

Luedemann A, von Malotky L, Erban A, Kopka J (2012) TagFinder: Preprocessing Software for the Fingerprinting and the Profiling of Gas Chromatography–Mass Spectrometry Based Metabolome Analyses. In NW Hardy, RD Hall, eds, Plant Metabolomics: Methods and Protocols. Humana Press, Totowa, NJ, pp 255-286

Maggio A, Barbieri G, Raimondi G, De Pascale S (2010) Contrasting Effects of GA3 Treatments on Tomato Plants Exposed to Increasing Salinity. Journal of Plant Growth Regulation **29:** 63-72

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2004) dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. Plant J **37:** 720-729

Martinoia E, Rentsch D (1994) Malate Compartmentation-Responses to a Complex Metabolism. Ann Rev Plant Physiol Plant Mol Biol **45:** 447-467

Martins, AO (2013) Impactos ecofisiológicos e metabólicos da alteração nos níveis de giberelina em tomate. Dissertação. Universidade Federal de Viçosa.

Masclaux-Daubresse C, Reisdorf-Cren M, Pageau K, Lelandais M, Grandjean O, Kronenberger J, Valadier M-H, Feraud M, Jouglet T, Suzuki A (2006) Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. Plant Physiology **140:** 444-456

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. Journal of experimental botany **51:** 659-668

Medeiros DB, Silva ECd, Santos HRB, Pacheco CM, Musser RdS, Nogueira RJMC (2012) Physiological and biochemical responses to drought stress in Barbados cherry. Braz J Plant Physiol **24:** 181-192

Meinzer FC, Goldstein G, Grantz DA (1990) Carbon isotope discrimination in coffee genotypes grown under limited water supply. Plant Physiology **92:** 130-135

Meyer EH, Tomaz T, Carroll AJ, Estavillo G, Delannoy E, Tanz SK, Small ID, Pogson BJ, Millar AH (2009) Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses Arabidopsis germination and growth and alters control of metabolism at night. Plant physiology **151:** 603-619

Mielke MS, Hoffmann A, Endres L, Fachinello JC (1995) Comparison of laboratory and field methods for the estimation of leaf area of wild fruit species. Sci Agri **52:** 82-88

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol **167:** 645-663

Nakamura T, Nomura M, Mori H, Jagendorf AT, Ueda A, Takabe T (2001) An isozyme of betaine aldehyde dehydrogenase in barley. Plant and Cell Physiology **42:** 1088-1092

Ning J, Li X, Hicks LM, Xiong L (2010) A Raf-Like MAPKKK Gene DSM1 Mediates Drought Resistance through Reactive Oxygen Species Scavenging in Rice. Plant Physiol **152:** 876-890

Nir I, Moshelion M, Weiss D (2014) The Arabidopsis GIBBERELLIN METHYL TRANSFERASE 1 suppresses gibberellin activity, reduces whole- plant transpiration and promotes drought tolerance in transgenic tomato. Plant, cell & environment **37:** 113-123

Noctor G, Mhamdi A, Foyer CH (2014) The Roles of Reactive Oxygen Metabolism in Drought: Not So Cut and Dried. Plant Physiology **164:** 1636-1648

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. The arabidopsis book **9:** 1-32

Nunes-Nesi A, Carrari F, Gibon Y, Sulpice R, Lytovchenko A, Fisahn J, Graham J, Ratcliffe RG, Sweetlove LJ, Fernie AR (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J **50:** 1093-1106

Ögren E, Öquist G (1985) Effects of drought on photosynthesis, chlorophyll fluorescence and photoinhibition susceptibility in intact willow leaves. Planta **166:** 380-388

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin Signaling: Biosynthesis, Catabolism, and Response Pathways. Plant Cell **14:** S61-S80

Park E-J, Jeknić Z, Sakamoto A, DeNoma J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. The Plant Journal **40:** 474-487

Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR, Anjum NA (2017) Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. Plant Physiology and Biochemistry

Pérez-López U, Miranda-Apodaca J, Mena-Petite A, Muñoz-Rueda A (2013) Barley growth and its underlying components are affected by elevated CO2 and salt concentration. Journal of plant growth regulation **32:** 732-744

Pinheiro HA, DaMATTA FM, Chaves AR, Loureiro ME, Ducatti C (2005) Drought tolerance is associated with rooting depth and stomatal control of water use in clones of Coffea canephora. Annals of botany **96:** 101-108

Porra R, Thompson W, Kriedemann P (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta (BBA)-Bioenergetics **975:** 384-394

Ramírez V, Coego A, López A, Agorio A, Flors V, Vera P (2009) Drought tolerance in Arabidopsis is controlled by the OCP3 disease resistance regulator. Plant J **58:** 578-591

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of plant physiology **161:** 1189-1202

Ribeiro DM, Araújo WL, Fernie AR, Schippers JH, Mueller-Roeber B (2012) Translatome and metabolome effects triggered by gibberellins during rosette growth in Arabidopsis. Journal of experimental botany**:** err463

Ribeiro DM, Araújo WL, Fernie AR, Schippers JHM, Mueller-Roeber B (2012) Translatome and metabolome effects triggered by gibberellins during rosette growth in Arabidopsis. J Exp Bot **63:** 2769-2786

Richards DE, King KE, Ait-ali T, Harberd NP (2001) How Gibberellin Regulates Plant Growth and Development: A Molecular Genetic Analysis of Gibberellin Signaling. Annu Rev Plant Phys **52:** 67-88

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. P Natl Acad Sci **104:** 19631-19636

Rocha M, Licausi F, Araújo WL, Nunes-Nesi A, Sodek L, Fernie AR, van Dongen JT (2010) Glycolysis and the Tricarboxylic Acid Cycle Are Linked by Alanine Aminotransferase during Hypoxia Induced by Waterlogging of Lotus japonicus. Plant Physiology **152:** 1501-1513

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmentally Modified Plant Systems. The Plant Cell Online **13:** 11-29

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, Miyao A, Hirochika H,

Kitano H, Ashikari M, Matsuoka M (2004) An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice. Plant Physiol **134:** 1642-1653

Saucedo M, Ponce G, Campos ME, Eapen D, García E, Luján R, Sánchez Y, Cassab GI (2012) An altered hydrotropic response (ahr1) mutant of Arabidopsis recovers root hydrotropism with cytokinin. Journal of experimental botany **63:** 3587-3601

Scholander PF, Hammel HT, Hemmingsen EA, Bradstreet ED (1964) Hydrostatic pressure and osmotic potentials in leaves of mangroves and some other plants. Proc. Natl. Acad. Sci. **52:** 119-125

Seki M, Umezawa T, Urano K, Shinozaki K (2007) Regulatory metabolic networks in drought stress responses. Curr Opin Plant Biol **10:** 296-302

Shan D-P, Huang J-G, Yang Y-T, Guo Y-H, Wu C-A, Yang G-D, Gao Z, Zheng C-C (2007) Cotton GhDREB1 increases plant tolerance to low temperature and is negatively regulated by gibberellic acid. New Phytol **176:** 70-81

Sharp RE (2002) Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. Plant, Cell and Enviroment **25:** 211-222

Sharp RE, Davis WJ (1989) Regulation of growth and development of plants growing with a restricted supply of water. In Plants Under Stress

Shulaev V, Cortes D, Miller G, Mittler R (2008) Metabolomics for plant stress response. Physiol Plantarum **132:** 199-208

Silva PEM, Cavatte PC, Morais LE, Medina EF, DaMatta FM (2013) The functional divergence of biomass partitioning, carbon gain and water use in Coffea canephora in response to the water supply: Implications for breeding aimed at improving drought tolerance. Environ Exp Bot **87:** 49-57

Soudry E, Ulitzur S, Gepstein S (2005) Accumulation and remobilization of amino acids during senescence of detached and attached leaves: in planta analysis of tryptophan levels by recombinant luminescent bacteria. Journal of experimental botany **56:** 695-702

Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. Current opinion in plant biology **15:** 282-292

Sun T-p, Gubler F (2004) Molecular Mechanism of Gibberellin Signaling in Plants. Annu Rev Plant Biol **55:** 197-223

Tuna AL, Kaya C, Dikilitas M, Higgs D (2008) The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environmental and Experimental Botany **62:** 1-9

Urano K, Maruyama K, Ogata Y, Morishita Y, Takeda M, Sakurai N, Suzuki H, Saito K, Shibata D, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. Plant J **57:** 1065-1078

Urquhart AA, Joy KW (1981) Use of phloem exudate technique in the study of amino acid transport in pea plants. Plant Physiology **68:** 750-754

Wang S-S, Liu Z-Z, Sun C, Shi Q-H, Yao Y-X, You C-X, Hao Y-J (2012) Functional characterization of the apple MhGAI1 gene through ectopic expression and grafting experiments in tomatoes. Plant Physiol **169:** 303-310

Wiese A, Christ MM, Virnich O, Schurr U, Walter A (2007) Spatio-temporal leaf growth patterns of Arabidopsis thaliana and evidence for sugar control of the diel leaf growth cycle. New Phytol **174:** 752-761

Wolters H, Juergens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet **10:** 305-317

Wu D, Cai S, Chen M, Ye L, Chen Z, Zhang H, Dai F, Wu F, Zhang G (2013) Tissue metabolic responses to salt stress in wild and cultivated barley. PLoS one **8:** e55431

Wyn Jones R, Storey R (1981) Betaines. In 'The physiology and biochemistry of drought resistance in plants'.(Eds LG Paleg, D Aspinall) pp. 171–204. In. Academic Press: Sydney

Yamaguchil S, Kamiya Y (2000) Gibberellin Biosynthesis: Its Regulation by Endogenous and Environmental Signals. Plant Cell Physiol **41:** 251 - 257

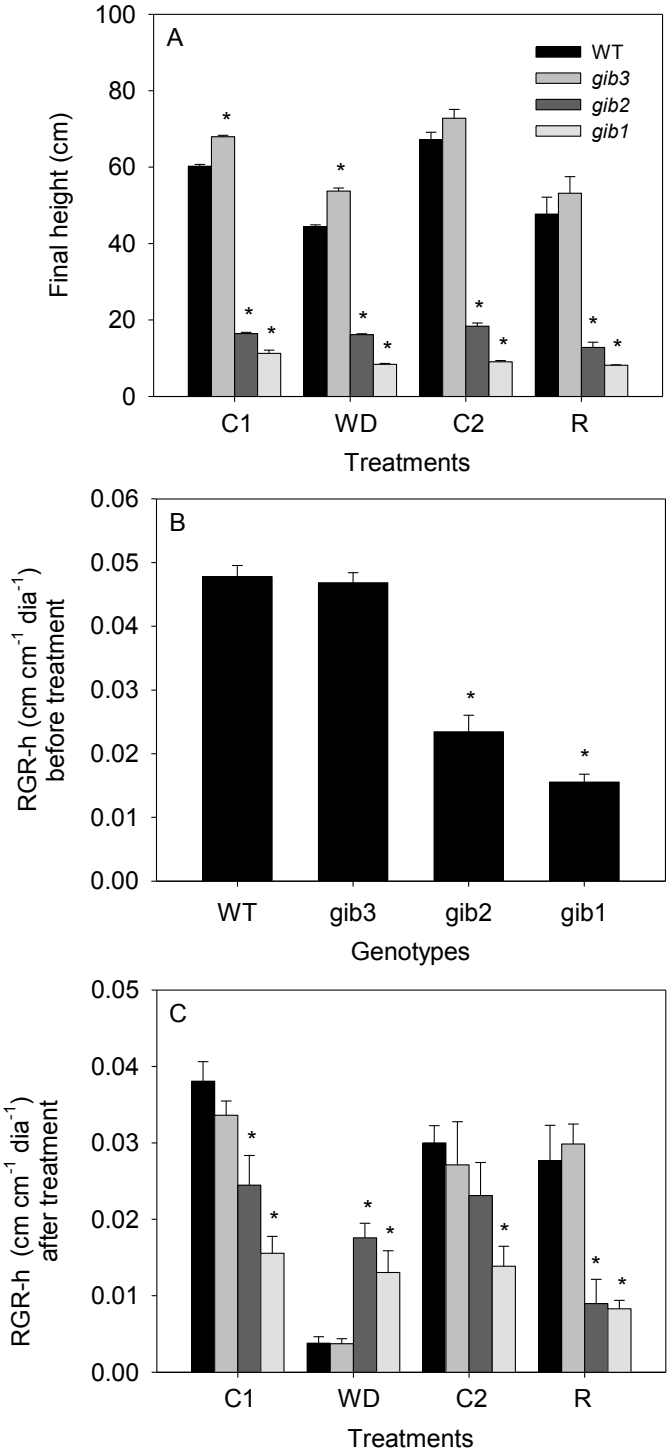
Yamauchi Y, Furutera A, Seki K, Toyoda Y, Tanaka K, Sugimoto Y (2008) Malondialdehyde generated from peroxidized linolenic acid causes protein modification in heat-stressed plants. Plant physiology and biochemistry **46:** 786-793

Yang R, Yang T, Zhang H, Qi Y, Xing Y, Zhang N, Li R, Weeda S, Ren S, Ouyang B, Guo Y-D (2014) Hormone profiling and transcription analysis reveal a major role of ABA in tomato salt tolerance. Plant Physiology and Biochemistry **77:** 23-34

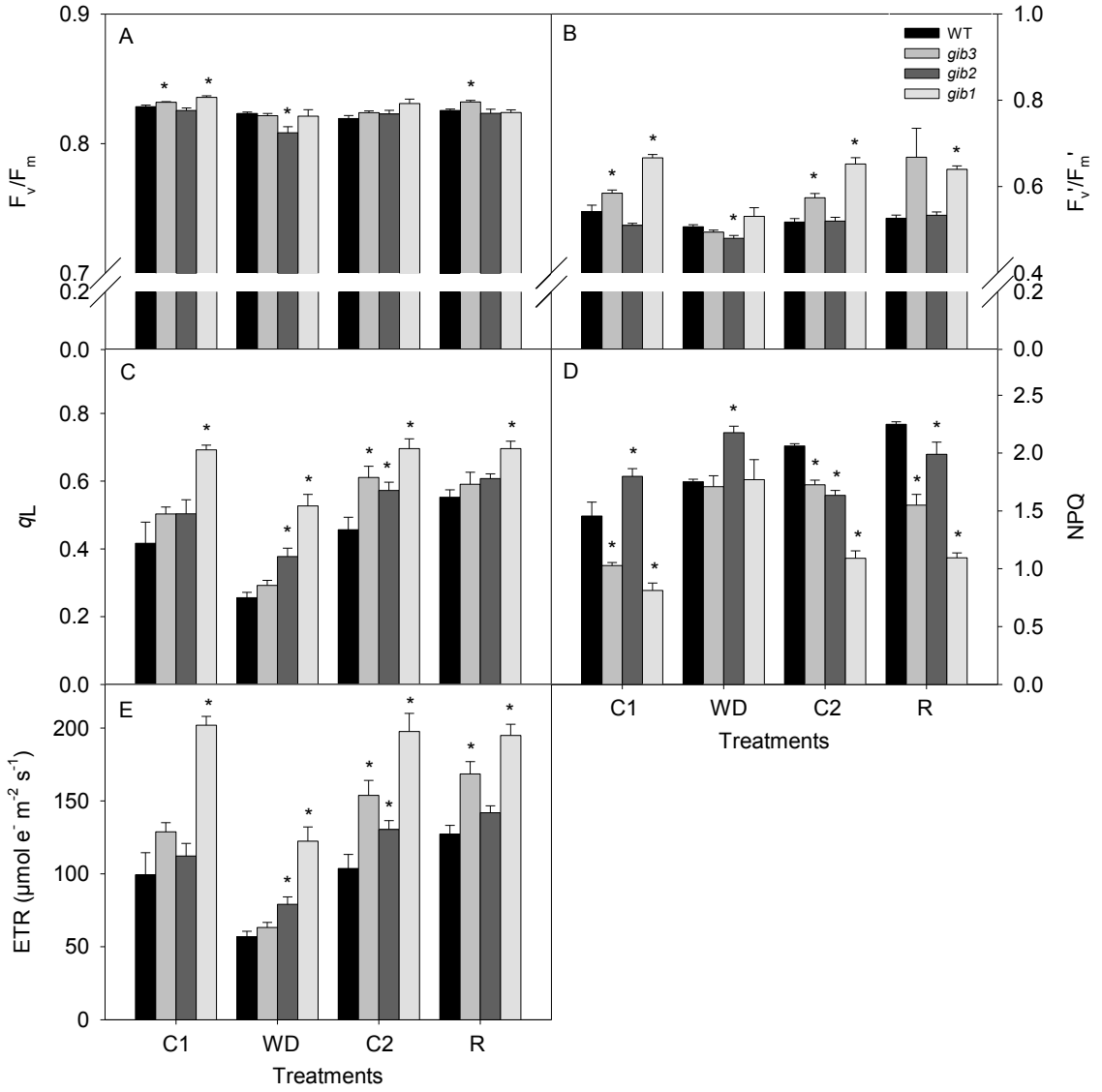
Zawaski C, Busov VB (2014) Roles of Gibberellin Catabolism and Signaling in Growth and Physiological Response to Drought and Short-Day Photoperiods in Populus Trees. PLoS ONE **9:** e86217

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) Elongates Uppermost Internode Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. Plant Cell **18:** 442-456

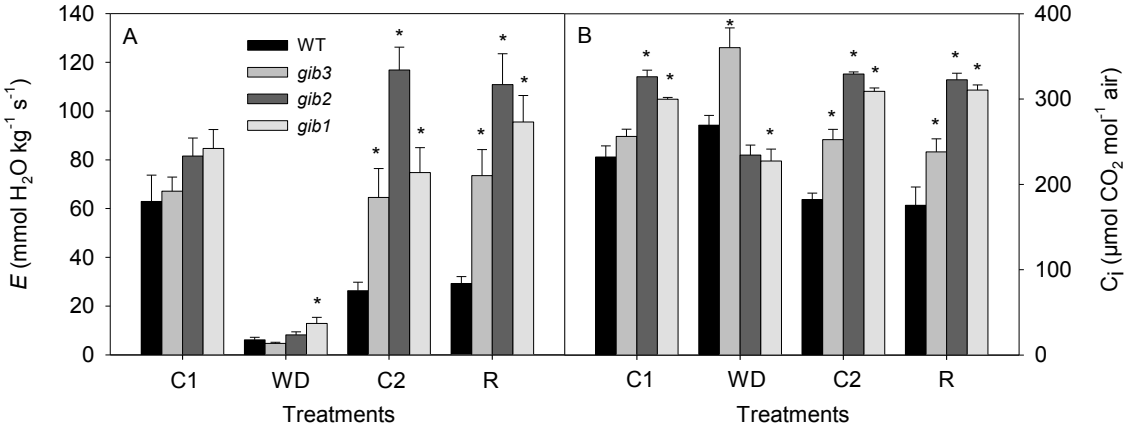
Supplementary data



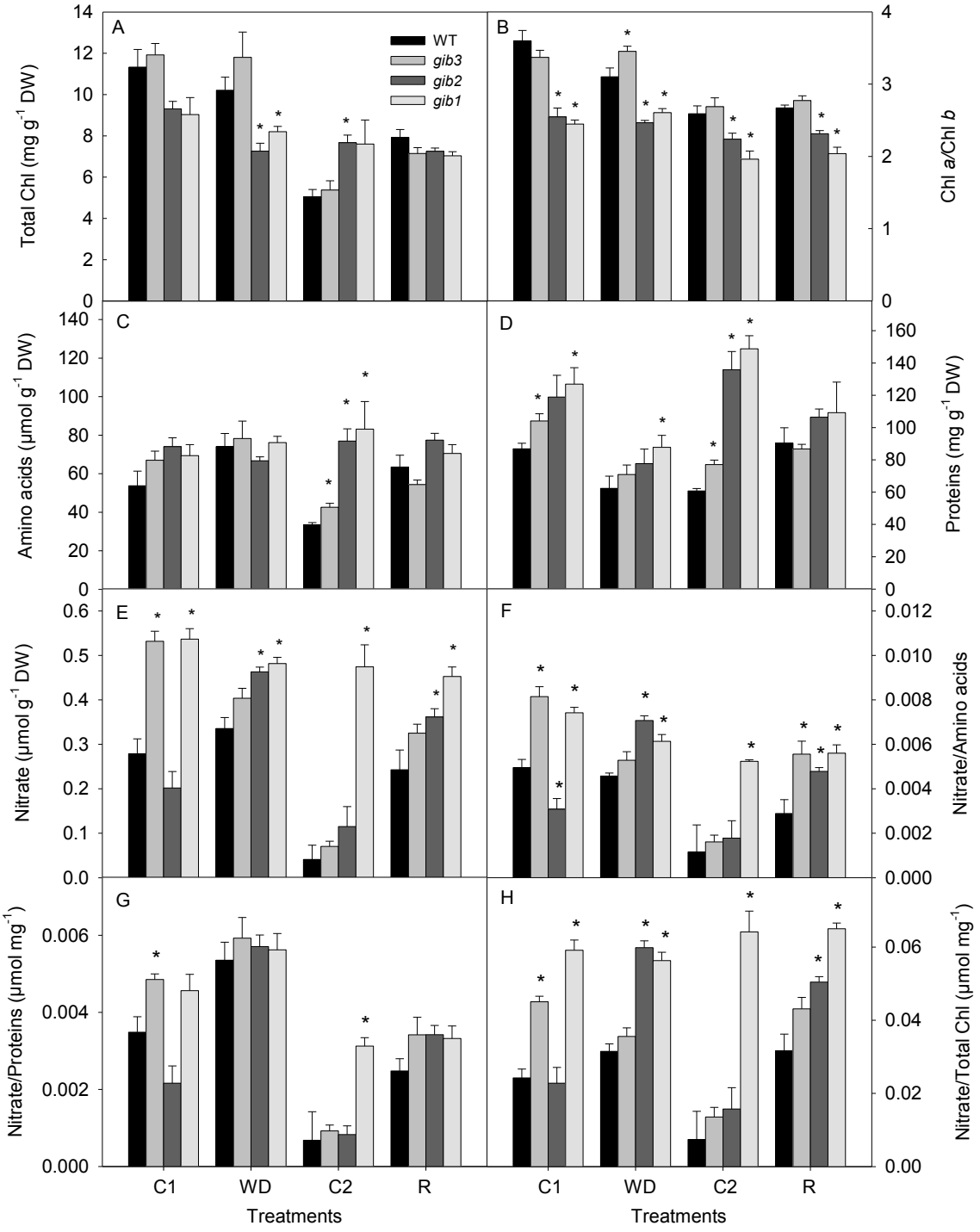
Supplementary figure S1. Variation in growth parameters related to height of tomato plants (Solanum lycopersicum cv. Moneymaker) in response to water deficit. (A) Final height of plants; (B) relative growth rate in height (RGR-h) of plants before application of treatments and; (C) relative growth rate in height (RGR-h) after application of water deficit. Values represent means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing mutants with the WT.



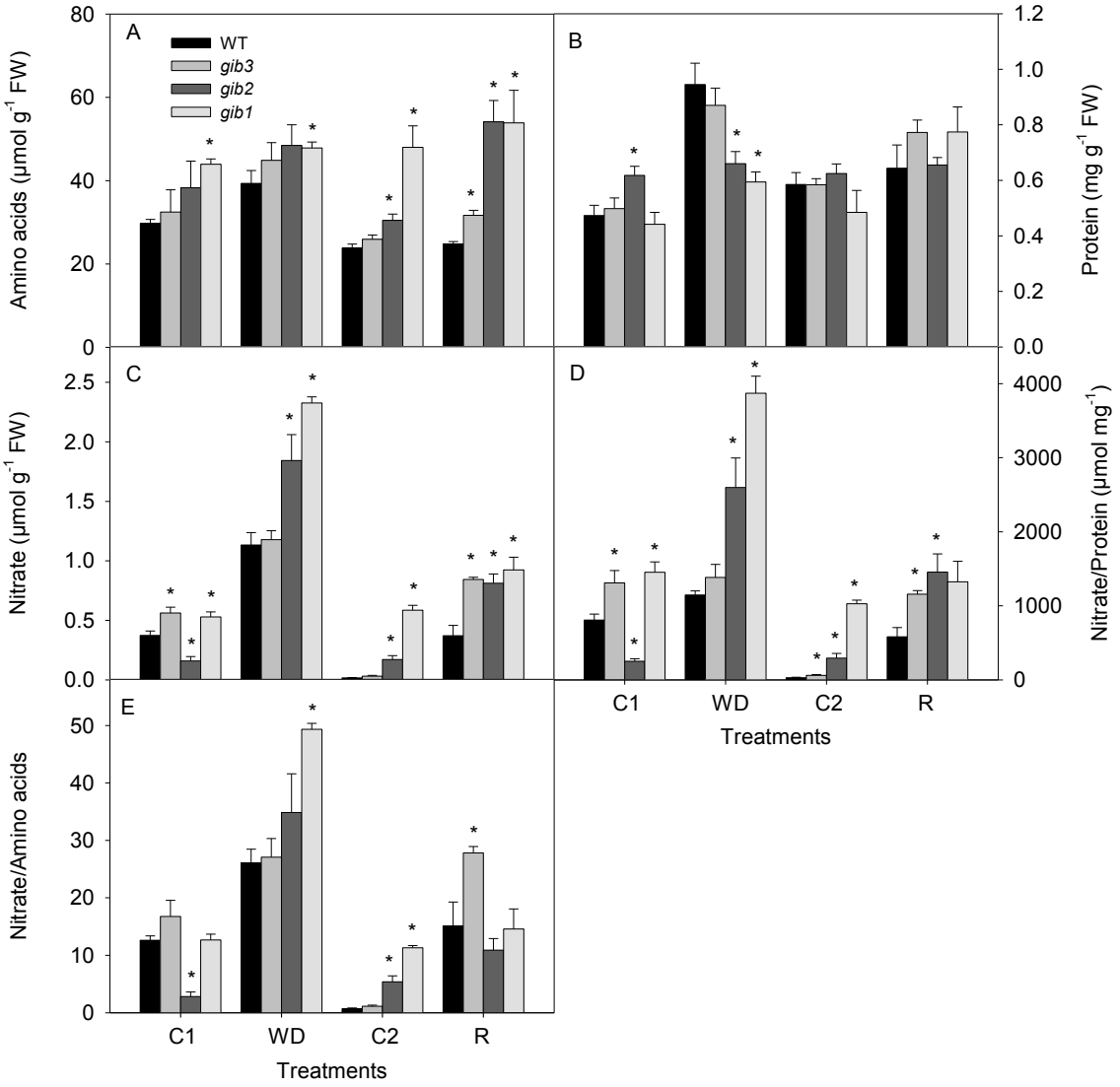
Supplementary figure S2. Variation in chlorophyll a fluorescence parameters in response to different GAs endogenous levels and water deficit in tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Maximum photochemical efficiency of FSII (F_v/F_m); (B) effective quantum yield of electron transport in FSII (F_v'/F_m'); (C) photochemical extinction coefficient (qL); (D) non-photochemical extinction coefficient (NPQ) and; (E) electron transport rate (ETR). Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing mutants with the wild type.



