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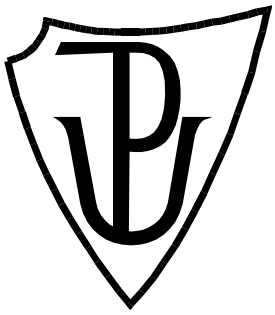

Study of blue light-induced de-etiolation in tomato: role of TFT proteins, PHOT1 and cytokinins

Ph.D. Thesis

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Study program: P1416 Biochemistry

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DECLARATION

I hereby declare that the submitted Ph.D. thesis is based on my own research carried out at the Department of Molecular Biology, Faculty of Science, Palacký University, Olomouc in the period September 2013 – November 2018. The thesis has been written by me with the use of literature cited in the References.

The data presented in this thesis are contained in the following publications:

Hloušková P. and Bergougnoux V. (2016). A substracted cDNA library identifies genes up-regulated during PHOT1-mediated early phase of de-etiolation in tomato (*Solanum lycopersicum* L.). *BMC Genomics* 17:291. Doi: 10.1186/s12864-016-2613-6.

Hloušková P., Černý M., Kořínková N., Luklová M., Minguet E. G., Brzobohatý B., Galuszka P. and Bergougnoux V. (2019). Affinity chromatography revealed 14-3-3 interactome of tomato (*Solanum lycopersicum* L.) during blue light-induced de-etiolation. *Journal of Proteomics* 193:44-61. Doi: 10.1016/j.jprot.2018.12.017.

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Mgr. PETRA HLOUŠKOVÁ

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Abstract

The PhD thesis is focused on understanding the involvement of phototropin 1 (PHOT1), 14-3-3 proteins (TFTs) and cytokinins in hypocotyl growth inhibition under exposure to blue light (BL) in tomato (*Solanum lycopersicum* L.). Upon BL illumination, reduction of hypocotyl growth rate occurs in two phases: PHOT1-mediated phase of rapid inhibition of the growth rate arising during the first 30 minutes of exposure to BL and subsequent CRY1-controlled establishment of the steady state growth rate. Using the suppression subtractive hybridization, 152 expressed sequence tags were identified to be rapidly accumulated in the hypocotyl of etiolated tomato seedlings within 30 minutes of BL illumination and thus potentially regulated by PHOT1. Concerning their molecular function, the identified genes were involved in chromatin remodeling, modification of cell wall and transcription/translation which constitute an important part of processes involved in de-etiolation and the establishment of photomorphogenesis.

Throughout the BL-induced inhibition of hypocotyl growth, TFT6 and TFT9 were shown to be the most abundant 14-3-3 isoforms. Similarly, isopentenyladenine (iP), an active cytokinin, accumulated in the elongation zone of hypocotyl under BL exposure. Whereas *TFT6* expression was not influenced or inhibited by iP, the abundance of *TFT9* mRNA significantly increased in a dose-dependent manner. Transcriptional activation assays using the luciferase reporter system performed in tomato leaf protoplasts confirmed that iP stimulates the transcription of the *TFT9* gene. Moreover, truncated versions of TFT9 promotor and site direct mutagenesis of cytokinin response elements (RR-B) proved that two binding sites at the position -1045 and -1106 are required for the iP-mediated regulation of *TFT9* gene. The co-transformation of protoplasts with plasmid

containing ARR1 resulted in enhanced expression of *TFT9* in absence of iP. Therefore, it was concluded that the stimulation of *TFT9* expression by iP is mediated via RR-B.

For a deeper understanding of the role of TFT6 and TFT9 in BL-mediated inhibition of hypocotyl elongation, the interaction partners of both isoforms were identified by pull-down assay coupled to LC-MS analysis. Exclusive for BL elongated hypocotyls, 115 and 37 putative interactors were found in TFT6 and TFT9 assays, respectively. Their annotation revealed that these proteins are involved in cell expansion, cell wall remodeling, metabolism, hormone signaling, transport, synthesis and protein folding. Other 14-3-3 isoforms were also identified, and several potential interactors were predicted to be also cytokinin-responsive. The yeast two-hybrid assay was used to validate the heterodimerization between different TFT isoforms and the interaction of TFTs with different proteins (PHOT1, PEPCK, GDSL and V-ATPase). The results indicate that 14-3-3 proteins could be implicated in the BL-mediated inhibition of hypocotyl growth through the binding and subsequent modulation of function/localization of several proteins involved in hypocotyl elongation.

In the last part of the thesis, amiRNA interference was used to generate tomato knock-down *phot1* mutant plants. The down-regulation of *PHOT1* caused distinct phenotypes in plant height and number of trichomes on the leaf and stem surface. The serration on the leaf margine and leaf flattening were also altered which might be associated with changes in transpiration and efficiency of photosynthesis. The fruits of mutant plants were darker green with lower number of seeds showing delayed germination. Proteomic analysis of fruit pericarp showed changes in proteins connected with organoleptic properties of fruit and carotenoid biosynthesis pathway which is in correlation with higher content of carotenoids, important components in human diet.

Keywords: de-etiolation, blue light, tomato, hypocotyl elongation, 14-3-3 proteins
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Abstrakt

Disertační práce je zaměřena na objasnění úlohy phototropinu 1 (PHOT1), 14-3-3 proteinů (TFT) a cytokininů v inhibici růstu hypokotylu na modrém světle u rajčete (*Solanum lycopersicum* L.). Modré světlo inhibuje prodlužování hypokotylu ve dvou fázích : během prvních 30 minut po ozáření dochází k rychlému zpomalení prodlužování, které je řízeno PHOT1, následně CRY1 reguluje rovnoměrný růst hypokotylu. Pomocí supresivní subtraktivní hybridizace bylo identifikováno celkem 152 genů, které byly akumulovány během 30 minut po ozáření modrým světlem a jsou tedy potencionálně regulované PHOT1. Nalezené geny jsou z hlediska funkce zapojeny do remodelace chromatinu, modifikace buněčné stěny a transkripce/translace, které představují důležitou část procesů podílejících se na de-etiolizaci.

TFT6 a TFT9 byly stanoveny jako dvě nejvíce exprimované isoformy 14-3-3 proteinů v elongační zóně hypokotylu rajčete na modrém světle. Aktivní cytokinin isopentenyladenin (iP) se rovněž akumuluje v elongační zóně na modrém světle. Zatímco iP nijak neovlivnil nebo inhiboval expresi *TFT6*, se zvyšující se koncentrací iP se zvyšovala i exprese *TFT9*, která byla potvrzena rovněž měřením luciferázové aktivity v protoplastech izolovaných z listů rajčete. Zkrácené verze TFT9 promotoru a cílená mutageneze regulátorů odpovědi cytokininů (RR-B) prokázala, že dvě vazebná místa pro RR-B v pozici -1045 a -1106 jsou zapojená ve stimulaci *TFT9* pomocí iP. Kotransformace protoplastů plasmidem zodpovědným za syntézu ARR1 ukázala zvýšenou expresi *TFT9,* a tedy, že stimulace exprese TFT9 pomocí iP je zprostředkována RR-B.

Pro hlubší pochopení úlohy TFT6 a TFT9 v procesu inhibice růstu hypokotylu na modrém světle byli pomocí pull-down assay a LC-MS identifikováni interakční partneři obou isoforem. Celkem bylo identifikováno 115 potenciálních vazebných partnerů pro TFT6 a 37 pro TFT9, specifických pro modré světlo. Jejich anotace odhalila proteiny

zapojené v buněčné expanzi, remodelaci buněčné stěny, metabolismu, hormonální signalizaci, transportu, syntéze a skládání proteinů. Identifikovány byly i jiné isoformy 14-3-3 proteinů a řada těchto nalezených potencinálních vazebných partnerů byla dříve identifikována jako cytokinin-responsivní. Dvouhybridní kvasinkový test prokázal vzájemnou heterodimerizaci isoforem TFT6/TFT9 a TFT4/TFT9 a interakci TFT protein s dalšími vazebnými partner (PHOT1, PEPCK, GDSL a V-ATPasou). Výsledky naznačují, že 14-3-3 proteiny se podílí na inhibici prodlužování hypokotylu na modrém světle skrze vazbu a následnou modulaci funkce řady proteinů zapojených do růstu hypokotylu.

Poslední část disertační práce byla zaměřena na přípravu mutanta *phot1* u rajčete pomocí amiRNA interference. Snížená exprese *PHOT1* výrazně ovlivbila výšku rostlin a počet trichomů na povrchu listu i stonku. Rovněž vroubkování na okraji listů a zploštění listů bylo rozdílné, což může být spojeno s pozměněnou hladinou transpirace a účinnosti fotosyntézy. Mutantní rostliny měly tmavě zelené plody s menším množstvím semen, u kterých je zpožděné klíčení. Proteomická analýza perikarpu plodů prokázala zvýšené množství proteinů zapojených v organoleptických vlastnostech plodů a v syntéze karotenoidů, což je v souladu s vyšším množstvím karotenoidů, důležitých složek potravy.

Klíčová slova: de-etiolizace, modré světlo, rajče, prodlužování hypokotylu, 14-3-3 proteiny
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CONTENT

INTRODUCTION

Throughout their lifecycle, plants are exposed to a variety of dynamic changes of environment. Due to their stationary nature, plant developed the way how to survive and thrive in these ever-changing environmental conditions. They have evolved the complex system of signaling pathways activated by external stimuli leading to the diverse plant responses. The perception and signal transduction of light play a pivotal role in the regulation of a wide array of physiological responses throughout the plant life-cycle, including seed germination, seedling photomorphogenesis, shade avoidance, circadian rhythms and flower induction. Although, the recent approaches have revealed quickly the components and mechanisms of signaling pathways, many questions are still unanswered. Indeed, the full understanding of plant signaling network is important for the improvement of agricultural traits such as the quality and quantity of crop yield.

The overall objectives of the present PhD thesis were to gain insights into understanding the blue-light (BL)-induced de-etiolation in tomato (*Solanum lycopersicum* L.), more precisely to uncover how 14-3-3 proteins, phototropin 1 (PHOT1) and cytokinins are involved in BL-mediated hypocotyl elongation. For this purpose, the work was divided into three main sections:

i) Study of PHOT1-mediated phase of BL-induced hypocotyl growth
ii) Elucidation of the role of 14-3-3 proteins and isopentenyladenine in BL-mediated hypocotyl growth
iii) Preparation and characterization of amiRNA-*phot1* mutant tomato plants

LITERATURE OVERVIEW

SOLANUM LYCOPERSIUM L.

Tomato (*Solanum lycopersicum* L.) is a plant species cultivated worldwide in greenhouses as well as in open fields. In 1694, Tournefort designated cultivated tomatoes as Lycopersicon, because in old German folklore, witches used tomatoes to evoke werewolves. Nowadays, the practice is called lycantropy. In the 18th century Carl Linnaeus conjured up binomial nomenclature to species name and renamed tomato to *Solanum lycopersicum* (Kiple and Ornelas, 2000). In 1768, tomato was entitled as *Lycopersicon esculentum* and moved to its own genus by Philip Miller. Nowadays, both names can be found in the literature. Tomato plants belong to the *Solanaceae* family and it originates from the Central and South America, where it was domesticated. After the Spanish colonization of Americas in the 16th century, tomatoes were brought to Europe. Recently, tomato is the seventh most important crop species with the worldwide production reaching almost 182 million tons in 2017. The largest producers of tomatoes are China, USA and India (Foolad, 2007;Bergougnoux, 2014). The interest of scientists and breeders grew up with the increasing popularity of tomato as major component in daily diet. The popularity of tomato relates to the fact that it can be eaten in multiple forms such as tomato preserves (whole peeled tomatoes, tomato puree, juice, paste), dried tomatoes (tomato powder, flakes) or tomato-based food (ketchup, tomato soup, sauce). In terms of human health, tomato is an excellent source of minerals, vitamins and antioxidants (Grierson and Kader, 1986; (Foolad, 2007). *S. lycopersicum* possesses numerous qualities allowing it to be a good model organism for several fields of studies. These features include: ease of cultivation, short life cycle, the diploid genome of average size (900 Mb; 12 chromosomes) and easy monitoring of pollination and hybridization (Lozano et al., 2009). Approximately 30% of the genes of tomato and other plants of the family *Solanaceae* are unique compared to Arabidopsis. Moreover, tomato has several traits, such as compound leaves, a sympodial shoot and fleshy fruit which cannot be studied in other model plants. Therefore, it is useful to investigate their biological importance (Van der Hoeven et al., 2002). Generation of tomato transgenic plants is focused on fruit quality, resistance to pesticides and disease and production of therapeutics or resistance to various environmental conditions (Bergougnoux, 2014).

DE-ETIOLATION

Undoubtedly, light is one of the crucial environmental factors affecting plant from the early development. Apart from acting as a source of energy, light is also an important information carrier and regulates various aspects of plant development, most notably seedlings de-etiolation – the transition from a pale heterotrophic seedling to a green photosynthetically competent one. The seedlings kept in the dark are characterized by the apical hook formation, unexpanded cotyledons and fast elongating hypocotyl. In opposite, the light treatment of dark-grown seedlings causes the opening of apical hook, expansion of cotyledons, and reduction in hypocotyl growth rate (Neff et al., 2000) (Fig. 1).

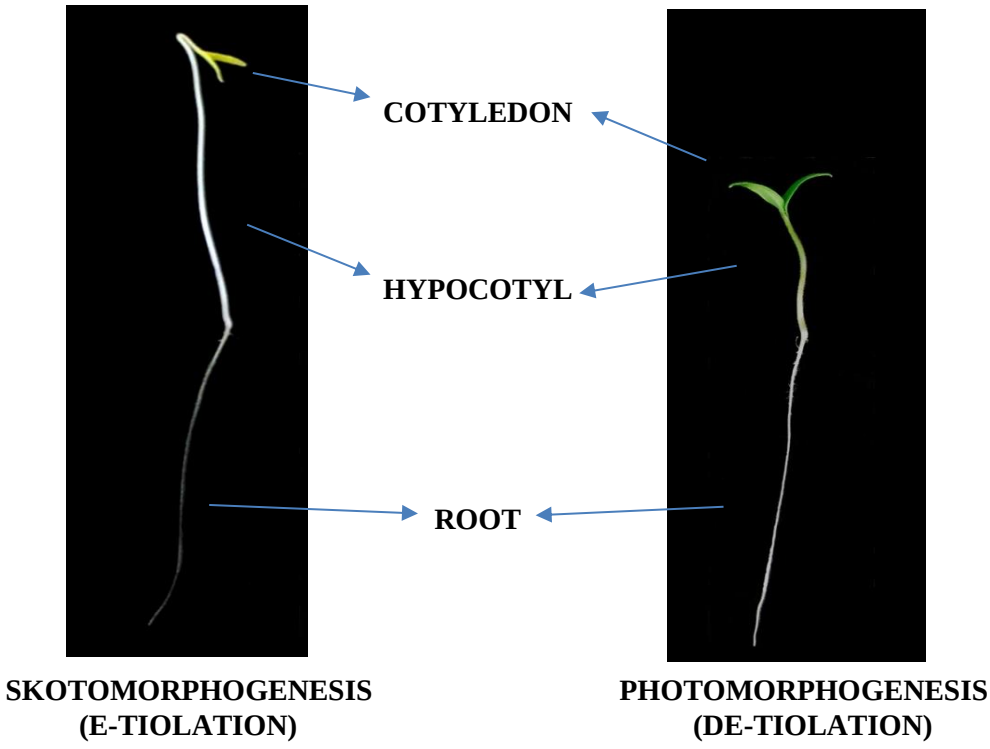

Figure 1. The contrasting phenotypes of dark- and light-grown tomato seedlings. Dark-grown seedlings undergo skotomorphogenesis (etiolation) characterized by elongated hypocotyls, closed and underdeveloped cotyledons and apical hook. Light-grown seedlings undergo photomorphogenesis (de-etiolation) and are characterized by short hypocotyls, open and expanded green cotyledons (modified according to (Nemhauser and Chory, 2002).

At the beginning of de-etiolation, the photons of incoming light are absorbed by plants via specific chromoproteins – photoreceptors, working individually or in combination. Plants have evolved the sophisticated system of photoreceptors able to absorb light to perceive changes in light wavelengths, intensity, duration or direction adjusting their growth, reproduction and survival (Fig. 2). If one considers the photosynthetically active radiation, mainly the blue (BL) and red (RL) parts of the spectrum are utilized by the plants.

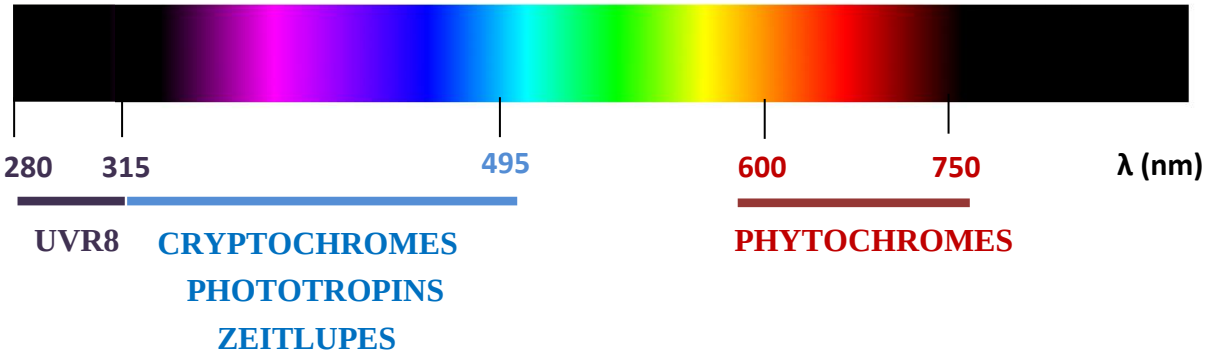

Figure 2. Photoreceptor-mediated light perception in plants. The plants perceive a visible part of the electromagnetic spectrum via various wavelength specific photoreceptors. Phytochromes absorb red and far-red light (600-750 nm); Cryptochromes, Phototropins and Zeitlupes absorb blue (450-495 nm) and UV-A light (315-400 nm); UVR8 absorbs UV-B (280-315 nm). Modified according to (Tilbrook et al., 2013).

Consistent with the remarkable changes in morphology and development, light induces the extensive reprogramming of transcriptome that is a consequence of the differential expression of at least 20% of the whole genome leading to the coordinate regulation of the major biochemical pathways upon transition from dark to light (Jiao et al., 2005). Transcriptional regulatory network plays a profound role in the control of light responses through the orchestrated activation and repression of downstream genes. In the case of blue light (BL), 20% of all transcription factors determinated in 6-day-old (do) seedling were responsive to BL (Jiao et al., 2003). Similarly, 44% and 25% of early far-red and red light induced genes, respectively, were classified as transcriptional factors (Lee et al., 2007). Some of those regulators are specific for a given light quality, while the others are involved in the regulation of signaling pathways downstream of several photoreceptors altering the overlapping set of genes which indicates the presence of integration signaling points and shared signaling components.

BLUE LIGHT PHOTORECEPTORS

The perception of blue light (BL; 450-495 nm) and ultraviolet-A radiation (UV-A; 315-400 nm) in higher plants is provided by three different types of photoreceptors: phototropins (PHOTs), cryptochromes (CRYs) and members of the Zeitlupe family (ZTL/FKF1/LKP2). Overall, seven blue-light receptors were identified and characterized so far. All of them consist of flavin as the chromophore (Banerjee and Batschauer, 2005).

PHOTOTROPINS

Phototropins are part of BL sensory system in plants that fine-tune the efficient use of solar energy for optimal photosynthesis and promote growth through a variety of responses. These responses include phototropism, chloroplast movement, stomatal opening, leaf flattening and positioning, and rapid inhibition of hypocotyl growth (Christie et al., 1998;Briggs et al., 2001;Jarillo et al., 2001;Kagawa et al., 2001;de Carbonnel et al., 2010).

The most phototropin containing species have two phototropins designated PHOT1 and PHOT2. PHOT1, originally named NON-PHOTOTROPIC HYPOCOTYL 1 (NPH1), was identified from the genetic screen of Arabidopsis mutants altered in phototropic response under BL illumination (Liscum and Briggs, 1995). PHOT2, initially named NPH1-like 1 (NPL1), was discovered both by genome sequence analysis as homolog of phot1 showing high structural and sequence similarity with identity of 58% along the protein sequence, and later by mutant screening for lack of chloroplast avoidance in high-light intensity (Jarillo et al., 2001;Kagawa et al., 2001).

Phototropins contain an N-terminal pair of photosensory domains named Light-Oxygen-Voltage (LOV1, LOV2) and a serine/threonine kinase domain at their C-terminus. Both LOV domains are composed of α/β scaffold and bind prosthetic flavin mononucleotide (FMN) as chromofore (Fig. 3). In the dark, FMN is non-covalently bound to the α/β scaffold of LOV domain with absorption spectrum at 450 nm (D450). BL irradiation induces the formation of covalent adduct between the C(4a) carbon of the FMN and the sulfur atom of cysteine residue of LOV domain with the absorption maxima at 390 nm (S390). The FMN-cysteinyl adduct formation evokes conformation changes including a tilt of the FMN isoalloxazine ring, rearrangement of glutamine side chain and hydrogen-bond network triggering the autophosphorylation of the serine-threonine kinase domain of the photoreceptor. The photoreceptor activation occurs within few microseconds upon BL illumination and converts back to D450 in a time range of second

or minutes (Christie et al., 1999;Salomon et al., 2000;Swartz et al., 2001;Kasahara et al., 2002;Nash et al., 2008).

In the etiolated seedlings, PHOT1 is associated with the inner surface of plasma membrane in its inactive state, mostly in the monomer form. Upon BL illumination, autophosphorylation and dimerization of phototropins occurs in a dose-dependent manner and a fraction is internalized into the cytoplasm via clathrin-mediated endocytosis. PHOT1 function and phototropism were not impaired in Arabidopsis when the PHOT1 internalization was prevented by myristoylation and farnesylation, predicting that PHOT1 signaling initiates at the plasma membrane. Concurrently, BL-induced autophosphorylation is probably the primary step of PHOT1 signaling since the kinase-inactive mutant *phot1*^{D806N} abolishes PHOT11 function and signaling cascade. Moreover, Bimolecular Fluorescence Complementation and photobleaching experiments using PHOT1 and *phot1* ^{D806N} further demonstrated that the inhibition of phosphorylation has no effect on PHOT1 dimerization. Recently, it was shown that PHOT1 recruiment to membrane microdomains is implicated in proper PHOT1 signaling (Sakamoto and Briggs, 2002;Inoue et al., 2008a;Kaiserli et al., 2009;Preuten et al., 2015;Xue et al., 2018).

Phototropin isoforms show diverse or overlapping functions depending on the regulated process (Briggs et al., 2001). Both phototropin isoforms are involved in phototropism, the BL-dependent reorientation of growth towards or away from light. The shoots show positive phototropic effect while the roots exhibit negative phototropism although gravitropism is more important in root growth. During phototropism, PHOT1 and PHOT2 show partially overlapping functions in a fluence rate-dependent manner. Whereas both play role under high BL conditions, the low-fluence BL effect is restricted to PHOT1 (Sakai et al., 2001). (Salomon et al., 1997) demonstrated the high degree of autophosphorylated phototropins on the irradiated side compared to the shaded one. Subsequently, the asymmetric activation of phototropins within the shoot initiates the lateral relocalization of auxin. However, the full mechanism of phototropic bending and how the gradient of activated PHOTs brings about the auxin gradient remains elusive. To date, several phot1-downstream signal transducers were identified to be essential for asymmetric distribution of auxin and induction of phototropic response. In the dark, PHOT1 is in its inactive, dephosphorylated state. The N-terminal LOV domain of PHOT1 binds the C-terminal coiled-coil domain of NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) which is inactive and phospohorylated (Motchoulski and Liscum, 1999). Both PHOT1 and NPH3 interact with PHYTOCHROME KINASE SUBSTRATE (PKS1)

(Lariguet et al., 2006). Moreover, PHOT1 physically interact with ATP binding cassette B 19 (ABCB19), an auxin efflux carrier, which enhances PIN-FORMED (PIN) protein stability at the plasma membrane (Blakeslee et al., 2007;Titapiwatanakun et al., 2009;Christie et al., 2011). Upon BL illumination, PHOT1 is autophosphorylated and promotes dephosphorylation of NPH3 which serves as a scaffold protein for the assembly of the complex of phototropin with the CULLIN3-RING LIGASE (CRL3) responsible for mono/multiubiquitination of phot1 resulting in the exclusion of PHOT1 from the plasma membrane which is substantial for phototropic response (Roberts et al., 2011). The active PHOT1 also phosphorylates ABCB19 leading the suppression of its transport activity and disruption of its stabilizing effect on PIN protein localization at the plasma membrane (Blakeslee et al., 2007;Titapiwatanakun et al., 2009;Christie et al., 2011).

In addition to the well-defined influence on stem phototropism, PHOT1 mediates root phototropism through enhancing the efficiency of root growth away from the soil (Sakamoto and Briggs, 2002;Galen et al., 2007).

Phototropins are also involved in the chloroplast photorelocation movement. Similar to phototropism, PHOT1 and PHOT2 exhibit partially overlapping functions in BL-induced chloroplast translocation. Whereas avoidance response from the strong BL is solely mediated by PHOT2, the accumulation response at the week BL is controlled by both phototropins (Kagawa et al., 2001;Sakai et al., 2001).

Stomatal opening is another process controlled equally by both PHOT1 and PHOT2. Stomata opening is associated with the phosphorylation of phototropins and the activation of the plasma membrane H^+-ATPase (PM H^+-ATPase) in guard cells in response to BL, creating an inside-negative electrical potential across the plasma membrane and driving K^+ uptake, leading to an entry of ions and metabolites in the cell, followed by water uptake, increasing the turgor pressure and then the stomata opening. The PM H^+-ATPase is activated by the phosphorylation of its C-terminus with a concomitant binding of the 14-3-3 proteins (Kinoshita et al., 2003;Padmanaban et al., 2004;Inoue et al., 2010).

In the case of leaf movement, another PHOT-mediated response, the BL-induced bending of leaves is mediated by the decrease of turgor pressure in the pulvinar motor cells of kidney bean, leading to the decrease of cellular volume. This response results from water efflux, brought about by ion effluxes which are driven by depolarization-activated channels of the plasma membrane, in response to the inactivation of the PM H^+-ATPase due to its dissociation with the 14-3-3 protein (Inoue et al., 2005).

Furthermore, PHOT1 acts in the primary phase of hypocotyl growth inhibition which starts within the 30 seconds after BL illumination and lasts about 30 minutes. During the rapid inhibition of hypocotyl growth mediated by BL, the depolarization of the plasma membrane was observed; 14-3-3 proteins were reported to play an important role during this process in etiolated barley shoots (Folta and Spalding, 2001;Klychnikov et al., 2007). (Folta et al., 2003) reported that BL induced the increase in cytosolic Ca^{2+} in etiolated seedlings. By using BAPTA, a specific chelator of Ca^{2+}, they proved that both the BL-induced Ca^{2+} increase and the rapid inhibition of hypocotyl elongation are similarly reduced. In addition, (Babourina et al., 2002) showed that only PHOT1, but not PHOT2, induced Ca^{2+} influx from apoplast to hypocotyl which suggested the importance of Ca^{2+} influx in phot1-dependent rapid inhibition of hypocotyl growth.

CRYPTOCHROMES

Cryptochromes are BL and UV-A photoreceptors controlling a variety of responses such as hypocotyl elongation, floral initiation, seed germination, circadian rhythms and apical dominance. Most plant species studied contain at least two cryptochrome isoforms marked as CRY1 and CRY2. In 1980, defect in hypocotyl growth of the *hy4* mutant restricted to BL was discovered (Koornneef, 1980). Over a decade later, another allele T-DNA insertion mutant in *hy4* was isolated showing no response to BL. Subsequently, HY4 was characterized as a protein with structural similarity to DNA photolyases missing the photolyase activity and was later renamed as cryptochrome 1 (Ahmad and Cashmore, 1993;Lin et al., 1996).

CRY proteins are composed of two conserved domains: an N-terminal Photolyase 6 Homologous Region (PHR) domain that binds the flavin adenine dinucleotide (FAD) chromophore, and a cryptochrome C-terminus (CCT) domain, which is important for their function and regulation (Fig. 3; (Yu et al., 2010). In the darkness, CCT and PHR domains form a closed, inactive state and the oxidized FAD is bound to the PHR domain. Within microseconds of BL irradiation, FAD is reduced and protonizated to FADH· which triggers a conformation change within the CCT domain to produce an open, active conformation that is required for the interaction with downstream signaling partners (Partch et al., 2005;Kottke et al., 2006;Bouly et al., 2007;Kondoh et al., 2011). The exact mechanism is not fully understood, but the ability of cryptochrome to bind ATP is disscussed (Burney et al., 2009;Cailliez et al., 2014;Müller et al., 2014). BL also induces cryptochrome phosphorylation contributing to its activation and biological function.

Phosphorylation of multiple serine residues might cause the electrostatic repulsion of the CCT domain from the surface of the PHR domain, resulting in the separation of the two domains to trigger or alter interaction between CRYs and their signaling partners (Liu et al., 2011).

CRYs are primarily localised to the nucleus and function mainly through the modulation of gene expression by two different ways. Firstly, CRYs regulate gene expression indirectly via interaction with SUPPRESSOR OF PHYA 1 (SPA1) which prevent forming of the E3 ubiquitin ligase complex COP1-SPA1-CUL4-DDB1 responsible for degradation of trancription factors that promote photomorphogenesis such as LONG HYPOCOTYL 5 (HY5) or HY5 HOMOLOG (HYH). Secondly, CRY can bind directly to transcription factor such CRY-interacting bHLH1 (CIB1) to promote expression of *Flowering locus T* (*FT*) (Liu et al., 2008). Although a fraction of CRYs were reported to be cytoplasmically localised to function in cotyledon expansion and control primary root growth (Wu and Spalding, 2007)

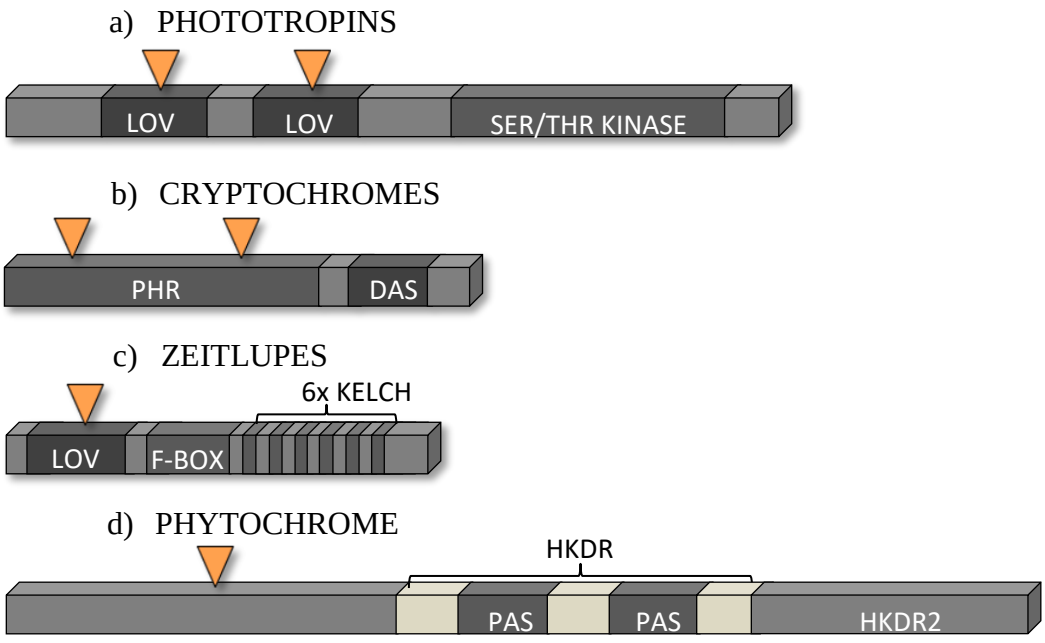

Figure 3. The schematic structures of the four classes of plant photoreceptors. The blue/UV-A-sensing phototropins contain two chromophores binding Light-oxygen and voltage domains (LOV1 and LOV2) and a C-terminal serine-threonine (SER/THR) kinase domain. The also blue/UV-A-sensing cryptochromes have an amino-terminal photolyase related domain (PHR) and a DQXVP-acidicSTATES (DAS) motif at their C-terminus. The blue/UV-A-sensing zeitlupes comprises of LOV domain, F-box and 6x Kelch repeat domain. The red/far red light-sensing phytochromes contain two carboxy-terminal histidine kinase domains (HKRD) and two Per-Amt-Sim domains (PAS) within the HKRD1 domain. Orange triangles represent the chromophores attachment sites in each of the photoreceptos (modified according to (Lin, 2002).

ZEITLUPES

Zeitlupe (ZTL), Flavin-binding kelch repeat F-box 1 (FKF1) and LOV kelch protein 2 (LKP2) are members of another family of BL photoreceptors named Zeitlupes. These proteins play a role in the circadian clock and the control of photoperiodic flowering (Ito et al., 2012;Suetsugu and Wada, 2013). Recent results proposed that proteins of this family function also as E3 ubiquitin ligases and target proteins for degradation in a light dependent manner. ZTL was first identified from genetic screens to isolate Arabidopsis mutants with an altered circadian clock (Kiyosue and Wada, 2000;Somers et al., 2000).

Structurally, the proteins comprises of a Light, Oxygen or Voltage (LOV) domain, an F box domain and a Kelch repeat domain (Fig. 3) (Ito et al., 2012). The recombinant LOV domain of the ZTL1, FKF1 and LKP2 binds the Flavin Mononucleotide (FMN) and undergoes a photocycle analogous to that of phototropin one, but the dark recovery of this photocycle is much slower compared to phototropins, taking days to return to the ground state (Zikihara et al., 2006). The F-boxes are associated with E3 ubiquitin ligases targeting proteins for proteasome degradation whereas Kelch repeats ensure protein:protein interactions and heterodimerization within the family (Takase et al., 2011).

ZTL contributes to the proteasome-dependent degradation of two circadian clock components: TIMING OF CAB1 (TOC1) and PSEUDO-RESPONSE REGULATOR 5 (PRR5) (Más et al., 2003;Kiba et al., 2007). The stability of ZTL protein is increased by BL-dependent binding of GIGANTEA (GI), the protein under circadian clock control (Park et al., 1999). In comparison with ZTL, FKF1 is rather a modulator of the circadian clock and induces flowering in long day conditions via modulating *CONSTANS* (*CO*) expression. FKF1 interacts with Cycling DOF factors (CDGs) that bind to the CO promotor to repress its transcription (Imaizumi et al., 2003;Más et al., 2003;Imaizumi et al., 2005). (Sawa et al., 2007) have shown that FKF1 also form a heterodimer with GI under BL. On the other hand, little is known about the role of LKP2. It was shown that over-expression of LPK2 leads to delayed flowering and that LKP2 interacts with TOC1, CO and GI (Schultz et al., 2001;Yasuhara et al., 2004;Fukamatsu et al., 2005;Kim et al., 2007). Nevertheless, further investigation is required.

UV-B RECEPTORS

Plant responses to ultraviolet radiation B (UV-B, 280-315 nm) are complex because UV-B photons are absorbed by numerous macromolecules such as nucleic acids, aromatic amino acids, proteins or phenolic compounds (Jenkins, 2009). The specific response depends on the UV-B wavelength, fluence rate and duration. Exposure to high fluence rates and short wavelengths of UV-B cause the stress responses, damage of DNA, proteins or inhibition of protein synthesis and photosynthesis while low fluence rates of UV-B mediate the regulatory function such as inhibition of stem growth, accumulation of flavonoids or regulation of gene expression (Kim et al., 1998;Ulm et al., 2004;Nawkar et al., 2013).

UV-B radiation is perceived by UV RESISTANT LOCUS 8 (UVR8), originally identified in a screen of Arabidopsis mutants hypersensitive to UV-B. It is formed by seven bladed β-propellers and differs from all known photoreceptors by the lack of external co-factor. The crystal structure analysis revealed that chromophore function is mediated by amino acis tryptophan (Trp) (Fig. 3) (Kliebenstein et al., 2002).

UVR8 protein shuttles between cytoplasm and nucleus upon UV-B radiation. Under light conditions devoided of UV-B, UVR8 appears as a homodimer in cytoplasm. After UV-B perception by tryptophan, the structure of salt bridges joining the dimer are disturbed and UVB8 monomer is activated, interacting via its C-terminal domain with the WD40 domain of the E3 ubiquitin ligase COP1 (Favory et al., 2009;Cloix et al., 2012). Function of UVR8 monomer may be to separate COP1-SPA complex from its association with CUL4-DB1, ensuring the repression of photomoprhogenesis in darkness. UVR8-COP-SPA induces the transcription factor HY5 which regulate the expression of UVR8 signaling pathway.

RED LIGHT PHOTORECEPTORS

The red part of light spectrum, and more specifically changes in red:far-red (R:FR) ratio are detected in plants by phytochromes (phys). PHYs regulate numerous light dependent physiological responses such as seed germination, seedling development or initiation of chlorophyll formation during the switch from heteroautotrophic to photoautotrophic state. PHYs also influence changes in the mode of growth and the timing of flowering (Masoner and Kasemir, 1975;Shinomura, 1997;Whitelam et al., 1998;Lin, 2000;Chen and Chory, 2011).

PHYs were initially identified as the photoreceptors mediating the plant photomorphogenesis in response to long-wavelength visible light (Butler et al., 1959). Several phytochrome proteins are encoded by plant genomes each with slightly diverged function and sensitivity to light. Arabidopsis has five phytochromes designated as PHYA-E, tomato genome encodes also five phytochrome genes referred to as *PHYA, PHYB1, PHYB2, PHYE,* and *PHYF* (Clack et al., 1994;Alba et al., 2000).

The PHYs are composed by an N-terminal photosensory domain combined with a C-terminal domain responsible for dimerization and localization (Fig. 3) (Nagatani, 2010). Through a covalent thioether linkage, PHYs bind to the linear tetrapyrrole bilin chromophore which enables the photoconversion from the inactive red-absorbing (Pr) and active far red-absorbing (Pfr) forms. The R:FR ratio received determines the ratio between active and inactive forms of PHY and conveys information about the photosynthetic energy available to ensure that the plant receives enough light for the growth (Rockwell et al., 2006;Franklin and Whitelam, 2007).

In the darkness, the PHYs are found in the cytoplasm whereas upon light illumination they translocate into the nucleus within minutes (Kircher et al., 2002). Accumulation of PHYs in the nucleus triggers the phosphorylation of the Phytochrome Interacting Factors (PIFs) leading to their subsequent rapid degradation via the ubiquitin/26S proteasome pathway (Al-Sady et al., 2006). PIFs are transcription factors that negatively regulate the photomorphogenesis by binding to the G-box motif of light-regulated genes (Chen and Chory, 2011;de Lucas and Prat, 2014).

COP/DET/FUS COMPLEX

Downstream of the photoreceptors, a group of CONSTITUTIVE PHOTOMORPHOGENISIS/DE-ETIOLATED/FUSCA (COP/DET/FUS) genes was identified and characterized as negative regulators of photomorphogenesis (Chory et al., 1989;Deng, 1994). The mutant seedlings of these genes exhibit light-grown phenotype when growm in the dark. Based on their biochemical and genetic characterization, COP/DET/FUS proteins are defined into three different protein complexes: the CONSTITUTIVE PHOTOMORPHOGENISIS 1/ SUPRESSOR OF PHYTOCHROME A-105 (COP1/SPA) complex, the COP9 signalosome (CSN) and the CONSTITUTIVE PHOTOMORPHOGENISIS 10/ DE-ETIOLATED 1/ UV-DAMAGED DNA BINDING PROTEIN 1 (COP10–DET1–DDB1 - CDD) complex (Lau and Deng, 2012).

The most studied gene from the COP/DET/FUS group is the *COP1*. It contains a WD40 domain and a coiled-coil domain that serve as protein-protein interaction domains and a RING finger domain, which is common to the E3 ubiquitin ligase function (Deng et al., 1992). It was shown that COP1 carries out his function by targeting the transcription factors promoting the de-etiolation (LONG HYPOCOTYL 5 - HY5; HY5-LIKE – HYH; LONG AFTER FAR-RED LIGHT 1 – LAF1 and LONG HYPOCOTYL IN FAR-RED 1 - HFR1) for degradation via the 26S proteasome pathway in the dark when it is localized in the nucleus. Upon exposure to light, COP1 is translocated to the cytoplasm and photomorphogenesis can take place (Fig. 4,5) (Osterlund et al., 2000;Saijo et al., 2003;Seo et al., 2003).

Moreover, the photoreceptors (PHYs and CRYs) were found to interact with COP1 via the WD40 domain, suppressing COP1-mediated degradation of transcription factors promoting the photomorphogenesis. Interestingly, it was shown that COP1 is essential for the degradation of phyA, phyB and cry2, thereby possibly desensitizing light signal transduction to prevent plants from an over-stimulation by light (Fig. 4) (Wang et al., 2001;Yang et al., 2001;Shalitin et al., 2002;Seo et al., 2004;Jang et al., 2010). In addition, COP1 was also found to act positively in photomorphogenic responses in UV-B light signaling. Indeed, *cop1* mutant exhibits impaired flavonoid biosynthesis and hypocotyl elongation in response to UV-B. In this regard, COP1 interacts with the UVR8 protein which then triggers downstream signaling events, including gene expression regulated by HY5 (Favory et al., 2009;Huang et al., 2012)(Fig 4).

Another important repressor of light signal transduction is the SPA1. The SPA1 protein shares a high structural similarity with COP1, having both a a carboxy-terminal WD40 and a coiled-coil domain. However, instead of a RING-finger motif, SPA1 carries a kinase-like domain (Hoecker et al., 1999) whose catalytic function has never been demonstrated (Fittinghoff et al., 2006). Additionally, SPA1 contains two nuclear localization sequences (NLSs) and the protein is constitutively localized in the nucleus (Hoecker et al., 1999). COP1 and SPA interact via their coiled-coil domains and act together in a tetrameric complex consisting of two COP1 proteins and a dimer of the SPA proteins to suppress photomorphogenesis (Fig. 5) (Hoecker and Quail, 2001;Saijo et al., 2003).

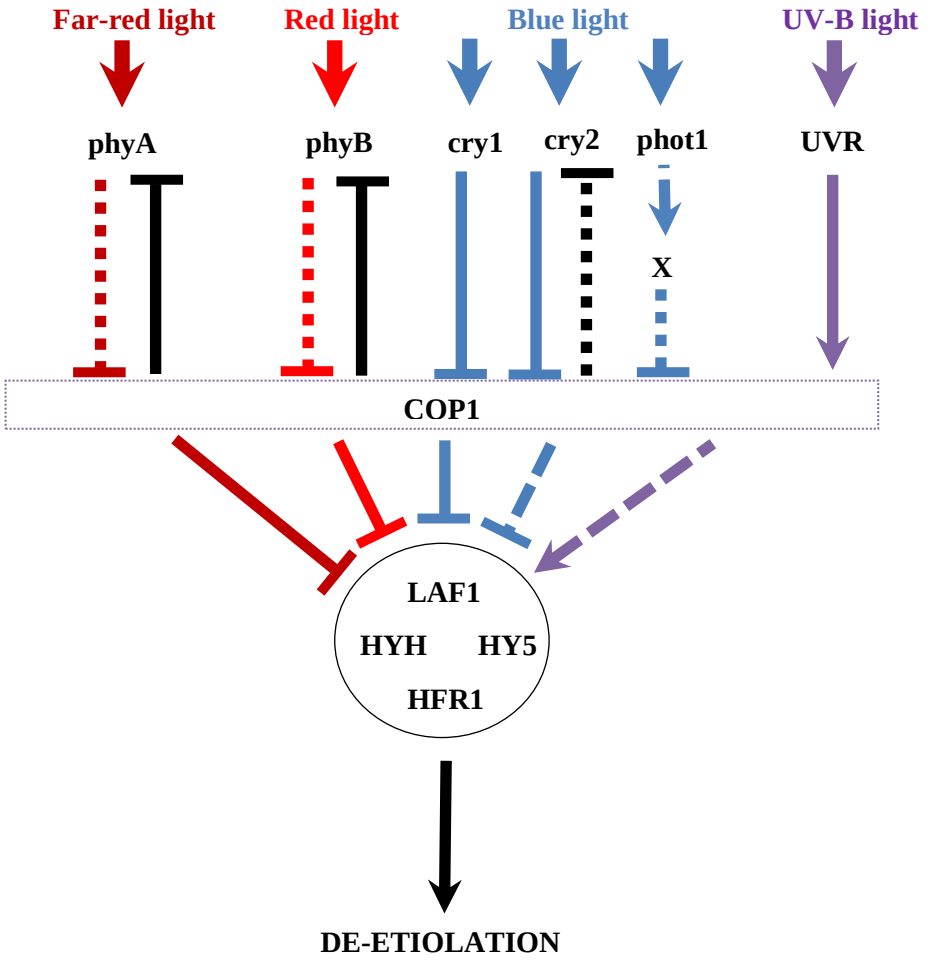

Figure 4. COP1 represents a central node integrating signals from various photoreceptors and controls many downstream targets ensuring the de-etiolation process. Phytochromes (PHYA and PHYB), cryptochromes (CRY1 and CRY2) and phototropins (PHOT1) are the major photoreceptors that perceive a part of the visible light. After perceiving light signal, the activated photoreceptors act to suppress COP1. Nevertheless, COP1 is essential for degradation of PHYA, PHYB and CRY2 to prevent plants from an over-stimulation by ligh. COP1 is a repressor of photomorphogenesis and functions as an E3 ubiquitin ligase that ubiquitinates transcription factors promoting de-etiolation (LAF1, HFR1, HY5, HYH). Thus, photoreceptor-mediated suppression of COP1 leads to the accumulation of these transcription effectors, resulting in de-etiolation. Under UV-B, UVR8 interacts with COP1 triggering transcription factors to promote photomorphogeneis. Modified according to (Huang et al., 2012;Lau and Deng, 2012).

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COP1 interacts also with the RING-finger domain of the ubiquitin-activating E2 variant COP10, another protein of the COP/DET/FUS group that mediates the repression of photomorphogenesis in darkness (Suzuki et al., 2002). COP10 associates with DET1 and DDB1 assembling a small protein complex called CDD complex (Yanagawa et al., 2004). Some proteins of the COP/DET/FUS group were found to interact with another multi-subunit complex, the COP9 signalosome (CSN) (von Arnim et al., 1997). Interestingly, in darkness COP1 is depleted in the nucleus in *csn* mutants compared to

wild type suggesting that the CSN plays an important role in COP1 nuclear import and/or retention in the dark (Chamovitz et al., 1996;von Arnim et al., 1997). CDD and CSN complexes act together in the ubiquitin-mediated proteasomal degradation of photomorphogenesis-promoting factors (Fig. 5) (Serino and Deng, 2003;Yanagawa et al., 2004;Yi and Deng, 2005).

