VITOR DE LAIA NASCIMENTO

REVIEWING THE FUNCTIONS OF ETHYLENE ON GROWTH AND CENTRAL METABOLISM IN TOMATO

Thesis presented to Universidade Federal de Viçosa, as part of the requirements for obtaining the Doctor Scientiae degree in Plant Physiology.

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“Be the change that you wish to see in the world.”

— Mahatma Gandhi

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BIOGRAPHY

VITOR DE LAIA NASCIMENTO, son of Hudson Luiz Nascimento and Sônia de Laia Nascimento, was born in Vitória, Espírito Santo State, Brazil in September 28th 1988. In 2007, he started the undergraduate course in Biology at the Universidade Federal do Espírito Santo (UFES), Vitória-ES, and achieved the bachelor degree in January of 2011. Next, in the same year, he started his Master course in Plant Biology, at the same university, where he achieved the master degree in February of 2013. Finally, in the same year (August of 2013) he started his PhD studies in Plant Physiology at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State, Brazil.

TABLE OF CONTENTS

ABSTRACT

NASCIMENTO, Vitor de Laia, D.Sc., Universidade Federal de Viçosa, March, 2017. **Reviewing the functions of ethylene in growth and central metabolism in tomato.** Adviser: Wagner Luiz Araújo

Ethylene is an unique phytohormone, is a gaseous hydrocarbon and consists of only two carbons. It is responsible for regulating various aspects of the plant life cycle, including seed germination, root initiation, root development, floral development, sexual determination, fruit ripening, senescence and responses to biotic and abiotic stresses. The biosynthetic pathway and the series of reactions to formation of ethylene are already well established. In addition, the ethylene signaling process is also well characterized in that receptors on the membranes recognize this gas and a signaling cascade is activated to the nucleus where ethylene responsive genes are expressed. Much is known about how phytohormones in general influence plant development or how it relates to the signaling and transduction of information that help these organisms to adapt to the most diverse conditions. On the other hand, little is known about its direct relationship with carbon metabolism. The main goal of this work was to provide an enhanced comprehension coupled with a revalidation of the functional role of ethylene as a growth-related phytohormone. Remarkably, the results described within this thesis further demonstrate that ethylene, plant growth and carbon metabolism are strictly associated. Interestingly, ethylene seems to act directly on plant growth by inhibit or, in absence of your perception, allow the increased growth in tomato plants. Thus, it was demonstrated that an exogenous application of this hormone is able to reduce plant growth coupled with several morphological and metabolic adjustments. By contrast, in tomato Never ripe mutant plants, that are ethylene insensitive, growth is fairly induced coupled with significant changes in carbon assimilation characterized by increases in photosynthesis and an extensive metabolic reprogramming. Finally, after revisiting the possible functions of ethylene as a plant growth regulator, we can conclude that a biphasic effect of ethylene occurs in tomato once opposite effects are exhibited on plant growth. It is reasonable to suggest that metabolic and biochemical mechanisms that govern these two phenomena are not necessarily opposites.

RESUMO

NASCIMENTO, Vitor de Laia, D.Sc., Universidade Federal de Viçosa, março de 2017. **Revisando as funções de etileno no crescimento e no metabolismo central em tomateiro.** Orientador: Wagner Luiz Araújo

O etileno é um hormônio único, um hidrocarboneto gasoso composto por apenas dois carbonos, e responsável pela regulação de vários aspectos do ciclo de vida vegetal, incluindo germinação de sementes, iniciação de raízes, desenvolvimento floral, determinação sexual, amadurecimento de frutos, senescência e respostas a estresses bióticos e abióticos. A via biossintetica e as reações para a formação do etileno já são bem estabelecidas. Além disso, o processo de sinalização do etileno também já é bem caracterizado, em que receptores nas membranas percebem este gás e uma cascata de sinalização é ativada até o núcleo, onde genes responsivos ao etileno são expressos. Apesar do conhecimento sobre como os fitormônios influenciam o desenvolvimento vegetal ou como se relacionam com a sinalização e transdução de informações que ajudam estes organismos a se adaptar às mais diversas condições, pouco se sabe quanto as relações diretas com o metabolismo do carbono. O objetivo central deste trabalho foi gerar uma maior compreensão e uma possível revalidação do papel funcional do etileno como um fitormônio relacionado ao crescimento. Os resultados obtidos nesta tese demonstram uma associação entre o etileno, o crescimento vegetal e o metabolismo do carbono. O etileno parece agir diretamente no crescimento vegetal por inibir ou, na ausência de sua percepção, permitir o maior de crescimento de plantas de tomate. Desta forma, foi demonstrado que aplicações de etileno exógeno é capaz de reduzir o crescimento de plantas acoplado com várias alterações morfológicas e metabólicas. Por outro lado, plantas mutantes Never ripe, que são insensíveis ao etileno, apresentam um crescimento acoplado a significativas mudanças na assimilação do carbono caracterizadas por aumento na fotossíntese e uma extensa reprogramação metabólica. Por fim, após revisar as possíveis funções do etileno como regulador do crescimento de plantas podemos concluir que o etileno induz um efeito bifásico em plantas de tomate com efeitos opostos no crescimento. É razoável sugerir que os mecanismos metabólicos e bioquímicos que governam estes fenômenos não são necessariamente opostos.

Overview

As sessile organisms, land plants are continuously exposed to a broad range of environmental changes. To cope with such changes they have developed molecular, biochemical, physiological, anatomical, and morphological mechanisms that may adjust their growth and productivity in response to these possible environmental changes. The plant hormones, or phytohormones, are compounds of crucial importance for both plant growth and development, particularly in response to such stress conditions. Although there are currently several definitions the concept that is more accepted is that phytohormones are organic substances produced by plants acting at very low concentrations (micro or nanomolar) (Chiwocha et al., 2003). The main growth-related phytohormones are auxins, brassinosteroids, cytokinins and gibberellins (GAs), whereas abscisic acid (ABA), ethylene, and jasmonic and salicylic acids are most commonly associated with stress responses (Wolters and Jürgens, 2009).

Among these various phytohormones, ethylene is a singular one once it is gaseous and consists of only two carbons. Moreover it has well known functions related to various aspects of the planst life cycle, including seed germination (Miransari and Smith, 2014; Wilson et al., 2014), root initiation and development (Ivanchenko et al., 2008; Lima et al., 2009; Huang et al., 2013), floral development (O'Neill, 1997; Wuriyanghan et al., 2009), sexual determination (Iwahori et al., 1970; Yamasaki et al., 2001; Salman-Minkov et al., 2008), fruit ripening (Giovannoni, 2001; Barry and Giovannoni, 2007; De Martinis et al., 2015), leaf senescence (Lim et al., 2007; Kim et al., 2014; De Martinis et al., 2015; Ueda and Kusaba, 2015) and response to biotic and abiotic stresses (Morgan and Drew, 1997; Wang et al., 2007; Lin et al., 2013; Steffens, 2014). Due to the several aspects related to ethylene, it is of high interest that controlling and understanding the possible effects of this phytohormone be achieved. Actually, it seems to be a commercial crusade on plants of agronomic and horticultural value once responses to ethylene can be either harmful or desirable, depending on the species, developmental stage and concentrations of ethylene (Chang, 2016). This will discussed subsequently in details.

The discovery of the biological function of ethylene dates back to the 19th century, when illuminating gas (coal gas) leaks was recognized to cause extensive damage to plants, as premature leaf senescence and plant defoliation

(Lin et al., 2009; Chang, 2016). In 1901 it was identified that the gas lamp compound that actively affected plant metabolism and growth was in fact ethylene (Neljubow, 1901). Cousins, in 1910, suggested that plants could produce a certain gas that would affect the maturation of neighboring plants; however, as ethylene could not be detected at that time it was only in 1934 that chemical evidence on the production of this phytohormone was obtained in apples (Gane, 1934; Yang and Hoffman, 1984). It is now known that this hormone is produced by all cells during plant development although the rates is highly variable, being the largest production associated with meristematic, stressed or maturing tissues (Lin et al., 2009). Indeed, it should be mentioned that ethylene was the first gaseous signaling molecule to be identified in any organism (Kende, 1998; Chang, 2016).

The “triple response” phenotype is easily induced in the laboratory and it is highly specific to ethylene. Since the demonstration of Neljubow it is known that etiolated dicotyledounous seedlings exhibit: (i) inhibition of hypocotyl and root elongation; (ii) radial swelling of hypocotyl and root cells; (iii) and exaggerated apical hook. Importantly, these features were used to identify mutants that are defective in the responses to ethylene mainly in Arabidopsis and tomato plants (Guzmán and Ecker, 1990; Klee, 2004). Several genes that act on biosynthesis, signal transduction and ethylene response pathways were identified based on mutant analyzes and models of these responses were created based on different possible gene interaction (Lin et al., 2009). It is important to mention that the biosynthesis and signaling pathways of ethylene are highly conserved in plants (Babula et al., 2006; Rzewuski and Sauter, 2008; Yang et al., 2015) and dates back to a green algae ancestor prior to the colonization of land at least 450 million years ago (Ju et al., 2015).

The complete elucidation of the ethylene biosynthetic pathway by Yang and Hoffman (1984) was a notable episode involved in the progress of ethylene studies on higher plants. Ethylene is synthesized from the carbons C3 and C4 of the amino acid methionine through three key enzymatic reactions: (i) the first step is the conversion of methionine into S-adenosyl-L-methionine (S-AdoMet) by the enzyme S-AdoMet synthetase; (ii) next, the convertion of S-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS) occurs; and (iii) finally the enzyme ACC oxidase (ACO) degrades the ACC for the

formation of bioactive ethylene. The step of forming ACC via S-AdoMet is considered the limiting step, meaning that the formation of ethylene is mainly controlled by the ACS enzyme (Yang and Hoffman, 1984; Alonso and Ecker, 2001).

Once it was described how ethylene is produced and that it freely diffuses to surrounding cells and tissues, understanding how the cell perceive and transduce the signals of this phytohormone has became the subject of intense research for more than three decades (Alonso and Ecker, 2001). Ethlyene signaling pathway has been elucidated mainly by genetic approaches in Arabidopsis (Bleecker et al., 1988; Chang et al., 1993; Schaller et al., 1995). The main components of this model include positive and negative regulators that culminate in transcriptional regulation that is basically described by: (i) ethylene binding occurs at the transmembrane receptors from ETR (ethylene receptors) family; (ii) this binding deactivates the receptor molecules and the CTR1 (constitutive triple response1) protein becomes inactive, regulating negatively the downstream ethylene response pathway; (iii) the absence of a positive regulatory signal from the receptors allows EIN2 (ethylene insensitive2) to function as a positive regulator of the pathway signaling downstream to the EIN3 family of transcription factors located in the nucleus; and finally, (iv) these transcription factors interacts with the GCC-box of ethylene-response genes and presumably turns them ‘on’ triggering the response to this phytohormone (Solano and Ecker, 1998; Wang et al., 2002).

As already described, ethylene acts in several processes in the development of plants and a significant portion of this diversity is related to the interaction of ethylene with other hormones, such as ABA, auxins, cytokinins and gibberellins. The interactions of ethylene and ABA is mainly reported to seed development and germination (Linkies et al., 2009; Linkies and Leubner-Metzger, 2012; Arc et al., 2013; El-Maarouf-Bouteau et al., 2014), floral development and transition of vegetative-to-reproductive stages (Trivellini et al., 2011; Zhu et al., 2011), and in response to water stress (Sharp and LeNoble, 2001; Salazar et al., 2015). Additionally, it is important to mention that in general ethylene acts as an antagonist of the effects of ABA (Gazzarrini and McCourt, 2001; Corbineau et al., 2014). The influence of auxins on ethylene biosynthesis and, consequently, on the control of process such as senescence and ripening is well-known (Grierson

et al., 1982; Tsuchisaka and Theologis, 2004). Another important phenomenon, which also depends on the action of auxins, is the formation of apical hooks observed during the triple response phenotype (Raz and Ecker, 1999; Vandenbussche et al., 2010). However, the main reported association between ethylene and auxin on plant development is the root formation and penetration in soil (Swarup et al., 2007; Ivanchenko et al., 2008; Lima et al., 2009; Santisree et al., 2011). Relationship between ethylene and cytokinin is mainly described during ethylene biosynthesis (Chae et al., 2003; Wang et al., 2004; Hansen et al., 2009). Moreover, together with ABA and auxin, a role for ethylene on the regulation of stomatal closure has been described (Tanaka et al., 2006; Acharya and Assmann, 2009). Finally, several mechanisms of interaction between ethylene and DELLA proteins (related to gibberellins) are described in different stages of plant development, from roots to flowers (Achard et al., 2003, 2007). It has recently been demonstrated that ethylene may also act negatively in the transition from flowers to fruits, a stage of plant development mainly dependent on auxins and gibberellins, because it changes the metabolism of gibberellins (Shinozaki et al., 2015).

As pointed above, much has been investigated concerning the functions of phytohormones on plant development. This fact apart, it remains rather unclear their direct relationship with energetic metabolism, particularly in autotrophic tissues. Thus, although the role of ethylene in plant growth and development has been previously revisited (Pierik et al., 2006; Dugardeyn and Van Der Straeten, 2008; Li, 2008) and the effects of ethylene on photosynthesis and growth have been controversially reported by demonstrating that ethylene is able to increase assimilation rate (Buhler et al., 1978; Pua and Chi, 1993; Khan, 2004) or decrease it (Kays and Pallas, 1980; Pallas Jr and Kays, 1982; Rajala and Peltonen-Sainio, 2001), how exactly ethylene affects plant metabolism remains unclear. It is important to highlight that ethylene induces multiple, and sometimes paradoxical, responses on plants in general and that such studies have clearly advanced our understanding of the effects of ethylene. However, our knowledge on how exactly ethylene impacts leaf primary metabolism and plant growth in general remains rather fragmented.

As mentioned above, phytohormones and in special ethylene, are required for several functions within plant development and it seems also to connect plant

growth and carbon metabolism yet our understanding of the precise molecular and metabolic basis of this phenomenon is poorly characterized. Thus, this thesis is largely focused on the evaluation of the possible roles of ethylene on the context of plant growth and leaf carbon metabolism as well as to investigate the functional role of the intermediaries of tricarboxylic acid cycle on well-known functions related to phytohormones such as fruit development. To this end, a range of complementary experimental approaches were used and therefore the thesis is organized as a compilation of three independent stand-alone chapters which the first one is presented as a review on the functions of organic acids on fruit development, a classical role of phytohormones, while we discuss in the two last chapters the impact of ethylene on plant growth and carbon central metabolism of tomato plants.

CHAPTER 1. Organic acid control of fruit development: revisiting the complexity of climacteric and non-climacteric fruits

Since the role of phytohormones during fruit development is a recurring theme in the study of plant biology, and given that we increasingly have evidence of the role of organic acids in plant development here we review some papers and hypothesize the possible role of organic acids in a classic scenario of studies with plant hormones. Our analysis of literature demonstrated that in a broad series of fruit during the development the content of organic acids in the early stages are high followed by clear reductions in the levels of such metabolites. Different organic acids can be accumulated in general, however citrate and malate seems to have main importance given that accumulation of these metabolites during development occurs in a broad range of fruits. Further demonstration of the importance of changes in organic acid levels in fruits by specific manipulation of genes associated in fruits or even during fruit maturation by using specific promoter following genetic manipulation, it seems necessarily to fully comprehension of this process.

CHAPTER 2. Exogenous ethylene treatment reduces plant growth via changes in primary metabolism and cell wall in tomato leaves

To gain further insights into the well-established function of ethylene as growth regulation we investigated the physiological, molecular, and biochemical

responses of tomato (Solanum lycopersicum) leaves following high concentrations of exogenous ethylene in the form of ethephon (CEPA - 2-chloroethylphosphonic acid). The results obtained demonstrate that plants treated with ethylene were characterized by growth inhibition which was associated with decreases in carbon assimilation due to both reduced photosynthesis rates and stomatal conductance, coupled with impairments in carbohydrates turnover. Furthermore, ethylene treatment culminated in increases of cell wall compounds (e.g. cellulose and lignin) and phenolic compounds indicating that changes in secondary metabolism are also occurring. Taken together, our results suggest that ethylene plays a more direct role in plant metabolism than previously thought, most likely by acting in reducing carbon assimilation and leading to altered central metabolism. The results obtained are discussed in the context of current models of ethylene effects on the regulation of both growth and metabolism.

CHAPTER 3. Physiological and metabolic bases of increased growth in the tomato ethylene insensitive mutant Never ripe: extending the functions of ethylene signaling in tomato plants

Although compelling evidence has demonstrated the importance of ethylene during tomato fruit development (Osorio et al., 2011), how ethylene impacts leaf primary metabolism and plant growth in general remains unclear. Here by performing a detailed morphological, physiological, and biochemical characterization of the ethylene signaling tomato mutant Never ripe (Nr) novel aspects of the influence of ethylene insensitivity on plant growth and primary metabolism in leaves is provided. Our results demonstrated that the ethylene signaling component Nr (SlETR3) plays an important role in mediating growth responses by impacting leaf metabolism as, highlighted by the changes in carbon assimilation, carbohydrates turnover, and an interesting reprogramming of metabolites levels. The results obtained are compared and contrasted with those previously obtained with tomato fruit and discussed within the current context of current models of the ethylene transduction signaling.

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Chapter 1: Organic acid control of fruit development: revisiting the complexity of climacteric and non-climacteric fruits

Introduction

True fruits are specialized plant organs found solely in angiosperms or flowering plants and this unique structure are believed to have evolved in order to improve seed dispersal and protection (McAtee et al., 2013; Karlova et al., 2014). Due to the natural diversity of angiosperms being composed from small herbs up to large and big trees coupled with its ability to grow in a variety of habitats, such as aquatic (including marine) and terrestrial including unusable agricultural areas, there is an increasing and intimate association between humans and angiosperms. This is particularly true considering the human connection with their fruits and seeds and other products of high economic and food value. Although fruits are usually characterized as structures derived from a mature ovary containing seeds, many structures frequently named as fruit are, indeed, composed of a variety of other flower tissues types (Seymour et al., 2013).

The countless types of fruits present in angiosperms can be didactically organized within a few broad categories by using combinations of characteristics such as: (i) dehiscence or indehiscence; (ii) fleshy or dry exterior; and (iii) free (apocarpous) or fused (syncarpous) carpels (Seymour et al., 2013). These variations are further exemplified, for instance, by fleshy fruits, that have evolved with an enlargement of seed surrounding tissues to create attractive flesh for seed dispersing animals, and by dry fruits, those ones with dry mesocarp that normally need to open itself in order to release the seeds inside via mainly abiotic dispersal mechanisms (McAtee et al., 2013). It is tempting to suggest that this high diversity between fruits is likely due to tight adaptations associated to specific fruits and their disperser. This fact apart, the existence of significant correlations between fruit type and habitat conditions in angiosperms (Bolmgren and Eriksson, 2005) indicates that the evolution of fruit fleshiness is more likely associated to changes in vegetation habitats than in dispersers itself. Accordingly, it is not surprising that fleshy fruit evolution is an important and continually recurring theme throughout flowering plant evolution. However, it is

important to mention that caution should be taken when performing assumptions with respect to the adaptive value of particular fruit traits (Seymour et al., 2013).

Developmental stages of fruits can be divided in time specific steps defined as: (i) fruit set; (ii) growth; (iii) maturation; and (iv) ripening, or senescence. The first step associated with fruit development, the fruit set, occurs during and after fertilization (McAtee et al., 2013), which can be defined as the transition of a quiescent ovary to a rapidly growing young fruit and depends on the successful completion of pollination and fertilization (De Jong et al., 2009). Without pollination and successful fertilization, the flower begins a terminal phase of senescence, achieving floral abscission. On the other hand, when pollination and fertilization take place, a cascade of events is triggered, leading to development of seeds and fruit growth (Fuentes and Vivian-Smith, 2009; McAtee et al., 2013). The second step involved in fruit development is the growth, wherein a signal, most likely derived from seeds, is able to induce neighboring tissues to expand, by both cell division and cell expansion (McAtee et al., 2013; Seymour et al., 2013). Accordingly, during this stage there is an accumulation of storage products and sugar accumulation in fruits (Carrari et al., 2006; Fait et al., 2008). Following this accumulation, the third step, called fruit maturation, initiates when the fruit growth stop reaching the competence to ripen but the ripening itself yet needs to start (McAtee et al., 2013). Lastly, fruit development involve ripening, or senescence, a complex process wherein several metabolic changes related to softening and flavor characteristics, or organoleptic traits, occurs (Li et al., 2011; Osorio et al., 2011; Fraige et al., 2015; Lira et al., 2016). Remarkably, the precise transition between all these stages of fruit development requires a high amount of energy to achieve the full fruit development. This energetic demand is directly provided by metabolic changes, with adjustments on abundance of different classes of carbon compounds (organic acids, amino acids and sugars) during development, from normal development towards mature fruits coupled with a generally brief stage of accelerated ripening that is normally associated with enhanced respiration (Miyasaka Almeida et al., 2002). Fleshy fruits are characterized by a broad range of sizes, shapes, and colors. Moreover, different species presents unique flavor characteristics that are of pivotal importance in several processes. Such aspects are attractive to frugivores enhancing seed dispersal, and furthermore have become an indispensable part of the human diet

(Barry and Giovannoni, 2007; Karlova et al., 2014). Fleshy fruits are quite diverse and as such it includes fruits as grapes (Vittis sp) and tomatoes (Solanum lycopersicum) which are derived from the ovary, the so called true fruits, whereas others such as apples (Malus domestica), pineapples (Ananas comosus), and strawberries (Fragaria x ananassa) which are derived from the receptacle tissues or from expansion of the sepals, are called pseudo or accessory fruits (Barry and Giovannoni, 2007). Comprehension of the principal steps of the development and the possible classifications of true fruits are fundamental to understanding the possible function and variability of the metabolism of these organs.

It is important to mention that fleshy fruits can be also categorized into two groups namely climacteric and non-climacteric fruits. This is based on physiological differences observed within their respiratory pattern during ripening. On one hand, climacteric fruits, such as apples, bananas (Musa x paradisiaca), papaya (Carica papaya), and tomatoes show a rapid increase in respiration and a burst of ethylene production at the onset of the ripening process (Cherian et al., 2014; Karlova et al., 2014). On the other hand, non-climacteric fruits, such as citrus (Citrus sp), grapes, melon (Cucumis melo), and strawberries do not show this respiratory burst and ethylene production remains at a basal level during fruit development (Cherian et al., 2014; Karlova et al., 2014). Take together this information about different classification related to development associated with metabolism help us to start the comprehension of the pattern of metabolism during the fruit formation.

Due to their economic importance, organoleptic traits are recurrent object of investigations seeking to improve fruit quality (Davies, 1966; Famiani et al., 2005; Chen et al., 2012; Etienne et al., 2014). Among the several characteristics that are clearly important for fruit quality (Valero and Serrano, 2010), as nutritional and sensorial quality of fruit (e.g. visual aspect, firmness, and taste), palatability is assumedly of major metabolic significance once this trait is mainly dependent on the balance between organic acids (acidity) and sugar (sweetness) levels (Kader, 2008). These metabolites are directly connected to central carbon metabolism being also involved in the biosynthesis of diverse compounds such as amino acids, vitamins and aroma volatiles, which influence fruit taste (Lin et al., 2015). Although fruit ripening and its regulation have been extensively studied in fruits with different characters (Giovannoni, 2001; Carrari and Fernie, 2006;

Ioannidi et al., 2009; Dai et al., 2013) much less is currently known about the complex role of organic acids play during this process. Here, we revisit this aspect by paying particular attention to organic acid recognizably known to be involved in fruit development for this we first discuss the roles of organic acids at general in plants, then we highlight the metabolic behavior of organic acids during fruit development with comments about the association of these compounds with sugar and phytohormones, and finally we propose some possible tools for the study of the relation of the organic acids metabolism during the fruit development.