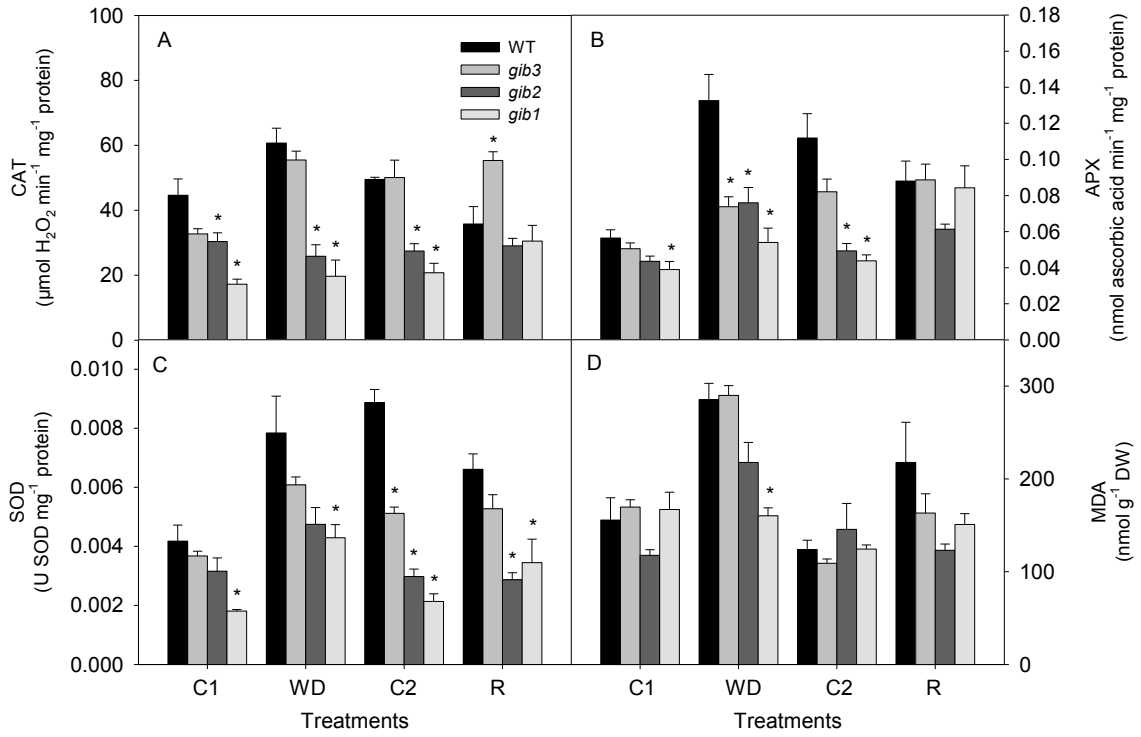
Supplementary figure S3. Variation in gas exchange parameters in response to different endogenous GAs levels in tomato plants (Solanum lycopersicum cv. Moneymaker) submitted to water deficit and after recovery. (A) Transpiration rate (E) and; (B) Internal CO_2 concentration (C_i). Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing mutants with the wild type.



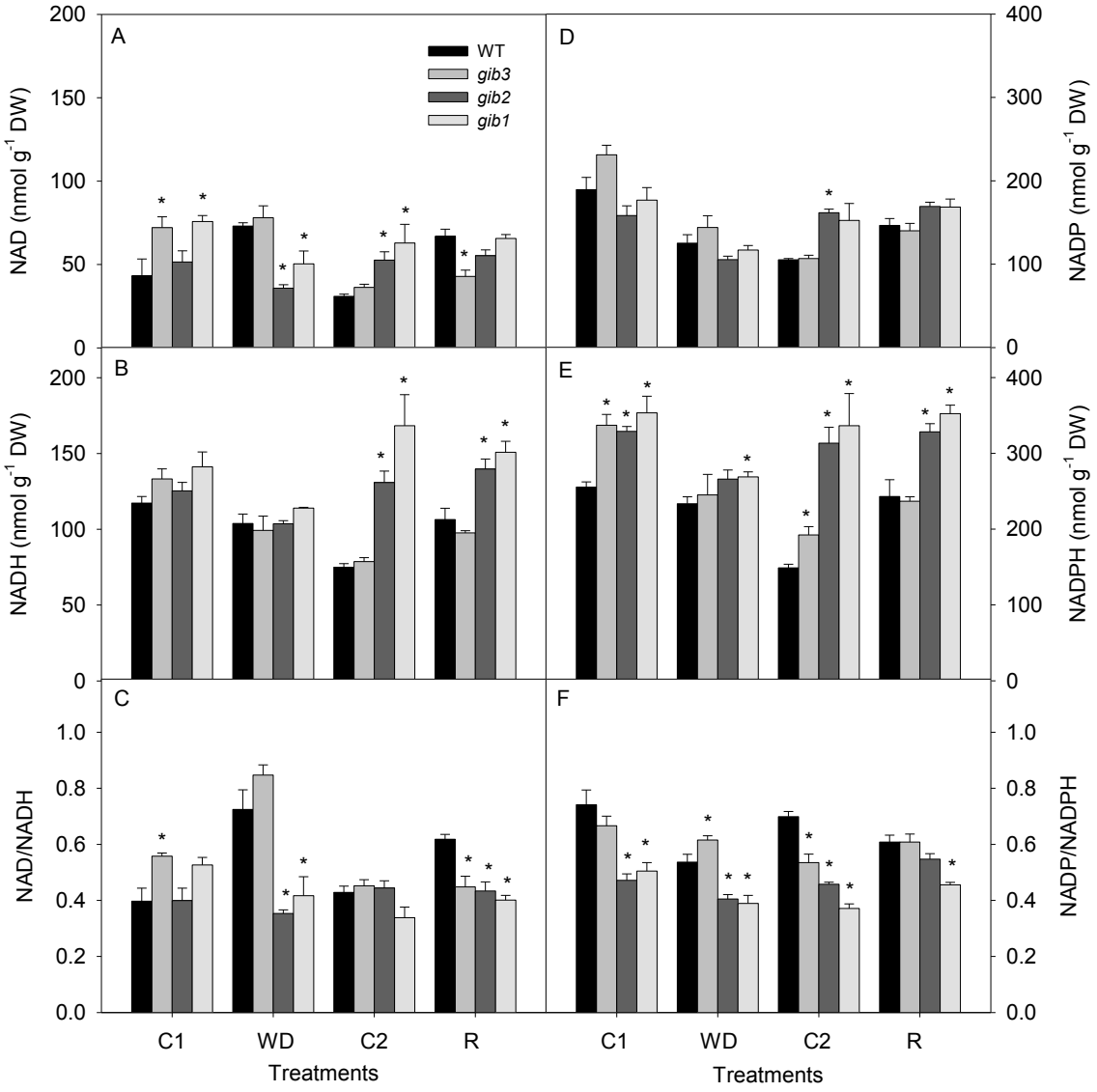
Supplementary figure S4. Changes in metabolite levels involved in nitrogen metabolism in leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with different levels of endogenous GAs under water deficit and after re-watering. (A) Total Chl (Chlorophyll); (B) Ratio between Chl a and Chl b; (C) Total soluble amino acids; (D) Total soluble proteins; (E) Nitrate; (F) Ratio between nitrate and amino acids; (G) Ratio between nitrate and proteins and; (H) Ratio between nitrate and total chlorophylls. Values are presented as means ± standard error of six plants. Asterisks indicates significant differences ($P<0.05$) by the Student's t test of mutant plants in relation to WT.



Supplementary figure S5. Changes in metabolite levels involved in nitrogen metabolism in roots of tomato plants (Solanum lycopersicum cv. Moneymaker) with different levels of endogenous GAs grown under water deficit and after re-watering. (A) Total soluble amino acids; (B) total soluble proteins; (C) nitrate; (D) Ratio between nitrate and proteins and; (E) Ratio between nitrate and amino acids. Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing to WT.



Supplementary figure S6. Variation in malonaldehyde content (MDA) and enzymes activity involved in antioxidative metabolism and cell damage in leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with different levels of endogenous GAs under water deficit and after watering. (A) Catalase (CAT); (B) ascorbate peroxidase (APX); (C) superoxide dismutase (SOD) and; (D) malonaldehyde (MDA). Values are presented as means ± standard error of six plants. Asterisks indicates significant differences ($P<0.05$) by the Student's t test of mutant plants in relation to WT.



Supplementary figure S7. Alteration of pyridine nucleotide content in leaves of tomato plants (Solanum lycopersicum cv. Moneymaker) with different GAs endogenous levels under water deficit and re-watering. (A) NAD^+; (B) NADH; (C) Ratio between NAD^+ and NADH; (D) $NADP^+$; (E) NADPH e; (F) Ratio between $NADP^+$ and NADPH. Values are presented as means ± standard error of six plants (n = 6). Asterisks indicates significant differences ($P<0.05$) by the Student's t test of mutant plants in relation to WT.

Tabela Suplementar S1. Leaf metabolites of all treatments used for the multivariate analysis and their contribution to the sepration of each principal component (PC).

	Metaolites	PC1	PC2
Amino acids	Alanine	0,99097	-1,88577
	Arginine	1,2178	0,11819
	Asparagine	-0,73132	-3,55097
	GABA	0,04887	-0,58078
	Glutamic acid	-0,07341	-0,44618
	Glutamine	1,12353	-2,03131
	Glycine	0,84569	-1,24974
	Hydroxyproline	2,82371	-0,85484
	Isoleucine	0,58745	-1,19673
	Lysine	0,66723	-1,76673
	Methionine	0,24859	-1,23825
	Phenylalanine	0,55093	-0,9848
	Proline	5,18608	-2,28029
	Serine	-0,35589	-0,90274
	Threonine	0,08904	-0,40896
	Tryptophan	0,32657	-1,40754
	Tyramine	-0,12666	-1,36075
	Valine	0,81562	-1,12803
	ß-alanine	0,62173	-1,28723
Organic acids	2-oxoglutaric acid	1,02801	0,50134
	Ascorbic acid	1,05335	0,3591
	Aspartic acid	-0,28937	-1,29989
	Dehydroascorbic acid	0,45496	2,66121
	Maleic acid	0,64936	-0,48739
	Malic acid	0,35074	0,13135
	Malonic acid	-0,81989	-1,11944
	Nicotinic acid	0,1855	-0,78617
	Succinic acid	0,01913	-0,34227
Sugars	Fucose	0,57421	0,36441
	Gentiobiose	0,71783	-0,30814
	Glucoheptose	1,78286	0,75138
	Glucose-6-phosphate	-1,02647	-0,95694
	Lyxose/Xylose	1,49246	-0,03622
	Maltose	1,07016	0,39485
	Rhamnose	0,63151	-0,1123
	Trehalose	0,91272	-0,05949
	Turanose	0,82695	0,13061
Others	Caffeic acid	0,99622	0,22402
	Galactinol	1,00223	-1,06274
	Glyceric acid	-0,39105	0,95112
	Glycerol	-0,11918	-0,55315
	Glycerol-3-phosphate	-0,21494	-1,14066
	Lactic acid	-0,17515	-0,45079
	Myo inositol	0,11032	-0,41047
	Phosphoric acid	-0,58705	-1,13627
	Putrescine	0,58066	-1,60481

Tabela Suplementar S2. Leaf metabolites of WD treatments used for the multivariate analysis and their contribution to the sepration of each principal component.

	Metaolites	PC1	PC2
Amino acids	Alanine	-1,05011	-3,84628
	Arginine	-1,41868	1,50342
	Asparagine	3,33489	-0,96472
	GABA	-0,45769	-0,54184
	Glutamic acid	0,68445	0,7653
	Glutamine	0,16478	-2,1092
	Glycine	-0,8081	0,36657
	Hydroxyproline	-1,13637	0,85582
	Isoleucine	0,17135	0,01785
	Lysine	-0,25585	-0,92448
	Methionine	-0,84848	-1,39435
	Phenylalanine	0,04977	-0,14001
	Proline	-0,84337	-0,23043
	Serine	-0,18629	-0,98854
	Threonine	-0,23845	-0,25962
	Tryptophan	0,02568	-1,167
	Tyramine	0,43837	-0,33731
	Valine	-0,50743	-0,27345
	ß-alanine	-0,45872	-0,28083
Organic acids	2-oxoglutaric acid	3,14634	-0,1482
	Ascorbic acid	0,39017	0,60212
	Aspartic acid	0,36375	-0,62609
	Dehydroascorbic acid	-1,50856	2,52077
	Maleic acid	0,37162	0,76276
	Malic acid	0,44875	0,87053
	Malonic acid	0,75227	0,81628
	Nicotinic acid	0,06329	0,3597
	Succinic acid	0,89219	0,78782
Sugars	Fucose	-0,70615	0,00754
	Gentiobiose	-0,35299	0,70519
	Glucoheptose	-1,11577	0,55783
	Glucose-6-phosphate	0,80349	-0,03324
	Lyxose/Xylose	0,02734	1,23906
	Maltose	-1,22459	0,22274
	Rhamnose	-0,46754	-0,03191
	Trehalose	-0,48597	-0,31551
	Turanose	-1,2524	-0,2979
Others	Caffeic acid	-0,50828	-0,35329
	Galactinol	-1,25512	-0,51787
	Glyceric acid	0,07181	0,31182
	Glycerol	0,18653	0,62178
	Glycerol-3-phosphate	1,18129	0,068
	Lactic acid	0,02812	0,15006
	Myo inositol	-0,04272	0,24414
	Phosphoric acid	0,93415	1,11177
	Putrescine	-1,35099	-0,79438

Tabela Suplementar S3. Root metabolites of all treatments used for the multivariate analysis and their contribution to the sepration of each principal component.

	Metaolites	PC1	PC2
Amino acids	Alanine	-0,25707	0,07567
	Asparagine	-0,34153	0,32682
	Aspartic acid	-0,45127	-0,55076
	GABA	-1,42539	0,05865
	Glutamine	0,46448	-1,01046
	Glycine	-1,31978	0,53631
	Homoserine	0,93767	2,06346
	Hydroxyproline	0,05062	-0,25103
	Isoleucine	-1,10704	-2,04534
	Methionine	0,75031	-0,10532
	Ornithine	1,09559	-0,21936
	Phenylalanine	-0,6353	-0,58612
	Proline	0,1885	-2,37644
	Pyroglutamic acid	-0,78088	-0,20916
	Serine	-1,64332	0,77668
	Threonine	-0,44111	-1,04194
	Tryptophan	1,12456	-0,54307
	Tyramine	-1,23924	0,19416
	Tyrosine	0,96033	-0,14829
	Valine	-1,23299	-1,88898
	ß-alanine	1,14144	-1,04167
Organic acids	2-oxoglutaric acid	-0,73469	-0,67793
	Citric acid	-0,58515	-0,37959
	Isocitric acid	0,17511	-1,9952
	Maleic acid	-0,00658	0,08808
	Malic acid	-1,81456	-1,84378
	Malonic acid	0,96206	-1,16114
	Nicotinic acid	1,22876	1,49814
	Succinic acid	-1,71378	0,15066
Sugars	Fructose	-1,12539	0,78402
	Fructose-6-phosphate	-0,03197	0,0735
	Gentiobiose	-0,68925	-0,38123
	Glucose	0,12581	1,03164
	Glucose-6-phosphate	-0,02038	-1,12637
	Mannitol	0,95572	0,62028
	Raffinose	1,45923	-0,19315
	Ribose	0,39541	0,37562
	Sucrose	-2,3957	0,36215
	Trehalose	-0,8688	0,27494
	Turanose	1,9112	-0,45432
Others	Galactinol	0,45625	-0,02172
	Galactonic acid	0,61169	-0,22762
	Galacturonic acid	0,24068	-0,29383
	Glycerol	1,31461	-0,19287
	Glycerol-3-phosphate	1,13537	-2,78192
	Myo inositol	-0,84791	0,00834
	Putrescine	-0,66138	0,18792
	Uracil	0,37833	1,0759
	Urea	0,01441	1,6973

Tabela Suplementar S4. Root metabolites of WD treatments used for the multivariate analysis and their contribution to the sepration of each principal component.

	Metaolites	PC1	PC2
Amino acids	Alanine	-0,0165	0,00051
	Asparagine	-0,82199	-1,45337
	Aspartic acid	0,5019	-0,21064
	GABA	-0,43735	-0,16108
	Glutamine	0,94451	-0,78533
	Glycine	-0,26915	-0,01068
	Homoserine	-0,49844	0,21289
	Hydroxyproline	0,47606	-1,60637
	Isoleucine	3,28037	-1,82611
	Methionine	0,10641	0,26004
	Ornithine	-0,12233	-0,18211
	Phenylalanine	2,0058	-1,66135
	Proline	-0,72598	-0,03819
	Pyroglutamic acid	-0,03061	0,16453
	Serine	0,06334	-0,15018
	Threonine	-1,44401	-2,7295
	Tryptophan	0,00328	-0,57662
	Tyramine	-0,88208	0,54242
	Tyrosine	-0,11647	0,05182
	Valine	-0,56458	-3,49998
	ß-alanine	1,43408	0,02075
Organic acids	2-oxoglutaric acid	1,27545	-1,08629
	Citric acid	0,67591	-0,19118
	Isocitric acid	0,39906	-1,51861
	Maleic acid	0,15629	-0,37026
	Malic acid	3,17485	-2,09717
	Malonic acid	0,55333	0,82116
	Nicotinic acid	-0,67533	-0,8943
	Succinic acid	0,91111	-1,01307
Sugars	Fructose	-0,12296	-0,17672
	Fructose-6-phosphate	-0,0501	-0,02948
	Gentiobiose	0,81349	-0,31396
	Glucose	-0,22395	-1,44642
	Glucose-6-phosphate	-0,99405	-0,25374
	Mannitol	0,00846	0,05434
	Raffinose	-0,00358	-0,06691
	Ribose	-0,36043	-0,26667
	Sucrose	0,18627	0,14127
	Trehalose	-0,00242	-0,25562
	Turanose	1,40322	-0,06809
Others	Galactinol	0,04453	0,07163
	Galactonic acid	-0,34122	-0,20617
	Galacturonic acid	-0,29324	-0,49639
	Glycerol	0,08865	-0,06024
	Glycerol-3-phosphate	0,15889	-1,46532
	Myo inositol	1,60321	-1,7848
	Putrescine	-2,36005	-4,15162
	Uracil	-0,11589	-0,87624
	Urea	-0,3482	-0,2011

Chapter 2

Root morphological and metabolic characterization in GA-deficient mutant tomato plants (Solanum lycopersicum L.)

Abstract

Gibberellins (GAs) belong to a class of plant hormones with multiple functions in the regulation of physiological processes associated with growth and development. In shoots and roots, GAs are synthesized in young and actively growing organs that can act locally or be transported to other tissues or organs. Understanding the role of GAs in roots came a few decades ago using plants that are severely deficient in GAs biosynthesis, either with genetic mutation in biosynthetic genes or using inhibitors of GAs biosynthesis or signaling. However, only few studies evaluate root metabolism in plants with reduced levels GAs and that associate it with root growth and morphology. Thus, the objective of this work was to evaluate the effects of the endogenous alteration in GA levels in tomato mutant (gib1, gib2 and gib3, deficient in GA biosynthesis) in root growth, morphology and metabolism. In summary, the low content of endogenous GAs affected the root growth and morphology. Additionally, the metabolism respiratory and labeled carbon allocation in leaves and roots were changed differently by the GAs. Plants gib2 and gib1 displayed higher root/shoot ratio compared to WT probably due to relocation of photoassimilates to root growth. The gib2 and gib1 mutants presented higher proportions of thick roots than WT and gib3, but the growth of fine roots (with smaller diameters) was the most pronounced. It was found lower shoot and root growth in gib2 and gib1 probably due the accumulation of amino acids in both organs of the mutant plants causing the reduction of tricarboxylic acid cycle intermediaries corroborating with the lower respiration rates. Therefore, our data suggest that the shoot responds differently from the root and that the root growth and primary metabolism are decoupled from the shoot in GA-deficient plants probably due to the higher GA sensitivity of roots. Taken together, this data suggest an important role of GAs in the metabolism, growth and root morphology of tomato plants.

Introduction

Gibberellins (GAs) belong to a class of plant hormones with multiple functions in the regulation of physiological processes associated by growth and development (Hedden and Kamiya, 1997; Olszewski et al., 2002; Sakamoto et al., 2004; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011). Physiologically, the main effect of GAs on superior plants is stimulating organ growth through cell stretching and, in some cases, cell division. In addition, GAs promote phase transition during development, such as between seed dormancy and germination, juvenile and adult growth phases, and vegetative and reproductive development (Hedden and Thomas, 2012).

In shoot and roots, GAs are synthesized in young and actively growing organs, such as developing leaves and expanding internodes (Smith et al., 1992), which are also sites of action of these GAs (Hedden, 2012). These organs contain high levels of gene expression of GA biosynthesis, indicating the synthesis of active GAs (Silverstone et al., 1997; Kaneko et al., 2003). It has been previously reported that GAs in Arabidopsis roots are mainly accumulated in the endoderm cells of stretching zone and the presence of an GA active transport mechanism has also been strongly suggested (Shani et al., 2013). However, GAs production in maturing leaves occurs due to the presence of the ent-caurene precursor that is synthesized in proplastids associated with the foliar vascular system (Silverstone et al., 1997), providing the GAs export to other parts of the plant by phloem (Eriksson et al., 2006). Currently, it is known that GAs can be transported cell-to-cell (or between tissues) through membrane transporters (Tal et al., 2016) or from one organ to another through xylem and phloem conducting vessels (Regnault et al., 2015; Regnault et al., 2016). Therefore, GAs can act in the places where they are synthesized or can be transported to other cells, tissues or organs promoting responses at short- and long-distances.

Additionally, the root metabolism can be altered as a function of the GAs endogenous concentration, the GAs exogenous application and also the lack of perception in GA signaling processes, causing changes in root growth. In Populus mutants, gai and rgl1, with deleted DELLA domain presenting higher levels of active GAs, it was observed a greater root growth and a larger number of roots probably due to increased respiration (Busov et al., 2006). The authors report that this fact was supported by monosaccharides decrease and citrate, asparagine and arginine accumulation. That is, the consumption of monosaccharides by respiration has caused

the accumulation of some organic acids from tricarboxylic acids cycle that are required for amino acids synthesis. Similarly, increased root respiration and carbon partitioning for roots was a possible reason for higher biomass partitioning in roots of tomato plants deficient in GA, gib3 and gib1 (Nagel and Lambers, 2002). Therefore, shoot and root growth and development, carbohydrates metabolism, organic acids and amino acids can be altered by GAs.

It is worth mentioning that the role of GAs in shoot and root growth and development of several species is well known since the "green revolution" which introduction of semi-annual varieties have been introduced and enormous improvements in productivity (Hedden, 2003). Understanding the role of GAs in roots came decades later using severely GAs deficient plants, either genetically mutated in biosynthesis genes or using GA inhibitors (Tanimoto, 2012). These studies provided valuable new information on a roots GAs responses. For example, when using mutant plants in GAs biosynthesis, plant active GA production is regulated locally, indicating that shoot regulation is different from root regulation. Furthermore, roots elongation and thickening these plants can be regulated by a lowest GA concentration and/or by a greater roots sensitivity to GA than to stem giving advantage when GA production is limited (Tanimoto, 2012). However, GAs regulation in root development and root elongation mechanisms have not yet been fully elucidated and few studies have been found.

Recently the importance of GA levels in response to water stress was studied (Chapter 1). In this study, were used tomato mutant plants deficient in GAs biosynthesis, gib3, gib2 and gib1, with moderate, intermediate and severe deficiency, respectively. It was verified that mutant plants with the lowest GAs content, gib2 and gib1, when submitted to water deficiency did not present an apparent wilt of severe water potential (-1.3 MPa). These plants maintained high water potential for a longer time in relation to control condition, and presenting a period of lower post-stress recovery. The tolerance of these plants with low GAs levels was attributed to several possible causes: (i) greater root growth over shoot, (ii) leaf architecture and morphology of mutant plants, (iii) pronounced accumulation of amino acids, mainly proline, in leaves and roots (Chapter 1). Other authors have observed similar responses in water and salt stress, which root system presented increases in relation to shoot, resulting in adequate plant water supply (Sharp et al., 1989; Albacete et al., 2008). However, it is believed that GAs lack in these mutant plants also altered the roots architecture and

morphology, which may possibly be another factor responsible for tolerance to water deficiency. For this, complementary studies of root growth, architecture, morphology and metabolism are necessary to elucidate the mechanisms involved in these tolerance responses. The growth and development of plants are known to be regulated by endogenous GA content (Olszewski et al., 2002; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011; Ribeiro et al., 2012). Many studies have investigated the role of GA in shoot growth processes and little importance has been given to the roots (Tanimoto, 2012). In this sense, to understand the role of GAs in root metabolism, growth and morphology, were used mutants tomato plants (Solanum lycopersicum cv. Moneymaker) in the GAs biosynthesis, gib3, gib2 and gib1, and biometric, morphologic and metabolic parameters were analyzed.

Material and methods

Description of genotypes used

The mutant tomato lines (Solanum lycopersicum L. cv. Moneymaker) deficient in GA biosynthesis used in this study were produced and characterized genotypically and phenotypically previously by (Koornneef et al., 1990). The mutants were obtained by immersion of seeds of commercial cultivar Money-maker in solution of ethyl methane sulfonate mutagenic agent (EMS). The genotypic analysis performed initially revealed that the mutations generated by EMS occurred at different loci, located on different chromosomes (Koornneef et al., 1990). The mutants named gib3, gib2 and gib1 have mutations in chromosomes seven, one and six, respectively (Koornneef et al. 1990). The characterization of gib1 and gib3 mutants revealed that these genotypes have reduced ent-copalyl diphosphate synthase enzyme activities, with a reduction of 94% in relation to wild type (WT) and ent-kaurene synthase, with a reduction of 93% compared to WT, respectively. These enzymes participate of first reactions of GAs synthesis located in the plastids. For gib2, although the level of activity reduction was not known, it must have affected the expression of the ent-kaurenoic acid oxidase enzyme located in the endoplasmic reticulum (Bensen and Zeevaart, 1990). Together, low gene expression and low activity of these three enzymes resulted in mutant plants with reduced GAs levels (Koornneef et al., 1990).

Experimental conditions

The experiment was performed at Viçosa (20º45'S, 650 m altitude), Minas Gerais, in a greenhouse of Department of Plant Biology of the Federal University of Viçosa.

Seeds from wild type and mutant lines gib1, gib2 and gib3 (highly, intermediate and moderately GA deficient) were kindly provided by Dr. Koornneef (Max Planck Institute for Plant Breeding Research, Cologne, Germany). These seeds were germinated in Petri dish (15 cm of diameter) containing two layers of germitest paper moistened with 10 μM GA_{4+7} solution. Subsequently, the plates were housed in a growth chamber BOD type (Forma Scientific, Inc, Ohio, EUA), under photoperiod of 12/12 hours (day/night), temperature 25/16 ^{o}C (day/night), relative humidity 65±5% and luminous intensity of 150 μmol photons m^{-2} s^{-1}, for a period of seven days. Each day, all germinated seeds, with a radicle of one centimeter length, were transferred to a 200 ml

plastic cups containing germicide paper moistened with Hoagland ½ strength nutrient solution (Hoagland and Arnon, 1950). The plants remained in a moist chamber in a growth room under controlled conditions for one week. In the following week the pots were transfered to a greenhouse in a humid chamber and stayed for another week until transplantation to the definitive styrofoam boxes. For about these two weeks, roots were dampened twice a day with Hoagland ½ strength solution.

After the appearance of two completely expanded leaves, the plantlets were transplanted to 5 L expanded polystyrene boxes, two plants per box, with Hoagland ½ strength nutrient solution, pH 6.0, aeration system and solution containing all the macro and micronutrients necessary for plants growth and development. After the transplant, the plants received nutrient solution ½ strength only in the first seven days and afterwards were provided a total strength solution until the end of the experiment. The nutrient solution was changed every 7 days. Adjustment of pH and correction of the nutrient solution volume in the boxes was performed daily. Biometric analyzes were performed throughout the experiment and the leaves and roots were collected only at the end of the experiment, after three weeks of planting in Hoagland full strength solution. At that time, the plants were six weeks old.

Growth analysis and sample harvesting

Plant growth was monitored every three days by measuring the shoot height. The plant height was monitored with a millimeter rule from the lap height to the apical bud throughout the experiment. The final height, shoot dry weight (leaves and stem), root dry weight, total leaf area and specific leaf area were determined at the end of the experiment. Subsequently, shoot and root relative growth rate (RGR) was calculated according to (Evans, 1972; Hunt, 1982).

Eight leaf discs (0,519 cm of diameter) were dried at 65 ºC until constant weight for determination of specific leaf area. The total leaf area was estimated from the specific leaf area using the dry weight of all leaves. The determination of both specific leaf area and total leaf area was performed following protocol described by (Mielke et al., 1995). The specific leaf area was calculated using equation:

$$\text{Specific leaf area } (m^2\ g^{-1}) = \frac{\text{leaf area of discs } (m^2)}{\text{dry weight of discs (g)}}$$

Root samples from some plants were collected in 30% ethanol to be scanned for future morphologic measurements with professional scanner Epson XL 10000 equipped with additional light unit (Supplementary figure S3). The WinRHIZO Pro 2007a program was used to calculate the root length, surface area, volume and ramifications. Leaves, stems and roots were separated and immediately taken to the oven with forced ventilation at 65 ºC to obtain the dry weight after constant weight. Values of dry weight, biomass partitioning of leaves, stems and roots and shoot-root ratio were calculated as described by (Benincasa, 1988).

