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São Paulo
2017
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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na Área de Botânica.

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São Paulo
2017
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84 páginas

Dissertação (Mestrado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Botânica.

1. Chloroplast 2. Tomato 3. Golden 2-Like 4. Light 5. Phytohormones

I. Universidade de São Paulo. Instituto de Biociências. Departamento de Botânica.

Comissão Julgadora:

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Prof. Dra. Maria Magdalena Rossi
Orientadora
Dedico este trabalho a todos que lutam pela sobrevivência da ciência brasileira. Que venham tempos melhores.
Não desistiremos.
“A scientist in his laboratory is not a mere technician:
he is also a child confronting natural phenomena
that impress him as though they were fairy tales.”

- Marie Curie
Agradecimentos

Agradeço ao Departamento de Botânica do Instituto de Biociências – USP pela estrutura acadêmica, e ao apoio financeiro da CAPES.

A realização deste trabalho só foi possível graças à dedicada orientação da Profª. Maria Magdalena Rossi e a co-orientação do Prof. Luciano Freschi, que sempre estiveram presentes e disponíveis quando precisei. Aprendi muito com vocês. Obrigada por todo o projeto, vocês são responsáveis por um grande crescimento pessoal e profissional durante esses quase 3 anos de laboratório.

Agradeço também as colaborações do Prof. Eduardo Purgatto com as análises de açúcares e ao Prof. Diego Demarco por todo o auxílio com o microscópio eletrônico.

Este trabalho só foi possível graças a todos do Laboratório de Genética Molecular de Plantas: As queridas GGDR (Giovanna Gramegna e Daniele Rosado), Bruninha Trench, Silvia Blanco e Bruno Lira. Muito obrigada por todo o apoio sempre, tanto no profissional quanto nas milhares de fotos de gatos compartilhadas. Agradeço também a todos os colegas do Laboratório de Fisiologia Vegetal, em especial ao Ricardo Bianchetti, Frederico Rocha e Aline Bertinatto; do Laboratório de Biologia Celular de plantas; e do Lignin Lab, em especial ao Prof. Dr. Igor Cesarino.

A todos que acompanharam essa jornada e foram de grande importância no suporte emocional: vocês são os melhores (e piores) amigos que eu poderia ter. Bios, nuzofers e gixers, valeu por tudo! Verena, Carlinha e Brunno, vocês são simplesmente essenciais. Não teria conseguido sem ter todos vocês comigo.

À família incrível que eu tenho, muito obrigada. Aurea, minha mãe que teve a honra de ter seu nome por toda a dissertação, obrigada por todo o apoio desde sempre. Vô, gostaria muito que pudesse ter visto esse capítulo da minha vida se concluindo. Obrigada por plantar a semente da ideia de trabalhar com genética de plantas muito antes de eu saber que me apaixonaria por esse tema.

Pela linda ilustração que decora minha capa e minha pele, muito obrigada Tiê Zerbeto!

Kami e Ivy, obrigada por deixarem as madrugadas de trabalho em casa bem melhores.

Aos compositores clássicos que me ajudaram durante a parte escrita, ao café que me permitiu trabalhar durante as manhãs e à terapia que me manteve relativamente sã.

Muito obrigada a todos que participaram da minha vida nesses quase 3 anos!
Resumo