On the functional diversity of organic acids: more than simple intermediates

Along fruit development the levels of organic acids and sugars are usually inversely related and as such during maturation whereas sugars accumulate, mainly due to sugars import or production from starch degradation, organic acids that accumulated in young fruits strongly decreases (Berüter, 2004; Fait et al., 2008; Fortes et al., 2011; Nardozza et al., 2013; Lin et al., 2015). This fact apart, many fruits, as apple, peach (Prunus persica), and citrus, are able to accumulate considerable amount of organic acids during ripening. For instance, citrate and malate are considered the most abundant organic acid present in ripe fruits being climacteric or non-climacteric, yet the total acidity is extremely variable between species (Osorio et al., 2012a; Etienne et al., 2013; Merchante et al., 2013a). Notably, the metabolism and accumulation of organic acids in fruits are under both genetic and environmental control and thus enhancing our current understanding of these factors and its interactions is of pivotal importance for fruit quality improvements (Etienne et al., 2013).

During the last decade we have witnessed intensive effort to enhance our understanding of potential alternative functions of tricarboxylic acid (TCA) cycle intermediates in addition to their recognized role as energetic intermediates in plants (Araújo et al., 2012a; Medeiros et al., 2015, 2016). It is equally important to mention that most of studies using transgenic approaches to investigate the role of TCA cycle intermediates have been mostly performed with vegetative organs such as leaves and roots (Fernie and Martinoia, 2009; Zell et al., 2010; Araújo et al., 2011a; Finkemeier et al., 2013). Although the significance of TCA cycle are highly variable depending on tissue and environmental factors, most

likely due to its direct link to organic acids export and photosynthesis regulation (Fernie et al., 2004; Araújo et al., 2014; Medeiros et al., 2015, 2016), the complex pathways through which organic acids are metabolized and precise details of how some of these are regulated in vivo remains insufficiently understood to date (Fernie et al., 2004; Sweetlove et al., 2007; Fernie and Martinoia, 2009; Meyer et al., 2010). Notably, the level of accumulation and metabolism of organic acids are extremely variable between species, developmental stages and tissue types (Sweetman et al., 2009).

Organic acids can support numerous and diverse functions in plants. For instance, Arabidopsis (Arabidopsis thaliana), soybean (Glycine max) and sunflower (Helianthus annuus), all C3 plants, can accumulate high levels of fumarate (Chia et al., 2000; Fahnenstich et al., 2007). Such high level of fumarate has been recently associated with the supply of carbon skeletons that allow growth (Zell et al., 2010). Similarly, malate has not only an important role during photosynthesis in CAM and C4 plants (Fernie and Martinoia, 2009; Zell et al., 2010) but it has also been associated with stomata regulation in different species (Nunes-Nesi et al., 2007; Fernie and Martinoia, 2009; Medeiros et al., 2015, 2016) wherein malate and fumarate accumulated in guard-cells induces the stomata opening (Kochevenko et al., 2012; Medeiros et al., 2016). Remarkably, malate and fumarate levels show similar diurnal changes to those of carbohydrates in some C3 plants, wherein they increase during the day and decrease during the night, suggesting that this organic acids can also function as transient carbon storage molecules (Fahnenstich et al., 2007). By analyzing the influence of photoperiod on organic acid accumulation it was expertly demonstrated that Arabidopsis mutant plants with low malate and fumarate levels were characterized by minor alterations in morphology, photosynthetic functions, or growth parameters when growing under long days (16 h day/ 8 h night). However, under short day conditions (8 h day/ 16 h night) those plants suffered a strong decrease in photosynthetic performance culminating with reduced biomass and a pale-green phenotype (Zell et al., 2010). It seems therefore reasonable to suggest that impairments in the supply of energy and carbon skeletons during a long night occurs in plants that are unable to accumulate high levels of malate and fumarate, suggesting a role of organic acids as storage carbon molecules (Zell et al., 2010). This fact apart, the contribution of organic

acids to metabolic process affecting fruit development remains to be elucidated. Dissecting these mechanisms is clearly required to fully understand the key components underlying the implications of organic acid metabolism on energetic processes in fruit development and metabolism.

Although the function of TCA cycle intermediates have been extensively demonstrated in distinct aspects of plant growth (Nunes-Nesi et al., 2008; Araújo et al., 2012a) and in response to stress conditions (Tschoep et al., 2009; Sweetlove et al., 2010; Nunes-Nesi et al., 2014), signaling functions have also been recently discovered for different TCA cycle intermediates, from animal to plant besides prokaryote cells (Templeton and Moorhead, 2004; Yang et al., 2012), such as citrate (Wellen et al., 2009; Gomez et al., 2010), fumarate (Hewitson et al., 2007; Yang et al., 2012) and succinate (Hewitson et al., 2007) in animal cells and also citrate (Gray et al., 2004; Clifton et al., 2005; Finkemeier et al., 2013), malate (Gray et al., 2004; Geigenberger and Fernie, 2014) and 2-oxoglutarate (Gray et al., 2004; Templeton and Moorhead, 2004) in cyanobacteria and plants. The precise mode of action of these metabolites is extremely variable, since they can affect not only the transcription via the inhibition of chromatin modifiers but also histone acetylation leading to the inhibition of mitogen-activated protein kinase (MAPK) signaling (Wellen et al., 2009; Gomez et al., 2010; Yang et al., 2012). In this vein, it has been demonstrate that citrate, regulates the accumulation of transcripts related to alternative respiratory pathways in both tobacco (Nicotiana tabacum) and Arabidopsis (Gray et al., 2004; Clifton et al., 2005). In addition, citrate, malate, and 2-oxoglutarate can affect nitrogen assimilation via controlling the abundance of Nitrate Reductase (NR) transcripts in tobacco (Müller et al., 2001). It is reasonable to assume therefore that TCA cycle intermediates are good candidate to play signaling roles in land plants. In good agreement, such molecules reflect both the metabolic and redox status of the cell and are known to be transported between compartments (Finkemeier et al., 2013).

It is important to mention that all genes coding for enzymes of the TCA cycle in tomato plants have been cloned and further investigation. Collectively this allowed the generation of a suite of transgenic plants in which the majority of the enzymes in the pathway are progressively decreased and these plants have provided an almost complete view of the distribution of control in this important

pathway (Araújo et al., 2012a) and organic acids might have important functions in control of several important process as connections of mitochondria with photosynthesis (Nunes-Nesi et al., 2008) and/or carbon to nitrogen metabolism (Araújo et al., 2014). The signaling importance of TCA cycle intermediates might rely also in how exactly the plant metabolism is reprogrammed following changes in their levels. For instance, although reduced expression of genes encoding for citrate synthase (CS) (Sienkiewicz-Porzucek et al., 2008), isocitrate dehydrogenase (ICDH) (Sienkiewicz-Porzucek et al., 2010; Sulpice et al., 2010), and 2-oxoglutarate dehydrogenase (OGDH) (Araújo et al., 2012b) in tomato plants shows no differences in both carbon assimilation and fruit yield, changes in mitochondrial ICDH resulted in reduced fruit diameter and fresh weight, probably associated to sink:source impacts (Sienkiewicz-Porzucek et al., 2010) whereas changes in OGDH resulted in early senescence coupled with significant alterations in metabolites pattern during fruit development (Araújo et al., 2012b). Moreover, reductions on the expression of succinyl-CoA ligase (SCoAL) (Studart-Guimarães et al., 2005) and fumarase (FUM) (Nunes-Nesi et al., 2007) resulted in reduction in both fruit yield and size. In the same vein, reductions on the expression of aconitase (ACO) (Carrari et al., 2003), malate dehydrogenase (MDH) (Nunes-Nesi et al., 2005) and succinate dehydrogenase (SDH) (Araújo et al., 2011b) were associated with enhanced fruit yield and SDH and MDH mutant plants were characterized by bigger fruits. Collectively, these data provide a range of evidence that the metabolic connections associated with TCA cycle are responsible for such changes and therefore an extensive metabolic reprogramming occurs when changes in TCA cycle takes place. It might be reasonable to assume that further demonstration of the importance of changes in organic acid levels in fruits by specific manipulation of genes associated in fruits or even during fruit maturation by using specific promoter following genetic manipulation it seems necessarily to fully comprehension of this process.

The metabolic behavior of organic acids during fruit development

Fleshy fruit development is often characterized by a breakdown of stored carbohydrates to sugars coupled with reductions in acidity alongside with increases in flavor and aroma volatiles (Klee and Giovannoni, 2011; Cherian et al., 2014). It is accepted that organic acids are important due to the control of fruit

growth via cell expansion through water uptake (Liu et al., 2007). Accordingly, organic acids accumulation during the early stages of fruit development is directly related to the supply of substrates for the maintenance of respiration processes during the development (Miyasaka Almeida et al., 2002; Seymour et al., 2013). It is important to mention that different species, including apple, berries citrus, kiwifruit (Actinidia deliciosa), grape, peach, pepper, and tomato, present a highly similar pattern in which high organic acids concentration are observed in the first stages of fruit development followed by clear reductions in the levels of such metabolites (Berüter, 2004; Famiani et al., 2005; Lombardo et al., 2011; Osorio et al., 2011; Nardozza et al., 2013; Fraige et al., 2015; Lin et al., 2015). Over the past two decades, much research efforts have been placed towards understanding the metabolic properties and organic acid behavior of several fleshy fruits (e.g. peach, strawberry, tomato, etc.) ranging from physiology and biochemistry to broad molecular and genetics approaches, and experimental techniques. Thus, over the following sections we will provide a detailed discussion about the organic acids behavior during fruit development as well as its relationship with other important metabolites.

a. Organic acids versus sugar metabolism

The ripening process in fleshy fruits are characterized by a series of coordinated changes in which both fruit biochemistry and physiology are drastically altered with several epigenetic alterations (Brady, 1987; Giovannoni et al., 2017). Although it is well known that these specific changes during ripening are typically variable according to the species as well as stages of maturation besides stress responses, often justified by changes secondary metabolism, thereby potentially increasing plant defenses and the concentrations of compounds involved in plant quality (Miller et al., 1998; Giovannoni, 2004; Baxter et al., 2005; Borsani et al., 2009; Ripoll et al., 2014). The main modifications observed are color, textural alteration, modification of sugars, organic acids, and volatile compounds (Giovannoni, 2004). Altogether, such modification are responsible to contribute to fruit flavor especially by adjusting the balance between sugar and organic acids (Chaimanee and Suntornwat, 1994). Accordingly, the major respiratory substrates present in most fruits are carbohydrates and organic acids and both their nature and concentration largely

affect taste characteristics and organoleptic quality (Seymour et al., 2013). As exception to this rule, few fruit such as avocado (Persea americana) contain high levels of lipids that does not seems to be utilized for respiration (Bennett et al., 1987).

The sugar accumulation during fruit development has been intensely studied in different species under different conditions. Throughout the ripening of the fruits an vast majority, fleshy fruits are characterized by increases in sugars contents whereas organic acids decrease, opening exceptions for citrus fruits, especially at the peak of maturity or ripening (Basson et al., 2010; Cao et al., 2013; Osorio and Fernie, 2013). However, the patterns and concentration change according to different species (Chen et al., 2004; Desnoues et al., 2014). In this same way, accumulation of sucrose, glucose, and fructose during ripening are especially observed in sweet fruits such as apples (Ackermann et al., 1992), litchi (Litchi chinensis) (Yang et al., 2013), mandarin (Yakushiji et al., 1996), melons (Burger et al., 2000), peach (Cirilli et al., 2015), strawberries (Treftz and Omaye, 2015), and watermelons (Citrullus spp.) (Liu et al., 2013). According to (Chen et al., 2004), the prerequisite for rapid accumulation of sugar in fruits is restricted to hexose catabolism and promotion of its synthesis In domesticated tomatoes fruits it is normally noted a higher accumulation of the hexoses with minor variations in sucrose content (Davies and Kempton, 1975; Carrari and Fernie, 2006).

It not surprising therefore that sucrose, glucose, and fructose, the most abundant carbohydrates and are widely distributed food components present in plants. Notably, the oxidation of such carbohydrates via glycolysis provides substrate for the participation of TCA cycle during the cell respiratory process, contributing not only to the generation of intermediate like organic acids but also contributing to cellular energy maintenance (Osorio et al., 2013; Osorio and Fernie, 2013). Remarkably, during the climacteric respiratory, there is a large increase in the rate of oxidation of substrates, mediated mainly by mitochondrial oxidases and as result, there is an increasing glycolytic flux. Interestingly, this enhanced glycolytic flux was associated with close relationship between the activities of key glycolytic enzymes (pyruvate kinase and phosphofructokinase) in different fruits such as apple, avocado, banana, and tomato (Rhodes and Wooltorton, 1967; Chalmers and Rowan, 1971; Bennett et al., 1987; Beaudry et al., 1989; Prabha and Bhagyalakshmi, 1998).

Similar to sugars, the organic acids are present in plants supporting several facets of cell metabolism and the accumulation of organic acids in plant cells is highly correlated with other metabolism pathways and appears to be under the control of many factors (Lin et al., 2016). The specific type and its levels are extremely dependent of species, development stages and tissue types (Araújo et al., 2011a). Although changes in content of organic acids are strongly dependent on the fruit in question it is important to highlight that the most abundant organic acids in many fruits are citrate and malate (Romero Rodriguez et al., 1992; Sweetman et al., 2009) and that the content of both can be variable over the different stages of fruit development (Table 1). The majority of the organic acids present in flesh fruits are not imported but rather synthesized in the flesh fruits mostly from imported sugars from glycolysis mediating starch as well as cell wall degradation (Etienne et al., 2013). This is in good agreement with findings showing that starch accumulation plays an important role in determining the soluble solids content (ºBrix index) of mature fruit (Schaffer and Petreikov, 1997; Redgwell et al., 1997; Baxter et al., 2005). It should be kept in mind that organic acids are equally important once they can play a plethora of roles in plants (Sweetman et al., 2009). Furthermore, organic acids present itself as highly valuable metabolites from a metabolic engineering perspective once the organic acid to sugar ratio defines a range of quality parameters at harvest time in fruits.

In recent studies proposed by (Lin et al., 2015), using integrative analyzes of metabolomics and transcriptome in ponkan fruits (Citrus reticulata) during fruit ripening, showed increase in sugars content followed by considerable reductions in the content of organic acids and perhaps more importantly that such behavior might be driven by sucrose phosphate synthase (SPS), asparagine transferases (AST), ATP-citrate lyase and glutamate decarboxylase (GAD) and sucrose metabolism shifted from synthesis to degradation, and was regulated by the activity balance between SPS and sucrose synthase (SS) gene products. In addition, both the glycolysis and the TCA cycle were accelerated during later maturation, indicating that the flux is somehow changing from sucrose metabolism to organic acid metabolism, with citrate degradation occurring mainly through the gamma-aminobutyric acid (GABA) and acetyl-CoA pathways (Lin et al., 2015). These data corroborate with the (Chen et al., 2012) that ponkan fruits

under hot air treatment (HAT) could activate citrate degradation via the GABA shunt pathway (especially by modulating ACO-IDH-GAD cascade), but not the glycolysis pathway.

Positive correlation between malate levels and the expression of genes involved in starch synthesis has been observed in pepper fruits meaning that malate metabolism most likely regulates transitory starch metabolism and that this process is probably conserved between climacteric and non-climacteric fruits (Osorio et al., 2012a). In good agreement, reduction of the activities of either the mitochondrial MDH (mMDH) or FUM in tomato fruits via targeted antisense approaches have demonstrated the physiological importance of malate metabolism in the activation state of ADP-glucose pyrophosphorylase (AGPase) that is correlated with the accumulation of transitory starch and also with the accumulation of soluble solids at harvest (Centeno et al., 2011). In agreement with that, we can suggest that perhaps cannot rule out a similar role of other organic acids during fruit development, and therefore it is reasonable to assume that manipulation of organic acid content might have unexpected responses associated with changes in fruit quality parameters and shelf life Since it is already known that changes in CS activity can severely compromise nitrogen metabolism and affect photosynthetic performance in tomato leaves (Sienkiewicz-Porzucek et al., 2008) citrate is a component that is found in considerable plant tissue content, can be found from 8% to 15% based on dry weight in plant, such as strawberries, lemons (Citrus lemon), spinach and haricot leaves (Popova and Pinheiro de Carvalho, 1998). Among the fruits, lemon and lime (Citrus aurantifolia) are the ones that accumulate most citrate is comprising as much as 8% of the dry fruit weight (Muller et al., 1996; Penniston et al., 2008).

In order to better understand the role of citrate in Pokan's fruits (Citrus reticulata), (Lin et al., 2016), they induced changes in citrate metabolism under different stresses such as low temperature (LT) and water stress (WS) and concluded that he expressions of transcripts of phosphoenolpyruvate carboxylase (PEPC), CS, ACO, and GAD were significantly increased in response to LT treatment, but showed no significant difference in WS compared to the control fruit. Thus, it can be concluded that LT may be the main factor influencing citrate metabolism during maturation in ponkan fruit. In the same hand, sweet orange (Citrus sinensis), a non-climacteric fruit, showed increased

activities of the enzymes of organic acid metabolism, as malic enzyme (ME), ICDH, ACO, and alcohol dehydrogenase, during the first 3 weeks of post-harvesting storage, concomitantly with the increase on the activity of enzymes involved in sugar catabolism, as hexokinase (HXK), SS, UDPG pyrophosphorylase, and PPi-dependent phosphofructokinase, during storage. Notably, these enzymes are necessary for organic acid use and for the subsequent oxidization of sugars in harvested sweet oranges (Echeverria and Valich, 1989). When considered together it seems reasonable to suggest that a very tight connection between sugar and organic acid metabolism is generally occurring during fruit development. How exactly this exquisite regulation occurs still remains to be tested in future investigation. The connection between organic acids and harvesting quality is also intriguingly in pineapple inflorescence. It turns out that pineapple is characterized by high contents of organic acids and this is primarily controlled by the activity of key enzymes such as CS, ACO, PEPC, MDH, and ME However, the ACO seems to link a greater role in the change of the acidity of this influence (Saradhuldhat and Paull, 2007).

Papaya fruit is a typical climacteric simple fruit and studies have revealed that the accumulation of sugar, especially sucrose occurs between 20 and 30 days before physiological maturation and, during this stage, there is a significant increase in acid invertase (AI) activity with lower SPS and SS enzyme activities in papaya mesocarp (Chan and Kwok, 1976; Hubbard et al., 1991; Zhou et al., 2000). In another hand, has been demonstrated that after harvesting, there was still sucrose synthesis, and more importantly the sucrose-phosphate synthase (SPS) activity is highly correlated with the sucrose content, indicating the importance of this enzyme during the process (Gomez et al., 2002). It is worth to mention that during this time has been also observed a steady decline in alcohol-insoluble solids, cell wall and several minerals coupled with an increase in total sugars (Selvaraj et al., 1982; Paull, R., & Chen, 1983). Papaya fruits has as main organic acids citrate, malate and ascorbate, however in low concentrations, in particular, citrate and malate have their content reduced over the course of ripening (Brekke et al., 1971; Selvaraj et al., 1982; Souza et al., 2014; Silva et al., 2015). Changes in enzymes involved in the metabolism of organic acids during the development of melon fruit have demonstrated a positive correlation between

the activities of the TCA cycle enzymes ACO and ME and citrate concentration (Tang et al., 2010).

Sugar and organic acid content in immature peach fruits showed that malate, quinate and shikimate concentrations were high at the beginning but declined at the end of fruit development. Thus, citrate concentration were maximal at immature fruits while mature fruits were characterized by increased sugar concentrations, especially sucrose (Wu et al., 2005). Interestingly, during ripening sucrose degradation in peach was accompanied by an increase of glucose and fructose and specific up and down regulation of transcripts encoding neutral invertases (NI), indicating differential or non-redundant functions of each putative NI isoform in peach (Borsani et al., 2009). PEPC was markedly induced, and may participate as a glycolytic shunt, since the malate level did not increase during postharvest ripening in the same fruits (Borsani et al., 2009). This suggests that alterations in genes involved in the metabolism of organic acids can determine the quality and extension of shelf-life of non-climacteric fruits, which have their physiological changes reduced after being detached from the plant.

The levels of malate is the predominant acid in many fruits, both climacteric and non-climacteric fruit, including plum (Prunus salicina), banana, tomato, grape and apple (Morvai and Molnár-Perl, 1992; Wu et al., 2007; Kortstee et al., 2007; Sweetman et al., 2009; Singh et al., 2009; Centeno et al., 2011), while citrate is predominantly in citric fruits like oranges, lime and lemon (Albertini et al., 2006), however it is found in main concentration in grape, guava (Psidium guajava), papaya, pineapple and strawberry (Clements, 1964; Singleton and Gortner, 1965; Wilson et al., 1982; Bartolomé et al., 1995; González-Aguilar et al., 2003; Sturm et al., 2003) (Table 2). Notwithstanding, this pattern of accumulation and degradation of organic acids are not directly associated with respiratory and climacteric characteristics of the fruit, since it is known that some climacteric fruits such as tomato appear to utilize malate during the respiratory burst (Goodenough et al., 1985; Singh and Singh, 2008), while banana and mango (Mangifera indica) continue to accumulate malate throughout ripening, even at the climacteric stage (Selvaraj and Kumar, 1989; Agravante et al., 1991), whilst non-climacteric fruits also display widely varying malate accumulation and degradation events (Saradhuldhat and Paull, 2007; Sweetman et al., 2009).

b. Organic acids versus phytohormones.

The phytohormones as signaling molecules have been extensively studied in different aspects of regulation of development and plant growth, however, in recent years has required to understand the relationship between phytohormones and metabolic changes in fruits and how to handle them can improve the quality of fruits and vegetables. For that, studies have been done focused on how phytohormones affect the metabolism of sugars and starch and how they can extend post-harvest life, besides understanding the modulations of organic acids (Bastías et al., 2011; Sagar et al., 2013a; Karlova et al., 2014; Kumar et al., 2014). In addition, understanding of the complex pathways through which organic acids are metabolized as well as how these are regulated in vivo and how phytohormone can interact with these pathways remains incomplete (Fernie et al., 2004). However, there are still few studies that try to associate phytohormones and metabolic changes and their involvement in the TCA cycle and mitochondrial respiration (Fernie et al., 2004; Sagar et al., 2013b,a; Kumar et al., 2014). The mitochondria are intimately involved in the seed germination (Macherel et al., 2007), flower development (Carlsson et al., 2008), and fruit ripening (Centeno et al., 2011) and also this one is closely linked with phytohormones (McAtee et al., 2013).