^{14}C-glucose feeding experiments

A total of 24 leaf discs (0.516 cm of diameter) and approximately 4 g of root tissues from the median region of the root system were collected from each genotype, from four plants of five weeks old. Samples were incubated in 5 mL of incubation medium (10 mM Mes-KOH, pH 6.5) containing 10 mM glucose supplemented with 1.4 MBq mmol^{-1} of ^{14}C labeled glucose ([U-^{14}C]-Glc). The samples were then incubated for 6 h and subsequently 1/3 of the samples were collected every 2 h, washed in the same incubation medium, without labeling, frozen in liquid N_2 and stored at -80ºC until further analysis. All incubations were carried out in a 100 mL sealed flask at 25 °C and shaken at 90 rpm. The evolved $^{14}CO_2$ was collected in 0.5 mL of 10% (w/v) KOH and quantified by counting in liquid scintillation. Samples incubation on labeled glucose followed (Centeno et al., 2011) with modifications.

Fractionation of ^{14}C-labeled material

Tissue fractionation was performed as described by (Fernie et al., 2001) with modifications. The frozen tissue was extracted successively for 20 min at 80°C in 2 mL 80% (v/v) ethanol, 2 mL of 50% (v/v) ethanol and 2 mL of 20% (v/v) ethanol. The supernatants were combined and dried under vacuum, the ethanol-soluble components were resuspended in 2 mL H_20 and separated into neutral, anionic and basic fractions. The pellets were suspended in 0.2 mL H_2O, digested and separated also in neutral, anionic and basic fractions. The recovery of radioactivity during fractionation was approximately 80% of that applied. The reliability of these fractionation techniques has been documented previously (Centeno et al., 2011).

Experimental design

The experiments were performed with a completely randomized design in a greenhouse condition, with ten replicates for each genotype, with each sample being considered as a replicate. The results were submitted to analysis of variance (ANOVA) and, to verify statistical difference between mutants lines and WT, the means were compared by Student t test at 5% of probability ($P<0.05$).

Results

Reduction in endogenous GA levels affects root growth

The shoot and root growth parameters, as height, relative growth rate (RGR) and root length, showed the lowest values in the lines with low endogenous GAs levels when compared to WT plants (Figure 1). At the end of the experiment, shoot height and root length of WT and mutant plants (Figure 1A and B) were determined. The tree mutant plants presented significant lower length of root and shoot when compared to WT, gib2 and gib1 plants presented the lowest GA levels. When comparing the length of the shoot with the root length, it is possible to notice greater root growth in all genotypes used, with a larger root length at the detriment of the shoot in gib2 and gib1.

During the experiment, measurements of shoot height and root length were performed with three days of intervals and the relative growth rate (RGR) was calculated (Figure 1C, D, E and F). The shoot RGR of the mutant plants was strongly reduced gib2 and gib1, under normal conditions of cultivation (Figure 1C). Interestingly, root RGR presented lower reductions when compared with shoot RGR, with significant reductions only for gib2 and gib1 genotypes (Figure 1D). It is noteworthy that, for gib2 and gib1 only, the reductions in the root RGR, was smaller when compared to shoot RGR.

After the transplant, it was observed that the plants showed approximately the same height and root length (Figure 1E and F). As the days went by, WT and gib3 plants grew similarly to each other and unlike gib2 and gib1, which were similar to each other. After the sixth day of the experiment, gib2 and gib1 presented lower height when compared to WT and thus continued until the twenty-seventh day (Figure 1E). gib3 plants differed statistically from WT only on the twenty-first day, also exhibiting a smaller growth in height. As for root length, the three mutants showed significantly lower length on the eighteenth day of analysis, and gib2 differed on day 15 (Figure 1F). The root growth of WT and mutant plants was higher comparable with the growth in height. These results indicate that the low endogenous content of GAs influences the shoot growth more than root growth.

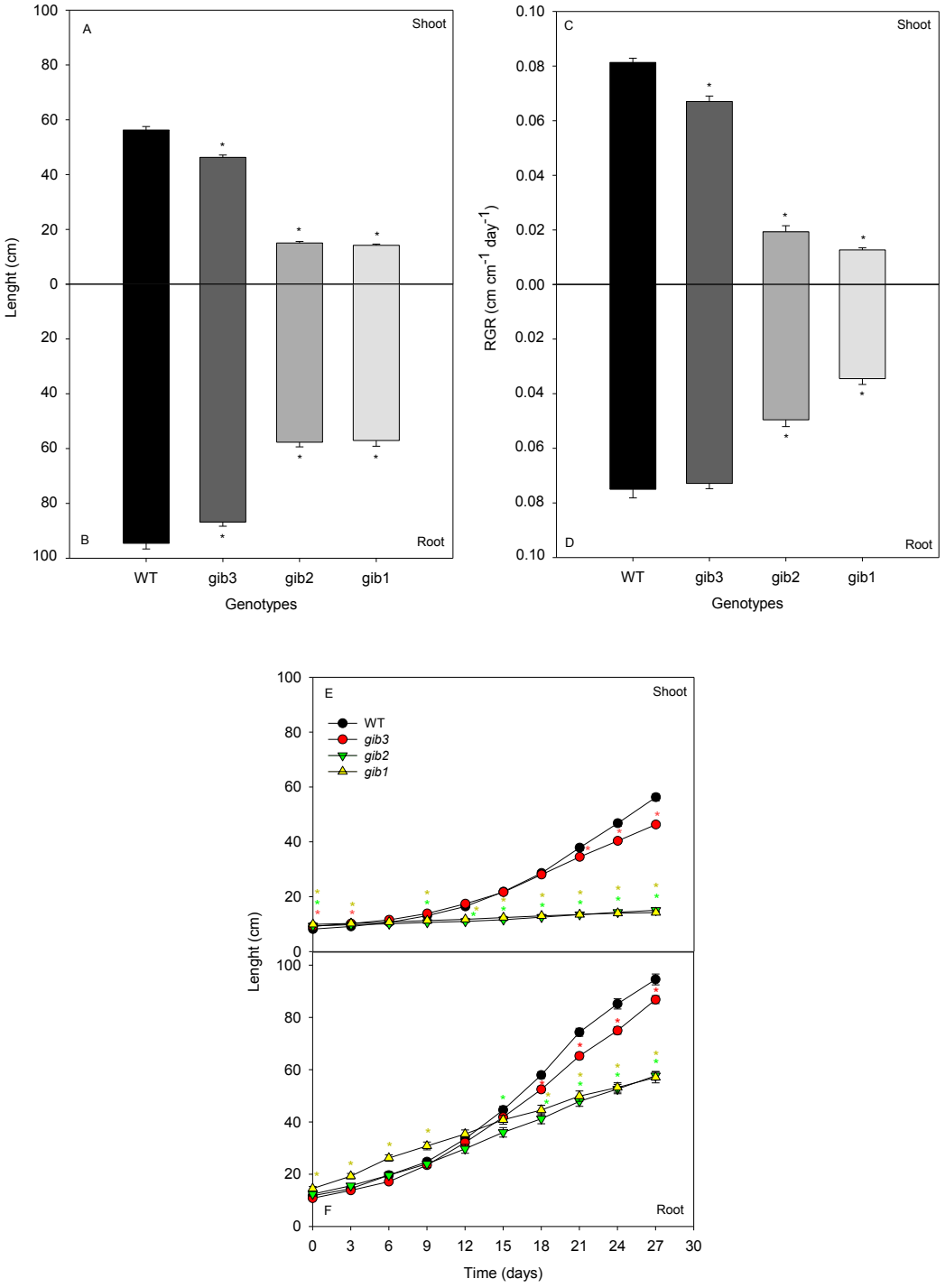


Figure 1. Variation in growth parameters related to shoot and roots length of tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis. (A) Final shoot length; (B) final root length; (C) relative growth rate (RGR) of shoot; (D) RGR of roots; (E) monitoring of shoot height growth at three-day intervals throughout the experiment; (F) monitoring the root elongation at three-day intervals throughout the experiment. Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing to WT.

At the end of the experiment, the dry weight of the plant organs as well as the whole plant (Supplementary Figure S1 and S2), root/shoot ratio and biomass partitioning between plant organs were determined (Figure 2). The mutant plants presented reduced dry weight of leaves, stems, roots and total dry weight. Increase in the root/shoot ratio and consequently the partitioning of root biomass was observed (Figure 2). Leaf, stem and root dry weight, and consequently total dry weight were significantly reduced in mutant plants in comparison to WT, with the most drastic reductions observed for gib2 and gib1 genotypes (Supplementary Figure S1 A, B, C and D).

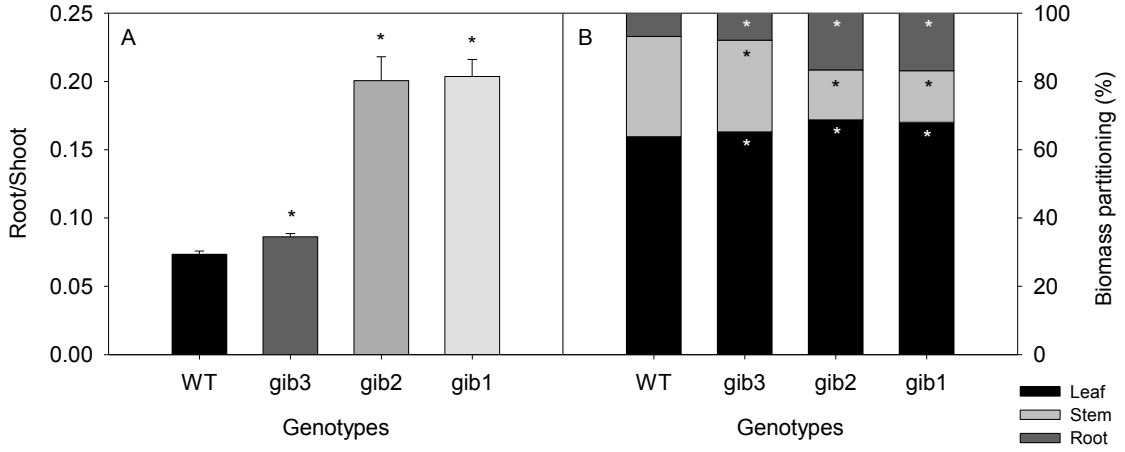


Figure 2. Variation in biometric parameters of tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in Hoagland complete solution. (A) ratio between root and shoot; (B) biomass partitioning where the bars represent the percentage of total dry weight subdivided between the components of plant. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 1.

Higher root/shoot ratio were found in the all mutant lines, most evidently in gib2 and gib1 plants, indicating a higher biomass partitioning in roots than shoot (Figure 2A). The biomass partitioning results confirm that gib3, gib2 and gib1 plants present high root biomass partitioning rate while WT presents lower biomass partitioning in this organ (Figure 2B). In the case of a whole plant, WT and gib3 have higher biomass partitioning in leaves, followed by stems and then in roots, while gib2 and gib1 allocate biomass mainly in leaves followed by roots and then stems. It is suggested, in the case of gib2 and gib1, that there is a reduction in the stems investment that is converted into greater root investment in these plants.

The results of specific leaf area were similar to those found for total leaf area in which mutant plants present smaller specific leaf area with significant reductions (Figure 3A). These results of specific leaf area indicate that plants with reduced GAs levels present thickening of the leaf limb. In the case of total leaf area was drastically lower in the mutant plants, mainly in gib2 and gib1 (Figure 3B), corroborating with the results of dry weight and growth.

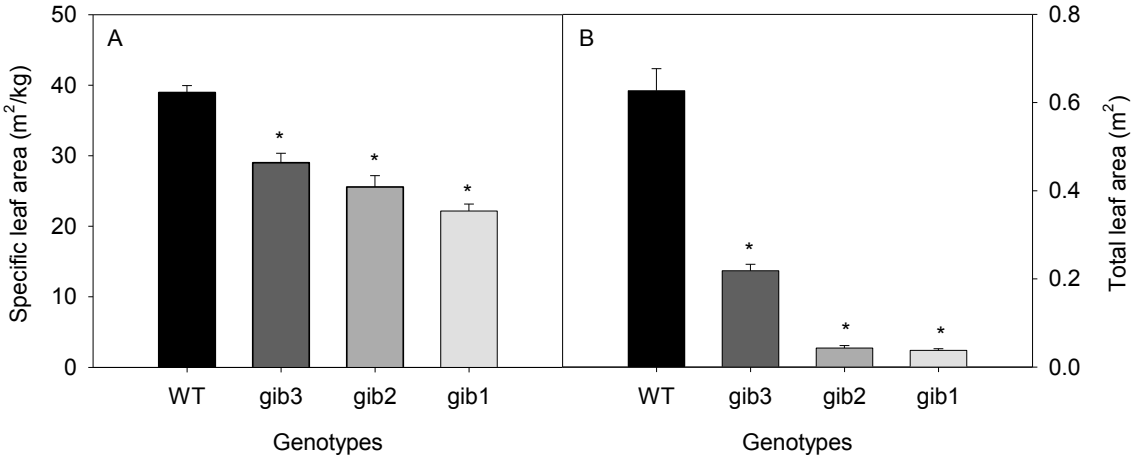


Figure 3. Changes in leaf area parameters from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in hydroponic system for four weeks. (A) Specific leaf area and (B) total leaf area measured at the end of the experiment. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 1.

As expected, total root length, surface area, total root volume and branches number were significantly reduced in the mutant lines. These four parameters showed a similar pattern among the genotypes, with gib3 being approximately 50% lower than WT and gib2 and gib1 with even smaller reductions than WT (Figure 4A, B and C). The branche number in the gib3 genotype was reduced, similar to the values observed for gib2 and gib1 in comparison to WT (Figure 4D).

The total length, surface area and total root volume were also determined according to root diameter, which these variables were calculated individually at intervals 0-1, 1-2, 2-3, 3-4, 4-5 and >5 mm of diameter (Supplementary Figure S1, S2 and S3). In the total root length in the range of 0-1 mm reductions are observed in the mutant genotypes, specifically the most GAs deficient lines, gib2 and gib1 (Supplementary Figure S1). In the diameters corresponding to the intervals 1-2 to 4-5 mm, the mutant genotypes presented similar pattern, and significant reductions were also observed at approximate levels of the three mutants when compared to WT

(Supplementary Figure S1 B, C, D and E). The lowest reductions in the total root length of the mutants occurred in the diameters 3-4, 4-5 and > 5 mm indicating that gib3, gib2 and gib1 plants, although they present most of their roots between the diameters of 0-2 mm, also have a large number of thick roots. It is noteworthy that in all diameters, root length was higher in WT plants. In addition, the four studied genotypes showed higher root length, especially in the smaller diameters, in decreasing order in the diameters 0-1, 1-2 and 2-3 mm.

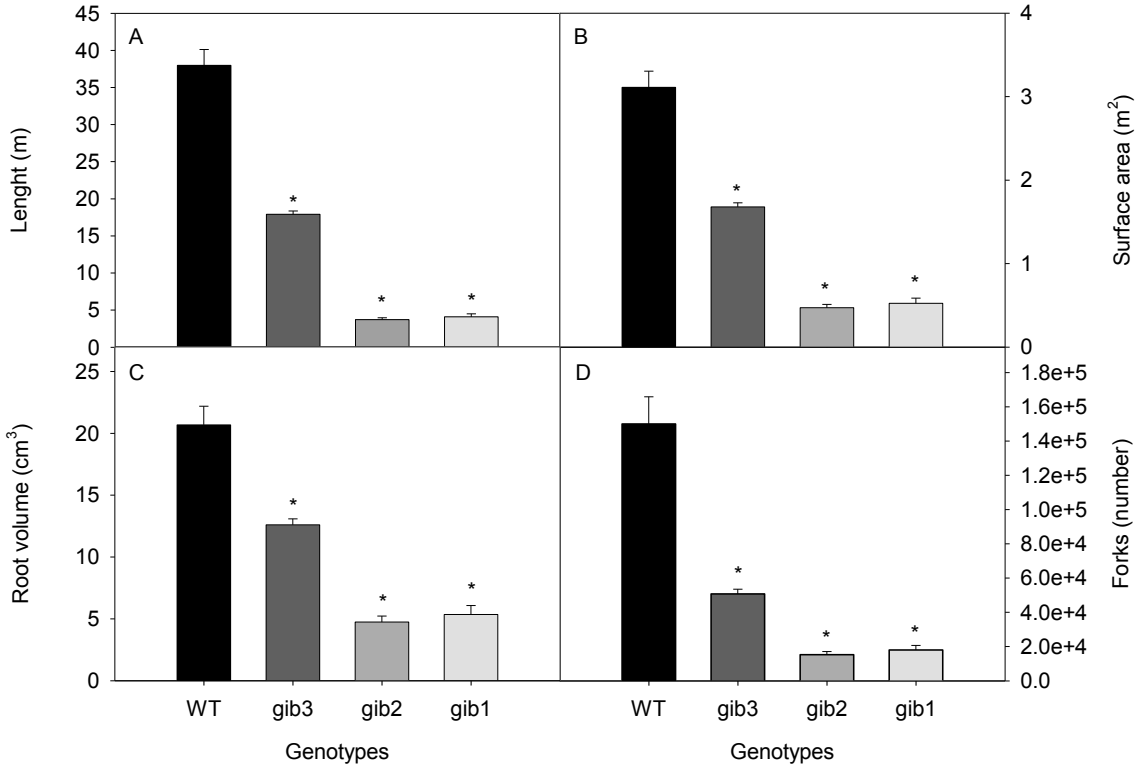


Figure 4. Variation in roots morphologic parameters from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in hydroponic system with Hoagland complete solution. (A) Total roots length; (B) root surface area; (C) total roots volume; (D) number of root branches. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 1.

The pattern of reduction in root surface area in mutant plants was identical to that observed in total root length for all diameters worked (Supplementary Figure S2). Interestingly, the roots volume of WT and mutant plants in the biosynthesis of GAs presented similar results to the parameters previously mentioned (Supplementary Figure S3).

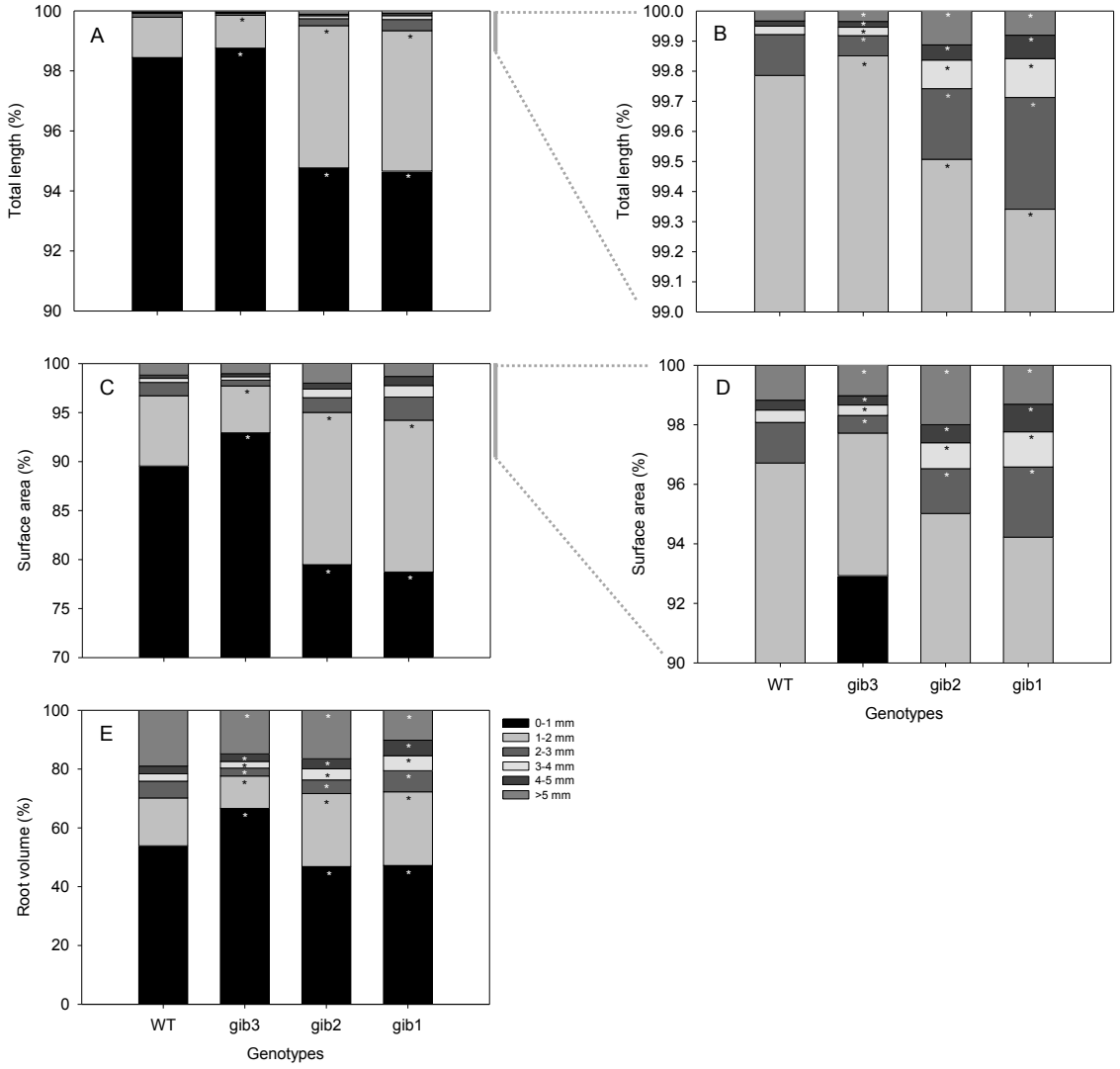


Figure 5. Roots morphologic parameters and proportional subdivision in all diameters analyzed from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in a Hoagland complete solution for four weeks. (A) and (B) Total roots length; (C) and (D) root surface area and; (E) total roots volume. (B) and (D) figures are interval enlargements of (A) and (C) figures, respectively, for better visualizations of the roots with the largest diameters. Values are presented as means of at least six plants. Statistical analysis as described in Figure 1.

In Figure 5, it is possible to visualize the proportion of thick and fine roots in WT and mutant plants in GAs biosynthesis. It can be observed that root length of WT and gib3 plants are composed mainly of fine roots, approximately 98.4% and 98.8% roots 0-1 mm in diameter and 1.3% and 1.1% of 1-2 mm, respectively (Figure 5A). The remaining 0.21% in WT and 0.14% in gib3 are distributed in thicker roots with diameters ranging from 2 mm to >5 mm (until 10 mm) (Figure 5B). For plants gib2 and gib1, the proportion of roots between 0-1 mm of diameter corresponds to 94.8% and 94.7%, respectively, and roots between 1-2 mm to 4.7% for both mutant genotypes

(Figure 5A). 0.46% and 0.66% of gib2 and gib1, respectively, are distributed in roots with diameters 2->5 mm (Figure 5B). Therefore, in gib2 and gib1, root lengths between 2 and >5 mm correspond to more than double of the same diameters evaluated in WT and gib3.

The proportion of roots in the surface area maintained the pattern observed for the root length (Figure 5C and D). WT plants are composed of 89.5%, 7.1% and 3.3% of roots with diameters of 0-1, 1-2 and 2-10 mm, respectively. Plants of gib3 mutant presented 92.9%, 4.8% and 2.3% of roots with 0-1, 1-2 and 2-10 mm in diameter. The gib2 has 79.5%, 15.6% and 4.9% in the same diameters mentioned above, while gib1 has 78.7%, 15.5% and 5.8%. Similarly, the pattern remained for root volume, but the percentage of thicker roots increased (Figure 5E). Roots comprised in the diameters of 0-1 and 1-2 mm comprised a total of 70.2% and 77.6% for WT and gib3, respectively. While for diameters larger than 2 mm, it was 29.8% and 22.4% for the same genotypes. For gib2 and gib1, 71.7% and 72.3% of the roots are between the diameters 0-2 mm, respectively, and 28.3% and 27.7% are roots larger than 2 mm.

Taken together, these biometric root data suggest that the low GA contents of mutant plants alter the proportion of fine and coarse roots. However, the roots from mutant plants still have higher length, surface area and volume in the smallest diameters, although differences between mutants and WT in larger diameters, such as diameters of 3-4, 4-5 and >5 mm, were found.

Carbohydrates allocation in mutant plants in GA biosynthesis

The mutants were characterized by a ^{14}C label incorporation and distribution rate similar in both leaves and roots of WT. There is a tendency of increase in the label redistribution in amino acids, starch in the starch/sugars ratio in leaves of mutant plants, while there is a tendency of reduction in sugars, organic acids and proteins, being significant only in proteins for the gib1 genotype. In the starch/sugars ratio, a significant difference was observed in gib2 when compared to WT, in addition to the increase tendency of the other mutants, suggesting that the glucose was being directed to starch synthesis in the mutants (Table 1).

In roots, the label redistribution rate in gib3 and gib2 was lower or very approximate to WT for all fractions analyzed, except for gib2 for amino acids and starch (Table 1). In gib1 plants, the label distribution was higher for all fractions when compared to WT, being significant for sugars and amino acids. The starch/sugars ratio

followed the same pattern of leaves, where mutants showed the highest values indicating higher glucose flux to starch synthesis than WT.

The CO_2 evolution was lower in mutant plants in the GAs biosynthesis in both organs, mainly in gib3 (Table 1). This suggests that gib3, gib2 and gib1 plants release lower CO_2 concentrations in both autotrophic (leaves) and heterotrophic (roots) tissues, indicating lower respiration in comparison to WT.

Table 1. Label redistribution after four hours incubation in [U^{14}C]-Glucose in leaves and roots of GAs mutant plants.

Parameter	**WT**	**gib3**	**gib2**	**gib1**
LEAVES		Bq g^{-1} FW		
Total uptake	367.14 ± 45.17	359.61 ± 26.03	334.88 ± 19.28	312.26 ± 10.85
Metabolised	362.56 ± 44.21	355.66 ± 25.97	329.55 ± 19.33	307.12 ± 10.51
CO_2 evolved*	0.1422 ± 0.008	0.0761 ± 0.006	0.1158 ± 0.009	0.1129 ± 0.016
Label incorporated in:		Bq g^{-1} FW		
Sucrose	139.19 ± 20.77	124.21 ± 2.88	111.44 ± 5.11	111.48 ± 2.95
Organic acids	136.72 ± 14.89	143.66 ± 12.48	112.50 ± 9.81	109.15 ± 1.83
Amino acids	12.73 ± 1.40	14.00 ± 1.18	15.96 ± 0.65	15.06 ± 0.85
Starch	42.27 ± 10.50	44.21 ± 11.51	57.70 ± 3.34	45.19 ± 7.58
Protein	2.85 ± 0.18	2.93 ± 0.34	2.78 ± 0.25	**1.77 ± 0.13**
Cell wall	28.80 ± 3.68	26.64 ± 1.41	29.16 ± 2.10	24.46 ± 1.70
Starch/Sucrose	0.33 ± 0.08	0.35 ± 0.09	**0.52 ± 0.02**	0.41 ± 0.07
ROOTS		Bq g^{-1} FW		
Total uptake	23.66 ± 6.62	21.25 ± 1.27	24.46 ± 4.13	29.09 ± 3.02
Metabolised	12.16 ± 2.95	10.98 ± 0.91	13.98 ± 2.54	18.12 ± 2.04
CO_2 evolved *	0.4805 ± 0.063	0.2268 ± 0.022	0.3796 ± 0.000	0.3572 ± 0.006
Label incorporated in:		Bq g^{-1} FW		
Sucrose	1.83 ± 0.22	1.41 ± 0.11	1.74 ± 0.27	**2.66 ± 0.31**
Organic acids	3.82 ± 0.79	3.43 ± 0.30	3.87 ± 0.46	5.92 ± 0.71
Amino acids	1.03 ± 0.26	1.03 ± 0.11	1.87 ± 0.32	**2.14 ± 0.23**
Starch	2.35 ± 0.73	2.02 ± 0.24	2.67 ± 0.44	3.37 ± 0.56
Protein	0.18 ± 0.05	0. 15 ± 0.01	0.18 ± 0.02	0.27 ± 0.02
Cell wall	2.95 ± 0.96	2.94 ± 0.26	2.83 ± 0.53	3.78 ± 0.49
Starch/Sucrose	1.21 ± 0.29	1.44 ± 0.11	1.52 ± 0.05	1.27 ± 0.14

Values are presented as mean ± SE of determinations on three individual plants per genotypes with two replicas per plant. Boldface type indicates values that were determined by the t test to be significantly different ($P<0.05$) from the wild type. FW, fresh weight. *as a % of ^{14}C supplied.