Os plastídios são organelas responsáveis por diversos aspectos essenciais do desenvolvimento das plantas como a fotossíntese, assimilação de nitrogênio e síntese de diversos compostos do metabolismo secundário. A diferenciação e atividade dos cloroplastos são altamente reguladas pela luz, e diversas proteínas e mecanismos envolvidos nestes processos têm sido caracterizados. Os fatores de transcrição GOLDEN 2-LIKE (GLKs) controlam a expressão de diversos genes relacionados à fotossíntese, biogênese e manutenção plastidial. *Solanum lycopersicum* possui duas cópias desses genes, *SlGLK1* e *SlGLK2*, e, embora sejam funcionalmente redundantes, seu padrão de expressão é diferente, uma vez que *SlGLK1* predomina nas folhas ao passo que *SlGLK2* é expresso apenas nos frutos, mais precisamente na região pedicelar. Durante o processo de domesticação do tomateiro, a seleção de variedades de amadurecimento uniforme resultou na fixação da mutação *uniform ripening* (*Slglk2*) na maioria das variedades cultivadas, resultando em mudanças na composição metabólica dos frutos. Neste contexto, este trabalho teve como objetivo geral caracterizar funcionalmente o gene *SlGLK2* visando compreender de que forma a luz (mediada por fitocromos) e os fitormônios (particularmente citocininas e auxinas), regulam a expressão deste gene e como a presença de *SlGLK2* afeta a qualidade nutricional dos frutos. Para isso, foi realizado um detalhado perfil transcricional de *SlGLK2* em frutos de plantas selvagens, *Slglk2* mutantes e deficientes para a percepção luminosa e para a sinalização hormonal. O efeito de *SlGLK2* sobre a qualidade nutricional foi avaliado caracterizando o metabolismo de carbono e de vitamina E. Adicionalmente, foi quantificada a atividade da proteína repórter GUS em plantas transgênicas que expressam o gene *uidA* sob controle de promotores responsivos à citocininas ou auxinas em plantas com genótipo *SlGLK2* ou *Slglk2* para analisar se a atividade hormonal é afetada pela presença de *SlGLK2*. Finalmente, com o intuito de verificar se a presença de *SlGLK2* é suficiente para reverter o fenótipo clorótico do mutante *aurea*, promovendo a diferenciação e maturação plastidial mesmo na ausência de fitocromos funcionais, foram geradas linhagens transgênicas sobreexpressando o gene *SlGLK2* em fundo genético *aurea-Slglk2*. A análise dos resultados permitiu concluir que o conteúdo de açúcares solúveis e vitamina E correlacionam com a expressão de *SlGLK2*, que a expressão de *SlGLK2* é reprimida por auxinas, que *SlGLK2* participa positivamente da sinalização de citocininas, e que a sua sobreexpressão reverte, parcialmente, o fenótipo dos frutos da mutante *aurea-Slglk2*. Os resultados obtidos nos levam a uma melhor compreensão da rede regulatória que interconecta o gene *SlGLK2*, os fitormônios e a luz promovendo a atividade plastidial e, por consequência, determinando a qualidade nutricional dos frutos de tomateiro, importante componente da dieta humana.
Abstract

Plastids are organelles responsible for several essential aspects for plant development, like photosynthesis, nitrogen assimilation and synthesis of several compounds of secondary metabolism. Chloroplasts differentiation and activity are highly regulated by light, and several proteins and mechanisms involved in these processes have been characterized. The GOLDEN 2-LIKE (GLK) transcription factors controls the expression of several genes related to photosynthesis, plastid biogenesis and maintenance. Solanum lycopersicum genome harbors two copies of this gene, SlGLK1 and SlGLK2 and, although they are functionally redundant, their expression pattern is different, once SlGLK1 predominates in leaves, while only SlGLK2 is expressed in fruit, precisely at the pedicel region. During tomato domestication, selection for varieties that ripened evenly resulted in the fixation of uniform ripening mutation (Slglk2) in most cultivated varieties, resulting in alterations in fruit metabolic composition. In this context, the objective of this work was to functionally characterize SlGLK2 gene aiming to understand in which way phytochrome mediated light and phytohormones, particularly auxins and cytokinins, regulates this gene expression, and how SlGLK2 presence affects fruit nutritional quality. To achieve this, a detailed transcriptional profile of SlGLK2 was performed in fruits of wild plants, Slglk2 mutant and plants deficient for light perception or hormonal signaling. The effect of SlGLK2 over nutritional quality was evaluated by characterizing carbon and vitamin E metabolism. Additionally, reporter protein GUS activity was quantified in transgenic plants that express uidA gene under control of promoters responsive to cytokinins or auxins in SlGLK2 or Slglk2 genotypes, to analyze if hormonal activity is affected by SlGLK2 presence. Finally, in order to verify if the presence of SlGLK2 is sufficient to reverse the chlorotic phenotype of the mutant aurea, promoting the differentiation and plastidial maturation even in the absence of functional phytochromes, transgenic lines were generated by overexpressing the SlGLK2 gene on aurea-Slglk2 genetic background. The integrated data analysis allowed us to conclude that the content of soluble sugars and vitamin E correlate with the expression of SlGLK2, that the expression of SlGLK2 is repressed by auxins, that SlGLK2 positively participates in the signaling of cytokinins, and that its overexpression partially reverts the phenotype of the aurea-Slglk2 mutant fruits. The results obtained in this work contributes to a better understanding of the regulatory network that interconnects SlGLK2 gene, phytohormones and light, promoting the plastidial activity and consequently, determining the nutritional quality of the tomato fruit, an important component of the human diet.
Introduction