With the advent of 'omics' approaches has enabled significant progress to be achieved in characterization of phytohormone responses in fruits in general (Osorio et al., 2011; Gapper et al., 2013). In addition, one important aspect of fruit development is the modulation and regulation of its metabolism, mainly driven by changes in sugars, organic acids and secondary metabolites immediately after fruit setting and partially recovering during or after ripening (Carrari et al., 2006). It is reasonable to assume therefore that fruit quality is likely controlled by an intrinsic and widely interplay between multiple phytohormone that control fruit development and ripening, resulting in large metabolic changes, although there are few studies that associate hormonal control on starch catabolism, increment of sugars and modulations in the TCA cycle (Bastías et al., 2011; McAtee et al., 2013; Sagar et al., 2013b; Kumar et al., 2014). In climacteric fruit occurs an increase in respiration and ethylene biosynthesis upon initiation of ripening, however on the other hand, in non-climacteric fruit the respiratory burst and rise in ethylene production are absent and is known that no single master control the

ripe one such as in climacteric fruit, but there are increase are different phytohormones like ethylene, abscisic acid (ABA), brassinosteroids have been suggested to promote ripening through complex interactions in this fruit, whereas auxin delays some ripening associated process (Kuhn et al., 2014; Fortes et al., 2015).

The phytohormone ethylene has been show to control many ripening-associated metabolic pathways, altering gene expression involved in senescence, defense signalizing and fruit ripening, being the last one autocatalytic ethylene production, changes in cell wall metabolism, carotenoid accumulation, chlorophyll degradation, synthesis of volatiles compounds such as modulation in sugars and acids contents (Giovannoni, 2001; Alexander and Grierson, 2002; Osorio et al., 2011; Pech et al., 2012; Chang, 2016). In melon, the ethylene has also been shown an important in the production of aroma volatiles as antisense suppression in ethylene production results in strong inhibition of aroma (Ayub et al., 1996). Peach is a climacteric fruit whose ripening process is controlled by ethylene with increase ethylene production and causes increased respiration, and changes in the chemical composition and the physical characteristics of the fruit with increase in citrate, malate and sugars (glucose and fructose) during the days after harvest and decrease in sorbitol and sucrose (Borsani et al., 2009).

In addition, recent studies have shown that strawberry fruit ripening, the ethylene is also involved in an organ-specific manner, controlling the levels of amino acids, glucose and fructose, and citrate and malate differentially in achene and the receptacle (Merchante et al., 2013b). In agreement, previous studies have reported the timely endogenous production of minor amounts of ethylene by the fruit as well as the differential expression of genes of the ethylene synthesis, reception, and signaling pathways during fruit development (Merchante et al., 2013b), generating controversial conclusions about this phytohormone in strawberry ripening (Trainotti et al., 2005; Iannetta et al., 2006; Sun et al., 2013). Thus, strawberry plants with altered sensitivity to ethylene could be used to unravel the role of ethylene in the fruit ripening process, as well as understanding modulation in metabolic pathways (Merchante et al., 2013b). In the same way in grape, a non-climacteric fruit, the ethylene seems to be also required for berry development and ripening (Chervin et al., 2004). Indeed,

according with during the grape ripening (Giovannoni, 2004; Leida et al., 2016), numerous transcriptional and metabolic changes are required in order to obtain colored, sweet and flavored berries and it has suggest that ethylene could triggering the onset of ripening and in fact, Ethylene Responsive Factor (VviERF045) affects phenolic secondary metabolism, key related to changes in epidermis and cuticle of the berry, cell expansion, a decrease in photosynthetic capacity in leaves, and the activation of several defense related genes as well as from the phenylpropanoids metabolism (Leida et al., 2016). Furthermore, recently has described role of polyamine catabolism in grape ripening is discussed, together with its putative interaction with other phytohormones. Moreover, other recent examples of cross-talk among the different phytohormones are presented, revealing a complex interplay of signals during grape development and ripening (Fortes et al., 2015). Additionally, these responses of ethylene in non-climacterics fruits is associated via cross-talk with other phytohormones such as ABA, auxin (Davies et al., 1997) and brassinosteroids (Symons et al., 2006), all of which are known to play a part in grape berry ripening.

In tomato fruit, in order to understand better the participation of ethylene in fruit ripening, several mutants lines have been generated that drastically affect the ripening processes of tomatoes fruits, for instance, ripening inhibitor (rin), non-ripening (nor), green ripe (Gr), green-flesh (gf), colorless non-ripening (Cnr), never ripe (Nr), high pigment 1 (hp1), high pigment 2 (hp2) and dark green (dg) (Lanahan et al., 1994; Mustilli et al., 1999; Vrebalov, 2002; Levin et al., 2003; Barry and Giovannoni, 2006, 2007). However, the complete understanding of the ripening regulation network is still not fully understood, the use of mutants has provided new insights on their respective roles and also hierarchical relations of regulation (Giovannoni, 2004). Indeed, according with (Osorio et al., 2011) Osorio et al., 2011, analyzed three dominant ripening mutants of tomato, nor, rin, and Nr, along the developmental and ripening periods and as results, could identify very strong correlation between ripening-associated transcripts and specific metabolite groups, such as organic acids, sugars, and cell wall-related metabolites, underlining the importance of these metabolic pathways during fruit ripening, revealing multiple ethylene-associated events during tomato ripening. The ethylene doesn’t act to regulate the ripening just alone, but it works in

conjunction with others phytohormones, known as cross-talk, specially auxin, ABA and cytokinin (McAtee et al., 2013; Kumar et al., 2014).

In apple one of the main organic acids is the malate (Table 2) and has recently been shown that ethylene is a regulating levels of such organic acid which in studies using the 1-methylcyclopropene (1-MCP) (inhibitor of ethylene perception) was observed that this compound delayed the loss of malate and citrate (Lu et al., 2013; Liu et al., 2016). The use of salicylic acid (MeSA) as a treatment for post-harvest in fruit sweet cherry demonstrated an effective and environmentally friendly tool to maintain sweet cherry quality during storage with enhanced bioactive compounds concentration and antioxidant activity and controlling the content of sugars and organic acids in the fruit (Giménez et al., 2015, 2016).

In recent studies suggest that ABA signaling may affect different aspects of fruit maturation (Sun et al., 2012). Overexpression of SlAREB1 resulted in increased content of organic acids, hexoses, hexose-phosphates, and amino acids in immature green, mature green, and red ripe fruits, and these modifications correlated with the up-regulation of enzyme-encoding genes involved in primary carbohydrate and amino acid metabolism, suggesting a possible role for this transcription factor in the regulation of fruit organoleptic properties (Bastías et al., 2011, 2014). In tomato, they are essential for fruit maturation and flavor, and they correlate with the expression of genes associated with ethylene and cell wall metabolism-related pathways (Carrari et al., 2006; Centeno et al., 2011; Osorio et al., 2012a). In addition, the suppression of the NCED1 (9-cis-epoxicarotenoid dioxygenase) gene, that catalyze the first step in ABA biosynthesis, results in down-regulation of several other genes involved in ripening process like, cell wall (polygalacturonases and pectinmethylesterase) (Sun et al., 2012; Yao et al., 2012). Similarly, the reduction of NCED in strawberry expression resulted in delayed fruit maturation (Jia et al., 2011). Most of the time, the reduction of the activity of these enzymes results in changes in the content of fruit sugars as well as in the firmness, causing the softening and damages, thus compromising fruit quality (Souza et al., 2009; de Oliveira and Vitória, 2011; Jha et al., 2013). Indeed, in papaya fruit, the loss of firmness was highly related to the hydrolytic enzyme activities and the sweet taste to the presence of simple sugars such as galactose liberated from the polysaccharide complexes (Yao et al.,

2012). In addition, a Integration ethylene and brassinosteroids (BR) can regulate the mitochondrial metabolism and in turn the respiratory process where, BR effect on the capacity of AOX-dependent electron transport, antagonized by ethylene (Mazorra et al., 2013; Mazorra Morales et al., 2014).

Currently, some studies have shown a wide participation of auxin in various processes formation and ripening of fruits, mainly via crosstalk between gibberellins and ethylene. Recent studies have demonstrate that tomato ripening can be regulated by exogenous auxin, once genes related to carotenoid metabolism, cell degradation and energy metabolism were strongly down-regulated by exogenous auxin furthermore, exogenous auxin altered the expression patterns of ethylene and auxin signaling-related genes that were induced or repressed in the normal ripening process, suggesting significant crosstalk between these two phytohormones during tomato ripening (Su et al., 2015; Li et al., 2016). Indeed, also in peaches, auxin has shown the ability to regulate the expression of a number of different genes moreover, many genes involved in biosynthesis and transport and, in particular, the signaling (receptors, Auxin Response Factors and Aux/IAA) of auxin had increased expression in the mesocarp during ripening (Trainotti et al., 2007). In addition, auxin is considered an important phytohormone for initiation of fleshy fruit development, since it was shown to play a key role in triggering fruit set upon flower fertilization (Chareonboonsit et al., 1985; Tang et al., 2015). Recent researches have showed an important participation of auxin in ripening as a modulator of the sugar levels in the fruit. SlARF4, an Auxin Response Factor is involved in the control of sugar metabolism during tomato fruit development an altogether, ARFs control of sugar content, an essential feature of fruit quality, and provide insight into the link between auxin signaling, chloroplastidic activity, and sugar metabolism in developing fruit (Sagar et al., 2013b,a).

In the last years, with the appearance of numerous mutants in the most different plant organisms of the most different phytohormones, and with the advancement of the omics, the level of understanding of the molecular, biochemical, transcriptional events of both climacteric fruits and non-climacteric fruits was greatly Discovered and restructured. However, studies directed to the direct effect of the phytohormone and the central metabolism during fruit formation and maturation are still little known. The works even presented often

low resolution and with comprehensive conclusions and for this emerging studies under new molecular technologies may help to understand the real direction of the involvement of each phytohormone in the pathway of central metabolism.

Understanding organic acids metabolism in fruit models

To increase our current understanding about how organic acids affect fruit metabolism we highlight two complementary approaches: (i) it is possibly a screening of ILs fruits searching phenotypes with increase in different TCA cycle intermediates to analyze relationship between developmental process and carbon metabolism (Schauer et al., 2006; Morgan et al., 2013); and (ii) engineering fruit-specific inhibitions in TCA cycle enzymes and analyze the phenotype resultant, linking with central metabolism (Centeno et al., 2011; Osorio et al., 2012b).

Until now, our knowledge about specific functions of organic acids in fruits is very restricted to experiments with more generalist focus. We have a number of examples in tomato research as described before. Changes in levels of FUM and MDH specifically during the fruit development lead to important alterations on carbon metabolism and fruit quality (Centeno et al., 2011) as well demonstrated the levels of ACO interfered directly in levels of important metabolites as citrate and malate during fruit development (Morgan et al., 2013). These two examples are according our suggestion to experimentation and it is important highlight that techniques using modern tools as fruit specific promoters are available to researchers (Fernandez et al., 2009).

It is also important that besides the changes in levels of organic acids in fruits, it seems necessary studying post harvesting physiology in more real environments, which means being in contact with companies that are selling fruits oversee and looking how some stress conditions, for instance low temperature as demonstrated in ponkan fruits (Lin et al., 2016), is affecting organic acid content and other important traits as discussed before.

Finally, coupling omics approaches with system biology, to analyze as demonstrated in tomato fruits (Osorio et al., 2011) and provide generation of plants, or fruits, more adapted to ever growing stress conditions since it is of general opinion that a fruit is clearly a response of what the plant has faced during their entire development. In summary, plenty of research still necessary to keep

increasing our knowledge in fruit metabolism during development and the pivotal importance of organic acid metabolism.

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TABLES

Table 1. Behavior of different sugars and organic acids present in the mesocarp of different fruit during growth and ripening in normal conditions of evaluation.

	Sugars			Organic Acids			
Fruits	Glucose	Fructose	Sucrose	Citrate	Malate	Fumarate	**Reference**
Apple	Increase	Increase	Increase	-	Increase	-	1–3
Banana	Increase	Increase	Decrease	-	Increase	-	4,5
Grape	-	-	-	Increase	Increase	-	6–8
Guava	Increase	No change	Traces	Increase	Traces	-	9
Kiwifruit	-	-	-	-	Increase	-	10
Lemon	Increase	Increase	Decrease	Increase	No change	-	11
Lime	No change	Increase	increase	Increase	Increase	-	11
Melons	Increase	Increase	Increase	-	-	-	12,13
Orange	Increase	Increase	Increase	Increase	No change	-	11
Papaya	No change	No change	Traces	Increase	-	-	14,15
Peach	Increase	Increase	Decrease	Decrease	Increase	-	16–18
Pineapple	Increase	Increase	Few changes	Increase	Few changes	-	19,20
Plum	No change	Increase	Decrease	Increase	Increase	Increase	21
Strawberry	Increase	Increase	Increase	Increase	Increase	-	22,23
Tomato	Increase	Increase	No change	-	Increase	Decrease	24
Watermelon	Increase	Increase	Increase	-	-	-	25

Table 2. The main sugars and organic acid in both climacteric and non-climacteric ripe fruit.

Fruits	Main Sugar	Main Organic Acid	Reference
Climacteric			
Apple	Fructose	Malate	2,3
Apricot	Glucose/Fructose	Malate/Citrate	26
Atemoya	Fructose/Glucose	Fumarate/Malate	27
Banana	Fructose	Malate	28
Blueberry	Glucose/Fructose	Citrate	29
Feijoa	Sucrose	Malate/Citrate	30,31
Fig	Glucose/Fructose	Citrate	32
Guava	Fructose	Citrate	33,34
Mango	Fructose	Citrate/Malate	35,36
Papaya	Glucose	Citrate	37
Peach	Glucose/Fructose	Malate/Citritate	16,17,38
Pear	Fructose/Sorbitol	Malate/Citrate	39,40
Non-climacteric			
Blackberry	Fructose	Isocitrate	41,42
Grape	Glucose	Malate/Tartarate	43,44
Lemon	Fructose	Citrate	11,45
Lime	Fructose	Citrate	11,45,46
Litchi	Sucrose/Glucose	Tartaric/Malate	47,48
Longan	Sucrose/Fructose	Malate/Oxalate	49–51
Orange	Fructose	Citrate	8,11,45,46
Pineapple	Sucrose/Fructose	Citrate	52
Ponkan	Sucrose/Fructose	Citrate/Quinate	11,53
Strawberry	Fructose/Glucose	Citrate	29

Reference of tables 1 and 2

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Chapter 2: Exogenous ethylene treatment reduces plant growth via changes in primary metabolism and cell wall in tomato leaves

INTRODUCTION

Comprehension of mechanisms that regulate plant growth and development is of key significance in attempt to increase crop yield and quality as well as to develop crops that are more stable and tolerant to stress in general. Phytohormones are one of the main players involved in the control of plant growth and development being also associated to plant responses to environmental conditions (Wolters and Jürgens, 2009; Depuydt and Hardtke, 2011). Ethylene, an intriguing and simple gaseous molecule, is a plant hormone that is classically associated with fruit ripening and stress-related (Wang et al., 2007, 2013; Lin et al., 2013). Yet, compelling evidence have been provided suggesting other functions to ethylene and it seems that this hormone is also related to growth (Millenaar et al., 2005; Pierik et al., 2006; Kim et al., 2012) and photosynthesis (Khan, 2004; Tholen et al., 2008; Iqbal et al., 2011; Arve and Torre, 2015).

Ethephon (2-chloroethylphosphonic acid - CEPA) is an ethylene-releasing compound used mainly in crops during reproductive stages to facilitate fruit harvesting (Hagemann et al., 2015). This compound is a direct ethylene source when applied to plants and it is able to elicit response that are identical to those induced by ethylene gas (Edgerton and Blanpied, 1968; Warner and Leopold, 1969; Yang, 1969). It is important to mention that several studies on the effects of ethylene on photosynthesis and growth have revealed contrasting results, as assimilation rate increased in green and white mustards (Brassica juncea and Sinapis alba) (Buhler et al., 1978; Pua and Chi, 1993; Khan, 2004) and decrease in peanuts (Arachis hypogaea) and wheat (Triticum aestivum) (Kays and Pallas, 1980; Pallas Jr and Kays, 1982; Rajala and Peltonen-Sainio, 2001). It has been demonstrated that decreases of photosynthesis rate through ethylene-releasing compounds was mainly due to stomatal effects on peanuts (Pallas Jr and Kays, 1982). Accordingly, Arabidopsis (Arabidopsis thaliana) mutants that overproduce ethylene (eto1) exhibit lower total leaf, rosette area, likewise number of leaves and flowering bud indicating that these changes were caused by a single factor, the amount of ethylene signal which was transferred though an ethylene signal transduction pathway (Ogawara et al., 2003). Notwithstanding, the exact reason

behind this behavior has not been defined yet and thus the most likely explanation is the biphasic model proposed by Pierik et al. (2006) in which the differential responses to ethylene occurs by showing that low levels of ethylene promote plant growth whereas high levels inhibit.

The connection between ethylene and cell wall has long been described, mainly in fruit softening (Osorio et al., 2013; Kumar et al., 2014), although it remains rather imprecise on general. Thus the inhibition of cell expansion in response to ethylene at both shoot and root levels is a well-described process (for a review see Vandenbussche et al. (2012). Accordingly, a direct relationship between ethylene and auxin in controlling growth has been described (Swarup et al., 2007). However, given that ethylene effects on cell size can occur in an extremely rapid manner an auxin-independent mechanism wherein ethylene directly affect cell wall dynamics has been proposed (Vandenbussche et al., 2012). Ethylene is also associated to cell wall degradation enzymes in several fruits, and it seems to be involved in both pedicel abscission zone and during fruit ripening by regulating the softness of these organs (Muñoz-Bertomeu et al., 2013; Hagemann et al., 2015; Villarreal et al., 2016).

Although ethylene induces multiple, and sometimes paradoxical, responses on plant growth it remains unclear how exactly ethylene affects plant carbon status. This fact apart, both the germination and seedling growth of alfalfa (Medicago sativa) were characterized by influence of ethylene most likely on α-amylase activity and metabolism of soluble carbohydrates (Kepczyńska and Zielińska, 2013). In a cross-talk with abscisic acid (ABA), it was clearly demonstrated that ethylene play important roles in regulating grain filling and starch accumulation through suppression of starch synthesis genes in rice (Oryza sativa) (Wuriyanghan et al., 2009; Zhu et al., 2011). Although these studies have clearly advanced our understanding of the effects of ethylene in carbon metabolism, they provided limited information concerning the general role of ethylene on the regulation of plant growth and leaf carbon metabolism. Once the growth and development of sink organs are also at least partly under control of carbon availability (Smith and Stitt, 2007) and also this availability is the result of an adequate balance between photosynthesis and accumulation of starch and other carbon-metabolites during the light period opposed to their remobilization at night to sink organ (Sulpice et al., 2014; Ribeiro et al., 2016) it seems

reasonable speculate that growth impacts of ethylene may be associate with changes in carbon metabolism in leaves.

To gain further insights into how ethylene impact growth and leaf metabolism we investigated the physiological, molecular, and biochemical responses of tomato (Solanum lycopersicum) leaves upon high concentrations of exogenous ethylene. Here we demonstrate that plants treated with ethylene were characterized by growth inhibition which was associated with decreases in carbon assimilation due to both reduced photosynthesis rates and stomatal conductance, coupled with impairments in carbohydrates turnover. Furthermore, ethylene treatment culminated in increases of cell wall compounds (e.g. cellulose and lignin) and phenolic compounds indicating that changes in secondary metabolism are also occurring. Taken together, our results suggest that ethylene plays a more direct role in plant metabolism than previously thought, most likely by acting in reducing carbon assimilation and leading to altered central metabolism. The results obtained are discussed in the context of current models of ethylene effects on the regulation of both growth and metabolism.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Seeds of the tomato (Solanum lycopersicum cv MicroTom) wild type were surface-sterilized with 5% sodium hypochlorite for 10 min, then washed with running distilled water and subsequently sowed in a tray with commercial substrate (Tropstrato HT©). Seven days after germination (or following the appearance of the first true leaf), seedlings were transferred to 1.5 L pots containing the same commercial substrate but supplemented with 5 g L^{-1} 4:14:8 NPK. Plants were grown in a greenhouse located in Viçosa (20°45′S, 42°15′W, 650 m above sea level), southeastern Brazil, with a minimum of 400 µmol photons m^{-2} s^{-1}. Plants were watered regularly and throughout the entire growth period the plants were maintained under naturally fluctuating conditions of light intensity, temperature and relative air humidity.

To examine the functional role of ethylene, fourteen days after sowing plants growing singly in pots were sprayed with 1mM ethephon (2-chloroethylphosphonic acid - CEPA) or deionized water (control plants). All

analysis and growth measurements were performed in plants of 28 days after sowing (fourteen days after the onset of treatment).

Determination of Growth Parameters

Plants were harvested in one cut to 1.0 cm above ground level, separating roots from shoots. The roots, after water wash of soil contamination, and shoots were placed in labelled paper bags and brought to a forced air circulation oven at 70 ℃ for 5 days, after which the dry weight of roots and shoots (stem and leaves) was determined. In addition, the total number of flowers per plant was determined. Leaf area (LA) was measured by digital image method using a scanner (Hewlett-Packard Scanjet G2410) and the images were after processed using the ImageJ software (Schneider et al., 2012).

Relative growth rate (RGR), which is the net dry weight increase per unit dry weight per day (mg g^{-1} d^{-1}), and their components ($\mathrm{RGR} = \mathrm{SLA\ x\ LMF\ x\ ULR}$) SLA is the specific leaf area (m^2 leaf area kg^{-1} leaf mass), by which the amount of fixed C available for growth can be converted from a leaf area to a leaf biomass basis; LMF is the leaf mass fraction (g leaf g^{-1} total plant mass), by which the amount of fixed C available for growth can be converted from a leaf mass to a total plant mass basis; and ULR is the unit leaf rate (g dry mass increase m^{-2} leaf area day^{-1}), is the rate of increase in plant biomass per unit leaf area, a variable closely related to the daily rate of photosynthesis per unit leaf area, were estimated according Hunt (1982) and Poorter (2002):

$$\mathrm{RGR} = \frac{(\ln \mathrm{M}_2 - \ln \mathrm{M}_1)}{\mathrm{t}_2 - \mathrm{t}_1}$$

$$\mathrm{SLA} = \frac{\mathrm{LA}}{\mathrm{LM}}$$

where M_1 and M_2 are the plant mass at times t_1 and t_2, respectively and LM is leaf mass. M_1 was measured 7 days after sowing and M_2 28 days after sowing.

Measurements of Photosynthetic Parameters

Gas exchange and chlorophyll a fluorescence analysis were performed simultaneously in the third fully expanded leaf from the apex of using a portable open-flow gas exchange system (Li-6400XT, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber (LI-6400-40; Li-Cor Inc.). Measurements of the net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (E) were conducted from 08:00 to 12:00 h (solar time), which is when A was at its maximum, under an ambient CO2 concentration (C_a) of 400 µmol mol^{-1} air (CO_2 injected from a cartridge) and a flow rate of 300 µmol s^{-1}. All measurements were conducted under artificial photosynthetic photon flux density (PPFD), saturating light of 1000 µmol photons m^{-2} s^{-1} (from a LED source) with 10% blue light in order to maximize the stomatal opening, at 25 ℃ and the leaf- to-air vapor pressure deficit was maintained at approximately 1.0 kPa. The CO_2 concentration and water vapor between the leaf and the reference chamber were automatically matched before the data were recorded. Dark respiration (R_D) was measured using the same gas exchange system described above after at least two hours during the nigh period in the same leaf previously used to determine gas exchange parameters.