Furthermore, COP1 and the SPA proteins were shown to associate with CUL4 based E3-ubiquitin ligase by binding to the DDB1 protein (Chen et al., 2010). The activity of DDB1- CUL4 complexes is regulated via the CSN, which rubylates and derubylates CUL4. Thus, rubylation seems to activate the DDB1-CUL4 complex (Chen et al., 2006). Taken together, an orchestrated synchronization of interaction and regulation between the vast number of proteins is required to ensure the proper photomorphogenesis (Fig.5).

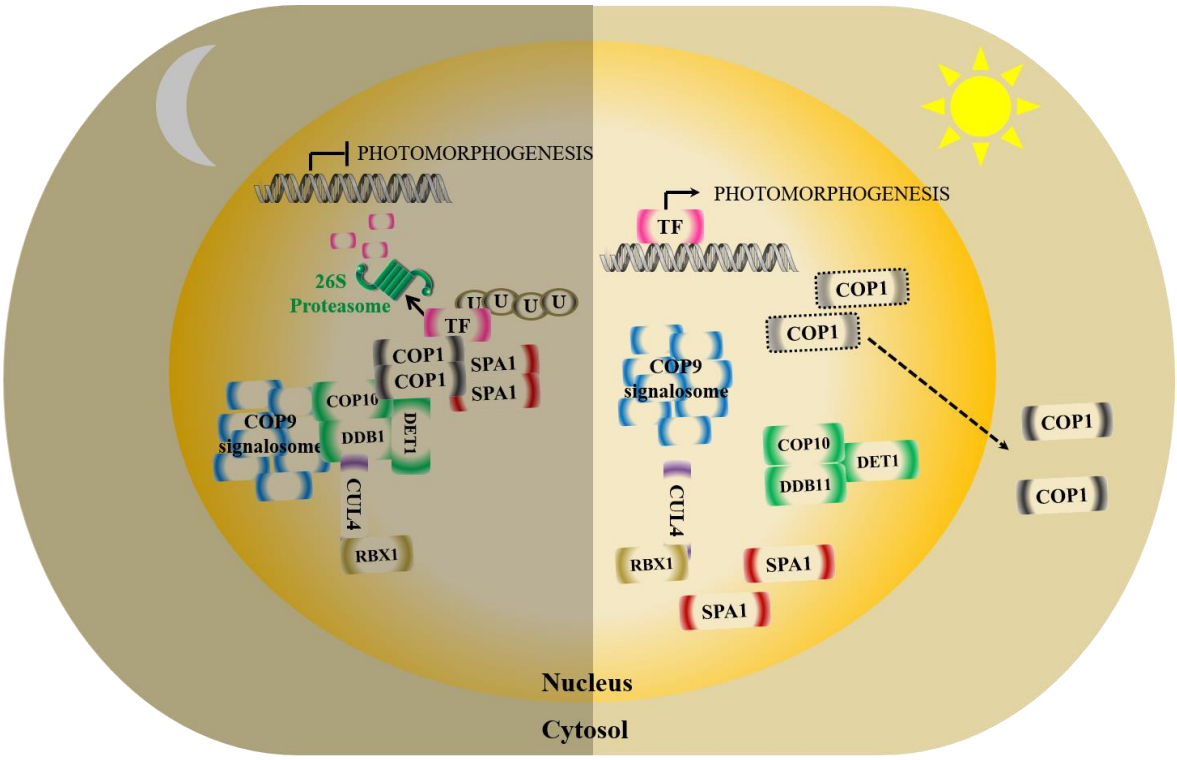

Figure 5. A simplified model of light signaling in plant cell during de-etiolation. In darkness, in nucleus of the cell, COP1/SPA1, CDD (COP10, DET1, DDB1) and COP9 signalosome protein complexes with CUL4/RBX1 co-operate to promote the degradation of photomorphogenesis-promoting transcription factors such as HY5, HYH, LAF1, HFR1 via the 26S proteasome. In this context, photomorphogenesis is repressed. Exposure to light triggers the dissociation of the protein complex COP1/SPA1/CDD/COP9 signalosome/CUL4-RBX1, COP1 is translocated to the cytosol, alllowing the expression of the photomorphogenesis-promoting transcription factors and photomorphogenesis (modified according to (Waters and Langdale, 2009).

HYPOCOTYL GROWTH INHIBITION

While studying de-etiolation, the attention is focused on the process in which light inhibits hypocotyl elongation of seedlings of higher plants because it is easily measured, and mutants affected in this response are available. The genetic screening of mutants altered in hypocotyl growth revealed that they are either impaired in a specific photoreceptor or their downstream targets.

The rapid decline of hypocotyl growth is most strongly induced by BL irradiation of dark-grown seedlings (Meijer, 1968). Within 30 seconds of BL illumination, the rapid growth rate of hypocotyl of seedlings grown in darkness is reduced reaching almost the complete inhibition after 30-40 minutes of BL irradiation. Subsequently, the hypocotyl growth rate raises back to a constant, steady-state growth rate persisting for several days (Fig. 6a). The phototropins are essential for the initiation of hypocotyl growth inhibition during the first 30 minutes whereas the cryptochromes and phytochromes are associated with the maintenance of growth inhibition (Fig. 6b; (Parks et al., 1998).

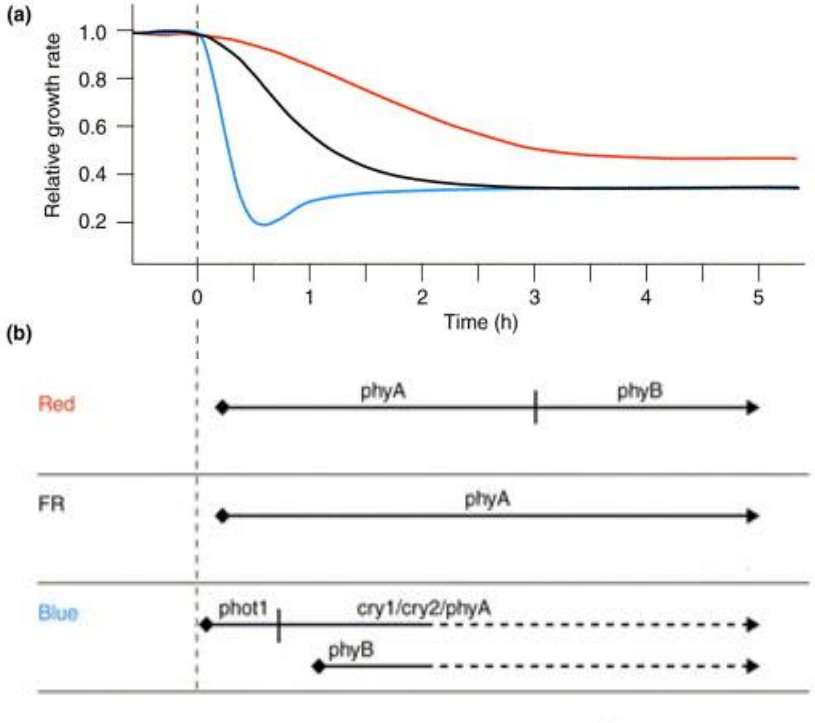

Figure 6. A model for growth rate regulation of hypocotyl as a function of light quality. a) In darkness, seedlings grow at a rapid rate (0.25 mm.h^{-1}), here shown normalized to 1.0. Upon irradiation with red, far-red (FR) or blue light (red, black and blue lines, respectively), growth rates decrease to different extends. b) The rate of hypocotyl growth at any point is controlled by the coordinated action of photoreceptors. The lines show a graphical representation of photoreceptor involvement on growth rate of hypocotyl in red, far red (FR) and blue light. Modified according to (Parks et al., 2001).

The red light also influenced the hypocotyl elongation growth rate. During the first three hours of RL illumination, the hypocotyl growth is regulated by PHYA. After that, PHYB participates in the establishment of the steady-state growth rate. Under far-red (FR) light, hypocotyl elongation is solely influenced by PHYA (Fig. 6b) (Parks et al., 2001).

14-3-3 PROTEINS

The 14-3-3 proteins are a family of acidic, regulatory proteins with a molecular mass of about 30 kDa. Since their first description in 1967 by Moore and Perez from bovine brain homogenate, 14-3-3s have been identified in all eukaryotic organisms analyzed so far. The nomenclature 14-3-3 was derived from the elution fraction (14^{th}) on DEAE-cellulose chromatography and their migration position (3.3) after subsequent starch electrophoresis (Moore and VJ, 1967;Rosenquist et al., 2001). The first mention of 14-3-3s in plants was reported for *Arabidopsis thaliana, Spinacea olerraca* and *Zea mays* in 1992 (de Vetten et al., 1992;Hirsch et al., 1992;Lu et al., 1992). 14-3-3 proteins are also referred to by different names due to the rediscovery of additional functions. For example, the GF14 (G-box Factor 14-3-3) has been identified as a protein binding to G-box motif of promotors in *A. thaliana* and *O. sativa*. They have been named also due to their organism of origin, like TFT (Tomato Fourteen Three-three) proteins of *S. lycopersicum*. They can be also found as GRFs (General Regulatory Factors) because they were characterized to regulate a vast number of cellular processes (Lu et al., 1992;Rooney and Ferl, 1995;Xu and Shi, 2006).

The 14-3-3 proteins are highly conserved through evolution from the yeast to humans. For instance, the amino acid sequences within Arabidopsis 14-3-3 isoforms share similarities ranging from 60% to 92% (Wu et al., 1997). The 14-3-3 proteins consist of three main regions: the amino-terminus, core and carboxy-terminus. Both amino- and carboxy-termini of 14-3-3 proteins endow divergence within the individual organism. Conversely, the central core of the sequence is conserved throughout 14-3-3s of all systems (reviewed in (Ferl et al., 2002). Individual 14-3-3 proteins may interact with each other to form homo- or hetero-dimers (Sluchanko and Gusev, 2012). Each monomer consists of nine antiparallel α-helices, forming an L-shaped structure. Multiple hydrophobic and polar contacts generate the formation of symmetrical dimer arrangement

via the first four helixes of both 14-3-3s. The remaining helices serve as the amphipathic central groove allowing binding of target proteins.

All eukaryotes possess multiple 14-3-3 gene paralogs. The absolute number of isoforms varies depending on the species. Thirteen isoforms are recognized in *A. thaliana* (DeLille et al., 2001;Rosenquist et al., 2001), eight in *O. Sativa* (Yao et al., 2007) or twelve in *S. lycopersicum* (Xu and Shi, 2006). Based on the distinct genomic organization, the 14-3-3 isoforms are phylogenetically delineated into two distinct evolutionary branches named epsilon and non-epsilon group (DeLille et al., 2001). The alignment of proteins sequences of several species predicts that the distribution into two groups existed before the divergence of monocots and dicots (Chandra et al., 2016). Each group is again clustered into two sub-groups with distinct intron-exon structural organization. Whereas the epsilon group contains six exons and five introns, or seven exons and six introns, the non-epsilon members show four exons and three introns or five exons and four introns (Sehnke et al., 2002).

The relatively large number of isoforms predicts the functional specifity of individual isoform. This fact is also endorsed by the subcellular localization of 14-3-3 proteins which can be in cytosolic, nucleolar (Bihn et al., 1997), chloroplastic (Sehnke et al., 2000) and mitochondrial (Bunney et al., 2001). The family-member specific expression pattern, individual regulatory mechanism as well as the abundance of 14-3-3 target proteins has raised the hypothesis of unique function for each 14-3-3 isoform (Roberts and de Bruxelles, 2002;van Heusden, 2005).

The interaction between 14-3-3 proteins and their targets requires the phosphorylation of a serine or threonine residue of the client protein. Several consensus 14-3-3 binding motifs were identified, including mode I [RSX(pS)XP], mode II [RX(F/Y)X(pS)XP] and mode III [(pS/pT) X_{1-2} – COOH] (Yaffe et al., 1997;Coblitz et al., 2005). In plants, the most commonly occurring motif is a variation of the mode I [LX(R/K)SX(pS/pT)XP]. Besides the conserved binding motifs, 14-3-3 proteins were found to bind to seemingly random amino acid sequences very different from those described for the consensus motifs. Further, several reports indicate that 14-3-3s may interact even with nonphosphorylated targets (Ottmann et al., 2007;Johnson et al., 2010;Taoka et al., 2011).

Besides the presence of specific binding motif, other factors contribute to the regulation of the interaction. These include divalent cations, polyamines, pH and C-terminal domain of 14-3-3. Stabilization of 14-3-3 binding to their targets is mediated by milimolar concentration of divalent cations such as Ca^{2+} or Mg^{2+} or micromolar

concentration of polyamines such as spermidine, spermine (Athwal and Huber, 2002;Garufi et al., 2007;Manak and Ferl, 2007). Consistently, a decreasing of pH from 7.6 to 6.0 increases the ability of 14-3-3 proteins to bind ligands (Athwal et al., 2000). Another regulatory mechanism is the autoinhibitory function of the C-teminal domain of 14-3-3s. The deletion of amino acid residues forming the C-terminal domain resulted in higher affinity towards the targets both in Arabidopsis and maize (Visconti et al., 2008;Pallucca et al., 2014).

The binding of 14-3-3 dimer can affect the conformation, activity or subcellular localization of client proteins. 14-3-3 dimers can also serve as an adaptor or scaffold protein in the formation of ternary complexes through a binding of two different client proteins (Roberts, 2003;Obsil and Obsilova, 2011). Thus, 14-3-3 proteins may regulate a plethora of cellular processes and signaling pathways. The involvement of 14-3-3s in plant primary metabolism, growth and cell division, hormone pathways and stomatal opening as well as their role during pathogen-plant interaction and response to biotic and abiotic stresses were nicely summarized in recent reviews (Denison et al., 2011;Cotelle and Leonhardt, 2015;Lozano-Durán and Robatzek, 2015;Keller and Radwan, 2015 ;Camoni et al., 2018).

14-3-3 PROTEINS IN DE-ETIOLATION

Aforementioned, on the molecular basis, the sensing and responding to light begins via activation of specific photoreceptors. The plant photoreceptors are classified into high and low light responsive. Whereas PHYB-E, CRY1 and PHOT2 are more specialized for high light responses, PHYA and PHOT1 accumulates in the hypocotyl of etiolated seedlings, ensuring sensing the light immediately after illumination (Kami et al., 2010).

Both PHYA and PHYB are important for de-etiolation respose as mutants affected in these genes display etiolated phenotype when grown in RL/FR (Khanna et al., 2006). Similarly, Arabidopsis *14-3-3 ν, μ, κ, χ* mutant plants are affected in the RL-mediated hypocotyl growth inhibition suggesting a link between 14-3-3 proteins and phytochrome signaling. Indeed, PHYB was found to be a binding partner of 14-3-3 κ (Mayfield et al., 2007;Shin et al., 2011;Adams et al., 2014). Moreover, within PHYB sequence, a phosphorylation cluster of signaling modulation (PCSM) motif was identified as crucial for the regulation of its activation. Besides PCMS, four phosphorylation sites (S596, S977, S1163 and T601) were further identified. These sites are constitutively phosphorylated in the presence or absence of light. Interestingly, S596 is present in all

phytochromes and is part of the 14-3-3 binding site (mode 1, R/KXXpS/pTXP). Thus, the phosphorylation of S596 might function as a 14-3-3 binding site, allowing the subsequent regulation of PHYB activity (Nito et al., 2013).The active PHYs translocate from the cytoplasm to nucleus where they are bound to basic helix–loop–helix (bHLH) transcription factors designated Phytochrome-Interacting Factors (PIFs). Their interaction with PHYs induces their phosphorylation causing a reduction in their ability to bind DNA and subsequent proteome degradation (Leivar et al., 2008;Casal et al., 2014). The seedling carrying the mutation in PIFs show constitutive-photomorphogenetic phenotype in darkness confirming that PIFs are family of important repressors of photomorphogenesis. A proteomic studies showed that PIF4 co-purifies with the 14-3-3 isoform ω whereas PIF1, PIF3 and PIF6 have been identified as a 14-3-3 κ interactors and PIF7 even binds six different 14-3-3 isoforms reflecting the specificity of 14-3-3s isoforms towards their interacting partners (Chang et al., 2009;Shin et al., 2011;Adams et al., 2014;Huang et al., 2018). Huang et al (2018) demonstrated that phosphorylation of PIF7 induces 14-3-3 proteins binding which retain PIF7 in cytoplasm to negatively regulate shade-induced hypocotyl elongation. To summarize, the photoactivation of phyB induces its movement to the nucleus, where it interacts with PIFs. Simoultanously, the PIFs are rapidly phosphorylated allowing the 14-3-3 protein binding and this interaction sequester the PIFs into the cytoplasm. In opposite, when plants are shaded or in the dark, phyB is in its inactive form, PIFs are accumulated in nucleus and influence the hypocotyl elongation.

Recent research showed that the function of PIF proteins is also modulated by the BL photoreceptors, CRYs and PHOTs (Sun et al., 2013;Ma et al., 2016).

BL photoreceptors also contribute to de-etiolation. Whereas *cry1* mutants exhibit longer hypocotyls under BL, no differences in hypocotyl length can be observed in *phot1* mutant (Kang et al., 2008). Nevertheless, a fine-tune analysis of the growth rate of the different mutants demonstrated that de-etiolation is a two-phase process involving PHOT1 during the first 30-45 min after illumination and requiring CRY1 to establish the stable steady-state growth (Parks et al., 2001;Bergougnoux et al., 2012). PHOT1 has been clearly identified as interacting partner of 14-3-3s in the stomatal guard cell. In Arabidopsis, a more comprehensive study demonstrated that the strongest interaction occurred between PHOT1 and the non-epsilon 14-3-3λ. Other member of this group (κ, φ and ν) exhibited lower binding affinities to the photoreceptor. In opposite, no interaction between PHOT1 and epsilon 14-3-3s was proved, indicating that PHOT1 specifically

binds to the non-epsilon 14-3-3s (Kinoshita et al., 2003;Sullivan et al., 2009). 14-3-3λ might also weakly and transiently bind to PHOT2 (Sullivan et al., 2009;Tseng et al., 2012).

Light-activated photoreceptors act to repress the function of CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), a key element of photomorphogenesis. In darkness, COP1 is nuclear-localized, forming a complex with SUPPRESSOR OF PHYA (SPA), CONSTITUTIVE PHOTOMORPHOGENETIC 9 SIGNALOSOME (CSN) and COP10-DDB1-DET1 (CDD) complexes (Saijo et al., 2003). This complex mediates the 26S proteasome degradation of photomorphogenesis-related transcription factors such as ELONGATED HYPOCOTYL5 (HY5), LONG AFTER FAR-RED LIGHT1 (LAF1) and LONG HYPOCOTYL IN FAR-RED1 (HFR1) (Ang et al., 1998;Osterlund et al., 2000;Seo et al., 2003;Jang et al., 2005). The translocation and inhibition of COP1 in the cytosol under light allow the expression of photomorphogenesis-related genes and subsequent de-etiolation. Affinity purification study revealed COP1 and COP8 bind to 14-3-3s (Shin et al., 2011); the interaction COP1/14-3-3κ was confirmed by Y2H assay (Adams et al., 2014). As 14-3-3 proteins affect the localization of their interacting partners within the cell compartments, 14-3-3s might regulate COP1 nucleocytoplasmic shuttling. To date, no information is available concerning the role of 14-3-3s in the regulation of COP1 nucleocytoplasmic shuttling in plants. In opposite, human ortholog of COP1 interacts with 14-3-3σ tumor suppressor to control its subcellular localization (Su et al., 2010). However, deeper investigations are required.

14-3-3 PROTEINS AND HORMONAL SIGNALING IN DE-ETIOLATION

Nevertheless, the modulation of the light quality alone does not induce a specific response of the plant. Not surprisingly, plant hormones are believed to interact with light perception and signaling during regulation of plant growth and development. Light-hormone interplay is supported by HY5 targeting many regulators of hormone signaling (Gangappa and Botto, 2016). Auxin promotes cell expansion (Rayle and Cleland, 1992). Its distribution is ensured by the polar auxin transport (PAT) driven by influx and efflux carrier proteins PIN-FORMED (PIN) and ATP-binding cassette family B (ABCB) (Friml and Palme, 2002). Light has a profound effect on PAT and endogenous auxin in the hypocotyl (BEHRINGER et al., 1992;Sorce et al., 2008;Sassi et al., 2013). Recent studies demonstrated that ABCB19, accounting for 80% of PAT in stem, contributes to etiolation

(Noh et al., 2001;Wu et al., 2010). Silenced epsilon 14-3-3 plants with defect in elongation when grown in darkness are severely affected in auxin distribution, PAT and PIN subcellular distribution (Keicher et al., 2017). Both PIN1 and ABCB19 are targets of 14-3-3s (Schoonheim et al., 2007;Shin et al., 2011). Light, through HY5, suppresses auxin signaling by stimulating the expression of AUXIN/INDOL-3-ACETIC ACID (AUX/IAA), negative repressor of transcription whose activity is regulated by auxin-mediated proteasome degradation (Paciorek and Friml, 2006;Gangappa and Botto, 2016). Photomorphogenic phenotypes are observed in *aux/iaa* mutants (*shy2/iaa3*, *axr2/iaa7* and *axr3/iaa17)* (Nagpal et al., 2000;Tian et al., 2002), and affinity purification assay revealed that IAA17 is a potential 14-3-3 interactor (Shin et al., 2011).

Gibberellins (GA)'s involvement in de-etiolation is evidenced by the photomorphogenetic phenotype of dark-grown seedlings with decreased GA content (Ait-Ali et al., 1999;O'Neill et al., 2000;Alabadí et al., 2004). Among DELLA proteins - GA-insensitive (GAI), REPRESSOR OF GA 1-3 (RGA) and RGA-LIKE (RGL1, RGL2 and RGL3), downstream repressors of giberellins, RGA and GAI are the main repressors controlling hypocotyl growth (Peng et al., 1997;Dill and Sun, 2001). In absence of GA, in the light, DELLA binds PIF3/4 which is unable to bind DNA, inhibiting cell elongation (de Lucas et al., 2008). In darkness, GA binds GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptor and form a complex with DELLA, leading to its degradation, and the subsequent release of PIF3/4 which in turn promotes the transcription of genes involved in cell elongation. RGA and RGL2 are potential binding partners of 14-3-3s (Shin et al., 2011;Adams et al., 2014). In parallel, endogenous GA content is regulated by REPRESSION OF SHOOT GROWTH (RSG) whose association with 14-3-3 leads to its cytoplasmic translocation and inactivation (Ishida et al., 2004).

Belonging to the first hormones associated with de-etiolation (Li et al., 1996;Szekeres et al., 1996), brassinosteroids (BR) are perceived at the plasma membrane (PM) by BRASSINOSTEROID INSENSITIVE1 (BRI1) which binds and phosphorylates 14-3-3s (Chang et al., 2009;Wu, 2011). In the absence of BR, BRI1 is inactivated by BRI1-KINASE INHIBITOR1 (BKI1); BRI1 activation requires the BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) and the dissociation of BKI1 which is phosphorylated and bound to 14-3-3s (Wang et al., 2011;Zhu et al., 2013). BR signaling culminates with the dephosphorylation of two transcription factors, BRASSINAZOLE RESISTANT1 (BZR1) and BZR2, and their nuclear localization (He et al., 2005). BZR1 interacts with PIF4 in the nucleus where they bind to the promoters of genes repressing

photomorphogenesis (Bai et al., 2012). In the light, BZR1 is phosphorylated and bound to 14-3-3s, resulting in its cytoplasmic retention and inability to bind DNA (He et al., 2005;Gampala et al., 2007).

In darkness, ethylene (ET) triggers the "triple response", i.e. the inhibition of hypocotyl elongation, radial growth of hypocotyl and exaggeration of the apical hook, ensuring growth through the soil without damage of the shoot meristem. Endogenous ET accumulation is enhanced with the deepness and compaction of soil (Zhong et al., 2014). In the light, ET is responsible for cotyledon greening and hook opening via cell elongation (Zhong et al., 2010;Zhong et al., 2014). ET is perceived by histidine kinase receptors. In absence of ET, the active receptors interact with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) to form a complex; CTR1 directly phosphorylates ETHYLEN-INSENSITIVE2 (EIN2), leading to its degradation and repression of the downstream signaling. CTR1 activity is stabilized by 14-3-3s (Chang and Shockey, 1999;Lancien et al., 2005). In the nucleus, EIN3/EIN-LIKE1 is degraded through ubiquitin-proteasome. In darkness, binding of ET inactivates the receptor and CTR1, resulting in EIN2 dephosphorylation and cleavage. EIN2 fragment translocates to the nucleus where it contributes to the stabilization and accumulation of EIN3/EIL1 and, consequently, induces transcription of ETHYLENE-RESPONSE FACTOR1 (ERF1) and other ethylene-responsive genes (Yang et al., 2015). EIN3 was identified as putative 14-3-3 interactor (Chang et al., 2009). ET endogenous content is controlled by the regulation of the ACC synthase by ETHYLENE OVERPRODUCER1 (ETO1) and ETO1-LIKE which were both shown to interact with 14-3-3s (Chang et al., 2009;Yoon and Kieber, 2013). Altogether, 14-3-3-mediated regulation of ethylene production relies on i) the 14-3-3 mediated-stability of ACS activity and ii) the 14-3-3 mediated-degradation of ETO1/EOLs, the negative regulator of ACS (Yoon and Kieber, 2013).

Cytokinin (CK) treatment of dark-grown seedlings mimics the de-etiolated phenotype of light-grown seedlings (Chory et al., 1991). Similar phenotype was observed with the dark-grown *altered meristem program* (*amp*) mutant with high endogenous CK (Chin-Atkins et al., 1996). The *cytokinin-insensitive 4* (*cin4*) mutant, allelic to the *cop10* mutant, links CK and light (Vogel et al., 1998). Mechanisms of CK-mediated de-etiolation are not fully understood, despite the importance of CK histidine kinase receptors (Riefler et al., 2006). Overexpression of *ARABIDOPSIS RESPONSE REGULATOR 2* (*ARR2*) gene, component of CK signaling, resulted in dark-grown

seedlings with short hypocotyls (Hass et al., 2004). ARR2 was found to interact with 14-3-3υ (Dortay et al., 2008;Braun et al., 2011).

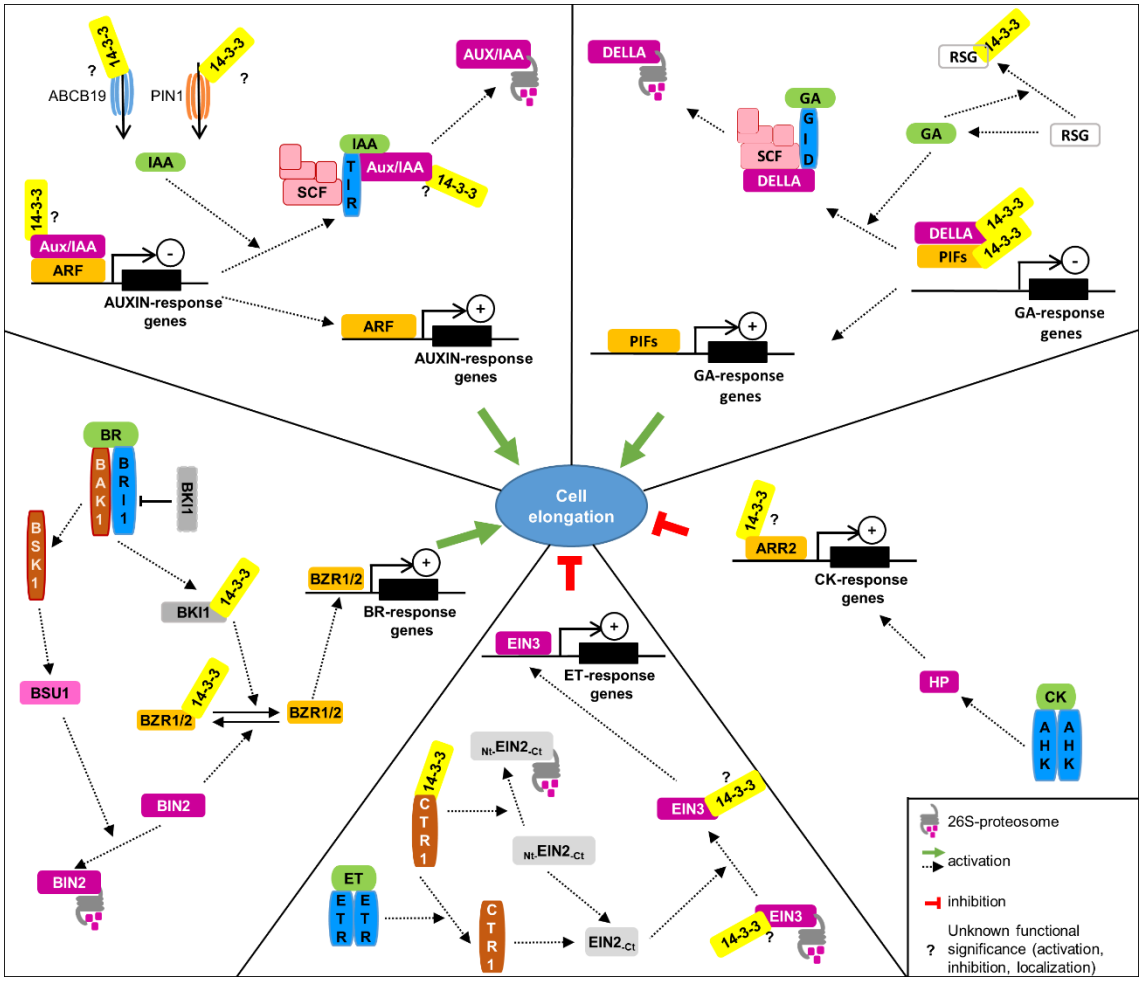

Figure 7: 14-3-3 proteins and hormonal regulation of cell elongation. Auxin (IAA), gibberellin (GA) and brassinosteroid (BR) participate in cell expansion observed during skotomorphogenesis. **A)** In the dark, the IAA maxima, generated by PIN1 and ABCB19, induces the 26S proteasome-mediated degradation of the repressor of transcription Aux/IAA, allowing the activation of the transcription factor Auxin Responsive Factor (ARF), and the subsequent expression of genes related to cell elongation. 14-3-3s bind ABCB19, PIN1 and Aux/IAA but the functional significance is unknown. Because interactions were determined in light conditions, it might be that the interaction with 14-3-3s reduces the IAA signaling by inducing the recycling of PIN1 and ABCB19 from plasma membrane to endosome. Binding to Aux/IAA could be required for the degradation. **B)** In the light, DELLAs, repressors of GA signaling, interact with PIF4 and repress its DNA-binding ability. Both are stabilized by their binding with 14-3-3s. In the dark, accumulation of GA triggers proteasome degradation of the DELLA repressors and allow accumulation of free PIF4, promoting PIF4-activated gene expression related to cell expansion. GA metabolism is regulated by REPRESSION OF SHOOT GROWTH (RSG). Its association with 14-3-3 results in its cytoplasmatic sequestration and inability to induce GA metabolism. **C)** BR activates the receptor BRASSINOSTEROID-INSENSITIVE 1 (BRI1), leading to its dimerization with BRI1-ASSOCIATED KINASE 1 (BAK1) and phosphorylation of BRI1 KINASE INHIBITOR (BKI1), which then dissociates from BRI1. Active BRI1 phosphorylates BRASSINOSTEROID SIGNALING KINASE 1 (BSK1) which activates the phosphatase BRI1 SUPPRESSOR 1 (BSU1). BSU1 dephosphorylates the BRASSINOSTEROID-INSENSITIVE 2 kinase (BIN2), triggering it proteasome-mediated degradation. The degradation of BIN2 induces the

disruption of interaction of BRASSINAZOLE RESISTANT1 (BZR1) and BZR2 with 14-3-3s and their transfer to the nucleus where they modulate the expression of BRs responsive genes. The phosphorylated BKI1 represent an acceptor for the 14-3-3 released from BZR1/2.
In opposite, ethylene (ET) and cytokinins (CK) inhibit cell elongation during skotomorphogenesis and photomorphogenesis, respectively. **D)** Soil deepness and compaction over the seeds induce the synthesis and accumulation of ET. The 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is the committed step in ET metabolism; its stability is controlled by ETHYLENE OVERPRODUCER1 (ETO1) and ETO1-LIKE (EOL) which are E3 ligases, targeting ACS for degradation. 14-3-3s affect directly the stability of ACS and indirectly by regulating the abundance ETO1/EOL. ET is perceived by ETHYLENE RECEPTORS (ETR), modulating the ability of CONSTITUTIVE TRIPLE RESPONSE (CTR1) to bind to 14-3-3s. When CTR1 is bound to 14-3-3, it drives ETHYLENE INSENSITIVE 2 (EIN2) for degradation; in opposite when CTR1 is free, it induces the cleavage of EIN2. The EIN2-$_{Cterm}$ fragment stabilizes the EIN3 transcription factor, inducing a transcriptional cascade. EIN3 can be also associated to 14-3-3s, but it is still unclear whether it participates in degradation or stabilization of EIN3. **E)** In the light, during de-etiolation, CK accumulate in the hypocotyl of the seedling, and bind to the HISTIDINE KINASE (AHK) inducing a phosphorelay cascade involving histidine phosphotransferase proteins (HP). Important event of the CK signaling are the Response Regulators (RRs). Type-B RRs are positive regulators of the CK response, directly influencing expression of CK response genes; in opposite, type-A RRs serve as negative feedback loop. The functional significance of the interaction between 14-3-3 and ARR2, a type-B RR, is unknown.

14-3-3 PROTEINS AND CELL EXPANSION

Cell expansion relies on the temporary imbalance between cell wall (CW) extensibility and cell turgor pressure (Le et al., 2005). Since 70's, the acid growth theory explains how auxin controls cell expansion. Auxin activates the plasma membrane (PM) H^+-ATPase which excretes protons in the apoplast, generating an osmotic gradient. This later is rectified by K^+ uptake through the inward-rectifying K^+-channels and concomitant water uptake, contributing to CW swelling (Hager, 2003). Auxin triggers the phosphorylation of the penultimate Thr residue of the H^+-ATPase C-terminal domain, allowing the interaction with 14-3-3 and its enzymatic activation (Takahashi et al., 2012). Whereas the binding 14-3-3/H^+-ATPases is known for long (Fuglsang et al., 1999;Svennelid et al., 1999), recent study demonstrated that non-epsilon 14-3-3s bind more efficiently the enzyme than epsilon isoforms (Pallucca et al., 2014). PM H^+-ATPase is also the target of BRs, as evidenced by physical interaction with and activation by BRI1 (Caesar et al., 2011). Whether it depends or not on BRI1-mediated phosphorylation of 14-3-3 remains unknown. Whereas the role of abscisic acid (ABA) in plant growth is often controversial (Humplík et al., 2015), ABA-mediated cell elongation inhibition correlates with the dephosphorylation of the penultimate Thr residue of the proton pump, and its consecutive inactivation (Hayashi et al., 2014).

CW determines the cell shape and form. It is composed of cellulose and hemicelluloses, embedded in a matrix of pectins, glycoproteins, proteoglycans, and other

low molecular weight compounds (Caffall and Mohnen, 2009). In Arabidopsis, the hypocotyl is composed of 20 epidermal cells which can expend up to 100 times their original size (Gendreau et al., 1997). This is possible due to CW modification requiring alteration of the existing CW, and *de novo* synthesis and deposition of new CW components (Darley et al., 2001). Among proteins involved in CW loosening are: expansins (pH-dependent loosening), xyloglucan endotransglucosylase/endohydrolases (XTHs), endoglucanases, or pectin-methylesterases (PMEs) (Darley et al., 2001;Bashline et al., 2014). All are targets of 14-3-3s (Schoonheim et al., 2007;Paul et al., 2009;Shin et al., 2011). Interestingly, BR modulate the expression and activity of XTH (Zurek et al., 1994;Azpiroz et al., 1998). Cellulose is synthesized by cellulose synthase A, which was identified as interactor of 14-3-3 (Bashline et al., 2014;Dou et al., 2015).

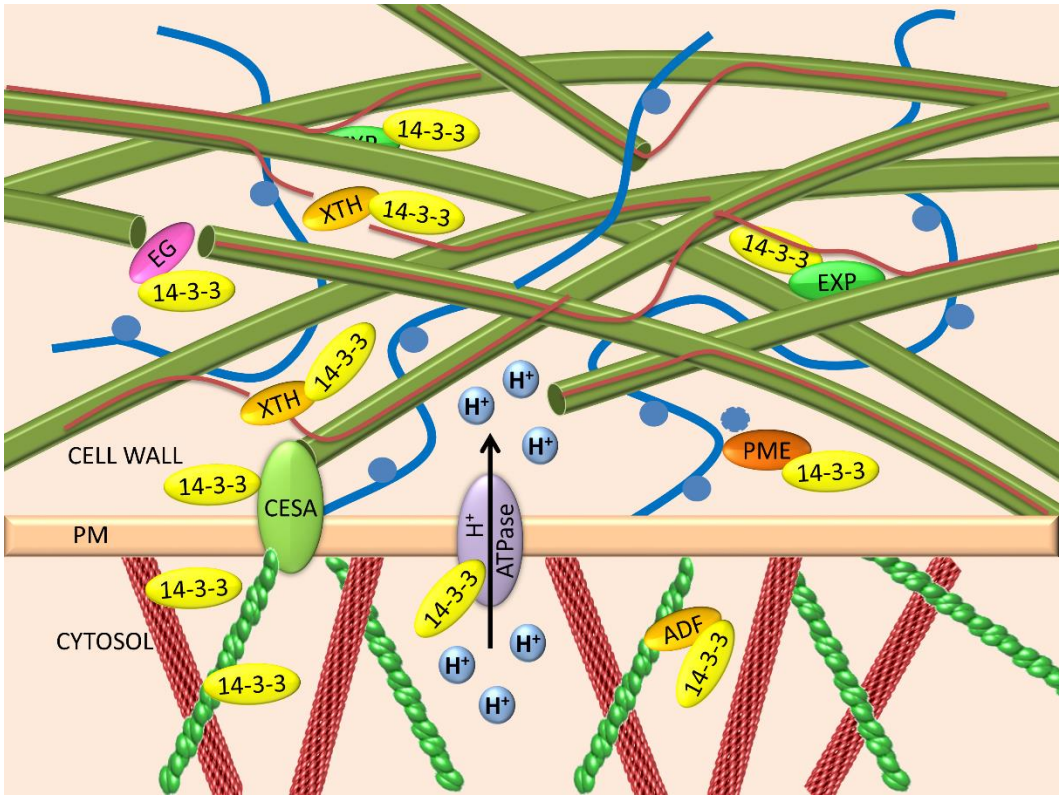

Figure 8. Targets of 14-3-3 proteins in cell expansion. Activated proton pumps (**H^+ ATPase**) in the plasma membrane lower the pH in the cell wall. It leads to the activation of expansins (**EXP**) that separate cellulose microfibrils from cross-linking polysaccharides which are then more accessible to cell wall loosening enzymes such as xyloglucan endotransglucosylase/hydrolase (**XTH**). **XTH** cleaves and religates xyloglucan or pectinmethylesterase (**PME**) which modifies the degree of methylesterification of pectins allowing the cellulose to slide. The extensibility of the cell wall is increased and turgor causes the cell expansion. Cellulose is cleaved by endoglucanase (**EG**) and synthetized by cellulose synthase (**CESA**) which is infuenced by actin trafficking the Golgi-derived vesicles containing **CESA**. **Actin** and **tubulin** also interact with 14-3-3 proteins as well as actin depolymerizing factors (**ADF**). Targets of 14-3-3 proteins are in bold.

Microtubules (MT) and actin filaments (AF) play important role in cell elongation. The current picture depicts MT being important for establishing and maintaining growth anisotropy, while AF delivering the material required for growth (Mathur and Hülskamp, 2002). The orientation of cellulose microfibrils has often been reported to be correlated with MT orientation (Verbelen et al., 2001). Synthesis of cellulose is influenced by AF which guide the trafficking of Golgi-derived vesicles containing CESA (Smith and Oppenheimer, 2005;Crowell et al., 2009;Gutierrez et al., 2009;Wightman and Turner, 2010). It was proposed that plant hormones could be involved in cytoskeleton modification (Foster et al., 2003;Lanza et al., 2012). Proteomic data indicate that 14-3-3 interacts with actin and tubulin (Chang et al., 2009;Paul et al., 2009;Shin et al., 2011;Dou et al., 2015). Tubulin- and actin-associated proteins are important for the dynamics, stability and organization of MT and AF, respectively (Carlier, 1998;Zhao et al., 2015;Krtková et al., 2016). COP1 is crucial for MT destabilization, targeting to degradation WDL3 protein, a member of the MT-associated protein (MAP) family involved in the control of hypocotyl cell elongation (Lian et al., 2017). The solely characterized interaction between 14-3-3 and MAP was reported for EDE1 (ENDOSPERM DEFECTIVE1), which is essential for microtubule organization in endosperm (Pignocchi and Doonan, 2011). Similarly, 14-3-3s were reported to interact with ACTIN DEPOLYMERIZING FACTORS (ADFs), among which ADF1 and ADF4 are involved in cell expansion (Bamburg, 1999;Dong et al., 2001;Henty et al., 2011;Zhao et al., 2015). The direct interaction between ADF1 and 14-3-3 in hypocotyls cells was demonstrated. Binding of 14-3-3λ suppressed the phosphorylation of ADF1 *in vivo*, influencing its localization (Zhao et al., 2015). Endoreduplication, characterized by a repetitive chromosal DNA synthesis without mitosis, plays a clear role in cell expansion (Larkins et al., 2001;Sugimoto-Shirasu and Roberts, 2003;Cheniclet et al., 2005). For long, it is assumed that endoreduplication could contribute to hypocotyl elongation (Gendreau et al., 1997;Bergougnoux et al., 2012). During the endoreduplication, the cell cycle oscillates between the G1/S phases and does not undergo the G2/M transition. Cyclin-dependent kinases (CDKs) and their interacting partners, cyclins (CYCs), are key regulators of the cell cycle. The initiation of endoreduplication requires the suppression of proteins of the CDK B-family (Boudolf et al., 2004). The D3-type cyclins are of particular interest in endoreduplication as they both maintain cells in mitotic cycle and restrain the transition to endocycling (Verkest et al., 2005). In humans and animals, 14-3-3s function at several key points in G1/S- and G2/M-transition. In most cases, the

association with 14-3-3s mediates cell cycle arrest. They are also implicated in the transcriptional regulation of CDK-inhibitors (Laronga et al., 2000;Boudolf et al., 2004). In opposite, in plants, the interaction between proteins involved in cell cycle and 14-3-3 is rarely reported. Nevertheless, as the cycle cycle progression is highly conserved among kingdoms, one might expect that 14-3-3 would contribute also in the control of cell cycle and endocyling.

TOMATO FRUIT DEVELOPMENT AND QUALITY

The tomato though wrongly commonly classified as a vegetable is a berry fruit. The fruit develops from the ovary in the base of the flower and contains seeds. The fruits are fleshy due to the pericarp walls and skin (Weier et al., 1982). From the economical and consumption point of view, tomato is the second most important vegetable in the world (Ibitoye et al., 2009).

The tomato fruit development can be divided into two phases: maturation and ripering (Burg and Burg, 1965). Fruit set is triggered right after fertilization. Rapid cell divisions lead to a progressive increase in pericarp cell number. When the immature green stage (IG) is reached, the cell division rapidly slow down and fruit growth relies on cell expansion leading to the significant increase in weight. At the end of maturation, the fruit enters the mature green stage (MG) and attains its final size. About two days after reaching MG stage, the tomato fruit undergoes an extensive metabolic reorganization and the fruit ripering begins (Ho and Hewitt, 1986). The change in color from green to yellow-orange is due to the chlorophyll degradation and this stage of fruit is called orange stage (O). The subsequent ripe stage (R) is characterized by the accumulation of carotenoids, conferring the typical color of the tomato fruit.

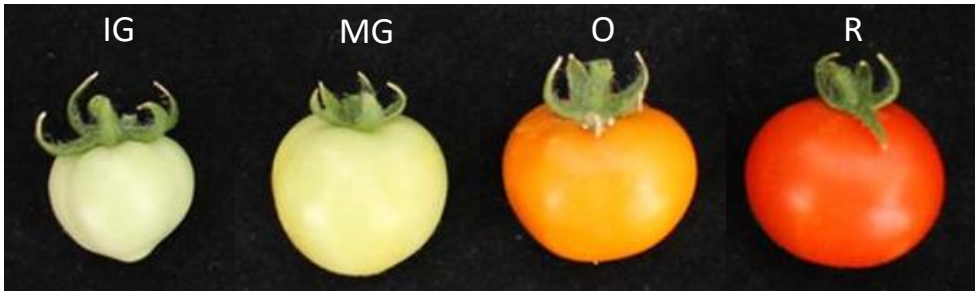

Figure 9. Different stages of tomato fruit development. Tomato fruit development can be divided into different stages: IG, immature green; MG, mature green; 0, orange; and R, red ripening stages are shown.

In particular, the fruit mainly accumulates the orange β-caroten and the red carotenoid lycopen. Another important group of pigment are flavonoids, providing the yellow pigmentation to the peel of the fruit. However, they are normally masked by carotenoids and they are noticed only when carotenoid synthesis is blocked (Fray and Grierson, 1993). In addition to the change in color, tomato fruits also increase the production of volatiles, the compounds that contribute to the final aroma and flavor. The volatiles derive from amino acid, fatty acids and carotenoids (Rambla et al., 2014). Finally, sugars are accumulated during tomato fruit ripening. The starch accumulated in green fruit is converted into glucose and fructose that contribute to the sweetness of the ripe fruit (Osorio et al., 2014).

Consumption of fruits provides to the consumers bioactive substances such as carotenoids (lycopene, lutein, β-carotene), phenolics (flavonoids, phenolic acid) and vitamins (C, E, B) with anti-inflammatory and anticancer effects, preventing chronic diseases such as obesity, diabetes, coronary heart disease, hypertension (reviewed in (Raiola et al., 2014). Taken together, the fruit quality is a result of interplay between many interacting biochemical, physiological and metabolic processes which are regulated by ontogenetic programme as well as by enviromental conditions. Despite increasing knowledge on the molecular and metabolic regulations of plant and fruit development, the improvement of fruit quality and quantity is still a challenge.