To determine in which fraction there was greater allocation of carbohydrates, the fractions were calculated as a percentage of what was metabolized by tissue, leaf or root (Table 2). In leaves of mutant plants, mainly gib2 and gib1, there was lower allocation of labeled glucose in sugars, organic acids and proteins, with significant difference only

in organic acids in gib2. Amino acids and starch had higher carbohydrate allocation in mutant plants, being significant in gib2 and gib1. It is noteworthy that the cell wall also presented higher glucose allocation in the mutant plants, but without significant differences when compared to WT. In roots, it was observed a greater allocation of glucose marked in sugars and amino acids in the mutants, with statistical differences in genotypes gib2 and gib1 compared to WT. In gib2 and gib1, the carbohydrate allocation to the cell wall was reduced, with a significant difference in the gib1 mutant. Organic acids, starch and root proteins of the mutants did not present significant differences when compared to WT (Table 2).

Tabela 2. Percentage of label incorporation in different fractions of leaves and roots of GAs mutant plants. Values are presented as a percentage of what was metabolized by tissue.

Parameters	**WT**	**gib3**	**gib2**	**gib1**
LEAVES				
Porcentage of metabolised		%		
Sucrose	37.73 ± 1,63	35.82 ± 1.53	33.65 ± 0.73	35.79 ± 0.70
Organic acids	38.22 ± 1.31	40.45 ± 1.08	**33.20 ± 0.27**	35.14 ± 1.08
Amino acids	3.57 ± 0.19	3.92 ± 0.13	**4.93 ± 0.13**	**4.88 ± 0.14**
Starch	14.54 ± 0.34	15.99 ± 0.62	**17.24 ± 0.24**	**16.74 ± 0.08**
Protein	0.85 ± 0.11	0.81 ± 0.03	0.83 ± 0.08	0.59 ± 0.03
Cell wall	8.00 ± 0.18	7.53 ± 0.22	8.71 ± 0.56	8.15 ± 0.35
ROOTS				
Porcentage of metabolised		%		
Sucrose	12.48 ± 0.35	12.94 ± 0.14	**13.37 ± 0.11**	**14.65 ± 0.51**
Organic acids	32.64 ± 1.69	31.13 ± 0.64	29.54 ± 1.85	32.89 ± 0.78
Amino acids	8.20 ± 0.86	9.54 ± 0.33	**14.27 ± 0.32**	**12.30 ± 0.45**
Starch	20.57 ± 0.48	19.30 ± 0.48	20.00 ± 0.28	19.47 ± 0.59
Protein	1.49 ± 0.18	1.44 ± 0.09	1.45 ± 0.08	1.51 ± 0.06
Cell wall	25.78 ± 1.20	25.79 ± 0.54	23.24 ± 0.65	**20.05 ± 1.15**

Values are presented as mean ± SE of determinations on three individual plants per genotypes with two replicas per plant. Boldface type indicates values that were determined by the t test to be significantly different ($P<0.05$) from the wild type. FW, fresh weight.

Discussion

Growth is increased and root morphology is altered by the reduction in GA levels

The gib2 and gib1 plants exhibit the dwarf phenotype characteristic of mutant lines that have a lower GA content also observed in other species (Fujioka et al., 1988; Magome et al., 2004; Sakamoto et al., 2004; Nir et al., 2013). These plants presented a much lower shoot length and also lower RGR compared to WT (Figure 1A, C e E). In contrast, the roots of gib2 and gib1 grew considerably more and grew faster compared to shoot, and the root RGR was increased in the mutant lines (Figure 1B, D and F). It is believed that the lower growth rate may lead to redirection of resources to support mechanisms that promote survival, since smaller plants with a more developed root system may be less vulnerable to stress (Achard et al., 2006). This indicates another line for future studies, since it is suggested that plants with reduced GAs levels can better withstand several extreme abiotic conditions (Nir et al., 2014).

Biomass is strongly reduced in plants with low GA levels (Figure 2). The higher values of root/shoot ratio in gib2 and gib1 plants in all applied treatments indicate higher biomass partitioning in roots than shoot (Figure 2E). In addition, for the whole plant, the highest biomass partitioning of the mutant plants occurred in leaves, roots and finally in stems (Figure 2F). Plants gib2 and gib1 have a dwarf phenotype due to the smaller investment in stems and these features have probably been directed to roots.

In addition, total root length, surface area and root volume data demonstrated that the low GA contents promoted a reduction of these parameters in the mutants (Figure 4). By analyzing the same parameters in each of the diameters worked (Figure 5 and supplementary figures S1, S2 and S3), it is possible to observe a change in the roots morphology of the mutants and a greater investment in roots with smaller diameters as in WT plants. Such morphological changes are related to the proportions of fine and thick roots, with gib2 and gib1 mutants having higher proportions of thick roots than WT and gib3 (Figure 5). However, despite the alteration of this proportion of thick roots, gib2 and gib1 maintained the growth of the great majority of their roots in the smaller diameters, roots responsible for the greater absorption of water and nutrients. Similarly, in studies with mutants in GA biosynthesis and with GA biosynthesis inhibitors, root thickening in pea and lettuce and finer roots were seen when treated with GA (Tanimoto, 1987, 1994).

Two hypotheses may explain the role of GAs in root lengthening and thickening. First, root elongation is regulated by significantly lower GAs concentration than stem. Second, the roots present a higher GA sensitivity in comparison to shoot. Long roots and short stems can be advantage when there is a limited production of GAs in plant (Tanimoto, 2012). It is noteworthy that the knowledge of molecular mechanisms of this greater sensitivity of roots to GA may also provide a new strategy to improve the root/shoot ratio for agricultural purposes, as happened in the called "green revolution" (Tanimoto, 2012).

Additionally, the total leaf area of the mutants is drastically reduced (Figure 3B). However, the leaves proportion of the mutants presented similar results and the roots proportion was increased, compared to WT (Figure 2). Therefore, even with reduced GAs levels, the proportion of mutants showed a greater investment in roots suggesting that shoot growth is decoupled from root growth because the responses are differentiated. In contrast, when analyzing growth and physiological responses of creeping bentgrass to changes in air and soil temperatures, it was found that soil temperature was more critical than air temperature for plant growth and roots may mediate shoot responses to heat stress (Xu and Huang, 2000). This response was due to direct inhibition of root growth and activity and, therefore, limitation of water and nutrient supplies to the shoot (Kramer, 1983), disruption of cytokinins synthesis in roots (Smart et al., 1991) and increasing abscisic acid transport from root to shoot (Udomprasert et al., 1995).

There are strong indications that root elongation and thickening can be regulated by a lowest GA concentration and/or by a greater roots sensitivity to GA giving advantage when GA production is limited (Tanimoto, 2012). Although the leaves of these mutants are smaller, they are thicker explaining the results of biomass partitioning to leaves. These leaf characteristics of the mutants have already been evidenced in previous studies (Koornneef et al., 1990). Corroborating with these results, gib2 and gib1 plants had a smaller specific leaf area (Figure 3A). The differences in leaves, stems and roots growth observed can be explained by (Tanimoto, 2012) using mutant plants also in GA biosynthesis, where the production of active GA in the plant is regulated locally, indicating that the shoot regulation is different from the root regulation. That is, even with reduced GAs amounts in roots, root growth and development is little affected proportionally.

Conversely, GA deficient Arabidopsis mutants showed reduced active GAs levels in root compared to WT, and this was associated with losses in root elongation (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2007; Willige et al., 2007). In transgenic tobacco plants with altered biosynthesis rate (GA20ox), catabolism (GA2ox1) and GA signaling (GAI), has been shown that GA have an inhibitory effect on adventitious roots and a stimulatory effect on root elongation of these plants (Niu et al., 2013). Reduced GA levels are necessary for maintenance of undetermined meristem cell fate (Sakamoto et al., 2001), whereas high GA concentrations promote cell differentiation and expansion (Jasinski et al., 2005; Shani et al., 2006). Increases in the GA endogenous content can induce significant increases in root elongation. On the other hand, the reduction of GA levels results in a decrease in the root growth rate (Lo et al., 2008). It is believed that reduced GA levels are required for the maintenance of undetermined meristematic cells (Sakamoto et al., 2001), while high GA concentrations promote cell differentiation and expansion (Shani et al., 2006). The regulation of the GA-dependent elongation is restricted to the root meristem and is not observed in the root elongation zone and root differentiation zone (Achard et al., 2009; Willige et al., 2011).

Shoot metabolism is not coupled to root metabolism

Leaf disks of WT plants and mutants in the GA biosynthesis were incubated in [U-^{14}C] -glucose for four hours and the evolution of CO_2 tended to decrease in mutants, it could be said that there was a tendency to reduce respiration (Table 1). Similarly, leaf dark respiration of GAs mutant plants using an infrared gas analyzer showed the same reduction pattern (Chapter 1). The consistency of the gas exchange and $^{14}CO_2$ uptake responses between intact plants and excised leaf discs suggests that the metabolic effects described above are due to a direct effect of endogenous GA reduction on the leaves. GAs biosynthesis is associated with respiration of whole plant cells, more specifically to 2-oxoglutarate, intermediate of the tricarboxylic acid (TCA) cycle. However, 2-oxoglutarate is not only an intermediate in the TCA cycle but also cofactor a variety of reactions, including the biosynthesis of amino acids and GAs, which can control biosynthesis rates (Araújo et al., 2014). Corroborating with the observations of these authors, 2-oxoglutarate significantly reduced in leaves the three mutants under well-watered conditions (Chapter 1). Others intermediate of TCA cycle as malate and succinate also reduced in gib2 and gib1. Inversely, the asparagine, glutamine, lysine,

isoleucine, serine and tryptophan increased indicating that 2-oxoglutarate may be being diverted to the synthesis of this amino acids.

We know that starch is the end product of photosynthesis and the predominant carbohydrate reserve in many species and is important for plant development (Gibon et al., 2004; Stitt and Zeeman, 2012). The carbon division alteration to starch and not to sugars, especially sucrose, may be the reason for the lower shoot growth of the mutant lines. In addition, it is suggested that proteins synthesis from amino acids is occurring at low rates and this accumulation of amino acids in the mutant plants caused in the reduction of TCA cycle activity corroborating with the lower organic acids and respiration rates, and lower shoot growth and leaf area in gib2 and gib1. According to (Hildebrandt et al., 2015), carbon skeletons from amino acids are converted into precursors or intermediates of the TCA cycle to support mitochondrial metabolism and ATP biosynthesis, while the oxidation of certain amino acids (such as leucine, isoleucine, valine and lysine) can directly supply electrons to the mitochondrial electron transport chain (Araújo et al., 2010; AraÚJo et al., 2012).

The reductions in CO_2 evolved at mutant roots, which can be called respiration, did not translate into lower root growth when compared to shoot. Probably because the shoots were being displaced to support the root growth, as the root/shoot ratio was higher (Figure 2A) and lower shoot RGR (Figure 1C and D). It is likely that most of the sucrose transported to roots have not been converted to reserve carbohydrates like starch (Stitt and Zeeman, 2012). Additionally, the allocation of labeled carbon to amino acids was significantly higher in gib2 and gib1 indicating that this source of alternative energy reserve was not being used, corroborating the low respiration rates found since the release of $^{14}CO_2$ and higher accumulation of glutamine, homoserine, methionine, ornithine, tryptophan, tyrosine and β-alanine (Chapter 1). The GA might modify primary metabolism mat the entry point of TCA cycle. In addition, it has been demonstrated by the over expression of genes associated with GA biosynthesis (GA 20-oxidase) or catabolism that GA (GA 2-oxidase) levels play key roles on transcription genes influencing plant growth (Biemelt et al., 2004; Dayan et al., 2010). Thus, accumulation of amino acids in the mutant plants may be caused in the reduction of TCA cycle intermediate, mainly 2-oxoglutarate, citrate, malate and succinate (Chapter 1), corroborating with the lower organic acids and respiration rates founded here, and lower root growth in gib2 and gib1 compared to WT.

In general, evolved CO_2 and incorporation of labeled carbon presented different values and patterns when comparing leaves and roots. In other works of the same research group, it can be observed that the metabolic profile of leaves and roots of the same genotypes followed the pattern found in the present study (Martins 2017, thesis; Chapter 1). Therefore, it is suggested that leaf metabolism is not coupled to root metabolism, as also observed for growth parameters. It is important to emphasize that, when says that the metabolism or root growth is decoupled from the shoot, it does not mean that they are independent organs, but mean that the responses between the organs are differentiated.

Conclusions

In summary, root growth and morphology appear to be less affected by the low content of endogenous GAs than shoot. The lack of GA changes the metabolism respiratory and labeled carbon allocation in leaves and roots differently, probably due to the higher GA sensitivity of roots. Additionally, the lower shoot and root growth in gib2 and gib1 was due the accumulation of amino acids in both organs of the mutant plants causing the reduction of TCA cycle intermediaries corroborating with the lower respiration rates. Therefore, our data suggest that the shoot responds differently from the root and that the root growth and primary metabolism are decoupled from the shoot in GA-deficient plants.

Acknowledgments

This work was supported by funding from the Max Planck Society (to ANN and WLA) and the National Council for Scientific and Technological Development (CNPq-Brazil, Grant 306355/2012-4 to ANN and Grant 483525/2012-0 to WLA) and the FAPEMIG (Foundation for Research Assistance of the Minas Gerais State, Brazil, Grant APQ-01357-14 to WLA). We also thank the scholarships granted by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES-Brazil) to RPOG. Research fellowships granted by CNPq-Brazil to ANN and WLA are also gratefully acknowledged.

Literature cited

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of Plant Responses to Environmentally Activated Phytohormonal Signals. Science **311:** 91-94

Achard P, Gusti A, Cheminant S, Alioua M, Dhondt S, Coppens F, Beemster GT, Genschik P (2009) Gibberellin signaling controls cell proliferation rate in Arabidopsis. Current biology **19:** 1188-1193

Albacete A, Ghanem ME, Martínez-Andújar C, Acosta M, Sánchez-Bravo J, Martínez V, Lutts S, Dodd IC, Pérez-Alfocea F (2008) Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (Solanum lycopersicum L.) plants. Journal of Experimental Botany **59:** 4119-4131

Araújo WL, Ishizaki K, Nunes-Nesi A, Larson TR, Tohge T, Krahnert I, Witt S, Obata T, Schauer N, Graham IA (2010) Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. The Plant Cell **22:** 1549-1563

Araújo WL, Martins AO, Fernie AR, Tohge T (2014) 2-Oxoglutarate: linking TCA cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis. Frontiers in plant science **5**

AraÚJo WL, Nunes-Nesi A, Nikoloski Z, Sweetlove LJ, Fernie AR (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant, Cell & Environment **35:** 1-21

Benincasa M (1988) Análise de crescimento de plantas: noções básicas. Funep

Bensen RJ, Zeevaart JAD (1990) Comparison of Ent-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and gib-1, gib-2, and gib-3 tomato plants. J Plant Growth Regul **9:** 237-242

Biemelt S, Tschiersch H, Sonnewald U (2004) Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. Plant Physiology **135:** 254-265

Busov V, Meilan R, Pearce DW, Rood SB, Ma C, Tschaplinski TJ, Strauss SH (2006) Transgenic modification of gai or rgl1 causes dwarfing and alters gibberellins, root growth, and metabolite profiles in Populus. Planta **224:** 288-299

Centeno DC, Osorio S, Nunes-Nesi A, Bertolo AL, Carneiro RT, Araújo WL, Steinhauser M-C, Michalska J, Rohrmann J, Geigenberger P (2011) Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. The Plant Cell **23:** 162-184

Dayan J, Schwarzkopf M, Avni A, Aloni R (2010) Enhancing plant growth and fiber production by silencing GA 2- oxidase. Plant Biotechnology Journal **8:** 425-435

Eriksson S, Böhlenius H, Moritz T, Nilsson O (2006) GA4 is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. The Plant Cell **18:** 2172-2181

Evans GC (1972) The quantitative analysis of plant growth, Vol 1. Univ of California Press

Fernie AR, Roscher A, Ratcliffe RG, Kruger NJ (2001) Fructose 2, 6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta **212:** 250-263

Fujioka S, Yamane H, Spray CR, Gaskin P, Macmillan J, Phinney BO, Takahashi N (1988) Qualitative and Quantitative Analyses of Gibberellins in Vegetative Shoots of Normal, dwarf-1, dwarf-2, dwarf-3, and dwarf-5 Seedlings of Zea mays L. Plant Physiol **88:** 1367-1372

Gao X-H, Xiao S-L, Yao Q-F, Wang Y-J, Fu X-D (2011) An Updated GA Signaling 'Relief of Repression' Regulatory Model. Mol Plant **4:** 601-606

Gibon Y, Blaesing OE, Hannemann J, Carillo P, Höhne M, Hendriks JHM, Palacios N, Cross J, Selbig J, Stitt M (2004) A Robot-Based Platform to Measure Multiple Enzyme Activities in Arabidopsis Using a Set of Cycling Assays: Comparison of Changes of Enzyme Activities and Transcript Levels during Diurnal Cycles and in Prolonged Darkness. The Plant Cell **16:** 3304-3325

Griffiths J, Murase K, Rieu I, Zentella R, Zhang Z-L, Powers SJ, Gong F, Phillips AL, Hedden P, Sun T-p (2006) Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. The Plant Cell **18:** 3399-3414

Hedden P (2003) The genes of the Green Revolution. Trends in Genetics **19:** 5-9

Hedden P (2012) Gibberellin Biosynthesis. In eLS. John Wiley & Sons, Ltd

Hedden P, Kamiya Y (1997) Gibberellin Biosynthesis: Enzymes, Genes and Their Regulation. Plant Physiol **48:** 431-460

Hildebrandt TM, Nesi AN, Araújo WL, Braun H-P (2015) Amino acid catabolism in plants. Molecular plant **8:** 1563-1579

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circular. California Agricultural Experiment Station **347**

Hunt R (1982) Plant growth curves. The functional approach to plant growth analysis. Edward Arnold Ltd.

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. Current Biology **15:** 1560-1565

Kaneko M, Itoh H, Inukai Y, Sakamoto T, Ueguchi- Tanaka M, Ashikari M, Matsuoka M (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? The Plant Journal **35:** 104-115

Koornneef M, Bosma TDG, Hanhart CJ, Veen JH, Zeevaart JAD (1990) The isolation and characterization of gibberellin-deficient mutants in tomato. Theor Appl Genet **80:** 852-857

Kramer P (1983) Water stress research: progress and problems [Plant physiology].

Lo S-F, Yang S-Y, Chen K-T, Hsing Y-I, Zeevaart JA, Chen L-J, Yu S-M (2008) A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. The Plant Cell **20:** 2603-2618

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2004) dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. Plant J **37:** 720-729

Mielke MS, Hoffmann A, Endres L, Fachinello JC (1995) Comparison of laboratory and field methods for the estimation of leaf area of wild fruit species. Sci Agri **52:** 82-88

Nagel O, Lambers H (2002) Changes in the acquisition and partitioning of carbon and nitrogen in the gibberellin- deficient mutants A70 and W335 of tomato (Solanum lycopersicum L.). Plant, Cell & Environment **25:** 883-891

Nir I, Moshelion M, Weiss D (2014) The Arabidopsis GIBBERELLIN METHYL TRANSFERASE 1 suppresses gibberellin activity, reduces whole- plant transpiration and promotes drought tolerance in transgenic tomato. Plant, cell & environment **37:** 113-123

Nir IDO, Moshelion M, Weiss D (2013) The Arabidopsis Gibberellin Methyl Transferase 1 suppresses gibberellin activity, reduces whole-plant transpiration and promotes drought tolerance in transgenic tomato. Plant Cell Environ**:** n/a-n/a

Niu S, Li Z, Yuan H, Fang P, Chen X, Li W (2013) Proper gibberellin localization in vascular tissue is required to regulate adventitious root development in tobacco. Journal of experimental botany **64:** 3411-3424

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin Signaling: Biosynthesis, Catabolism, and Response Pathways. Plant Cell **14:** S61-S80

Regnault T, Davière J-M, Achard P (2016) Long-distance transport of endogenous gibberellins in Arabidopsis. Plant Signaling & Behavior **11:** e1110661

Regnault T, Davière J-M, Wild M, Sakvarelidze-Achard L, Heintz D, Carrera Bergua E, Lopez Diaz I, Gong F, Hedden P, Achard P (2015) The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. Nature Plants **1:** 15073

Ribeiro DM, Araújo WL, Fernie AR, Schippers JHM, Mueller-Roeber B (2012) Translatome and metabolome effects triggered by gibberellins during rosette growth in Arabidopsis. J Exp Bot **63:** 2769-2786

Sakamoto T, Kamiya N, Ueguchi-Tanaka M, Iwahori S, Matsuoka M (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes & Development **15:** 581-590

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, Miyao A, Hirochika H, Kitano H, Ashikari M, Matsuoka M (2004) An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice. Plant Physiol **134:** 1642-1653

Shani E, Weinstain R, Zhang Y, Castillejo C, Kaiserli E, Chory J, Tsien RY, Estelle M (2013) Gibberellins accumulate in the elongating endodermal cells of Arabidopsis root. Proceedings of the National Academy of Sciences **110:** 4834-4839

Shani E, Yanai O, Ori N (2006) The role of hormones in shoot apical meristem function. Current opinion in plant biology **9:** 484-489

Sharp RE, Davies WJ, Sharp RE, Davies. WJ (1989) Regulation of growth and development of plants growing with a restricted supply of water Plants under Stress. Cambridge University Press

Silverstone AL, Chang Cw, Krol E, Sun Tp (1997) Developmental regulation of the gibberellin biosynthetic gene GA1 in Arabidopsis thaliana. The Plant Journal **12:** 9-19

Smart CM, Scofield SR, Bevan MW, Dyer TA (1991) Delayed leaf senescence in tobacco plants transformed with tmr, a gene for cytokinin production in Agrobacterium. The Plant Cell **3:** 647-656

Smith VA, Knatt CJ, Gaskin P, Reid JB (1992) The distribution of gibberellins in vegetative tissues of Pisum sativum LI Biological and biochemical consequences of the le mutation. Plant physiology **99:** 368-371

Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. Current opinion in plant biology **15:** 282-292

Sun T-p, Gubler F (2004) Molecular Mechanism of Gibberellin Signaling in Plants. Annu Rev Plant Biol **55:** 197-223

Tal I, Zhang Y, Jørgensen ME, Pisanty O, Barbosa IC, Zourelidou M, Regnault T, Crocoll C, Olsen CE, Weinstain R (2016) The Arabidopsis NPF3 protein is a GA transporter. Nature communications **7**

Tanimoto E (1987) Gibberellin-dependent root elongation in Lactuca sativa: recovery from growth retardant-suppressed elongation with thickening by low concentration of GA3. Plant and cell physiology **28:** 963-973

Tanimoto E (1994) Interaction of gibberellin A3 and ancymidol in the growth and cell-wall extensibility of dwarf pea roots. Plant and Cell Physiology **35:** 1019-1028

Tanimoto E (2012) Tall or short? Slender or thick? A plant strategy for regulating elongation growth of roots by low concentrations of gibberellin. Annals of Botany **110:** 373-381

Udomprasert N, Li PH, Davis DW, Markhart AH (1995) Effects of root temperatures on leaf gas exchange and growth at high air temperature in Phaseolus acutifolius and Phaseolus vulgaris. Crop science **35:** 490-495

Ueguchi-Tanaka M, Nakajima M, Katoh E, Ohmiya H, Asano K, Saji S, Hongyu X, Ashikari M, Kitano H, Yamaguchi I (2007) Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. The Plant Cell **19:** 2140-2155

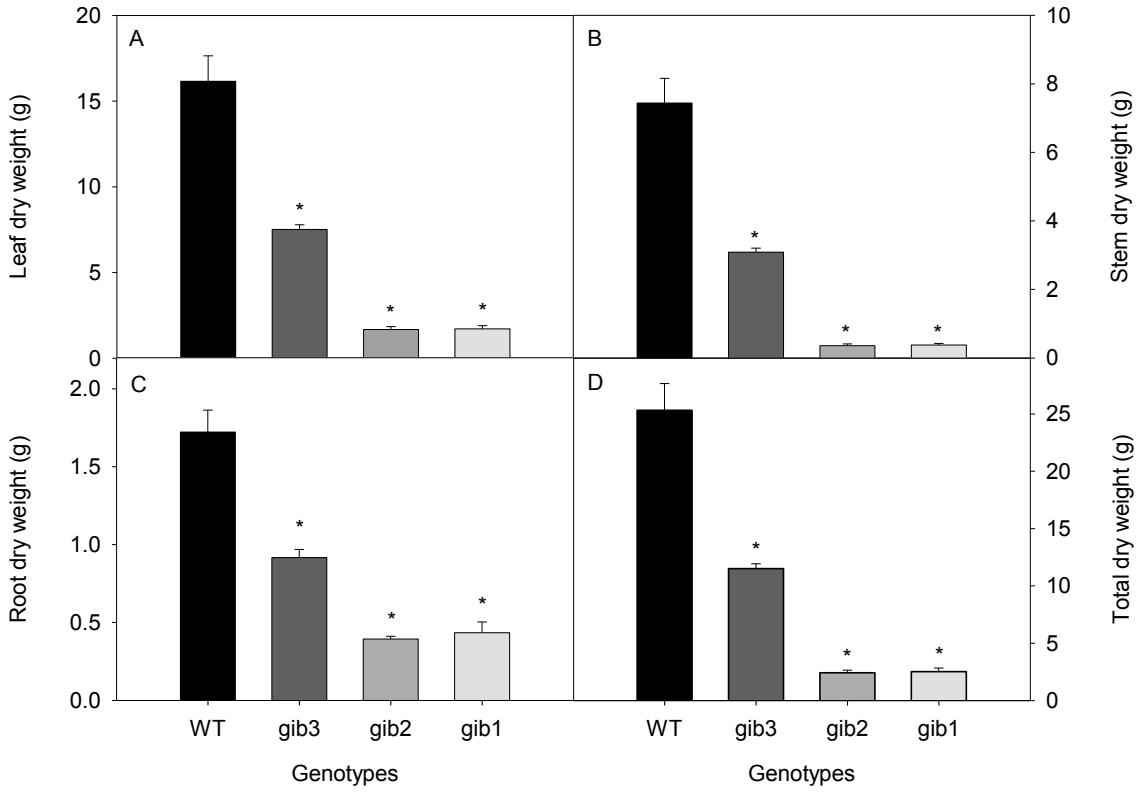
Willige BC, Ghosh S, Nill C, Zourelidou M, Dohmann EM, Maier A, Schwechheimer C (2007) The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of Arabidopsis. The Plant Cell **19:** 1209-1220

Willige BC, Isono E, Richter R, Zourelidou M, Schwechheimer C (2011) Gibberellin regulates PIN-FORMED abundance and is required for auxin transport–dependent growth and development in Arabidopsis thaliana. The Plant Cell **23:** 2184-2195