The initial fluorescence (F_0) was measured by illuminating previously dark adapted leaves with weak modulated measuring beams (0.03 µmol m^{-2} s^{-1}). Saturating white light pulses (8000 µmol photons m^{-2} s^{-1}) was applied for 0.8 s to obtain the maximum fluorescence (F_m), from which the variable-to-maximum Chl fluorescence ratio, was then calculated: $F_v/F_m=[(F_m - F_0)/F_m)]$. In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured with the application of a saturating white light pulse (8000 µmol m^{-2} s^{-1}, 0.8 s) to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off and a far-red illumination (2 µmol m^{-2} s^{-1}) was applied to measure the light-adapted initial fluorescence (F_0'). The capture efficiency of excitation energy by open photosystem (PS) II reaction centers (F_v'/F_m') was estimated following Logan et al. (2007) and the actual PSII photochemical efficiency (δPSII) was estimated as $\delta PSII = (F_m' - F_s)/F_m'$ (Genty et al., 1989). In addition, the coefficients of photochemical quenching (qP) and non-photochemical quenching (NPQ) and the electron transport rate (ETR), were determined as previously described (Martins et al., 2014; Medeiros et al., 2016).

Determination of Metabolite Levels

All sampling procedures were carried out on the third fully expanded source leaves (same leaves used for gas exchange analysis). Leaves were harvested in different time points along the light/ dark cycle (end of day and end of night). Leaves were flash-frozen in liquid nitrogen and stored at −80 °C until further analyses. Metabolite extraction was performed by rapid grinding in liquid nitrogen and immediate addition of the appropriate extraction buffer. The levels of photosynthetic pigments, starch, sucrose, fructose, glucose, protein and amino acids in the leaf tissues were determined exactly as described previously (Cross et al., 2006). Malate and fumarate was determined according to Nunes-Nesi et al. (2007).

Cell wall extraction, lignin and cellulose content quantification

Leaf samples were freeze-dried and homogenized to a fine powder less than 60 mesh. Isolation of cell wall residue (CWR) was performed by sequentially extractions with hot (i) water, (ii) 100% ethanol, (iii) chloroform, and (iv) acetone as previously described Giordano et al. (2014) wherein in each step the samples was incubated, for 30 min, 750 rpm, and temperatures of (i) 98, (ii) 76, (iii) 59, and (iv) 54 ºC, between the steps the samples was centrifuged (3 min, 10000 g), the supernatant was removed, and, at the end, the precipitates were dried at a speed vacuum concetrator (at least 3 hours). The extracted CWR was used for determination of total lignin and cellulose.

Lignin content was measured according to Foster et al. (2010) with modifications. Briefly, CWR samples were incubated for 30 min at 70 ºC in 100 µL 25% (v/v) acetyl bromide in glacial acetic acid and 4 µL of perchloric acid. Samples were cooled to room temperature and centrifuged (15 min, 10000 g). The supernatant was transferred to a new tube. The pellet was washed with 500 µL of acetic acid and centrifuged (3 min, 10000 g). The supernatants and the washing phase were combined and transferred to a new tube containing 200 µL NaOH (2 M). Final volume was adjusted to 2 mL with acid acetic. Lignin content was determined by measuring absorbance at 280 nm, calculated with an extinction coefficient of 18.126 and expressed in % of CWR.

Approximately 4 mg of cell wall residue was used to determine the amount of cellulose based on a colorimetric assay was performed as in Van Acker et al. (2013) and expressed in % of CWR.

Analysis of total phenolic compounds

Phenolic content in leaves was measured with Folin-Ciocalteau reagent (Sigma-Aldrich). Freeze dried samples (20 mg) were washed with chloroform and methanol. Supernatant was collected and washed with milliQ water. The upper phase was used for quantification. Reaction was performed with 7,5 µL of sample; 67,5 µL milliQ water; 37,5 µL Folin (1 M); 187,5 µL de Na2CO3 (20%). Samples were incubated at room temperature for 40 min with agitation. Determination of phenolic compounds was performed using a microplate reader (ELISE Versamax Molecular Devices, Sunnyvale, CA), at 725 nm, wherein tannic acid was used for standard curve, adapted from Ainsworth and Gillespie (2007).

Correlation and statistical analysis

The data was obtained from experiments using a completely randomized design. All statistical analyses were carried out using the algorithm embedded into Microsoft Office Excel®. Data are expressed as the mean ± standard error (SE). The term significant is used here only when the change in question has been confirmed to be significant ($P < 0.05$) by the performance of Student's t test. The original data is additionally given as Supplemental Table 1. Correlation analysis (Pearson parametric method) was calculated using all morphological and physiological data obtained including data obtained from treated and control plants.

RESULTS

In order to investigate the growth and developmental responses following ethylene treatments, we started by analyzing the growth of treated plants growing side by side with control plants and a clear reduction in growth of the aerial part of the treated plants was observed (Fig. 1) and as such 30% reduction in plant height compared with non-treated plants was observed (Fig. 2 A). Close examination of plants cultivated in presence of CEPA revealed at least 30% reduction in shoot and root dry weight, as well as in total plant dry weight in

ethylene treated plants compared to control ones (Table 1). Moreover, ethylene treated plants were characterized by reduced number of leaves (20%), leaf area (35%), culminating with 10% inhibition on RGR coupled with a 14% reduction in both ULR and SLA although an increase on LMF (13%) was observed. This can be explained, at least partially, by the higher reduction on total dry weight than on leaf dry weight and demonstrate that ethylene inhibition of growth affect drastically carbon partitioning on tomato shoot. This assumption is further supported by the strong reduction on stem dry weight (over 60%) despite the absence of changes in root to shoot ratio following CEPA treatment (Table 1). To better illustrate the correlation between RGR and its components, correlations graphs were created (Fig. 2B and C) showing high correlations between RGR and ULR (Fig. 2B) and SLA (Fig. 2C). Collectively, these results indicate that both ULR, which is related to biochemical changes, and SLA, which is related to structural/morphological alterations, could explain that inhibition of growth observed in ethylene treated plants. Furthermore, all flowers in CEPA treatment was aborted as expected in a high ethylene treatment.

Given that most of plant biomass is derived from photosynthesis we next investigated the photosynthetic performance by analyzing gas exchange and chlorophyll a fluorescence. This experiment revealed that ethylene-treated plants exhibited drastically reduction on gas exchange parameters as observed by reduced assimilation rates in close agreement with reductions in stomatal conductance and transpiration rates despite no changes in internal CO_2 concentration (Table 2). Analysis of other parameters of gas exchange revealed that ethylene treated plants were not impacted in dark respiration (Table 2). Taken together, these data indicate that the photosynthesis is reduced in CEPA treated plants by a mechanism that restricts CO_2 uptake via the stomata. We next determined chlorophyll a fluorescence in vivo using a pulse amplitude modulation and, with the exception of the maximum photochemical efficiency of PSII (F_v/F_m), all other parameters analyzed including F_v'/F_m', δPSII, qP, NPQ and ETR, exhibited an increase in ethylene-treated plants when compared with control which indicates that the photosynthetic apparatus is most likely disturbed.

To further investigate the functional link between ethylene and growth impairment we next performed a metabolic characterization in leaf tissues. First, we analyzed the levels of pigments and observed increases mostly in chlorophyll

b that led to simultaneous increases in total chlorophyll and reduction in chlorophyll a/b ratio (Table 3). Additionally, we observed that ethylene treated were characterized by strong inhibition of starch synthesis during the light period and as such control plants accumulated more starch at the end of the day (Fig. 3A), and this compound was consumed during the night period reaching similar levels in both ethylene treated and control plants. Given that the starch turnover was altered following CEPA treatment we further determined the sugar levels in leaves. The levels of sucrose (Fig. 3B) and glucose (Fig. 3C) followed a similar pattern as starch and increases of these sugars at the end of the light period were observed. Remarkably, the ethylene-treated plants were unable to fully degrade glucose (Fig. 3C) and fructose (Fig. 3D) by the end of the dark period, reaching higher values than those observed in control plants. Evaluation of compounds related to nitrogen metabolism revealed that there were no changes in the levels of total amino acids and soluble proteins (Fig. 3E and F) in both time points analyzed. In agreement with the results observed for R_d (Table 2) the levels of tricarboxylic acid (TCA) cycle intermediates, namely malate (Fig. 3G) and fumarate (Fig. 2H), were invariant between control and ethylene treated plants.

We next extend this metabolic study to investigate whether the growth and metabolic perturbation here could be associated with modifications of cell wall by the determination of cellulose and lignin, as well as total phenolic compounds. These studies revealed that cellulose increase more than 80% in leaves of ethylene treated plants when compared to control plants (Fig. 4A). Total lignin content in leaves following ethylene treatment revealed an increase (higher than 50%) when compared to control plants (Fig. 4B). Not only cell wall compound was enhanced but also an increased in cellulose/lignin ratio in ethylene-treated plants was observed (Fig. 4C). Total phenolic content in leaves was 30% higher after ethylene treatment (Fig. 4D).

Finally, as it is always the case with experiments using pharmacological chemicals, we cannot rule out the possibility that they could have potential secondary effects. For this reason, we carried out a broad correlation analysis using all analyzed parameters in an attempt to determine which changes were most closely associated with the changes in ethylene. When evaluating the strength of these correlations and their significances, it becomes apparent that in a universe of 1128 possible correlations we detected a total of 287 correlations

that are possible associated with the phenotypes here observed (Fig. 5). Among the correlations observed 179 were positive and 108 were negative, including some expected as positive height with RGR, A_N with g_S, and negative cell wall compounds with RGR, glucose at end of night with growth parameters as well some were less intuitive, such as those involving the chlorophyll fluorescence and content with cell wall compounds (Fig. 5).

DISCUSSION

Plant biomass accumulation is the result of mainly the balance of carbon fixation by photosynthesis versus carbon consumption by respiration and others metabolic pathways during the whole plant development. In this vein, although the pivotal role of ethylene in plant growth and development has been previously recognized (Pierik et al., 2006) the function of this hormone in controlling primary metabolism remains largely unknown, particularly in leaves. Although interactions between ethylene and growth seems to be strongly affected by ethylene concentrations (Ogawara et al., 2003; Pierik et al., 2006) our comprehension of how ethylene impact primary metabolism is still fragmentary and therefore we decided to investigate how exactly ethylene affects both growth and metabolism. Our results demonstrated that high concentrations of exogenous ethylene inhibit tomato growth by leading not only to modifications in leaf morphology as observed by changes in SLA and cell wall compounds (Table1; Fig. 4) but also by directly impacting gas exchange parameters (Table 2). This in good agreement with the possible actions mode of ethylene (Pallas Jr and Kays, 1982; Huang et al., 2013). During fruit ripening, ethylene induces the modification of cell wall, acting directly in degradation of this structure and softening of fruits (Villarreal et al., 2016) despite that in leaves we observe an increase in such compounds (Fig. 4), most likely as a protection. In good agreement with photochemical apparatus, chlorophyll a fluorescence (Table 2) and content (Table 3), that exhibit enhanced values in ethylene-treated plants clearly indicating that the ethylene perception in leaves occurs in a very exquisite manner in attempt to protect from a possible stress.

The amount of carbon available for growth is the result of an adequate balance between photosynthesis and accumulation of starch and other carbon-metabolites during the light period opposed to their remobilization at night to sink

organ (Sulpice et al., 2014; Ribeiro et al., 2016). Accordingly, the growth and development of sink organs are also at least partly under control of carbon availability (Smith and Stitt, 2007). The reduced growth observed in ethylene treated plants was followed by a similar and reduced pattern of carbohydrates accumulation during the light period in comparison to control plants (Fig. 2). It was observed that exogenous ethylene promoted a reduction in both A and g_S most likely due to both stomatal and biochemical limitations to the photosynthesis. Although we cannot formally exclude the possibility that ethylene application mimics a stress situation our results are highly indicative that this is also occurring in tomato treated plants, as it can be observed by the changes in sugars as well as in lignin content leading to growth reductions. This could be explained, at least partially, given that carbohydrates, mainly starch, produced during the light period must be remobilized to sustain growth at night period (Gibon et al., 2009; Sulpice et al., 2009; Pantin et al., 2011). In such way, it is reasonable to assume that high concentrations of ethylene impact primary metabolism by decreasing carbon levels in source organs and consequently culminating in growth inhibition.

We observed positive correlations between carbohydrates accumulation during the day and gas exchange parameters (Fig. 4) and it is important to mention that the effects of ethylene on both photosynthesis and stomatal response is still controversial. As such, it seems to be specie dependent and also variable depending on the growth conditions (Vandenbussche and Van Der Straeten, 2007). Briefly, ethylene induces stomatal closure via hydrogen peroxide synthesis in Arabidopsis (Desikan et al., 2006), a behavior described since de 1980's in several species (Pallas Jr and Kays, 1982; Madhavan et al., 1983; Taylor and Gunderson, 1986; Gunderson and Taylor, 1991). It seems reasonable to suggest that our results go on this direction given that we also observed changes in total chlorophyll content coupled with impairments in chlorophyll a fluorescence and phenolic compounds (Fig. 4). Remarkably, ethylene can also induce stomatal opening in cross-talk with ABA, auxin and/or cytokinin in Arabidopsis and fababean (Vicia faba) (Levitt et al., 1987; Merritt et al., 2001; Tanaka et al., 2005, 2006). These paradoxical behavior of stomata in response to ethylene is most likely associated with a differential signaling response at the guard cells (Pierik et al., 2006; Iqbal et al., 2011).

Interestingly, guard cells exhibit double responses to sucrose and hexoses concentration, more specifically the action of hexokinase (HXK) mediating g_s (Daloso et al., 2016). It has been shown that overexpression of HXK specifically in guard cells induces higher and lower g_s under low and high light intensities, respectively (Lugassi et al., 2015) and it is tempting to speculate that somehow the ethylene maybe it is associate with sugars and acting in control of stomatal responses. Although ethylene alone is able to promote stomatal closure in connection with other hormones ethylene can induce stomatal opening or, in other words, it can alleviate the closure effects. Thus, an unique and as yet relatively few understood cross-talk mechanism must be achieved in the presence of multiple hormonal stimuli (Acharya and Assmann, 2009). When analyzing the organic acids from TCA, malate and fumarate, no differences between the treatments were observed (Fig. 2). However, fumarate levels determined at end of the day seems to be positively correlated with growth-related parameters such as leaf area, leaf mass and root mass (Fig. 4). This is similar to what was recently observed in peppers (Capsicum chinense) accessions (Rosado-Souza et al., 2015). Several reports have already suggested that fumarate play an important function as a regulator of stomata function (Nunes-Nesi et al., 2007; Araújo et al., 2011a,b).

Plants treated with CEPA showed a strong decrease in the rate of leaf expansion and biomass accumulation coupled with strong negative correlations between cellulose and lignin and growth-related parameters (Fig. 4). Thus, it is reasonable to assume that ethylene seems to inhibit both stem and leaf growth simultaneously inducing lignification, which reinforced the cell wall, and increase of cellulose content (Fig. 3). The exact mechanism behind such response is not immediately apparent however similar results have been reported in Arabidopsis, mung bean and tobacco and in all instance is has been related to cell wall lignification (Faivre-Rampant et al., 1998; Almagro et al., 2009; Huang et al., 2013). This fact apart, the increase in total phenolic compounds in ethylene treated plants (Fig. 3) seems to be in good agreement with the growth inhibition effects and may explain, at least partially, the relocation of carbon from primary to secondary metabolites such as phenolic compounds which are directly involved in stress responses (Caretto et al., 2015). It is important to mention that these behavior has been previously described naturally in carrots and in wound-

induced in lettuce (Lactuca sativa) (Sarkar and Phan, 1979; Ke and Saltveit, 1989). Taken together these results demonstrate that high concentrations of ethylene perturb the optimal structure of leaves by changes in both primary and secondary metabolism, especially in carbohydrate and cell wall that ultimately lead to metabolic responses similar to stress situation such as increase on phenolic compounds.

Our model of growth inhibition is quite different of others, for instance the demonstrated in growth inhibition of Arabidopsis by Se, which is more related to starch consumption at night, and energy expenditure in that period, than with decrease in the photosynthetic rate (Ribeiro et al., 2016). Here we found that metabolic changes in light period and responses quite similar to a stress condition are more important to ethylene inhibition of growth, linking this phytohormone to control of growth and stress responses. Also, give the stomatal conductance responses indicated that further experimentation in guard cell is required to fully elucidate the function of ethylene in this highly-specialized cell. It seems reasonable conclude that ethylene in high concentrations affects growth both in ULR and in SLA leading to the decrease of carbon metabolism with lower CO_2 assimilation rates, and consequently a lower accumulation of starch and soluble carbohydrates during the day light period in opposition to the minor changes in carbohydrates that are exhibit during the night no difference in respiration rates what suggest that ethylene action in growth inhibition it more related to light period and assimilation of carbon. Further experimentation, e.g. metabolites flux, and profile, analysis of cross-talk with other phytohormones, and stomatal responses to regulators, are required to complete comprehension of the exactly role of ethylene in control of photosynthesis and carbon metabolism.

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TABLES

Table 1. Growth and morphological parameters in control and ethylene-treated plants with 28 days after sowing. Values are presented as average ± SE of at least 10 independent biological replicates per treatment; bold letters in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the control.

Parameters	Control	CEPA
Dry weight of leaves (g)	0,96 ± 0,03	**0,73 ± 0,04**
Dry weight of stem (g)	0,46 ± 0,02	**0,17 ± 0,01**
Dry weight of roots (g)	0,49 ± 0,03	**0,29 ± 0,02**
Dry weight of shoot (g)	1,42 ± 0,05	**0,91 ± 0,05**
Total dry weight (g)	1,91 ± 0,08	**1,21 ± 0,07**
Leaf area (cm2)	166,89 ± 6,83	**108,93 ± 6,63**
Number of leaves	9,41 ± 0,14	**7,25 ± 0,21**
Relative growth rate (mg g^{-1} day^{-1})	202,82 ± 0,4	**180,75 ± 1,15**
Unit leaf rate (g m^{-2} day^{-1})	25,81 ± 0,29	**21,99 ± 0,61**
Leaf area ratio (m kg^{-1})	18,91 ± 0,21	19,05 ± 0,25
Leaf mass fraction (g g^{-1})	0,36 ± 0,003	**0,41 ± 0,006**
Specific Leaf Area (cm2 g-1)	173,68 ± 3,19	**148,79 ± 5,43**
Root:shoot ratio	0,34 ± 0,01	0,32 ± 0,01
Number of flowers	9,33 ± 0,96	**NA**

Table 2. Gas exchange and chlorophyll a fluorescence parameters in control and ethylene-treated plants. Values are presented as average ± SE (n = 10) obtained in third fully expanded leaf from the apex of with 28 days after sowing; bold letters in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the control.

Parameters	Control	CEPA
A (µmol CO_2 m^{-2} s^{-1})	22,57 ± 0,66	**15,27 ± 0,76**
R_D (µmol CO_2 m^{-2} s^{-1})	1,22 ± 0,18	1,3 ± 0,17
g_S (mol H_2O m^{-2} s^{-1})	0,27 ± 0,022	**0,16 ± 0,03**
E (mol H_2O m^{-2} s^{-1})	2,69 ± 0,15	**1,86 ± 0,19**
C_i (µmol CO_2 mol air^{-1})	230,40 ± 8,68	209,24 ± 16,31
F_v'/F_m'	0,61 ± 0,01	**0,64 ± 0,01**
F_v/F_m	0,84 ± 0,001	0,83 ± 0,002
δPSII	0,38 ± 0,02	**0,44 ± 0,01**
qP	0,61 ± 0,03	**0,69 ± 0,01**
NPQ	2,61 ± 0,08	**2,79 ± 0,04**
ETR	166,72 ± 9,07	**195,75 ± 3,48**

A, net CO_2 assimilation rate; R_D, dark respiration; g_s, stomatal conductance; E, transpiration rate; F_v/F_m, maximum PSII photochemical efficiency; F_v'/F_m', actual PSII photochemical efficiency; δPSII, actual PSII photochemical efficiency; qP; photochemical quenching; and NPQ, non-photochemical quenching.

Table 3. Photosynthetic pigments content changes at midday. Values are presented as average ± SE (n = 10) obtained in third fully expanded leaf from the apex of with 28 days after sowing; bold letters in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different ($P<0.05$) from the control. FW, fresh weight.

Parameters	Control	CEPA
Chlorophyll *a* (mg g^{-1} FW)	2,31 ± 0,08	2,46 ± 0,07
Chlorophyll *b* (mg g^{-1} FW)	0,95 ± 0,03	**1,21 ± 0,04**
Total chlorophyll (mg g^{-1} FW)	3,26 ± 0,10	**3,67 ± 0,11**
Chlorophyll *a*/*b* ratio	2,50 ± 0,05	**2,04 ± 0,05**

FIGURE LEGENDS

Figure 1. Photographs showing phenotypic changes in plants with 28 days after sowing. **(A)** Representative plants exhibiting contrasting phenotypes between control and CEPA treatment, fourteen days after treatment, **(B)** superior view of a representative control plant, and **(C)** superior view of a representative CEPA treated plant.

Figure 2. Growth and morphological parameters in control and ethylene-treated plants. **(A)** Height of plants during 4 weeks of experiment; **(B)** unit leaf rate and **(C)** specific leaf area as function of relative growth rate. In **(B)** and (**C)** each point represents a value from an individual measurement. Control, black bars and circles; CEPA, white bars and circles. Values are presented as average ± SE (n = 8). Asterisks (*) in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different ($P<0.05$) from the control.

Figure 3. Metabolism variation along diurnal cycle. Metabolites, **(A)** starch, **(B)** sucrose, **(C)** glucose, **(D)** fructose, **(E)** proteins, **(F)** amino acids, **(G)** malate, and **(H)** fumarate were obtained in third fully expanded leaf from the apex of plants with 28 days after sowing. Leaves were harvest in two points: at the end of the day (white sectors) and the end of the day (grey sectors). Control, black bars; CEPA, white bars. Values are presented as average ± SE (n = 8). Asterisks (*) in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different ($P<0.05$) from the control.

Figure 4. Cell wall compounds and total phenolic content in leaves of control and ethylene-treated plants. **(A)** Cellulose, **(B)** lignin, **(C)** cellulose/lignin ratio, and **(D)** total phenolic compounds. Control, black bars; CEPA, white bars. Values are presented as average ± SE (n = 8). Asterisks (*) in CEPA treatment plants indicate values that were determined by the Student's t-test to be significantly different ($P<0.05$) from the control.

Figure 5. Pearson correlation analysis. Significant correlation coefficients ($P<0.05$) are indicated by dark, medium, and light shading, with positive and negative correlations being distinguished by red and green, respectively.