Efforts have been done to increase the content of carotenoids in tomato fruits to potentially provide benefits to human health. The genes of carotenoid biosynthesis (Fig. 10) were primarily targeted for their overproduction. The plant carotenoids are mainly produced in plastids by methylerithritol 4-phosphate (MEP) pathway which also provides precursors for other isoprenoids (side chain of chlorophyll, plastoquinones, tocopherols) (Rohmer et al, 2007). Overexpression of *E. coli 1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE* (*DXS*) in tomato fruits caused increased levels of carotenoids (Enfissi et al., 2005). The loss of function mutant of *PHYTOENE SYNTHASE 1* (*PSY1*) gene showed complete lack of carotenoids in the ripe fruit confirming that PSY1 is the only enzyme involved in introducing substrate into the carotenoid synthesis patway during fruit ripering. In opposite, *PSY1* overexpression signicantly increased the carotenoid content (Fray and Grierson, 1993;Fraser et al., 2007). Mutant plants in *CAROTENE ISOMERASE* (*CrtISO*) also showed decreased in carotenoids (Isaacson et al., 2002). The cyclization of lycopene is another important step in the carotenoid biosynthetic pathway. One route leads to β-caroten, violaxanthin, zeaxanthin and

neoxanthin, precursors of abscisic acid and strigolactones. The second one leads to α-carotene and lutein. These steps are maintained by lycopene cyclases (LCY). Up-regulation of *LCY-B2* in tomato resulted in high level of β-carotene; conversely null mutation of the same gene led to the abolishment of carotene and increase in lycopene. Similarly, the overexpression of *LCY-E* increased the endogenous content of δ-carotene in *delta* mutant (Ronen et al., 1999;Ronen et al., 2000).

Beyond doubt, phytohormones, including ethylene, auxin and abscisic acid are involved in the regulation of carotenoid accumulation in tomato fruits. The ripering process relates to the increased accumulation of carotenoids which depends on the transcriptional regulation of ethylene production. RIPERING INHIBITOR (RIN), TOMATO AGAMOUS-like 1 (TAGL1) and FRUITFULL 1, 2 have been shown to regulate the ethylene-dependent carotenoid production since their corresponding mutants have altered ethylene and carotenoid content (Giovannoni et al., 1995;Vrebalov et al., 2009;Bemer et al., 2012;Shima et al., 2013). Suppression of *ETHYLENE RESPONSE FACTOR 6* (*ERF6*) enhanced both carotenoids and ethylene production (Lee et al., 2012). The role of ethylene in carotenoid accumulation is also reflected by the tomato ethylene-insensitive mutant *Never ripe* (*Nr*) which miss carotenoid accumulation (Lanahan et al., 1994). The down-regulation of *AUXIN RESPONSE FACTOR 4* (*ARF4*) resulted in a dark-green fruits and increased size of chloroplasts and carotenoids content (Jones et al., 2002). Recently, it was demonstrated that the role of ARF4 in fruit plastid accumulation was rather mediated by the up-regulation of *GOLDEN-LIKEs* (*GLKs*). Likewise, the overexpression of *GLK2* led to the elevated fruit pigmentation and plastid compartment size. GLK2 is the closest relative to ARABIDOPSIS PSEUDO RESPONSE REGULATOR2-like (APRR2-like). *APRR2* overexpression increases the plastid area and number, as well as endogenous carotenoids content (Fitter et al., 2002;Pan et al., 2013;Sagar et al., 2013). Moreover, ABA-deficient mutant *flacca* and *sitiens* exhibit impaired content of carotenoids and increased size and number of plastids (Galpaz et al., 2008).

In tomato, the *high pigmentation* (*hp*) mutants present defects in carotenoids synthesis. The *hp1* mutant has been characterized to bear a mutation in the UV-DAMAGED DNA-BINDING PROTEIN 1 homolog (DDB1), *hp2* mutation occurs in *DE-ETIOLATED* gene (*DET*) and *hp3* is deficient in zeaxantin epoxidase. All these mutations showed increased size and number of plastids (Mustilli et al., 1999;Liu et al., 2004;Galpaz et al., 2008;Powell et al., 2012;Pan et al., 2013;Nguyen et al., 2014).

Interestingly, both DET1 and DDB1 are part of the light signaling pathway leading to de-etiolation predicting that photomorphogenesis-related genes could function in fruit nutritional quality improvement. The role of light in the fruit nutritional quality was also demonstrated for transgenic plants affected in the BL photoreceptor CRY2. Indeed, the fruits of such plants showed higher content of pigments, especially lycopene. Apart from the DET and DDB1, silencing of another negative regulator of photomorphogenesis CONSTITUTIVELY PHOTOMORPHOGENIC1-like (COP1-like) increased the lycopene content in fruits. In opposite, RNAi-medited repression of the positive regulators HY5 induced the reduction of endogenous carotenoid content (Davuluri et al., 2004;Liu et al., 2004;Davuluri et al., 2005;Giliberto et al., 2005).

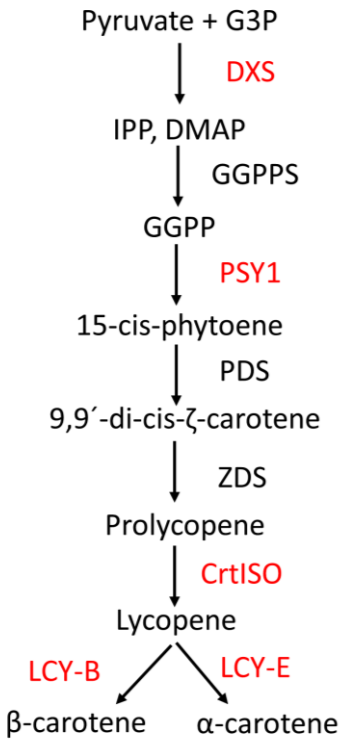

Figure 10: Carotenoid synthesis pathway. Genes targeted for overproduction of carotenoids in tomato fruits are indicated in red. CrtISO, carotene isomerase; DMAP, dimethylallyl diphosphate; DXS, 1-deoxy-d-xylulose 5-phosphate synthase; G3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl pyrophosphate synthase; IPP, isopentenyl diphosphate; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase; PDS, phytoene desaturase; PSY1, phytoene synthase 1; ZDS, ζ-carotene desaturase.

MATERIAL AND METHODS

PLANT MATERIAL

Experiments were conducted on wild-type (wt) tomato *Solanum lycopersicum* (L.) seedlings of the Rutgers, Ailsa Craig and Microtom cultivars. Antisense *tft6* tomato lines were obtained by RNAi in the background Ailsa Craig and kindly provided by provided by Prof. M. Roberts from Lancaster University (UK). Antisense *phot1* tomato lines were generated by amiRNA interference in the cv. Rutgers background in the frame of the present study.

CULTIVATION

The seeds were surface-sterilized for twenty minutes in 3% sodium hypochloride (VWR chemicals, France) and rinsed extensively with sterile distilled water prior to sowing. Subsequently, the seeds were sown on the basal Murashige and Skoog medium (Murashige and Skoog, 1962) in square Petri dishes (120 x 120 mm). Before autoclaving the pH of the medium was adjusted to 6.1 with KOH and solidified with 0.7% (w/v) Phytoagar (Calbiochem, Germany). The Petri dishes were placed vertically in darkness or irradiated by continuous BL illumination provided by fluorescent tubes (TL-D 36W/18-Blue, Philips; total photon fluence rate 10 μmol m^{-2} s^{-1}) at a temperature of 23°C in growth chamber (Weiss Gallenkamp, Great Britain).

For germination assay, the seed germination, defined as radicle protrusion, was scored for nine days in the dark and under BL illumination. Four independent biological replicates containing 30 seeds were counted.

For hypocotyl growth measurement, the dark-grown germinated seeds were transferred to fresh MS medium and further grown in the dark or exposed to continuous blue light as already mentionned. Dishes were regularly scanned and the length of hypocotyl was determined with ImageJ (https://imagej.nih.gov/ij/). Three independent biological replicates containing 30 seedlings were measured.

For library preparation and gene expression, germinated seeds were transferred to fresh MS medium in the dark for 3 additional days to a culture chamber maintained at 23 °C. For BL-induced de-etiolation, dishes containing 3-day-old etiolated seedlings were transferred for 30, 60 or 120 min under BL provided by fluorescent lamps (BL; TL-D 36W/Blue, Phillips; total photon fluence rate 10 μmol.m^{-2}.s^{-1}). For analysis of cytokinin response, germinated seedlings were transferred on Murashige and Skoog medium

supplemented with 0–10 μM isopentenyladenine (iP, Olchemim, Czech Republic). The seedlings were further grown in darkness for 3 additional days. Afterwards, the elongation zone of the hypocotyl was excised for RNA extraction and gene expression.

RNA EXTRACTION AND SUBTRACTIVE LIBRARY CONSTRUCTION

For all experiments, the elongating zone of the hypocotyl of 3-day-old etiolated seedlings was excised from the rest of the seedling, either under green safety light (dark control) or under BL after 30 min of exposure to BL. The elongating zone corresponding to the portion of hypocotyl situated beneath the hook and cotyledons was limited to the upper third of the hypocotyl as described in (Bergougnoux et al., 2014). Samples were immediately frozen in liquid nitrogen and stored at −80 °C before RNA isolation. Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen). Remaining traces of DNA were removed with a recombinant DNAseI (Takara) and RNA were subsequently purified by a phenol:chloroform:isoamyl alcohol (25:24:1) step. PolyA$^+$ mRNA were purified using the Straight A's mRNA isolation system (Novagen). Quantity and quality of mRNA were checked by spectrophotometer and electrophoresis. The suppression subtractive hybridization (SSH) library was constructed according to the instructions of the PCR-Select cDNA Subtraction Kit (Clontech). The principle of SSH library is illustrated by the figure 11. In order to identify genes up-regulated by the exposure to BL, subtractive hybridization was performed using cDNA from hypocotyls exposed for 30 min to BL as tester against cDNA from control hypocotyls (not exposed to BL) as driver. The subtraction efficiency was evaluated by a PCR reaction amplifying a region of the tomato EF1α gene and the PCR product was analyzed after 15, 20, 25, 30, and 35 cycles.

CLONING, SCREENING FOR DIFFERENTIAL EXPRESSION, SEQUENCING AND ANALYSIS

Secondary SSH-PCR products were inserted into pGEM-T Easy Vector (Promega) and cloned into Escherichia coli DH5α strain. A blue–white screening was performed in order to obtain a bank of subtracted ESTs. White colonies were picked and grown in 96-well microtiter plates in a lysogeny broth medium containing ampicillin (100 mg.L^{-1}). Screen for differentially expressed ESTs was performed by dot blot hybridization as described in the PCR-select cDNA subtraction kit (Clontech). For this purpose, plasmids were

isolated, quantified and transferred to Hybond-N+ nylon membranes. Membranes were prepared in duplicates with equal amounts of plasmids and were hybridized either with the BL-specific tester probe or the dark-specific driver probe, both DIG-labelled. Detection was performed with an anti-digoxigenin antibody coupled with a horse radish peroxidase.

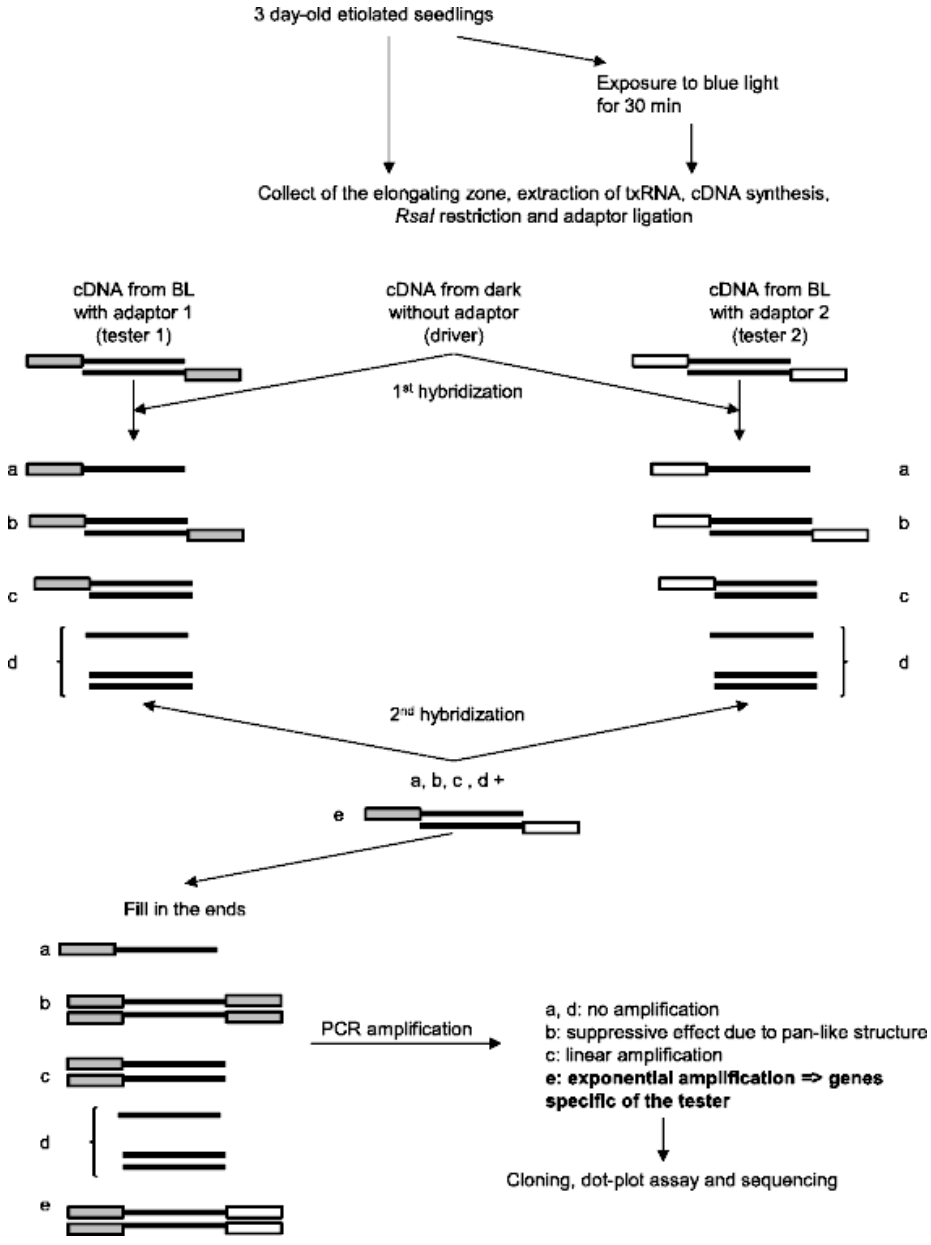

Figure 11. Schematic representation of the suppression subtractive hybridization adapted to the present study from (Diatchenko et al., 1996).

Cold detection was performed by enhanced chemiluminescent detection and exposure to X-ray films. Autoradiographies were scanned and the intensity of the dots was determined using ImageJ software. The dot intensity of a specific clone obtained with the BL-specific probe was compared to that obtained with the dark-specific probe. All clones showing higher intensity with BL-specific probe compared to dark-specific probe were selected and sequenced by Macrogen (Korea). A total of 168 ESTs were found to be differentially expressed. Their sizes ranged from 128 to 1387 bp, with an average size of 447 bp. Because during the process of library preparation the cDNA were restricted by *Rsa*I, the first step of the analysis was to retrieve the full-length of the gene and the corresponding protein for downstream analyses by BLAST against the tomato database at SolGenomics Network. The gene ontology annotation was performed with Blast2GO according to plant-specific Gene Ontology terms (Conesa et al., 2005). Concurrently, the functional annotation was performed by Mercator/MapMan which allows attributing DEGs a functional pathway (Lohse et al., 2014; Klie and Nikoloski, 2012).

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

To confirm the differential expression of the selected ESTs, total RNA was extracted from the elongating zone of 3-day-old etiolated seedlings exposed or not to BL as previously described. Reverse transcription was performed from 1 µg of the total RNA according to the instructions of the PrimeScript kit (Takara), followed by subsequent RNaseH treatment (Takara) and purification on a Macherey-Nagel column to remove any compounds which could have an inhibitory activity during subsequent steps. For quantitative real-time PCR, cDNA samples were diluted by 5-fold and used in a reaction containing SYBR Premix ExTaq (Takara) PCR Master Mix and 200 nM of each primer (Table 1). Three technical repeats were run for each sample on the Mx3000P thermocycler (Stratagene) in a two-step amplification program. The initial denaturation at 94 °C for 10 s was followed by 40 cycles of 94 °C for 5 s and 60 °C for 20 s. A dissociation curve was obtained for each sample.

Table 1. Primers used in quantitative real-time PCR

Identification	Description of the gene	Primers	Primer efficiency
A-E4	Mitogen-activated protein kinase	F: 5′- GAAGATGAGAAACCACAAGCG R: 5′- CATTCTGAGGAACTTGGAGAGG	90%
C-G1	Importin subunit alpha1a	F: 5′- GAACTCATTTTGTGCCCCATC R: 5′- GCTGAGGGATTGGAAAAGATTG	92%
E188	Intracellular Ras-group-related LRR protein 9	F: 5′- GAGAGGCAGGATTGGAGATTG R: 5′- TCCGCATCCTTCAACATCTTC	94%
E-E3	Polyadenylate-binding protein RBP47	F: 5′- TCCTAATGAGCCTAACAAACCTG R: 5′- TCCGTCTTATTGCCTTCCAC	92%
VHA-A1	V-ATPase catalytic subunit A1	F: 5’- CGAGAAGGAAAGCGAGTATGG R: 5’- TCATTCACCATCAGACCAGC	107%
B-D5	Vacuolar H+-ATPase V0 sector	F: 5′- GCAGTCATTATCAGTACCGGG R: 5′-TCTAACACCAGCATCACCAAC	89%
B-E2	*Pectin acetylesterase*	F: 5’-CACACCCACAAAGAGAAACAG R: 5’-TTCCAAGAATGCCCCTTCAG	103%
12.	*XTH*	F: 5’-AGAGGTGGGCTTGAGAAAAC R: 5’-GAACCCAACGAAGTCTCCTATAC	93%
B-D9	*26S proteasome*	F: 5’-TCTTGTCCTCTTTCTGTTCCTTATC R: 5’-AATCCTTGCCTCACTTCCAG	95%
C-A3	Histone H2B	F: 5′- TTGGTAACAGCCTTAGTTCCTC R: 5′- AAAGCCTACCATCACTTCTCG	89%
PP2ACS	*PROTEIN PHOSPHATASE 2A catalytic subunit*	F: 5’- CGATGTGTGATCTCCTATGGTC R: 5’- AAGCTGATGGGCTCTAGAAATC	98%

For expression study of TFTs and PHOT1, the elongation zone of the hypocotyl was rapidly harvested under BL or safety green light for BL-irradiated and darkness-grown seedlings, respectively, then frozen in liquid nitrogen. Total RNA was isolated using the ZR Plant RNA MiniPrep kit (Zymo Research, USA) according to the manufacturer’s instructions. RNA was treated with 2u of Turbo DNAse (Ambion, USA) for 45 min at 37°C. Reverse transcription was performed from 2 μg of the total RNA using RevertAid H minus transcriptase (ThermoScientific, USA). cDNA was diluted 50 times and used in a reaction containing GreenMaster SYBR Premix with high ROX (Jena Biosciences, Germany) and 300 nM of forward and reverse primer (Table 2). qPCR reactions were carried out on a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) as follows: an initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, then 1 min at 60°C. Melting curve analysis was performed after 40 cycles to verify primer specificity.

All primers were designed using IDT qPCR assay design and OligoAnalyzer with default parameters taken from http://eu.idtdna.com/scitools/Applications/

RealTimePCR/). Their specificities were checked by BLAST (http//blast.ncbi.nlm.nih.gov) against the tomato database. The primer sequences and efficiencies are presented in Tables 1 and 2.

Table 2. Primers used for analysis of expression profile by qRT-PCR.

Gene	Accession*	Primer sequence	Primer efficiency
PP2ACS	Solyc05g006590	F: 5′-CGATGTGTGATCTCCTATGGTC	98%
		R: 5′-AAGCTGATGGGCTCTAGAAATC	
Tip41like	Solyc10g049850	F: 5′-GGTTCCTATTGCTGCGTT	97%
		R: 5′-CGAAGACAAGGCCTGAAA	
EF1a	X14449	F: 5’- CCCAAGAGGCCATCAGACAA	113%
		R: 5’-CAACAGGGACAGTTCCAATACCA	
TFT1	Solyc11g010470	F: 5′-AACACATCCGATACGACTTGG	108%
		R: 5′-CCCATAGTGTCCAGTTCAGC	
TFT2	Solyc12g057110	F: 5′-TGGCTGAATTTAAGACCGGAG	113%
		R: 5′-AAGTTGAAGCCAGTCCAAG	
TFT3	Solyc04g074510	F: 5′-CTGGCTCTCAACTTCTCTGTG	106%
		R: 5′-TCCCAATGTATCCAACTCAGC	
TFT4/ TFT11	Solyc02g063070	F: 5′-GACTTGCCCTTAACTTTTCCG	101%
		R: 5′-CCTTGTAAGATTCCTCACCCAG	
TFT5	Solyc04g012120	F: 5′-GTGCTTGTAATCTCGCCAAAC	104%
		R: 5′-GCATATCCGAGGTCCACAAAG	
TFT6	Solyc11g010200	F: 5′-GCTCCTACACATCCAATCCG	108%
		R: 5′-GATTACAGGCACGATCAGGAG	
TFT7	Solyc04g074230	F: 5′-AATGGTTGAAGCAATGAAGGC	100%
		R: 5′-AGACAAGATCCGCCATGAAG	
TFT8	Solyc12g010860	F: 5′-GGCTTCATCCAAAGAACGTG	109%
		R: 5′-ATCAACCATCTCATCGTAGCG	
TFT9	Solyc07g053260	F: 5′-TGAATTGACTGTGGAGGAAAG	103%
		R: 5′-AATAGTCTCCCTTCATCTTGTG	
TFT10	Solyc04g076060	F: 5′-GAAAATCTCAGCCGTGAACAG	110%
		R: 5′-AGATTCCGTTCCTCAACAGTG	
TFT12	Solyc05g012420	F: 5′-TTTCGTTACCTCGCTGAGTTC	102%
		R: 5′-GTTCAAAGCAAGACCAAGACG	
PHOT1	Solyc11g072710	F: 5′-GTGTACGCAGAAATAATGG	97%
		R: 5′-CAGGGAGGTTCTGAAGATCA	

* Accession numbers were obtained from Sol Genomics Network (https://solgenomics.net/)

Each sample was analyzed in three to five independent biological replicates and in technical triplicates. Each independent biological replicate represented a pool of 25 explants. The cycle threshold value was determined for each sample. Three reference

genes, *PP2Acs*, *EF1α* and *Tip41like* were used for expression normalization of genes of interest (Dekkers et al., 2012); these genes were determined as being the most stable reference genes in our conditions (data not shown). Normalized relative quantities were obtained using the efficiency corrected $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). In order to exclude the effect of biological (intra-assay) variation on the statistical significance of results, $\log_{10}$ transformation of normalized relative quantities, mean centering and autoscalling were performed (Willems et al., 2008). The relative quantification was determined in comparison to the expression of *TFT1* gene in dark control sample and/or untreated samples or sample in wt. Presented data show the mean ± standard error of the mean (SEM) of three to five independent biological replicates. Statistical significance was supported by non-parametric Kruskal-Wallis Anova & Median test analysis followed by a post-hoc multiple comparison of mean rank (Statistica 12, Statsoft).

BAFILOMYCIN A1 TREATMENT

Sterile cultures were obtained as described in (Bergougnoux et al., 2009). After germination in darkness, germinated seeds were transferred on a Murashige and Skoog medium containing varying concentrations of bafilomycin A1. For condition of darkness, dishes were wrapped in aluminum foil and placed in the culture chamber; for light conditions, dishes were cultivated in a culture chamber illuminated with BL (total photon fluence rate 10 $\mu mol.m^{-2}.s^{-1}$). After 5 days, the length of the hypocotyl was measured to the nearest millimeter with a ruler. The graph represents the mean ± SEM; an average of 45 seedlings were measured for each condition. The non-parametric Kruskal-Wallis ANOVA (Statistica 12) was performed in order to support the statistical significance of the data.

PROTEOMICS AND LC-MS/MS ANALYSIS

Total proteins of leaves and fruits were extracted with trichloroacetic acid (TCA)/acetone precipitation followed by phenol extraction, diluted with 1.5 ml digestion buffer (2 M urea, 50 mM ammonium bicarbonate) and digested with an immobilized trypsin (Promega) overnight. Tryptic digests corresponding to 5 µg of protein extract were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and an UHR

maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted up to a 120 min witha 4% to 40% acetonitrile gradient; spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s. The measured spectra were extracted by Bruker's Data Analysis 4.1 (e.g. Cerna et al., 2017). Recalibrated MGF files were searched against tomato protein sequence database (ITAG 2.4) by Sequest HT and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys/His/Leu/Arg methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation. Results were integrated by Proteome Discoverer 2.0 (Thermo), and the quantitative differences were evaluated by calculating normalized numbers of peptide spectral matches (PSM) (Černý et al., 2013). Differently expressed proteins were targeted in experimental replicates via an SRM-based analysis (TSQ Quantiva, Thermo). Only proteins with at least three detectable proteotypic peptide were considered for the analysis and the resulting data were evaluated in Skyline 3.1 (MacCossLab Software, https://skyline.gs.washington.edu) and the statistical significance was validated by t-test ($p<0.05$).

HYPOCOTYL PROTEIN EXTRACTION, RECOMBINANT TFT9 AND TFT6, AND PULL-DOWN ASSAY

For the identification of putative interactors of TFT6 and TFT9, cytoplasmic protein fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific, USA) according to manufacturer's instructions. With the modification that buffers were supplemented with plant protease and phosphatase inhibitors (Sigma-Aldrich). Each sample corresponded to the pool of hypocotyl of 30 etiolated seedlings grown in the dark for 3 days and exposed or not for 2h to blue light (BL; L-D 36W/18-Blue, Philips; total photon fluence rate 10 μmol m^{-2} s^{-1}).

For pull-down assay, recombinant proteins were prepared for TFT9 and TFT6, both fused to histidine-tag. For this purpose, the coding regions of TFT9 (Solyc07g053260) and TFT6 (Solyc11g010200) were cloned into the C-terminal His-vector PQE60 (Qiagen, Germany) and N and the C-terminal His-vector PET28b+ (Novagen), respectively. The constructs were introduced into *E. coli* strain BL21 (DE). Expression of recombinant His-tagged proteins was induced for 4h at 37°C by addition of 1 mM isopropylthio-*β*-galactoside to overnight-grown bacterial culture. Cells were collected by centrifugation

for 20 minutes at 4500 x*g* at 4°C, then resuspended in His-binding buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 10 mM imidazole, 0.03% Triton X-100). Lysis of bacterial cells was performed by repeated freezing/thawing cycles in liquid nitrogen and at 42°C, respectively. The cell lysate was clarified by centrifugation at 12,000 x*g* at 4°C for 20 minutes. The supernatant containing expressed His-tagged TFT protein was immobilized on nickel-charged His-Affinity Gel using His-Spin Protein Miniprep (Zymo Research, USA) and incubated with cytoplasmic proteins extract from excised elongating zones of etiolated hypocotyls or hypocotyls illuminated by BL for 2 hours. Two controls were included: the cytoplasmic protein extract without immobilized bait, and the immobilized bait incubated with protein extraction buffer. Cytoplasmic proteins extracted from elongation zone of etiolated seedling hypocotyls exposed or not to BL were incubated with immobilized TFT9 or TFT6 for 1 h at 4°C and unbound fractions were washed with two volumes of wash buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 50 mM imidazole, 0.03% Triton X-100). The bound proteins were eluted with 150 µl of the elution buffer containing 50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride and 250 mM imidazole), diluted with 1.5 ml digestion buffer (2 M urea, 50 mM ammonium bicarbonate), digested overnight with an immobilized trypsin (Promega) for further analysis on LC-MS/MS (as described above). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD006123 and 10.6019/PXD006123.

PREDICTION OF TOMATO CYTOKININ-RESPONSIVE PROTEINS

Prediction of tomato cytokinin-responsive proteins based on orthology with arabidopsis CK-responsive protein (Černý et al., 2016) was done as followed: tomato sequences were used as query in GreenPhyl v4 to retrieve arabidopsis homolog sequences inferred from phylogeny and best blast mutual hit (BBMH) matching sequences (Conte et al., 2008). Further the Arabidopsis accessions were searched into the database of CK-responsive proteins described by Černý et al. (2016). When an arabidopsis accession was found to be CK-responsive, the tomato homolog sequence was annotated as CK-responsive as well.

HYPOCOTYL PROTEOMICS

Plants for analysis of light-dependent changes in hypocotyl proteome were cultivated as described above with the following modifications: seeds of cultivar Micro-Tom were stratified at 4°C for 5 days and cultivated at 24°C in an LED-equipped growth chamber (Polyklima, Germany) under (i) continuous blue light (20 μmol m^{-2} s^{-1}), (ii) white light (80 μmol m^{-2} s^{-1}), or (iii) darkness. Hypocotyls of at least 15 seedlings per replicate were excised and flash-frozen, and the total protein was extracted and processed as described above. For comparative analyses, peptide concentration was determined by a colorimetric peptide assay (ThermoScientific, USA) and then aliquots corresponding to 5 μg of peptide were analyzed by LC-MS/MS (described above). Changes in protein abundance were deemed significant if the corresponding amounts of detected peptides were significantly ($p < 0.05$) changed at least twofold in both biological replicates.

YEAST TWO-HYBRID ASSAY

The ORFs of TFT6 (Solyc11g010200), TFT9 (Solyc07g053260), Phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690), V-ATPase subunit A (Solyc12g055800) and phototropin 1 (PHOT1; Solyc11g072710) were fused to the GAL4-activating domain (AD) or GAL4-binding domain (BD) via cloning into pGADT7 or pGBKT7 vectors (Clontech, USA), respectively. The *Saccharomyces cerevisiae* Y2H GOLD yeast strain (Clontech, USA) was co-transformed with bait and prey using the polyethylene glycol/lithium acetate method. Transformants were selected on synthetic defined (SD) media lacking leucine and tryptophan (SD-Leu-Trp). Four individual colonies were grown overnight in liquid cultures (SD-Leu-Trp) at 30°C and 10-fold dilutions were dropped on SD-Leu-Trp and selective media lacking leucine, tryptophan and histidine (SD-Leu-Trp-His) and SD-Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazol (3-AT).

SUBCELLULAR LOCALIZATION OF TFTS

The ORFs of *TFT6* and *TFT9* genes were fused to yellow fluorescent protein in the pEarlyGate104 vector. Both constructs were inserted into *Agrobacterium tumefaciens* C58 cells. Transient expression in leaves of 4-week-old *Nicotiana benthamiana* was performed as described by Sparkes et al. (2006). To prevent silencing, *A. tumefaciens* C58 carrying a construct that expresses the silencing suppressor P19 was added to the

mixtures. The ratio of cells carrying P19:effector was 1:5. Mixtures were incubated for 3 h in darkness at room temperature before infiltration. Three days after infiltration, leaf circle sections were collected and analyzed using a confocal Zeiss LSM 780 microscope.

PROTOPLAST ISOLATION AND DUAL LUCIFERASE REPORTER ASSAY

The sequence of TFT9 promotor and its truncated versions were cloned into PGL3 vector (Promega, USA) to obtain a *Firefly* luciferase reporter system. Leaves of tomato plants 4 weeks old were used for protoplasts transformation as described by Mishra et al. (2002). In brief, 10^6 protoplasts were transformed with 20 µg of plasmid DNA mixture consisting of TFT-reporter vector and vector driving the expression of *Renilla* luciferase. This latter allowed normalizing the assay. Incubation was in darkness at room temperature for 2 h. Subsequently, 10 µM iP was added to the reaction for 16 h. The protoplasts were lysed in passive lysis buffer before determination of *Firefly* and *Renilla* LUC activities (Dual-Glo Luciferase Assay System, Promega, USA) by measuring luminescence with a luminometer (BioTek, USA).

PROMOTER SEQUENCE ANALYSIS AND SITE-DIRECTED MUTAGENESIS

The prediction of *cis*-acting element binding sites was performed by PlantPAN2.0 (http://plantpan2.itps.ncku.edu.tw/; Chow et al., 2015). The 1.4 kb sequences upstream of the start codon were retrieved from EnsemblPlants (http://plants.ensembl.org/index.html). A Q5® Site-Directed Mutagenesis Kit (New England Biolabs, USA) was used to create specific, targeted changes in two-component response regulators sites in double-stranded plasmid DNA containing a truncated TFT9 promoter sequence.

amiRNA CONSTRUCT DESIGN AND TOMATO FRUIT TRANSFORMATION

The web MicroRNA Designer platform (WMD3; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) currently supports 280 plant species including *Solanum lycopersicum* (ITAG2.3) and designs 21mer sequences directed against the gene of interest, based on good hybridization properties to the target mRNAs while minimizing possible off-target effects to other genes in the tomato genome (Ossowski et al. 2008). Currently, 21mers from the reverse complement of the target transcript(s) are considered effective amiRNA candidates, if they have an ‘‘A’’ (sometimes also ‘‘U’’) at position 10 and display 5’ instability (Warthmann et al., 2008). The amiRNA construct, pRSphot1, was engineered from pRS300, replacing the 21 bases of the natural Arabidopsis miR319a as well as the partially complementary region of the miRNA* in a pBSK vector using overlapping PCRs as described by Schwab et al. (2006) (Figure 12A-B).

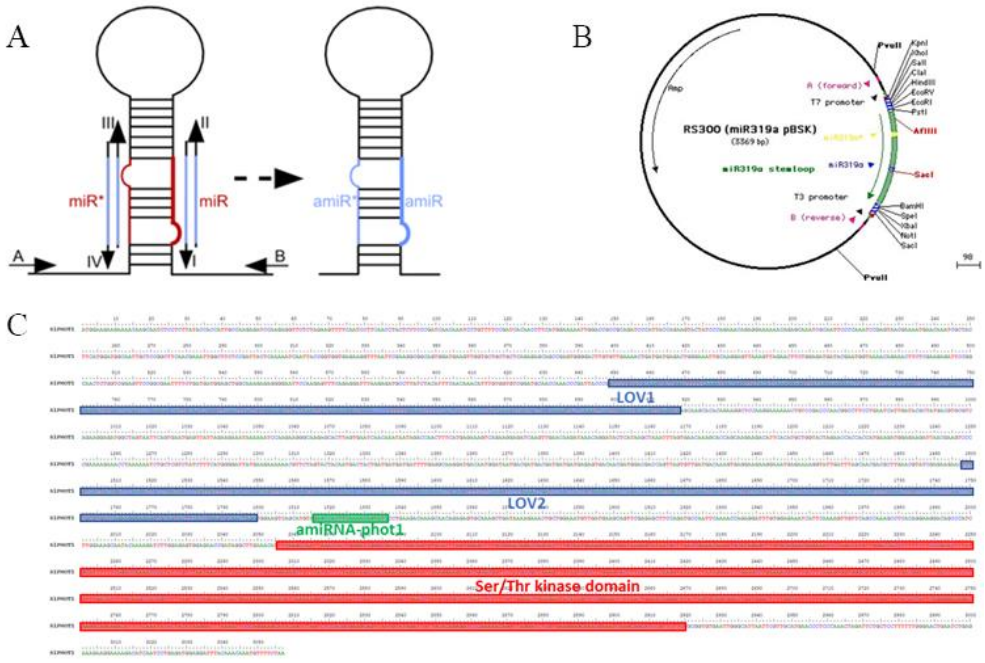

Figure 12. Construction of pRSphot1. A) Principle of cloning strategy. The original miRNA and miRNA* sequences (in red) will be replaced by the artificial miRNA sequences (blue) designed for the gene of interest, using overlapping PCR. Arrows indicate the position an orientation of oligonucleotides used in combination for the PCR amplification. A and B are universal primers that were designed with *Asc*I and *Not*I restriction sites, respectively, to allow cloning of the artificial microRNA precursor into the final binary vector; I to IV designed the oligonucleotides specific of the mRNA of interest. **B) Map of the pRS300 vector containing the ath-miRNA319a precursor and its miRNA* in the backbone pBSK. C) Localization of the amiRNA target on the sequence of Sl*phot1* (Solyc11g072710).** The two LOV domains and the Ser/Thr kinase domain have been indicated as well as the target sequence for amiRNA as determined by WMD3 (http://wmd3.weigelworld.org/).

WMD3 was used to design amiRNA sequences (21mers) for *SlPHOT1* based on the ITAG2.3 tomato genome annotation. WMD3 suggested several suitable amiRNA candidates that were further validated by BLAST on the ITAG2.3 tomato genome for their plant specificity and more particularly for their specificity towards *SlPHOT1*. Only one candidate was further considered to target *PHOT1* mRNA (Fig. 12C). The plasmid information for pRS300 was selected, and all appropriate primer sequences, needed for customization of pRSphot1, were retrieved using the primer design function of WMD3. Four oligonucleotide sequences required for engineering pRSphot1 into the Arabidopsis endogenous miR319a precursor by site-directed mutagenesis were thus obtained. Two more oligonucleotides, primers A and B, were based on the template plasmid sequence, located at each extremity of miR319a precursor and had *Asc*I and *Not*I restriction sites, respectively, to allow cloning into the binary vector pSK36. The oligonucleotides used are provided in the Table 3.

Table 3. Oligonucleotides used to prepare the pRSphot1 construct.

Oligonucleotides	Sequence	Comment
Primer A (*Asc*I)	TTGGCGCGCCCCAAACACACGCTCGGA	Forward
Primer B (*Not*I)	ATAGTTTAGCGGCCGCCCCATGGCGATGCCTTAA	Reverse
primer I	gaTATACTGTTTTGAAGTGGCTCtctctctttgtattcc	miR-s
primer II	gaGAGCCACTTCAAAACAGTATAtcaaagagaatcaatga	miR-a
primer III	gaGAACCACTTCAAATCAGTATTtcacaggtcgtgatatg	miR*-s
primer IV	gaAATACTGATTTGAAGTGGTTCtctacatatatattcct	miR*-a

All PCRs were performed with high fidelity *Pfu* DNA Polymerase (Thermo Fisher Scientific) in a volume of 50 µl according Schwab et al. (2006). Three fragments were PCR amplified from the template clone pRS300 using the six primers in combination: A+II (272 bp), I+IV (206 bp) and III+B (340 bp). The three resulting fragments were gel purified (Promega) and then fused by one PCR with the two flanking primers A and B on a mixture of 1 µl of each previous PCR as template. The fusion product of 448 bp was again gel purified (Promega), cloned into pGEM®-T Easy Vector after A-tailing (Promega), sequence verified, excised with *Asc*I/*Not*I and transferred into the binary vector pSK36 (Ikeda et al., 2006). In pSK36, the expression of the transgene is driven by the promoter of the Cauliflower mosaic virus 35S. The amiRNA plant expression vector was transformed into *Agrobacterium tumefaciens* strains LBA4404 and use to transform immature tomato fruits as described by Yasmeen et al. (2009). Mutant plants were selected based on their phenotype expected to be affected in their growth response to

light. The presence of the transgene was validated by PCR. The effect of the amiRNA expression on the transcription and traduction of PHOT1 was determined both at the gene level by qPCR and at the protein level by Western-blot.

LIGATION-MEDIATED POLYMERASE CHAIN REACTION (LM-PCR)

LM-PCR was performed to identify the integration site of amiRNA construct in tomato genome and thus the possible interruption of gene expression. The protocol of Thole et al. (2009) was used with the following modifications: gDNA was isolated using Invisorb spin plant mini kit (Stratec, Germany) according to the manufacturer´s instruction. DNA was digested by *SspI* restriction enzyme (Thermo Scientific, USA). Sequences of adapters and primers used are given in Table 4.

Table 4. Adapters and primers used for LM-PCR.

Name	Description	Sequence (5´- 3´)
ADP2	Used with ADP3 to generate the *SspI*	ATACCTGCCCAA
ADP3	Used with ADP2 to generate the *SspI*	CTAATACGACTCACTATAGGGCTCG
AP1	Adapter primer used with LB1 for first	GGATCCTAATACGACTCACTATAGG
AP2	Nested adaptor primer used with LB2 for second PCR	TATAGGGCTCGAGCGGC
LB1	amiRNA primer near LB used with AP1 for first PCR	AGGGCGACACGGAAATGTTG
LB2	Nested RNA primer near LB used with AP2 for second PCR	ATTGTCTCATGAGCGGATAC

PROTEIN ISOLATION AND WESTERN BLOT

Hypocotyls of 4-d-old etiolated seedlings were homogenized in buffer containing 0.1 M Tris-HCl, pH 8.5, 4% SDS, 2 mM phenylmethylsulfonyl fluoride, and 1:100 protease inhibitor cocktail (Sigma, USA) in a ration 1:1,5 (tissue:buffer). The homogenate was then heated at 80°C for 3 minutes and centrifuged at 14,000 rpm for 15 min at 4°C. Total protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, USA). 50 µg of protein extract was mixed with 4x sample buffer and reducing reagent (Thermo Scientific, USA) and boiled for loading on 4%-12% BisTris Plus gel (Thermo Scientific, USA) to separate the proteins in MOPS running buffer supplemented with antioxidant reagent (Thermo Scientific, USA). Proteins were transferred to polyvinylidene fluoride membranes (Millipore) following standard procedures described

by Towbin et al. (1979). Antigen and antibodies directed against tomato PHOT1 were synthesized and produced in rabbit, respectively, by Eurogentec (Belgium). The antigen (H_2N-GKTETSRRDSGNSGRC-$CONH_2$) was selected in the N-terminal of the protein, before the LOV1 domain, and for its specificity to PHOT1 (Solyc11g072710; Fig. 13). Anti-PHOT1 antibodies were diluted 1:500 in TBST buffer (20mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20) and incubated with membrane with gentle shaking overnight at 4°C. The goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma-Aldrich) was used as secondary antibody and diluted 1:5000 in TBST buffer for 1 hour at room temperature. Detection was done according instruction of the Pierce™ Fast Western Blot Kit, ECL Substrate (Thermo Fischer Scientific) and Gel Doc™ EZ System (Biorad).

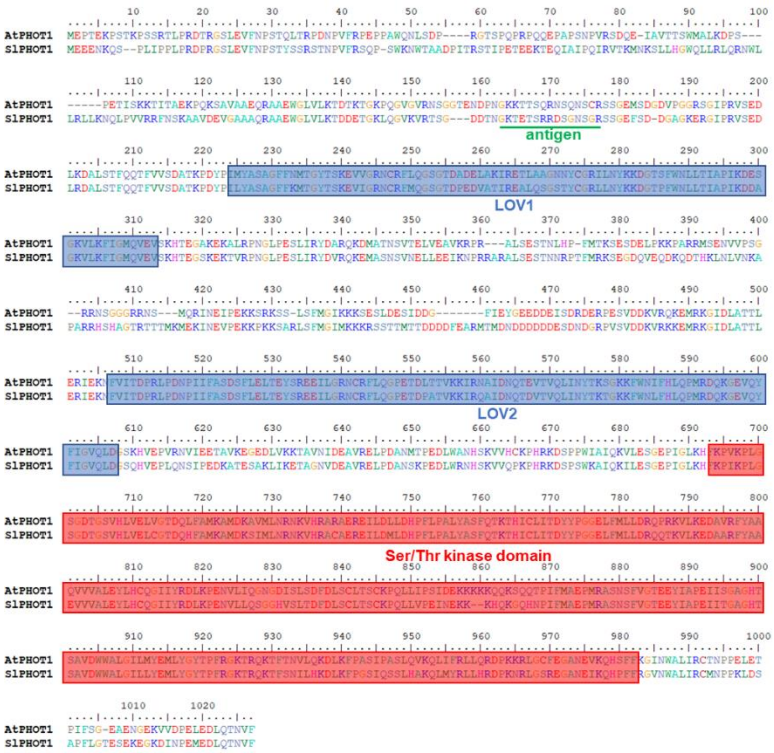

Figure 13. Alignment of Arabidopsis (At) and tomato (Sl) PHOT1. The two LOV domains and the Ser/Thr kinase domain have been indicated as well as the antigen produced to obtain anti-serum directed against tomato PHOT1.

DETERMINATION OF PIGMENTS IN FRUITS USING HPLC

HPLC analysis of pigments was performed according to the method described in Pogson et al. (1996) with some modifications. Dry weight tomato fruits (0.2 g) were homogenized with a mortar in 80% acetone. Homogenate was centrifuged at 6 000 x*g* for 3 minutes at 4˚C. Supernatant was taken and filtered through 0.45 µm PTFE syringe filter

(Acrodisc, Waters, Milford, MA, USA) into dark vials. The amount of 100 µl was injected into the HPLC system (Alliance e 2695 HPLC System, Waters, Milford, MA, USA.) and signal was detected at 440 nm using UV/VIS detector. A LiChroCART RP-18 (5 µm; 4.6 x 250 mm) Column (Merck & Co., Inc., Kenilworth, NJ, USA) was used. The analysis was performed by gradient reverse – phase analysis (1.5 ml/min at 25 °C). The analysis started with isocratic elution using the mobile phase composed of acetonitrile, methanol and 0.1 M Tris in the ratio 87:10:3 (v:v:v) for 10 min and was followed by a 2-min linear gradient using mobile phase composed of a mixture of methanol and n-hexane in the ratio 4:1 (v:v). The amount of pigments in samples, except lycopene, was determined using their conversion factors (Färber and Jahns, 1998) and the amount of pigments per g of dry weight was calculated. To quantify the concentration of lycopene, a calibration curve was obtained by plotting the peak area at 440 nm for various concentrations of lycopene standard (Sigma-Aldrich).

STATISTICAL ANALYSIS

Statistical significance of the results was supported either by the nonparametric Kruskal-Wallis Anova followed by a post-hoc multiple comparison of mean rank or by the one-way Anova (Statistica v.12, StatSoft).

CHAPTER I

PHOT1-MEDIATED EARLY STEP OF HYPOCOTYL GROWTH INHIBITION

Upon exposure to blue light, reduction of hypocotyl growth rate occurs in two phases. The first phase is characterized by a rapid inhibition of the growth rate and is mediated by phot1. After 30 minutes of exposure to BL, one may observe a partial recovery in the growth rate and establishment of a steady state growth rate. This later phase is controlled by cry1. These photorecetors trigger complex signaling networks, including modulation of enzymatic activity, dynamic regulation of gene expression, and protein-protein interaction. Although some information is available for cry1-mediated de-etiolation, less attention has been given to the phot1 phase of de-etiolation. Thus, we focus our interest on the first 30 min following the exposure to BL when phot1 is required to induce the hypocotyl growth inhibition. The suppression subtractive hybridization method was used to investigate the molecular mechanisms of phot1-induced de-etiolation in tomato hypocotyl (*Solanum lycopersicum* L.). Our library generated 152 expressed sequence tags that were found to be rapidly accumulated upon exposure to BL and consequently potentially regulated by phot1. Annotation revealed that biological functions such as modification of chromatin structure, cell wall modification, and transcription/translation. These processes comprise an important part of events contributing to the establishment of photomorphogenesis in young seedlings.