1.1. Plastid biogenesis, differentiation and maintenance

Plastids are organelles with a great diversity of shapes and functions, and they are found in all photosynthetic eukaryotes. Besides being responsible for photosynthesis, depending on the plant development stage, these organelles are also responsible for other functions, such as synthesis of amino acids, fatty acids, nitrogenous bases, pigments and hormones. Additionally, they also participate on the assimilation of sulfur and nitrogen (Jarvis & López-Juez 2013).

Land plants have many plastids that play different functions (Figure 1). Proplastids are found mainly in meristematic tissues, and are the precursors of other plastids. Amyloplasts accumulates starch and are found mainly in storage organs, such as roots, seeds and tubercles, having a key role in energy storage and gravitropism. Gerontoplasts, which are mostly found in senescent tissues, originate from the disassembly of photosynthetic machinery and macromolecule degradation, playing a main role in nutrient recycling and remobilization towards sink organs. Chloroplasts synthetize and accumulate chlorophyll and maintain all the machinery responsible for light capture and photosynthetic activity, allowing atmospheric carbon fixation, on which most forms of life depend. Chromoplasts are found in flowers of different species and also in fleshy fruits, in which they perform various functions, including synthesis and accumulation of a wide spectrum of metabolites, many of them with nutraceutical relevance, i.e. antioxidants. In this regard, great effort has been made to understand minutely the mechanisms responsible for differentiation and maintenance of plastid structure and metabolism, aiming the improvement of nutritional quality of edible fruits (Jarvis & López-Juez 2013).

Among the exogenous factors that influence the differentiation of proplastids into chloroplasts, the light signal has a prominent role ensuring this transition only in appropriate conditions for photosynthetic activity. The main photoreceptors involved in the light perception that regulates this conversion are the PHYTOCHROMES (PHYs). Structurally, PHYs are homodimeric proteins whose unit is composed of an apoprotein bound to a tetrapyrrole chromophore, i.e. phytochromobilin (Gyula et al. 2003). In the darkness PHYs are found inactive in the cytoplasm while, in the presence of light, phytochromobilin undergoes an isomeric alteration that changes the structure of the apoprotein, directing the active PHYs to the cellular nucleus (Bae & Choi 2008). Within the nucleus, they promote degradation of PHYTOCHROME INTERACTING FACTORS (PIFs), negative regulators of light signal transduction. In turn, PIFs repress the expression of ELONGATED HYPOCOTYL 5 (HY5), a positive regulator of photomorphogenesis, and GOLDEN 2-LIKE (GLKs) transcription factors (Song et al. 2014) involved in plastidial biogenesis and activity.
maintenance (Fitter et al. 2002). Additionally, HY5 positively regulates *Arabidopsis thaliana* GLK2 (Lee et al. 2007). Together, HY5 and GLKs activate transcription of several proteins related to the photosynthetic machinery and, consequently, chloroplast differentiation (Jarvis & López-Juez 2013).