FIGURES

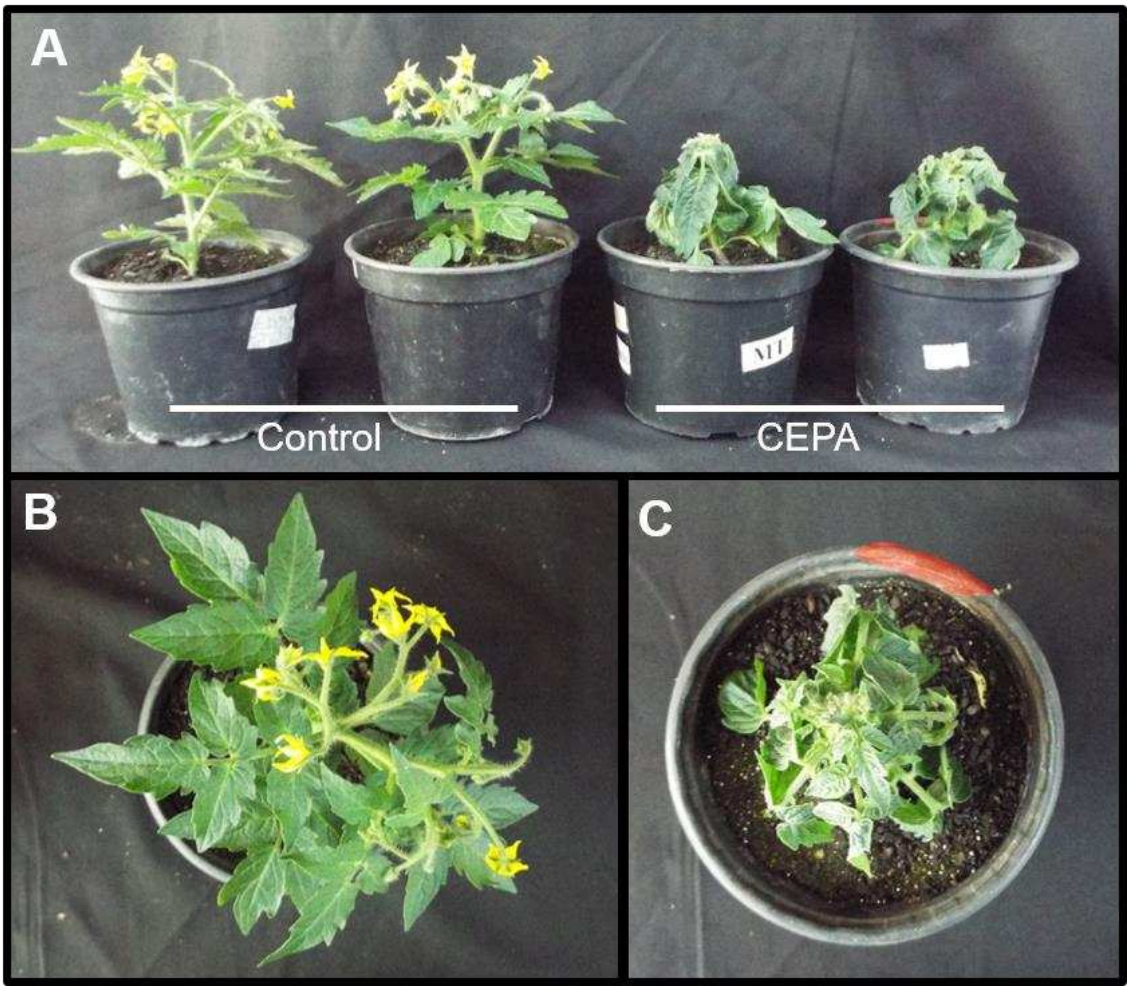

Figure 1.

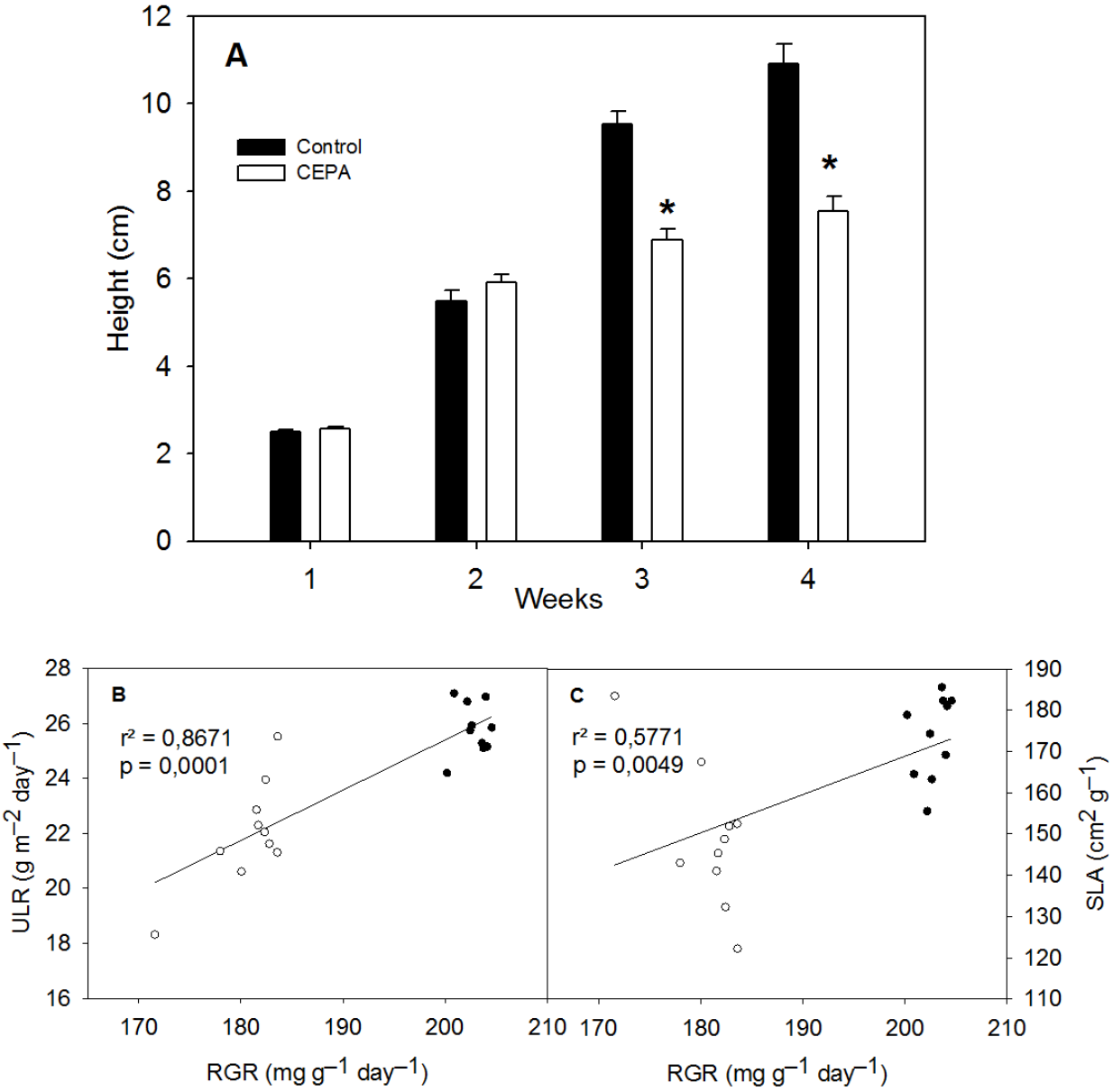
A
Control
CEPA
Height (cm)
Weeks
*
*
B
r² = 0,8671
p = 0,0001
ULR (g m⁻² day⁻¹)
RGR (mg g⁻¹ day⁻¹)
C
r² = 0,5771
p = 0,0049
SLA (cm² g⁻¹)
RGR (mg g⁻¹ day⁻¹)

Figure 2.

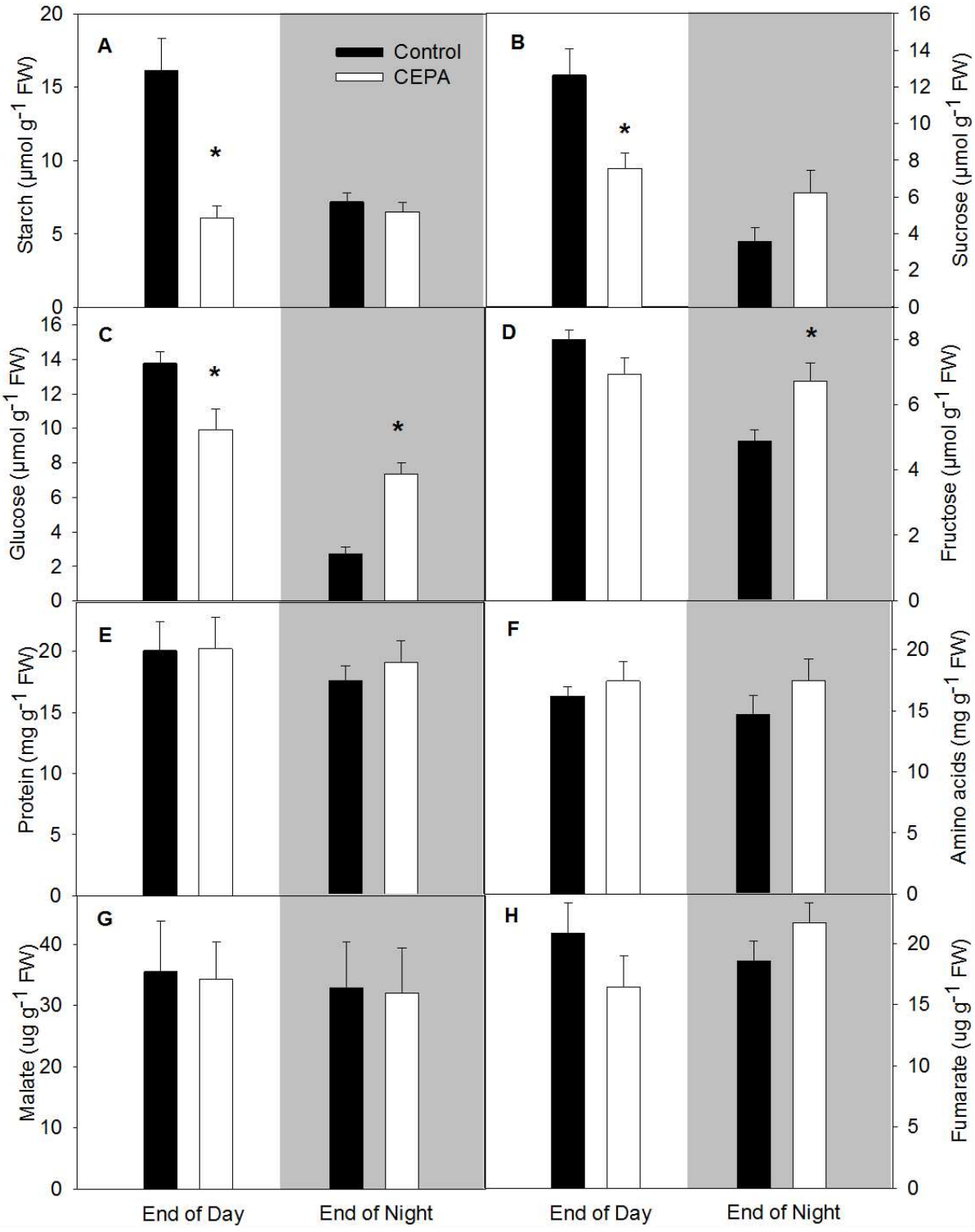
A
B
C
D
E
F
G
H
Control
CEPA
Starch (µmol g-1 FW)
Sucrose (µmol g-1 FW)
Glucose (µmol g-1 FW)
Fructose (µmol g-1 FW)
Protein (mg g-1 FW)
Amino acids (mg g-1 FW)
Malate (ug g-1 FW)
Fumarate (ug g-1 FW)
End of Day
End of Night
End of Day
End of Night

Figure 3.

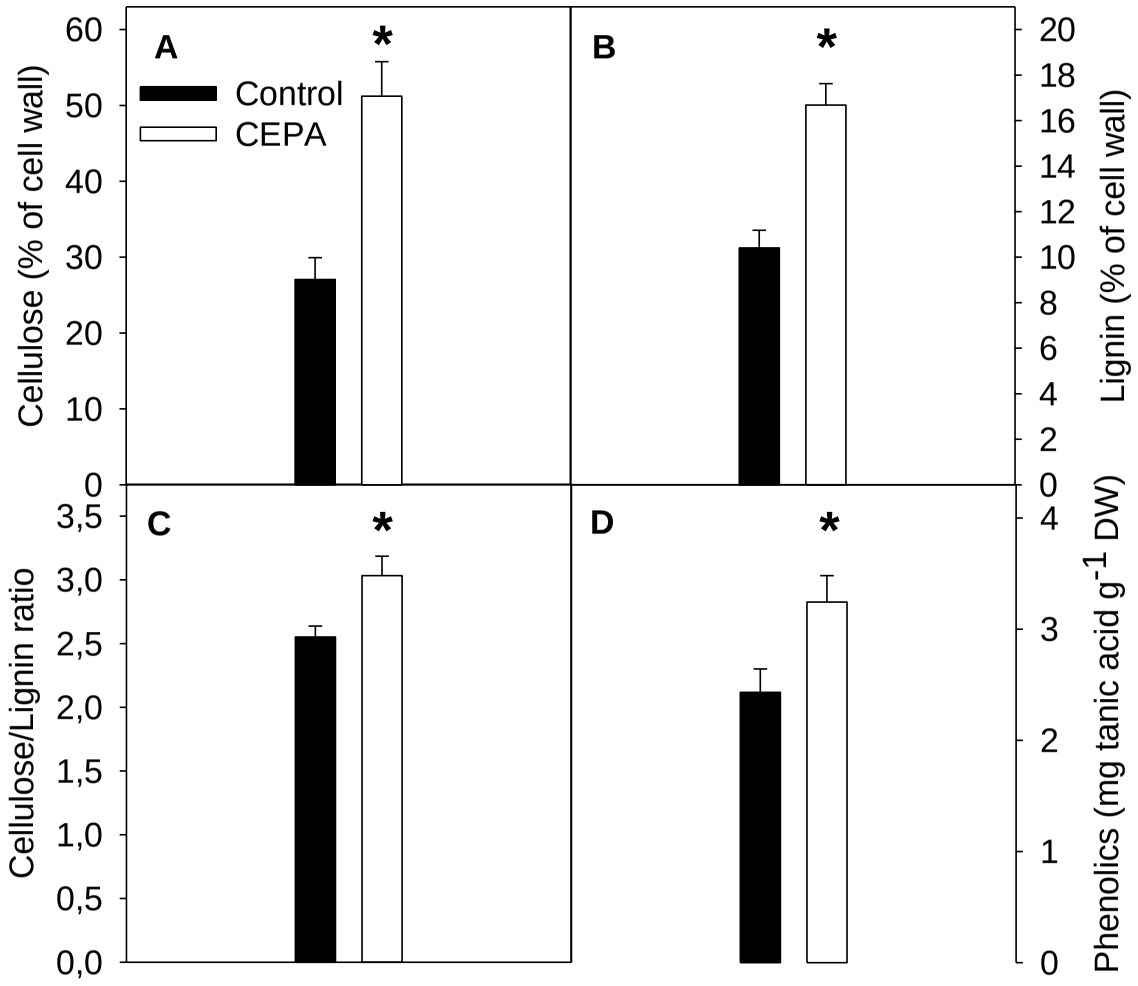
A
B
C
D
Control
CEPA
*
Cellulose (% of cell wall)
Lignin (% of cell wall)
Cellulose/Lignin ratio
Phenolics (mg tanic acid g-1 DW)
60
50
40
30
20
10
0
20
18
16
14
12
10
8
6
4
2
0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
4
3
2
1
0

Figure 4.

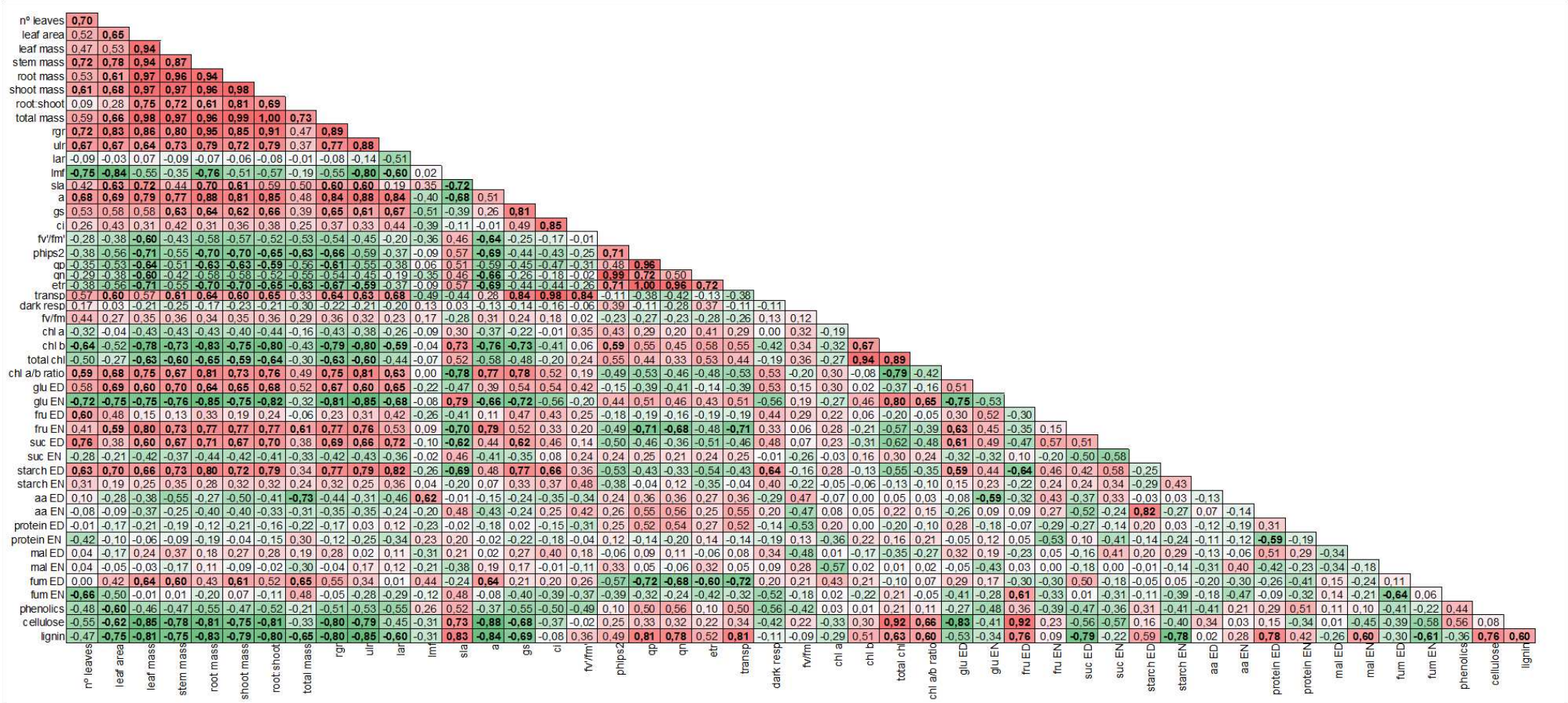
nº leaves
leaf area
leaf mass
stem mass
root mass
shoot mass
root:shoot
total mass
rgr
ulr
lar
lmf
sla
a
gs
ci
fv'/fm'
phips2
qp
qn
etr
transp
dark resp
fv/fm
chl a
chl b
total chl
chl a/b ratio
glu ED
glu EN
fru ED
fru EN
suc ED
suc EN
starch ED
starch EN
aa ED
aa EN
protein ED
protein EN
mal ED
mal EN
fum ED
fum EN
phenolics
cellulose
lignin

Figure 5.

Chapter 3: Physiological and metabolic bases of increased growth in the tomato ethylene insensitive mutant Never ripe: extending the functions of ethylene signaling in tomato plants

INTRODUCTION

During the last decades we have witnessed remarkable advances in understanding the functions of phytohormones and how they affect plant development (Bishopp et al., 2006; Benková and Hejátko, 2009; Wolters and Jürgens, 2009). The usage of mutants in hormone biosynthesis, perception and signal transduction has played a large role in this progress (Reid, 1993; Gazzarrini and McCourt, 2003). In this vein, recent work has demonstrated that ethylene is not only important for the regulation of plant growht and metabolism, but also that it plays a pivotal role in stress tolerance (Lin et al., 2013; Silva et al., 2014; Zhang et al., 2014). The simplicity of the triple ethylene response (inhibition of hypocotyl and root elongation, enhanced horizontal growth, and exaggerated curvature of the apical hook in etiolated seedlings) allowed for the rapid identification of ethylene mutants (Guzmán and Ecker, 1990). A series of ethylene insensitive mutants was thus discovered in Arabidopsis (Arabidopsis thaliana), including etr1 (ethylene receptor 1), a dominant mutant insensitive to ethylene. This mutation affects an ethylene receptor, which is thus unable in binding of the ethylene molecule (Chang et al., 1993; Schaller and Bleecker, 1995). By further genetic and biochemical analysis of ethylene insensitive mutants has been instrumental for the elucidation of the ethylene signal transduction pathway (Stepanova and Ecker, 2000; Chen et al., 2005; Schaller, 2012). The currently accepted model includes positive and negative regulators that culminate in transcriptional regulation: (i) ethylene binds to the N-terminal transmembrane domain of the receptors, and a copper co-factor is required for the ETR (ethylene receptors) family; (ii) this binding deactivates the receptor molecules and the CTR1 (constitutive triple response1) protein (a negative regulator) becomes inactive (iii) EIN2 (ethylene insensitive2) is activated, signaling downstream to the EIN3 family of transcription factors (such as ERF1 – ethylene response factor1) located in the nucleus; and finally, (iv) ERF1 interacts with the GCC-box cis-regulatory element of ethylene-response genes and presumably activates their transcription (Stepanova and Ecker, 2000; Wang et al., 2002).

In tomato (Solanum lycopersicum) six ethylene receptors, SlETR1 to SlETR6, have been characterized (Klee and Tieman, 2002). SlETR3 is, for historical reasons, known as Never ripe (Nr) (Klee, 2002). A single amino acid substitution within the sensor domain on the Nr protein precludes ethylene binding and therefore signaling of this phytohormone is impaired (Wilkinson et al., 1995). Notably, Nr is the ortholog of the Arabidopsis ETR1 receptor (Wilkinson et al., 1995; Yen et al., 1995; Hackett et al., 2000), which has been extensively characterized and whose functions are associated with control of germination (Chiwocha et al., 2005; Wilson et al., 2014), responses to salt and other abiotic stresses (Zhao and Schaller, 2004; Wang et al., 2010), vegetative growth (Ogawara et al., 2003; Tholen et al., 2004), regulation of flowering time and leaf senescence (Grbic and Bleecker, 1995; Ogawara et al., 2003) and cross-talk with other hormones (Collett et al., 2000; Ghassemian et al., 2000; Chiwocha et al., 2005). The Nr mutant was first discovered and reported in tomato in the 1960s and, to date, the vast majority of studies on this mutant has been focused on either fruit development and metabolism or plant yield (Hobson, 1967; Tucker and Grierson, 1982; Harriman et al., 1991; Osorio et al., 2011). Despite the recent association of several phytohormones, e.g. abscisic acid (ABA), auxin, cytokinin, and gibberellin (GA), with carbon assimilation and metabolism (Zhu et al., 2011; Novák et al., 2012; Ribeiro et al., 2012b; Černý et al., 2013), a causal link between ethylene and the regulation of photosynthesis and carbon metabolism remains missing (Khan, 2004; Tholen et al., 2007, 2008; Iqbal et al., 2011; Nazar et al., 2014; Arve and Torre, 2015).