Hloušková P, and Bergougnoux V. (2016). A substracted cDNA library identifies genes up-regulated during PHOT1-mediated early phase of de-etiolation in tomato (*Solanum lycopersicum* L.). BMC Genomics 17:291. doi: 10.1186/s12864-016-2613-6

RESULTS AND DISCUSSION

CONSTRUCTION OF THE SUBTRACTED CDNA LIBRARY AND ANALYSIS

In order to study the molecular events of the rapid inhibition of tomato hypocotyl growth observed within the first 30 min following exposure to BL, a SSH library was constructed and screened for genes whose expression is stimulated by BL. Two contrasting mRNA samples were extracted. One sample, extracted from the elongating zone of the hypocotyl of seedlings grown in darkness and exposed for 30 min to BL (10 $\mu mol.m^{-2}.s^{-1}$), potentially containing differentially expressed genes, was used as the tester. The second sample, isolated from the elongating zone of the hypocotyl of seedlings grown only in darkness, constituted the driver that should express transcripts common to both samples and eliminated during the process of subtraction. Due to technical limitation, 500 putative subtracted clones were randomly picked and used in cDNA dot-plot array for differential screening. Clones were considered for sequencing when they hybridized only to the BL-specific probes or showed higher intensity with the BL-specific probe than with dark-specific probe.

In these conditions, we determined that 168 ESTs were potentially differentially expressed. After BLAST analysis, 17 sequences from 168 were found to be redundant, bringing to 151 the number of expressed sequence tag (ESTs) encoding proteins. The ontology annotation was performed using Blast2GO according to plant-specific Gene Ontology terms (Conesa et al., 2005). Computational analysis using the software Blast2GO enabled annotation of the expressed sequences according to the terms of the three main Gene Ontology vocabularies (i.e., cellular compartment, molecular function and biological process; Fig. 14). Concerning molecular function, the most represented categories were those of binding and catalytic activities (Fig. 14B).

Regarding cellular compartments, the most represented were ribosome, plastid, and nucleus, together accounting for more than 50% of total annotations (Fig. 14C). When taking into consideration the most relevant level of distribution for the biological process (i.e., level 8, as shown in Fig. 14A), more than 40 categories were found for the biological process vocabulary (data not shown). The number of categories was therefore simplified to level 2 of the distribution (Fig. 14D).

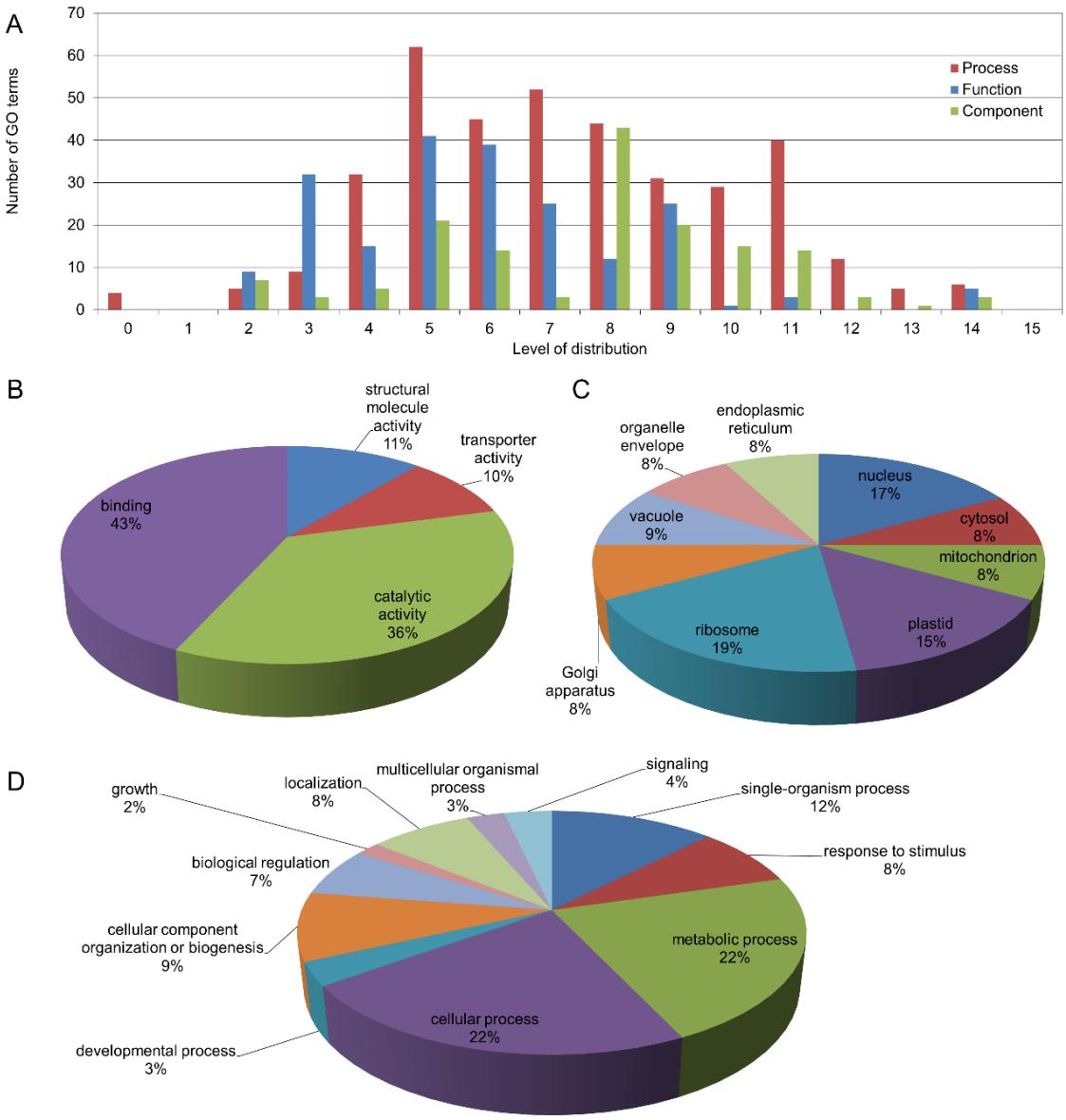

Figure 14. Gene Ontology terms distribution. Number of Gene Ontology terms per level of distribution (A), Gene Ontology terms distribution by molecular functions (B), cellular components (C), and biological processes (D) vocabularies. In (B), binding and catalytic activities were the most represented molecular functions. In (C), the most represented categories were ribosome, followed by nucleus and plastid. In (D), the most abundant categories were metabolic and cellular processes.

The functional annotation was performed with Mercator, using the last updated version of the tomato annotation (ITAG2.4) (Lohse et al., 2014). Identical description of the ESTs was obtained when the annotation was performed by Blast2Go, KOG attribution or Blast against the specific annotated tomato genome ITAG2.4 (Table 5).

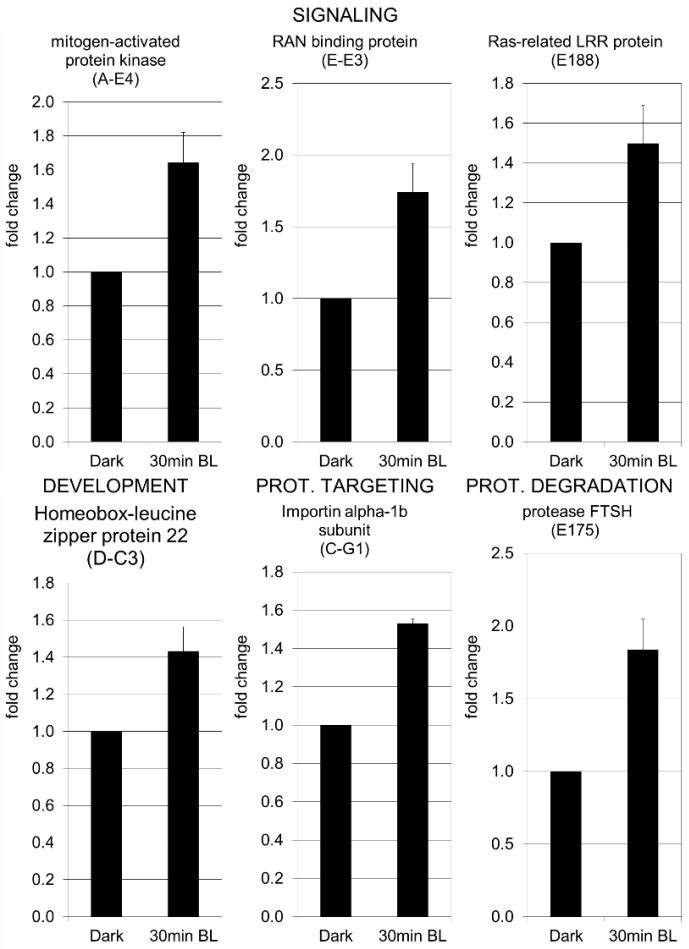

Figure 15. Analysis by quantitative real-time PCR of expression of selected genes belonging to different functional categories. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the *pp2ase* gene as reference gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results.

Table 5. Gene ontology and functional annotation of the expressed sequence tags (ESTs) up-regulated by 30 min of exposure to blue light. Annotation was performed using BlastP against the tomato genome annotation (ITAG2.4). Blast (RSP-blast) against the clusters of orthologous eukaryotic genes database (KOG), Blast and annotation by Blast2GO and finally description by BIN code with Mercator/Mapmann are not shown and they are accessible from: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-016-2613-6#export-citations

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Photosynthesis				
D-G4	Solyc05g053810.2.1	XP_004239849	662	Serine hydroxymethyltransferase
Minor carbohydrates				
C-F4	Solyc01g095470.2.1	XP_004229976	387	Aldose 1-epimerase family protein
D-H8	Solyc07g055300.2.1	XP_004243268	174	Alpha alpha-trehalose-phosphate synthase (UDP-forming)
187	Solyc03g097210.2.1	XP_010318288	445	Kinase pfkB family protein
Glycolysis				
B-A6	Solyc05g014470.2.1	XP_010320554	510	Glyceraldehyde 3-phosphate dehydrogenase
Fermentation				
80	Solyc02g084640.2.1	XP_010316396	300	Aldehyde dehydrogenase-dependent
Mitochondrial electron transport/ATP synthesis				
B-B3	Solyc00g042130.1.1	XP_004253362	613	ATP synthase subunit alpha
C-D5	Solyc01g103220.2.1	XP_004230430	391	Cytochrome c
E196	Solyc12g055760.1.1	XP_004252620	288	ATP synthase subunit epsilon mitochondrial
E-G1	Solyc04g080570.2.1	XP_004238283	381	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase
Cell wall				
12	Solyc01g099630.2.1	NP_001233858	438	Xyloglucan endotransglucosylase_hydrolase 5
B-E2	Solyc01g102350.2.1	XP_004230372	627	Pectinacetylesterase like protein
B-G3	Solyc04g081300.2.1	XP_010320423	472	Endoglucanase 1
D-D4	Solyc01g091530.2.1	XP_004229828	251	Fasciclin-like arabinogalactan protein 13 IPR000782 FAS1 domain
E182	Solyc07g017600.2.1	XP_004242901	364	Pectinesterase
E192	Solyc02g068530.2.1	XP_004233254	693	UDP-N-acetylglucosamine pyrophosphorylase
E-F8	Solyc10g074920.1.1	XP_010327704	600	Mannan endo-1 4-beta-mannosidase
Lipid metabolism				
A-B12	Solyc01g098110.2.1	XP_010312252	640	Hydrolase alpha_beta fold family protein

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Lipid metabolism				
C-C8	Solyc06g007130.2.1	NP_001233791	484	Omega-3 fatty acid desaturase
E176	Solyc05g008580.2.1	XP_004238876	658	Phosphatidylserine synthase 2
Amino acid metabolism				
15	Solyc09g092390.2.1	XP_004247887	691	Adenosylhomocysteinase
33	Solyc01g101060.2.1	NP_001234425	688	S-adenosylmethionine synthase
C-G5	Solyc01g109850.2.1	XP_004230896	445	Diaminopimelate decarboxylase
D-B8	Solyc09g092380.2.1	NP_001234012	210	Adenosylhomocysteinase
D-D9	Solyc09g008280.1.1	XP_010325713	331	S-adenosylmethionine synthase
D-E5	Solyc10g085630.1.1	XP_004249576	644	Fatty acid oxidation complex subunit alpha
D-F4	Solyc03g044330.1.1	XP_004234664	265	Acetolactate synthase
Stress				
A-D3	Solyc04g054810.2.1	XP_004237415	518	Pollen allergen Phl p 11
B-E9	Solyc01g103450.2.1	XP_004230445	368	Chaperone DnaK
C-E10	Solyc07g065840.2.1	NP_001234439	361	Heat shock protein 90
C-G9	Solyc01g059930.2.1	XP_004229006	460	Universal stress protein UspA
C-H6	Solyc09g010630.2.1	XP_004246402	976	heat shock protein 70
D-E12	Solyc05g052970.2.1	XP_004239912	539	Dehydration-responsive family protein
D-G8	Solyc09g098540.2.1	XP_004247978	325	Chitinase-like protein
E162	Solyc10g011870.2.1	XP_004248066	299	Reticulon family protein
E-D5	Solyc06g076520.1.1	AAD30452	369	class I heat shock protein Hsp20
E-F6	Solyc01g079610.2.1	XP_004229405	187	DNAJ chaperone
F-A11	Solyc04g008540.2.1	NP_001234096	258	Tobamovirus multiplication protein (Fragment)
Redox				
C-G7	Solyc02g084710.2.1	XP_004232447	221	Thioredoxin h1

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Redox				
D-D11	Solyc08g081530.2.1	XP_010325306	545	Reductase
Miscellaneous enzyme families				
B-E4	Solyc07g019460.2.1	XP_004242931	544	Cytochrome P450 NADPH-reductase
C-C9	Solyc04g007400.2.1	XP_004236988	359	Tropinone reductase II
E158	Solyc05g006510.1.1	XP_004238733	311	Glycosyltransferase family GT8 protein
E-A1	Solyc03g121180.2.1	XP_004235632	402	GDSL esterase_lipase
RNA: processing, binding, regulation of transcription				
38	Solyc04g007970.2.1	NP_001274294	394	Ubiquitin-conjugating enzyme E2
A-B3	Solyc08g081000.2.1	XP_010325408	313	Lysine-specific demethylase 5D, Transcription factor jumonji
B-A2	Solyc02g082340.2.1	XP_004232589	197	RNA polymerase I-specific transcription initiation factor RRN3
B-C7	Solyc06g070900.2.1	NP_001233815	283	TCP family transcription factor
B-F8	Solyc03g025850.2.1	NP_001234231	690	Remorin 1
C-B10	Solyc09g089870.2.1	XP_010326757	327	Transcription factor
C-C7	Solyc11g069340.1.1	XP_004251126	350	23S rRNA (Uracil-5-)-methyltransferase
D-C7	Solyc12g100060.1.1	XP_004252868	315	Zinc finger A20 and AN1 domain-containing stress-associated protein 6
D-E9	Solyc04g081880.2.1	XP_004238382	241	Ribonuclease P protein subunit p25
D-F3	Solyc03g093160.2.1	XP_004235160	597	LUC7-like 2
E181	Solyc07g042180.2.1	XP_004243695	671	Polyadenylate-binding protein
E-C11	Solyc08g076530.2.1	XP_004245957	356	RNA recognition motif-containing protein
DNA: synthesis/chromatin structure				
A-E2	Solyc10g053970.1.1	XP_004248700	720	SEC14-like protein
C-A3	Solyc05g055440.1.1	XP_004239714	411	Histone H2B
E-A3	Solyc01g108500.2.1	XP_004230808	404	Polyadenylate-binding protein
E-C5	Solyc09g010400.2.1	XP_004246465	307	Histone H2A

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Protein: synthesis, postranslational modification, targeting, degradation				
Synthesis				
13	Solyc03g096360.2.1	XP_004235258	151	60S ribosomal protein L33-B
27	Solyc12g005330.1.1	XP_004251488	667	50S ribosomal protein L2
32	Solyc01g009100.2.1	XP_004228473	352	Ribosomal protein L30
47	Solyc12g005330.1.1	XP_004251488	667	50S ribosomal protein L2
48	Solyc06g073310.2.1	XP_004241407	610	Ribosomal L9-like protein
A-B6	Solyc02g070310.2.1	XP_004233141	614	Ribosomal protein L32
A-F6	Solyc07g065170.2.1	XP_004244061	893	40S ribosomal protein S8
A-G3	Solyc10g086020.1.1	XP_004246693	567	30S ribosomal protein S12
B-B7	Solyc06g071720.1.1	XP_004241546	311	60S ribosomal protein L27A
B-D10	Solyc04g074300.2.1	XP_004237924	577	30S ribosomal protein S5
B-D7	Solyc11g072260.1.1	XP_004234944	481	40S ribosomal protein S13
B-E8	Solyc01g088370.2.1	XP_00422	515	Eukaryotic translation initiation factor 3 subunit B
C-E8	Solyc03g058350.2.1	XP_010317931	354	Translation initiation factor
C-H4	Solyc08g062800.2.1	XP_004253094	417	ATP-dependent RNA helicase eIF4A
D-B5	Solyc12g096700.1.1	XP_004253036	274	Ribosomal L9-like protein
D-G12	Solyc02g070350.2.1	XP_004233136	148	Ribosomal protein S4-like protein
E172	Solyc06g074300.2.1	XP_004241322	326	Ribosomal protein
F-A1	Solyc05g054580.2.1	XP_004239781	394	60S acidic ribosomal protein P0
Postranslational modification				
D-A8	Solyc04g015130.2.1	XP_010319220	645	Ribosomal protein S6 kinase alpha-3
D-C11	Solyc10g084770.1.1	XP_004249535	450	Nuclear factor related to kappa-B-binding protein related
D-F8	Solyc03g083520.2.1	XP_004234945	295	Calmodulin
D-G7	Solyc05g013990.2.1	XP_004239159	238	T-complex protein 1 subunit epsilon

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Targeting				
C-G1	Solyc01g060470.2.1	XP_004228994	688	Importin alpha-1b subunit
E186	Solyc01g100860.2.1	XP_010313724	187	ADP-ribosylation factor
E195	Solyc07g066090.2.1	XP_010324226	399	SEC14 cytosolic factor family protein
Degradation				
3	Solyc01g079940.2.1	XP_004229430	176	Xylanase inhibitor (Fragment)
B-A8	Solyc06g083620.2.1	XP_004242383	339	26S protease regulatory subunit 4
B-D9	Solyc02g083710.2.1	XP_010316429	222	26S proteasome non-ATPase regulatory subunit 4
D-D12	Solyc02g069090.2.1	XP_004233221	219	Cathepsin B
E175	Solyc10g086550.1.1	XP_004249560	464	Cell division protease ftsH homolog 3
E179	Solyc02g083710.2.1	XP_010316429	229	26S proteasome non-ATPase regulatory subunit 4
E-D1	Solyc12g042060.1.1	XP_004252280	365	ATP-dependent clp protease ATP-binding subunit
E-E8	Solyc04g078540.2.1	XP_004238129	733	Cathepsin B-like cysteine proteinase
Signalling				
40	Solyc08g077730.2.1	XP_004245835	754	1-phosphatidylinositol-4-phosphate 5-kinase-like protein
A-E4	Solyc01g080240.2.1	XP_004229459	496	Mitogen-activated protein kinase 16
C-C5	Solyc12g010790.1.1	XP_004251797	184	Ras-related protein Rab-25
D-E7	Solyc10g084280.1.1	XP_004249500	285	Calmodulin-binding protein
E157	Solyc01g100350.2.1	XP_004230294	601	ADP-ribosylation factor-like protein 3
E188	Solyc03g112270.2.1	XP_004236264	1387	Lrr, resistance protein fragment
E-E3	Solyc03g120280.1.1	XP_004235706	392	RAN binding protein 3
F-A4	Solyc03g120280.1.1	XP_004235706	397	RAN binding protein 3
Cell cycle				
E-A8	Solyc09g075760.2.1	XP_004247372	581	Cyclin T1

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Vesicle transport				
79	Solyc01g103480.2.1	XP_010314473	539	Coatomer subunit delta, Clathrin adaptor
B-A4	Solyc12g098950.1.1	XP_004252947	616	Novel plant SNARE 11
Cytoskeleton				
A-H7	Solyc04g081490.2.1	NP_001234807	502	Tubulin beta-1 chain
B-H8	Solyc04g077020.2.1	XP_004238046	611	Tubulin alpha-3 chain
F-B8	Solyc08g006890.2.1	XP_004244533	517	Tubulin alpha-3 chain
Development				
54	Solyc08g076920.2.1	XP_004245896	317	ADP-ribosylation factor GTPase-activating protein 3
C-B2	Solyc01g099840.2.1	XP_004230256	353	Auxin-repressed protein
D-C3	Solyc02g091930.2.1	XP_004231956	458	Homeobox-leucine zipper protein 22
D-F6	Solyc10g085060.1.1	XP_010312300	399	Defective in exine formation
E-H12	Solyc03g006360.2.1	XP_004234112	569	Auxin-repressed protein
Transport				
4	Solyc07g053970.2.1	XP_004243353	588	Two-pore calcium channel 2
9	Solyc10g054590.1.1	XP_004248676	355	V-type proton ATPase 16 kDa proteolipid subunit
65	Solyc04g005570.2.1	XP_004237046	377	Transmembrane emp24 domain-containing protein 10
82	Solyc07g053970.2.1	XP_004243353	588	Two-pore calcium channel 2
A-B4	Solyc01g094690.2.1	BAO18622	728	Aquaporin
B-D5	Solyc02g084360.2.1	XP_004232469	520	V-type proton ATPase 16 kDa proteolipid subunit c2
B-E11	Solyc01g094690.2.1	BAO18622	700	Aquaporin
B-G5	Solyc06g074820.2.1	BAO18632	555	Aquaporin-like protein
B-H3	Solyc02g094280.2.1	XP_004231762	517	Oligopeptide transporter
E169	Solyc01g073690.2.1	XP_010322009	513	V-type ATP synthase subunit D
E198	Solyc04g008340.2.1	XP_004236909	128	Zinc transporter

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
No ontology				
E-A2	Solyc04g079560.2.1	XP_004238196	327	Lysine_histidine transporter
55	Solyc02g092360.2.1	XP_010316023	140	Beta-glucanase-like protein
A-A7	Solyc11g056680.1.1	XP_004229610	204	LRR receptor-like serine_threonine-protein kinase, RLP
A-E11	Solyc02g094430.2.1	XP_010315903	428	Hydrolase alpha_beta fold family protein
D-A10	Solyc01g094920.2.1	XP_004229933	618	Receptor-like kinase
D-C1	Solyc09g011030.2.1	XP_004246423	185	Hsp70 nucleotide exchange factor fes1
D-C2	Solyc03g123390.2.1	XP_004235544	299	Hydrolase
D-E4	Solyc01g044360.2.1	XP_004228963	353	Importin beta-3
D-G1	Solyc04g015020.2.1	XP_004236656	147	Proline-rich protein
Unknown				
21	Solyc06g065980.2.1	XP_004241781	590	Nucleic acid binding protein
71	Solyc01g100760.2.1	XP_004230326	325	Susceptibility homeodomain transcription factor
A-C3	Solyc06g082930.2.1	XP_010323105	766	Frigida-like
A-F11	*****	XP_004249026	534	
B-B2	Solyc04g082200.2.1	XP_004238411	730	Dehydrin
B-C1	Solyc06g067910.2.1	XP_004241713	413	Os01g0611000 protein
B-C2	Solyc10g086560.1.1	XP_010312356	527	Unknown Protein
B-D1	Solyc07g008670.2.1	XP_010323341	660	AT1G74160 protein (Fragment)
B-F5	Solyc08g067030.2.1	XP_004245192	443	Os01g0611000 protein (Fragment)
B-G10	Solyc08g015780.2.1	XP_004244785	455	F-box_ankyrin repeat protein SKIP35
B-H10	Solyc12g088960.1.1	XP_004252777	542	Transmembrane protein 147
B-H12	Solyc02g092790.2.1	NP_001234443	522	Arabinogalactan
D-A1	Solyc04g074730.1.1	XP_004237948	385	Unknown Protein
D-B10	Solyc01g081200.2.1	XP_004229523	249	Unknown Protein

Table 5. (continued)

DEGs	ITAG2.4 IDs	NCBI_protein ID	Seq. length (bp)	ITAG2.4. Full Description
Unknown				
E-B7	Solyc10g047210.1.1	XP_004248804	596	Unknown Protein
E-D7	Solyc06g061130.2.1	XP_010322870	276	Unknown Protein
E-E4	Solyc04g009700.2.1	XP_004236809	371	Dual specificity phosphatase catalytic domain containing protein expressed
E-E5	Solyc06g073900.2.1	XP_004241358	307	Unknown Protein
E-E9	Solyc06g061200.1.1	AAP83840	389	Glycine-rich protein TomR2
F-C1	Solyc02g076820.2.1	XP_004243287	361	Light-dependent short hypocotyls 1

The Table 6 shows the number of sequences which enter the different categories. Twenty-seven sequences could not be annotated. The functional annotations “Protein: synthesis, targeting, postranslation modification, degradation” and “RNA: processing, transcription, regulation of transcription” were the most represented, including 33 and 12 sequences, respectively. More detailed information can be found in Table 5. For all genes tested, qPCR confirmed the differential expression detected by the screening of the cDNA library, meaning the up-regulation of the expression of ESTs as soon as 30 min after exposure to BL (Fig. 15).

Table 6. Functional categories of up-regulated genes.

BIN Categories	Number of sequences
Photosynthesis	1
Minor carbohydrates	3
Glycolysis	1
Fermentation	1
Mitochondrial electron transport / ATP synthesis	4
Cell wall	7
Lipid metabolism	3
Amino acid metabolism	7
Stress	11
Redox	2
Miscellaneous enzyme families	4
RNA: processing, transcription, regulation of transcription	12
DNA: synthesis/chromatin structure, repair	4
Protein: synthesis, targeting, postranslation modification, degradation	33
Signalling	8
Cell organisation	3
Cell cycle	1
Cell, vesicle transport	2
Development	5
Transport	12
No ontology	8
Unknown	19

TRANSLATION AND TRANSCRIPTION

Sixteen differentially expressed sequences were predicted to encode proteins involved in translation, RNA processing and modification (ribosomal proteins), transcription (eukaryotic initiation factors), and chromatin structure and dynamics (Histone 2B – H2B, Histone H2A). In our study, we found by SSH screening and confirmed by qPCR analysis that H2B is up-regulated during PHOT1-mediated de-etiolation in tomato, indicating that it could play a role during the establishment of photomorphogenesis in tomato (Fig. 16A). Histones form the protein core of the nucleosome around which the DNA helix is wrapped. In this compact state, histones block the association of transcription factors to their binding sites, thus repressing transcription. Several post-translational modifications (acetylation, methylation or phosphorylation) of histone "tails" can influence nucleosome compaction and access to DNA.

Moreover, their spatio-temporal regulation as well as their ability for cross-talk renders the regulation of gene expression even more complex (Fisher et al., 2011). In plants, chromatin remodeling plays an important role during plant growth and development, especially in response to light. Indeed, a large-scale reorganization of chromatin can be observed during the floral transition in *Arabidopsis* (Tessadori et al., 2007). During de-etiolation, the perception of light induces a remarkable reprogramming of gene expression that leads the heterotrophic seedling to become an autotrophic organism which will be able to complete its life cycle. In darkness, the photomorphogenic repressor DET1 binds to the H2B tails of the nucleosomes surrounding the genes which are repressed in this condition. When light is perceived, the H2B acetylation concomitant with the release of DET1 enables the activation of genes involved in photomorphogenesis (Benvenuto et al., 2002). It would be interesting in the near future to validate the potential involvement of H2B in the control of de-etiolation in tomato and thereby to follow the relationship between gene expression and H2B enrichment during this process. Finally, we could identify and confirm that the subunit RPN10/PSMD4 of the 26S proteasome regulatory complex is up-regulated during de-etiolation (Fig. 16B). For this reason, it is tempting to hypothesize that light-regulated histone expression/modification and ubiquitin-proteasome-mediated protein degradation might interact during tomato de-etiolation.

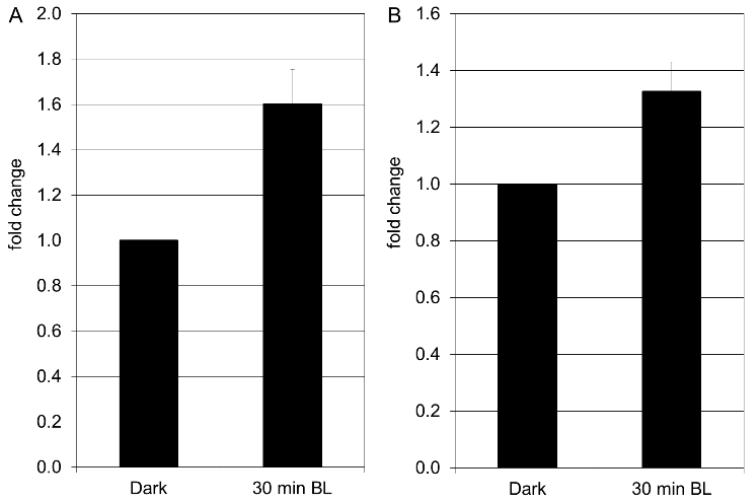

Figure 16. Analysis by qPCR of the expression of two genes encoding proteins involved in translation and transcription: Histone 2B (A) and 26S proteasome regulatory complex, subunit RPN10/PSMD4 (B). The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the *pp2ase* gene as refernce gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results.

CELL WALL MODIFICATION

In our study, seven tomato EST encoding proteins involved in cell wall modification were found to be up-regulated in the etiolated hypocotyl of tomato seedlings exposed for 30 min to BL: pectin acetylesterase, pectinesterase, xyloglucan endotransglucosylase-hydrolase 1 (XTH), or endoglucanase. This suggested that de-etiolation induced a strong modification of the cell wall structure and/or composition. Plant cell walls consist of a complex network of cellulose microfibrils embedded in a matrix of hemicelluloses (mainly xyloglucans), pectins, and glycoproteins (Carpita and Gibeaut, 1993). During cell maturation, cell walls lose the ability to expand (Van Volkenburgh et al., 1985). Growth cessation is accompanied by cell wall tightening (Kutschera, 1996). Various modifications of cell wall structure during maturation have been proposed, including changes in hemicellulose. For example, the maturation of pea tissues is characterized by an increase in the total amount of xyloglucan (Baumann et al., 2007). Xyloglucan endotransglucosylase/hydrolases (XTH) are enzymes capable of modifying xyloglucan during cell expansion. They comprise a subgroup of the glycoside hydrolase family 16. XTH proteins characterized to date have endotransglycosylase (XET) or hydrolase (XEH) activities towards xyloglucans, or both. Their phylogenic study indicates that they are organized into three groups: I/II, III-a, and III-b. Only members of the III-a group are strict XEH (Baumann et al., 2007).

Transgenic tomatoes with altered levels of XTH gene showed higher XET activity, lower hemicellulose depolymerization and reduced fruit softening during ripening. This suggests that XET could have a role in maintaining the structural integrity of the cell wall (Miedes et al., 2010; Miedes et al., 2011). Thus, whereas some XTH members are critical in promoting cell wall expansion, others are required for wall strengthening in cells that have completed the expansion process (Nishikubo et al., 2011). The analysis by qPCR of the XTH identified by SSH screening confirmed that it is up-regulated by BL (Clone 12; Fig.17A). Based on the aforementioned literature, we can assume its role in cell wall strengthening during de-etiolation. Pectins, comprising another important cell wall component, are synthesized in the *cis*-Golgi, methyl-esterified in the medial-Golgi, substituted in the *trans*-Golgi, and then secreted into the cell wall. Zhao and co-authors (2008) reported that de-esterification of methyl-esterified pectin may also be associated with growth cessation in both grasses and dicotyledons and may contribute to wall tightening by strengthening pectin–calcium networks. Pectin acetylation is another modification of pectins which probably occurs between the Golgi and the cell wall during pectin exocytosis. Its occurrence and function are poorly understood. The degree of O-acetylation of pectin changes during growth and differentiation of plant tissues, but also in response to environmental conditions. Pectin acetylesterases trigger the deacetylation of pectin. The overexpression of the black cottonwood (*Populus trichocarpa*) *PAE1* gene in tobacco has been shown to impair the cellular elongation of floral organs. Thus, it appears that pectin acetylesterases function as an important regulator of pectin acetylation status to affect the physiochemical properties of the cell wall's polysaccharides and consequently to affect cell extensibility (Gou et al., 2012). The confirmation by qPCR that pectin acetylesterase (B-E2; Fig.17B) is up-regulated by BL supports the hypothesis that they are actors of the inhibition of cell expansion which occurs during de-etiolation.

Based on our data and the analysis of the literature, we can hypothesize that exposure to BL rapidly induces changes in cell wall properties, namely extensibility. It would be thus interesting to validate this hypothesis through physico-chemical measurement of the cell wall of tomato seedlings' hypocotyl during BL-induced de-etiolation.

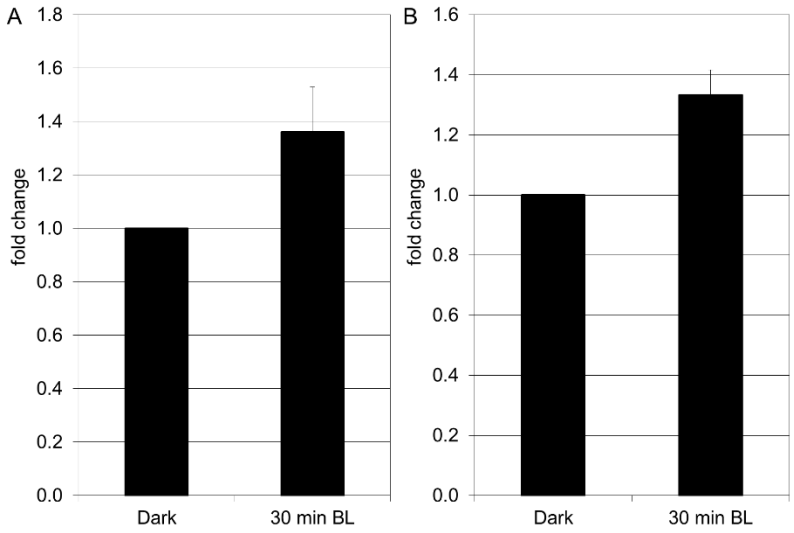

Figure 17. Analysis by qPCR of the expression of two genes encoding proteins involved in cell wall modification: xyloglucan endotransglucosylase-hydrolase/XTH (A) and pectin acetylesterase (B). The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the *pp2ase* gene as refernce gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results.

ROLE OF VACUOLAR H^+-ATPASE DURING DE-ETIOLATION

In tomato, three ESTs encoding vacuolar H+-ATPase (V-ATPase: 9, B-D5, E169) subunits were found to be up-regulated during PHOT1-mediated inhibition of hypocotyl growth. This was confirmed by qPCR for the V-type H^+-ATPase subunit c2 (B-D5; Fig.18A). Considering the role of V-ATPase during de-etiolationis important if one considers that hypocotyl growth in darkness does not require the division of cortical or epidermal cells and cells elongate along an acropetal spatial and temporal gradient (Gendreau et al., 1997). Cell expansion is achieved by: i) increase in cell ploidy via endoreduplication, and ii) osmotic water uptake into the vacuole, creating the turgor pressure necessary for the irreversible extension of the cell wall caused by the synthesis, incorporation, and cross-linking of new cell wall components. The cell expansion is restricted by cell wall extensibility (Cosgrove, 2005; Perrot-Rechenmann, 2010). V-ATPases are potentially involved in creating or regulating turgor pressure. They represent a major fraction of the total tonoplast proteins. V-ATPases also are present in the *trans*-Golgi network (TGN), where they are essential for its proper function (Dettmer et al., 2006). Whereas inhibition of the tonoplast-localized V-ATPase does not affect cell expansion, inhibition of that which is TGN-localized is enough to restrict cell expansion (Brux et al, 2008). Moreover, the *det3* mutant, a

possible negative regulator of photomorphogenesis affected in V-ATPase function, was originally proposed to be impaired in vacuolar solute uptake resulting in adequate turgor pressure for cell expansion (Schumacher et al., 1999). Recent evidence has shown that a cell wall defect in the mutant is responsible for its reduced hypocotyl cell expansion (Brux et al., 2008). Together, these data indicate that V-ATPase plays a role in cell wall integrity/synthesis through its function in the TGN-mediated secretory pathway, thereby participating in the restriction of cell expansion.

In eukaryotes, V-ATPase consists of at least 12 distinct subunits organized in two large subcomplexes: the cytosolic V1 and membrane Vo subcomplexes. The cytosolic V1 complex is constituted of subunits A through H and catalyzes the hydrolysis of ATP which is associated with the pumping of protons into a compartment via the membrane-bound Vo complex. The Vo complex includes three integral proteins, named subunits a, c, c'', and one hydrophilic subunit d (Padmanaban et al., 2004). In tomato, two isoforms of the subunits A (A1 and A2) were isolated. Whereas VHA-A2 isoform was found to be specifically expressed in roots, VHA-A1 isoform was ubiquitously expressed in all tissues and up-regulated by salinity stress (Bageshwar et al., 2005). The analysis of expression of the *VHA-A1* isoform in the elongating zone of the tomato hypocotyl during BL-induced de-etioation revealed the accumulation of *VHA-A1* transcripts during the time-course of the experiment (Fig. 18B). When tomato seedlings were grown in BL on a medium containing varying concentrations of bafilomycin A1, a specific inhibitor of V-ATPases, the length of hypocotyl increased with increased concentration of bafilomycin A1 (Fig. 18C).

These results indicated that in tomato, like in barley, BL induces accumulation of V-ATPase as well as its activation (Klychnikov et al., 2007). Both events appear to be required to trigger the restriction of cell expansion occurring during de-etiolation. To conclude, we found a strong evidence that V-ATPases play a role during BL-mediated inhibition of hypocotyl growth. It nevertheless would be interesting to verify if V-ATPase participates in elaborating the turgor pressure required for cell expansion or if it contributes to cell wall integrity.

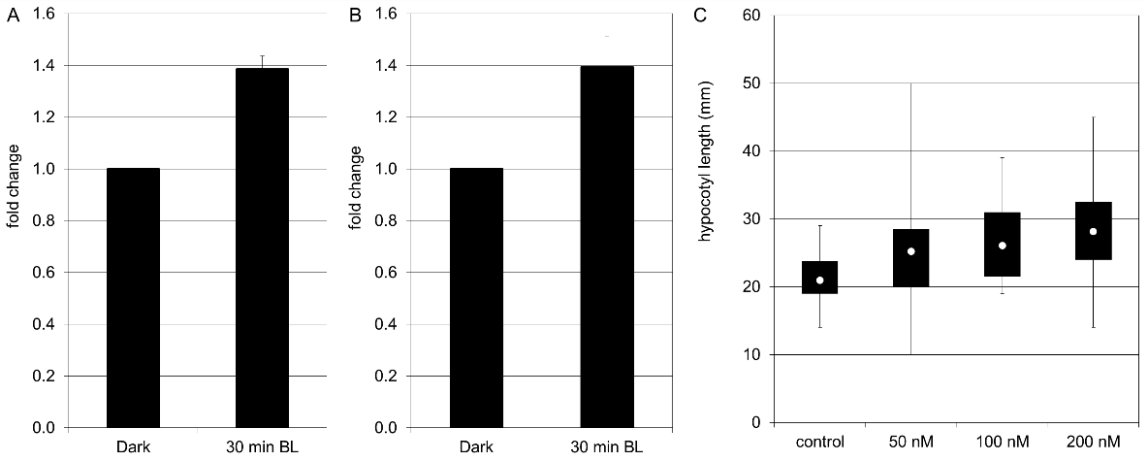

Figure 18. Involvement of V-H$^+$-ATPase during de-etiolation of tomato seedlings. A) Analysis by qPCR of V- ATPase subunit c2 (B-D5) during de-etiolation. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the *pp2ase* gene as reference gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results. **B)** Analysis by qPCR of VHA-A1 subunit during de-etiolation. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the *pp2ase* gene as refernce gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results. **C)**. Effect of bafilomycin A1 on hypocotyl growth of tomato seedlings grown in BL. Germinated seeds were grown either in darkness or under constant BL on Murashige and Skoog medium containing varying concentrations of BafA1. After 5 days of growth, the length of hypocotyl was measured with a ruler to the nearest millimeter. The data are presented as boxes and whiskers. The whiskers represent the range of the data; the white dot within the box indicates the median value, while the boxes' lower and upper boundaries indicate the first and third quartiles, respectively. An average of 45 plantlets coming from independent replicates was measured. The non-parametric Kruskal-Wallis Anova with multiple comparison of mean rank was used for statistical significance of the data (Software: Statistica 12); a: statistically different from the control condition with p-value ≤ 0.01.

CHAPTER II

14-3-3 PROTEINS AND CYTOKININS INVOLVEMENT IN HYPOCOTYL GROWTH INHIBITION UNDER BLUE LIGHT

The phytohormones, cytokinins (CKs) largely contribute to the hypocotyl growth inhibition during de-etiolation. Notably, reversible phosphorylation is a key event of cell signaling, allowing proteins to become active or generating a binding site for specific interaction. 14-3-3 proteins are phosphopeptide-binding proteins regulating a variety of plant responses from seed seed germination to flowering. The expression, hormonal regulation, and proteomic network under the control of 14-3-3s were addressed in tomato (*Solanum lycopersicum* L.) during blue light (BL)-induced photomorphogenesis. The ancestral epsilon TFT9 isoform and the non-epsilon TFT6 isoform were specifically investigated due to their phylogenetic divergence and their high expression during tomato de-etiolation. The multidisciplinary approach demonstrated that *TFT9* expression, but not *TFT6*, was regulated by CKs and identified *cis*-regulating elements required for this response. Our study revealed more than 130 potential TFT6/9 interactors. Their functional annotation predicted that TFTs regulate the activity of proteins involved notably in cell wall strengthening or primary metabolism. Several interactors were also predicted to be CK-responsive. We demonstrated that 14-3-3s are important actors of BL-induced de-etiolation, mediating CK signaling pathway, coordinating cell expansion inhibition and steady-state growth rate establishment, and reprograming from heterotrophy to autotrophy.

Hloušková P., Černý M, Kořínková N, Luklová M, Minguet EG, Brzobohatý B, Galuszka P, Bergougnoux V. (2019). Affinity chromatography revealed 14-3-3 interactome of tomato (Solanum lycopersicum L.) during blue light-induced de-etiolation. Journal of Proteomics 193:44-61.doi: 10.1016/j.jprot.2018.12.017

RESULTS

EXPRESSION OF 14-3-3S IN HYPOCOTYL ELONGATION ZONE DURING BL-INDUCED DE-ETIOLATION

The phylogenic analysis of tomato 14-3-3s revealed their organization into the epsilon group including TFT7, 8, 9 and 12 and the non-epsilon group encompassing TFT1, 2, 3, 4, 5, 6, 10 and 11 (Fig. 19). As in Arabidopsis, this was supported by the exon-intron structure: with structures 7-6 and 4-3 for the epsilon and non-epsilon members, respectively (DeLille et al., 2001). The non-epsilon group can be divided into three sub-groups. In tomato, like in Arabidopsis, the hypocotyl of seedlings grown in the dark elongates along an acropetal gradient, with the portion beneath the cotyledon and hook forming the elongation zone of hypocotyl (Bergougnoux et al., 2012).

The expression profile of the 12 tomato isoforms was determined in the elongation zone by quantitative real-time PCR (qPCR) during BL-mediated de-etiolation (Fig. 20). Unfortunately, the high homology between *TFT4* and *TFT11* did not allow us to design specific primers to distinguish these two isoforms. Interestingly, *TFT12* was not detectable under our study conditions, which was consistent with a predicted expression restricted to pollen both in Arabidopsis and tomato (Keicher et al., 2017).

The non-epsilon *TFT6* and the two epsilon isoforms *TFT9* and *TFT7* were the most abundant in the elongation zone of hypocotyl. Our observation was supported by the proteomic analysis done on the hypocotyl of seedlings grown under different light regimes (Table 7). Indeed, both TFT6 and TFT9 proteins were found to be highly accumulated in the hypocotyl of tomato seedlings grown under continuous BL, with TFT6 being the most accumulated.

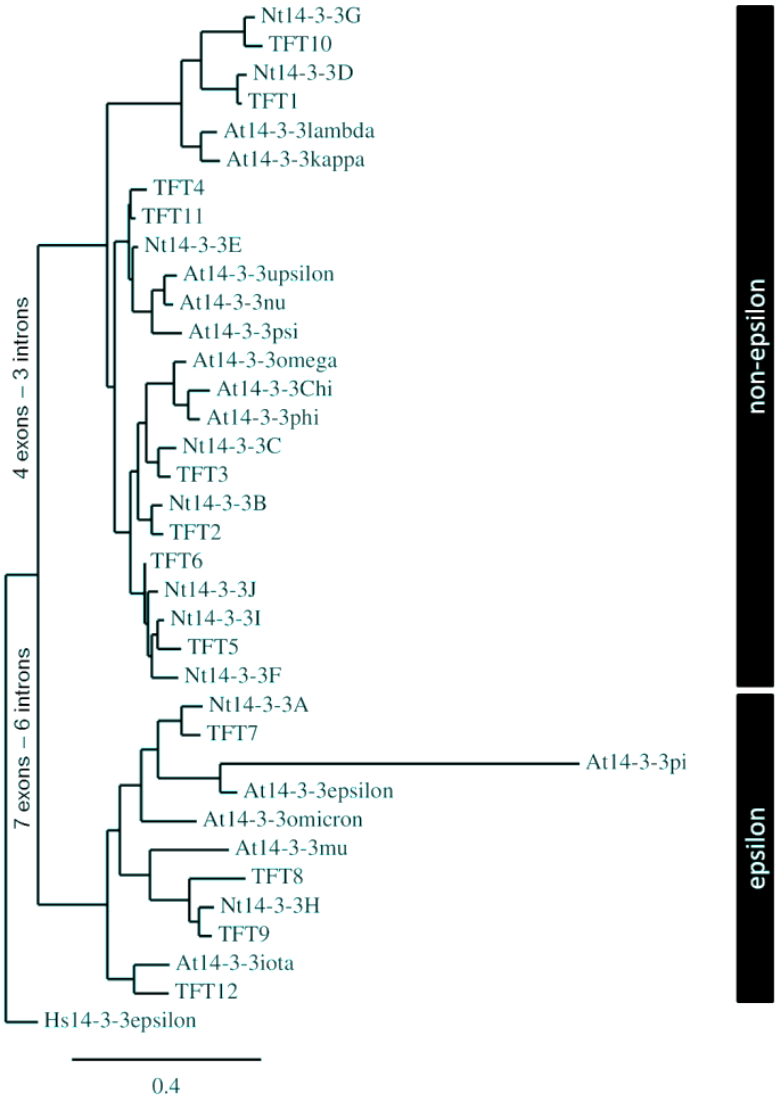

Figure 19. Phylogenic tree of 14-3-3 proteins, including proteins of Arabidopsis (At), tobacco (Nt), and tomato (TFT). One human 14-3-3 protein (Hs14-3-3epsilon) was used to root the tree. The tree was created using workflow Phylogeny.fr (Dereeper *et al*., 2008).