![Figure 1: Plastid diversity and interconnections.](image)

Proplastids are able to differentiate into other plastids, according to physiological stimuli of the cell; amyloplasts accumulates starch; chloroplasts are photosynthetic plastids; gerontoplasts are formed during senescence by disassemble of photosynthetic machinery and autophagy; and chromoplasts accumulates other pigments such as carotenoids. Adapted from Jarvis & López-Juez (2013).

In relation to endogenous factors, plastidial biogenesis and differentiation is also influenced by different phytohormones. Auxins and cytokinins, for example, regulates plastidial differentiation in different plant organs. At the seedling stage, cytokinins play a key role in chloroplast differentiation and division during de-etiolation, whereas auxin has an inhibitory effect, preventing, for example, the development of chloroplasts in the roots (Cortleven & Schmulling 2015; Kobayashi et al. 2017).

1.2. **Importance of plastid metabolism for nutritional quality of *Solanum lycopersicum* fruits**

Because of its nutritional importance, widespread consumption in the western population and economic importance, the tomato (*Solanum lycopersicum*) has been configured as the model species for the study of fleshy fruits development and ripening (Giovannoni 2004).

The development of the tomato fruit begins after pollination with a phase of intense cell division comprising the immature green stages (IG), followed by a period of cell expansion until the fruit reaches its final size at mature green stage (MG). During these green stages, the fruits have active photosynthetic machineries (Carrara et al. 2001). Although fruits in general are sink organs,
requiring more photoassimilates than they produce, local photosynthesis in tomatoes seems to be responsible for up to 20% of the total carbon in ripe fruits (Cocaliadis et al. 2014).

From MG stage onwards, the fruits become responsive to ethylene, which triggers ripening, turning fruits from MG to breaker stage (Br), when the fruit starts to change color from green to yellow. During this process, a progressive conversion of chloroplasts into chromoplasts is observed involving several biochemical alterations that will contribute to the definition of the color, flavor, aroma and texture of the ripe fruit (five days after Br onwards, Br+5). Gradual chlorophyll degradation leads to loss of green coloration, and the released phytol is recycled and used, at least in part, for tocopherol (vitamin E, VTE) production (Almeida et al. 2016). Carotenoid biosynthetic route is intensely stimulated during ripening, resulting in accumulation of lycopene, responsible for the intense red color of ripe tomato fruits (Giovannoni 2004). The induction of several aromatic amino acid decarboxylases leads to increased production of volatile compounds, such as phenylethanol and phenylacetaldehyde, which confer the characteristic aroma of tomatoes (Tieman et al. 2006; Tieman et al. 2007). Fruit softening is induced by cell wall hydrolases, changing fruit texture (Fischer & Bennet 1991). Finally, the degradation of starch and cell wall increases the content of soluble sugars and organic acids, determining the texture and density of tomato puree, i.e. the °Brix, a feature of great industrial importance considering that two-thirds of the world's production is consumed as processed tomato (Carrari & Fernie 2006; Sila et al. 2009). All the described changes determine the nutritional and industrial quality of the fruit (Giovannoni 2004; Fraser et al. 1994; Egea et al. 2011).

The most valuable nutraceutical compounds in fleshy fruits, e.g. carotenoids and tocopherols, are synthetized in chloroplast/chromoplast. Therefore, chloroplast abundance and activity in the green stages of the fruits collaborate not only for the synthesis of part of the photoassimilates necessary to the organ development, but also determine future capacity of these fruits to produce nutraceutical compounds in the chromoplasts. In this way, the increment in the number of active chloroplasts, as well as the enhancement of plastidial metabolism, appears as a key target for of fleshy fruit yield and quality improvement (Isaacson et al. 2002; Nashilevitz et al. 2010).