There is a growing body of evidence showing that ethylene biosynthesis and signalling are likely required to maintain proper function of energetic associated pathways. For instance, the importance of ethylene during seed germination has been demonstrated under both optimal and stress conditions, mostly in cross-talk with ABA, GA and/or jasmonates, in several species (Stewart and Freebairn, 1969; Linkies et al., 2009; Linkies and Leubner-Metzger, 2012; Arc et al., 2013; Silva et al., 2014; Yoong et al., 2016). Another interesting feature of ethylene was the demonstration that it affects flowering time and vegetative-to-reproductive transition in Arabidopsis (Ogawara et al., 2003). Notably, this developmental regulation occurs via the modulation of DELLA proteins and interaction with both GA and auxin (Achard et al., 2003, 2007). Moreover,

ethylene also impacts floral transition and starch accumulation in rice (Oryza sativa) (Wuriyanghan et al., 2009; Zhu et al., 2011). Although ethylene is classically associated to growth inhibition (Pierik et al., 2006; Huang et al., 2013), it has been also connected to growth promotion, most likely in association with GA and auxin (Pierik et al., 2004, 2009; Dugardeyn et al., 2008; Muday et al., 2012). Ethylene insensitivity, does not impact growth parameters in Arabidopsis, petunia, and tobacco (Tholen et al., 2004) but can negatively affect the activity of Rubisco in tobacco (Tholen et al., 2007). Collectivelly, these results indicate that ethylene might play a pivotal role in the regulation of energy balance in plants likely by acting in different steps of metabolic pathways in general.

Connections between hormone metabolism and respiratory metabolism (Navet et al., 2003; Zhu et al., 2014), in particular with TCA cycle enzymes, have already been described (Carrari et al., 2004; Araújo et al., 2012; Cavalcanti et al., 2014). For instance, it has recently been demonstrated that GA levels impact growth by uncoupling carbon and nitrogen metabolism and as such the whole metabolism seems to be affected following conditions of unbalanced hormone levels (Ribeiro et al., 2012b,a). Furthermore, it has also been shown that changes in endogenous cytokinin content affects carbon metabolism leading to higher consumption of sucrose and starch in Arabidopsis (Černý et al., 2013, 2014) and that starch metabolic-related genes are regulated by exogenous abscisic acid and ethylene in rice (Zhu et al., 2011). Although compelling evidence has proven the importance of ethylene during tomato fruit development (Osorio et al., 2011), how ethylene impacts leaf primary metabolism and plant growth in general has not been thoroughly explained. Here, we assess aspects of the influence of ethylene insensitivity on plant growth and primary metabolism in leaves through morphological, physiological and biochemical analysis of the tomato Nr mutant. Our results demonstrated that impaired ethylene signaling increases plant growth through changes in leaf metabolism, carbon assimilation, carbohydrates turnover, and reprogramming of metabolites levels. These results suggest that this effect is fairly specific with a detailed characterization revealing that major effects are on photosynthetic performance and metabolic reprogramming following impairments on ethylene signaling. The results obtained are compared and contrasted with those previously obtained with tomato fruit and we discuss these results within the current context of current models of ethylene signaling.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Seeds of the tomato (Solanum lycopersicum cv Ailsa Craig) wild type (WT) and its isogenic ethylene insensitive mutant Never ripe (Nr) were surface sterilized with 5% sodium hypochlorite for 10 min, then washed with running distilled water and subsequently sowed in a tray with commercial substrate (Tropstrato HT©).

Seven days after germination (or following the appearance of the first true leaf), seedlings were transferred to 3.5 L pots containing the same commercial substrate but supplemented with 5 g L^{-1} 4:14:8 NPK. Plants were grown in a greenhouse located in Viçosa (20°45′S, 42°15′W, 650 m above sea level), southeastern Brazil, with a minimum of 400 µmol photons m^{-2} s^{-1}. Plants were watered regularly and throughout the entire growth period the plants were maintained under naturally fluctuating conditions of light intensity, temperature and relative air humidity.

Determination of Growth Parameters

Dry matter accumulation in shoot and roots was determined at the vegetative and reproductive stages. After fourteen weeks, the plants were harvested in one cut to 1.0 cm above ground level, separating roots from shoots. The roots, after water wash of soil contamination, and shoots were placed in labelled paper bags and brought to a forced air circulation oven at 70 ℃ for 5 days, after which the dry weight of roots and shoots (stem, leaves and fruits) was determined. To estimate the fruit set percentage, at least five randomly chosen flowers were marked after anthesis per branch on each plant, according to Rosado-Souza et al. (2015).

Relative growth rate (RGR), which is the net dry weight increase per unit dry weight per day (mg g^{-1} d^{-1}), and their components ($\mathrm{RGR} = \mathrm{SLA} \times \mathrm{LMF} \times \mathrm{ULR}$), were estimated by using an independent group of plants in their vegetative stage. Briefly, specific leaf area ($\mathrm{SLA} = \mathrm{m}^2\ \mathrm{leaf\ area} \times \mathrm{kg}^{-1}\ \mathrm{leaf\ mass}$), by which the amount of fixed C available for growth can be converted from a leaf area to a leaf biomass basis; leaf mass fraction ($\mathrm{LMF} = \mathrm{g\ leaf} \times \mathrm{g}^{-1}\ \mathrm{total\ plant\ mass}$), by which the amount of fixed C available for growth can be converted from a leaf mass to a total plant

mass basis; and the unit leaf rate ($\mathrm{ULR} = \mathrm{g\ dry\ mass\ increase\ x\ m^{-2}\ leaf\ area\ day^{-1}}$), is the rate of increase in plant biomass per unit leaf area, a variable closely related to the daily rate of photosynthesis per unit leaf area (Poorter and Pothmann, 1992; Poorter, 2002) were determined. Roots, leaves and stem were treated according described before to determine dry weight. Leaf area (LA) was measured by digital image method using a scanner (Hewlett-Packard Scanjet G2410) and the images were processed after using the ImageJ software (Schneider et al., 2012). From these measurements, RGR and SLA was calculated using the classical approach (Hunt, 1982):

$$\mathrm{RGR} = \frac{(\ln \mathrm{M_2} - \ln \mathrm{M_1})}{\mathrm{t_2} - \mathrm{t_1}}$$

$$\mathrm{SLA} = \frac{\mathrm{LA}}{\mathrm{LM}}$$

where M_1 and M_2 are the plant mass at times t_1 and t_2, respectively and LM is leaf mass. M_1 was measured 7 d after germination and M_2 21 d after germination (or 3-week-old plants). Once RGR is assumedly the relation of SLA, LMF and ULR we could estimate what growth component is more important in this context of ethylene insensitivity.

Leaf Anatomy Analysis

Sections of the terminal leaflet of third fully expanded leaf were hand cut from the widest part of the leaf using a razor blade and stored in 100% methanol for epidermal analysis, or in FAA 70% solution for 24h for transverse analysis. First, the sections were mounted on a microscope slide, and cleared using a solution of 85% (w/v) lactic acid (Kalve et al., 2014a; Zsogon et al., 2015). Slides were placed in a water bath at 95 ℃ overnight. Sections from the abaxial surface were viewed using a photomicroscope (Zeiss Scope A1) and images captured using a digital camera (Zeiss Axiocam 105 color). Measurements of stomatal density and index (the ratio of stomata to stomata plus other epidermal cells) were determined in at least five different fields of view per leaflet from three different plants using AnatiQuanti (Aguiar et al., 2007) and ImageJ (Schneider et

al., 2012) software. After 70% FAA storage, leaf tissue samples were transferred to ethanol 70% for 24h. Tissues samples were dehydrated in a progressively increasing ethanol concentrations (from 70% to 100%) and later embedded in historesin (Leica), sectioned by ultramicrotome Leica (5 µm), and stained with toluidine blue. Four subsamples for each cross-section were selected for measurement of the area occupied by leaf thickness and palisade and spongy mesophyll cell sizes. Transversal sections were observed using a photomicroscope (Olympus AX70) and images captured using a digital camera (Zeiss AxioCam HRc). Measurements of leaf and mesophyll thickness were performed on images using the ImageJ software.

Measurements of Photosynthetic Parameters

Gas exchange and chlorophyll a fluorescence analysis was performed simultaneously in the third fully expanded leaf from the apex of 3-week-old plants (vegetative stage) using a portable open-flow gas exchange system (Li-6400XT, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber (LI-6400-40; Li-Cor Inc.). Measurements of the net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (E) were conducted from 08:00 to 12:00 h (solar time), which is when A was at its maximum, under an ambient CO_2 concentration (C_a) of 400 µmol mol^{-1} air (CO_2 injected from a cartridge) and a flow rate of 300 µmol s^{-1}. All measurements were conducted under artificial photosynthetic photon flux density (PPFD), saturating light of 1000 µmol photons m^{-2} s^{-1} (from a LED source) with 10% blue light in order to maximize the stomatal opening, at 25 ℃ and the leaf-to-air vapor pressure deficit was maintained at approximately 1.0 kPa. The CO_2 concentration and water vapor between the leaf and the reference chamber were automatically matched before the data were recorded. Dark respiration (R_D) was measured using the same gas exchange system described above after at least two hours during the nigh period in the same leaf previously used to determine gas exchange parameters.

The initial fluorescence (F_0) was measured by illuminating previously dark adapted leaves with weak modulated measuring beams (0.03 µmol m^{-2} s^{-1}). Saturating white light pulses (8000 µmol photons m^{-2} s^{-1}) was applied for 0.8 s to obtain the maximum fluorescence (F_m), from which the variable-to-maximum

Chl fluorescence ratio, was then calculated: $F_v/F_m=[(F_m - F_0)/F_m)]$. In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured with the application of a saturating white light pulse (8000 µmol m^{-2} s^{-1}, 0.8 s) to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off and a far-red illumination (2 µmol m^{-2} s^{-1}) was applied to measure the light-adapted initial fluorescence (F_0'). The capture efficiency of excitation energy by open photosystem (PS) II reaction centers (F_v'/F_m') was estimated following Logan et al. (2007) and the actual PSII photochemical efficiency (δPSII) was estimated as $\delta PSII = (F_m' - F_s)/F_m'$ (Genty et al., 1989). In addition, the coefficients of photochemical quenching (qP) and non-photochemical quenching (NPQ) and the electron transport rate (ETR), were determined as previously described (Martins et al., 2014; Medeiros et al., 2016).

Photosynthetic light-response curves (A/PPFD) were obtained by decreasing the PPFD in 12 steps from 1500 to 0 µmol photons m^{-2} s^{-1} at 25 ºC (Martins et al., 2014). The A/PPFD curves were obtained at same conditions described before.

The responses of A to C_i were determined at 1000 µmol photons m^{-2} s^{-1}, at 25 ºC, under ambient O_2 following the protocol described in Long and Bernacchi (2003). Briefly, A/C_i curves were initiated at a C_a of 400 µmol mol^{-1} and once the steady state was reached, C_a was decreased stepwise down to 50 µmol mol^{-1} air. Upon completion of the measurements at low C_a, it was returned to 400 µmol mol^{-1} air to restore the original A. Next, C_a was increased stepwise to 1600 µmol mol^{-1} air (Martins et al., 2013). The maximum rate of carboxylation (V_{cmax}) and maximum rate of carboxylation limited by electron transport (J_{max}) were then estimated by fitting the mechanistic model of CO_2 assimilation using the Excel spreadsheet provided by Sharkey et al. (2007). Corrections for the leakage of CO_2 into and water vapor out of the leaf chamber of the LI-6400 have been applied to all gas-exchange data, according to Rodeghiero et al. (2007).

Determination of Metabolite Levels

All sampling procedures were carried out on the third fully expanded source leaves (same leaves that analysis of photosynthetic parameters) from 3-week-old plants, during vegetative stage. Leaves were harvested in different time points along the light/ dark cycle (middle of day, end of day and end of night).

Leaves were flash-frozen in liquid nitrogen and stored at −80 °C until further analyses. Metabolite extraction was performed by rapid grinding in liquid nitrogen and immediate addition of the appropriate extraction buffer.

The levels of photosynthetic pigments, starch, sucrose, fructose and glucose, protein and amino acids (Cross et al., 2006). The levels of others metabolites were quantified by an established gas chromatography-mass spectrometry (GCMS) protocol (Roessner et al., 2001; Lisec et al., 2006).

Chromatograms were exported from Leco ChromaTOF software (version 3.25) to R software. Peak detection, retention time alignment, and library matching were performed using Target Search R-package (Cuadros-Inostroza et al., 2009). Metabolites were quantified by the peak intensity of a selective mass. Metabolites intensities were normalized by dividing the fresh-weight, followed by the sum of total ion count and global outlier replacement as described elsewhere (Giavalisco et al., 2011).

qRT-PCR Analysis

Total RNA was isolated using the Trizol® reagent (Ambion, Life Technology) following the manufacturer's manual. The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a spectrophotometer. DNAse-treated RNA (1-2 µg) was used for cDNA synthesis using Superscript™ III reverse transcriptase (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions. The expression of genes was assessed using PCR in real time (Step One PlusTMReal Time PCR System, Applied Biosystems, CA, USA) with the SYBR green fluorescence detection (Applied Biosystems®), using the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX kit.

The primers used here were designed using the QUANTPRIME software (Arvidsson et al., 2008) and detailed primer information are described in the Supplemental Table 1. Data analyses were performed exactly as described by Caldana et al. (2007). The average CT of the reference control gene was used for the relative expression analyses. For the normalization of the gene expression data, their CT values were subtracted from the CT of the mean reference gene (ΔCT). To calculate the ΔΔCT, the ΔCT of interest was subtracted from the ΔCT of the control (reference) condition. These results were transformed to a log2

scale to obtain the fold-change values. The relative expression levels were normalized using the constitutively expressed gene Ubiquitin3 (Wang et al., 2008) and the reference gene was measured using three replicates in each PCR run, and their mean cycle threshold was used for relative normalized expression analyses.

Statistical analysis

The data was obtained from the at least two experiments (with similar results) conducted in a completely randomized design. Data are expressed as the average ± standard error (SE). The term significant is used here only when the change in question has been confirmed to be significant ($P<0.05$) with Student's t test. All statistical analyses were performed using the algorithm embedded into Microsoft Excel® (Microsoft, Seattle).

RESULTS

Nr is not expressed in fruits and others tissues exhibit low expression of this receptor in mutant plants

Given that the Nr receptor has been extensively characterized during tomato fruit development, its functions have been mainly associated with fruit ripening. Here, we first confirmed that the Nr gene is constitutively expressed in several tissues and not only in fruits (Fig. 1A). We showed that this mutation (low expression of Nr – Soly09g075440) occurs in other organs beyond fruits and confirmed the low expression of Nr in different tissues by using a qRT-PCR (Fig. 1A). Thus, mature and young leaves, petioles, flowers, seedlings, stem and roots were analyzed. Notably, all tissues investigated in the Nr mutant exhibit less than 50% of WT expression of this gene.

Nr influence on the expression of genes related to ethylene signaling and biosynthesis

In order to understand the behavior of other components of the ethylene signaling pathway in the ethylene insensitive mutant Nr we investigated, in the same leaves used for biochemical and physiological analysis, the expression of all ETR family (ETR1-6) and EIN3 genes (Fig. 1B), wherein no difference was observed between the genotypes. Next, we determined the expression of some

components of the ethylene biosynthesis pathway including ACO1, ACO3, ACS2 and ACS4 (Fig. 1B). Our data revealed that ACO1, ACO3 and ACS2 were expressed at similar levels in Nr and WT plants whilst ACS4 is upregulated (3-fold increase) in Nr plants.

Vegetative growth rate is increased in Nr plants

Once we observed that there is a lower expression of the Nr in several plant tissues we asked what is the physiological function of this gene. In order to investigate the growth and developmental responses in Nr plants, we first analyzed plant growth. Despite no visible aberrant phenotypes during vegetative and reproductive growth (Fig. 1C and D) we observed that the ethylene insensitive plants were consistently taller than their WT counterparts (Fig. 2A). This difference was maintained from the first measurement in seven-day-old plants until the end of the experiment (14 weeks-old plants). Leaf thickness, palisade and spongy mesophyll structure as well as stomatal index and stomatal density did not differ between genotypes (Fig. 2B and Table 1). During the vegetative stage, Nr plants were characterized by enhanced growth parameters (e.g. stem diameter, total leaf area, and total dry weight) in relation to their WT counterpart as shown in Table 1; on the other hand, no differences were observed in seeds size and mass, root:shoot ratio, and leaf area ratio (Table 1). To better understand this growth difference, the relative growth rate (RGR) and its two major components (i) specific leaf area (SLA) and (ii) leaf mass fraction (LMF) were determined as well as unit leaf rate (ULR) (Table 1). Nr plants have higher RGR and URL than WT in spite of no changes in SLA and LMF (Table 1). To better illustrate the correlation between RGR and its components, correlations graphs were created (Fig. 2C and 2D) showing the absence of significant correlation between RGR and SLA (Fig. 2D) and a correlation higher than 80% between RGR and ULR (Fig. 2C). Collectively, these results indicate that ULR, which is related to biochemical changes, is likely more important than structural/morphological (SLA and LMF) alterations to explain that enhanced growth observed in Nr plants.

Photosynthetic rate is increased in Nr, possibly by higher carboxylation rates

Given the growth differences observed between the genotypes studied and that most of plant biomass is derived from photosynthesis, we next characterized the photosynthetic performance of Nr by analyzing gas exchange and chlorophyll fluorescence parameters. A was increased in Nr plants but no changes were found in all others parameters analyzed (Table 2). We next characterized the photosynthetic performance by analyzing gas exchange under different photosynthetic photon flux density (PPFD) (A/PPFD curves and g_s/PFD curves; Fig. 3A and 3B) and we observed that mutant plants exhibited unaltered A and g_s irrespective of the irradiance. Thus, no changes in saturation irradiance (I_s), light-saturated A_N (A_{PPFD}), compensation irradiance (I_c) and light use efficiency were observed between WT and Nr plants (Supplemental Table 2). Additionally, the response of A to the internal leaf CO_2 concentration (C_i) (A/C_i curves; Fig. 3C) was obtained, which was further converted into responses of A to chloroplastidic CO_2 concentration (A/C_c curves; Fig. 3D). Interestingly, whereas Nr plants were characterized by higher A with increments in CO_2 (Fig. 3), we noticed that under ambient CO_2 concentration (400 µmol mol^{-1}), C_i, C_c, g_s and mesophilic conductance (g_m) estimations were similar between genotypes (Table 3). In addition, the maximum carboxylation velocity (V_{cmax}) and maximum capacity for electron transport rate (J_{max}) were significantly higher (65% and 25%, respectively) in ethylene-insensitive plants (Table 3). Moreover, the lower J_{max}:V_{cmax} ratios observed in Nr plants (Table 3) suggest that differences in A were more likely related to higher carboxylation than electron transport rates (Table 3).

Higher starch consumption and sucrose turnover in Nr

To enhance our understanding of the consequences of changes in growth and in photosynthetic capacity among the genotypes we next performed a detailed metabolic analysis in leaves of WT and Nr plants. First, we determined the levels of some carbon and nitrogen related metabolites in samples harvested at the middle of light period. There were no changes in the levels of sucrose and starch, or in total amino acids and protein despite an increase in chlorophyll b leading to simultaneous reduction in chlorophyll a/b ratio at the vegetative stage (Table 4). In addition, we measured the levels of starch and soluble sugars (glucose, fructose, and sucrose) as well as total proteins and total amino acids at

the end of the day (ED) and at the end of the night (EN) (Fig. 4). The levels of starch are similar at the ED and, as expected, starch decreases during the night (EN) in both genotypes but a higher reduction was observed in Nr plants and as such WT plants showed 45% of reduction while Nr plants showed 75% of reduction of starch (Fig. 4A). Soluble sugars showed different diel pattern wherein sucrose and fructose were characterized by an interesting turnover showing that sucrose levels are higher in Nr (Fig. 4B) whereas fructose was lower (Fig. 4C) in Nr at ED. At the EN, both sucrose and fructose levels are similar between genotypes (Fig. 4B and 4C) showing that sucrose and fructose were consumed quickly and slowly, respectively, in Nr during the night. On the other hand, glucose levels were invariant between genotypes in the time points analyzed here (Fig. 4D). We also did not observe differences in the levels of total amino acids and total proteins (Fig. 4E and 4F).

In order to further explore the consequences of ethylene insensibility on primary metabolism we decided to extend the analysis by studying the major primary metabolic pathways, by using an established gas chromatography-mass spectrometry (GCMS) protocol (Lisec et al., 2006) on leaves harvested at the middle of light period. These studies revealed that among the 45 successfully annotated compounds related to primary metabolism, considerable changes occurred in the levels of a wide range of amino acids, organic acids, and sugars (Fig. 5). Analysis of the 17 individual amino acids identified here has revealed an increase in levels of 7 amino acids (alanine, arginine, asparagine, glutamine, serine, tryptophan and tyrosine) yet only glycine has decreased (Fig. 5A). When considering the levels of organic acids, only the levels of 2-oxoglutarate, malate and threonate were increased in Nr plants (Fig. 5B). Lastly, in relation to sugars and sugars alcohol, we detected increases in fructose, glucose, idose, mannose, and myo-inositol, while ribose decreases (Fig. 5C). The full data set from these metabolic profiling studies are additionally available in Supplemental Table 3.

The Nr mutation increases fruit set and yield without changes in carbon partitioning

The enhanced growth observed in Nr plants during the vegetative stage was maintained in the reproductive stage, in which Nr plants presented a higher number of leaves, shoot and root dry weight, as well as increased dry weight of

leaves, stem, and fruits, with no differences in shoot to root ratio (Table 5). The fruit set rate was also higher in Nr than in WT as well as the total dry weight; however, when analyzing the carbon partitioning between vegetative and reproductive part, we did not observe differences between the two genotypes (Table 5).

DISCUSSION

Tomato, an important model for fruit development, has been studied intensively with respect to fruits genetics, physiology and biochemistry. However, the importance of phytohormones on leaf metabolism and its connection to plant growth is less well understood. Our results demonstrated that in tomato, the ethylene receptor protein Never ripe (Nr) is notably involved with both growth and primary metabolism of leaves. Here, we first demonstrated that the Nr mutation leads to lower expression of the Nr gene not only in fruits as previously described (Wilkinson et al., 1995; Payton et al., 1996), but also in roots, stem, leaves and flowers of tomato plants (Fig. 1A). In addition, we found no change in the expression levels in mature leaves of the other ethylene receptors (ETR family) or in the ethylene signaling cascade components (EIN3) (Fig. 1B). This suggests that there is no redundancy or compensation between ethylene receptors, and provides further evidence that the morphological and physiological changes observed here are due only to the Nr mutation. Furthermore, we analyzed the expression of genes associated with ethylene biosynthesis and showed that there is no difference in three out of four genes analyzed despite a 3-fold increase in the expression in ACS4 (Fig. 1B). ACS4 is important for tomato fruit ripening (Lincoln et al., 1993; Hoogstrate et al., 2014), considering that Nr is a key player in this process, they may be associated at the genetic level and therefore an impairment in the Nr receptor induces the expression of ACS4. Although ethylene biosynthesis induces the expression of Nr in tomato fruits during ripening, it does not impact Nr expression in leaves during senescence (Lashbrook et al., 1998) and thus it seems plausible that the reduction in perception due to reduced Nr expression in leaves might lead to an altered expression of genes from ethylene biosynthesis. Taken together, these data provide evidences that this ethylene insensitive mutant can be assumed as a good model to study plant growth and leaf metabolism in response to ethylene. It is important to mention that this mutant

Nr has been already used in studies with roots (Clark et al., 1999; Kim et al., 2008; Vidoz et al., 2010) and shoots (Castagna et al., 2007; Gratão et al., 2012; Poór et al., 2015) wherein results demonstrate the role of ethylene in response to abiotic stress.