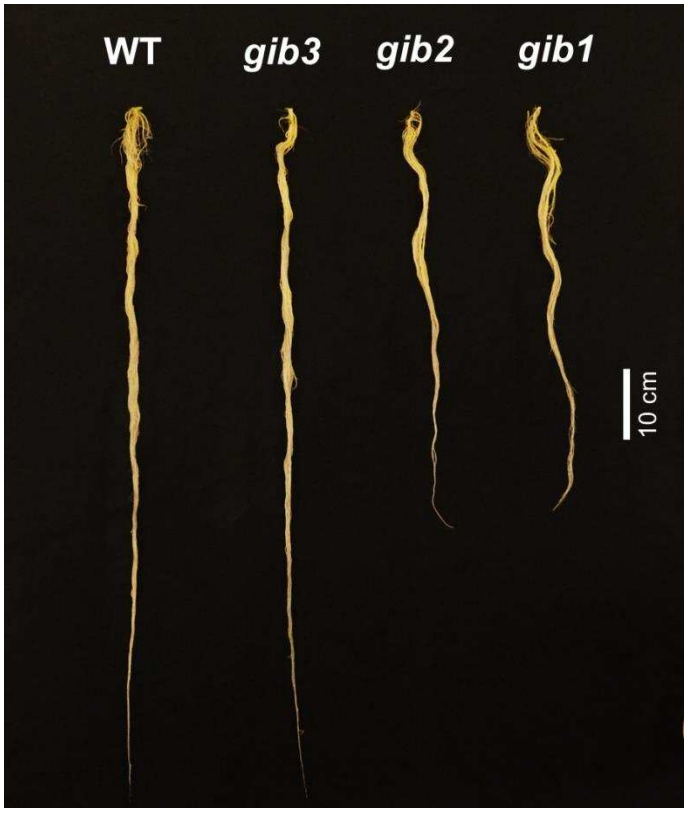
Xu Q, Huang B (2000) Growth and physiological responses of creeping bentgrass to changes in air and soil temperatures. Crop Science **40:** 1363-1368

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) Elongates Uppermost Internode Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. Plant Cell **18:** 442-456

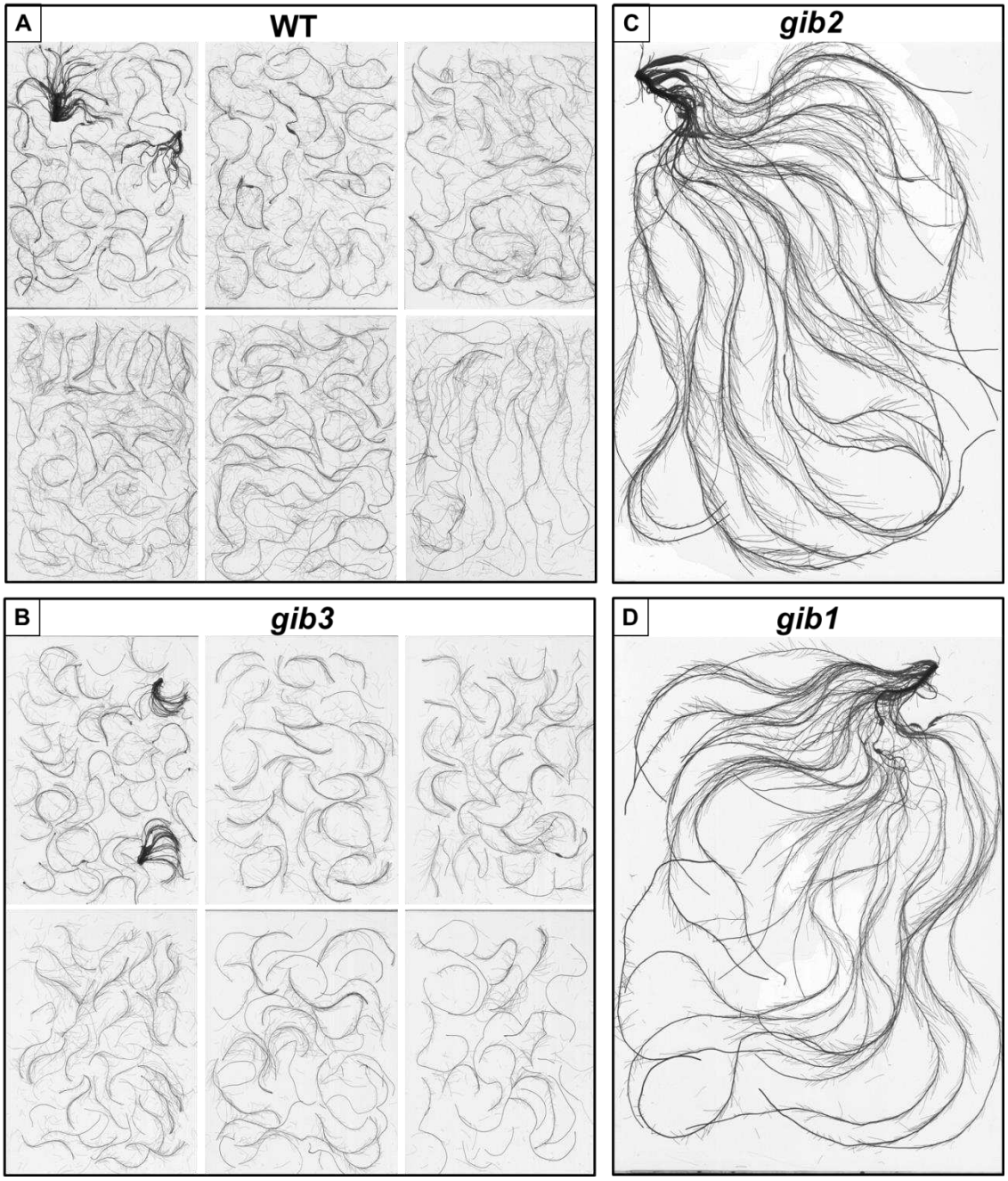
Supplementary data



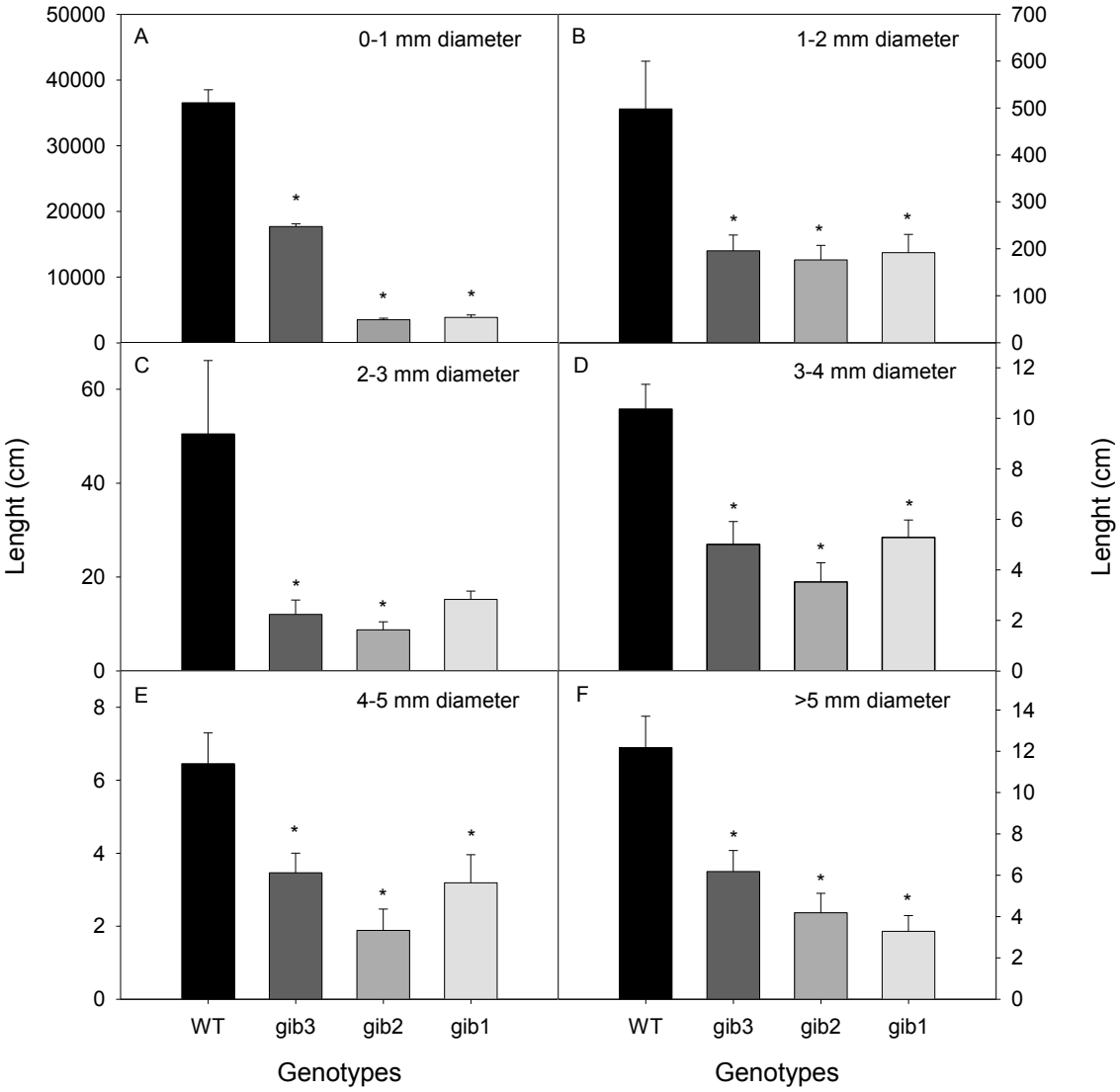
Supplementary figure S1. Variation in biometric parameters of tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in Hoagland complete solution. (A) Leaf dry weight; (B) stem dry weight; (C) roots dry weight and; (D) total plant dry weight. Values are presented as means ± standard error of at least six plants. Asterisks indicates significant difference by Student's t test at 5% probability ($P<0.05$) among genotypes in the same treatment, always comparing to WT.



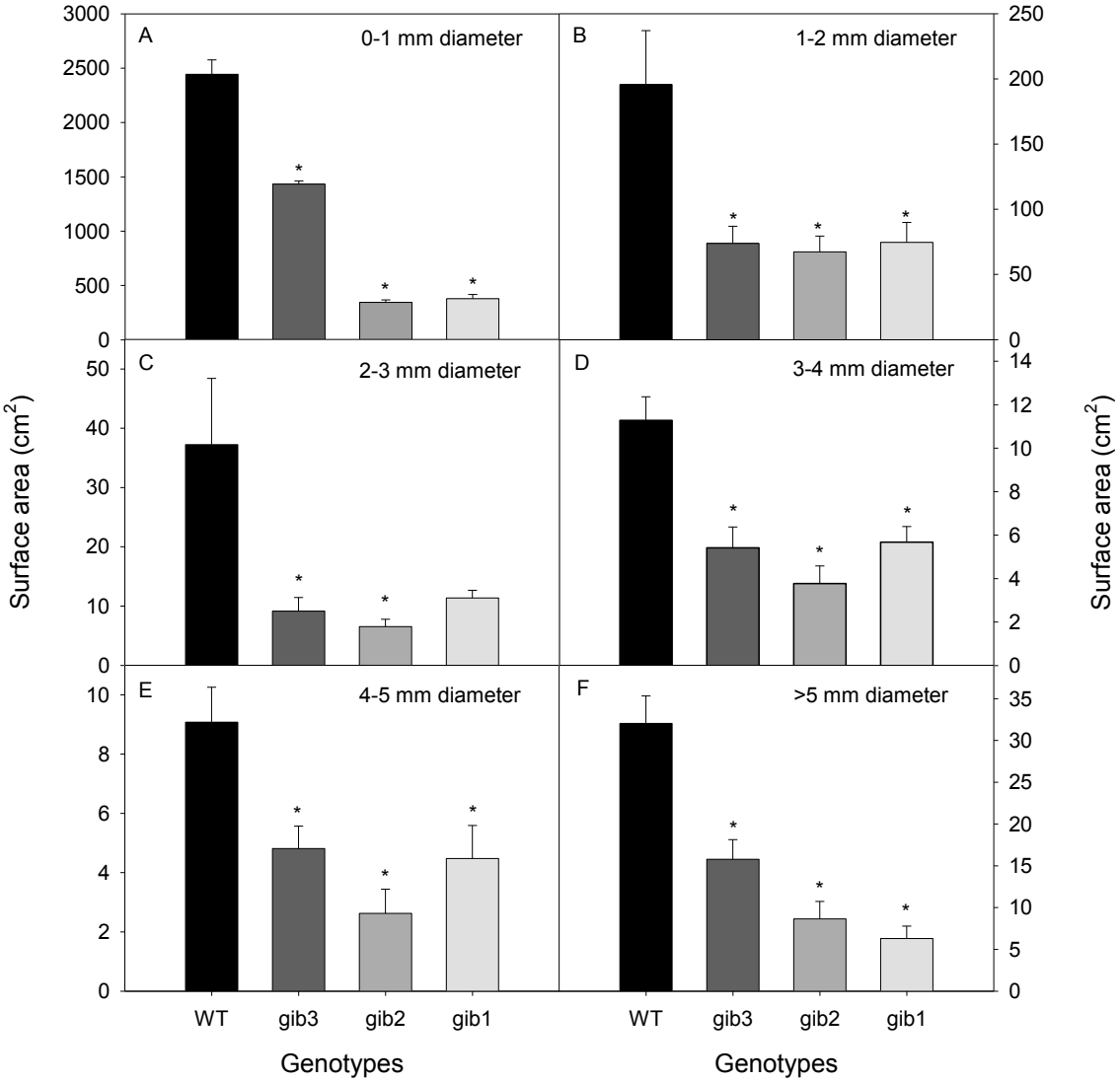
Supplementary figure S2. Root visual aspect tomato plants (Solanum lycopersicum cv. Moneymaker) with low levels of endogenous GAs, gib3, gib2 and gib1, at the end of experiment.



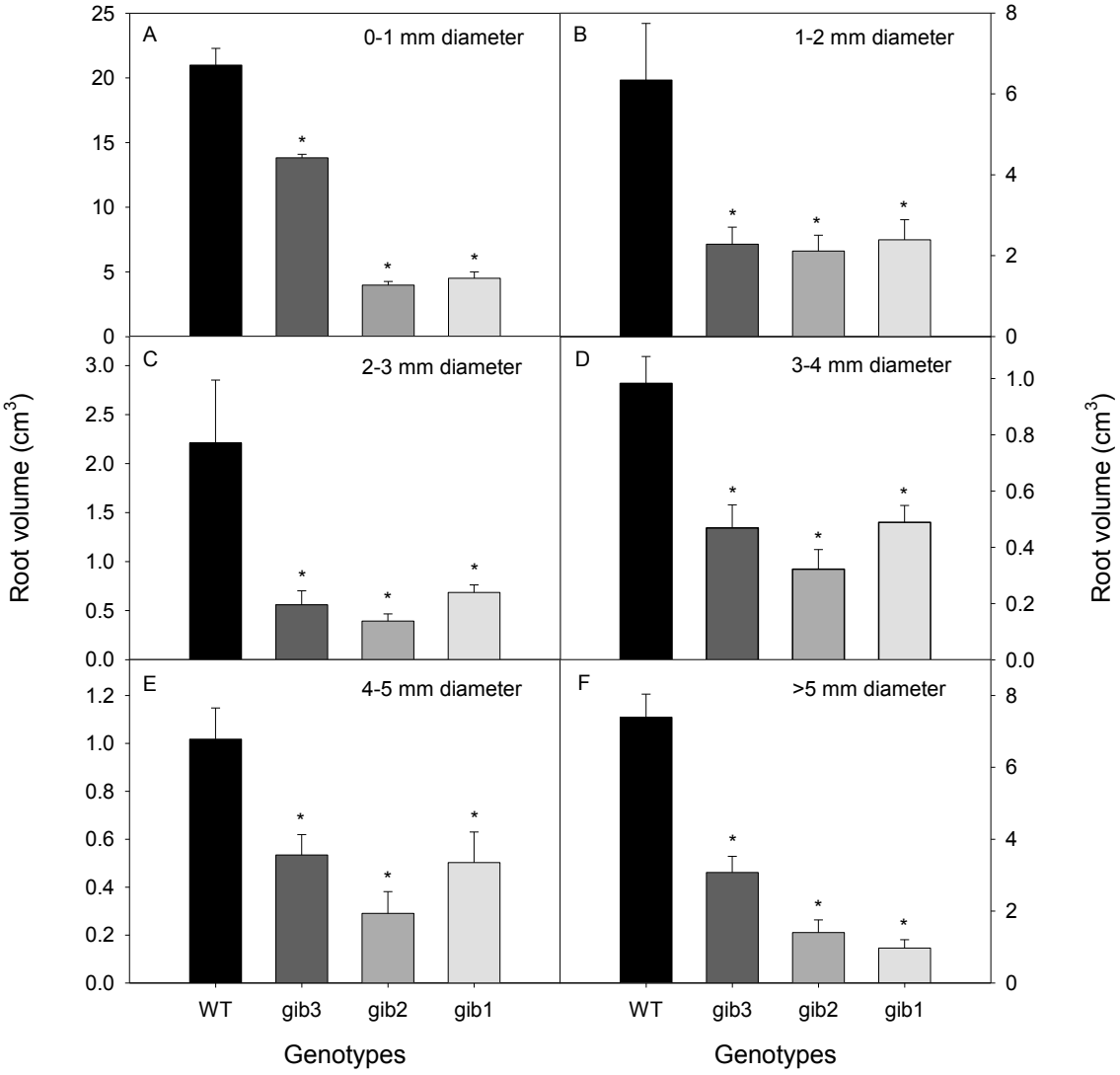
Supplementary figure S3. Root system images of tomato plants (Solanum lycopersicum cv. Moneymaker) obtained with WinRHIZO Pro 2007a attached to a scanner. (A) Root fragments of WT plant; (B) root fragments of gib3 plant; (C) root of gib2 plant and; (D) root of gib1 plant. Root of WT and gib3 plants were fragmented in six parts and each one scanned separately. Each image has 40 cm length x 30 cm width.



Supplementary figure S4. Total root fractions length, separated by diameter, from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in hydroponic system with Hoagland complete solution for four weeks. (A) Total root length with diameters from 0 to 1 mm; (B) total root length with diameters from 1 to 2 mm; (C) total root length with diameters from 2 to 3 mm; (D) total root length with diameters from 3 to 4 mm; (E) total root length with diameters from 4 to 5 mm; (F) total root length with diameters >5 mm. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S1.



Supplementary figure S5. Total root fractions surface area, separated by diameter, from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in hydroponic system with Hoagland complete solution for four weeks. (A) Root surface area with diameters from 0 to 1 mm; (B) root surface area with diameters from 1 to 2 mm; (C) root surface area with diameters from 2 to 3 mm; (D) root surface area with diameters from 3 to 4 mm; (E) root surface area with diameters from 4 to 5 mm; (F) root surface area with diameters >5 mm Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S1.



Supplementary figure S6. Total root fractions volume, separated by diameter, from tomato plants (Solanum lycopersicum cv. Moneymaker) WT and mutants in GAs biosynthesis grown in hydroponic system with Hoagland complete solution for four weeks. (A) Root volume with diameters from 0 to 1 mm; (B) root volume with diameters from 1 to 2 mm; (C) root volume with diameters from 2 to 3 mm; (D) root volume with diameters from 3 to 4 mm; (E) root volume with diameters from 4 to 5 mm; (F) root volume with diameters >5 mm. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S1.

Chapter 3

Reciprocal grafting promote shoot and root growth in GA-deficient tomato plants

Abstract

Little known about the regulation of the hormones, RNAs, proteins transport from biosynthesis places to tissues and organs that are require for growth. To investigate the signaling transport from shoot to root and vice versa, it was performed reciprocal grafts of WT and tomato mutants lines (Solanum lycopersicum) gib3, gib2 and gib1, with are moderate, medium and highly GA-deficient mutants, respectively. Biometric, morphologic and metabolic parameters were analyzed in leaves and roots. In this study it was suggested that some signal, as precursor or active GA, derived from shoot might be transported to the roots, and vice versa, under optimal growth conditions for tomato plants. This conclusion was possible due to reversion of the root and shoot phenotype of GA-deficient mutants, especially gib2 and gib1. In addition, of the quantified metabolites in leaves and roots, amino acids in mutant rootstock were been used to growth probably. However, further studies are still needed to identify the transported molecule between the organs of GA-deficient tomato plants.

Introduction

The gibberellins (GAs) are an important family of diterpenoid compounds, of which only few member such as GA_1 and GA_4, with multiple functions in the regulation of physiological processes associated by growth and development (Hedden and Kamiya, 1997; Olszewski et al., 2002; Sakamoto et al., 2004; Sun and Gubler, 2004; Zhu et al., 2006; Gao et al., 2011; Regnault et al., 2016). During plant life cycle, GAs influence seed germination, vegetative growth, flowering and fruit development (Hedden and Thomas, 2012). GAs also respond to intrinsic (development) and extrinsic (environmental) stimuli, which can regulate their biosynthesis, inactivation, perception or signal transduction, acting at various GA pathway points (Hedden and Thomas, 2012).

The GAs can be synthesized in young and actively growing organs, such as developing leaves and expanding internodes, but also in roots (Smith et al., 1992), which are also sites of action of these GAs (Hedden, 2012). It has been previously reported that GAs in Arabidopsis roots are mainly accumulated in the endoderm cells of stretching zone and the presence of an GA active transport mechanism has also been strongly suggested (Shani et al., 2013). However, GAs production in maturing leaves occurs in plastids associated with the foliar vascular system (Silverstone et al., 1997), exporting GAs to other parts of the plant by phloem (Eriksson et al., 2006). Currently, it is known that GAs can be transported cell-cell (or between tissues) through membrane transporters (Tal et al., 2016) or from one organ to another, as from root to leaves (and vice versa), through xylem and phloem conducting vessels (Regnault et al., 2015; Regnault et al., 2016). The optimal levels of active GAs are controlled by regulatory mechanisms that involve genes encoding GA biosynthesis enzymes. Moreover, little is known about the regulation of GA transport from biosynthesis places to tissues and organs that require GAs for growth.

The grafting and micrografting technique is an excellent approach to study the long-range GAs signaling in plants (Bidadi et al., 2014; Notaguchi and Okamoto, 2015; Spiegelman et al., 2015; Regnault et al., 2016). However, for the study of short-range GAs transport, experiments with the exogenous GAs application are more indicated to show the precursors and bioactive GAs movement (Dayan et al., 2012; Shani et al., 2013). Using grafting experiments with pea WT on mutants in the GAs biosynthesis, it was shown the influence of the root system on the shoot with the GAs supply by

reversing the plant phenotype (Dodd, 2005). In addition, it has recently been shown through micrografting and biochemical approaches that the GA_{12} precursor is mobile GA transported over long distances through the Arabidopsis vascular system (Regnault et al., 2015; Regnault et al., 2016).

Some carriers of plant hormones at short distances have been identified in Arabidopsis, yeast and oocytes (Kanno et al., 2012) The Arabidopsis NRT1/PTR FAMILY (NPF) proteins, initially characterized as nitrate or di/tri-peptide transporters, ware also identified as plant hormone transporter like auxin (AtNPF6.3/CHL1/NRT1.1) (Krouk et al., 2010), abscisic acid (NPF4.6/NRT1.2, AtNPF6.3/CHL1/NRT1.1, AtNPF2.10/GTR e AtNPF3.1) (Huang et al., 1999; Krouk et al., 2010; Chiba et al., 2015; Tal et al., 2016), jasmonoyl-isoleucine (AtNPF2.10/GTR1) (Chiba et al., 2015), and more recently, GAs (AtNPF2.10/GTR1, AtNPF3.1) (Chiba et al., 2015; Tal et al., 2016). The AIT3 proteins, a member of the NRT1/PTR family, can transport abscisic acid as well as GA_3 in yeast (Kanno et al., 2012). AtSWEET13 and AtSWEET14 are some Arabidopsis SWEET proteins transport GA when expressed in yeast and Xenopus oocytes (Kanno et al., 2016).

The long-range traffic of signaling molecules by conducting vessels such as hormones, RNAs and proteins have been identified and characterized in plants (Giavalisco et al., 2006; Lough and Lucas, 2006; Omid et al., 2007; Bidadi et al., 2014; Spiegelman et al., 2015). Recently studies, demonstrated that ABA can be transported over long distances through the tomato and pea vascular system (McAdam et al., 2016). It was observed that GAs are transported in Arabidopsis by xylem and phloem (Regnault et al., 2015; Regnault et al., 2016). FLOWERING LOCUS T (FT) protein is best characterized at long-range, from leaves to apex, in several species with control function the flowering beginning (Corbesier et al., 2007; Tamaki et al., 2007). CLAVATA3/ESR-embryo surrounding region-related (CLE) peptide plays a central role in cell-cell communication and controls the division/differentiation of meristematic tissue cells through plant hormone-regulated signaling pathways such as auxin and cytokinin (Hejátko et al., 2009). CLE6 acts at long-range and is one of the genes coding for CLE peptides, is highly expressed in hypocotyls and roots, especially in stem cells. The systemic effect of CLE6 was verified since it was transported via xylem from root to shoot and partially compensated for the dwarfism associated with GA deficiency that was demonstrated by grafting with GA deficient mutant and plants overexpressing CLE6 (Bidadi et al., 2014). In addition, it was verified the phloem traffic from WT

SlCyp1 protein (graft) to tomato dgt mutant (plants that have low auxin sensitivity due SlCyp1 gene mutations) resulting in large root transcriptome changes and recovery of the auxin response capacity, restoring the lateral roots development and the xylem vessels formation. Additionally, it has been shown that this protein is upregulated by the increase in light intensity that correlates with the increase of specific root growth and reduction of the shoot-root ratio (Spiegelman et al., 2015). It is worth noting that grafting is an important tool for the study of root-to-shoot communication, especially for studies aimed at identifying molecules and/or mechanisms associated to abiotic stresses tolerance.

In previous chapter, we have shown that GA levels is important in response to water stress in tomato plants, however it was not clear if the observed phenotypes were consequence of alteration in signals coming from the roots or shoots. In this study we used tomato mutant plants deficient in GAs biosynthesis, gib3, gib2 and gib1, with moderate, intermediate and severe deficiency, respectively. The tolerance of mutants plants to water deficit was verified and was attributed to several causes: (i) greater root growth over shoot, (ii) leaf architecture and morphology of mutant plants, (iii) pronounced accumulation of proline in leaves and roots (Chapter 1). However, it is believed that the high root growth and root/shoot ratio alteration of these mutant plants were the important factors responsible for tolerance. Therefore, it is possible a differential regulation between the shoot and root in which compounds/molecules may be moving in a regulated manner promoting tolerance. To investigate this hypothesis, complementary studies of aerial and root growth and metabolism are required. Thus, I performed reciprocal grafts of WT and mutant tomato lines (Solanum lycopersicum) in the GAs biosynthesis, gib3, gib2 and gib1 and analyzed biometric, morphologic and metabolic parameters.

Material and methods

Description of genotypes used

The mutant tomato plants (Solanum lycopersicum L. cv. Moneymaker) deficient in GAs biosynthesis used in this study were produced and characterized genotypically and phenotypically previously by (Koornneef et al., 1990). The mutants were obtained by immersion of seeds of commercial cultivar Money-maker in solution of ethyl methane sulfonate mutagenic agent (EMS). The genotypic analysis performed initially revealed that the mutations generated by EMS occurred at different loci, located on different chromosomes (Koornneef et al., 1990). The mutants named gib3, gib2 and gib1 have mutations in chromosomes seven, one and six, respectively (Koornneef et al. 1990). The characterization of gib1 and gib3 mutants revealed that these genotypes have reduced ent-copalyl diphosphate synthase enzyme activities, with a reduction of 94% in relation to wild type (WT) and ent-kaurene synthase, with a reduction of 93% compared to WT, respectively. These enzymes participate of first reactions of GAs synthesis located in the plastids. For gib2, although the level of activity reduction was not known, it must have affected the expression of the ent-kaurenoic acid oxidase enzyme located in the endoplasmic reticulum (Bensen and Zeevaart, 1990). Together, low gene expression and low activity of these three enzymes resulted in mutant plants with reduced GAs levels (Koornneef et al., 1990).

Experimental conditions

The experiment was performed at Viçosa (20º45'S, 650 m altitude), Minas Gerais, in green house, on Department of Plant Biology of Universidade Federal de Viçosa.

Wild type tomato plants and mutants deficient in the biosynthesis of GAs, gib1, gib2 and gib3 (highly, intermediate and moderately deficient) were used, kindly provided by Dr. Koornneef (Max Planck Institute for Plant Breeding Research, Colônia, Germany).