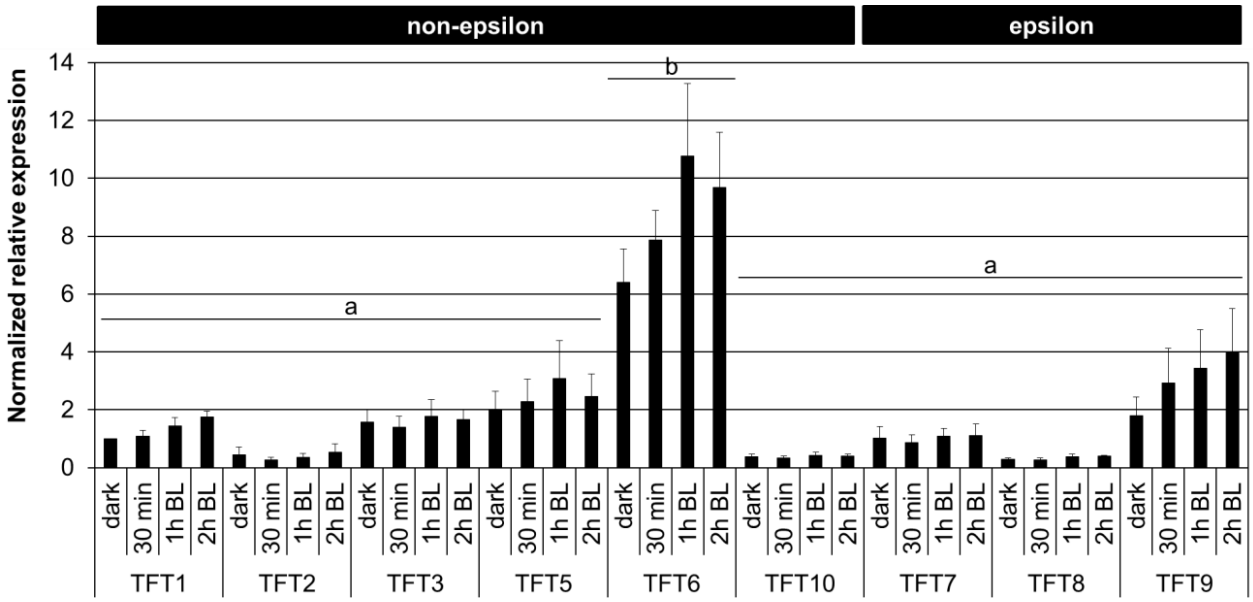

Figure 20. Expression profile of the tomato *14-3-3/TFT* genes in the elongation zone of hypocotyl during BL-induced de-etiolation. The graph represents the average of three to five independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three refernce genes: *PP2Acs* (Solyc05g006590), *Tip41-like* (Solyc10g049850) and *EF1α* ((X14449). Log_{10} transformation, mean centering and autoscalling were performed as described by Willems *et al*. (2008). Results were expressed as fold change relative to the expression of the *TFT1* gene in the elongation zone of seedlings grown in darkness. TFTs are grouped according to their relation to epsilon and non-epsilon group, as indicated by boxes. A non-parametric Kruskal-Wallis Anova & Median test analysis was performed followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft); identical letters indicate no significant difference.

Consequently, we focused our attention on TFT9 (epsilon isoform) and TFT6 (non-epsilon), whose expression was high and stimulated by exposure to BL, thus leading to the hypothesis that they could play a particular role in the process. Moreover, under our study conditions, we observed that tomato plants deficient in *TFT6* expression were shorter than the control plants when grown under continuous BL (10 μmol m^{-2} s^{-1}; Fig. 21). This supported the hypothesis that TFT6 plays a role in light-controlled growth and might be required for cell expansion in light, contributing thus to the establishment of the steady-state growth rate.

Table 7. Proteomic of hypocotyl of tomato seedlings grown under different light regimes. Statistically significant results (t-test; $p \leq 0,05$ are in yellow), the ratios between different light regimes>2,5 are in bold.

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
11S globulin CRU4	Solyc09g025210.2.1	R.ADVYNPQAGR.F [305, 314]	1,8	0,5	1,2	0,0	1,4	0,3	0,02	0,29	0,16
11S globulin seed storage protein 2	Solyc09g072560.2.1	R.GGESFLLSPQR.R [467, 477]	**5,4**	0,3	1,5	0,6	**4,0**	1,5	0,00	0,23	0,03
14-3-3 like	Solyc03g034180.2.1	K.EAAENTLLAYK.S [144, 154]	**23,7**	9,0	1,1	0,4	**21,3**	10,5	0,00	0,29	0,00
1-aminocyclopropane-1-carboxylate oxidase	Solyc06g073580.2.1	K.SFSYTEYLK.I [326, 334]	**7,6**	2,4	1,5	0,4	**5,1**	1,5	0,00	0,02	0,00
1-Cys peroxiredoxin	Solyc03g096040.2.1	K.VTYPIIADPNR.E [93, 103]	**8,2**	3,7	1,7	0,6	**5,3**	3,8	0,00	0,06	0,02
30S ribosomal protein S10	Solyc01g096580.2.1	K.VLNITTR.K [57, 63]	**2,6**	0,4	1,4	0,1	1,8	0,1	0,02	0,15	0,02
30S ribosomal protein S19	Solyc02g082000.2.1	R.FIPLK.- [146, 150]	1,0	0,8	0,7	0,3	1,5	1,1	0,46	0,06	0,15
3-oxoacyl-reductase	Solyc06g071910.2.1	K.VEAPVVIVTGASR.G [75, 87]	**17,3**	13,7	1,6	0,3	**11,0**	8,6	0,00	0,00	0,00
40S ribosomal protein S17-like protein	Solyc05g055230.1.1	K.ILEEVAIIPSK.R [33, 43]	**7,3**	6,1	1,9	1,7	9,0	11,4	0,00	0,13	0,27
40S ribosomal protein S18	Solyc06g073370.2.1	K.LRDDLER.L [106, 112] (missed 1)	**4,8**	0,3	**2,1**	0,2	2,3	0,0	0,00	0,04	0,14
40S ribosomal protein S28	Solyc02g021400.1.1	R.EGDILTLLESER.E [47, 58]	**19,6**	5,8	1,0	0,8	**20,3**	17,4	0,00	0,47	0,03
40S ribosomal protein S6	Solyc08g006040.2.1	R.ISQEVSGDSLGEEFK.G [31, 45]	**6,9**	2,6	1,7	0,7	4,7	3,4	0,00	0,03	0,07
40S ribosomal protein S7-like protein	Solyc06g069090.2.1 / Solyc03g119360.2.1	K.AVVIHVPYR.L [59, 67]	**11,3**	5,5	**2,0**	1,6	**5,9**	5,4	0,00	0,04	0,04
40S ribosomal protein SA	Solyc06g072120.2.1	R.YVDIGIPANNK.G [156, 166]	**6,2**	0,7	1,3	0,3	**4,8**	1,6	0,00	0,15	0,00
50S ribosomal protein L15	Solyc05g009370.2.1	K.ILGDGELSVK.L [197, 206]	**28,7**	5,1	1,3	0,1	**22,1**	3,0	0,00	0,16	0,00
50S ribosomal protein L2	Solyc12g005330.1.1	K.GVVTEIIHDPGR.G [42, 53]	**7,0**	0,2	1,0	0,6	**8,0**	4,5	0,00	0,16	0,04
60s acidic ribosomal protein-like protein	Solyc11g067100.1.1	K.GKDITELIAAGR.E [47, 58] (missed 1)	**6,7**	1,8	**2,1**	0,5	**3,2**	0,0	0,00	0,04	0,04
60S ribosomal protein L10	Solyc06g082650.2.1	K.DAFHLR.V [82, 87]	**12,8**	10,7	1,8	1,0	**6,5**	2,5	0,00	0,14	0,03

Table 7. (continued…)

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
60S ribosomal protein L12	Solyc11g065670.1.1	K.IGPLGLSPK.K [31, 39]	**6,8**	2,4	1,3	0,6	**6,5**	4,8	0,00	0,21	0,04
60S ribosomal protein L23	Solyc09g005720.2.1	K.LDQYAILK.Y [67, 74]	**5,3**	1,2	1,2	0,2	**4,3**	0,2	0,00	0,25	0,03
60S ribosomal protein L3	Solyc01g104590.2.1	K.LTAFLGYK.A [42, 49]	**10,4**	7,4	**2,6**	1,4	5,5	5,8	0,00	0,02	0,16
60S ribosomal protein L7	Solyc08g075090.2.1	K.ILQLLR.L [413, 418]	**9,9**	6,9	0,8	0,3	**11,9**	4,5	0,00	0,33	0,04
Aberrant pollen transmission 1	Solyc04g076540.2.1	R.FAVSDLR.L [1961, 1967]	1,4	0,3	1,3	0,5	1,1	0,4	0,01	0,04	0,39
Actin	Solyc03g078400.2.1	R.VAPEEHPVLLTEAPLNPK.A [97, 114]	**24,2**	11,0	**2,1**	1,3	**11,3**	7,1	0,00	0,01	0,01
Adenosine kinase	Solyc10g086190.1.1	R.ITVITQGADPVVVAEDGK.V [255, 272]	**3,7**	2,1	**3,0**	0,6	1,2	0,5	0,00	0,00	0,13
Adenosylhomocysteinase	Solyc09g092380.2.1	R.AEFGPSQPFK.G [43, 52]	**3,5**	0,8	1,3	0,2	**2,6**	0,3	0,00	0,14	0,00
ADP, ATP carrier protein 3	Solyc07g053830.2.1	K.TAAAPIER.V [97, 104]	**9,3**	3,5	**2,1**	0,5	4,9	2,7	0,00	0,03	0,06
Akonitate hydratase	Solyc07g052350.2.1	R.ILLESAIR.N [141, 148]	**3,3**	0,8	1,8	0,3	1,8	0,7	0,01	0,01	0,15
Alanine aminotransferase 2	Solyc01g007940.2.1	K.GILESLR.R [357, 363]	**7,9**	1,5	1,3	1,0	7,5	4,3	0,00	0,20	0,13
Alcohol dehydrogenase	Solyc11g010960.1.1	K.AFGANVTVISTSPSK.K [203, 217]	**12,3**	9,4	1,2	0,6	**10,2**	8,3	0,00	0,25	0,00
Alpha-1,4-glucan protein synthase	Solyc04g005340.2.1	K.DINALEQHIK.N [116, 125]	**2,6**	1,1	1,2	0,5	2,1	0,1	0,03	0,23	0,13
Ascorbate peroxidase	Solyc06g005150.2.1	K.EGLLQLPSDK.A [199, 208]	**4,1**	1,4	1,1	0,5	**3,7**	2,1	0,00	0,36	0,01
Aspartate aminotransferase	Solyc07g032740.2.1	K.LIFGADSPAIK.E [85, 95]	**8,7**	4,0	1,8	0,7	**4,8**	2,5	0,00	0,01	0,00
Aspartic proteinase nepenthesin-1	Solyc01g096450.2.1	K.SFVPIASGR.Q [89, 97]	**4,4**	0,4	**2,0**	0,2	**2,3**	0,1	0,00	0,01	0,05
ATP synthase beta subunit	Solyc01g007320.2.1	R.LLDFTEK.L [353, 359]	**9,5**	1,5	0,8	0,6	**12,5**	9,9	0,01	0,30	0,04
ATP synthase delta subunit	Solyc01g087120.2.1	K.VESELLDLIAASK.K [80, 92]	**66,0**	37,3	1,2	0,8	**55,0**	38,2	0,00	0,31	0,01
ATP synthase subunit 1	Solyc11g039980.1.1	R.AAELTSLLESR.I [6, 16]	**2,4**	0,3	1,8	0,7	1,4	0,4	0,01	0,15	0,12

Table 7. (continued…)

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
ATP-binding cassette transporter	Solyc11g067000.1.1	R.LPVFFK.Q [604, 609]	**11,7**	5,3	1,7	0,5	**6,9**	3,6	0,00	0,01	0,00
Beta-xylosidase 4	Solyc10g047030.1.1	R.LGFFDGNPK.S [367, 375]	1,8	0,3	1,5	0,2	1,2	0,4	0,00	0,03	0,06
Calmodulin	Solyc10g077010.1.1	R.VFDKDQNGFISAAELR.H [91, 106] (missed 1)	**97,7**	63,9	1,9	1,4	**52,2**	34,4	0,00	0,08	0,00
Chaperonin Cpn10	Solyc07g042250.2.1	K.YAGTEVEFDGSK.H [127, 138]	**5,1**	1,3	0,6	0,3	**8,8**	5,4	0,00	0,19	0,01
Chlorophyll a/b binding protein	Solyc02g070980.1.1	K.SAPSSSPWYGPDR.V [41, 53]	**670,8**	185,4	**2,0**	1,0	**337,8**	150,7	0,00	0,02	0,00
Chlorophyll a/b binding protein	Solyc09g014520.2.1	K.NLAGDIIGTR.T [97, 106]	**829,4**	547,2	1,1	0,4	**920,5**	857,5	0,00	0,20	0,02
Cytochrome c	Solyc01g103220.2.1	K.QGPNLNGLFGR.Q [36, 46]	**5,8**	4,2	1,8	0,3	3,4	2,8	0,00	0,03	0,20
Dihydrolipoyl dehydrogenase	Solyc01g100360.2.1	K.EGFEIGIAK.T [464, 472]	**12,3**	7,2	1,7	1,2	**7,1**	6,3	0,00	0,05	0,02
Elongation factor 1- alpha	Solyc06g005060.2.1	R.LPLQDVYK.I [235, 242]	**7,8**	0,4	1,1	0,1	**7,0**	1,1	0,00	0,31	0,02
Elongation factor 1-beta	Solyc07g016150.2.1	K.SPSAEYVNASR.W [45, 55]	**6,2**	2,8	**2,0**	0,3	3,0	0,7	0,00	0,02	0,07
Elongation factor beta-1	Solyc11g072190.1.1	K.TYISGDQLTK.D [24, 33]	**4,9**	0,9	1,8	0,5	2,8	0,8	0,00	0,05	0,08
Embryonic protein DC-8	Solyc09g008770.2.1	K.AGVIGTIIK.S [71, 79]	**12,7**	1,8	1,7	0,4	**8,0**	3,0	0,00	0,06	0,01
Enolase	Solyc09g009020.2.1	R.IEEELGSEAVYAGASFR.K [421, 437]	**12,8**	3,8	1,3	0,5	**10,0**	2,6	0,00	0,12	0,00
Enolase	Solyc10g085550.1.1	K.VNQIGSVTESIEAVK.M [352, 366]	**3,4**	1,5	1,2	0,3	**2,9**	1,2	0,00	0,13	0,00
Fructose-bisphosphatase aldolase	Solyc05g008600.2.1	R.GILAIDESNATAGK.R [64, 77]	**5,4**	1,0	1,8	0,3	**3,1**	0,7	0,00	0,06	0,01
GDP-mannose 3',5'-epimerase	Solyc01g097340.2.1	K.QLETNVSLK.E [151, 159]	**11,3**	8,1	1,7	0,8	**9,4**	10,8	0,00	0,17	0,03
GDSL esterase lipase	Solyc05g013690.2.1	R.LLITAGQVDK.A [237, 246]	**33,9**	20,1	0,9	0,1	**38,6**	26,8	0,01	0,44	0,02

Table 7. (continued…)

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
GDSL esterase_lipase	Solyc02g071700.2.1	R.ANYPPYGVDFPDGPTGR.F [50, 66]	**5,1**	0,5	1,6	0,1	**3,1**	0,5	0,00	0,02	0,01
Glucose ribitol dehydrogenase	Solyc01g098850.2.1	K.IALVTGGDSGIGR.A [90, 102]	**10,2**	2,3	1,3	0,4	**7,7**	0,5	0,00	0,27	0,05
Glutamine synthetase	Solyc11g011380.1.1	R.TLPGPVTSPAELPK.W [38, 51]	**6,5**	0,2	1,7	0,4	**3,9**	0,8	0,00	0,01	0,01
Glutaredoxin	Solyc06g005260.2.1	K.EIVSGNPVAVFSK.T [7, 19]	**2,4**	2,7	**2,2**	2,0	1,1	1,5	0,02	0,02	0,35
Glyceraldehyde-3-phosphate dehydrogenase	Solyc05g014470.2.1	K.TLLFGEK.A [69, 75]	**5,2**	2,1	1,2	0,0	4,4	1,8	0,00	0,29	0,06
Glycine-rich RNA-binding protein	Solyc01g109660.2.1	R.NITVNEAQSR.G [75, 84]	**7,1**	0,6	**2,3**	0,2	3,1	0,1	0,00	0,02	0,08
Glyceraldehyde-3-phosphate dehydrogenase, cytos.	Solyc06g071920.2.1	K.KVVISAPSK.D [119, 127] (missed 1)	**3,2**	0,3	1,5	0,7	**2,4**	1,4	0,00	0,01	0,01
Guanine nucleotide-binding protein	Solyc03g119040.2.1	R.LWDLQAGTTAR.R [89, 99]	**70,7**	52,6	1,7	0,6	**41,8**	32,1	0,00	0,11	0,04
H+-ATPase	Solyc03g113400.2.1	R.RAEIAR.L [919, 924] (missed 1)	**5,0**	3,7	1,6	0,9	**3,2**	2,7	0,00	0,07	0,03
Heat shock protein 70	Solyc08g082820.2.1	K.FDLTGIPPAPR.G [490, 500]	**3,2**	1,1	2,1	1,9	2,5	1,6	0,00	0,25	0,14
Histone H2A	Solyc12g005270.1.1	K.AGLQFPVGR.I [22, 30]	**5,6**	2,2	1,5	0,0	**3,8**	1,5	0,00	0,09	0,03
Lactoylglutathione lyase	Solyc02g080630.2.1	K.VVNLAIQELGGK.I [287, 298]	1,9	0,0	1,0	0,4	2,0	0,7	0,04	0,36	0,16
Legumin 11S-globulin 3	Solyc03g005580.2.1	K.FFLAGNPQR.G [204, 212]	**9,3**	7,5	0,9	0,8	**10,3**	12,2	0,00	0,42	0,04
Legumin 11S-globulin B	Solyc09g090150.2.1	K.TNDEAITSALAGR.L [434, 446]	**2,8**	1,8	0,9	0,2	**3,2**	2,0	0,00	0,18	0,00
Leucine aminopeptidase 2	Solyc12g010040.1.1	K.FQNPLLQK.L [106, 113]	**5,9**	2,6	1,1	0,6	**6,8**	3,6	0,00	0,13	0,04
Leucine-rich repeat protein kinase	Solyc05g008860.2.1	K.VVSVSIPR.K [67, 74]	1,6	0,8	1,0	0,4	1,6	1,0	0,01	0,25	0,05
Malate dehydrogenase	Solyc03g115990.1.1	K.LFGVTTLDVVR.A [234, 244]	2,0	1,8	0,9	0,7	2,3	2,7	0,06	0,38	0,13

Table 7. (continued…)

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
Nucleoside diphosphate kinase	Solyc01g089970.2.1	R.GLVGEIISR.F [15, 23]	**9,9**	2,4	1,3	0,3	**7,8**	2,2	0,00	0,23	0,01
Pectinesterase	Solyc03g123630.2.1	K.SNTIITASR.N [330, 338]	**4,2**	0,2	**2,0**	0,1	**2,1**	0,0	0,00	0,03	0,04
Peptidyl-prolyl cis-trans isomerase	Solyc01g111170.2.1	K.TAENFR.A [31, 36]	1,8	0,0	**2,0**	0,3	0,9	0,1	0,02	0,00	0,48
Peroxiredoxin	Solyc10g082030.1.1	K.SGGLGDLNYPLISDVTK.S [161, 177]	**25,1**	2,7	0,7	0,2	**39,5**	7,1	0,00	0,23	0,04
Peroxisomal multifunctional enzyme 2	Solyc04g078920.2.1	K.SDAIFDQLK.L [9, 17]	**12,0**	8,5	**2,2**	1,4	**5,4**	4,3	0,00	0,02	0,01
Phosphoenolpyruvate carboxykinase	Solyc04g076880.2.1	K.GSFITSSGALATLSGAK.T [160, 176]	**13,5**	5,2	**2,7**	1,3	6,2	4,9	0,01	0,11	0,10
Phosphoglycerate kinase	Solyc07g066610.2.1	K.GVTTIIGGGDSVAAVEK.V [424, 440]	**5,3**	0,5	0,8	0,0	**6,6**	0,8	0,01	0,29	0,02
Pyruvate dehydrogenase E1	Solyc05g006520.2.1	K.GFGVEAYGADR.K [372, 382]	**4,0**	2,4	**3,0**	1,8	1,3	1,0	0,00	0,00	0,24
Ribosomal L9-like protein	Solyc06g073310.2.1	K.FLDGIYVSEK.G [177, 186]	**6,0**	3,9	0,7	1,0	8,6	12,4	0,00	0,34	0,11
RUBISCO	Solyc02g085950.2.1	K.AYPQAWVR.I [149, 156]	**40,6**	26,3	1,4	0,2	**28,3**	14,0	0,00	0,06	0,00
RUBISCO activase 1	Solyc10g086580.1.1	K.NFLTLPNIK.V [146, 154]	**43,0**	20,9	1,5	0,7	**28,8**	5,8	0,00	0,11	0,03
Susceptibility homeodomain transcription factor	Solyc01g100760.2.1	K.TPTGLAR.T [124, 130]	**5,4**	3,4	1,4	0,7	**3,8**	2,8	0,00	0,07	0,01
TFT10	Solyc04g076060.2.1	K.NVIGSLR.A [60, 66]	1,8	1,1	1,0	0,6	1,8	0,8	0,08	0,46	0,02
TFT3	Solyc04g074510.2.1	K.LIPSATSGDSK.V [113, 123]	N/A		N/A		N/A				
TFT4	Solyc02g063070.2.1	K.TADVEELTVEER.N [34, 45]	**31,9**	20,5	0,8	0,9	38,4	48,0	0,00	0,38	0,06
TFT5	Solyc04g012120.2.1	K.VVAALNGEELTVEER.N [30, 44]	**15,3**	11,1	0,7	0,3	**19,5**	7,2	0,00	0,20	0,00
TFT6	Solyc11g010200.1.1	K.AAQDIANAELAPTHPIR.L [157, 173]	**215,6**	56,6	1,3	0,2	**168,4**	42,0	0,00	0,02	0,00

Table 7. (continued…)

		Peptides	Average ratio + SD (2 biological replicates)						*t*-test		
Protein name	**Gene**	**[*shared*/unique]**	**B:D**		**B:W**		**W:D**		**B:D**	**B:W**	**W:D**
TFT9	Solyc07g053260.2.1	R.NLLSVGYK.N [45, 52]	**27,2**	4,2	1,7	0,3	**15,6**	3,7	0,00	0,00	0,00
Tubulin beta chain 8	Solyc03g025730.2.1	K.LAVNLIPFPR.L [252, 261]	23,3	28,1	**3,1**	4,3	7,5	7,8	0,06	0,13	0,06
UDP-glucose 6-dehydrogenase	Solyc02g088690.2.1	K.NLFFSTDVEK.H [64, 73]	**11,4**	1,3	1,4	0,4	**8,6**	3,1	0,00	0,20	0,02
Uncharacterized protein	Solyc08g006650.2.1	R.DIPDPLGFTR.A [28, 37]	**6,5**	5,6	1,8	1,0	**3,2**	1,4	0,00	0,15	0,03
Vicilin	Solyc09g082340.2.1	R.EVEEIFQR.Q [523, 530]	**2,2**	0,6	1,0	0,0	**2,1**	0,6	0,01	0,44	0,02
V-type proton ATPase subunit A	Solyc12g055800.1.1	R.LGDLFYR.L [577, 583]	**2,0**	1,1	**2,0**	1,1	1,0	0,7	0,01	0,01	0,48
V-type proton ATPase subunit B	Solyc01g111760.2.1	R.TVSGVAGPLVILDK.V [13, 26]	**3,6**	0,9	1,1	0,3	**3,2**	0,1	0,00	0,21	0,03
V-type proton ATPase subunit D	Solyc01g073690.2.1	K.SDALTVQFR.Q [22, 30]	**10,3**	8,5	1,7	0,9	**5,4**	2,3	0,01	0,21	0,02
V-type proton ATPase subunit E	Solyc08g008210.2.1	K.LQLVEAEK.K [40, 47]	**3,4**	2,0	0,9	0,5	**3,7**	2,5	0,00	0,31	0,02
Xylose isomerase	Solyc07g006650.2.1	K.YGLIGEFK.L [218, 225]	**7,2**	0,0	1,7	0,1	**4,3**	0,2	0,00	0,03	0,01

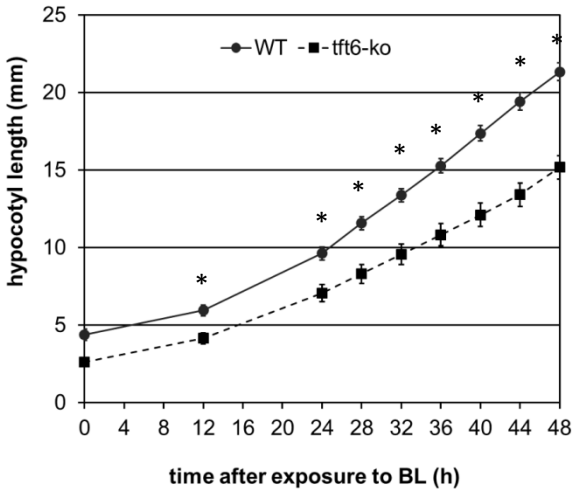

Figure 21. Growth of etiolated hypocotyl of Alisa Craig and *tft6-ko* mutant after exposure to BL. Germinated seedlings grown for 2 days in the dark where transferred to continuous BL (total fluence rate 10 µmol.m^{-2}.s^{-1}). The length of hypocotyl was determined by ImageJ after scanning of the dishes. Three independent biological replicates containing at least 30 seedlings were measured. *:statistically significant between the WT and tft6 (p <0,05;Statistica 12; StatSoft).

IDENTIFICATION OF TFT6/9 INTERACTORS

The 14-3-3/TFT proteins are important actors of the phosphorylation/dephosphorylation signaling pathway cascade. The accumulation of *TFT6* and *TFT9* transcripts in response to BL exposure in the elongation zone of etiolated hypocotyl led us to hypothesize that they are part of the regulatory network triggering de-etiolation. In the present study, we initiated the interactomic study of these two phylogenetically distinct tomato isoforms belonging to the non-epsilon group (TFT6) and the epsilon group (TFT9). For this purpose, the cytoplasmic proteins were extracted from the elongation zone of hypocotyl of etiolated seedlings exposed or not for 2h to BL. Proteins not identified in dark-grown seedlings or significantly accumulated in BL-exposed seedlings were determined as putative BL-dependent TFT interactors. Altogether, more than 870 proteins were found in hypocotyl pull-down assays (false discovery rate, FDR = 1%). Of these, only 133 were exclusive for BL-elongated hypocotyls or significantly increased compared to the darkness-grown hypocotyl elongation zone (absolute ratio > 2.0, $p < 0.05$; Table 7). Most of the proteins identified as potential TFT interactors contained high-scoring 14-3-3 binding sites as determined by 14-3-3-Pred (Madeira et al., 2015). Of these 133 proteins, 18 were found to be nonspecific to one or the other isoform (representing nearly 50% of

the putative TFT9 interactors), 96 were specific to the non-epsilon TFT6, and 19 were specific to the epsilon TFT9 (Fig. 22a). Thirty-three percent of the client proteins identified in the present study have already been reported as client proteins of 14-3-3s in other species (Table 8). The protein–protein interaction network generated by STRING v10 (Szklarczyk et al., 2015), as well as the functional enrichment based on gene ontology and KEGG metabolite pathways, allowed functional annotation of the identified proteins (Fig. 22b-c).

To a large extent, TFT6-interacting proteins belong to the functional category "metabolism" (35% of the sequences). Client proteins belonging to the "cell organization" and "hormone metabolism and signaling" functional categories were found specifically to interact with TFT6. It can also be noted that seven hypothetical TFT6 interactors have a role in cell wall modification and synthesis. In contrast, no particular functional categories could be related to binding with TFT9. Nevertheless, up to 25% of the proteins interacting with TFT9 were related to "transport" and identified as subunits of the vacuolar H^+-ATPAse (V-ATPase).

The functional relevance of the putative TFT interactors identified in cv. Rutgers was supported by a proteomic profiling performed on Micro-Tom tomato seedlings grown for 7 days under various light (continuous BL, white light/WL, or in darkness/D). Altogether, more than 900 hypocotyl proteins were identified in a liquid chromatography–mass spectrometry shotgun analysis of two biological replicates. Only about half of the putative TFT interactors were detectable in the untargeted analysis, however, and just four TFTs were present among the high-scoring proteins. A more sensitive, selected-reaction monitoring-based protein quantitation was therefore employed, and it provided reliable information for 98 putative interactors (Table 8).

Table 8. Putative interactors of TFT6 and/or TFT9 identified by LC/MS analysis in the hypocotyl of etiolated tomato seedlings exposed to blue light.

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Cell wall							
Solyc03g123630	Pectinesterase*	FLARDItFQNT	366	x		no	AT3G14310.1
Solyc02g088690	UDP-glucose 6-dehydrogenase*	TAWNSDtLPIY	46	x		-	not found
Solyc10g047030	Beta-xylosidase 4	RTYRFYtGPTV	621	x		no	AT5G64570.1
Solyc04g015560	Beta-D-glukosidase	NIVRSMsEMVP	196	x		-	not found
Solyc04g005340	Alpha-1,4-glucan protein synthase*	PFFQSAtLPKD	298	x		no	AT3G02230.1
Solyc01g097340	GDP-D-mannose-epimerase	LQTRSFtFIDE	245	x		yes	AT5G28840.2
Solyc07g006650	Xylose isomerase*	VRKRYQsFDSE	351	x	x	no	AT5G57655.2
Metabolism							
TCA or Krebs cycle							
Solyc03g115990	Malate dehydrogenase*	DVVRANtFVAQ	248	x		yes	AT3G47520.1
Solyc01g005560	Isocitrate dehydrogenase*	GLAHRAtLDNN	347	x		yes	AT1G65930.1
Solyc07g052350	Aconitase	EFGKFYsLPAL	124	x		-	not found
Solyc07g055840	Citrate synthase*	LRGRSPsTETD	487	x		-	not found
Solyc03g111140	Malate synthase	HFMRDYsDLLI	335	x	x	no	AT5G03860.2
Glycolysis							
Solyc05g006520	Pyruvate dehydrogenase E1*	PVERIRsLILA	323	x		no	AT1G59900.1
Solyc01g100360	Dihydrolipoyl dehydrogenase	IPAACFtHPEI	444	x		no	AT3G16950.2
Solyc05g008600	Fructose-bisphosphatase aldolase*	AGKRLAsIGLD	82	x		no	AT2G01140.1
Solyc09g009020	Enolase*	-	-	x		no	AT2G36530.1
Solyc10g085550	Enolase*	-	-	x		-	not found
Calcin cycle							
Solyc05g014470	Glyceraldehyde-3-phosphate dehydrogenase*	RGGRAAsFNII	207	x	x	-	not found
Solyc07g066610	Phosphoglycerate kinase*	YLVGAVsTPKR	263	x		no	AT1G56190.1
Solyc02g085950	RUBISCO*	GLKSTAsFPVS	34	x	x	-	not found
Solyc10g086580	RUBISCO activase 1	ITKNFLtLPNI	150	x		-	not found
Solyc02g070980	Chlorophyll a/b binding protein*	RKAVAKsAPSS	42	x		-	not found

Table 8. (continued…)

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Metabolism							
Calcin cycle							
Solyc09g014520	Chlorophyll a/b binding protein*	-	-	x		no	AT5G01530.1
Amino acids							
Solyc08g065220	Glycine dehydrogenase*	QQVRSIsVEAL	67	x		yes	AT4G33010.1
Solyc11g011380	Glutamine synthetase*	MRSKARtLPGP	39	x	x	no	AT5G37600.1
Solyc10g081510	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase*	MARGNAsVPAM	103	x		yes	AT5G17920.2
Solyc12g010040	Leucine aminopeptidase 2*	IFTKFQsSPIW	30	x		no	AT4G30920.1
Solyc01g007940	Glutamate-glyoxylate aminotransferase	TEGAMYsFPQI	391	x		-	not found
Solyc07g032740	Aspartate aminotransferase*	MADRIIsMRQQ	277	x		-	not found
Solyc09g092380	Adenosylhomocysteinase*	LYGCRHsLPDG	247	x		-	not found
Nucleotide metabolism							
Solyc10g086190	Adenosine kinase	-	-	x		-	not found
Solyc11g068830	Uridine 5'-monophosphate synthase	DLRLIVsYPSI	43	x		no	AT3G54470.1
Redox							
Solyc06g005150	Ascorbate peroxidase*	-	-	x		yes	AT1G07890.8
Solyc06g005260	Glutaredoxin	-	-	x		no	AT5G40370.1
Solyc03g096040	1-Cys peroxiredoxin	NKGHKVtYPII	95	x	x	no	AT1G48130.1
Solyc10g082030	Peroxiredoxin	HISQSIsVPSA	31	x		no	AT5G06290.1
Others							
Solyc07g005390	Aldehyde dehydrogenase*	PVIRINsVEEG	404	x		yes	AT2G24270.4
Solyc04g076880	Phosphoenolpyruvate carboxykinase*	IKVRIVsARAY	251		x	no	AT4G37870.1
Solyc02g080630	Lactoylglutathione lyase	CSNRFFtLPRL	36	x		no	AT1G11840.6
Solyc04g078920	Peroxisomal multifunctional enzyme 2	-	-	x		no	AT5G42890.1
Solyc06g071910	3-oxoacyl-reductase	YASRNItVNAI	252	x		-	not found
Solyc04g082780	Cinnamoyl CoA reductase-like	GTVRDPsDDKY	42	x		no	AT2G33590.1
Solyc01g098850	Glucose ribitol dehydrogenase	TTKRGRsYPPV	37	x		yes	AT1G54870.1

Table 8. (continued…)

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Metabolism							
Others							
Solyc01g089970	Nucleoside diphosphate kinase	-	-	x		yes	AT4G09320.1
Solyc11g010960	Alcohol dehydrogenase*	NFSRRAtGEKD	35	x		-	not found
Solyc10g077010	Calmodulin	-	-	x		-	not found
Solyc05g008860	Leucine-rich repeat protein kinase	PSMRHIsDALD	704		x	no	AT2G01210.1
Storage proteins/Remobilization of resources							
Globulin							
Solyc09g072560	11S globulin seed storage protein 2	IRQNSLsLPNF	96	x	x	-	not found
Solyc09g025210	11S globulin CRU4	TTVNSLtLPIL	323	x	x	no	AT5G44120.3
Solyc11g072380	Vicilin	GVMRVVsKGGF	58	x		no	AT2G28490.1
Solyc09g090150	Legumin 11S-globulin B	RRGRAKtDCRI	45		x	-	not found
Solyc09g082340	Vicilin	CQRRCQsEQQG	105		x	no	AT3G22640.1
Solyc03g005580	Legumin 11S-globulin 3	RALRSKtECQI	48		x	-	not found
Lipidic catabolism							
Solyc12g010920	Oleosin	-	-	x	x	no	AT3G01570.1
Solyc02g086490	Oleosin	-	-		x	-	not found
Solyc02g071700	GDSL esterase_lipase	CCQISKtMPEG	293	x		-	not found
Solyc05g013690	GDSL esterase lipase	PKEKHFsEGLY	168		x	-	not found
Solyc08g078160	Oleosin	-	-		x	-	not found
Others							
Solyc09g008770	Embryonic protein DC-8	KERRDQsQRDV	34	x		no	AT2G36640.1
Cell cytoskeleton							
Solyc03g078400	Actin*	PPERKYsVWIG	340	x		no	AT5G09810.1
Solyc04g011500	Actin 4	PPERKYsVWIG	340	x		no	AT3G12110.1
Solyc03g025730	Tubulin beta chain 8*	QQYRALtVPEL	285	x		-	not found
Transport							
Solyc01g087120	ATP synthase delta subunit	AGRIRStLPHL	10	x		yes	AT5G13450.1

Table 8. (continued…)

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Transport							
Solyc01g007320	ATP synthase beta subunit*	ALRRARtRVEA	600	x		yes	ATCG00480.1
Solyc11g039980	ATP synthase subunit 1	-	-	x		-	not found
Solyc04g018100	Sodium/hydrogen exchanger 7	GCLRDQsTEQL	642	x		-	not found
Solyc01g103220	Cytochrome c	-	-	x		no	AT4G10040.1
Solyc07g053830	ADP, ATP carrier protein 3*	ALWRGNtANVI	150	x		no	AT4G28390.1
Solyc11g067000	ATP-binding cassette transporter	LGARSQtMVEY	1104	x		-	not found
Solyc03g113400	Plasma membrane H^+-ATPase (LHA1)*	TRSRSWsFVER	786	x		-	not found
Solyc12g055800	V-type proton ATPase subunit A*	GYVRKVsGPVV	28		x	-	not found
Solyc01g111760	V-type proton ATPase subunit B*	MTRRDHsDVSN	385		x	no	AT4G38510.5
Solyc01g073690	V-type proton ATPase subunit D	SLKRGIsLGSA	226		x	no	AT3G58730.1
Solyc08g008210	V-type proton ATPase subunit E	-	-		x	-	not found
Solyc08g081910	V-type proton ATPase subunit E	-	-		x	no	AT1G64200.1
Solyc04g049330	V-type proton ATPase subunit G1	MLLRHVtTVKN	107	x	x	no	AT3G01390.2
Solyc12g056110	V-type proton ATPase subunit E	-	-	x	x	-	not found
Solyc03g097790	V-type proton ATPase subunit C	SRYWVVsLPVQ	9	x	x	no	AT1G12840.1
Solyc05g056020	V-type proton ATPase subunit G 2	MLLRQVtTVKN	107	x	x	-	not found
Ribosome							
Solyc01g096580	30S ribosomal protein S10	-	-	x	x	-	not found
Solyc09g005720	60S ribosomal protein L23	PKYPRVsAPGR	61	x	x	no	AT2G39460.2
Solyc05g009370	50S ribosomal protein L15	FKARAFsTSAK	216	x		no	AT3G25920.1
Solyc06g073370	40S ribosomal protein S18*	TGRRGKtVGVS	145	x		-	not found
Solyc02g082000	30S ribosomal protein S19	-	-	x		-	not found
Solyc08g006040	40S ribosomal protein S6	GERRRKsVRGC	98	x		-	not found
Solyc05g055230	40S ribosomal protein S17-like protein	VIERYYsKMTL	22	x		no	AT5G04800.4
Solyc03g119360	40S ribosomal protein S7-like protein	PKERNNtEYKL	163	x	x	-	not found
Solyc06g069090	40S ribosomal protein S7-like protein	PKERNNtEYKL	163	x		no	AT1G48830.2
Solyc06g072120	40S ribosomal protein SA	QLQTSYsEPRL	118	x		-	not found

Table 8. (continued…)

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Ribosome							
Solyc06g082650	60S ribosomal protein L10	-	-	x		-	not found
Solyc12g005330	50S ribosomal protein L2	GPARFRsLDFG	31	x		-	not found
Solyc06g083820	60 ribosomal protein L14	VQKRRAsLNDF	101	x		yes	AT2G20450.1
Solyc01g104590	60S ribosomal protein L3	TKKRVVtLRQS	341	x		-	not found
Solyc06g073310	Ribosomal L9-like protein	FGSRKAtASIR	70	x		-	not found
Solyc11g067100	60s acidic ribosomal protein-like protein	GREKLAsVPSG	64	x		no	AT2G27710.4
Solyc11g065670	60S ribosomal protein L12*	ERDRKKtKNIK	96	x	x	-	not found
Solyc08g075090	60S ribosomal protein L7	GGHRMNtAEML	196	x		-	not found
Solyc02g021400	40S ribosomal protein S28	-	-	x		-	not found
Protein profiling							
Solyc08g082820	Heat shock protein 70*	CSRRGNsLVVL	10	x		no	AT5G28540.1
Solyc07g042250	Chaperonin Cpn10*	NNSRSFsRLVV	45	x		yes	AT5G20720.3
Solyc03g007890	Heat shock protein 90	DKIRFEsLTDK	50	x		yes	AT5G52640.1
Solyc04g081570	Chaperone protein htpG	LRERVVtDPED	729	x		yes	AT4G24190.1
Solyc06g083620	26S protease regulatory subunit 4	-	-	x		no	AT4G29040.1
Solyc01g105710	Peptidyl-prolyl cis-trans isomerase*	-	-	x		no	AT5G64350.1
Solyc04g072250	Heat shock protein Hsp20	PPSLSTtTPEG	211	x		-	not found
Solyc07g016150	Elongation factor 1-beta*	-	-	x		yes	AT1G30230.2
Solyc11g069790	RuBisCO large subunit-binding protein	GIARKVtITKD	370	x		yes	AT2G28000.1
Solyc11g072190	Elongation factor beta-1	-	-	x		no	AT5G19510.1
Solyc10g051380	Glycine-rich RNA-binding protein*	LDGRNItVNEA	79	x		-	not found
Solyc01g109660	Glycine-rich RNA-binding protein*	LDGRNItVNEA	78	x		yes	AT2G21660.2
Solyc01g096450	Aspartic proteinase nepenthesin-1	QPLRIKtTPLL	275	x		no	AT5G07030.1
Solyc03g119040	Guanine nucleotide-binding protein	VPRRRLtGHGH	61	x		yes	AT1G18080.1
Solyc12g005270	Histone H2A*	MAGRGKtLGSG	7	x		-	not found
Solyc09g059210	Heat shock protein Hsp20	-	-	x		-	not found
Solyc07g065840	Heat shock protein 90	DKIRFEsLTDK	50	x		yes	AT5G56030.1

Table 8. (continued…)

Category / Accession	Protein description*	Putative binding motive	Position of pS/pT	Interact with TFT6	Interact with TFT9	Potential cytokinin responsive based on homology with Arabidopsis[a]	Arabidopsis homolog sequence[b]
Protein profiling							
Solyc08g079170	Heat shock protein 70	MIARALtRKGT	330	x		no	AT1G62740.1
Solyc06g005060	Elongation factor 1- alpha*	ERERGItIDIA	72		x	-	not found
Solyc01g111170	Peptidyl-prolyl cis-trans isomerase*	NFVKKHtGPGI	100		x	no	AT2G21130.1
Solyc06g036290	Heat shock protein 90	DKIRFEsLTDK	50		x	-	not found
Solyc01g067590	Sucrose non-fermenting 4-like protein	PRRLVWsGPDN	264		x	-	not found
Solyc11g008420	SKP1-like protein	-	-	x		-	not found
Solyc03g005220	Histone H2A*	-	-	x		-	not found
Hormone biosynthesis and signaling							
Solyc06g073580	1-aminocyclopropane-1-carboxylate oxidase*	PKYKSFsYTEY	329	x		-	not found
Solyc12g099380	Two-component response regulator *	MEERSDtTTLK	186	x		-	not found
Solyc11g008560	AP2-like ethylene-responsive TF	ASLRRKsSGFS	359	x		-	not found
Solyc07g043450	CK glukosidase	FACRISsFGLP	32	x		-	not found
Solyc06g081980	Pyridoxal biosynthesis protein	GGVARMsDPQL	79	x		no	AT5G01410.1
14-3-3s/TFTs							
Solyc11g010200	TFT6	IGARRAsWRII	63		x	no	AT1G78300.1
Solyc03g034180	14-3-3 like	IGARRAsWRII	61	x	x	yes	AT5G38480.1
Solyc07g053260	TFT9	VGSRRAsWRIL	62	x		no	AT2G42590.2
Solyc02g063070	TFT4	IGARRAsWRII	63		x	yes	AT5G38480.1
Solyc04g076060	TFT10	AAWRIVsSIEQ	74	x		yes	AT5G10450.1
Solyc04g074510	TFT3	IGARRAsWRII	65	x		no	AT1G35160.2

Each identified TFTs binding partner was tested for the presence of 14-3-3 binding motive using 14-3-3Pred (Madeira et al., 2015): the putative binding motif as well as the position of putative core phospo-serine (pS) or phosho- threonine (pT) is indicated. * identified as 14-3-3s interactor in different species (Paul et al. 2009, Swatek et al. 2011, Shin et al. 2011, Diaz et al. 2011, Dou et al. 2015, Jiang et al. 2005, Pauly et al. 2007, Schoonheim et al. 2007, Chang et al. 2009, Kim et al. 1997, Braun et al. 2011, Arabidopsis Interactome Mapping Consortium 2011). [a] Identification of cytokinin responsive tomato proteins based on their homology with Arabidopsis CK-responsive proteins (Černy et al., 2016). [b] Arabidopsis homolog sequences were predicted by GreenPhyl V4 based on phylogeny and best blast mutual hit (BBMH) matching sequences (Conte et al., 2008)

Our results confirmed that most of the putative TFT6/9 interactors identified are proteins involved in light-dependent growth. By comparing their relative amounts to those from the D-grown hypocotyls, we found that 87 and 64 were significantly accumulated in BL- and WL-grown hypocotyls, respectively. Finally, 34 proteins were significantly more accumulated in BL-grown seedlings than in those grown in WL (Table 7). Nevertheless, 17 of them – including TFT9 and TFT6 – did not meet the selected two-fold threshold criteria. With the exception of TFT10, all detected 14-3-3 proteins were significantly accumulated in response to the BL and/or WL irradiation.