Both initiation and development of leaves and fruits are extremely dependent on hormone balance and the importance of auxins, gibberellin and cytokinin has been expertly demonstrated (McAtee et al., 2013; Kalve et al., 2014b; Shwartz et al., 2016). By contrast, ethylene function has been unequivocally associated to hypocotyl elongation (Dugardeyn and Van Der Straeten, 2008), leaf senescence (Lim et al., 2007; Lira et al., 2014) and fruit ripening (Lelievre et al., 1997; Osorio et al., 2013; Seymour et al., 2013) and less attention has been paid to its role in leaf metabolism in general. Considering the importance of ethylene for plant development coupled with the low expression of Nr in tomato mutants (Fig. 1A) and the absence of compensatory effects by the other ethylene receptors (Fig. 1B) it is tempting to suggest that the growth enhancement of the mutant plants is likely associated with metabolic changes. In fact, the ethylene-insensitive mutant displayed higher carbon assimilation than WT but with no differences in g_S, C_i, R_D and chlorophyll parameters (Tables 2 and 3). In addition, despite the absence of changes in response to light intensity (Figs. 3A and B), it was observed a number of changes in response to CO_2 concentration (Figs 3C and D) including increases in V_{cmax} and decreases in J_{max}/V_{cmax} ratio without changes in g_m (Table 3). In agreement with the higher photosynthetic rates (Table 2) on the ethylene insensitive mutant, levels of glucose and fructose are higher at midday (Fig. 5C). Thus, our data allows us to infer that this increase in carbon assimilation and ultimately in growth is most likely due to enhanced activity of the enzymes of Calvin-Benson cycle. It is important to mention, however, that ethylene insensitivity did not promote growth alterations in other species such as Arabidopsis, Petunia and tobacco (Tholen et al., 2004, 2007, 2008) but led to reductions in V_{cmax} (Tholen et al., 2007, 2008). Although the reasons behind such contrasting results are not immediately evident we suggest that: (i) reduced expression of Nr gene in those plants is not as big as in the Nr tomato mutant; (ii) reduced expression of Nr in those plants was followed by changes in the expression of other receptors which means that a different regulatory control might exist in other species; and (iii) perhaps more

importantly, tomato is probably a more sensitive species to ethylene changes due to source:sink relation in this fleshy-fruit species. As such it seems unlikely that fruit demand for assimilates is completely met by carbohydrates produced in source leaves of the other species. It should be kept in mind that changes observed in sugars as well as in organic acids might, at least partially, explain the higher growth rates verified in Nr plants. It seems plausible that an interaction between ethylene sensing and these compounds might exist and it will be important to further establish the functional significance of this connection in future studies.

Increased plant growth can be the result of biochemical and/or anatomical changes (Nunes-Nesi et al., 2016). Since relative growth rate (RGR) is a function of the other growth related parameters ULR (biomass increase per unit leaf area and time) and LAR (leaf area per unit plant mass) which, in turn, can be further analyzed as the product of SLA and LMF (Poorter and van der Werf, 1998), the quantification of these variables allowed us to infer which one is actually more correlated with growth rate. Our analyses of growth-related parameters (Fig.2A; Tables 1 and 5) demonstrated that the higher growth observed in Nr are most likely associated with metabolic and biochemical changes (ULR) rather than structural and morphoanatomical changes (LAR). This assumption is further supported by the absence of anatomical changes (Fig.2B; Table 1) coupled with major changes in CO_2 response curves and derived parameters (Fig. 3C and D; Table 3). Taken together, our data suggest that the main variable involved in the variation in RGR in response to ethylene insensitivity in tomato plants is ULR. It has been extensively demonstrated that increases in growth are correlated with increases in LAR (for a review see: Poorter, 2002; Poorter and van der Werf, 1998). By contrast, it is usually assumed that some stresses (e.g. drought and salt) that lead to growth reductions are more related to reductions in ULR coupled with reductions in photosynthetic rates (Poorter, 2002). Given that we found a different trend here, it is tempting to speculate that under optimal conditions a mutation that culminated with impairments in the perception of ethylene, a hormone known to mediate stress response in plants, may act also enhancing growth according the biphasic model of ethylene function (Pierik et al., 2006). Whether this response will be maintained under field conditions remains to be investigated and therefore caution should be taken when interpreting the results

presented here in the context of growth enhancement with biotechnological applications (Zsögön et al., 2017).

It has been extensively demonstrated that plant growth is highly dependent on carbon availability (Smith and Stitt, 2007; Pantin et al., 2011) as well as that the starch synthesized and accumulated during the light period must be remobilized to allow continuous export of sucrose and growth during the night (Gibon et al., 2009; Pilkington et al., 2015). Altogether, this provides a highly efficient manner for the allocation of photosynthates to maximize vegetative growth (Stitt and Zeeman, 2012; Sulpice et al., 2014). Therefore, despite no changes in starch accumulation during the day, the higher consumption of starch in Nr during the night (Fig.4A) indicates that the reduced perception of ethylene somehow impacts the enzymes responsible for starch degradation. We cannot formally exclude the possibility that changes in growth here observed might be associated with increased efficiency of photosynthates allocation that maximize vegetative growth, however it seems highly tempting to suggest that changes in starch metabolism might explain, at least partially, growth enhancements in Nr plants under optimal conditions. Moreover, higher sucrose levels at the end of the day coupled with higher A in Nr plants could be explained by the recently described function of sucrose as a modulator of the stomatal opening in response to light (Talbott and Zeiger, 1998; Daloso et al., 2016; Santelia and Lawson, 2016). Furthermore, during the night consumption of sucrose is higher in Nr since the levels are similar to WT at the end of dark period (Fig. 4B). It seems therefore reasonable to suggest that in response to an alteration in ethylene perception there is a substantial adjustment of starch and sugars turnover that result in a distinct growth pattern. While the precise nature of the connection between ethylene signaling and sugars metabolism could not be resolved in the present study, it remains an exciting topic for future research. Concerning the metabolic profile of leaves, at middle of the day, we showed that changes in several amino acids and sugars are different from those exhibited during Nr fruit development with an increase pattern on these metabolites in leaves and decrease in fruits (Osorio et al., 2011) as well as in organic acids, mainly the increase in malate levels, are similar from those observed in tomato fruits (Osorio et al., 2011). Interestingly, the increase in 2-oxoglutarate levels associated with increase in glucose and fructose observed here is quite similar to observed in tomato plants

mutants to 2-Oxoglutarate Dehydrogenase Complex (Araújo et al., 2012). Despite that, these changes on metabolite levels are likely associated to a metabolic adjustment allowing enhanced growth due to higher carbon fixation. Whether this is associated with transcriptional changes remains to be further investigated.

CONCLUDING REMARKS

Here, we provide evidence that altered ethylene perception in tomato, besides its well-known effects on fruit ripening, leads to favorable pleiotropic effects on vegetative plant growth, fruit set and yield. In spite of a significant increase in vegetative and reproductive growth, carbon flow is apparently not altered by ethylene signaling. Reduced ethylene signaling can stimulate plant growth, which falls in line with the observation that high ethylene concentrations are inhibitory to growth (Kieber et al., 1993; Chae et al., 2003; Huang et al., 2013) It is noteworthy that ethylene might be required by a plant to cope with a stress episode; so whether our results are extensive to stress situations (e.g. drought, nutrients) remains an open question. The connection between energy metabolism and hormone-mediated control of growth is only beginning to be unveiled (Araújo et al., 2012; Ribeiro et al., 2012a; Cheng et al., 2015; Nam et al., 2016). It is however tempting to speculate that the pathways of energy metabolism and ethylene biosynthesis and perception are tightly regulated at the leaf level in a suitable manner allowing the plant to prioritize aerial growth under optimal conditions.

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TABLES

Table 1. Phenotype, growth, and morphological parameters in WT and Nr mutant plants under optimal growth conditions in vegetative stage (3-weeks-old). Values are presented as average ± SE of at least 8 independent biological replicates per genotype; bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Parameters	WT	Nr
Stomatal index (%)	15,6 ± 0,37	17,23 ± 1,13
Stomatal density (per mm^2)	85,79 ± 3,95	101,82 ± 8,35
Internode length (cm)	5,69 ± 0,72	**8,44 ± 0,35**
Stem diameter (mm)	9,64 ± 0,28	**10,55 ± 0,14**
Total leaf area (cm^2)	1527,20 ± 80,45	**1789,21 ± 89,74**
Dry weight of leaves (g)	6,61 ± 0,37	**8,28 ± 0,32**
Dry weight of stem (g)	3,66 ± 0,23	**5,30 ± 0,20**
Dry weight of roots (g)	2,02 ± 0,29	**2,78 ± 0,16**
Dry weight of shoot (g)	10,27 ± 0,56	**13,58 ± 0,46**
Total dry weight (g)	12,29 ± 0,79	**16,36 ± 0,45**
Leaf length (cm)	31,47 ± 2,64	34,56 ± 1,39
Root:shoot ratio	0,19 ± 0,02	0,21 ± 0,02
10 seeds area (mm^2)	63,58 ± 1,77	60,58 ± 1,68
1000 seeds weight (g)	2,81 ± 0,04	2,70 ± 0,24
Leaf area ratio ($m\ kg^{-1}$)	17,64 ± 1,03	16,93 ± 1,07
Relative growth rate ($mg\ g^{-1}\ day^{-1}$)	205,73 ± 4,36	**224,04 ± 5,28**
Specific leaf area ($m\ kg^{-1}$)	29,92 ± 0,98	28,55 ± 0,75
Leaf mass fraction ($g\ g^{-1}$)	0,54 ± 0,02	0,51 ± 0,01
Unit leaf rate ($g\ m^{-2}\ day^{-1}$)	14,55 ± 0,81	**17,59 ± 0,86**

Table 2. Gas exchange and chlorophyll a fluorescence parameters in WT and Nr plants. Values are presented as average ± SE (n = 10) obtained in third fully expanded leaf from the apex of 3-week-old plants (vegetative stage); bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Parameters*	WT	Nr
A (µmol H2O m^{-2} s^{-1})	20,89 ± 0,92	**22,92 ± 0,97**
g_s (mol H2O m^{-2} s^{-1})	0,681 ± 0,049	0,735 ± 0,057
Ci (µmol CO2 mol air^{-1})	326,171 ± 3,424	327,107 ± 2,663
R_D (µmol CO2 m^{-2} s^{-1})	1,177 ± 0,118	1,455 ± 0,137
E (mol H2O m^{-2} s^{-1})	4,932 ± 0,146	6,118 ± 0,131
ETR (mmol m^{-2} s^{-1})	157,509 ± 5,297	155,808 ± 3,286
F_v/F_m	0,800 ± 0,010	0,804 ± 0,010
F_v'/F_m'	0,600 ± 0,010	0,599 ± 0,007
δPSII	0,360 ± 0,013	0,358 ± 0,007
qP	0,598 ± 0,155	0,595 ± 0,093
NPQ	0,670 ± 0,024	0,652 ± 0,010

*A, net CO_2 assimilation rate; g_s, stomatal conductance; R_D, dark respiration; E, transpiration rate; F_v/F_m, maximum PSII photochemical efficiency; F_v'/F_m', actual PSII photochemical efficiency; δPSII, actual PSII photochemical efficiency; qP; photochemical quenching; and NPQ, non-photochemical quenching.

Table 3. Photosynthetic parameters derivate from CO2-response curves in WT and Nr mutant plants. Values are presented as average ± SE (n = 8) obtained in third fully expanded leaf from the apex of 3-week-old plants (vegetative stage); bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Parameters*	WT	Nr
V_{cmax} (µmol m^2 s^{-1})	53,97 ± 1,31	**89,32 ± 6,9**
J_{max} (µmol m^2 s^{-1})	86,24 ± 2,39	**108,62 ± 2,12**
J_{max}:V_{cmax}	1,61 ± 0,072	**1,25 ± 0,064**
g_m (mol CO_2 m^2 s^{-1} bar^{-1})	0,14 ± 0,003	0,14 ± 0,004
Ci (µmol CO_2 mol^{-1})	332,49 ± 4,66	334,54 ± 5,87
Cc (µmol CO_2 mol^{-1})	179,85 ± 2,98	178,46 ± 2,06

*V_{cmax}, maximum carboxylation capacity based; J_{max}, maximum capacity for electron transport rate; g_m, mesophyll conductance to CO_2 estimated according to the Harley method; Ci, substomatal CO_2 concentration; Cc, chloroplastidic CO_2 concentration.

Table 4. Biochemical changes at middle of the day. Values are presented as average ± SE (n = 8) obtained in third fully expanded leaf from the apex of 3-week-old plants (vegetative stage); bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT. FW, fresh weight.

Parameters	WT	Nr
Sucrose (µmol g^{-1} FW)	9,73 ± 1,02	9,27 ± 0,98
Starch (µmol g^{-1} FW)	7,30 ± 0,40	6,63 ± 0,63
Amino acids (µmol g^{-1} FW)	18,22 ± 0,56	18,02 ± 1,16
Protein (mg g^{-1} FW)	20,05 ± 1,20	20,59 ± 2,03
Chlorophyll a (mg g^{-1} FW))	5,37 ± 0,17	5,85 ± 0,35
Chlorophyll b (mg g^{-1} FW)	1,61 ± 0,08	**1,95 ± 0,10**
Total chlorophyll (mg g^{-1} FW)	6,98 ± 0,25	7,80 ± 0,45
Chlorophyll a/b ratio	3,35 ± 0,08	**2,99 ± 0,05**

Table 5. Phenotype, growth, and morphological parameters in WT and Nr mutant plants under optimal growth conditions in reproductive stage (14-weeks-old). Values are presented as average ± SE of at least 8 independent biological replicates per genotype; bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Parameters	WT	Nr
Nº of leaves	20,36 ± 0,78	**25,75 ± 1,58**
Dry weight of leaves (g)	16,83 ± 1,15	**22,84 ± 1,79**
Dry weight of stem (g)	20,36 ± 1,56	**27,77 ± 1,69**
Dry weight of fruits (g)	15,71 ± 1,42	**21,57 ± 1,79**
Dry weight of shoot (g)	37,19 ± 2,45	**50,61 ± 3,16**
Dry weight of roots (g)	4,49 ± 0,44	**6,45 ± 0,43**
Root:shoot ratio	0,12 ± 0,01	0,13 ± 0,02
Fruit set (%)	58,79 ± 2,73	**74,25 ± 4,37**
Total dry weight (g)	57,98 ± 3,59	**78,12 ± 3,95**
Vegetative dry weight (g)	41,11 ± 2,46	**57,07 ± 2,78**
Carbon partioning (vegetative %)	71,21 ± 1,93	73,15 ± 1,32
Carbon partioning (reproductive %)	28,79 ± 1,93	26,87 ± 1,32

FIGURE LEGENDS

Figure 1. Genotypic and phenotypic characterization of the ethylene insensitive NR mutants. Relative transcript expression of **(A)** Nr (ETR3) in whole-plant: mature leaves (ML), young leaves (YL), petioles (PE), seedlings (SL), stem (ST) roots (RT) and flowers (FL), and **(B)** genes involved in ethylene signaling (ETR1-6 and EIN3) and biosynthesis (ACO1, ACO3, ACS2 and ACS4) on mature leaves was determined. Data are normalized with respect to the average response calculated for the corresponding WT. WT, black bars; Nr, white bars. Data presented are average ± SE (n = 5). Asterisks (*) indicate that the values from ethylene insensitive mutants were determined by the Student's t-test to be significantly different ($P<0.05$) from the WT. Photographs showing representative plants in **(C)** vegetative (3-weeks-old) and **(D)** reproductive (5-weeks-old) stages.

Figure 2. Growth and morphoanatomical parameters in WT and Nr mutant plants under optimal growth conditions. **(A)** Height of plants during 14 weeks of experiment; **(B)** thickness of cell layers; **(C)** unit leaf rate and **(D)** specific leaf area as function of relative growth rate. In **(C)** and (**D)** each point represents a value from an individual measurement. WT, black circles; Nr, white circles. Values are presented as average ± SE (n = 8). Asterisks (*) indicate that the values from ethylene insensitive mutants were determined by the Student's t-test to be significantly different ($P<0.05$) from the WT.

Figure 3. Net photosynthesis (A) curves in response to changes in photosynthetically active photon flux density (PPFD) and to substomatal (Ci) or chloroplastidic (Cc) CO_2 concentrations in WT and Nr plants. **(A)** Assimilation rates as function of PPFD intensities, **(B)** stomatal conductance (g_s) as function of PPFD intensities, **(C)** A/Ci curves and **(D)** A/Cc curves. WT, black circles; Nr, white circles. Data presented are average ± SE obtained using third fully expanded leaf from the apex of 3-week-old plants (vegetative stage). Asterisks (*) indicate that the values from ethylene insensitive mutants were determined by the Student's t-test to be significantly different ($P<0.05$) from the WT

Figure 4. Metabolism variation along diurnal cycle. Metabolites, **(A)** starch, **(B)** sucrose, **(C)** fructose, **(D)** glucose, **(E)** amino acids, and **(F)** proteins were

obtained in third fully expanded leaf from the apex of 3-week-old plants (vegetative stage). Leaves were harvest in two points: at the end of the day (white sectors) and the end of the day (grey sectors). WT, black bars; Nr, white bars. Values are presented as average ± SE (n = 5). Asterisks (*) indicate that the values from ethylene insensitive mutants were determined by the Student's t-test to be significantly different (P<0.05) from the WT

Figure 5. Relative metabolite content in leaves of WT and Nr plants. **(A)** Amino acids, **(B)** organic acids, and **(C)** sugars and sugar alcohols were determined by GC-MS as described in "Materials and Methods". The full data sets from these metabolic profiling studies are additionally available in Supplemental Table 3. Data are normalized with respect to the average response calculated for the corresponding WT (to allow statistical assessment, individual plants from this set were normalized in the same way). WT, black bars; Nr, white bars. Values are presented as average ± SE (n = 5). Asterisks (*) indicate that the values from ethylene insensitive mutants were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

FIGURES

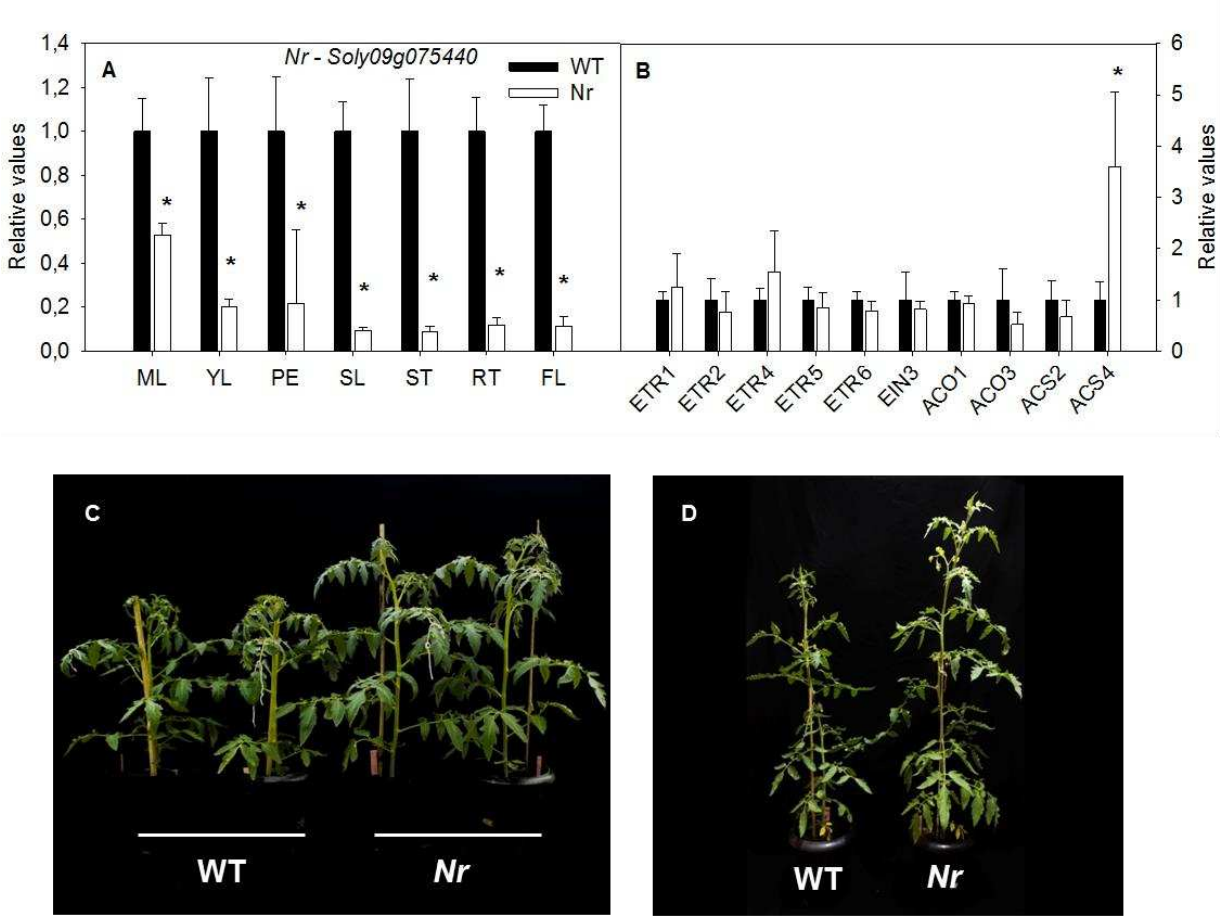

Figure 1.