In this work, we focused on tocopherol nutraceutical compound, since it is a subject of special interest in our research group. Moreover, tomato fruit carotenogenesis has been extensively characterized (Liu et al. 2015). Since 2011, our group have published several papers characterizing the biosynthesis of tocopherols in S. lycopersicum, including all the enzyme-encoding genes and the limiting steps for its production (Almeida et al. 2011; Quadrana et al. 2013). Moreover, we have revealed the mechanisms by which some of these limiting steps are regulated (Almeida et al. 2015; Almeida et al. 2016; Quadrana et al. 2013; Lira et al. 2016).
Tocopherols are lipophilic antioxidants that, together with tocotrienols, are collectively called VTE. They are synthetized in the chloroplasts and accumulate in plastoglobuli (Vidi et al. 2006). These two families of metabolites are composed by a polar head and a hydrophobic side chain, and exist in four different forms each (α, β, γ and δ) (Figure 2) (Mène-Saffrané & Dellapenna 2010).

The benefits of VTE for human health are related to its antioxidant and anti-inflammatory properties (Rizvi et al. 2014) and includes decreased risk of mortality from thromboembolism in women (Booth et al. 2004), inhibition of cancer growth (Stone et al. 2004; Jiang et al. 2004), protection against neurodegenerative disorders, such as Alzheimer’s disease (Morris et al. 2015) and dementia (Cherubini et al. 2005), among others. Tocopherol forms have different bioavailability and bioactivity, being α-tocopherol the form with the highest VTE activity in mammals (Mène-Saffrané & Dellapenna 2010). However, it is worth to mention that γ-tocopherol is also well absorbed in human tissues, and has properties that are important to human health that are not shared with α-tocopherol, with epidemiologic studies describing positive relations between increased γ-tocopherol serum concentration and lower risk of prostate cancer and cardiovascular diseases (Jiang et al. 2001).

In plants, tocopherols are fundamental players of the photoprotective machinery particularly involved in controlling the level of singlet oxygen (\(^{1}O_2\)) in photosystem II (PSII), and the extent of lipid peroxidation in thylakoid membranes (Triantaphylidès & Havaux 2009; Rastogi et al. 2014; Miret & Munné-Bosch 2015). Beyond photoprotective roles, tocopherol is
also involved in seed longevity, seedling germination (Sattler et al. 2004; Mène-Saffrané et al. 2010), and photoassimilate export regulation (Almeida et al. 2016).

Tocopherol metabolism (Figure 3) is highly linked to chlorophyll and carotenoid metabolic pathways and, in recent years, it has been characterized in tomato (Almeida et al. 2011; Almeida et al. 2016; Quadrana et al. 2013; Guyer et al. 2014; Lira et al. 2016). The precursors for tocopherol biosynthesis are derived from two plastidial secondary metabolism pathways, methylerythritol phosphate (MEP) and shikimate (SK) and the description below highlights the main steps for which the catalyzing enzyme encoding genes have shown to be transcriptional regulated and will be studied in the present work (Quadrana et al. 2013). The product of two \textit{1-DEOXY-D-XYLULOSE-5-P SYNTHASE} (DXS) paralog genes catalyze the first step of MEP route, \textit{DXS(2)} in green tissues and \textit{DXS(1)}, whose expression is enhanced during ripening. Geranylgeranyl-2P (GGDP) is the MEP intermediate from which carotenoids are synthesized and, in green tissues, is also converted by \textit{GERANYLGERANYL DIPHOSPHATE REDUCTASE} (GGDR) into phytol-2P, a precursor of both, chlorophyll and tocopherol biosynthesis.

\textbf{CHLOROPHYLL SYNTHASE} (CHLG) catalyzes the reaction between chlorophyllide \textit{a} and phytol-2P to produce chlorophyll \textit{a} in photosynthetic organs. While, \textit{PHEOPHYTYNASE} (PPH) and \textit{PHEOPHYTYNASE-LIKE1} (PPHL1) are responsible for chlorophyll degradation and recycling, respectively.