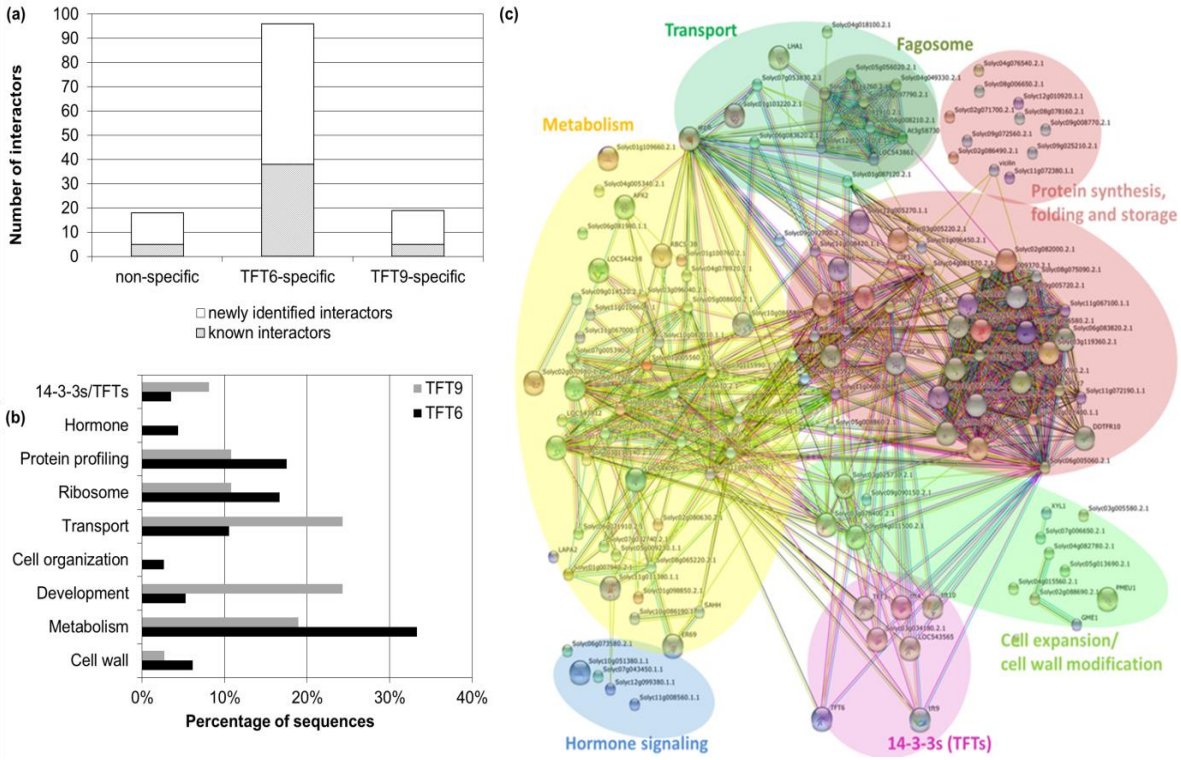

Figure 22. Identification of potential interactors of TFT6 and/or TFT9 (a) and their functional characterization (b). In (a), "non-specific" indicates interactors which were present in the interactomes of both TFT6 and TFT9. The number of already known (gray shaded) and newly identified (white) interactors are also indicated. In (b), the data are expressed as percentages of the total number of proteins identified in the interactome of either TFT6 or TFT9. (c) Visualization of interacting network. Protein–protein interaction network was determined by STRING v10 (Szklarczyk *et al*., 2015). Each node represents a protein, and each line represents an interaction.

14-3-3s form homo- and heterodimers. In our study, we determined that both TFT isoforms possibly interacted with other TFT isoforms. Specifically, TFT6 and TFT4 were identified among the possible client proteins of TFT9 and the interactome of TFT6

contained TFT10 and TFT3. A yeast two-hybrid assay confirmed the interactions between TFT9 and TFT6 and between TFT9 and TFT4. Nevertheless, no interaction between TFT6 and TFT4 could be observed, which is in agreement with the fact that the TFT6 interactome did not contain TFT4 (Fig. 23a). The subcellular localization of the three proteins indicated that whereas TFT6 and TFT9 are localized both in cytoplasm and the nucleus, TFT4 seemed to be restricted to cytoplasm (Fig. 23b–d).

ISOPENTENYLADENINE (IP) AFFECTS *TFT6* AND *TFT9* EXPRESSION

De-etiolation relays on a complex hormonal regulatory network. While auxin, gibberellins, ethylene and abscisic acid stimulate growth, cytokinins strongly limit cell expansion. In tomato, we demonstrated that iP, a free active form of CKs, accumulated in the elongation zone of etiolated seedlings in response to BL exposure (Bergougnoux et al., 2012). Moreover, under the present study conditions, exogenously applied iP resulted in shortening of the hypocotyls in a dose-dependent manner (Fig. 24a), further supporting the role of CKs in hypocotyl growth inhibition (Chory et al., 1994). Two Arabidopsis 14-3-3s (GF14ψ and GF14α) were identified as early CK-response proteins (Černý et al., 2011). Consequently, we addressed the question whether in tomato, *TFT* expression might be regulated by CKs. Therefore, the accumulation of *TFT6* and *TFT9* transcripts was monitored in the hypocotyl elongation zone of seedlings grown on medium supplemented with iP at different concentrations (Fig. 24b). *TFT6* expression was not significantly affected by exogenous iP, whatever the concentration used. In contrast, *TFT9* transcripts significantly accumulated in the presence of exogenous iP in a dose-dependent manner. The Arabidopsis ortholog of *TFT9* (At2g42590.2) was not predicted to be CK-responsive in earlier studies (Table 8), probably due to the difference of material analyzed (Černý et al., 2011; 2016). Taken together, our data indicate a CK-dependent (*TFT9*) and CK-independent (*TFT6*) regulation of gene expression during BL-induced de-etiolation in tomato.

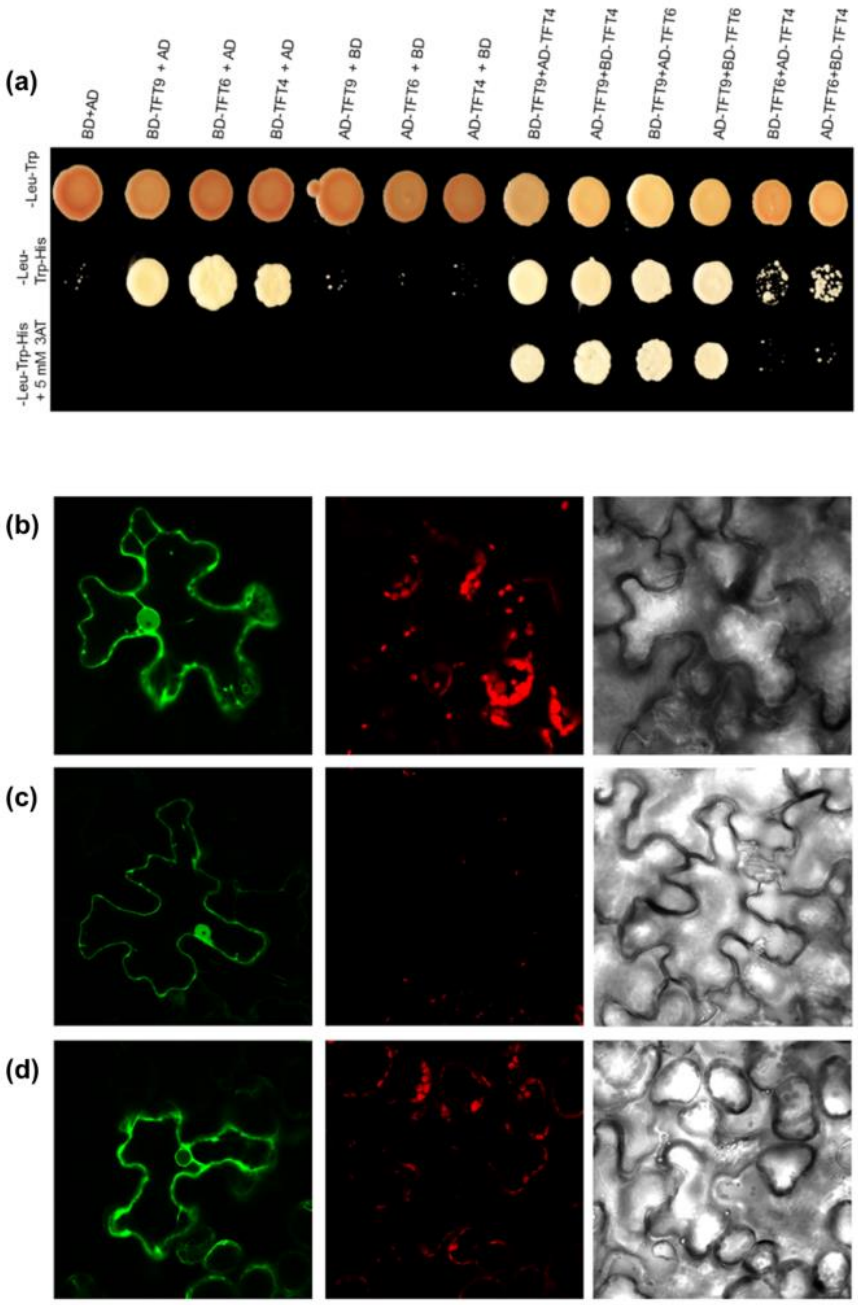

Figure 23. TFT interaction (a) and subcellular localization (b–d). (a) Interaction between TFT9 and TFT6/TFT4 as shown by yeast two-hybrid assays. Representative images are presented and include the respective controls and reciprocal tests. Yeasts transformed with plasmids expressing TFTs fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (-Leu-Trp), leucine, tryptophan and histidine (-Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control. Confocal laser-scanning subcellular localization of TFT9 (b), TFT6 (c), and TFT4 (d) in tobacco leaves.

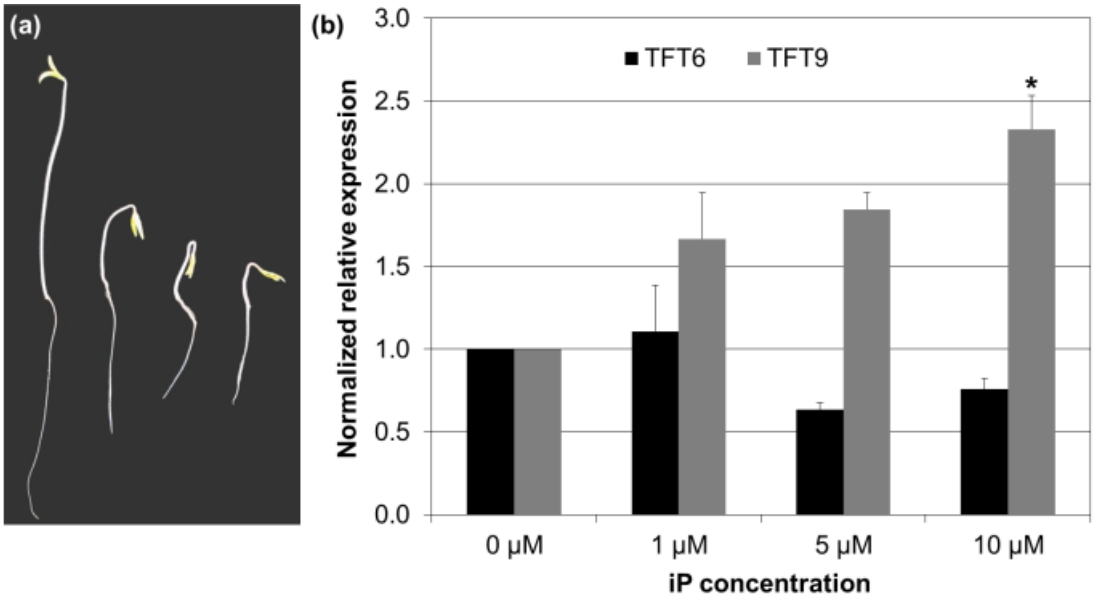

Figure 24. Effect of exogenous isopentenyladenine (iP) on the darkness-grown seedlings of tomato (a), and the expression of *TFT6* and *TFT9* genes in the elongation zone of hypocotyl of seedlings grown in darkness (b). In (a), the seedlings were grown in darkness on Murashige and Skoog medium containing different concentrations of iP. From left to right: control, 1 µM, 5 µM and 10 µM. In (b), three independent biological replicates were analyzed; the bars represent the standard errors of the means. Normalization was done in relation to three housekeeping genes: *PP2Acs* (Solyc05g006590), *Tip41-like* (Solyc10g049850) and *EF1α* (X14449). Log transformation, mean centering and autoscalling were performed as described by Willems *et al*. (2008). Results were expressed as fold change relative to the expression observed in the control sample. A non-parametric Kruskal-Wallis Anova & Median test analysis was performed followed by a post-hoc multiple comparison of mean rank (*: statistically significant from the control sample with $p < 0.05$; Statistica 12, StatSoft).

TFT9 EXPRESSION IS STIMULATED BY IP VIA BINDING OF TWO-COMPONENT RESPONSE REGULATORS (RRS)

We analyzed the upstream region (1.2 kb) of the *TFT9* gene by PlantPAN2.0 using the Arabidopsis database as a reference. We could predict the presence of 21 *cis*-regulating element binding sites related to the type B-cytokinin response regulator (RRB). A transcriptional activation assay was designed in order to confirm the iP-stimulated expression of *TFT9* and identify the binding motifs responsible for this activation. For this purpose, the 1.2 kb native promoter sequence and different truncated versions were fused to the luciferase reporter gene and transfected into tomato protoplasts (Fig. 25a). The addition of iP increased the luciferase activity by 40% compared to mock-treated protoplasts (Fig. 25b). Truncation of the promoter between -1224 and -1158 resulted in a two-fold increase of the luciferase activity, indicating that this region might contain a repressor element. A further deletion of the region between -1158 and -929 resulted in a complete loss of CK stimulatory effect. Two core cytokinin response motifs were present at the positions -1106 and -1035, both localized on the same strand. To decipher their role

or roles in iP-mediated regulation of *TFT9* expression, these two sites were point-mutated (Fig. 25c): mutation in either or both of these binding sites abolished the CK stimulatory effect, suggesting that both sites are required for CK-mediated regulation of *TFT9* expression.

To further validate the possible RRB-mediated activation of *TFT9* expression, the promTFT9::luciferase reporter was co-transformed with the pEarlygate201:ARR1 vector driving the expression of *ARR1*, an Arabidopsis RRB (Fig. 25d). In the absence of exogenous iP, the co-expression of *ARR1* resulted in an increase in luciferase activity by 40%. Removal of the hypothetic "repressor" sequence did not significantly modify the ARR1-stimulated activation of TFT9 promoter. The co-transformation of *ARR1* with promTFT9::luciferase having either one or two mutated RRB binding sites did not result in increased luciferase activity. The same experiment was performed with the constitutively active ARR1DDK, a truncated version of ARR1 from which the receiver domain was removed (Fig. 25e). No increase in luciferase assay could be observed compared to the native form of the ARR1. Based on our results, it might be concluded that CK-regulated expression of *TFT9* is mediated by RRB and requires the two binding sites situated 1106 and 1035 upstream from the start codon.

TFT6 AND TFT9 INTEGRATE CK SIGNALING PATHWAY

Based on the phylogenetic relationship and the best blast mutual hit (BBMH), 74 of the TFT6/9 interactors were identified as orthologs to Arabidopsis genes (Conte et al., 2008). Of them, 21 were predicted to be functional orthologs of known Arabidopsis cytokinin-responsive proteins (Černý et al., 2016; Table 8). These putative CK-responsive TFT interactors are involved in processes such as cell wall modeling, metabolism, transport, and protein profiling. Furthermore, TFT6 might bind with Solyc12g099380, a two-component response regulator homologous to Arabidopsis ARR2 (B-type cytokinin response regulator). Because TFT6 was found to be involved in hypocotyl growth in light conditions, one might assume that TFT6 is involved in a feedback regulation of the CK signaling pathway. In any case, our data demonstrated that TFTs are not only regulated by CKs, but also are part of the CK signaling during tomato de-etiolation.

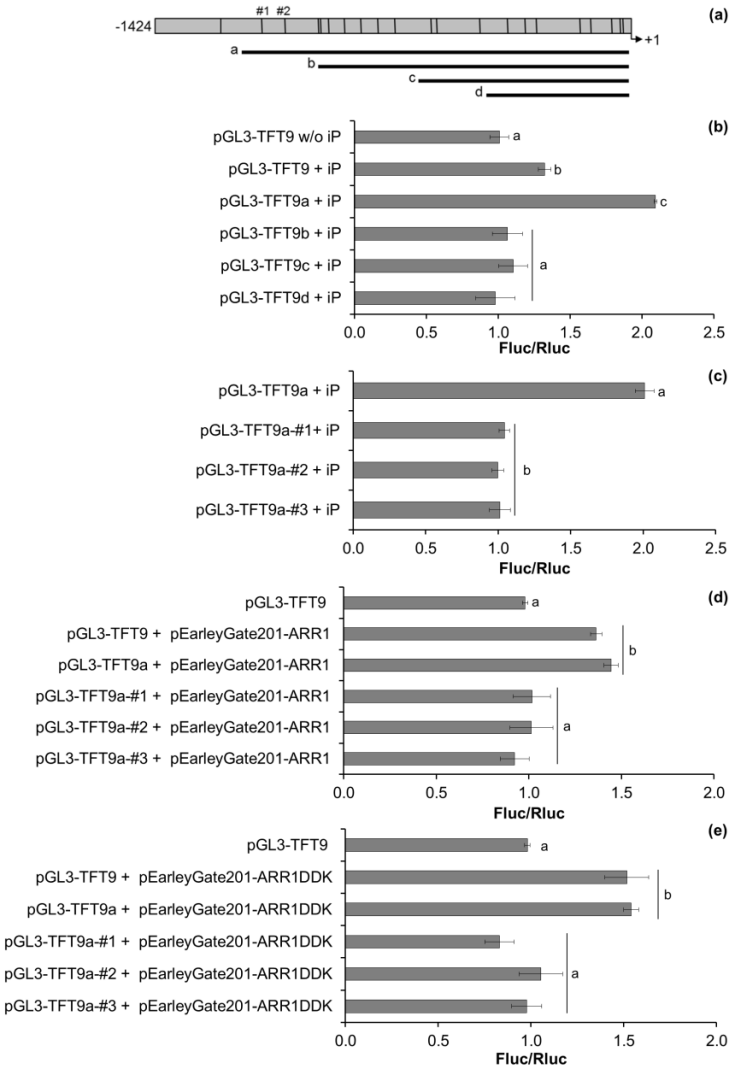

Figure 25. Identification of cytokinin-response region in the *TFT9* promoter by dual luciferase reporter assay. (a) Schematic representation of the different version of the promoter used for the reporter assay. The upper box represents the initial promoter sequence (1424 bp); the arrow followed by "+1" shows the start of the coding sequence of the *TFT9* gene. The lines inside the box indicate the positions of the CK *cis*-regulating elements as identified by PlantPAN2.0. #1 and #2 show the two *cis*-regulating elements which were mutated by point-mutation PCR. The lines beneath the promoter, annotated a–d, depict the four truncated versions of the promoter. b) Dual luciferase assay showing the activity of different truncated versions of *TFT9* promoter. Tomato leaf protoplasts were polyethylene glycol-transformed with different truncated versions of the *TFT9* promoter cloned into the pGL3 vector; the luciferase activity was measured after 16 h of incubation with 10 μM iP in darkness. The letters refer to the different truncated promoters as described in (a). (c) Dual luciferase assay showing the activity of point-mutated *TFT9* promoter. Two *cis*-regulating elements were point-mutated alone (#1 at position -1106 and #2 at position -1035) or in combination (#3). The luciferase assay was performed as described in (b). Dual luciferase assay showing the activity of point-mutated *TFT9* promoter in the presence of the Arabidopsis ARR1 in its native (ARR1; d) or constitutively active (ARR1DDK; e) forms. The luciferase assay was performed as described in (b). Statistical significance was supported by one-way ANOVA and Tukey post-hoc (Statistica 12, StatSoft); different letters indicate that the difference between samples was statistically significant.

VALIDATION OF INTERACTION BETWEEN TFT9 AND THREE PUTATIVE INTERACTORS

Three of the putative TFT9 interactors were further investigated: phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690) and V-ATPase subunit A (V-ATPaseA; Solyc12g055800) (Fig. 25). The analysis of their expression by qPCR indicated that they were all induced by exposure to BL, reinforcing the hypothesis that they might take part in BL-induced de-etiolation (Fig. 26a). The transcription of *PEPCK* and *GDSL* was also stimulated in the hypocotyl of seedling growing in the dark in the presence of 1 µM iP, suggesting that they might be a down-stream target of CK signaling. Such regulation was not observed for the V-ATPaseA, suggesting that different mechanism(s) might be responsible for its regulation during BL-induced de-etiolation (Fig. 26b). Finally, the Y2H assay confirmed that all 3 proteins physically interact with TFT9, confirming and validating thus our interactome data obtained by pull-down assay (Fig. 26c).

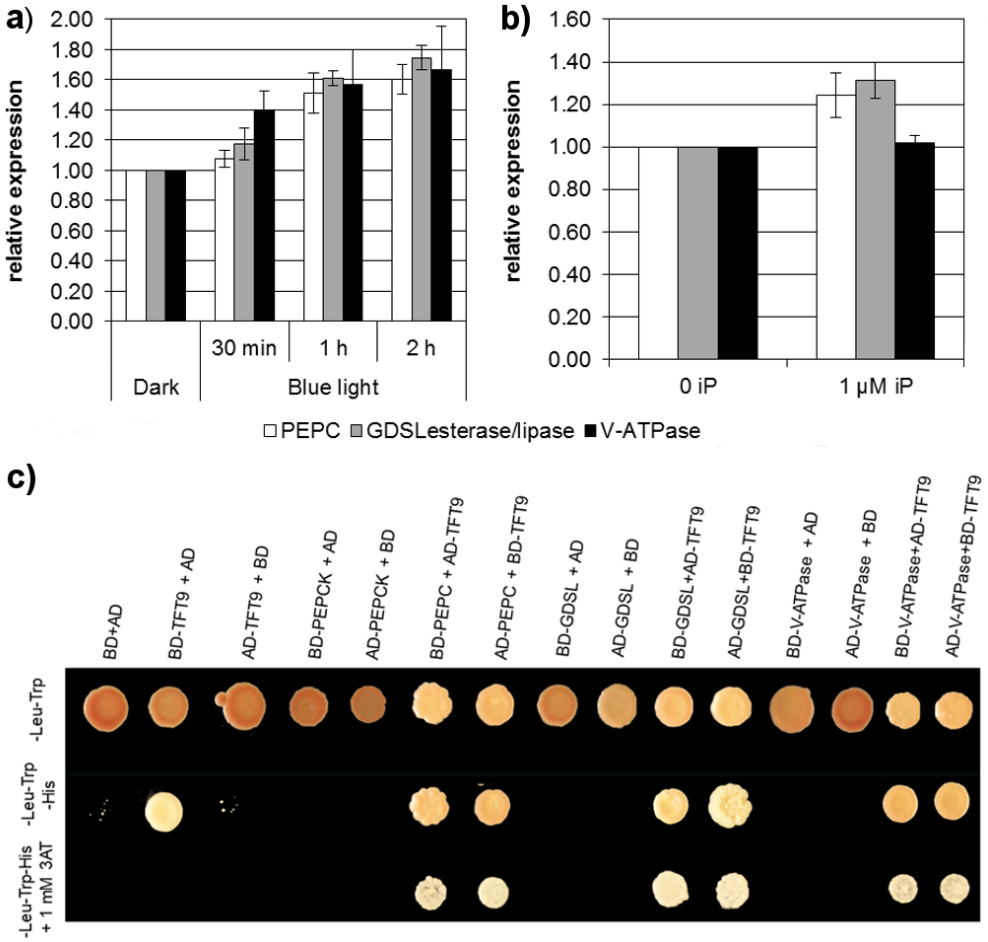

Figure 26. Characterization of 3 putative interactors of TFT9 during BL-induced de-etiolation: phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690) and V-ATPase subunit A (Solyc12g055800). a) Analysis of expression during BL-induced de-etiolation. The graph represents the average of three independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three housekeeping genes: PP2Acs (Solyc05g006590), Tip41-like (Solyc10g049850) and EF1α(X14449). Log transformation, mean centering and autoscalling were performed as described by Willems et al. (2008). Results were expressed as fold change relative to the expression of the same gene in the elongation zone of seedlings grown in darkness. **b) Effect of exogenous cytokinin (1 µM iP) of gene expression in the elongation zone of hypocotyl of dark-grown seedlings.** The graph represents the average of three independent biological replicates, with bars showing the standard errors of the means. Normalization and quantification were performed as in a). Results were expressed as fold change relative to the expression of the same gene in the elongation zone of seedlings grown in the absence of iP. **c) Yeast-two-hybrid assay showing the interaction between TFT9 and different putative interactors.** Representative image is presented and includes the respective controls and reciprocal tests. Yeasts transformed with plasmids expressing TFT9 and a putative interactor (PEPCK, GDSL or V-ATPAse) fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (-Leu-Trp), leucine, tryptophan and histidine (-Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control.

DISCUSSION

TOMATO 14-3-3S ARE REGULATED BY AND ACT DOWNSTREAM FROM CKS

The tomato genome contains 12 isoforms of the 14-3-3 protein family, named TFTs, which are differentially expressed among diverse tissues and have isoform-specific functions (Roberts and de Bruxelles, 2002). Except of *TFT12* which is predicted to be specifically expressed in flower and pollen, all tomato TFT isoforms were detected in the elongation zone of hypocotyl. The abundance of the non-epsilon members was much more important than that of the epsilon members, which was in agreement with the expression of *14-3-3* genes in different species (Keicher et al., 2017). Nevertheless, in the present study, we evidenced both at the transcriptional and protein level that the non-epsilon type *TFT6* and the epsilon type *TFT9* were the most abundantly accumulated in the elongation zone of hypocotyl of etiolated seedlings during BL-induced de-etiolation. It is accepted for a long time that cytokinins (CKs) are mediators of photomorphogenesis and that iP, one of the three active CKs, specifically accumulated in the elongation zone of tomato hypocotyl during de-etiolation (Chory et al., 1994; Bergougnoux et al., 2012). The hormonal regulation of 14-3-3 proteins has previously been reported in Arabidopsis (Lochmanová et al., 2008; Černý et al., 2011; Černý et al., 2016). In tomato, whereas the expression of the non-epsilon *TFT6* gene was insensitive to CK, the hormone strongly regulated the expression of the epsilon *TFT9* gene. The *in silico* analysis of the *TFT9* promoter predicted an enrichment in type-B RR binding sites; two of them at the positions -1035 and -1106 were determined to be essential for the CK-mediated regulation of *TFT9* expression. This was in agreement with the activation of CK-responsive genes requiring the combination of two RBS in Arabidopsis (Ramireddy et al., 2013). The activation of *TFT9* promoter in the presence of the Arabidopsis *ARR1* in tomato protoplasts grown in absence of exogenous CK demonstrated that TFT9 is a primary target of the CK signaling pathway leading to the restriction of hypocotyl growth. Surprisingly, the use of the constitutively active truncated ARR1 protein (Sakai et al. 2001) did not significantly increase the response. This might be related to the polymorphism between Arabidopsis and tomato. Indeed, the Arabidopsis ARR1 recognizes a specific extended motif (Ramireddy et al., 2013) which is different from the two binding sites essential for the

activation of the tomato *TFT9* promoter. Consequently, this might result in an imperfect or limited activation of the tomato promotor by the Arabidopsis ARR1. The *in silico* analysis of *TFT6* promoter also revealed the presence of two RR binding sites in the same orientation and in close vicinity, suggesting a potential regulation by CKs. Nevertheless, an enrichment in RR binding sites in the surroundings of the active sites has been reported as a common trait of CK-regulated genes (Ramireddy et al., 2013). Whereas this was the case in the *TFT9* promoter, it was not observed for the *TFT6* promoter.

Not only TFTs are regulated by CK during BL-induced de-etiolation and photomorphogenesis, but they also are involved in the down-stream events of CK signaling pathway. Indeed, our proteomic investigation determined that a set of putative TFT6/9 interactors are orthologs to known Arabidopsis CK-responsive proteins (Černý et al., 2016) with a role or roles in cell wall modelling, metabolism, or protein profiling. Interestingly, a tomato ortholog of Arabidopsis ARR2, a type-B response regulator, was found to be a potential target of TFT6. Our observation is consistent with the Arabidopsis 14-3-3 interactom (Braun et al., 2011). In Arabidopsis, in the presence of CKs, in contrast to ARR1, ARR2 is phosphorylated and its stability is decreased, driving it to degradation via the 26S proteasome, (Kim et al., 2012; Adams et al., 2014; Shull et al., 2016). Whether the binding with TFT6 leads to stabilization or degradation is not known. Nevertheless, this suggests a role in feedback regulation of CK signaling pathway to desensitize the plant to CKs, keeping the response within a certain range and allowing the establishment of the steady-state growth rate (Fig. 27). Indeed, the analysis of tomato *tft6-ko* plants revealed that TFT6 is required to ensure proper growth in the light. In a different context, the role of TFT6 in promoting growth was also demonstrated during low phosphorus stress (Xu et al., 2012).

TOMATO 14-3-3S FORM HOMO- AND HETERODIMERS

It is well known that 14-3-3s/TFTs form hetero- and homodimers. In our interactomic study, we were able to identify different TFTs among the potential TFT6/9 binding partners, indicating that TFT6 and TFT9 form heterodimers. We cannot answer the important question as to which hetero- or homodimers mediate which responses. Although it is increasingly apparent that 14-3-3s are important, the question of which specific biological roles are associated with the distinct isoforms remains unresolved, especially during BL-induced de-etiolation and photomorphogenesis.

All 14-3-3s examined so far exhibit a highly conserved nuclear export signal (I/LxxxLxxxLxL) in their C-terminal region (Ferl et al., 2002). Both TFT6 and TFT9 were found to localize in nucleus and in cytoplasm in infiltration tobacco cells. Nevertheless, we cannot ascertain that this subcellular localization reflects their specific localization or their localization after interaction with putative endogenous tobacco binding proteins as this was already suggested (Paul et al., 2005).

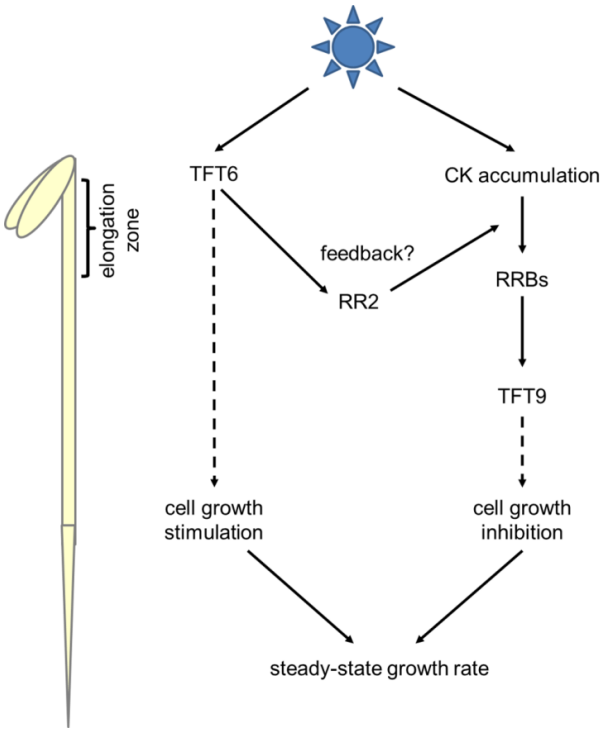

Figure 27. Schematic representation of the interaction between TFTs and cytokinins (CK) during BL-induced deetiolation and photomorphogenesis of etiolated tomato seedling

TOMATO 14-3-3S INTEGRATE HORMONE METABOLISM AND SIGNALING PATHWAYS

Plant hormones form a complex network regulating a plethora of physiological processes, including hypocotyl growth (Vandenbussche et al., 2005; Černý et al., 2016). 14-3-3s have been proved to be important mediators in signaling cascades, notably in brassinosteroid signaling pathway. The 14-3-3 interactom currently available suggests 14-3-3s to participate in processes mediated by almost any phytohormone (Jaspert et al., 2011; Camoni et al., 2018). In tomato, we determined that TFT6 might interact with both CKs and ethylene biosynthesis and signaling. Ethylene has long been reported to play a

role during hypocotyl growth, being an inhibitor in the dark and promoter in the light, and especially under blue light (Ecker et al., 1995; Smalle et al., 1997; Vandenbussche et al., 2007; Yu et al., 2013). The 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, catalyzing the production of ACC, is stabilized by 14-3-3s (Yoon & Kieber, 2013). In the present study, we identified ACC oxidase as a putative interactor of 14-3-3/TFT. ACC oxidase catalyzes the last step of ethylene synthesis. Its binding to 14-3-3 might contribute to ethylene production and accumulation during light exposure. Finally, exogenous CKs increased ethylene biosynthesis in hypocotyl, suggesting a crosstalk between the two hormones (Cary et al., 1995). It is noteworthy that in Arabidopsis the aforementioned ARR2 is a target of both CKs and ethylene (Haas et al., 2004). If such a dual targeting exists in tomato, this might contribute to establishment of the steady-state growth rate observed during de-etiolation and photomorphogenesis.

TOMATO 14-3-3S REGULATE H^+-ATPASES

By generating a proton gradient across plasma membranes, plasma membrane H^+-ATPase (PM-ATPase) provides the driving force for nutrient uptake, phloem loading, water movements, stomatal closure and opening (Comparot et al., 2003). In darkness-grown seedlings, the accumulation of auxin induces activation of the PM-ATPase (Takahashi et al., 2012), leading to acidification of the cell wall (CW) and activation of enzymes responsible for CW loosening. Activation of the pump leads to hyperpolarization of the PM, activation of K^+-inward rectifying channels, water uptake, and subsequent cell expansion (Perrot-Rechenmann, 2010; Chapman et al., 2012). The activity of PM-ATPase varies from an auto-inhibited state where ATP hydrolysis is poorly coupled to H^+ transport and an up-regulated state with a close relationship between hydrolysis and transport (Falhof et al., 2016). Up-regulation of the PM-ATPase requires phosphorylation of its penultimate residue and subsequent binding of 14-3-3 protein that stabilizes the pump in this state (Fuglsang et al., 1999; Svennelid et al., 1999; Rosenquist et al., 2001; Cosgrove, 2014). The phosphorylation of two other sites inactivates the pump, but whether or not this is related to 14-3-3 binding remains unclear (Falhof et al., 2016). Upon exposure to light, one might expect inactivation of the proton pump. In our study, the PM-ATPase subunit LHA1 was identified as a TFT6 interactor. It is hardly conceivable that the binding of TFT6 on PM-ATPase results in the complete inactivation of the enzyme. In fact, when the *tft6*-knockout plants were grown in the light, they had

shorter hypocotyls compared to wild-type plant, suggesting that the functional TFT6 contribute to hypocotyl elongation. For this reason, the binding TFT6 to the PM-ATPase might contribute to a low activity of the enzyme, thus ensuring the establishment of the steady-state growth rate of the hypocotyl whose growth is not inhibited but only restricted during de-etiolation and photomorphogenesis.

In our study, V-ATPases, another type of H^+-ATPase, were found also to interact with TFT6/9. In plants, they are active both at the vacuole and the *trans*-Golgi network (TGN) (Dettmer et al., 2006; Luo et al., 2015). They are multisubunit complexes organized into two domains: the V_1 domain, responsible for ATP hydrolysis, and the V_0 domain, responsible for H^+ translocation (Cipriano et al., 2008). We previously had demonstrated that transcripts encoding different V-ATPase subunits accumulated during de-etiolation in tomato (Hloušková and Bergougnoux, 2016). In the present study, nine subunits of the V-ATPase were found in the interactome of the two tomato 14-3-3s. As they form a complex, the binding of one subunit to the 14-3-3 can result in co-precipitation of the full complex. To our knowledge, only the barley V-ATPase subunit A was found specifically to interact with 14-3-3. The role of V-ATPase in cell expansion is rather controversial. Initially identified as a negative regulator of photomorphogenesis, BL has been shown to induce the activation of V-ATPase and its binding to 14-3-3 (Schumacher et al., 1999; Klychnikov et al., 2007). Inhibition of the TGN-localized V-ATPase restricted cell expansion, making V-ATPase essential for trafficking, transport, and deposition of cellulose required for cell wall strengthening (Brüx et al., 2008; Ferjani et al., 2013). It also has been proposed that V-ATPase might bind to actin in order to constrain cell size (Ma et al., 2012).

TOMATO 14-3-3S REGULATE CELL WALL (CW) REMODELING ENZYMES

Plant CW is composed of cellulose, which forms microfibrils embedded in the hemicellulose/pectin matrix. Pectins contribute greatly to CW strengthening (Harholt et al., 2010). In the present study, several TFT interactors were related to CW remodeling, such as pectinesterase (PME), GDP-D-mannose-3,5-epimerase (GME), UDP-glucose dehydrogenase (UGD), and alpha-1,4-glucan-protein synthase (GPS). PME catalyzes hydrolysis of the methyl ester group of pectin chain; highly methyl-esterified pectins are very plastic, whereas low methyl-esterification results in stiffer CW. In Arabidopsis, the

high activity of PME has been shown to restrict cell elongation (Derbyshire et al., 2007). GME participates in biosynthesis of such CW polysaccharides as glucomannans and pectin (Reiter and Vanzin, 2001). Its partial inactivation causes structural alterations of pectin network and results in growth defects (Gilbert et al., 2009; Voxeur et al., 2011). UGD catalyzes the formation of UDP-glucuronic acid, the main precursor for the synthesis of hemicelluloses and pectic polymers (Lunn et al., 2013; Behmüller et al., 2014). Finally, GPS is highly similar to the reversibly glycosylated polypeptide proteins observed in Arabidopsis and involved in the synthesis of CW components (Rautengarten et al., 2011). Consequently, it appears that 14-3-3/TFTs participate in the inhibition of cell expansion through the regulation of different enzymes involved in synthesis and modification of the CW constituents.

TOMATO 14-3-3S REGULATE PROTEIN FOLDING

The switch from skoto- to photomorphogenesis requires a deep reprogramming of the cell, marked by the activation of transcription and synthesis of new proteins. In this context, we identified a huge number of ribosomal proteins as TFT interactors. Whereas ribosomal proteins are highly abundant and constitute a common artifact in proteome analyses, all those identified here contained a high-scoring TFT-binding motif and their orthologs had been reported to associate with 14-3-3 proteins (Chang et al., 2009; Shin et al., 2011; Swatek et al., 2011). It might be hypothesized that in tomato 14-3-3s regulate translation while associating with ribosomes. Also, several chaperones of the heat shock protein (Hsp) family were identified as potential TFT interactors. Hsp proteins are responsible for protein folding, assembly, translocation, and degradation; their involvement during photomorphogenesis has been demonstrated (Cao et al., 2003; Cazalé et al., 2009).

TOMATO 14-3-3S ARE INVOLVED IN RESERVE MOBILIZATION AND PRIMARY METABOLISM

De-etiolation and the subsequent photomorphogenesis are marked by the switch from heterotrophy to autotrophy. In our conditions, several proteins related to storage were identified as TFT-interacting proteins. These proteins belong to oleosins and globulin family (vicilin and legumin). Oleosins are proteins involved in stabilization of lipid droplets, composed essentially of triacylglycerols (Fang et al., 2014). The

phosphorylation of oleosin induces their degradation via the 26S proteasome signaling pathway, allowing the mobilization of oil storage to provide carbon and energy for growth of the seedling (Parthibane et al., 2012; Deruyffelaere et al., 2015). Vicilin and legumin accumulate in protein bodies during seed maturation. They are mobilized during germination and early seedling development to provide amino acides required for protein synthesis (Tiedemann et al., 2000). Globulins are phosphorylated during germination. Like for oleosins, their phosphorylation might address them for degradation and subsequence resource mobilization (Quiroga et al., 2013; Wang et al., 2007).

Several enzymes putatively regulated by 14-3-3/TFTs were found to be involved in the primary metabolism which includes the Calvin cycle, glycolysis, Krebs/TCA cycle, amino acid metabolism, chloroplast electron transport chain, and sucrose and starch metabolism. The role of 14-3-3s in the primary metabolism has been reviewed by Diaz et al. (2011). Our study on tomato hypocotyl demonstrated that TFT6 and TFT9 contribute to mobilization of resources from storages and initiation of the primary metabolism, which is characteristic for the transition from heterotrophy to autotrophy.

PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) AND GDSL-ESTERASE/LIPASE (GDSL): TWO INTERESTING PUTATIVE INTERACTORS OF THE EPSILON TFT9 ISOFORM

We identified and confirmed by Y2H assay the interaction of TFT9 with a PEPCK (Solyc04g076880) and GDSL (Solyc05g013690) (Fig. 26c). PEPCK are ubiquitous in flowering plants catalyzing the reversible decarboxylation of oxaloacetate to produce phosphoenolpyruvate (PEP) and CO_2. During the gluconeogenesis, PEPCK is responsible for the production of sugars from storage lipids and proteins (Rylott et al., 2003). The resulting sugars constitute both a source of energy for the growing plants and a source of material for new cell wall component deposition (Rylott et al., 2003; Penfield et al., 2004). The activity of PEPCK is regulated by many factors, including light and phosphorylation. In many plant species, PEPCK activity was found to be higher in tissue grown in the dark compared to tissue illuminated by light (Shen et al., 2017). Interestingly, in maize, the activity was found to be related to the presence of the non-phosphorylated form of the PEPCK (Chao et al., 2014). Among the four phosphorylated residues in the N-terminal region of the PEPCK, the Ser^{55} was found to play an important in this reversible phosphorylation. Interestingly, it is localized inside a putative 14-3-3 binding site. Therefore, the light-induced reduction of PEPCK activity might be related to the

inhibitory role of 14-3-3 as it was demonstrated for nitrate reductase, sucrose phosphate synthase and glutamine synthetase (Lea et al., 2001).

GDSL represent a class of newly identified lipolytic enzymes. They function in plant development, morphogenesis, synthesis of secondary metabolites and defense response (Chepyshko et al., 2012). In the tomato genome, we identified 224 putative GDSL which are organized into 3 major clades: I, II and III. One of the TFT9 putative interactors (Solyc05g013690) was identified to belong to the clade Id and to present a high homology with rice and Arabidopsis fucosidases which release the fucosyl moiety from xyloglucan-derived oligosaccharides (Chepyshko et al., 2012; Torre et al., 2002). During etiolation, CW loosening correlates with the increase in the xyloglucan cleavage driven by cellulases with low pH optimum. The resulting fucose-containing nonasaccharide inhibits the growth induced by auxin, protons, gibberellins and fusicoccin that stabilizes the interaction between 14-3-3 and its partner (Marre et al., 1979; Fry et al., 1993; Augur et al., 1993). In pea, α-fucosidase is expressed only in elongating tissue (Augur et al., 1993); in Arabidopsis, AtFXG1 is detectable in leaves of different age, with higher activity in younger, developing leaves (Torre et al., 2002). Both examples pinpoint the relation with cell elongation. In the present study, we confirmed that the GDSL encoded by Solyc05g013690 interact with TFT9. Its expression was stimulated both by exposure to BL and exogenous CK. Further investigation will be required to: 1) determine whether the GDSL has a fucosidase activity, 2) the function of fucosylated oligosaccharides in the control of hypocotyl elongation, 3) the functional significance of the binding to 14-3-3.

We provided evidence that tomato 14-3-3s participate in BL-mediated early seedling development, starting with the regulation of the BL-photoreceptor PHOT1 (Fig. 28). The interactome of two isoforms, TFT6 and TFT9, predicted that 14-3-3s might affect an array of client proteins involved in cell elongation, metabolism, protein synthesis, and hormone pathways. The functional significance of these interactions needs to be further addressed in the future. The Figure 29 depicts the different processes potentially regulated by 14-3-3s during tomato de-etiolation. We have demonstrated that 14-3-3s are potential components of the CK signaling pathway, which in turn regulates their expression. Moreover, the interactomic data provide highly valuable, novel targets

for detailed mechanistic studies that will deal with understanding the role or roles of 14-3-3s during photomorphogenesis.

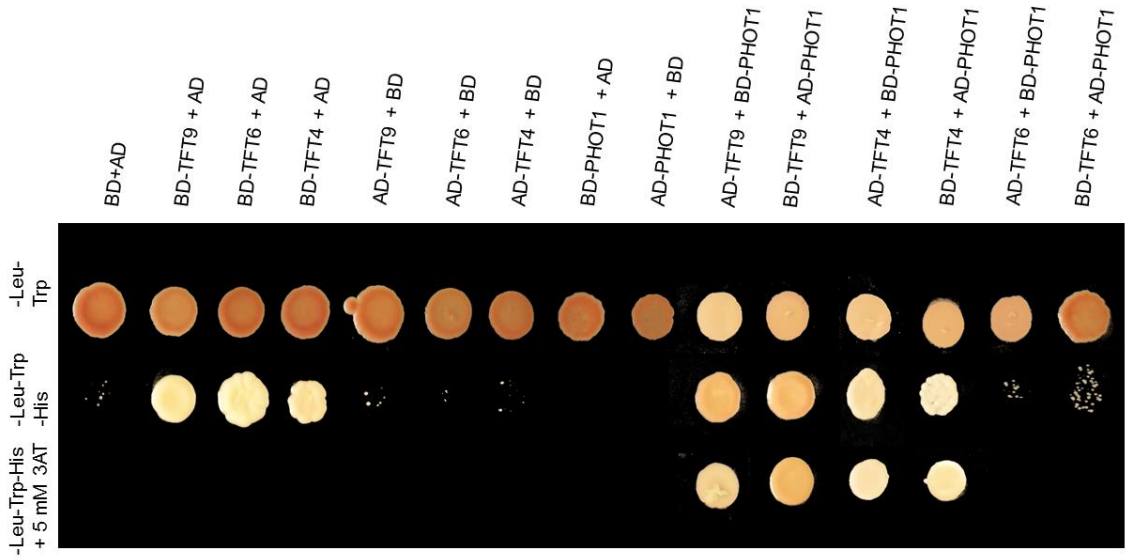

Figure 28. Yeast-two-hybrid assay showing the interaction between TFT9, TFT4 or TFT6 and phototropin1 (PHOT1, Solyc11g072710). Representative image is presented and includes the respective controls and reciprocal tests. Yeasts transformed with plasmids expressing TFT9, TFT4, TFT6 and PHOT1 fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (-Leu-Trp), leucine, tryptophan and histidine (-Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control.