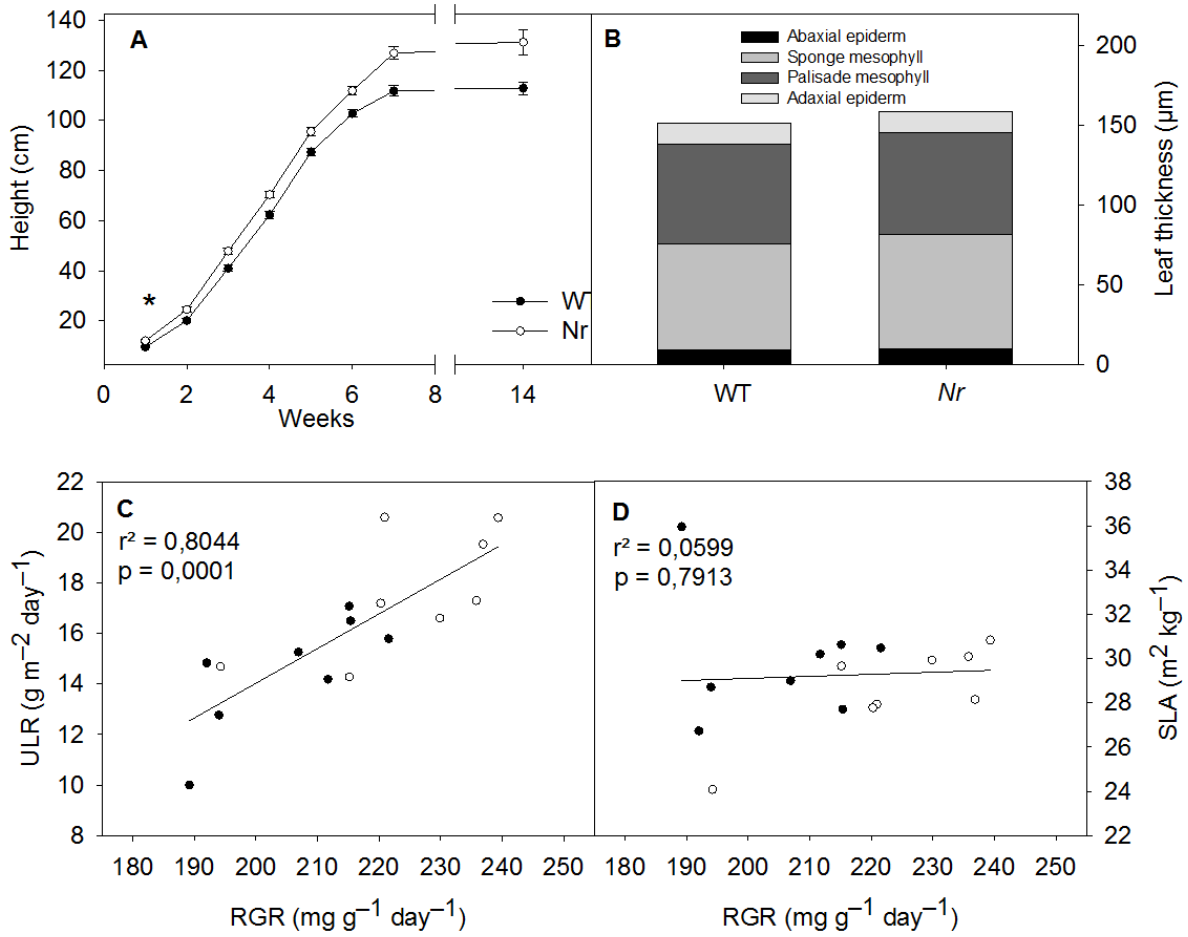
A
B
C
D
Height (cm)
Weeks
Abaxial epiderm
Sponge mesophyll
Palisade mesophyll
Adaxial epiderm
Leaf thickness (µm)
WT
Nr
r² = 0,8044
p = 0,0001
r² = 0,0599
p = 0,7913
ULR (g m⁻² day⁻¹)
SLA (m² kg⁻¹)
RGR (mg g⁻¹ day⁻¹)
RGR (mg g⁻¹ day⁻¹)

Figure 2.

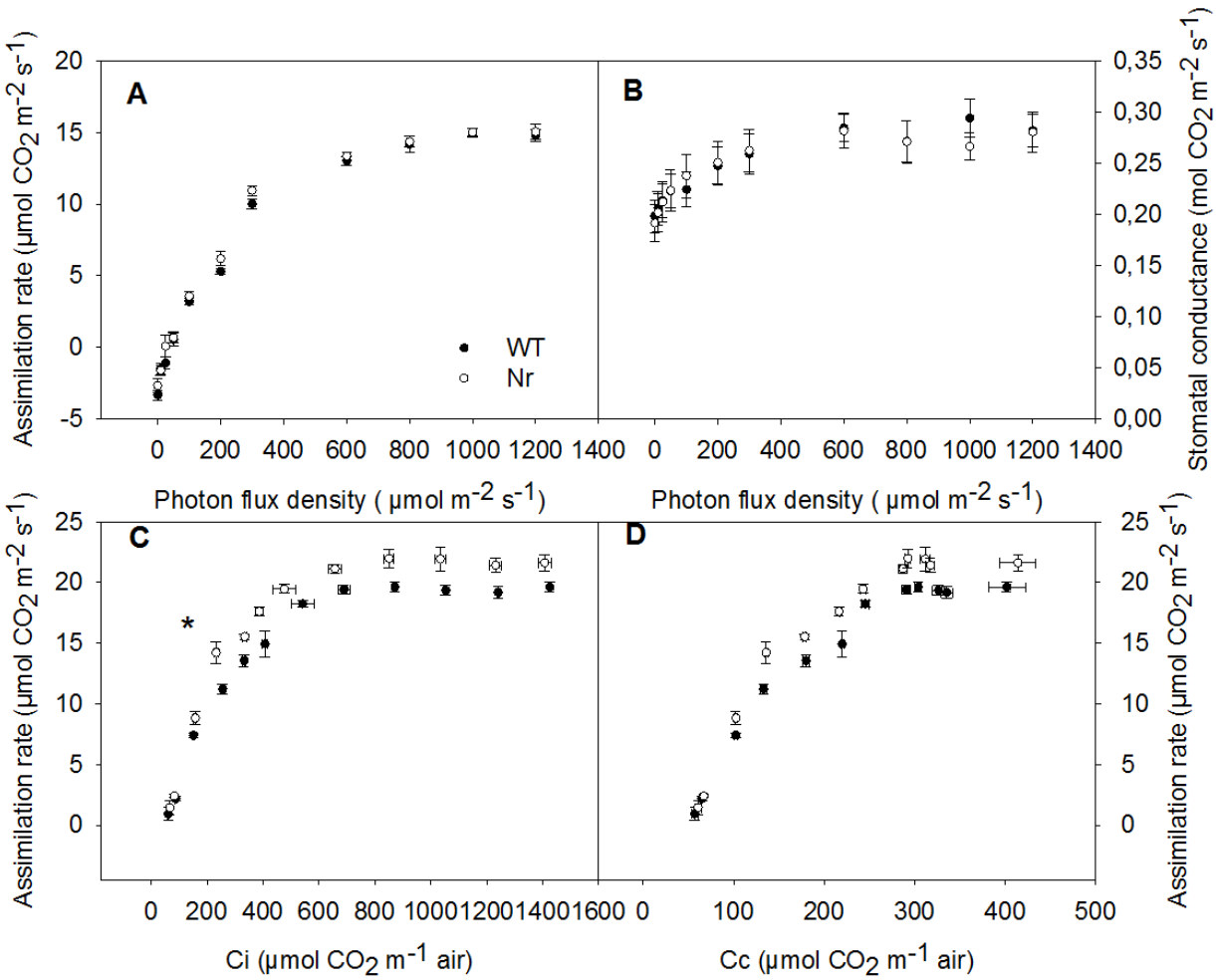
A
B
C
D
WT
Nr
Assimilation rate (µmol CO2 m-2 s-1)
Stomatal conductance (mol CO2 m-2 s-1)
Photon flux density (µmol m-2 s-1)
Ci (µmol CO2 m-1 air)
Cc (µmol CO2 m-1 air)
*

Figure 3.

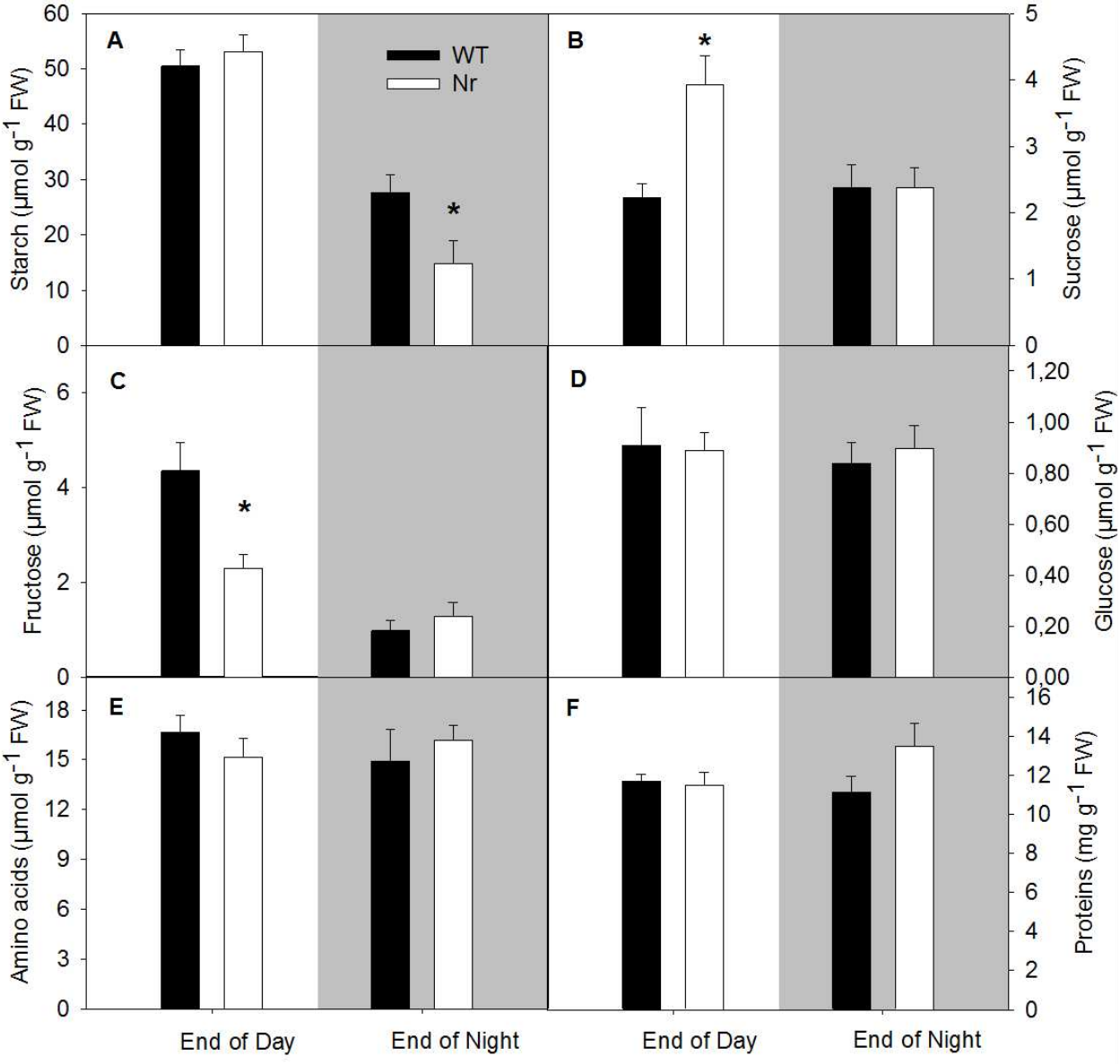
A
B
C
D
E
F
WT
Nr
Starch (µmol g-1 FW)
Sucrose (µmol g-1 FW)
Fructose (µmol g-1 FW)
Glucose (µmol g-1 FW)
Amino acids (µmol g-1 FW)
Proteins (mg g-1 FW)
End of Day
End of Night
End of Day
End of Night

Figure 4.

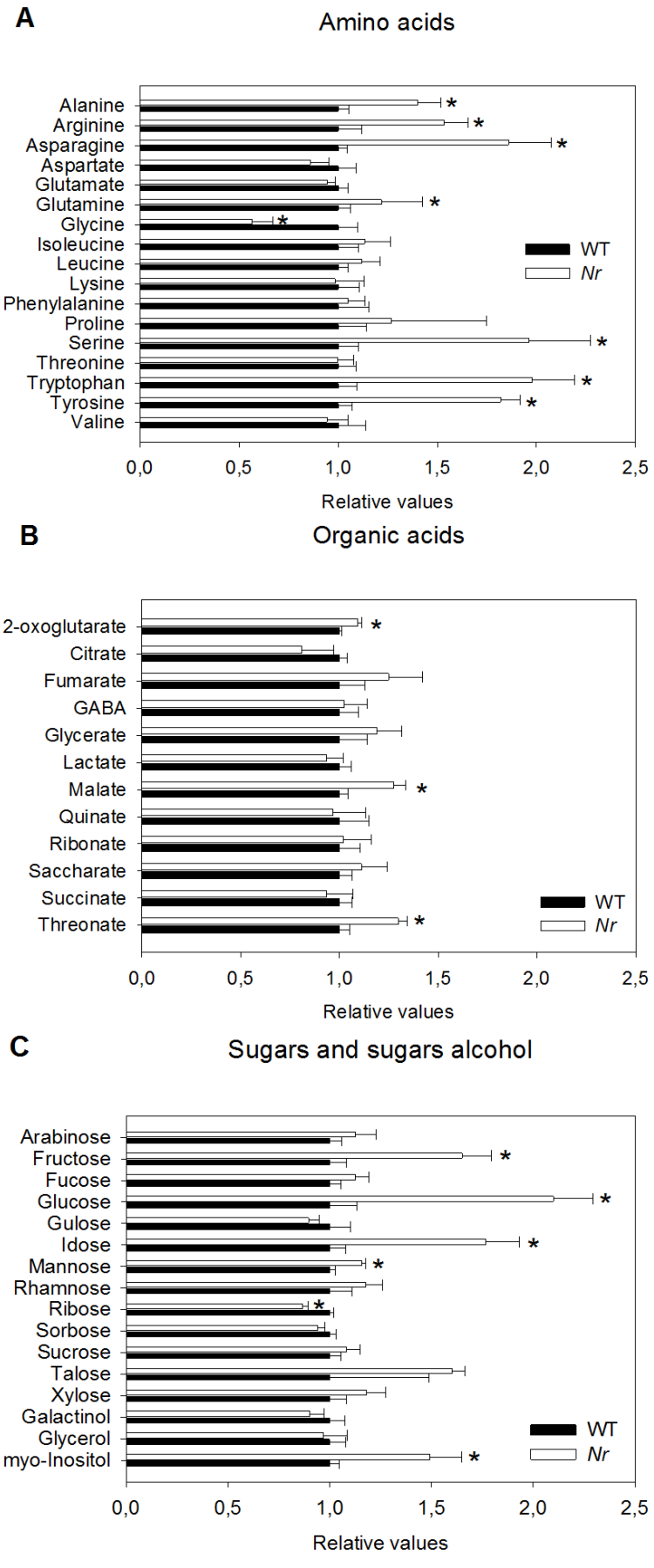
A
Amino acids
Alanine
Arginine
Asparagine
Aspartate
Glutamate
Glutamine
Glycine
Isoleucine
Leucine
Lysine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine
WT
Nr
0,0
0,5
1,0
1,5
2,0
2,5
Relative values
B
Organic acids
2-oxoglutarate
Citrate
Fumarate
GABA
Glycerate
Lactate
Malate
Quinate
Ribonate
Saccharate
Succinate
Threonate
WT
Nr
0,0
0,5
1,0
1,5
2,0
2,5
Relative values
C
Sugars and sugars alcohol
Arabinose
Fructose
Fucose
Glucose
Gulose
Idose
Mannose
Rhamnose
Ribose
Sorbose
Sucrose
Talose
Xylose
Galactinol
Glycerol
myo-Inositol
WT
Nr
0,0
0,5
1,0
1,5
2,0
2,5
Relative values

Figure 5.

SUPPLEMENTAL INFORMATION

Supplemental Table 1. Primers utilized to qRT-PCR

SGN ID	NAME	FORWARD	REVERSE
Solyc12g011330	**ETR1**	GGCATTCCTGGACGTGCAAATG	TTTGGTTACCATCCTGCTAACACC
Solyc07g056580	**ETR2**	TGGCATTCCTGGTCGCTTAAATG	CTTCGTTACCATCCTGCTAAACCC
Solyc09g075440	**ETR3 (Nr)**	TGTTCCCTATGCCAAGTGATGGC	ACACCCAGTATCTCTAACCTGGAC
Solyc06g053710	**ETR4**	TGTGCAGAAAGCTGGTTCAGTTG	AGTTGAAGCCCAAGAACGACAGC
Solyc11g006180	**ETR5**	AGGAAGTAACGGAGGGCTTGAG	TCCCTGCATCATCTGAACAAGC
Solyc09g089610	**ETR6**	GCAGGAAGTTGGTTCATTTGATGC	GGGAATTAACGGCTGCCTTTGG
Solyc01g014480	**EIN3**	TTGCCTCCTAGGCAGCTATGTG	TGGTGCAGCTTTCAGGTACATTC
Solyc02g036350	**ACO1**	AAATCATGAAGGAGTTTGCTGATAAA	TTTTCACACAGCAAATCCAACAG
Solyc07g049550	**ACO3**	ACGGGAAGTACAAGAGCGTGAT	CTAGTGACATCCGAGTCCCATCT
Solyc01g095080	**ACS2**	TGGAGAAAACAAGAGGAGGAAGA	GGCACCACCAGCCATAACA
Solyc05g050010	**ACS4**	CCATCTTGTTTGCGACGAAATA	CGATGCTAACGAATTTTGGAGAA

Supplemental Table 2: Photosynthetic parameters derivate from light-response curves in WT and Nr mutant plants. Values are presented as average ± SE (n = 7) obtained in third fully expanded leaf from the apex of 3-week-old plants (vegetative stage); bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Parameters*	WT	Nr
A_{PPFD} (µmol CO_2 m^2 s^{-1})	17,99 ± 0,184	17,732 ± 0,50
I_c (µmol m^2 s^{-1})	40,68 ± 8,30	42,41 ± 6,07
I_s (µmol m^2 s^{-1})	578,25 ± 8,05	520,58 ± 29,65
1/ϕ (µmol photons mol-1 CO^2)	21,36 ± 1,03	20,35 ± 0,71

*A_{PPFD}: Net CO2 assimilation rate saturated by light; I_c: light compensation point; I_s: light saturation point; 1/ϕ: Light use efficiency

Supplemental Table 3. Relative metabolite content in WT and Nr plants in vegetative growth (4-week-old). Data are normalized with respect to the mean response calculated for the corresponding WT (to allow statistical assessment, individual plants from this set were normalized in the same way). Data are presented as means ± SE (n = 5) and bold letters in Nr plants indicate values that were determined by the Student's t-test to be significantly different (P<0.05) from the WT.

Amino acids	WT	Nr
Alanine	1,00 ± 0,05	**1,40 ± 0,11**
Arginine	1,00 ± 0,12	**1,53 ± 0,12**
Asparagine	1,00 ± 0,05	**1,86 ± 0,21**
Aspartate	1,00 ± 0,09	0,85 ± 0,10
Glutamate	1,00 ± 0,05	0,94 ± 0,04
Glutamine	1,00 ± 0,06	**1,22 ± 0,21**
Glycine	1,00 ± 0,10	**0,56 ± 0,10**
Isoleucine	1,00 ± 0,10	1,13 ± 0,13
Leucine	1,00 ± 0,05	1,11 ± 0,09
Lysine	1,00 ± 0,11	0,98 ± 0,15
Phenylalanine	1,00 ± 0,15	1,05 ± 0,08
Proline	1,00 ± 0,14	1,26 ± 0,48
Serine	1,00 ± 0,10	**1,96 ± 0,031**
Threonine	1,00 ± 0,09	0,99 ± 0,08
Tryptophan	1,00 ± 0,10	**1,98 ± 0,09**
Tyrosine	1,00 ± 0,07	**1,81 ± 0,10**
Valine	1,00 ± 0,14	0,94 ± 0,11
Organic acids		
Threonate	1,00 ± 0,02	**1,29 ± 0,05**
2-oxoglutarate	1,00 ± 0,01	**0,88 ± 0,02**
Citrate	1,00 ± 0,04	0,81 ± 0,16
Fumarate	1,00 ± 0,13	1,25 ± 0,17
GABA	1,00 ± 0,10	1,02 ± 0,12
Glycerate	1,00 ± 0,14	1,19 ± 0,12
Lactate	1,00 ± 0,06	0,93 ± 0,08

Malate	1,00 ± 0,04	**1,27 ± 0,06**
Quinate	1,00 ± 0,15	0,97 ± 0,16
Ribonate	1,00 ± 0,10	1,02 ± 0,14
Saccharate	1,00 ± 0,06	1,11 ± 0,13
Succinate	1,00 ± 0,06	0,93 ± 0,14
Sugars		
Arabinose	1,00 ± 0,06	1,12 ± 0,10
Fructose	1,00 ± 0,08	**1,65 ± 0,14**
Fucose	1,00 ± 0,05	1,12 ± 0,07
Glucose	1,00 ± 0,13	**2,09 ± 0,19**
Gulose	1,00 ± 0,10	0,89 ± 0,05
Idose	1,00 ± 0,08	**1,76 ± 0,16**
Mannose	1,00 ± 0,03	**1,15 ± 0,02**
Rhamnose	1,00 ± 0,11	1,17 ± 0,08
Ribose	1,00 ± 0,02	**0,87 ± 0,03**
Sorbose	1,00 ± 0,03	0,94 ± 0,03
Sucrose	1,00 ± 0,06	1,08 ± 0,07
Talose	1,00 ± 0,48	1,60 ± 0,06
Xylose	1,00 ± 0,08	1,18 ± 0,10
Sugars alcohol		
Galactinol	1,00 ± 0,08	0,90 ± 0,07
Glycerol	1,00 ± 0,08	0,97 ± 0,12
myo-Inositol	1,00 ± 0,05	**1,49 ± 0,16**

Concluding remarks and outlook

The main goal of this work was to provide an enhanced comprehension coupled with a revalidation of the functional role of ethylene as a growth-related phytohormone. That being said, our results described in chapters 2 and 3 provides compelling evidence that growth impacts associated with ethylene are related also to significant changes in carbon metabolism. Remarkably, the results described within this thesis further demonstrate that ethylene, plant growth and carbon metabolism are strictly associated.

Interestingly, ethylene seems to act directly on plant growth by inhibit or, in absence of your perception, allow the increased growth in tomato plants. Thus, it was demonstrated that an exogenous application of this hormone is able to reduce plant growth coupled with several morphological and metabolic changes. For instance, carbon assimilation and stomatal conductance was reduced in ethylene-treated plants leading to a several reductions in carbon metabolism and impacting the energetic demand to plant growth. In association, we found interestingly alterations in amount of cell wall and phenolic compounds in leaves as well in photochemical apparatus demonstrating a very exquisite manner in attempt to protect from a possible stress. Undoubtedly some further experiments are suggested to improve our understanding on ethylene relations with stomatal conductance and guard cell responses, as cross-talk with ABA and sugars, and analysis of function of other phytohormones, e.g. auxin, cytokinin and GA, in association with ethylene in control of growth mediating changes in morphology of tomato leaves.

By contrast, in tomato Nr mutant plants, that are ethylene insensitive, growth is fairly induced coupled with significant changes in carbon assimilation characterized by increases in photosynthesis and an extensive metabolic reprogramming. It is worth to mention that we do not detected changes in morphoanatomy of Nr plants in opposition to increase in biochemical stage of photosynthesis and in central metabolism. Besides that, we suggest some analysis to better understand the role of ethylene in regulation of growth, as hormonal profile and/or expression of genes related to other phytohormones to check the possible cross-talk of ethylene with other regulators, metabolic flux and/or radiolabeled incorporation analysis to check the partitioning of carbon.

Finally, it is reasonable to suggest that the metabolic and biochemical mechanisms that govern these two phenomena are not necessarily opposites and we can conclude here that the biphasic effect of ethylene occurs in tomato once opposite effects are exhibited on plant growth. We suggest that studying the effect of ethylene on carbon growth and metabolism in other species and also in adverse conditions seems to be the next natural step after revisiting the possible functions of ethylene as a plant growth hormone.