Tocopherols are produced by the condensation of phytol-2P and the homogentisate (HGA). The latter is synthesized by two possible \textit{4-HYDROXYPHENYLPYRUVATE DIOXYGENASES} (HPPD) of SK pathway in tomato, being HPPD2 the most expressed in all tomato tissues/organs (Zouine et al. 2017). The \textbf{HOMOGENTISATE PHYTYL TRANSFERASE} (VTE2) produces 2-methyl-6-phytylquinol (MPBQ) from phytol-2P and HGA. The conversion of MPBQ to 2,3-dimethyl-5-phytylquinol (DPBQ) is done by \textit{2,3-DIMETHYL-5-PHYTYLQUINOL METHYL TRANSFERASE} (VTE3), while \textit{γ-} and \textit{δ-} tocopherols are synthetized by \textit{TOCOPHEROL CYCLASE} (VTE1) from DPBQ and MPBQ, respectively. Further, \textit{α-} and \textit{β-} tocopherols are converted from \textit{γ-} and \textit{δ-} forms, respectively, by \textit{TOCOPHEROL γ-METHYL TRANSFERASE} (VTE4). During tomato fruit ripening, GGDR is downregulated and the GGDP is directed towards carotenoid biosynthesis (Quadrana et al. 2013; Almeida et al. 2015). However, tocopherol content increases during ripening, fed by the recycling of chlorophyll degradation-derived phytol by \textit{PHYTOL KINASE} (VTE5) and \textit{PHYTYL-PHOSPHATE KINASE} (VTE 6), which produce phytol-2P for further condensation with HGA (Almeida et al. 2011, Almeida et al. 2016).
Figure 3: Simplified tocopherol biosynthetic pathway.

MEP, shikimate, chlorophyll and tocopherol metabolism are circled in red, purple, green and blue respectively. Dotted arrows indicate that intermediate steps were omitted. Enzymes: CHLG: CHLOROPHYLL SYNTHASE; DXS: 1-DEOXY-D-XYLULOSE-5-P SYNTHASE; GGDR: GERANYLGERANYL DIPHOSPHATE REDUCTASE; HPP: 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE; PPH: PHEOPHYTINASE; PPHL1: PHEOPHYTINASE LIKE-1; VTE1: TOCOPHEROL CYCLASE; VTE2: HOMOGENITISATE PHYTYL TRANSFERASE; VTE3: 2,3-DIMETHYL-5-PHYTYLQUINOL METHYL TRANSFERASE; VTE4: TOCOPHEROL Γ-METHYL TRANSFERASE; VTE5: PHYTOL KINASE; VTE6: PHYTYL-PHOSPHATE KINASE. Metabolites: Chlide a: chlorophyllide a; Chl a: chlorophyll a; DXP: 1-Deoxy-D-xylulose-5-P; GGDP: geranylgeranyl-2P; HGA: Homogentisate; HPP: hydroxyphenylpyruvate; MPBQ: 2-Methyl-6-phytylquinol; Pheide a: pheophorbide a; Phein a: pheophytin.

1.3. The role of GOLDEN 2-LIKE genes in chloroplast maintenance and activity.

Several transcriptional factors are involved in chloroplast differentiation. Among them, GLK proteins are one of the key factors, with great importance for both, plastidial biogenesis and activity maintenance (Fitter et al. 2002). In A. thaliana, there are 2 loci that encodes for GLK proteins, AtGLK1 and AtGLK2. Although they have a slightly different transcriptional pattern, they are functionally redundant since only the double mutant, Atglk1Atglk2, is deficient in plastid
development. AtGLK1 and AtGLK2 proteins induce expression of several genes involved in the formation of the photosynthetic apparatus, and respond to retrograde signals of chloroplasts, being able to coordinate the expression of nuclear genes related to photosynthesis and optimize this process according to environmental conditions (Waters et al. 2009).

In *S. lycopersicum* genome there are also two copies of GLKs: *SlGLK1* and *SlGLK2*. While *SlGLK1* is mostly expressed in cotyledons, sepals and leaves, *SlGLK2* is predominantly expressed in fruits, concentrated at the pedicelar region, resulting in a phenotype called “green shoulder”, decreasing in a longitudinal gradient until the base of the fruit (Powell et al. 2012; Nguyen et al. 2014).