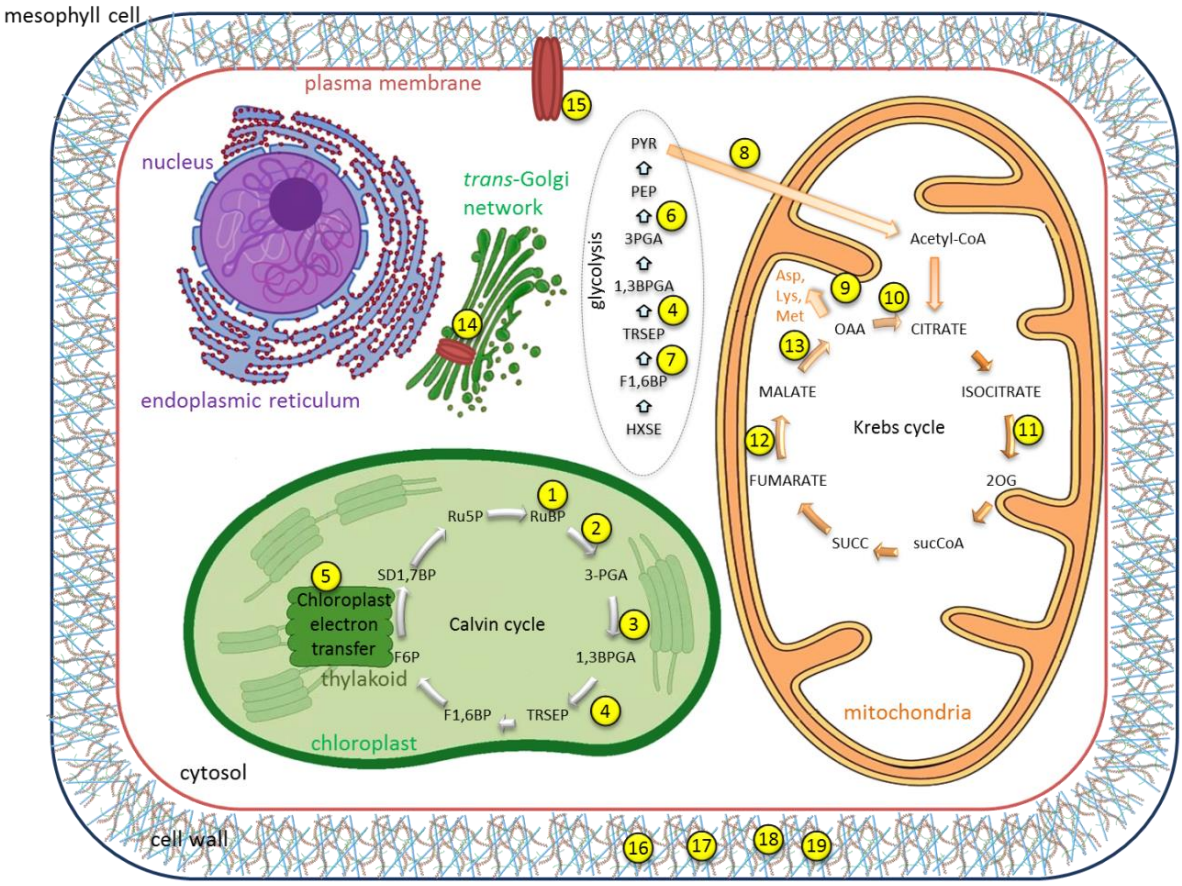

Figure 29. Tomato 14-3-3s/TFTs during blue light-mediated photomorphogenesis. For the sake of simplifying the figure, substrates and some reaction steps have been omitted, and the size of organelles is not respected. Only enzymes which were identified as putative interactors of 14-3-3/TFT are indicated by numbers. 1, Rubisco activase 1 (Solyc10g086580); 2,Rubisco (Solyc02g085950); 3,Phosphoglycerate kinase (Solyc07g066610); 4,Glyceraldehyde-3-phosphate dehydrogenase (Solyc05g014470); 5,chlorophyl a/b binding protein (Solyc02g070980, Solyc09g014520); 6,Enolase (Solyc09g009020, Solyc10g085550); 7,Fructose-bisphosphatase aldolase (Solyc05g008600); 8,Pyruvate dehydrogenase E1 (Solyc05g006520); 9,Aspartate aminotransferase (Solyc07g032740); 10,Citrate synthase (Solyc07g052350); 11,Isocitrate dehydrogenase (Solyc01g005560); 12,Malate synthase (Solyc03g111140); 13,Malate dehydrogenase (Solyc03g115990); 14,V-type proton ATPase (Solyc12g055800, Solyc01g111760, Solyc01g073690, Solyc08g008210, Solyc08g081910, Solyc04g049330, Solyc12g056110, Solyc03g097790, Solyc05g056020); 15,H+-ATPase (Solyc03g113400); 16,Pectinesterase (Solyc03g123630); 17,UDP-glucose 6-dehydrogenase (Solyc02g088690); 18, Alpha-1,4-glucan protein synthase (Solyc04g005340.2.1); 19, GDP-D-mannose-3,5-epimerase (Solyc01g097340).
Abbreviations (listed alphabetically): 1,3BPGA, 1,3-bisphosphoglycerate; 2OG, 2-oxoglutarate; 3PGA, 3-phosphoglycerate; acetyl-CoA, acetyl coenzyme A; Asp, aspartic acid; F1,6BP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; HXSE, hexose (glucose and/or fructose); Met, methionine; Lys, lysine; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; SD1,7BP, sedoheptulose 1,7-bisphosphate; SUCC, succinate; succCoA, succinyl coenzyme A; TRSEP, triose phosphate, collectively dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.

CHAPTER III – MUTANT LINES OF *PHOT1*

The last goal of the thesis was to generate transgenic lines of tomato knocked-down for the blue light photoreceptor PHOT1. Using amiRNA interference, we isolated two tomato lines in the cv. Rutgers background with decreased level of PHOT1. The lowering of PHOT1 abundance affected several phenotypic features, starting with the plant height. Indeed, the transgenic plants were higher compared to wt plants, presented a reduced serration of the leaf margin and curled-leaf phenotype. Moreover, a higher number of trichomes on leaf's surface and stem was observed. All these features indicated a putative protective role during water restriction. This hypothesis is supported by the proteomic study conducted on the leaves of the amiRNA-*phot1* lines showing the upregulation of catalase, aquaporin and protein disulfide isomerase that are known to play a role during drought stress tolerance. The tolerance to drought stress of such plants was analyzed by deep phenotyping while applying different watering regimes on plants and measuring temperature of leaves, gasometric and chlorophyll fluorescence parameters and antioxidant enzymatic activity.

Furthermore, the fruits of the mutants were also affected, harboring a darker green color than WT fruits. This was associated with higher amount of chlorophylls and carotenoids. This later trait of character is in agreement with the prediction that manipulation of the light signalling pathway affects the fruit nutritional quality as it was reported for *cry2* overexpressor, mutants in *det1*, *ddb1*, *cop1*. The proteomic analysis of fruits showed higher abundance of proteins involved in carotenoid biosynthesis and influencing the organoleptic properties of fruits, such as endo β-mannase modifying the fruit firmness, heat shock protein vis1 influencing the juice viscosity, ADH2 and esterase affecting flavor.

RESULTS

CHARACTERIZATION OF TWO TOMATO amiRNA-*phot1* MUTANT LINES

Knocking-down of *PHOT1* gene in tomato was obtained by using the amiRNA methodology that offers a higher specificity than the classical RNA interference. Immature fruits of tomato were infiltrated with *Agrobacterium tumefaciens* LA4404 containing the amiRNA precursor (also sometimes referred to as amiRNA stem loop) in the pSK36 binary vector, conferring resistance to kanamycin *in planta*. Unfortunately, the selection of mutant plants by kanamycin was not efficient in tomato. Therefore, plants with altered PHOT1 abundance were selected based on their phenotype, and more specifically based on their taller stature that is expected in case of photoreceptor mutants. Two independent lines were retrieved from this screen.

In order to validate that the observed phenotype was independent for the place of insertion of the transgene, we used the method of ligation-mediated polymerase chain reaction (LM-PCR) to determine the place of insertion of the transgene in the genome. We found that the transgene was inserted in the chromosome 12 at the position 12 239 242. The analysis of the genome in this region indicated the presence of two genes: myosin 9-like factor (Solyc12g019920.1) located 155 404 bp downstream of the insert and the MYB44-like transcription factor (Solyc12g019910.1) located on the reverse strand only 490 bp upstream of the insert (Fig. 30).

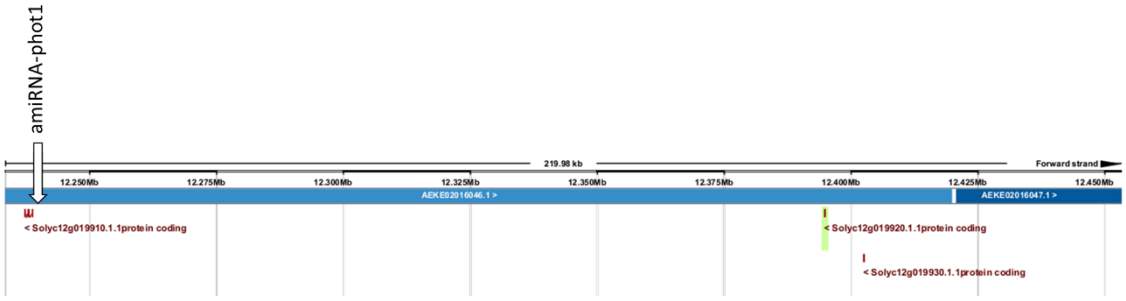

Figure 30. Graphical representation of the region of tomato genome where the transgene (amiRNA-*PHOT1*) was introduced in the tomato line. Note that the insertion occurred in close vicinity to Solyc12g019910.1, encoding a MYB44-like transcription factor.

The close vicinity between MYB44-like transcription factor and the transgene led to the hypothesis that insertion of the transgene might influence the expression of *MYB44-like*. Therefore, qRT-PCR analysis was used to check *MYB-44-like* expression. The expression was not detected neither in wt nor in the mutant line (data not shown). Our results are supported with data available from public databases (Genevestigator; (Hruz et al., 2008) and tomato eFP browser; (Winter et al., 2007)) where no expression of this transcription factor was reported. Also we concluded that the observed phenotypes of the amiRNA-*phot1* mutant line were solely the results of the silencing of the *PHOT1* gene.

PHOT1 RNA/PROTEIN LEVEL VERIFICATION

The expression of *SlPHOT1* was investigated by qRT-PCR analysis in leaves of wild-type and mutant plants. Gene expression of *PHOT1* in mutant lines was decreased to appox. 50% compared to the wt (Fig. 31).

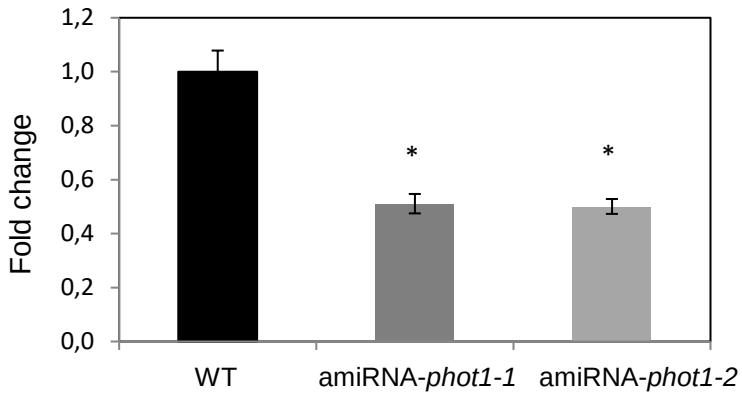

Figure 31. Expression profile of the tomato *PHOT1* gene in the leaves of cv. Rutgers (wt) and amiRNA-*phot1* lines. The graph represents the average of four independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three housekeeping genes: *PP2Acs* (Solyc05g006590), *Tip41-like* (Solyc10g049850) and *EF1α* (X14449). Log_{10} transformation, mean centering and autoscalling were performed as described by Willems *et al*. (2008). Results were expressed as fold change relative to the expression of the *PHOT1* gene in the wt leaves. * Significantly different to wt in Kruskal-Wallis Anova followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

Furthermore, western blot analysis of wild-type protein extract probed with specific anti-phot1 antibody detected a 110-kD band which is the molecular mass reported for tomato PHOT1 (Knieb et al., 2004). While a band of the same size was also detected in the amiRNA-*phot1* lines, its intensity was less than 30% that of the wild-type one (Fig.32)

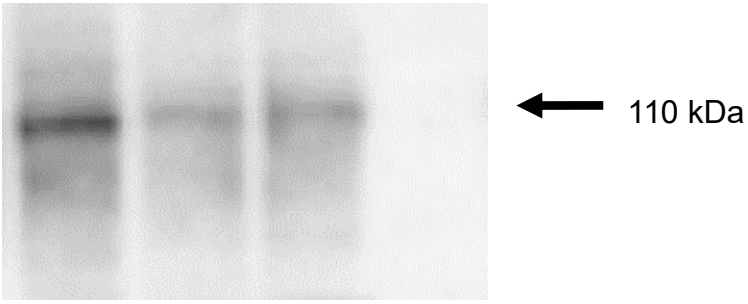

Figure 32. Western blot of PHOT1 in cv. Rutgers and amiRNA-*phot1* tomato lines. Immunodetection of PHOT1 was performed in protein extracts isolated from 3-day-old etiolated tomato seedlings. From left – WT; amiRNA-*phot1-1;* amiRNA-*phot1-2.*

PHENOTYPIC CHARACTERIZATION

The downregulation of *PHOT1* in tomato caused considerable changes in several features of plant phenotype. The plant height was altered the most noticeably. After 20 days, mutant plants demonstrated 30% increase in plant high compared to wild-type plants (Fig.33, 34a).

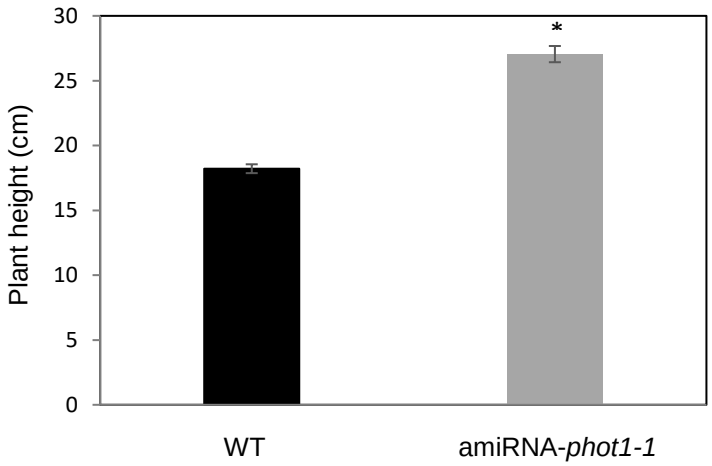

Figure 33. Height (cm) of 20-day-old plant of cv. Rutgers and amiRNA-*phot1*mutant line. The graph represents the average height of 10 plants, with bars showing the standard errors of the means. *Difference between wt and mutant significant at $p<0.05$ in Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

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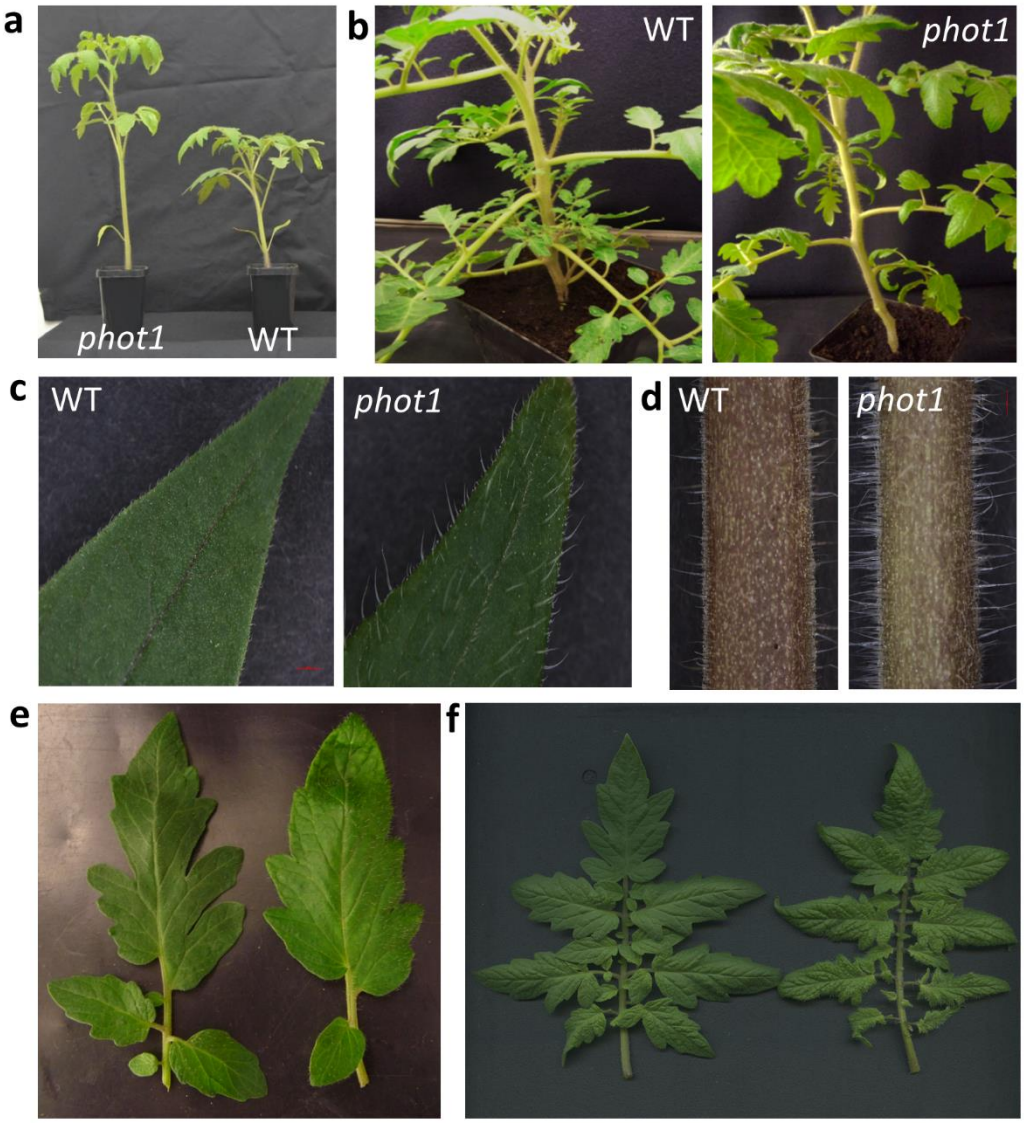

Figure 34. Phenotypic characterization of the cv. Rutgers and amiRNA-*phot1* mutant line. a) plant height – 20-day-old plant watered to 80% field capacity, b) stem branching, c) leaf trichomes, d) stem trichomes, e) representative leaflet, f) representative leaf.

Furthermore, the stem branching was reduced in mutant plant in comparison with wt, indicative of a stronger apical dominance in the mutant line (Fig. 34b). Higher amount of trichomes on both leaf and stem surface was observed in mutant plants (Fig. 34c, d). Interestingly, the mutant was also characterized by altered morphology of leaflets with reduced serration of the leaf margin (Fig. 34e, f). Finally, difference in leaf flattening were observed in transgenic line compared to the wt. The leaves of amiRNA-*phot1* gently curled downwards, whereas the wt exhibited flatter leaves (Fig. 35)

Figure 35. Leaf flattening in cv. Rutgers and amiRNA-*phot1* mutant line.

Because of the involvement of PHOT1 in de-etiolation of hypocotyl, the hypocotyl elongation was followed for eight days after sowing both in WT and amiRNA-*phot1* mutant line. In the dark, the hypocotyl of mutant was slightly shorter compared to wt till one week after sowing (Fig. 36a), then the hypocotyl growth rate rapidly increased in the mutant (Fig. 37a). Under BL, no significant change in hypocotyl length was determined between mutant and wt (Fig. 36b), however, from eighth day after sowing, the hypocotyl growth rate of mutant increased (Fig. 37b).

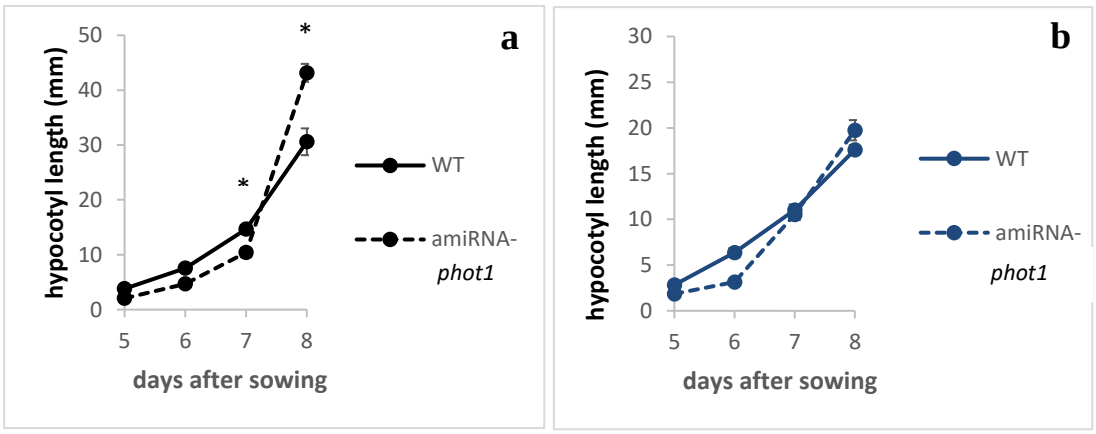

Figure 36. Analysis of hypocotyl of cv. Rutgers (line) and amiRNA-*phot1* (dash line) mutant line. Hypocotyl length in the dark (a) or in BL (10 µmol $m^{-2}s^{-1}$) (b) from fifth to eighth day after sowing. The graph represents the average hypocotyl length of four independent biological replicates (n=20) with bars showingthe standard errors of the means. *Difference between WT and mutant significant at $p<0.05$ asd described by Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

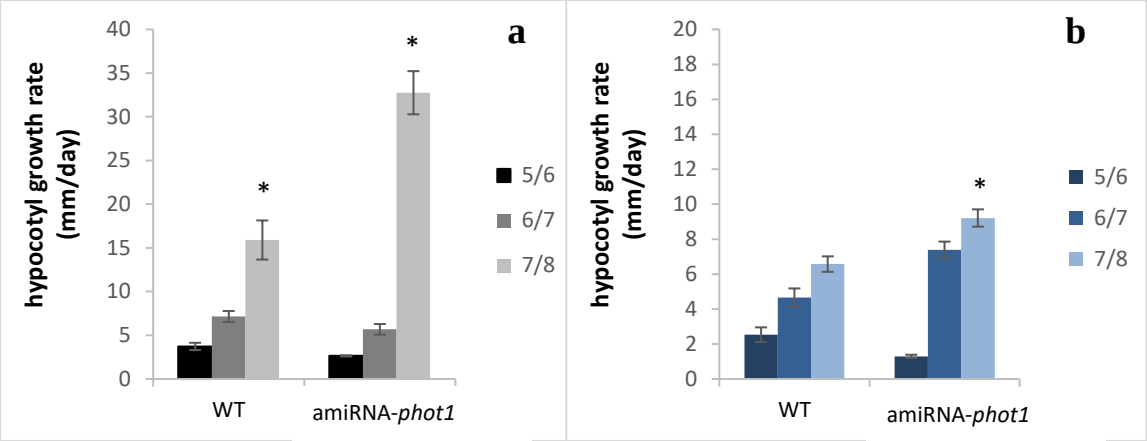

Figure 37. Analysis of hypocotyl growth rate of cv. Rutgers and amiRNA-*phot1* mutant line. Hypocotyl growth rate was evaluated as average increases in hypocotyl length at 24 h intervals in seedlings from fifth to eighth day after sowing in dark (a) and BL (10 µmol $m^{-2}s^{-1}$) (b). The graph represents the average hypocotyl growth rate of four independent biological replicates (n=20) with bars showing the standard errors of the means. *Difference between WT and mutant significant at $p<0.05$ in Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

The phenotypic difference between amiRNA-*phot1* line and wt was also observed in fruits. The amiRNA-*phot1* fruits were darker green compared to wild-type (Fig. 38). Whereas the fruit was bigger, the total number of fruits per plant were decreased in mutant (Fig. 39a, b). The fruits of mutant plant had also decreased number of seeds (Fig. 39c, 40).

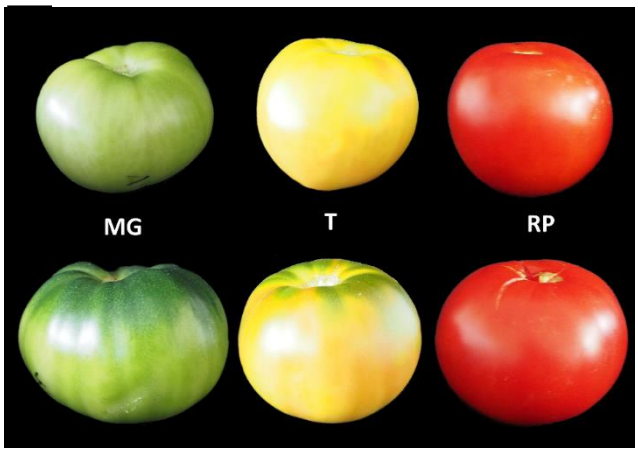

Figure 38. Tomato fruit ripering stages of cv. Rutgers (up) and amiRNA-*phot1* mutant (down). The collected stages include mature green (MG - 30 day post-anthesis (DPA), turning (T – 37 DPA) and red ripe (R-46 DPA).

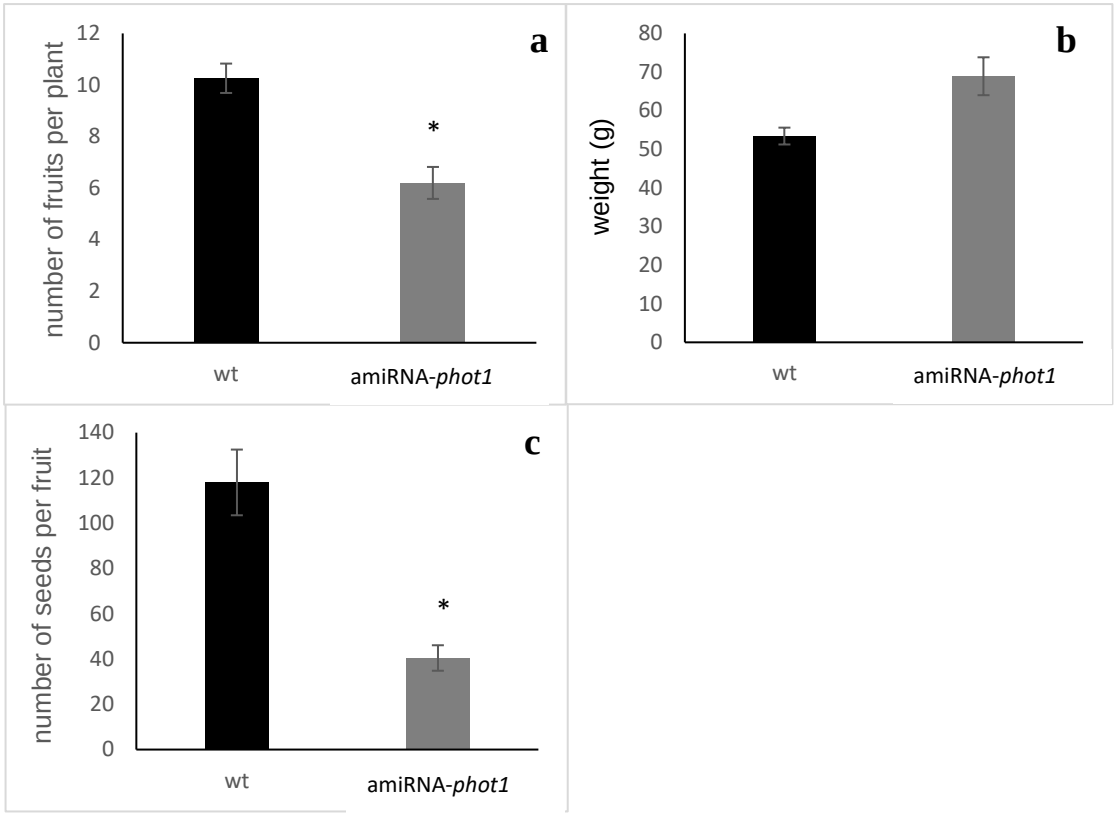

Figure 39. Fruit characterization of cv. Rutgers and amiRNA-*phot1* mutant plant. a) Average number of fruits per plant b) Average fruit weight c) Average number of seeds per fruit. The graphs represent the average value from fifteen plants with bars showing the standard errors of the means. *Difference between wt and mutant significant at $p<0.05$ in Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

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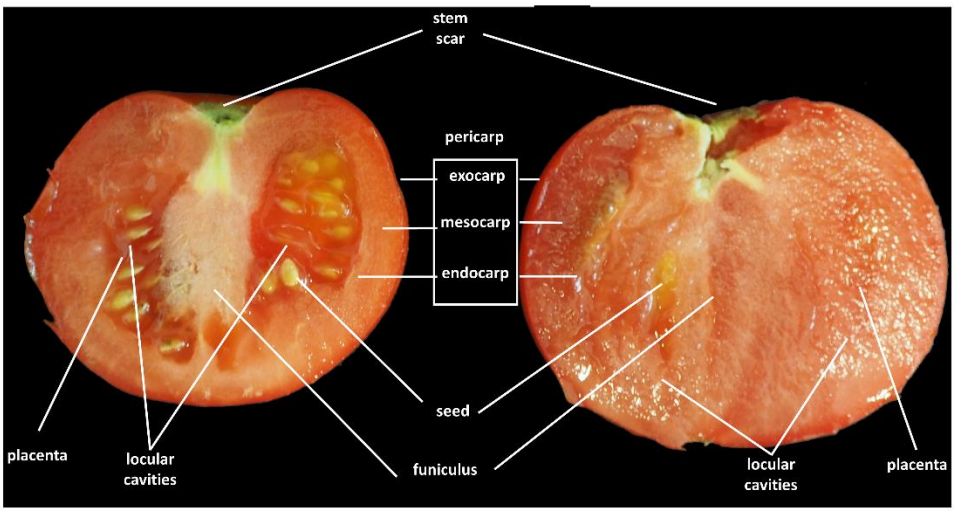

Figure 40. Transverse section of tomato fruit of cv. Rutgers (left) and amiRNA-*phot1* line (right). The fruits are internally divided into sections called locular cavities separated by the funiculus. The seeds are localized in placenta. The most external part is the pericarp which is composed from exocarp, mesocarp and endocarp.

During our work with amiRNA-*phot1* mutant we noted that amiRNA-*phot1* seeds germinated poorly in darkness. To examine the potential effect of PHOT1 on the dormancy-to-germination transition, we measured the germination frequencies of amiRNA-*phot1* line and corresponding wt seeds in the dark and under blue light conditions. When seeds were imbibed and kept in darkness, the mutant was delayed by 1 day in its germination compared to wt. Nevertheless, the time to reach the maximum germination frequency was shorter as both gentypes reached 80% of germinated seeds on the 8th day of experiment (Fig. 40a). When seeds were imbibed and germinated under constant BL illumination, the mutant showed lower germination frequency than WT throughout the duration of the experiment (Fig. 40b).

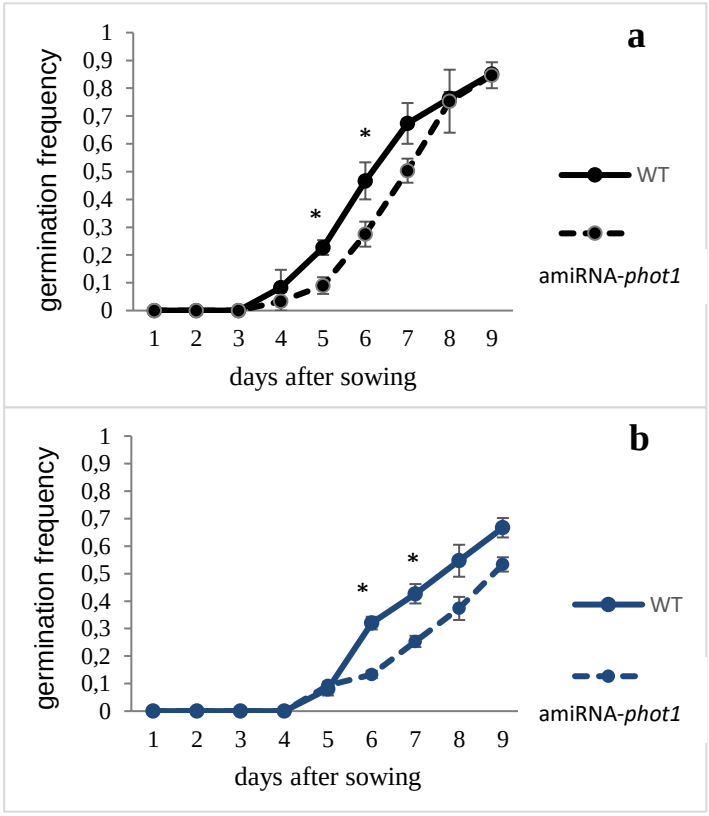

Figure 40. Induction of germination of cv. Rutgers and amiRNA*phot1* line in the dark (a) and in blue light (10 µmol $m^{-2}s^{-1}$) (b). Germination frequency was determined during nine days after sowing. The graph represents the average germination frequency of four independent biological replicates (n=50) with bars showing the standard errors of the means. *Difference between WT and mutant significant at $p<0.05$ in Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

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The proteomic characterization of tomato fruits and mature leaves from amiRNA-*phot1* line and corresponding wt (cv. Rutgers) was achieved by LC-MS/MS analysis of proteins extracted from pericarp harvested at two stages of fruit development: mature green and red ripe. This analysis allowed a comparison of the protein repertoire in wt and mutant line, determining the quantitative variations in each of them (Tab. 9, 10). Twenty-nine proteins in fruits and fifty-three proteins in leaves showed significant difference in amiRNA-*phot1* compared to wt in all biological replicates with validation on two unique peptides (Tab. 9).

The chlorophyll and carotenoid contents were determined in mature green and red ripe fruits of both mutant and wt using HPLC. We observed that the darker green color of mutant fruit could be related of higher content of chlorophylls. In both, green and red fruits, higher content of carotenoids were found in amiRNA-*phot1*, predicting better nutritional quality of mutant fruits (Fig. 41).

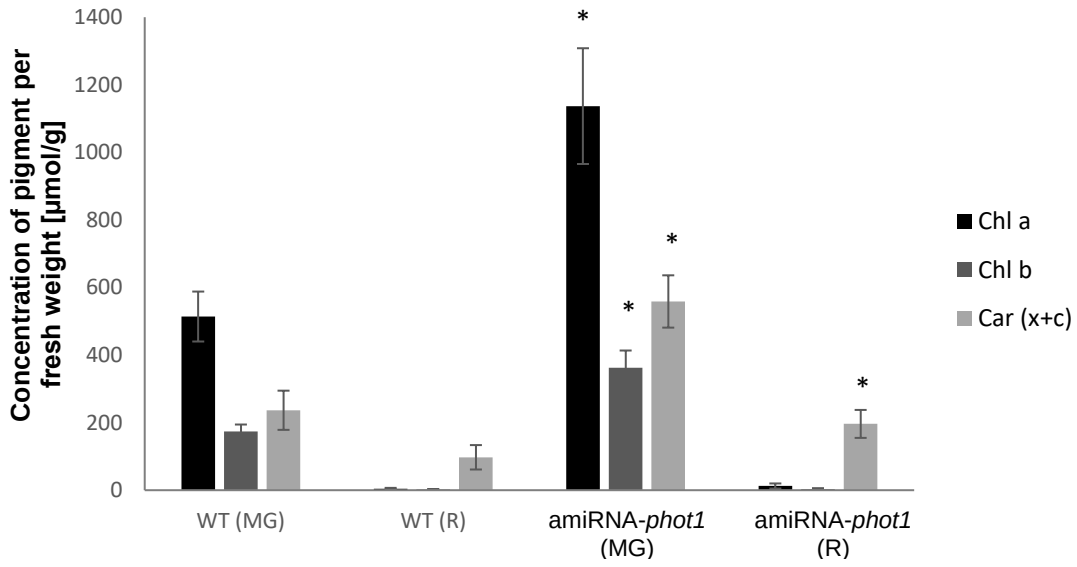

Figure 41. Concentration of chlorophyll a, chlorophyll b and carotenoids in mature green (MG) and red ripe (R) fruits of cv. Rutgers and amiRNA-*phot1* line. The graph represents the average pigment concentration of four independent biological replicates with bars showing the standard errors of the means. *Difference between WT and mutant significant at $p<0.05$ in Kruskal-Wallis ANOVA followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft).

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Table 9. List of the differently expressed proteins between amiRNA-*phot1*fruits and cv. Rutgers in mature green and red ripe stage. Difference between WT and mutant significant at p<0.05 is in red. AA-amino acid; SIG-signaling; CW-cell wall; DEV-development; GLY-glycolysis; STR-stress signaling; RNA – RNA processing; PROT-proteins; LIP-lipid metabolismus; TCA-Krebs cycle; PS-photosynthesis; TRANS-transport; REDOX-redox potential; DIV-division; HOR – hormones; SMET- secondary metabolism.

Accession	Protein description	BIN category	MT/WT green	MT/WT red	peptide
Solyc11g011920	Glutamate decarboxylase	AA	1,85	0,58	K.VLHELDMLPAR.V
Solyc08g014340	Cysteine synthase	AA, SIG	1,32	1,37	K.AFGAELVLTDPAK.G
Solyc01g008710	inactive endo-beta-mannanase	CW	0,52	0,10	K.VAYKDDPTILSWELINEPR.C
Solyc05g014280	small heat shock protein 1	CW;STR;RNA;PROT	1,14	0,76	R.TPQMAPVGLWDR.F
Solyc09g025210	alcohol dehydrogenase-2	DEV	0,21	0,09	K.YGLIGEFK.L
Solyc09g072560	Legumin 11S-globulin	DEV	0,15	0,007	R.GDAQVQVVDHTGQQVMNDR.V
Solyc09g082330	SM80.1 Vicilin	DEV	0,19	0,02	K.SNNPYLFESQR.F
Solyc09g090150	11S storage globulin	DEV	0,13	0,02	K.TNDEAITSALAGR.L
Solyc12g014250	Phosphoenolpyruvate carboxylase	GLY	0,73	1,05	R.FLDILQDLHGEDLK.G
Solyc01g005560	Isocitrate dehydrogenase	TCA;LIP	1,07	1,59	K.LILPFLELDIK.Y
Solyc06g009050	Adenine nucleotide alpha hydrolases-like superfamily protein	RNA;PROT	1,09	0,65	K.EKSVNDVVVEIVEGDAR.H
Solyc02g078570	Epoxide hydrolase	HOR	0,93	0,63	K.FPGIEDYISSGVLK.S
Solyc12g009250	Chaperonin	HOR;STR;PROT	1,20	1,40	K.EKPSFGAVIAVGPGPLDEEGK.R
Solyc09g015000	Heat-shock protein,	HOR;STR;PROT	0,68	0,45	K.AEMENGVLTVTVPKEEEK.K
Solyc07g044860	psbXphotosystem II 23 kDa protein	PS;TRANS	0,81	0,40	K.EVEYPGQVLR.Y
Solyc03g005590	Regulator of chromosome condensation (RCC1)	DEV;STR;RNA;TRANS;DIV	0,22	0,19	K.AGEQGLDYIAFK.T
Solyc05g014280	small heat shock protein 1	CW;STR;RNA;PROT	1,39	0,81	R.IALPENIDFEK.I
Solyc12g042060	ATP-dependent Clp protease ClpA	STR;PROT	0,98	0,58	R.GSGFVAVEIPFTPR.A
Solyc06g060260	stromal ascorbate peroxidase 7	REDOX	0,36	0,93	R.MGLNDKEIVALSGAHTLGR.S
Solyc01g109300	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	SMET	1,64	3,65	R.GIPSYWIDSEQR.V
Solyc11g018550	ascorbate peroxidase 6	REDOX	0,21	0,89	K.EIVALSGAHTLGR.S
Solyc01g108560	Acetyl esterase SlCXE3	LIP	1,10	0,59	K.SGWPGTVEVVEVK.D
Solyc05g054760	dehydroascorbate reductase 1	LIP;REDOX	1,78	1,27	K.IFPTFVSFLK.S
Solyc03g115110	ATP synthase subunit gamma	TRANS	1,03	1,55	K.DIELVMTELQK.N
Solyc09g064450	NAD(P)-binding Rossmann-fold superfamily protein	TRANS	1,25	1,61	R.FIQVSSLGASPASSSR.L
Solyc09g098150	Metacaspase	PROT	1,28	1,87	K.SLPLSTLIEILK.Q
Solyc11g069000	T-complex protein 1 subunit beta	PROT	1,29	1,46	K.DSFLDEGFILDK.K
Solyc07g032640	Oxygen-evolving enhancer protein 1	PS	1,65	1,12	K.DGIDYAAVTVQLPGGER.V

Table 10. List of the differently expressed proteins between amiRNA-*phot1* leaves and cv. Rutgers leaves. Difference between WT and mutant significant at p<0.05. AA-amino acid; SIG-signaling; CW-cell wall; DEV-development; GLY-glycolysis; STR-stress signaling; RNA – RNA processing; PROT-proteins; LIP-lipid metabolismus; TCA-Krebs cycle; PS-photosynthesis; TRANS-transport; REDOX-redox potential; DIV-division; HOR – hormones; SMET- secondary metabolism; FE-fermentation; MIT-mitochodrial transport.

Accession	Protein description	BIN category	MT/WT ratio	peptide
Solyc03g113800	Betaine aldehyde dehydrogenase	FE, SMET,STR	0,28	LGPVISR
Solyc10g051390	RNA-binding glycine-rich protein-1b	RNA	0,35	GFGFVTFK
Solyc01g079880	Asparagine synthetase	MIT, METAL	1,40	ITSDALVNR
Solyc07g043490	UDP-glucosyltransferase family 1 protein	HOR, STR	0,61	ISGFPISIVTIK
Solyc11g021130	30S ribosomal protein S7 chloroplastic	PROT	1,47	TETNPLSVLR
Solyc10g055810	Endochitinase	STR	2,07	AIGVDLLNNPDLVATDPVISFK
Solyc03g062720	Galactose mutarotase-like	not assigned	0,68	GTGIQGLR
Solyc03g119080	Beta-glucosidase	CHO met	0,57	GPSIWDTFLK
Solyc03g052980	ATP dependent RNA helicase	RNA	1,68	MLDMGFEPQIR
Solyc08g041870	Aspartate aminotransferase	MIT, AA	1,54	ISLAGLSAAK
Solyc01g104170	Ankyrin repeat domain-containing protein 2	RNA, CO	1,83	LNNQQDVLK
Solyc01g088080	T-complex protein theta subunit	PROT	48,88	TLAENAGLNAMEIISSLYAEHASGNVK
Solyc08g066110	Profilin	RNA, DNA, CO	1,72	KGPGGITIK
Solyc11g013810	Nitrate reductase	N-metabolism	1,78	EYTMEDILTQLK
Solyc02g067180	Cystathionine gamma synthase	AA	0,69	VFIETILPK
Solyc04g050930	Violaxanthin de-epoxidase	SECMET	0,61	LFGNALPIR
Solyc06g069090	40S ribosomal protein S7-like protein	PROT	2,69	TLTSVHDAILEDLVVPAEIVGK
Solyc05g055230	40S ribosomal protein S17-like protein	PROT	1,88	ILEEVAIIPSK
Solyc07g052480	Isocitrate lyase	GLU	1,93	GLATLLSEAMAAGK
Solyc01g079090	Protoporphyrinogen oxidase	TETSYN	1,30	VISANYPNLMVTEAR
Solyc03g112060	Quinolinate synthase A	VITMET, SIG	1,97	MNSLSSLLR
Solyc01g096450	Aspartic proteinase nepenthesin-1	RNA, PROT	1,65	ILFDVPNSR
Solyc05g056400	Protein disulfide isomerase	REDOX, STR	1,50	LDATANDIPK
Solyc06g074890	Trigger factor	not assigned	2,13	EIVTSTLADYVK
Solyc11g066160	Histone H4	DNA, CO	1,74	IFLENVIR
Solyc07g055080	Proteasome subunit alpha type	PROT	0,65	LYKEPIPVTQLVR
Solyc01g100870	ADP-ribosylation factor	PROT	0,61	ILMVGLDAAGK
Solyc12g008590	Profilin	CO	1,72	KGPGGITIK
Solyc06g068090	Phospholipase D	PS;LIPID;RNA	1,38	IIVVDSALPSGELEK
Solyc10g051340	Adenylyl cyclase-associated protein	SMET;RNA;DNA	0,74	VLVEFR
Solyc02g067080	UDP-D-glucose dehydrogenase	CW	1,56	AADLTYWESAAR
Solyc01g006300	Peroxidase	peroxidases	0,70	GVMQQAQSTDVR
Solyc01g111660	Aquaporin-like protein	TRANS	1,58	EVIEEGQVQQHGK

Table 10. (continued)

Accession	Protein description	BIN category	MT/WT ratio	peptide
Solyc12g094620	Catalase	REDOX, STR	1,60	TWPEDLLPLIPVGR
Solyc03g113400	H-ATPase	CHO MET;TETSYN;TRANS	0,67	ALNLGVNVK
Solyc01g087040	Thylakoid lumenal protein	PS	1,94	ASAEYIYDVPDGWK
Solyc08g079850	Subtilisin-like protease	PROT;CELLTRA	2,52	GPSVASPGILKPDIIGPGVNVLAAWPTSVDNNK
Solyc08g082400	Genome sequencing data contig C313	not assignated	2,23	NPPPPPPK
Solyc11g066110	Inner membrane protein oxaA	PROT; DIV	0,63	LGGAKPAVSGDAGGIISAGR
Solyc12g009960	Eukaryotic translation initiation factor 4	PROT	0,68	ELSTNPQLVAR
Solyc02g064950	CBS domain-containing protein-like	HOR	0,66	VVGDLMTPAPLVVR
Solyc02g080630	Lactoylglutathione lyase	AA;biodegradation	1,41	GGSSVIAFVK
Solyc01g108630	Nitrite reductase	N-MET	0,58	GVVLPDVPEILK
Solyc11g009080	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	AA;TRA	1,39	AFDSILAEVR
Solyc01g088300	Germin-like protein 6	N-MET;RNA;STR;TRA	0,63	FFTVDNIPGLNTLGISIGR
Solyc03g119860	Peptidyl-prolyl cis-trans isomerase	CC	0,19	FSNLVSGAAGVSYR
Solyc03g120640	Photosystem I reaction center subunit VI-1	PS	0,27	FFETFAAPFTK
Solyc09g059170	Anthocyanidin 3-O-glucosyltransferase	SMET	0,60	FIEDLVYEMK
Solyc01g099830	60S ribosomal protein L22-2	CHO MET; PROT	1,82	AGALGDSVTVTR
Solyc01g100360	Dihydrolipoyl dehydrogenase	TCA;LIP;RED;TRA	0,61	SLGVDILTGFGTVLGPQK
Solyc08g067100	Aspartic proteinase nepenthesin-1	RNA;PROT	1,43	TAGLIGLGR
Solyc08g008210	V-type proton ATPase subunit E	PROT;TRANS	0,68	QIQQMVR
Solyc10g083490	Cell division protein ftsZ	DIV	1,71	AVQAQEGIAALR

DISCUSSION

The data presented here constitute the first characterization of tomato plants with downregulated *PHOT1* gene using the amiRNA methodology. PHOT1 has been characterized to be involved in developmental responses such as hypocotyl growth inhibiton and mRNA stabilty and, in combination with PHOT2, in phototropism, chloroplast accumulation, stomatal opening, leaf flattening and movement (reviewed in (Christie, 2007). In our amiRNA-*phot1* transgenic plants, we observed alterations in several of these features.