The seeds were germinated in Petri dish (15 cm of diameter) containing two layers of germiteste paper towels moistened with 10 μM GA_{4+7} solution. Subsequently, the plates were housed in a growth chamber BOD type (Forma Scientific, Inc, Ohio, EUA), under photoperiod of 12/12 hours (day/night), temperature 25/16 ^{o}C (day/night), relative humidity 65±5% and luminous intensity of 150 μmol photons m^{-2} s^{-1}, for a

period of seven days. Each day, germinated seeds with a radicle emission of one centimeter in length were transferred to vase of 0.08 dm^3 containing Tropstrato HT® commercial substrate for vegetables and remained in growing chambers until cotyledonary leaves expansion. The vase were kept in a greenhouse and, after the appearance of three completely expanded leaves, the seedlings were transplanted to vessels of 1.4 dm^3 Containing substrate for vegetables supplemented with the 4:14:8 NPK granulated (equivalent at 4% of $(NH_4)^+(NO_3)^-$, 14% of P_2O_5, 8% of K_2O) in the proportion of 0.5 kg of NPK to 10.0 kg of substrate. Plants were cultivated for six weeks in a greenhouse and were watered daily in order to keep the substrate moisture of vase close to the field capacity until the end of experiment. One week after transplantation, the grafts were performed. The experiment was conducted under optimum conditions to the growth of plants to observe the different phenotypes generated and to collect biometric data. Leaf and root samples were collected also to determine some metabolites below described.

Grafting technique

The communication between root and shoot of the different plant genotypes was evaluated using the grafting technique. Grafting followed as described by (Spiegelman et al., 2015). Slotted grafting was performed one week after transplantation when plants had 2-3 fully expanded leaves. A slit was made 5 mm below the cotyledonary leaves and grafts were supported using Solanaceae silicon clips for grafting (Supplementary figure S1 A). The grafting site was wrapped with film paper (Supplementary figure S1 B) and the grafted plants covered with a clear plastic bag, immediately after grafting, for seven days. It is possible to visualize the grafted site cicatrization approximately one week after grafting technique (Supplementary figure S1 C).

The grafts were performed and 10 combinations of plants were generated from the four genotypes. Each of the mutants was individually grafted with WT which the WT was scion at one time and rootstock at another. Are they: WT (scion)/WT (rootstock), WT/gib3, gib3/WT, gib3/gib3, WT/gib2, gib2/WT, gib2/gib2, WT/gib1, gib1/WT e gib1/gib1. WT/WT, gib3/gib3, gib2/gib2 e gib1/gib1 combinations were self-grafited and used as control of the other combinations. To observe the effect of the grafting technique on the plants, the ungrafted mutant and WT genotypes were included as a second control. Non-grafted and self-grafited plants were included as controls. The comparison between the non-grafted and self-grafited plants, with the biometric and

biochemical parameters evaluated, were in the supplementary data. Six plants of each combination or genotype were used, each plant being considered a repetition.

Phenotype and growth analysis

The record of plant growth after grafting was done every seven days until the end of the experiment using a digital camera.

Growth in shoot height was determined during and at the end of experiment. Plant growth was monitored every three days. For this, height of plants was monitored with help of a millimeter rule from the lap height to the apical bud throughout the experiment. The final height, shoot dry weight (leaves and stem), root dry weight, total leaf area and specific leaf area were determined at the end. Subsequently, relative growth rate in height (RGR-h) was calculated by (Evans, 1972; Hunt, 1982).

Eight leaf discs of 0,212 cm^2 (0,519 cm of diameter) were stove dried at 65 ºC until constant dry weight for determination of specific leaf area. The total leaf area was estimated from the specific leaf area using the dry weight of all leaves. The determination of both specific leaf area and total leaf area was performed following protocol described by (Mielke et al., 1995). The specific leaf area was calculated using equation:

$$\text{Specific leaf area } (m^2\ g^{-1}) = \frac{\text{leaf area of discs } (m^2)}{\text{dry weight of discs (g)}}.$$

Leaf samples were collected, immediately frozen in liquid nitrogen and stored at -80 ºC until analysis. Leaves, stems and roots were separated and immediately taken to the stove with forced ventilation at 65 ºC to obtain the dry weight (DW) after constant weight. Values of dry weight, biomass partitioning of leaves, stems and roots and shoot-root ratio were calculated by (Benincasa, 1988).

Ethanolic extraction

For the determination of the metabolites, leaf and root samples were collected in the middle of the light period and immediately frozen in liquid nitrogen and stored at -80ºC until extraction. Aliquots of approximately 30 mg of fresh macerated mass of both tissues were used for biochemical analysis. The samples were subjected to hot ethanol extraction by the addition of 250 µL of 98% ethyl alcohol to the 1.5 mL microtubes

containing the macerate. The samples were homogenized and incubated for 20 min at 80 °C with shaking of 822 g. After this time the microtubes were centrifuged at 17,000 g for 10 min at 4 °C. The supernatant was collected and stored in a new 1.5 mL tube. The resulting precipitate was subjected to two further extractions as described above, using 150 and 250 µl of 80% and 50% ethyl alcohol, respectively. The supernatants obtained in these steps were combined and, as well as the resulting precipitate, stored at -20 °C for analyzes of the metabolites as previously described (Nunes-Nesi et al., 2007). The levels of chlorophyll a, chlorophyll b, were determined immediately and subsequently malate, fumarate, and total amino acids were quantified, all quantified from the ethanolic extract. Total proteins were determined from the precipitate.

Determination of pigments, amino acid, protein, malate and fumarate

Leaf and root samples were submitted to hot ethanol extraction above described for metabolic determination. Photosynthetic leaf pigments will be determined according to the methods by (Porra et al., 1989). Proteins and amino acids (Gibon et al., 2004), malate and fumarate contents (Nunes-Nesi et al., 2007) were determined in leaves and roots.

Experimental design

The data were obtained from completely randomized experiments in a greenhouse system. Ten combinations of plants were generated by graft from the four genotypes (WT/gib3, gib3/WT, WT/gib2, gib2/WT, WT/gib1 and gib1/WT), which four combinations are self-grafted plants (WT/WT, gib3/gib3, gib2/gib2 and gib1/gib1). The self-grafted plants are controls from grafted plants. The non-grafted gib3, gib2, gib1 and WT were included as self-grafting controls. Thus, the non-grafted plants are controls from self-grafted plants. Six plants of each combination or genotype were used, each plant being considered a repetition. The results were submitted to analysis of variance (ANOVA) and, to verify statistical difference between combinations and control (non-grafted or self-grafted), the means were compared by Tukey test at 5% of probability ($P<0.05$) with the aid of the algorithms present in Genes program.

Results

The shoot phenotype is partially reversed in WT scion grafted by rootstock gib2 or gib1

Self-grafted plants exhibited a shoot phenotype similar to non-grafted plants during six weeks of greenhouse cultivation, always comparing with their corresponding genotype (example: WT/WT grafted plants with non-grafted plants WT, gib3/gib3 with gib3, and so on). However, it was possible to observe that self-grafted plants presented reduced growth in comparison to non-grafted ones (Figure 1). WT scion grafted onto GA-biosynthetic mutants (gib3, gib2 or gib1) rootstock, are WT (scion)/gib3 (rootstock), WT/gib2 and WT/gib1, showed the aerial phenotype similar to WT/WT control plants.

When reverse grafting, whose mutant plants were used as scion and WT rootstock (gib3/WT, gib2/WT and gib1/WT), it was observed that the shoot phenotype was similar to its respective self-grafted control. In this case, the comparison was between gib3/WT grafted with gib3/gib3 self-grafted plants, gib2/WT with gib2/gib2 and gib1/WT with gib1/gib1. However, mainly the gib2/WT and gib1/WT grafts exhibited a partial reversion of the shoot phenotype evidenced by the higher growth in relation to their self-grafted controls gib2/gib2 and gib1/gib1, respectively. These shoot alterations were more discrepant in plants from two weeks after grafting and especially in older plants (Figure 1B and C).

When analyzing the final height of the graft combinations, similar results to the shoot phenotype were observed (Figure 2A). WT/WT, gib3/WT, WT/gib2 and WT/gib1 plants showed the highest height, with a reduction in WT/gib3. In gib2/WT and gib1/WT the lowest heights and significant were observed when compared to the previous grafts, however, similar to gib2/gib2 and gib1/gib1, respectively. In the height-relative growth rate (RGR-h), in the period after grafting to the end of the experiment, similar patterns to final height were observed (Figure 2B). Plants WT/WT, WT/gib3, gib3/WT, gib3/gib3, WT/gib2 and WT/gib1 were those that grew at higher speeds during the experiment and gib2/WT, gib2/gib2, gib1/WT and gib1/gib1 the ones that grew more slowly. Interestingly, WT/gib2 and WT/gib1 had the highest RGR-h while gib2/gib2 and gib1/gib1 had the smallest RGR-h.

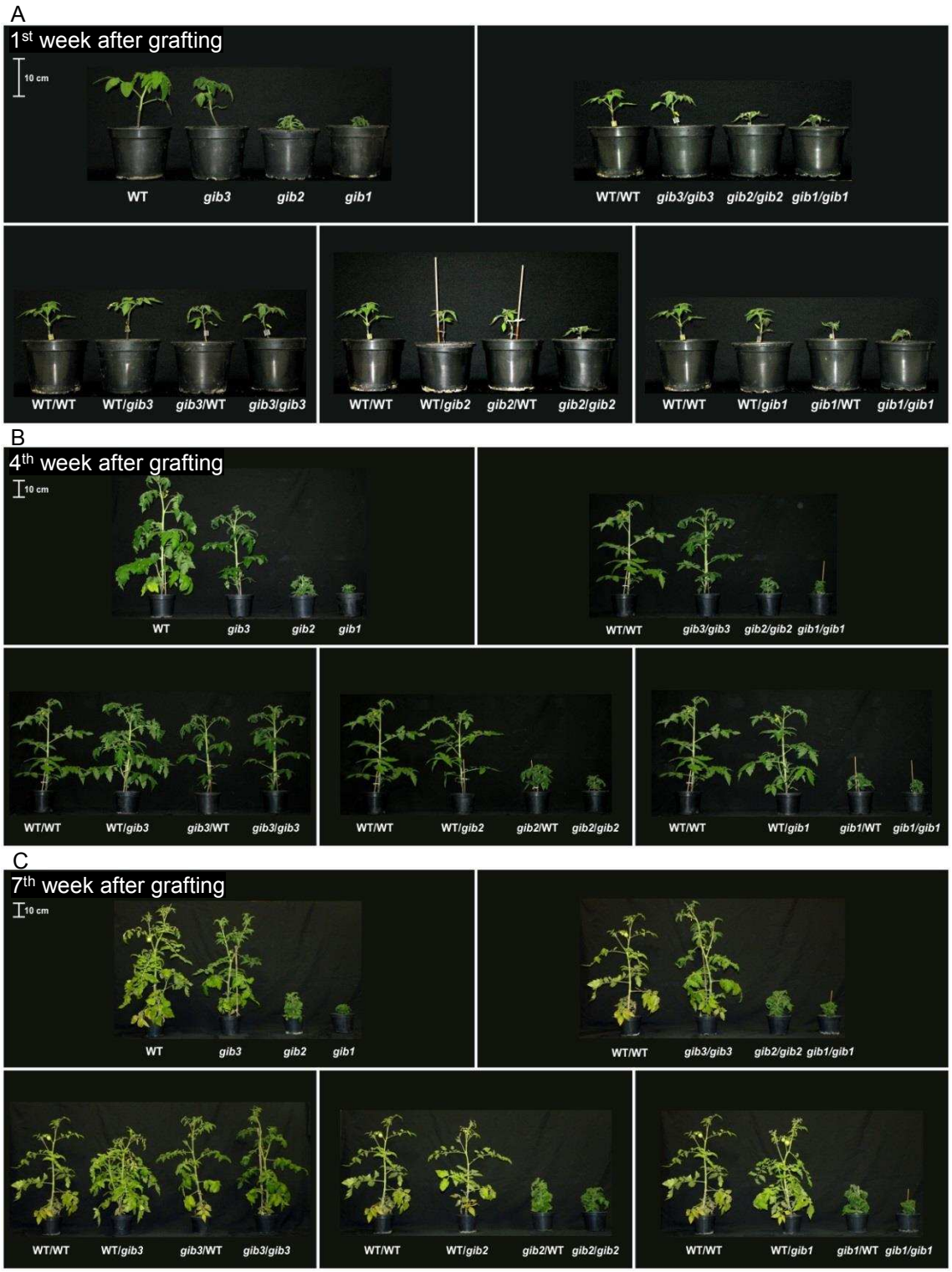


Figure 1. Visual aspect of non-grafted, self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker) under optimal growth conditions at green house. (A) First week after grafting; (B) Fourth week after grafting and; (C) Seventh week after grafting. Representative vertical bars equal to 10 cm.

At the end of the six weeks of greenhouse cultivation, the total leaf area and specific leaf area were determined (Figure 2C and D). Total leaf area presented higher values in WT/WT, WT/gib3, gib3/WT, WT/gib2 and WT/gib1 with significant reductions in gib3/gib3. The most drastic reductions in total leaf area occurred in plants gib2/WT, gib1/WT, gib2/gib2 and gib1/gib1 (Figure 2C). The specific leaf area was the biometric parameter related to shoot that presented similarity in all genotypes. The gib1/WT presented higher values of specific leaf area while WT/WT, gib2/gib2 and gib1/gib1 presented the lowest values (Figure 2D). Intermediate values of specific leaf area of the other combinations were found, not differing from gib1/WT nor from WT/WT, gib2/gib2 and gib1/gib1. Interestingly, in the grafts which the mutant genotypes were scion lower specific leaf area was observed, except for the combination gib1/WT which was the highest value. The biometric data of self-grafted plants (WT/WT, gib3/gib3, gib2/gib2 and gib1/gib1) and non-grafted plants (WT, gib3, gib2 and gib1) were compared to each other to know the influence of grafting on growth (Supplementary Figures S2 and S3). As some differences can be observed among them, the self-grafted plants were used as a comparison of the WT scion grafted onto GA-biosynthetic mutant rootstock.

Root growth is completely reversed in GA-biosynthetic mutants rootstock grafted onto WT scion

The leaves, stems, roots dry weight and the total dry weight presented a similar pattern among genotypes (Figure 3A, B, C and D). Corroborating with data of shoot growth and leaf area above mentioned, leaf and stem weight were higher in WT/WT followed by gib3/WT, WT/gib2 and WT/gib1 (Figure 3A and B). Reduction was observed in WT/gib3 and gib3/gib3 plants and more drastic reductions in gib2/WT, gib2/gib2, gib1/WT and gib1/gib1, with statistical differences compared to WT/WT plants, in both leaf and stem dry weight. As well as on the leaves dry weight, the significant reductions in stems dry weight were found in gib2 and gib1 scion.

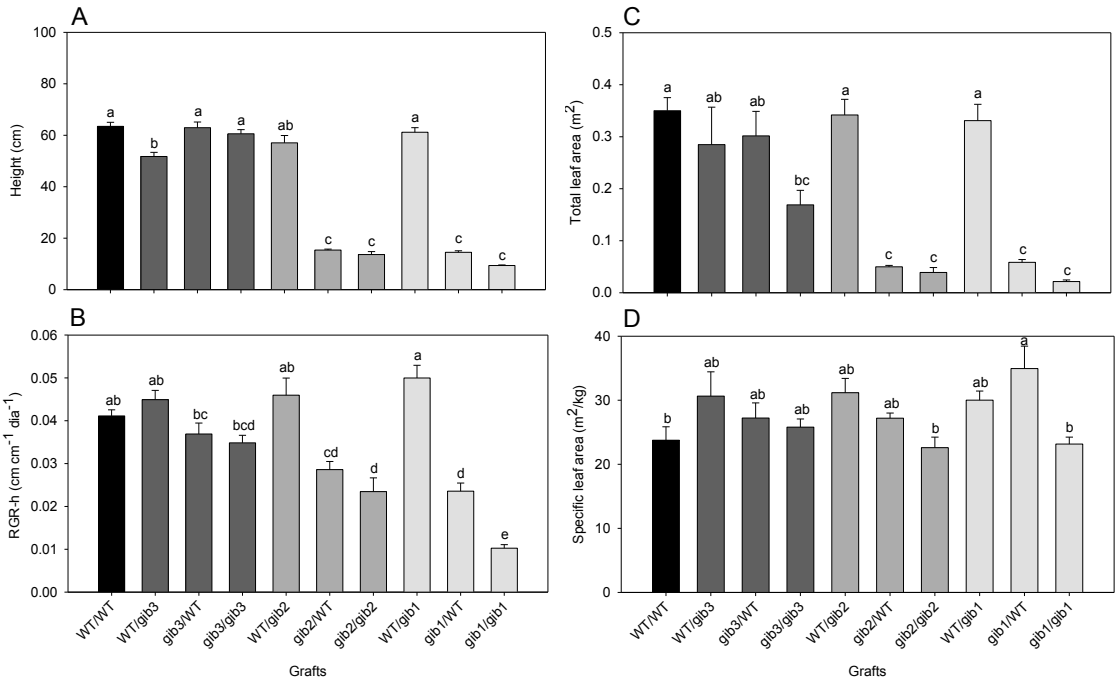


Figure 2. Variation in growth parameters related to height and leaf are parameters of self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker) under optimal growth conditions. (A) Final height of plants; (B) relative growth rate in height (RGR-h) of plants; (C) total leaf area and; (D) specific leaf area. Values represent means ± standard error of at least six plants. Letters indicates significant difference by Tukey test at 5% probability ($P<0.05$) between grafted combinations and self-grafited control.

Concerning root dry weight, the strong reductions were observed in gib2 and gib1 scion (gib2/WT, gib2/gib2, gib1/WT and gib1/gib1) when compared to the other grafting (Figure 3C). The high values of the WT/gib2 and WT/gib1 and WT/WT, without differences between them, indicated completely reversal in root growth by observation root dry weight.

In gib2/gib2 and gib1/gib1 self-grafting plants, higher root/shoot ratio was observed indicating root mass accumulation in comparison of shoots, and the smaller in plants with WT or gib3 scion (Figure 3D). Also high values of this ratio were found in gib2/WT and gib1/WT, but smaller than gib2/gib2 and gib1/gib1. The biomass partitioning results corroborate with the results of higher partitioning in roots of gib2/WT and gib1/WT plants, especially in gib2/gib2 and gib1/gib1 self-grafted (Figure 3F). This higher biomass partitioning in roots these combinations is due to the lower partitioning in stems, since the partitioning in leaves was very high in these plants.

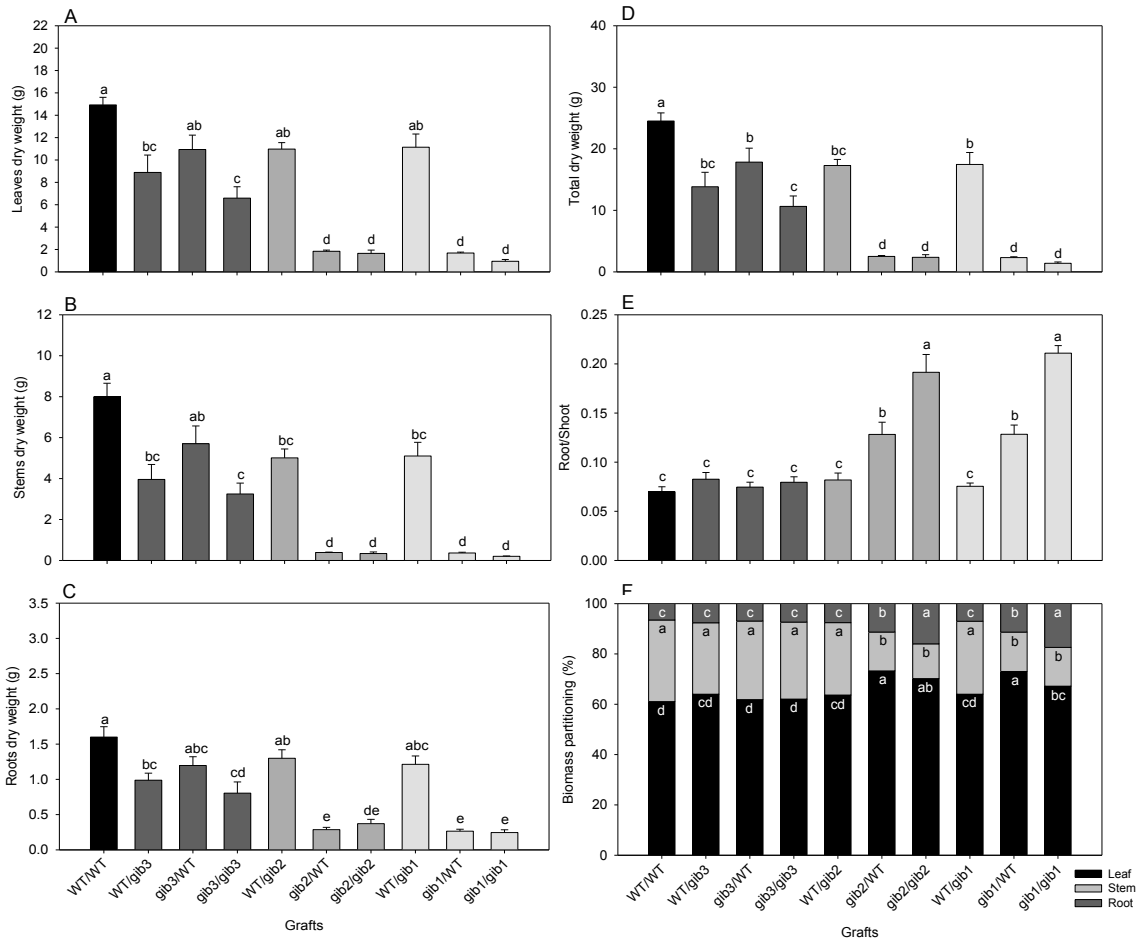


Figure 3. Biometric parameters variation of self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker) under optimal growth conditions. (A) Leaf dry weight; (B) stem dry weight; (C) root dry weight; (D) total dry weight of plant; (E) ratio between root and shoot; (F) biomass partitioning which bars represent the percentage of the total dry weight of each organ. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 2.

Photosynthetic pigments predominate in leaves of WT and gib3 scion

The content of leaf photosynthetic pigments was quantified in all grafting combinations as well as in those non-grafted plants (Figure 4 and supplementary figure S4). The amount of chlorophyll (Chl) a and Chl b was significantly higher in plants WT/gib3, gib3/WT, gib3/gib3, WT/gib2 and WT/gib1 (Figure 4A and B). Lower values of these pigments were found in gib2/WT, gib2/gib2, gib1/WT, gib1/gib1 and also in WT/WT. In all combinations, the Chl a is highly accumulated in leaves in comparison with Chl b. Consequently, total Chl followed the same pattern of accumulation observed, whose plants WT and gib3 scion had the largest accumulations (Figure 4C). The Chl a/Chl b ratio did not show many statistical differences between the grafted plants worked (Figure 4D). A higher ratio can be observed in plants WT/gib2, WT/gib1

and gib1/WT, indicating a greater accumulation of Chl a compared to Chl b. The lowest values were found in gib2/gib2.

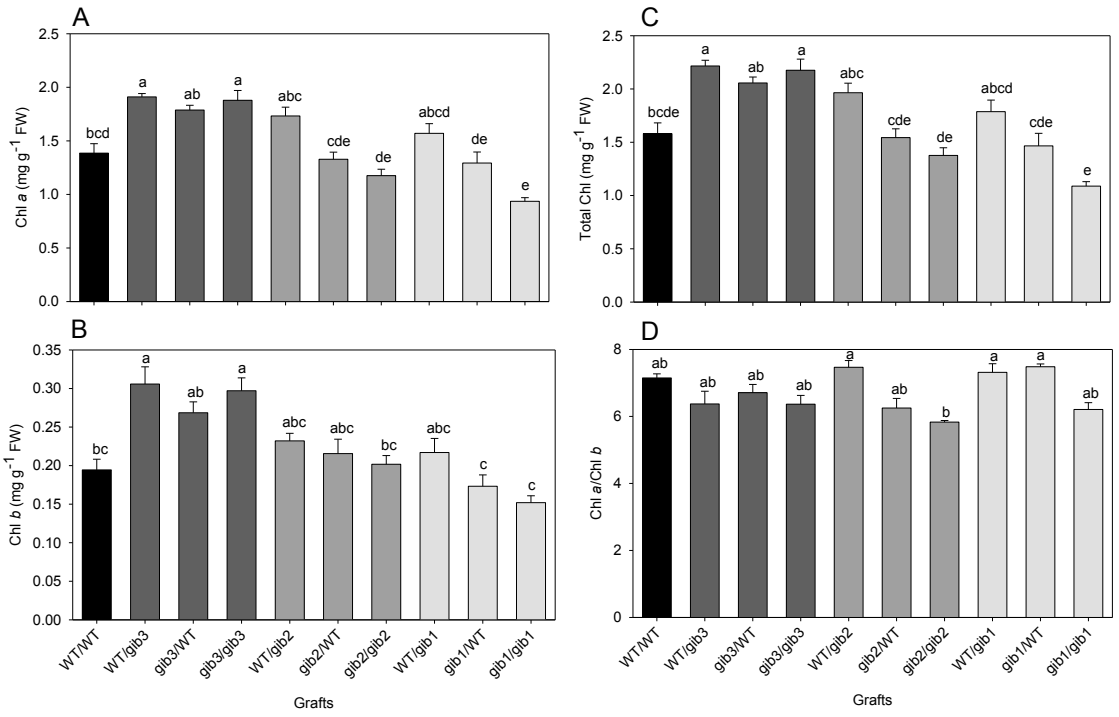


Figure 4. Photosynthetic pigments variation in leaf of self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Chlorophyll (Chl) a; (B) Chl b; (C) total Chl and; (D) ratio between Chl a and Chl b. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 2.

Nitrogen metabolism and organic acids changes in leaves and roots

Among the metabolites involved in nitrogen metabolism, the proteins and amino acid levels in leaves and roots of grafted and non-grafted plants were determined (Figure 5 and supplementary figure S5). Leaf protein content presented higher values in gib3 scion or gib3 rootstock (Figure 5A). The gib1 scion or rootstock showed lower values of total proteins, but they did not differ significantly from WT/WT and gib1/gib1. In roots, the total protein content was variable but can be noted that WT/gib3 differ significantly of WT/WT (Figure 5B). However, it can be observed that leaves showed nearly double protein content present in roots in all combinations.

The amino acid content in leaves did not present statistical differences in any grafts combination (Figure 5C). In roots, the highest levels of amino acids were observed in gib1/gib1, gib2/gib2, followed by gib2/WT, gib1/WT and the lowest levels in WT/WT. Significant reductions in amino acids content were observed in WT/gib2

and WT/gib1 when compared to controls gib2/gib2 and gib1/gib1, but did not differ to WT/WT. Unlike protein content, the amino acid content in leaves and roots is similar.