Characteristics that facilitate harvesting and shipping, and increase shelf-life had great importance during tomato domestication (Giovannoni 2001). In this context, in order to select fruits that ripen evenly, the uniform ripening mutation was fixed, selecting the mutant allele of *SlGLK2* (*Slglk2*), which is found in most cultivated tomato varieties nowadays. *Slglk2* allele has a single base insertion at the coding region, originating a premature stop codon and a truncated protein with only 80 amino acids (Figure 4), while the wild protein encoded by the wild allele has 310 amino acids (Powell et al. 2012).

Fixation of *Slglk2* mutation had metabolic consequences, as mutant fruits has lower amount of chlorophyll and soluble sugars (Powell et al. 2012; Nguyen et al. 2014). Also, overexpression of *SlGLK2* in mutant background results in fruits with higher content of starch, soluble sugars and carotenoids (Powell et al. 2012; Nguyen et al. 2014). There are no reports of *SlGLK2* effect on other nutraceutical compounds, such as VTE.

Regarding *SlGLK*s expression regulation, it has been shown that *SlGLK2* expression is higher in fruits that developed in the presence of light when compared to dark grown fruits (Powell et al. 2012). This observation is in agreement with the results obtained in *A. thaliana*, which demonstrated the transcriptional downregulation and post translational inactivation of AtGLK proteins triggered by dark-induced senescence (Garapati et al. 2015; Rauf et al. 2013; Sakuraba et

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**Figure 4: Comparison between wild *SlGLK2* and mutant *Slglk2* alleles.**

Detail from nucleotide number 210 of the coding region of wild (above) and mutant (below) alleles. The insertion of an adenine caused a frameshift, resulting in a premature stop codon (circled), creating a non-functional truncated protein. Adapted from Powell et al. (2012).
Kobayashi et al. (2012) demonstrated that auxins repress, while cytokinins induce chloroplast development in *A. thaliana* roots. The mechanism involves cytokinin-mediated transcriptional induction of *HY5* and *AtGLK2*, which in turn coordinate the expression of key genes of chloroplast biogenesis. In tomato, Sagar et al. (2013) demonstrated that the downregulation of *SiARF4* expression, an auxin induced repressor of auxin response, leads to the upregulation of *SiGLK1*, resulting in the increment of chloroplast number and chlorophyll accumulation. Additionally, senescence delay and maintenance of plastid activity has been largely described in “evergreen” plants with high endogenous cytokinin content, but the role of *GLK* genes has not been addressed in these studies (Thomas & Ougham 2014).

In this context, the importance of *GLK* genes for plastid function is evident, however, data on the effect of *SiGLKs* on fruit quality and their regulatory mechanisms are fragmented, and the studies were mostly restricted to the model species *A. thaliana*. Thus, the present work intended to improve the knowledge about *SiGLK2*, specially about their role on tomato fruit development and ripening.
Conclusions

1. *GLK* genes are a novelty of Embryophyta clade.
2. The duplication that originated *SIGLKs* occurred in Solanaceae lineage prior to the divergence between *Solanum lycopersicum* and *Solanum tuberosum*.
3. The *SIGLK2* transcriptional regulation by light is, at least in part, mediated by PHYs.
4. Auxins downregulate *SIGLK2* expression in fruits.
5. *SIGLK2* wild allele positively affects cytokinin signaling in fruits.
6. *SIGLK2* promotes the differentiation of chloroplasts with highly stacked thylakoids in a PHY-independent manner in fruits.
7. *SIGLK2* promotes chlorophyll biosynthesis in immature green stages of fruit development, which is proportional to tocopherol content in ripe fruits.
8. *SIGLK2*-mediated chlorophyll and tocopherol increment are explained, at least in part, by the transcription upregulation of the biosynthetic enzyme encoding genes.
9. *SIGLK2* alters carbon metabolism inhibiting starch accumulation at immature stages of fruit development.
10. *SIGLK2* overexpression promotes the accumulation of higher levels of soluble sugar in ripe fruit contributing to higher °Brix.
11. *SIGLK2* has a negative effect on productivity traits such as fruit weight and aerial biomass.
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