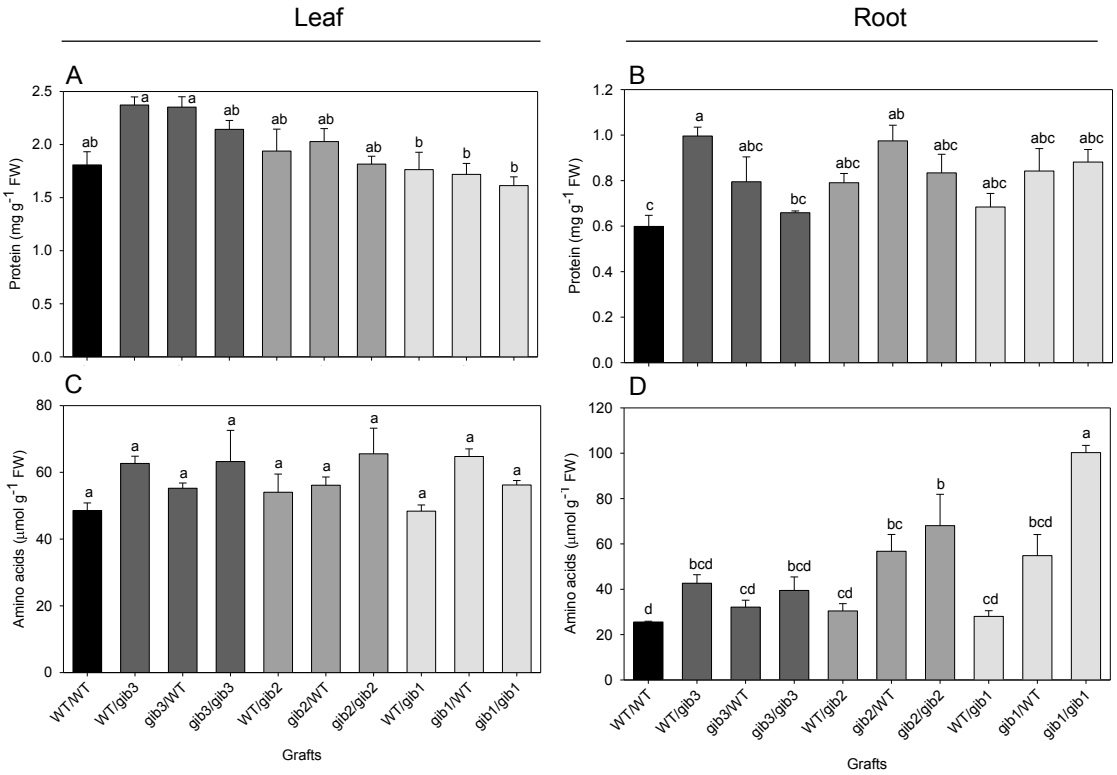


Figure 5. Metabolite levels changes involved in nitrogen metabolism in leaf and root of self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Leaf protein content; (B) root protein content; (C) leaf amino acids content and; (D) root amino acids content. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 2.

The concentrations of two organic acids in leaves and roots, malate and fumarate were also determined (Figure 6 and Supplementary figure S6). The malate content in leaf was higher in the WT/WT followed by gib3/gib3, gib2/WT, gib2/gib2, WT/gib1 and gib1/WT (Figure 6A). The lowest values of leaf malate were found in gib1/gib1. Root malate levels were higher in WT/WT followed by WT/gib1, WT/gib2, gib3/gib3, gib3/WT and WT/gib3 (Figure 6B). Malate content in root significantly reduced in gib2/WT and gib1/WT compared to WT/WT and significantly increased in WT/gib2 and WT/gib1 compared to respective controls, gib2/gib2 and gib1/gib1. Minor and discrepant malate values in roots were observed in gib2/WT and gib1/WT followed by gib2/gib2 and gib1/gib1. It has also been found that leaf malate concentrations are twice as high as those found in roots.

The behavior of fumarate levels observed in leaf was the same observed for the levels of malate in roots for all grafted plants (Figure 6C). Leaf fumarate levels were higher in WT/WT followed by WT/gib1, WT/gib2, gib3/gib3, gib3/WT and WT/gib3 (Figure 6B). Lower values of fumarate in leaves, which were significantly different from WT/WT, were observed in gib2/WT and gib1/WT followed by gib2/gib2 and gib1/gib1. In roots, the fumarate content presented similar leaf pattern, but in much lower concentrations (Figure 6D). Fumarate content in root significantly reduced in gib2/WT and gib1/WT compared to WT/WT and significantly increased in WT/gib2 and WT/gib1 compared to respective controls, gib2/gib2 and gib1/gib1.

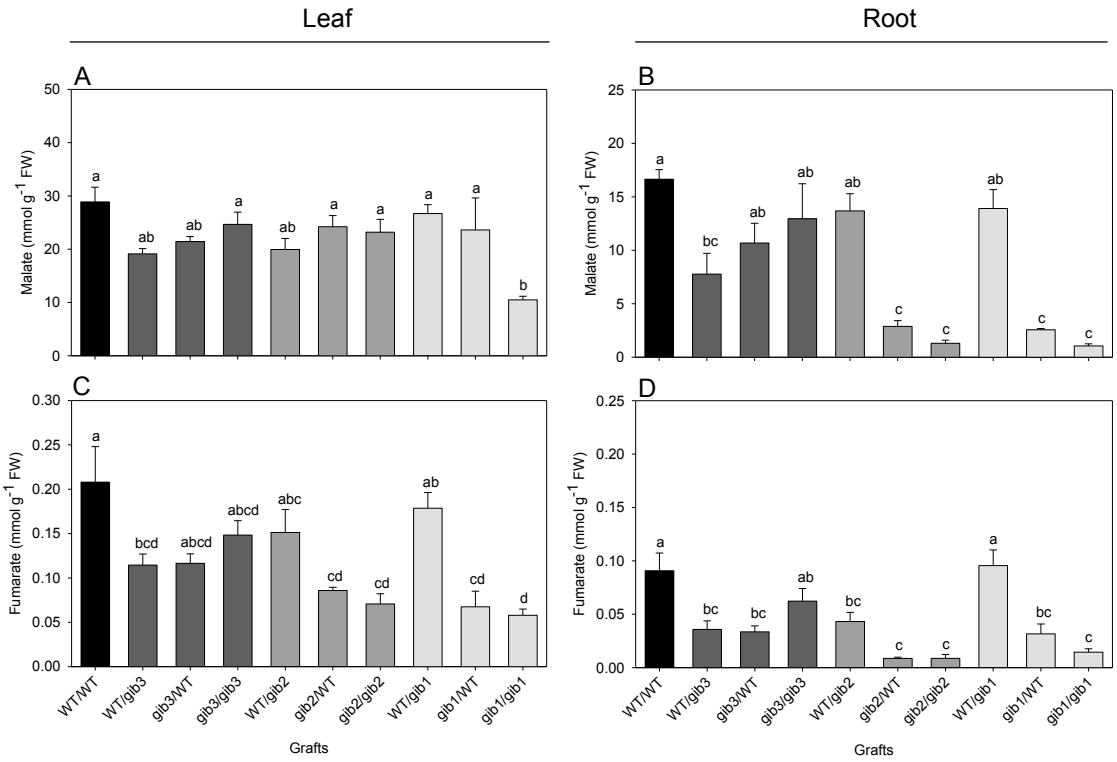


Figure 6. Organic acids levels changes in leaf and root of self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Leaf malate content; (B) root malate content; (C) leaf fumarate content and; (D) root fumarate content. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in Figure 2.

Discussion

WT rootstock-derived GAs promote partial reversal of the shoot phenotype of gib2 and gib1 scion

Some biometric and morphological analyzes in shoot of WT plants grafted onto GAs biosynthesis mutant (gib3, gib2 and gib1) were determined. The gib2/WT and gib1/WT grafted plants showed a tendency to increase when compared to their gib2/gib2 and gib1/gib1 controls, respectively, in the majority of shoot analyzes and naked eye by photographic records. Among these analyzes, we can mention: height, RGR-h, total leaf area, leaf dry weight, stems dry weight and total dry weight (Figure 2 and 3). Therefore, it was partial reversal of shoot growth of gib2 and gib1 scion in well-watered conditions. It is possible that this partial reversal may have been caused by the same signal or GA transport from WT rootstock. Some studies support the idea of local transport and GAs long-range in plants (Katsumi et al., 1983; Shani et al., 2013). In addition, although micrografting performed was identify GA predominantly transported in Arabidopsis, GA_{12} (Regnault et al., 2015), the first GA formed in the biosynthesis pathway and precursor of the other GAs. It has been suggested that GA_{12} is transported from the root to the shoot by the xylem and from leaves to other tissues, as growing parts of apices and roots, by the phloem (Regnault et al., 2015). The WT rootstock was a GAs donors for the shoot, confirming the existence of an acropter GAs movement partially restoring the growth of gib2 and gib1 scion.

Similar results were found in works with grafting between WT and GAs biosynthesis mutant plants. In a study, many micrografting were made between WT hypocotyls and GAs deficient mutants of Arabidopsis, with the GA biosynthesis pathway altered in the initial (ga1-3 mutant), intermediary (kao1 kao2 mutant) and final (ga20ox1 ga20ox2 Ga20ox3 and ga3ox1 ga30x2 mutants) (Regnault et al., 2015; Regnault et al., 2016). In the case of ga1-3 scion and kao1 kao2 scion the growth was restored by WT rootstock (used as GA donor), and in the other mutants no differences were observed when compared to their self-grafted controls. Thus, the enzyme ent-kaurenoic acid oxidase (KAO, deficient in the double mutant kao1 kao2) catalyzes the conversion of ent-kaurenoic acid to GA_{12}, intermediate substrate of GA_{20}-oxides (GA_{20}ox, deficient in the triple ga20ox1 ga20ox2 ga20ox3 mutant) and GA quantification data, the authors concluded that GA_{12}, the common precursor of all other GAs, is the most transported form as a transmitted signal. To date, it is difficult to infer

which GA is being shoot transported because the mutants gib1, gib3 and gib2, have deficiencies in the enzymes ent-copalyl diphosphate synthase, ent-kaurene synthase and ent-kaurenoic acid oxidase, respectively, steps initials of the GAs biosynthesis pathway, before to the formation of the first GA, GA_{12}. In addition, quantification data of the GA metabolites may give greater support to suggest which GA may have been transported. In addition, it is possible that some metabolite, RNAs, protein or peptide associated with GAs and organ growth may be involved.

In a recent work with Arabidopsis, a gene (CLE6) encoding a small peptide member of the CLAVATA/ESR-RELATED (CLE6) gene family has been identified, both gene and peptide can be transported and influence on shoot and root growth (Bidadi et al., 2014). GA deficient mutant plants (ga3ox1 ga3ox2) and overexpressing the CLE6 gene showed growth in both shoot and root. And when ga3ox1 ga3ox2 plants overexpressing CLE6 were grafted onto mutant ga3ox1 ga3ox2 plants, and vice versa, the phenotype of the GA-deficient mutant was restored, including the floral organs growth. In addition, the CLE6 promoter activity in the root stele suggests a transport of the CLE6 peptide from the root to the shoot via xylem promoting the growth of this organ and demonstrating its systemic effect under the GA action. Our observations of increase in dry weight of floral organs and fruits of plants WT/gib2 and mainly in WT/gib1 when compared to self-grafted WT/WT control, suggest the involvement of root metabolism influencing this high dry weight in WT scion (Supplementary Figure S7). Therefore, it is possible that some molecule has been transported from mutant rootstock to the WT scion inducing these observed reproductive responses. However, further studies of grafting with this plants are required during the reproductive phase.

The non-complete reversal of shoot of gib2/WT and gib1/WT plants may be due to lower GAs biosynthesis in roots in detriment to shoot, in this case, in WT rootstock. Underlying this hypothesis, it has been shown that root growth is controlled by lower GA concentrations than those required for shoot growth (Tanimoto, 1994, 2012). In addition, it is also suggested that roots have greater GAs sensitivity compared to shoot (Tanimoto, 2012). So even though these GAs are transported via xylem from the roots to the shoot of these grafted plants, GA concentrations are still lower than those required to completely restore the shoot phenotype.

Interestingly, we observed the highest RGR-h in WT scion grafted onto gib3, gib2 or gib1 rootstocks when compared to self-grafted WT (Figure 2B). We also verified that this happened in WT and gib3 self-grafted plants, presenting high and

significant values when compared to the non-grafted WT and gib3, respectively. Therefore, this increase in plant height growth occurred only in grafted plants with WT scion (normal endogenous GA content) and gib3 scion (low endogenous GA content). This probably happened because the hypocotyl cut for grafting between WT plants and GA deficient mutants stimulated the GAs production in the cotyledonary leaves for grafted place healing and restoration of the physiological connections. Consequently, this stimulus in the GAs synthesis in leaves may have generated a GAs accumulation in shoot and provoked the observed shoot growth. According to (Asahina and Satoh, 2015), when plants are incised the GAs produced in leaves are required for cell division during the cortex tissues meeting of injured hypocotyls, and cotyledons are required for the normal maintenance of GA levels in the hypocotyl in Cucumber and tomatoes. In addition, microelements such as zinc, manganese and boron from the root can contribute to the incised tissues meeting (Asahina and Satoh, 2015). The GAs requirement for the assembly of tissues into cut hypocotyls was also evident in GA-deficient tomato mutants, gib1 (Asahina et al., 2002).

WT scion grafted onto gib2 and gib1 rootstock showed normal root growth

The ability of plants to carry possibly GAs from leaves to roots under well-watered conditions was shown here through grafting between WT plants and GA deficient mutants (Figure 3). It is believed that this hypothesis can observed a complete reversal of the root phenotype in plants WT scion grafted onto gib2 or gib1 rootstock (WT/gib2 and WT/gib1). These grafted plants had higher and significant roots dry weight, higher root/shoot ratio and high biomass partitioning in roots. It is known that the GAs can move to short-range (cell-to-cell transport) and long-range inside the plants, in both directions, a movement that can be acropter (from the root to the shoot) or basipet (from the shoot to the root) (Regnault et al., 2015). Besides GAs, other molecules like RNAs, protein and/or peptides can be long-range transported. In the same way that we hypothesized the signal or GAs transport to the shoot of gib2/WT and gib1/WT plants, our hypothesis here is that high GAs synthesized concentrations (precursor or active GA) in the shoot were being transported by phloem from the WT scion (GA donor) to the gib2 and gib1 mutants roots (GA receptor organ). Also in this case, we do not rule out the idea of transporting another molecule via phloem.

In the same work that identified the predominant GA_{12} transport in Arabidopsis, cited above (Regnault et al., 2015), it was observed that WT scion was able to

significantly increase the root length of ga1-3 and kao1 kao2 mutants compared to their self-grafted controls. Thus, endogenous GA_{12} can be transported in both directions, from shoot to root and from root to shoot. Moreover, applying GA to the shoot of Arabidopsis slightly enhanced the primary root elongation (Bidadi et al., 2010). The authors concluding that the shoot GA precursor or active GA is transported to roots where, in combination with active GA produced by the root itself, the root is supplied with enough GA for normal growth.

In tomato plants, grafting studies aiming the study of long distance GAs transport have not yet been observed. However, it is already possible to find studies with abscisic acid transport (ABA) and auxin signaling and its consequences on the growth and morphology of tomato plants (Spiegelman et al., 2015; McAdam et al., 2016). Reciprocal grafts of WT and ABA-biosynthetic mutant (wilty of pea and sitiens of tomato) plants showed reversal of root growth in wilty and sitiens due to the ABA from leaves WT scion, show when using deuterium-labelled ABA (McAdam et al., 2016). In another work, the role of a cyclophilin 1 protein (SlCyp1) transported from the WT scion to mutant plants auxin-insensitive (dgt) rootstock was result in great changes in the root transcriptome and recovered the responsiveness of the dgt mutant with consequent restoration of lateral root development and formation of xylem vessels Additionally, showed that were trafficking of SlCyp1 from scion to the rootstock is up-regulated by elevated light intensities, the that finding correlates with a specific increase in root growth and a reduction in shoot-to-root ratio. Similar results have been found here, where gib2/WT and gib1/WT showed high and significant root/shoot ratio but reduced when compared to their controls self-grafted, gib2/gib2 and gib1/gib1.

The change in the root/shoot ratio is important for agricultural production, practical methods to change the shoot/root ratio have long been awaited, as demonstrated by the green revolution whose partial suppression of shoot growth resulted in more cereal production (Hedden, 2003). The cereals used in the 'green revolution' were semi-dwarfs due to reduced internode elongation. The characteristics of internodes shortening, dwards plants and increased root/shoot ratio were also observed in the gib2 and gib1 mutants used in this work. When gib2 and gib1 scion were grafted onto WT rootstocks these characteristics were partially maintained when compared to gib2/gib2 and gib1/gib1 (Figure 3). Therefore, we further emphasize that root elongation occurs at low GAs concentrations because they are more sensitive to GAs, that concentrations are not sufficient to promote stem growth (Tanimoto, 2012).

WT grafted onto GA deficient plants cause few changes in the primary metabolism of leaves and roots

To observe if the metabolism is altered in leaves and roots of WT scion and mutant rootstocks, and vice versa, were determined the pigments, proteins, amino acids, malate and fumarate contents. gib2 and gib1 non-grafted and self-grafted showed dark green leaves that did not directly provide to the chl content (Figure 4). These results can be explained by the leaf morphology and architecture characterized by the high limb thickness. In addition, gib2 and gib1 scion presented higher total Chl contents compared to their self-grafted control (Figure 4C). The lower Chl content is characteristic to mutants with the lowest content of GAs, gib2 and gib1 (Chapter 1), indicating that the WT rootstock was not able to reverse this phenotype when the grafting was performed.

Protein and amino acid content in gib2/WT plants accumulated in roots compared to WT/WT (Figure 5B and D). Conversely, in WT/gib2 and WT/gib1 the root accumulation was reduced when compared to its self-grafted controls gib2/gib2 and gib1/gib1. Therefore, the root metabolism of these plants is mainly affected by the accumulation of proteins and amino acids in WT rootstock and reduction in gib2 and gib1 rootstock. The amino acids and amides are the first stable products of inorganic N assimilation (Oaks, 1994) and are the building blocks for proteins. An increase in the protein synthesis rate leads to the depletion of free amino acids, and when protein degradation occurs, an increase in amino acid content (Causin, 1996). When there is a decrease in shoot growth there is an amino acids accumulation in the cytoplasm that are transported to roots through the phloem (Causin, 1996). It is possible that the lower growth in mutant scion may have increased the amino acid content and these were transported to WT rootstocks contributing to the high amino acid contents found and explaining the maintenance of high protein contents too. In addition, the high root/shoot ratio of gib2/WT and gib1/WT indicates the use of amino acids root synthesized as the carbon source for root growth. Similarly, reduction in gib2 and gib1 rootstocks indicate the use of amino acids root synthesized for root growth and, conversely, the roots amino acids were not kept high because there was not amino acids transport from the shoot because these were being used in biosynthesis place (WT scion) also for growth.

Malate and fumarate contents accumulated in gib2 and gib1 rootstock (WT/gib2 and WT/gib1 compared to their respective gib2/gib2 and gib1/gib1 controls) (Figure 5B and D). Conversely, in gib2/WT and gib1/WT the root accumulation was reduced when compared to its WT/WT self-grafts control. Malate can serve as an alternative source of

carbon and can be metabolized to promote growth (Martinoia and Rentsch, 1994). Otherwise, malate can be transported to the guiding cells, thus contributing to the stomatal opening by decreasing the osmotic and water potential (Nunes-Nesi et al., 2007; Fernie and Martinoia, 2009; Araújo et al., 2011). Thus, the reduced malate content in WT rootstock indicates utilization for growth corroborating our results with high root/shoot ratio in grafted plants gib2/WT and gib1/WT (Figure 3E). In addition, the WT rootstock malate was possibly being transported to the mutants shoot contributing to the stomatal opening, since these mutants show greater stomatal conductance (chapter 1).

The changes observed in the grafts between WT plants and gib3 mutant and their self-grafted controls were small and in many cases did not differ statistically for the parameters analyzed in this work. Such plants have identical phenotypes, morphology, growth, accumulation and dry weight partitioning as well as the pigments and quantified metabolites of shoot and root. The major changes observed occurred in gib2 and gib1 scion and rootstock, therefore a greater focus was given to them. Likewise, in WT plants grafted with gib2 and gib1 mutants, future analyzes of metabolite GA determination, hormonal profile and complementation of the metabolites by Liquid chromatography coupled to mass spectrometer (CG-MS) are necessary to support our hypothesis of GAs transport at long-distances.

Conclusions

In this study we suggested that some signal, as precursor or active GA, or molecule derived from shoot may be being transported to the roots, and vice versa, under well-watered conditions. This was possible due to reversion of the root and shoot phenotype of GA-deficient mutants, especially gib2 and gib1. In addition, although proteins, amino acids, malate and fumarate accumulate in greater amounts in the shoot, the roots present a greater alteration of the these metabolites contents between scion and stocks. The analysis for determination of GA metabolites, hormonal profile and metabolic profile of these plants are necessary to support our findings. In addition, grafting of WT plants and GA-deficiency mutant under water deficit can identify the factor responsible for the tolerance possibly observed in some combination and will explain partially the tolerance of gib2 and gib1 plants to water deficiency found in chapter 1.

Acknowledgments

This work was supported by funding from the Max Planck Society (to ANN and WLA) and the National Council for Scientific and Technological Development (CNPq-Brazil, Grant 306355/2012-4 to ANN and Grant 483525/2012-0 to WLA) and the FAPEMIG (Foundation for Research Assistance of the Minas Gerais State, Brazil, Grant APQ-01357-14 to WLA). We also thank the scholarships granted by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES-Brazil) to RPOG. Research fellowships granted by CNPq-Brazil to ANN and WLA are also gratefully acknowledged.

Literature cited

Araújo WL, Nunes-Nesi A, Osorio S, Usadel B, Fuentes D, Nagy R, Balbo I, Lehmann M, Studart-Witkowski C, Tohge T, Martinoia E, Jordana X, DaMatta FM, Fernie AR (2011) Antisense Inhibition of the Iron-Sulphur Subunit of Succinate Dehydrogenase Enhances Photosynthesis and Growth in Tomato via an Organic Acid–Mediated Effect on Stomatal Aperture. Plant Cell **23:** 600-627

Asahina M, Iwai H, Kikuchi A, Yamaguchi S, Kamiya Y, Kamada H, Satoh S (2002) Gibberellin Produced in the Cotyledon Is Required for Cell Division during Tissue Reunion in the Cortex of Cut Cucumber and Tomato Hypocotyls. Plant Physiology **129:** 201-210

Asahina M, Satoh S (2015) Molecular and physiological mechanisms regulating tissue reunion in incised plant tissues. Journal of Plant Research **128:** 381-388

Benincasa M (1988) Análise de crescimento de plantas: noções básicas. Funep

Bensen RJ, Zeevaart JAD (1990) Comparison of Ent-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and gib-1, gib-2, and gib-3 tomato plants. J Plant Growth Regul **9:** 237-242

Bidadi H, Matsuoka K, Sage-Ono K, Fukushima J, Pitaksaringkarn W, Asahina M, Yamaguchi S, Sawa S, Fukuda H, Matsubayashi Y, Ono M, Satoh S (2014) CLE6 expression recovers gibberellin deficiency to promote shoot growth in Arabidopsis. The Plant Journal **78:** 241-252

Bidadi H, Yamaguchi S, Asahina M, Satoh S (2010) Effects of shoot-applied gibberellin/gibberellin-biosynthesis inhibitors on root growth and expression of gibberellin biosynthesis genes in Arabidopsis thaliana. Plant Root **4:** 4-11

Causin HF (1996) The central role of amino acids on nitrogen utilization and plant growth. Journal of Plant Physiology **149:** 358-362

Chiba Y, Shimizu T, Miyakawa S, Kanno Y, Koshiba T, Kamiya Y, Seo M (2015) Identification of Arabidopsis thaliana NRT1/PTR FAMILY (NPF) proteins capable of transporting plant hormones. Journal of plant research **128:** 679-686

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT Protein Movement Contributes to Long-Distance Signaling in Floral Induction of Arabidopsis. Science **316:** 1030-1033

Dayan J, Voronin N, Gong F, Sun T-p, Hedden P, Fromm H, Aloni R (2012) Leaf-induced gibberellin signaling is essential for internode elongation, cambial activity, and fiber differentiation in tobacco stems. The plant cell **24:** 66-79

Dodd IC (2005) Root-To-Shoot Signalling: Assessing The Roles of 'Up' In the Up and Down World of Long-Distance Signalling In Planta. Plant and Soil **274:** 251-270

Eriksson S, Böhlenius H, Moritz T, Nilsson O (2006) GA4 is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. The Plant Cell **18:** 2172-2181

Evans GC (1972) The quantitative analysis of plant growth, Vol 1. Univ of California Press

Fernie AR, Martinoia E (2009) Malate. Jack of all trades or master of a few? Phytochem **70:** 828-832

Gao X-H, Xiao S-L, Yao Q-F, Wang Y-J, Fu X-D (2011) An Updated GA Signaling 'Relief of Repression' Regulatory Model. Mol Plant **4:** 601-606

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Towards the proteome of Brassica napus phloem sap. PROTEOMICS **6:** 896-909

Gibon Y, Bläsing OE, Palacios- Rojas N, Pankovic D, Hendriks JH, Fisahn J, Höhne M, Günther M, Stitt M (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post- translational activation of ADP- glucose pyrophosphorylase in the following light period. The Plant Journal **39:** 847-862

Hedden P (2003) The genes of the Green Revolution. Trends in Genetics **19:** 5-9

Hedden P (2012) Gibberellin Biosynthesis. In eLS. John Wiley & Sons, Ltd

Hedden P, Kamiya Y (1997) Gibberellin Biosynthesis: Enzymes, Genes and Their Regulation. Plant Physiol **48:** 431-460

Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. Biochemical Journal **444:** 11-25

Hejátko J, Ryu H, Kim G-T, Dobešová R, Choi S, Choi SM, Souček P, Horák J, Pekárová B, Palme K, Brzobohatý B, Hwang I (2009) The Histidine Kinases CYTOKININ-INDEPENDENT1 and ARABIDOPSIS HISTIDINE KINASE2 and 3 Regulate Vascular Tissue Development in Arabidopsis Shoots. The Plant Cell **21:** 2008-2021

Huang N-C, Liu K-H, Lo H-J, Tsay Y-F (1999) Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. The Plant Cell **11:** 1381-1392

Hunt R (1982) Plant growth curves. The functional approach to plant growth analysis. Edward Arnold Ltd.

Kanno Y, Hanada A, Chiba Y, Ichikawa T, Nakazawa M, Matsui M, Koshiba T, Kamiya Y, Seo M (2012) Identification of an abscisic acid transporter by functional screening using the receptor complex as a sensor. Proceedings of the National Academy of Sciences **109:** 9653-9658

Kanno Y, Oikawa T, Chiba Y, Ishimaru Y, Shimizu T, Sano N, Koshiba T, Kamiya Y, Ueda M, Seo M (2016) AtSWEET13 and AtSWEET14 regulate gibberellin-mediated physiological processes. Nature Communications **7**

Katsumi M, Foard D, Phinney B (1983) Evidence for the Translocation of Gibberellin A3 and Gibberellin-Like Substances in Grafts between Normal, Dwarf1 and Dwarf5 Seedlings of Zea mays L. Plant and Cell Physiology **24:** 379-388

Koornneef M, Bosma TDG, Hanhart CJ, Veen JH, Zeevaart JAD (1990) The isolation and characterization of gibberellin-deficient mutants in tomato. Theor Appl Genet **80:** 852-857

Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K (2010) Nitrate-regulated auxin transport by NRT1. 1 defines a mechanism for nutrient sensing in plants. Developmental cell **18:** 927-937

Lough TJ, Lucas WJ (2006) INTEGRATIVE PLANT BIOLOGY: Role of Phloem Long-Distance Macromolecular Trafficking. Annual Review of Plant Biology **57:** 203-232

Martinoia E, Rentsch D (1994) Malate Compartmentation-Responses to a Complex Metabolism. Ann Rev Plant Physiol Plant Mol Biol **45:** 447-467

McAdam SA, Brodribb TJ, Ross JJ (2016) Shoot- derived abscisic acid promotes root growth. Plant, cell & environment **39:** 652-659

Mielke MS, Hoffmann A, Endres L, Fachinello JC (1995) Comparison of laboratory and field methods for the estimation of leaf area of wild fruit species. Sci Agri **52:** 82-88

Notaguchi M, Okamoto S (2015) Dynamics of long-distance signaling via plant vascular tissues. Frontiers in Plant Science **6:** 161

Nunes-Nesi A, Carrari F, Gibon Y, Sulpice R, Lytovchenko A, Fisahn J, Graham J, Ratcliffe RG, Sweetlove LJ, Fernie AR (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J **50:** 1093-1106

Oaks A (1994) Primary nitrogen assimilation in higher plants and its regulation. Canadian Journal of Botany **72:** 739-750

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin Signaling: Biosynthesis, Catabolism, and Response Pathways. Plant Cell **14:** S61-S80

Omid A, Keilin T, Glass A, Leshkowitz D, Wolf S (2007) Characterization of phloem-sap transcription profile in melon plants. Journal of Experimental Botany **58:** 3645-3656

Porra R, Thompson W, Kriedemann P (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta (BBA)-Bioenergetics **975:** 384-394

Regnault T, Davière J-M, Achard P (2016) Long-distance transport of endogenous gibberellins in Arabidopsis. Plant Signaling & Behavior **11:** e1110661

Regnault T, Davière J-M, Wild M, Sakvarelidze-Achard L, Heintz D, Carrera Bergua E, Lopez Diaz I, Gong F, Hedden P, Achard P (2015) The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. Nature Plants **1:** 15073

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, Miyao A, Hirochika H, Kitano H, Ashikari M, Matsuoka M (2004) An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice. Plant Physiol **134:** 1642-1653

Shani E, Weinstain R, Zhang Y, Castillejo C, Kaiserli E, Chory J, Tsien RY, Estelle M (2013) Gibberellins accumulate in the elongating endodermal cells of Arabidopsis root. Proceedings of the National Academy of Sciences **110:** 4834-4839

Silverstone AL, Chang Cw, Krol E, Sun Tp (1997) Developmental regulation of the gibberellin biosynthetic gene GA1 in Arabidopsis thaliana. The Plant Journal **12:** 9-19

Smith VA, Knatt CJ, Gaskin P, Reid JB (1992) The distribution of gibberellins in vegetative tissues of Pisum sativum LI Biological and biochemical consequences of the le mutation. Plant physiology **99:** 368-371

Spiegelman Z, Ham B-K, Zhang Z, Toal TW, Brady SM, Zheng Y, Fei Z, Lucas WJ, Wolf S (2015) A tomato phloem-mobile protein regulates the shoot-to-root ratio by mediating the auxin response in distant organs. The Plant Journal **83:** 853-863

Sun T-p, Gubler F (2004) Molecular Mechanism of Gibberellin Signaling in Plants. Annu Rev Plant Biol **55:** 197-223

Tal I, Zhang Y, Jørgensen ME, Pisanty O, Barbosa IC, Zourelidou M, Regnault T, Crocoll C, Olsen CE, Weinstain R (2016) The Arabidopsis NPF3 protein is a GA transporter. Nature communications **7**

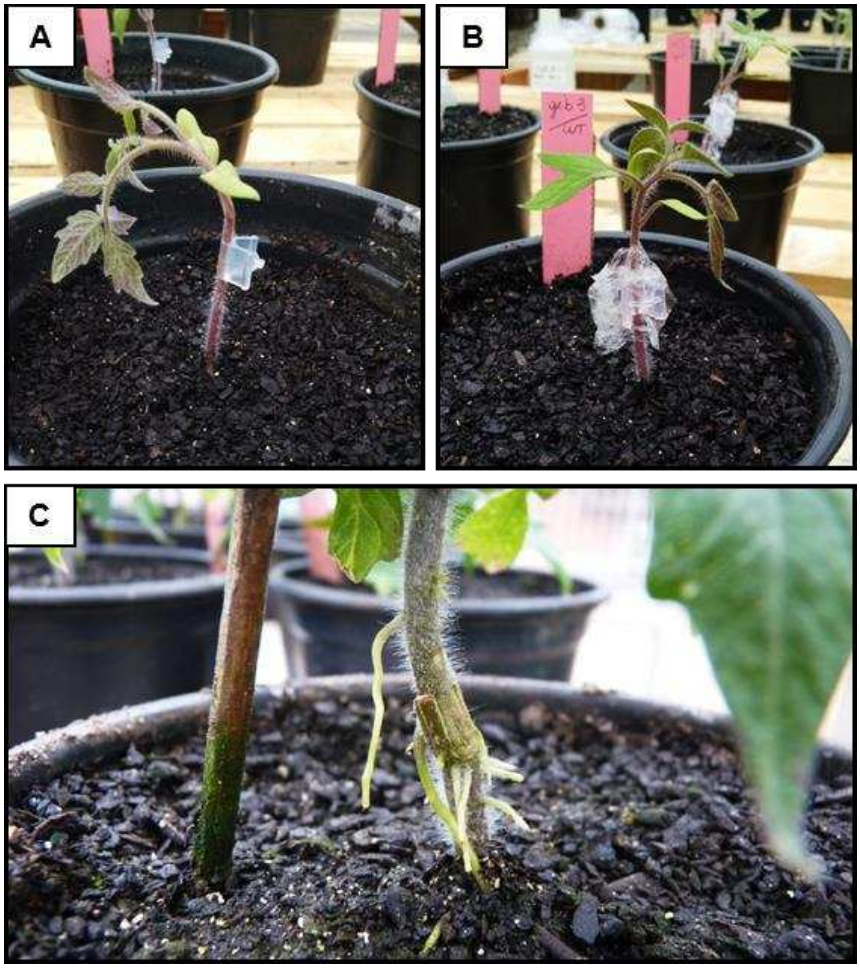
Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a Protein Is a Mobile Flowering Signal in Rice. Science **316:** 1033-1036

Tanimoto E (1994) Interaction of gibberellin A3 and ancymidol in the growth and cell-wall extensibility of dwarf pea roots. Plant and Cell Physiology **35:** 1019-1028

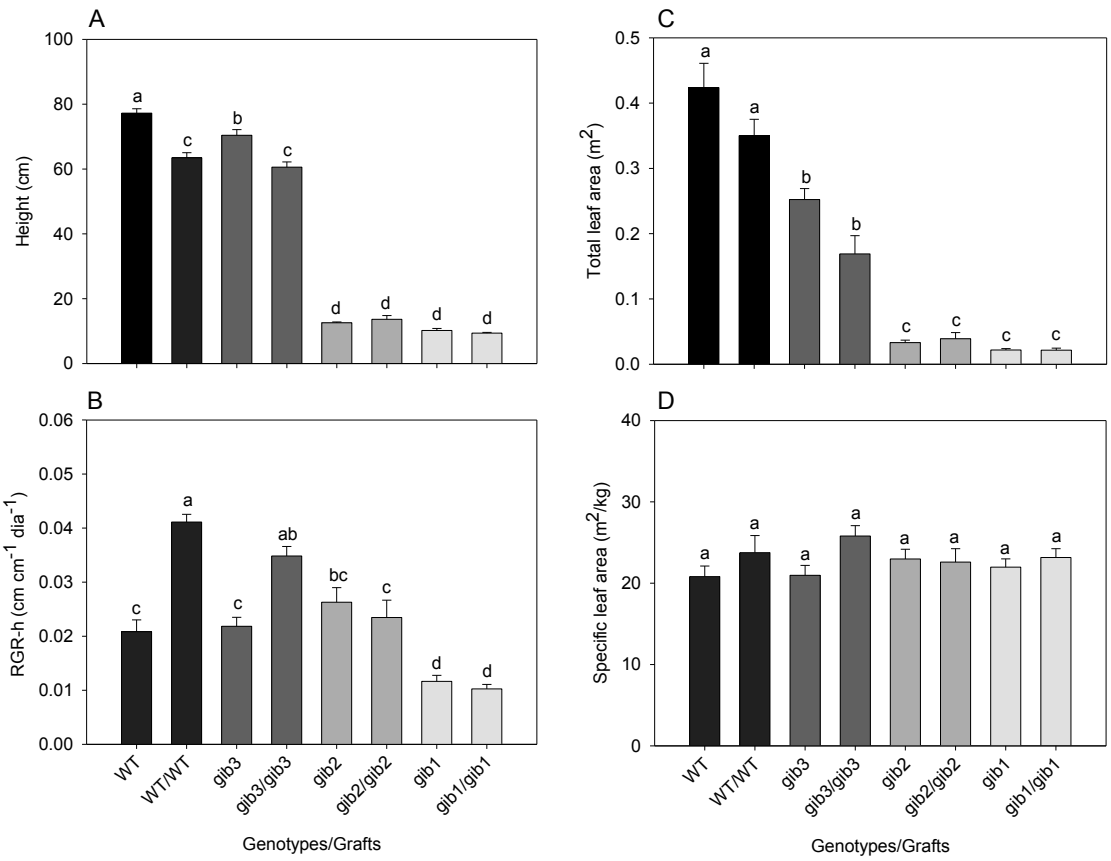
Tanimoto E (2012) Tall or short? Slender or thick? A plant strategy for regulating elongation growth of roots by low concentrations of gibberellin. Annals of Botany **110:** 373-381

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) Elongates Uppermost Internode Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. Plant Cell **18:** 442-456

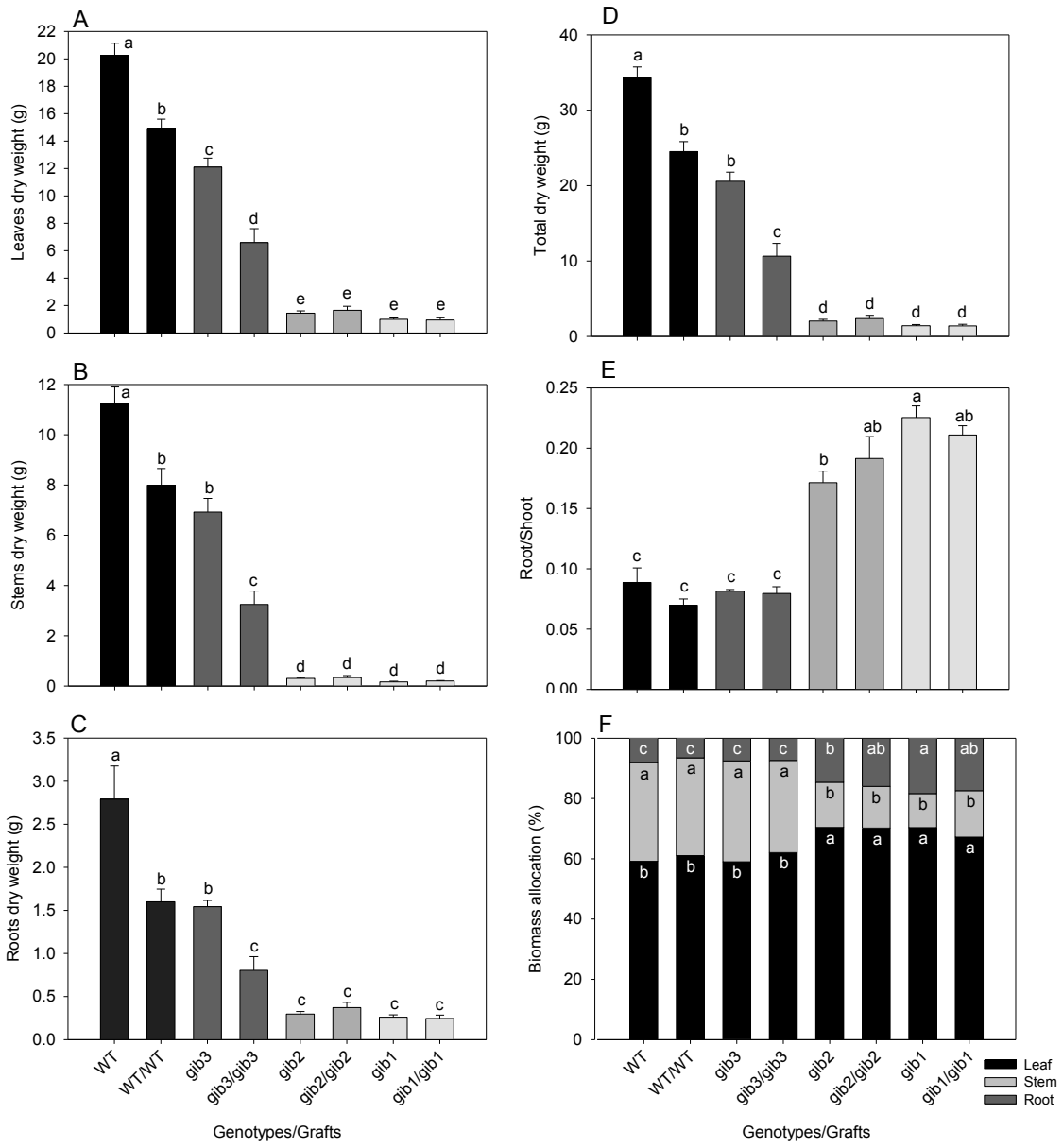
Supplementary data



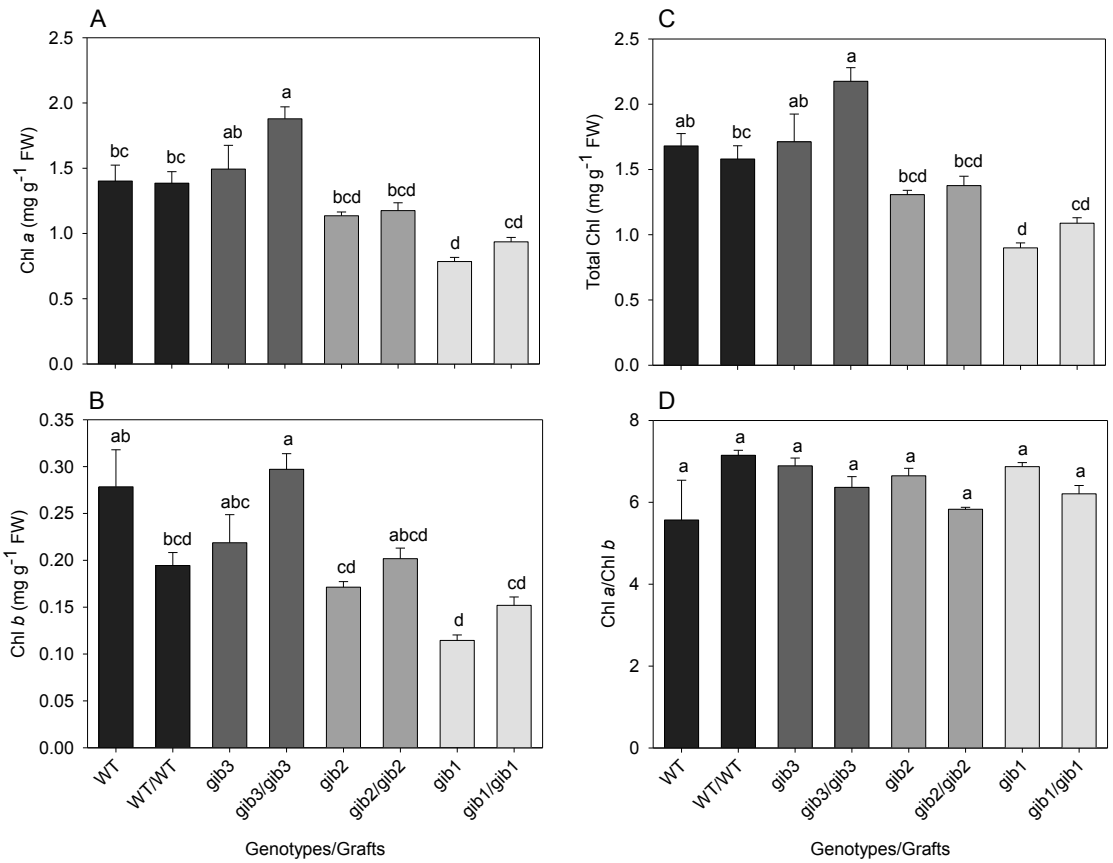
Supplementary figure S1. Illustrative images of some grafting step in tomato plants (Solanum lycopersicum cv. Moneymaker) at green house. (A) Plant with Solanaceae silicon clips at first day after grafting; (B) grafted plant with silicon clips and wrapped with film paper and; (C) cicatrization grafted site after one week.



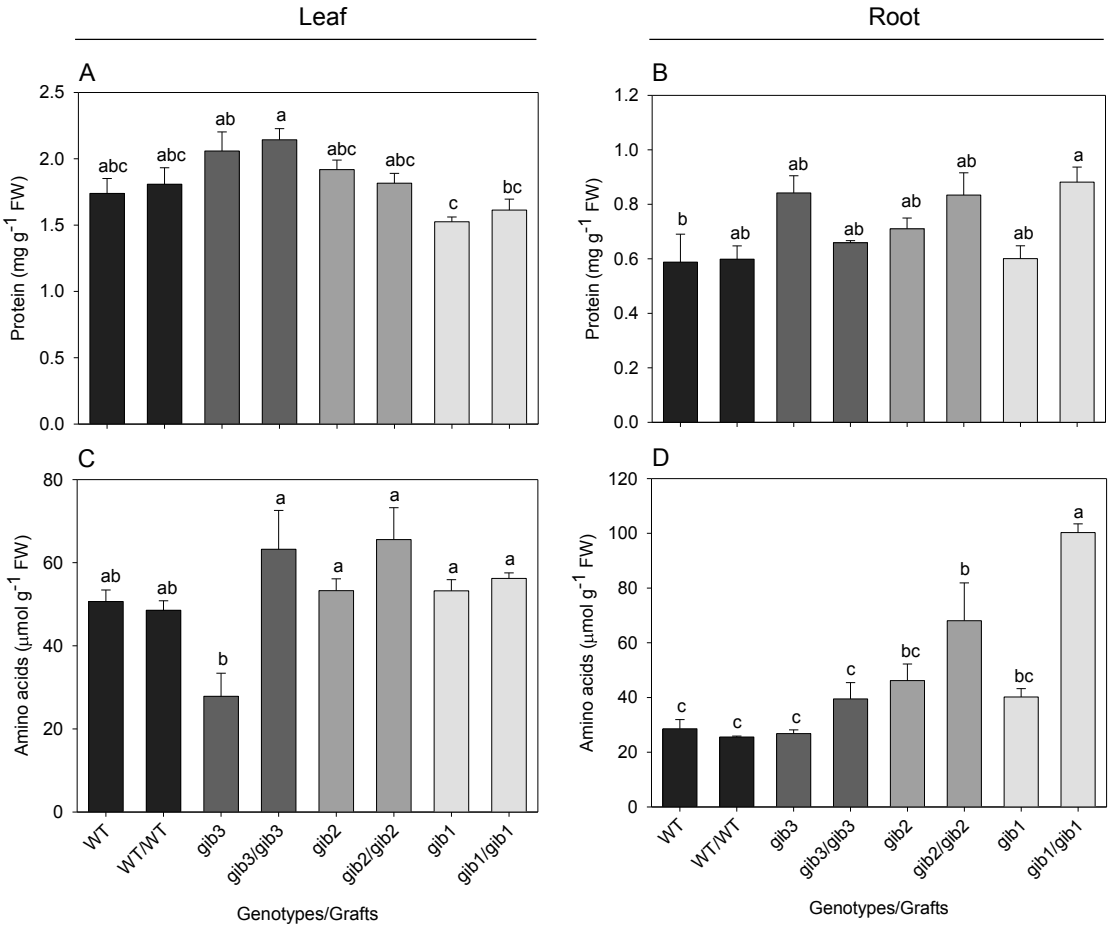
Supplementary figure S2. Variation in growth parameters related to height and leaf are parameters of non-grafted and self-grafted tomato plants (Solanum lycopersicum cv. Moneymaker) under optimal growth conditions. (A) Final height of plants; (B) relative growth rate in height (RGR-h) of plants; (C) total leaf area and; (D) specific leaf area. Values represent means ± standard error of at least six plants. Letters indicates significant difference by Tukey test at 5% probability ($P<0.05$) between self-grafited and non-grafted control.



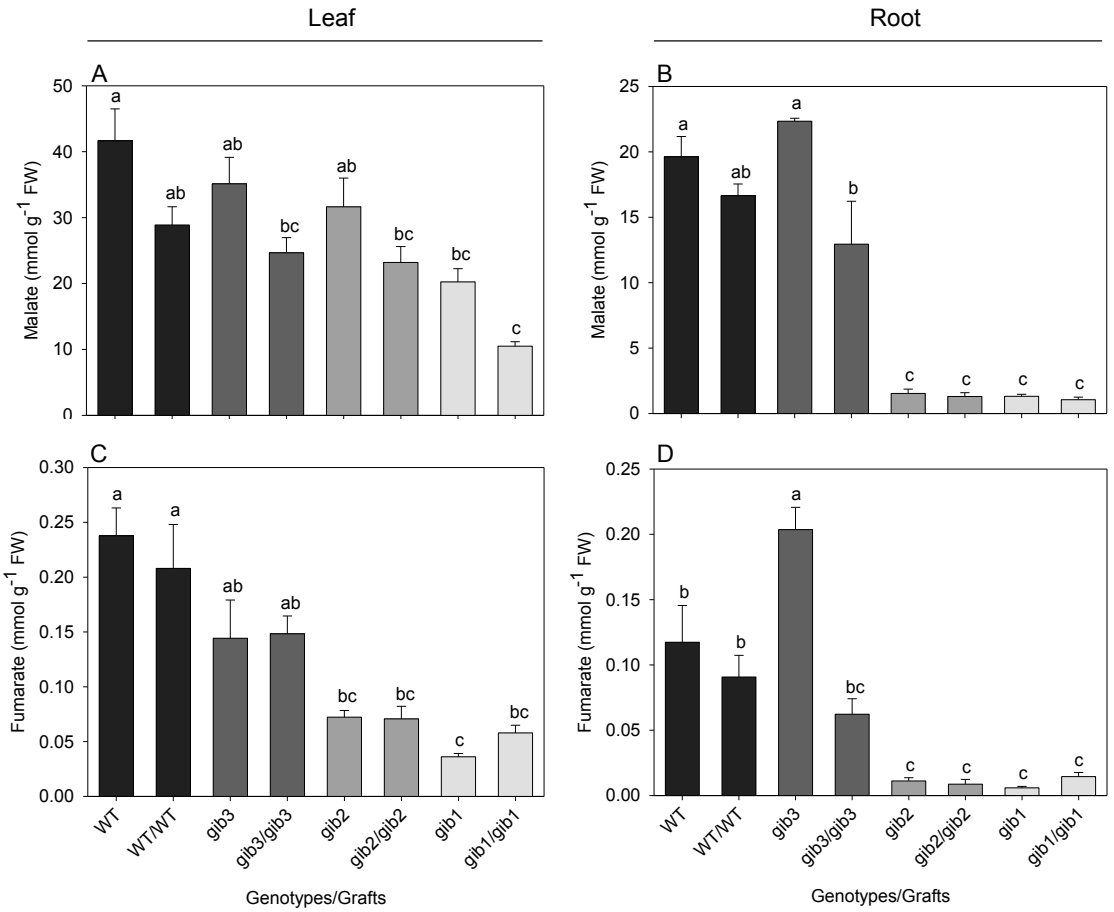
Supplementary figure S3. Biometric parameter variations of non-grafted and self-grafted tomato plants (Solanum lycopersicum cv. Moneymaker) under optimal growth conditions. (A) Leaf dry weight; (B) stem dry weight; (C) root dry weight; (D) total dry weight of plant; (E) ratio between root and shoot; (F) biomass partitioning which bars represent the percentage of the total dry weight of each organ. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S2.



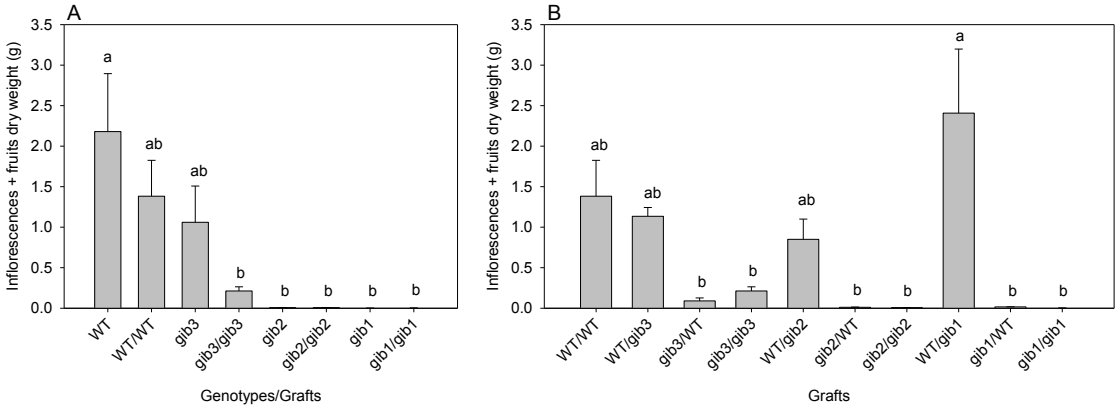
Supplementary figure S4. Photosynthetic pigments variation in leaf of non-grafted and self-grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Chlorophyll (Chl) a; (B) Chl b; (C) total Chl and; (D) ratio between Chl a and Chl b. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S2.



Supplementary figure S5. Metabolite level changes involved in nitrogen metabolism in leaf and root of non-grafted and self-grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Leaf protein content; (B) root protein content; (C) leaf amino acids content and; (D) root amino acids content. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S2.



Supplementary figure S6. Organic acids level changes in leaf and root of non-grafted and self-grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Leaf malate content; (B) root malate content; (C) leaf fumarate content and; (D) root fumarate content. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S2.



Supplementary figure S7. Dry weight of inflorescences and fruits from non-grafted, self-grafted and grafted tomato plants (Solanum lycopersicum cv. Moneymaker). (A) Comparison between non-grafted and self-grafted plants and; (B) comparison between self-grafted and grafted plants. Values are presented as means ± standard error of at least six plants. Statistical analysis as described in supplementary figure S2.

Concluding remark

Concluding remark

This work focused in understands the relationship between gibberellins (GAs), growth and tolerance to water deficit in tomato plants, at leaf and root level. First, the results obtained in this study indicated that reduced endogenous levels of GAs lead to physiological and metabolic adjustment due to water deficit that promotes greater tolerance to water deficit. Under this stress condition, the GAs promoted tolerance for turgidity maintenance, accumulation of malate, amino acids, lower shoot growth and higher roots biomass partitioning in leaves and roots. Together with the data of (Nir et al., 2014) and Martins (2013) suggest the GA influence in plant growth contributing to water stress conditions.

The root morphology and architecture of GAs deficient plants probably contribute for the tolerance to water deficit due plants alter the proportion of fine and thick roots. We also suggest that decoupling of growth and metabolism in shoot and root may have contributed to greater tolerance. These root characteristics were important for soil water absorption and possibility governed the adaptability of mutant plants to water deficit, including others stress conditions. In general, roots from GAs deficient mutant plants with larger diameters were present in less quantity in all plants in comparison to smaller diameter, and in mutants plants presented smaller thick roots. These data corroborate with (Ubeda-Tomás et al., 2009) that found GA increase the root secondary growth.

Together, the morphological and biochemical changes in leaves and roots promoted tolerance to water deficit due the osmotic adjustment which avoided water loss and a better absorption and use of soil water with lower leaf desiccation due to the reduced endogenous levels of GAs. It was observed that the nitrogen metabolism is favored in comparison to carbon metabolism under optimal and stress conditions in GA-deficient mutants due accumulation and carbon allocation to amino acids while organic acids have decreased their concentration. In addition, the proline accumulation was has an inverse response to GA endogenous content under water deficit, differently than observed in plants under salt stress (Tuna et al., 2008; Per et al., 2017). The proline accumulation in leaves and roots was associated with reduction in organic acids under water deficit conditions. We can suggest that several stressful, together GA content, may regulate the proline metabolism differently in leaves and roots. Therefore, studies

to verify the tolerance of these plants to other stresses, such as salt stress, are necessary to understand broadly the effects of GAs on plants.

Taken together, the results presented in this thesis suggest an important role of GAs in regulating plant growth and metabolism with different responses between root and shoot contributing for the tolerance to water deficiency. In addition, the transport of signal molecules and GAs (precursors or actives forms) seem to occur in order to influence organ growth. To support these findings and better clarify shoot-root interconnection, hormone profile and metabolic profile analysis are still required in optima and stressful conditions.

Literature cited

Martins, AO (2013) Impactos ecofisiológicos e metabólicos da alteração nos níveis de giberelina em tomate. Dissertação. Universidade Federal de Viçosa.

Nir IDO, Moshelion M, Weiss D (2014) The Arabidopsis GIBBERELLIN METHYL TRANSFERASE 1 suppresses gibberellin activity, reduces whole-plant transpiration and promotes drought tolerance in transgenic tomato. Plant, Cell & Environment **37:** 113-123

Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR, Anjum NA (2017) Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. Plant Physiology and Biochemistry

Tuna AL, Kaya C, Dikilitas M, Higgs D (2008) The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environmental and Experimental Botany **62:** 1-9

Ubeda-Tomás S, Federici F, Casimiro I, Beemster GT, Bhalerao R, Swarup R, Doerner P, Haseloff J, Bennett MJ (2009) Gibberellin signaling in the endodermis controls Arabidopsis root meristem size. Current Biology **19:** 1194-1199