Apparently, the hypocotyl growth rate was higher in seedling with reduced level of PHOT1 compared to wt, supporting the role of PHOT1 in hypocotyl growth inhibition (Parks et al., 2001). The amiRNA-*phot1* plants harbored a higher stature compared to the wt cv. Rutgers throughout the plant life. Such effect of modulation of BL photoreceptors on the overall growth of the plants has already been reported for different species. Whereas tomato antisense *cry1* plants were taller than the WT, the overexpression of *cry2* resulted in smaller plants (Ninu et al., 1999;Giliberto et al., 2005). Similarly, the fresh weight of Arabidopsis *phot1-5* plants grown under natural conditions (70 μmol $m^{-2}s^{-1}$) was higher (Takemiya et al., 2005). Two-fold increase in the growth and biomass was observed in *phot1* mutants in algae (patent n. WO 2016/197136-A2). On the other hand the rice *phot1* plants were smaller compared to wt (Goh et al., 2009) suggesting slightly different role of phot1 in monocotyledonous and dicotyledonous plants.

Light is an important environmental factor to which plants respond by regulating growth and development of leaves. Thus, it is probable that the photoreceptor´s mutants will be affected in leaves. The altered serration of the leaf margin was already reported in *nps1* mutant in tomato generated by ethyl methansulfonate-mutagenesis (EMS), showing amino acid transition in a highly conserved α helix prior to LOV2 domain of PHOT1 (Sharma et al., 2014). Under monochromatic red light, the leaves curl downwards, whereas flat leaves develop under monochromatic blue light. Previously, it was reported that phototropins mediate the leaf flattening under BL (Inoue et al., 2008b;Kozuka et al., 2013). Accordingly, a phototropin-deficient mutant in Arabidopsis showed a curled-leaf phenotype (Sakai et al., 2001;Sakamoto and Briggs, 2002;de Carbonnel et al., 2010). This was also true for the tomato amiRNA-*phot1* lines obtained in the frame of the current study. The potential role of auxin in leaf flattening is also discussed since the auxin-insensitive mutants *massugu1* (*msg1*) and *auxin resistant 4* (*axr4*) exhibit a leaf curled

phenotype. Likewise, decreased or increased expression of *INDOLE-3-ACETIC ACID CARBOXYL METHYLTRANSFERASE 1* (*IAMT1*) caused downward and upward leaf phenotype, respectively (Hobbie and Estelle, 1995;Watahiki and Yamamoto, 1997;Qin et al., 2005). (Kozuka et al., 2013) came with the idea that *NPH3*, downstream component of the PHOT1 signaling pathway, may establish an auxin gradient along the abaxial–adaxial axis within leaves similarly as PINs do in hypocotyl during phototropism. PIN1 protein promotes basipetal auxin flux and is stabilized at the basal side of hypocotyl cells by an intrinsic auxin transporter ABCB19 (Noh et al., 2003). Under BL illumination, disruption of ABCB19 activity by PHOT1 phosphorylation reduces the auxin flux leading to auxin accumulation at the bending zone of hypocotyl. Subsequently, auxin is redistributed towards the shaded side of hypocotyl by PIN proteins (Ding et al., 2011;Christie et al., 2011).

In a similar manner, the auxin involvement in shoot branching is discussed (Müller and Leyser, 2011). The role of all photoreceptors in shoot branching was studied in the model *Physcomitrella*. Authors found out that CRYs and PHYs initiate the process of branching, whereas the position of branching is determined by PHOTs and PHYs (Uenaka et al., 2005). The changes in PHOT1 abundance in the tomato amiRNA-*phot1* lines might be responsible for the limited branching in comparison with wt plants. The role of the light quality, especially the ratio red:far-red, on bud outgrowth (branching) or dormancy has been reported in many crops. Fewer investigations have been carried out concerning the role of blue light on this process, with very often opposite responses depending on the species, or even on the varieties among species (Leduc et al., 2014). Whereas there are no doubt that PHOT1 is involved in tomato branching, further studies are needed to determine how PHOT1 might regulate auxin's (and other hormones) fluxes, and consequently participate in apical dominance.

The tomato amiRNA-*phot1* mutant also showed a hairy phenotype characterized by a huge number trichomes both on leaf and stem. Such hairy phenotype could represent an adaptation mechanism to better tolerate water deprivation. Indeed, the important population on trichomes on aerial parts of the plant might limit the water loss by transpiration, prevent leaf overheating or protect leaf from UV-related photoinhibition either by reflection of UV radiation or absorption by pigments (Palliotti et al., 1994; Guerfel et al., 2009; Mo et al, 2016; Galmez et al., 2007; Holmes and Keller et al., 2002; Ehleringer and Mooney et al., 1978). Our proteomic study of leaves support the hypothesis that the tomato amiRNA-*phot1* plants could be more tolerant to drought stress.

Indeed, proteins with role in drought tolerance such as aquaporins, protein disulfide isomerase or catalase were more abundant in the transgenic tomato (Devi et al., 2012;Zargar et al., 2017;Zhang et al., 2018). In Arabidopis, the defect in *PHOT1* stimulated the foraging behavior of the root to avoid the soil surface, constituting a mechanism of adaptation to drought stress (Galen et al., 2007). To confirm the role of PHOT1 in drought stress tolerance, a deeper phenotypic analysis will be required, involving different watering regimes and measurement of several parameters such as the temperature of leaves, chlorophyll fluorescence, gasometric parameters and antioxidant enzymatic activity.

Changes in physical properties of the fruits of tomato amiRNA-*phot1* plants were not surprising. Indeed, tomato mutants affected in light signaling pathways showed similar feature. Whereas mutants in negative regulators of photomorphogenesis *det1*, *ddb1*, *cop1* and *cry2* exhibits darker fruits, the positive regulator *hy5* mutant had pale green fruits. The change in pigmentation coincides with the nutritional quality of fruits. The darker green phenotype means the higher content of chlorophyll and carotenoids (Mustilli et al., 1999;Liu et al., 2004;Giliberto et al., 2005). Carotenoids are antioxidants which help to prevent some type of cancers and degenerative diseases (Bartley and Scolnik, 1995). Proteomic analysis was done to investigate the protein differences between amiRNA-*phot1* and wt plants. The fruit of the amiRNA-*phot1* plants were characterized by the presence in hight amount of the 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), enzyme of the MEP pathway responsible for the synthesis of the carotenoid precursors. It was already demonstrated that the upregulation of HDR leads to the increased level of carotenoids both during tomato fruit ripening and Arabidopis seedling de-etiolation (Botella-Pavía et al., 2004). Caseinolytic protease (Clp) was shown to be downregulated in the fruit of the amiRNA-phot1 mutant. Interestingly, the gene encoding this enzyme was found to be accumulated in the hypocotyl of tomato illuminated by blue light. Despite the different organs, and the different level (transcriptional vs. traductional), these results evidenced that this enzyme is regulated by blue light in a PHOT1-dependent manner. CLP is involved in the regulation of the MEP pathway, probably through the degradation of HDR (Flores-Pérez et al., 2008;Moreno et al., 2018). The dehydroascorbate reductase (DHAR) is another interesting enzyme identified in our proteomic study whose abundance was increased in the fruit of amiRNA-phot1 plants. This enzyme is involved in ascorbate production. Ascorbic acid (Asc) is a major antioxidant in plants that detoxifies reactive oxygen species (ROS) and maintains

photosynthetic function. Expression of dehydroascorbate reductase (DHAR), responsible for regenerating Asc from an oxidized state, regulates the cellular Asc redox state, which in turn affects cell responsiveness and tolerance to environmental ROS. Supression of DHAR resulted in the loss of chlorophyll a, lower rate of CO2 assimilation and premature leaf aging (Chen and Gallie, 2006). Despite its functions in photoprotection, Asc acts as a cofactor for enzymes such as violaxanthin deepoxidase, ethylene-forming enzyme, 2-oxoacid-dependent dioxygenases required for the synthesis of abscisic acid (ABA) and gibberellic acid (Chen and Gallie, 2006; Gest et al., 2013). The HPLC analysis of chlorophyll and carotenoid content confirmed the higher level of both pigments in mutant compared to wt predicting better nutritional quality of the fruit.

Moreover, the proteome analysis of the fruit of amiRNA-*phot1* plants revealed changes in proteins linked to the organoleptic properties of the fruit. Reduced level of endo-β-mannase predicts higher firmness of fruit, reduced heat shock protein vis1 higher viscosity of fruits, reduced alcohol dehydrogenase 2 could affect the balance of flavor aldehydes and alcohols and reduced acetyl esterase CXE3 could influence the flavor volatiles (Speirs et al., 1998;Ramakrishna et al., 2003;Wang et al., 2009;Goulet et al., 2012). The downregulation of legumin and vicilin, two tomato allergens, was detected as well (Bässler et al., 2009). The altered level of phosphoenolpyruvate carboxylase (PEPC), which permits the sythesis of organic acids to provide the turgor pressure for cell expansion, can be connected with the fruit size (Guillet et al., 2002). As well as the changed amount of glutamate dehydrogenase 3 (GAD3) can cause lower content of γ-aminobutyric acid, participating in cell elongation (Renault et al., 2011;Takayama et al., 2015).

CONCLUSION

Light is an important environmental factor affecting plants alltrough their life spam. In the frame of the current study, we focused our interest on the blue part of the light spectrum and on the specific blue light (BL) photoreceptor PHOTOTROPIN 1 (PHOT1). PHOT1 has been described to play an important role in plethora of physiological responses, including phototropism, chloroplast movement, leaf bending, hypocotyl growth. Most of the studies related to BL and PHOT1 are conducted on the plant model Arabidopsis and only a minor amount of knowledge is available for plants species of economical importance, such as tomato (*Solanum lycopersicum* L.). Understanding how BL regulates tomato responses to environment and the specific role of PHOT1 will bring about tools for bioingeneering of tomato for better adaptation to environment pressure.

The current study started with simple theoretical questions: How is BL-induced de-etiolation regulated in tomato? What is the role of PHOT1 in this process? In tomato, like in Arabidopsis, BL-induced de-etiolation is a sequential process depending first on PHOT1 during the first 30–40 min of exposure to BL, with CRY1 being later responsible for the establishment of the steady-state growth rate (Bergougnoux et al., 2012; Folta and Spalding, 2001). Whereas CRY1-mediated de-etiolation has been characterized at the molecular level in Arabidopsis (Folta et al., 2003), no information is available concerning the PHOT1-mediated phase of de-etiolation. Therefore, we developed a suppression subtractive hydridization assay to revealed genes that were up-regulated by BL during the PHOT1-mediated phase of de-etiolation. We identified 152 genes quickly up-regulated by BL. Their annotation revealed deep changes in chromatin modelling, transcription, and translation, but also in cellular processes and signaling such as cell wall integrity/synthesis, cytoskeleton, and trafficking/secretion.

In general, cell signaling mechanisms often involve posttranslational modifications (PTMs) of proteins, affecting their conformation, activity, stability, and/or localization (Wold, 1981). The reversible phosphorylation of the serine, threonine or tyrosine residue has been probably the most extensively studied (Olsen et al., 2006). Very often, however, phosphorylation of the protein alone is not enough to modulate protein function. Rather, phosphorylation is required to ensure the binding of interactors which will themselves regulate protein function. Among the plant phosphopeptide-binding proteins, 14-3-3 proteins (14-3-3s) are the best characterized (Chevalier et al., 2009). They have recently attracted attention due to their remarkable capacity to affect a wide

array of physiological, developmental, and cellular processes such as primary metabolism, hormone signaling, response to light, cell growth and division, pathogen–plant interaction, and response to biotic and abiotic stresses (Cotelle et al., 2015; Denison et al., 2011; Lozano-Durán and Robatzek, 2015; Camoni et al., 2018). The altered hypocotyl length of Arabidopsis 14-3-3 mutants grown in RL or in darkness suggests a role for 14-3-3 proteins in the control of hypocotyl elongation (Mayfield et al., 2007; Zhao et al., 2015). However, their possible involvement in plant response to BL is not yet clear. In tomato, 12 isoforms have been identified and are referred to as Tomato Fourteen Three (TFTs). Earlier study of antisense transgenic tomato plants had demonstrated that at least two isoforms were involved in light-mediated plant development (Roberts et al., 2002). In the present study, we focused on events arising during establishment of the steady-state growth rate under BL exposure while giving special attention to the role of 14-3-3/TFTs in that process. We also described how two tomato TFTs are involved in the BL-mediated response of tomato hypocotyl, and their regulation by cytokinins. Using immobilized recombinant tomato TFTs, more than 130 proteins related to a large variety of cellular processes were identified as potential TFT interactors. Their functional annotation predicted that TFTs might regulate the activity of proteins involved notably in cell wall strengthening or primary metabolism. Several potential interactors were also predicted to be CK-responsive. For the first time, the 14-3-3 interactome linked to de-etiolation was investigated and evidenced that 14-3-3s might be involved in CK signaling pathway, cell expansion inhibition and steady-state growth rate establishment, and reprograming from heterotrophy to autotrophy.

In the meantime, we generated tomato transgenic plants with knocked-down expression of *PHOT1* via the amiRNA methodology and the *Agrobaterium tumefaciens*-mediated transfornation of immature tomato fruits. From the phenotypic characterization of the amiRNA-*phot1* mutant we evidenced roles of tomato PHOT1 in leaf development, response to water deprivation, fruit development and maturation. Deeper analysis of the mutant is required in order to get a more precise insights concerning the role of PHOT1 in these processes.

In the frame of the present thesis, a huge amount of data has been collected to understand how BL modulates tomato development throught PHOT1. High-throughput RNA-sequencing, advanced phenotyping, generation of transgenic plants via CRISPR-Cas9 guided RNA methodology for specific candidates: these are all aspects that have to be developed in the future to complete this work.

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ABREVATIONS

3-AT	3-amino-1,2,4-triazol
ABA	Abscisic acid
ABCB19	ATP-binding cassette B19
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ADF	Actin depolymerizing factor
AF	Actin filament
AHK	Arabidopsis histidine kinase
amiRNA	Artificial microRNA
AMP	Altered meristem program
ANOVA	Analysis of variance
APPR2	Arabidopsis pseudo response regulator 2
ARF4	Auxin response factor 4
ARRs	Arabidopsis response regulators
AUX/IAA	Auxin/indol-3-acetic acid
BAK1	BRI1-associated receptor kinase 1
BBMH	Best blast mutual hit
BKI1	BRI1 kinase inhibitor 1
BL	Blue light
BR	Brassinosteroids
BRI1	Brassinosteroid insensitive 1
BSU1	BRI1 supressor 1
BZR1, 2	Brassinazole resistant 1, 2
CCT	Cryptochrome C-terminus
cDNA	Complementary DNA
CDGs	Cycling DOF factors
CDKs	Cyclin dependent kinase
CESA	Celulose synthase A
CIB1	Cry-interacting bHLH1
CIN4	Cytokinin-insensitive 4
CK	Cytokinin
CO	Constans
COPx	Constitutive photomorphogenesis
CRLx	Cullin 3-ring ligase
CRYs	Cryptochromes

CrtISO	Carotenoid isomerase
CSN	COP9 signalosome
CUL4	Cullin 4
CYCs	Cyclins
CW	Cell Wall
DEAE	Diethylaminoethyl
DDB1	UV-damaged DNA binding protein 1
DET1	De-etiolated 1
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
DXS	1-deoxy-D-xylulose-5-phosphate synthase
EDE1	Endosperm defective 1
EF1α	Elongation factor α
EG	Endoglucanase
EIL1	ETO1-like 1
EIN2	Ethylene insensitive 2
ERFx	Ethylene response factors
EST	Expressed sequence tag
ET	Ethylene
ETO1	Ethylene overproducer 1
FAD	Flavinadenindinucleotide
FKF1	Flavin-binding kelch repeat F-box 1
FMN	Flavinmononucleotide
FT	Flowering locus T
GA	Gibberellin
GAI	GA-insensitive
gDNA	genomic DNA
GDSL	GDSL-esterase/lipase
GF14	G-box factor 14-3-3
GI	Gigantea
GID1	Gibberellin insensitive dwarf 1
GLK2	Golden like 2
GRFs	General regulatory factors
HKDR	Histidine kinase domain
HFR1	Long hypocotyl in far red light 1
His	Histidine
	Long hypocotyl 4, 5

iP	isopentenyladenine
IG	Immature green
KOH	Pottasium hydroxide
LAF1	Long after far red light 1
LC	Liquid chromatography
LCY1,3	Lycopene cyclase 1, 3
Leu	Leucine
LKP2	LOV kelch protein 2
LM-PCR	Ligation-mediated PCR
LOV1,2	Lov oxygen and voltage domain
MAP	MT-associated protein
MEP	Methylerithritol 4-phosphate
MG	Mature green
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Mediator RNA
MT	Microtubule
MS	Mass spectrometry
NPH1,3	Nonphototropic hypocotyl 1, 3
NPL1	NPH1-like 1
NR	Nitrate reductase
O	Orange
ORF	Open reading frame
PAT	Polar auxin transport
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PHOT1, 2	Phototropin 1, 2
PHR	Photolyase 6 Homologous Region
PIFs	Phytochrome Interacting Factors
PINs	Pinoids
PM	Plasma membrane
PME	Pectin methylesterase
PP2Acs	Protein phosphatase 2A catalytic subunit
PRR5	Pseudo response regulator 5
PKS1	Phytochrome kinase substrate 1
PSM	Peptide spectral matches
PSY1	Phytoene synthase 1
qTOF	Quadrupole time of fligh

R	Red
RGA	Repressor of GA 1-3
RGL1	RGA-like 1
RL	Red light
RIN	Ripering inhibitor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR-B	Response regulator B
RSG	Repression of shoot growth
SEM	Standard error of the mean
SPA1	Supressor of PHYA
SSH	Suppression subtractive hybridization
TAG1	Tomato agamous like 1
TFTs	Tomato 14-3-3 proteins
TIP41	Type 2A phosphatase aktivátor TIP41
TOC1	Timing of CAB1
Trp	Trypthofane
UPLC	Ultra performance liquid chromatography
Ura	Uracil
UV-A, B	Ultra-violet A, B
UVR8	UV resistant locus 8
V-ATPase	Vacuolar ATPase
WDL3	WVD2-like
XTH	Xyloglucan transglucosylase hydrolases
ZTL1	Zeitlupe 1

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SUPLEMENTAL DATA

Supplemtal data 1: Nucleic acid sequences of the 168 ESTs found to be putatively up-regulated 30 min after exposure to BL

>12
ACCTCGCTTTATCAGTGCAATAGTTATAAACAGTGTATTTTTGACGAACCCAACGAAGTCTCCTATACTGTAATGCATCTAAATCTTGGAAGGCCT
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>F-C1
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ATTTGAAGAAAATGCCTCTGATTGAACATGACTGCTAATTAATTAATTAGCAGAAAAAAAGGAAATCAAGGCACTTATATATATAAAAAAAACAAC
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A subtracted cDNA library identifies genes up-regulated during PHOT1-mediated early step of de-etiolation in tomato (Solanum lycopersicum L.)

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Abstract

Background: De-etiolation is the switch from skoto- to photomorphogenesis, enabling the heterotrophic etiolated seedling to develop into an autotrophic plant. Upon exposure to blue light (BL), reduction of hypocotyl growth rate occurs in two phases: a rapid inhibition mediated by phototropin 1 (PHOT1) within the first 30–40 min of illumination, followed by the cryptochrome 1 (CRY1)-controlled establishment of the steady-state growth rate. Although some information is available for CRY1-mediated de-etiolation, less attention has been given to the PHOT1 phase of de-etiolation.

Results: We generated a subtracted cDNA library using the suppression subtractive hybridization method to investigate the molecular mechanisms of BL-induced de-etiolation in tomato (Solanum lycopersicum L.), an economically important crop. We focused our interest on the first 30 min following the exposure to BL when PHOT1 is required to induce the process. Our library generated 152 expressed sequence tags that were found to be rapidly accumulated upon exposure to BL and consequently potentially regulated by PHOT1. Annotation revealed that biological functions such as modification of chromatin structure, cell wall modification, and transcription/translation comprise an important part of events contributing to the establishment of photomorphogenesis in young tomato seedlings. Our conclusions based on bioinformatics data were supported by qRT-PCR analyses the specific investigation of V-H^+-ATPase during de-etiolation in tomato.

Conclusions: Our study provides the first report dealing with understanding the PHOT1-mediated phase of de-etiolation. Using subtractive cDNA library, we were able to identify important regulatory mechanisms. The profound induction of transcription/translation, as well as modification of chromatin structure, is relevant in regard to the fact that the entry into photomorphogenesis is based on a deep reprograming of the cell. Also, we postulated that BL restrains the cell expansion by the rapid modification of the cell wall.

Keywords: Blue light, De-etiolation, Suppression subtractive hybridization, Tomato (Solanum lycopersicum L.)

Background

Light is one of the most important environmental factors influencing plants throughout their life spans. Blue and red/far-red portions of light can be considered as the most active rays within the light spectrum for regulating plant growth and development. As sessile organisms, plants have evolved highly sophisticated unique photoreceptors to sense light. They possess three main classes of photoreceptors: phytochromes (PHY), cryptochromes (CRY), and phototropins (PHOT), capable of absorbing red/far-red, blue, and blue light, respectively [1]. Not only is light the primary source of energy for photosynthesis, but it also regulates numerous physiological responses, such as shade avoidance, flowering, germination, tropisms, and de-etiolation [2]. De-etiolation occurs during early seedling development. In dicotyledonous plants, the hypocotyl

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(embryonic stem) connects the two cotyledons (embry-onic leaves) to the root. When germinated in darkness in the soil, the hypocotyl expands toward the surface in order to place the shoot apical meristem in an environment suit-able to ensure photoautotrophic growth. When the seed-ling emerges from the soil, it perceives light; the hypocotyl stops growing, the cotyledons unfold and green, the chlo-roplasts differentiate, and finally photosynthetic growth is initiated [3]. As almost all of the hypocotyl's cells are formed during embryogenesis; only a few cell divisions occur in the hypocotyl during etiolation, being limited to the development of stomata [3]. For example, in Arabi-dopsis, the hypocotyl consisting of only 20 epidermal cells elongates more than 100-fold its embryonic length [3].

Hypocotyl de-etiolation is regulated by the three mentioned photoreceptor families. Nevertheless, at equal irradi-ances, blue light (BL) is more effective than red light as it inhibits growth more quickly and to a greater extent [4]. Using cry1 Arabidopsis mutant defective in BL-induced de-etiolation, studies have demonstrated that CRY1 is the BL receptor involved in the control of hypocotyl elong-ation [5, 6]. Using computer-assisted electronic image capture, however, Parks and co-authors [7] demonstrated that in cry1 seedlings hypocotyl growth inhibition begins to develop within approximately 30 sec of BL irradiation and reaches the same maximum level displayed by wild-type seedlings after approximately 30 min of BL treat-ment. At this point, cry1 seedling growth accelerates, soon attaining the growth rate observed for darkness-grown seedlings. This experiment demonstrated that BL-mediated hypocotyl inhibition in Arabidopsis occurs in two genetically independent phases [7]. A few years later, while applying the same method to different Arabidopsis photoreceptor mutants, Folta and Spalding [8] identified PHOT1 as being involved in the rapid phase of BL-mediated hypocotyl growth inhibition.

The PHOT1 signaling pathway has been studied extensively in the phase of stomata opening. In response to BL, plasma membrane H^+-ATPases in the guard cells are activated. This induces a negative electrical potential across the plasma membrane and drives K^+ uptake. Ions and metabolites enter the cell concomitantly with water uptake, thereby increasing turgor pressure and resulting in the opening of the stomata. The plasma membrane H^+-ATPase is activated by phosphorylation of its C-terminus with a concomitant binding of the 14-3-3 proteins [9]. By comparison, the mechanisms involved in PHOT1-mediated de-etiolation are still poorly understood. Nevertheless, genetic, biochem-ical, and physiological studies have begun to delineate the signaling pathway initiated after the onset of BL excitation. Evidence has accumulated to prove that excitation of PHOT1 induces a rapid activation of Ca^{2+} channels at the plasma membrane, leading to an increased concentration of cytosolic Ca^{2+} [10], [11]. To our knowledge, few events acting downstream of PHOT1 have been identified during de-etiolation [8], [12]. Therefore, it remains challenging to identify the PHOT1-signaling pathway during de-etiolation. All analyses to date have been performed on plant models, most notably in Arabidopsis. Little or no information is available from important crop species. For several years, we have focused on understanding the role of BL in the growth and development of tomato (Solanum lycopersicum L.), an economically important crop [13]. We previously demonstrated that in etiolated tomato seedlings exposed to BL the reduction of the hypocotyl growth rate is a two-step process [14]. Based on the knowledge coming from studies on Arabidopsis, we hypothesized that the first rapid inhib-ition might be triggered by PHOT1, and that the steady-state rate of growth might be established by CRY1.

Suppression subtractive hybridization (SSH) is a powerful approach which allows the comparison of two samples (tester and driver) and the identification of differentially regulated genes [15]. Indeed, SSH is a combination of normalization which equalizes the abundance of cDNA within the target population and subtraction which excludes sequences common to both the tester and the driver [15]. Therefore, SSH identifies not only abundant differentially expressed genes but also rare transcripts which were enriched during the process. This latest category is of high interest as it can represent a pool of unknown genes. This method does not require an in-depth knowledge of the genome under study and can thus be applied easily to non-model species [16], [17], [18], [19]. In the present study, we used the SSH approach to identify the molecular mechanisms of the PHOT1-mediated rapid inhibition of hypocotyl elongation in tomato. Our current results provide evidence that a complex network is quickly activated by exposure to BL in order to induce de-etiolation.

Methods

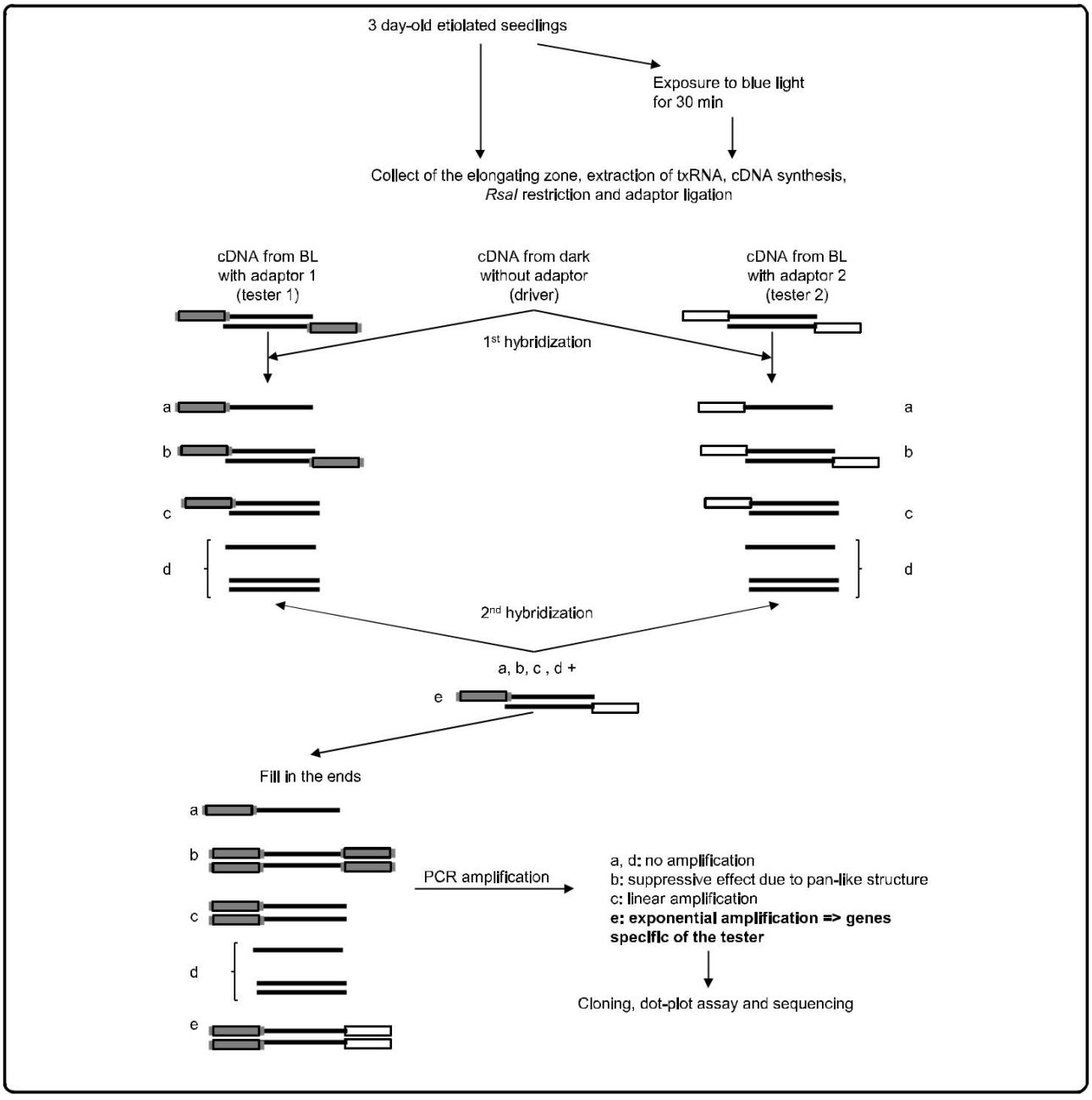
Plant materials and light treatment

The tomato cultivar Rutgers was used in this study. Sterile cultures were obtained as described in [20]. After germination in darkness at 23 °C, germinated seeds were transferred in the dark for 3 additional days to a culture chamber maintained at 23 °C. For BL-induced de-etiolation, dishes containing 3-day-old etiolated seedlings were transferred for 30 min under BL provided by fluores-cent lamps (BL; TL-D 36 W/Blue, Phillips; total photon fluence rate 10 $\mu mol.m^{-2}.s^{-1}$).

RNA extraction and subtractive library construction

For all experiments, the elongating zone of the hypo-cotyl of 3-day-old etiolated seedlings was excised from the rest of the seedling. either under green safety light (dark control) or under BL after 30 min of exposure to BL. The elongating zone corresponding to the portion

of hypocotyl situated beneath the hook and cotyledons was limited to the upper third of the hypocotyl as described in [14]. Samples were immediately frozen in liquid nitrogen and stored at −80 °C before RNA isola-tion. Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). Remaining traces of DNA were removed with a recombinant DNAseI (Takara) and RNA were subsequently purified by a phenol:chloroform:isoamyl alcohol (25:24:1) step. PolyA$^+$ mRNA were purified using the Straight A's mRNA isolation system (Novagen). Quantity and quality of mRNA were checked by spectrophotometer and electrophoresis. The suppression subtractive hybridization (SSH) library was constructed according to the instructions of the PCR-Select cDNA Subtraction Kit (Clontech). The principle of SSH library is illustrated by the Fig. 1. In order to identify genes up-regulated by the exposure to BL, subtractive hybridization was performed using cDNA from hypocotyls exposed for 30 min to BL as tester against cDNA from control hypocotyls (not exposed to BL) as driver. The subtraction efficiency was evaluated by a PCR reaction amplifying a region of the tomato EF1α gene and the PCR product was analyzed after 15, 20, 25, 30, and 35 cycles.



Cloning, screening for differential expression, sequencing and analysis

Secondary SSH-PCR products were inserted into pGEM-T Easy Vector (Promega) and cloned into Escherichia coli DH5α strain. A blue–white screening was performed in order to obtain a bank of subtracted ESTs. White colonies were picked and grown in 96-well microtiter plates in a ly-sogeny broth medium containing ampicillin (100 $mg.L^{-1}$). Screen for differentially expressed ESTs was performed by dot blot hybridization as described in the PCR-select cDNA subtraction kit (Clontech). For this purpose, plasmids were isolated, quantified and transferred to Hybond-N+ nylon membranes. Membranes were prepared in duplicates with equal amounts of plasmids and were hybridized either with the BL-specific tester probe or the dark-specific driver probe, both DIG-labelled. Detection was performed with an anti-digoxigenin antibody coupled with a horse radish peroxidase. Cold detection was performed by enhanced chemiluminescent detection and exposure to X-ray films. Autoradiographies were scanned and the intensity of the dots was determined using ImageJ software. The dot intensity of a specific clone obtained with the BL-specific probe was compared to that obtained with the dark-specific probe. All clones showing higher intensity with BL-specific probe compared to dark-specific probe were selected and sequenced by Macrogen (Korea). A total of 168 ESTs were found to be differentially expressed. Their sizes ranged from 128 to 1387 bp, with an average size of 447 bp. Because during the process of library preparation the cDNA were restricted by RsaI, the first step of the analysis was to retrieve the full-length of the gene and the corresponding protein for downstream analyses by BLAST against the tomato database at SolGenomics Network. The gene ontology annotation was performed with Blast2GO according to plant-specific Gene Ontology terms [21]. Concurrently, the functional annotation was performed by Mercator/ MapMan which allows attributing DEGs a functional pathway [22], [23].

Quantitative real time-PCR

To confirm the differential expression of the selected ESTs, total RNA was extracted from the elongating zone of 3 day-old etiolated seedlings exposed or not to BL as previ-ously described. Reverse transcription was performed from 1 μg of the total RNA according to the instructions of the PrimeScript kit (Takara), followed by subsequent RNaseH treatment (Takara) and purification on a Macherey-Nagel column to remove any compounds which could have an in-hibitory activity during subsequent steps. For quantitative real-time PCR, cDNA samples were diluted by 5-fold and used in a reaction containing SYBR Premix ExTaq (Takara) PCR Master Mix and 200 nM of each primer. Three tech-nical repeats were run for each sample on the Mx3000P thermocycler (Stratagene) in a two-step amplification pro-gram. The initial denaturation at 94 °C for 10 s was followed by 40 cycles of 94 °C for 5 s and 60 °C for 20 s. A dissociation curve was obtained for each sample. Three independent biological repeats were analyzed for each sam-ple. Each independent biological replicate represents a pool of 20 to 25 explants. Cycle threshold values were normal-ized in respect to the PP2Acs gene [24]. The analysis of different usual housekeeping genes was performed and confirmed that PP2Acs gene is the most stable in the condi-tions of the present study (data not shown). Differences in cycle numbers during the linear amplification phase be-tween the samples. After determination of primer effi-ciency, the Pfaffl method was used to determine the fold change in gene expression [25]. The relative quantification was made compared to the dark control sample. The re-sults are expressed in term of fold change and represent the average ± standard error on mean (SEM) of 3 independent biological replicates. Primers were designed using default parameters of IDT qPCR assay design and checked with OligoAnalyzer tool (http://eu.idtdna.com/scitools/Applications/RealTimePCR/). Their specificity was checked by blast restricting to tomato database. Sequences of primers and their efficiency are given in Table 1.

The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results.

Bafilomycin A1 treatment

Sterile cultures were obtained as described in [19]. After germination in darkness, germinated seeds were trans-ferred on a Murashige and Skoog medium containing varying concentrations of bafilomycin A1. For condition of darkness, dishes were wrapped in aluminum foil and placed in the culture chamber; for light conditions, dishes were cultivated in a culture chamber illuminated with BL (total photon fluence rate 10 $\mu mol.m^{-2}.s^{-1}$). After 5 days, the length of the hypocotyl was measured to the nearest millimeter with a ruler. The graph represents the mean ± SEM; an average of 45 seedlings were measured for each condition. The non-parametric Kruskal-Wallis ANOVA (Statistica 12) was performed in order to support the stat-istical significance of the data.

Results and discussion

Construction of the subtracted cDNA library and analysis

In order to study the molecular events of the rapid in-hibition of tomato hypocotyl growth observed within the first 30 min following exposure to BL, a SSH library was constructed and screened for genes whose expression is stimulated by BL. Two contrasting mRNA samples were extracted. One sample, extracted from the elongating zone of the hypocotyl of seedlings grown in darkness and exposed for 30 min to BL (10 $\mu mol.m^{-2}.s^{-1}$), poten-tially containing differentially expressed genes, was used

Table 1 Primers used in quantitative real-time PCR

Identification	Description of the gene	Primers	Primer efficiency
A-E4	Mitogen-activated protein kinase	F: 5′- GAAGATGAGAAACCACAAGCG	90 %
		R: 5′- CATTCTGAGGAACTTGGAGAGG	
C-G1	Importin subunit alpha1a	F: 5′- GAACTCATTTTGTGCCCCATC	92 %
		R: 5′- GCTGAGGGATTGGAAAAGATTG	
E188	Intracellular Ras-group-related LRR protein 9	F: 5′- GAGAGGCAGGATTGGAGATTG	94 %
		R: 5′- TCCGCATCCTTCAACATCTTC	
E-E3	Polyadenylate-binding protein RBP47	F: 5′- TCCTAATGAGCCTAACAAACCTG	92 %
		R: 5′- TCCGTCTTATTGCCTTCCAC	
VHA-A1	V-ATPase catalytic subunit A1	F: 5'- CGAGAAGGAAAGCGAGTATGG	107 %
		R: 5'- TCATTCACCATCAGACCAGC	
B-D5	Vacuolar H + -ATPase V0 sector	F: 5′- GCAGTCATTATCAGTACCGGG	89 %
		R: 5′-TCTAACACCAGCATCACCAAC	
B-E2	Pectin acetylesterase	F: 5'-CACACCCACAAAGAGAAACAG	103 %
		R: 5'-TTCCAAGAATGCCCCTTCAG	
12.	XTH	F: 5'-AGAGGTGGGCTTGAGAAAAC	93 %
		R: 5'-GAACCCAACGAAGTCTCCTATAC	
B-D9	26S proteasome	F: 5'-TCTTGTCCTCTTTCTGTTCCTTATC	95 %
		R: 5'-AATCCTTGCCTCACTTCCAG	
C-A3	Histone H2B	F: 5′- TTGGTAACAGCCTTAGTTCCTC	89 %
		R: 5′- AAAGCCTACCATCACTTCTCG	
PP2ACS	PROTEIN PHOSPHATASE 2A catalytic subunit	F: 5'- CGATGTGTGATCTCCTATGGTC	98 %
		R: 5'- AAGCTGATGGGCTCTAGAAATC	

as the tester. The second sample, isolated from the elongating zone of the hypocotyl of seedlings grown only in darkness, constituted the driver that should express transcripts common to both samples and eliminated during the process of subtraction. Due to technical limitation, 500 putative subtracted clones were randomly picked and used in cDNA dot-plot array for differential screening. Clones were considered for sequencing when they hybridized only to the BL-specific probes or showed higher intensity with the BL-specific probe than with dark-specific probe. In these conditions, we determined that 168 ESTs were potentially differentially expressed. After BLAST analysis, 17 sequences from 168 were found to be redundant, bringing to 151 the number of expressed sequence tag (ESTs) encoding proteins. The ontology annotation was performed using Blast2GO according to plant-specific Gene Ontology terms [21]. Computational analysis using the software Blast2GO enabled annotation of the expressed sequences according to the terms of the three main Gene Ontology vocabu-laries (i.e., cellular compartment, molecular function and biological process; Fig. 2). Concerning molecular function, the most represented categories were those of binding and catalytic activities (Fig. 2b). Regarding cellular compartments, the most represented were ribosome, plastid, and nucleus, together accounting for more than 50 % of total annotations (Fig. 2c). When taking into consideration the most relevant level of distribution for the biological process (i.e., level 8, as shown in Fig. 2a), more than 40 categories were found for the bio-logical process vocabulary (data not shown). The num-ber of categories was therefore simplified to level 2 of the distribution (Fig. 2d). The functional annotation was performed with Mercator, using the last updated version of the tomato annotation (ITAG2.4) [22]. Identical description of the ESTs was obtained when the annotation was performed by Blast2Go, KOG attribution or Blast against the specific annotated tomato genome ITAG2.4 (Additional file: 1 Table S1). The Table 2 shows the number of sequences which enter the different categories. Twenty seven sequences could not be annotated. The functional annotations "Protein: synthesis, targeting, postranslation modification, degradation" and "RNA: processing, transcription, regulation of transcription" were the most represented, including 33 and 12 se-quences, respectively. More detailed information can be found in Additional file 1: Table S1. For all genes tested, qPCR confirmed the differential expression detected by

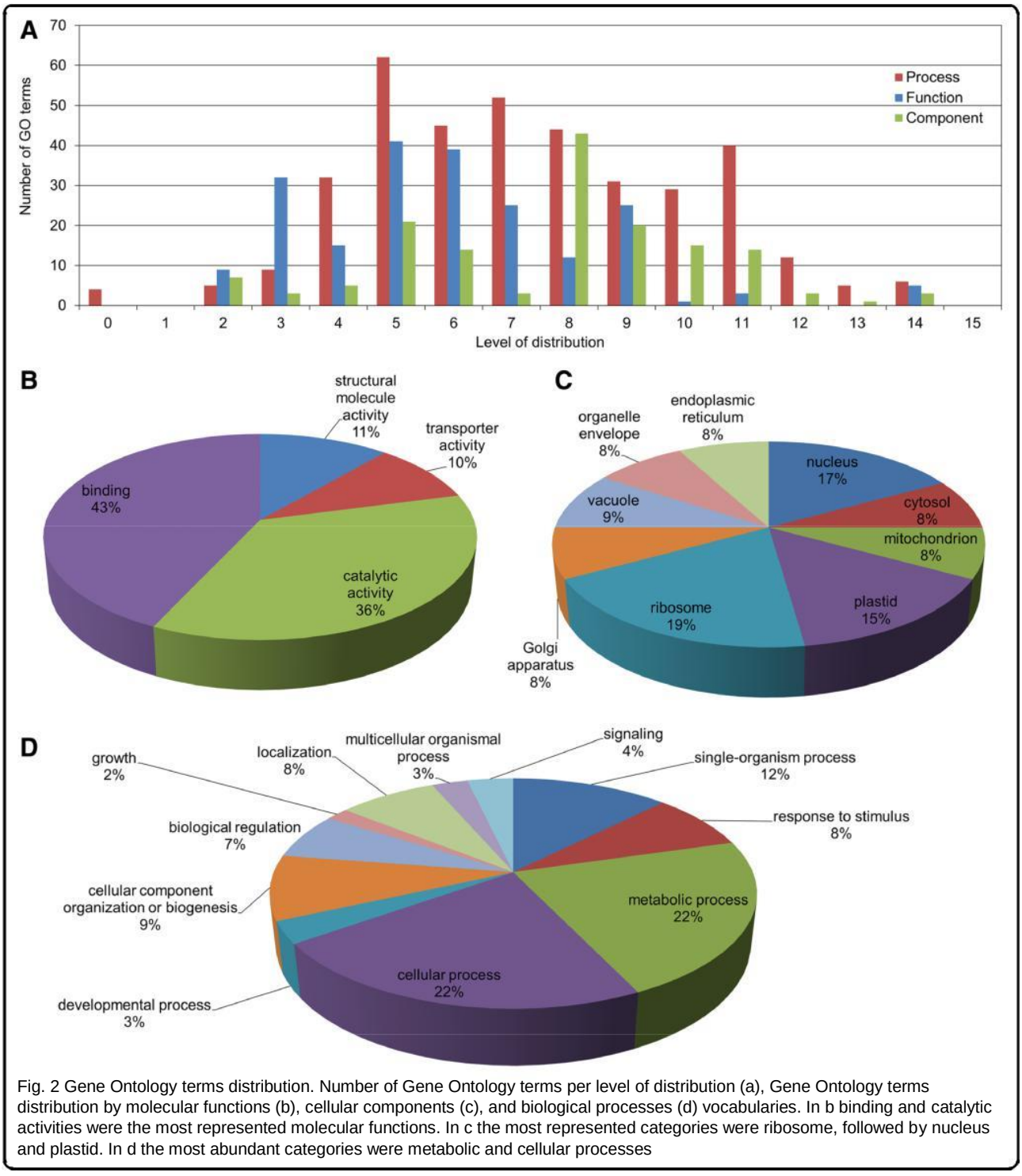


Fig. 2 Gene Ontology terms distribution. Number of Gene Ontology terms per level of distribution (a), Gene Ontology terms distribution by molecular functions (b), cellular components (c), and biological processes (d) vocabularies. In b binding and catalytic activities were the most represented molecular functions. In c the most represented categories were ribosome, followed by nucleus and plastid. In d the most abundant categories were metabolic and cellular processes

the screening of the cDNA library, meaning the up-regulation of the expression of ESTs as soon as 30 min after exposure to BL (Fig. 3). Below, we discuss the potential involvement of various genes in the rapid inhibition of hypocotyl growth induced by 30 min of exposure to BL and mediated by PHOT1.

Translation and transcription

Sixteen differentially expressed sequences were predicted to encode proteins involved in translation, RNA processing and modification (ribosomal proteins), transcription (eukaryotic initiation factors), and chromatin structure and dynamics (Histone 2B – H2B, Histone H2A).

Table 2 Functional categories of up-regulated genes

BIN categories	Number of sequences
Photosynthesis	1
Minor carbohydrates	3
Glycolysis	1
Fermentation	1
Mitochondrial electron transport/ATP synthesis	4
Cell wall	7
Lipid metabolism	3
Amino acid metabolism	7
Stress	11
Redox	2
Miscellaneous enzyme families	4
RNA: processing, transcription, regulation of transcription	12
DNA: synthesis/chromatin structure, repair	4
Protein: synthesis, targeting, postranslation modification, degradation	33
Signalling	8
Cell organisation	3
Cell cycle	1
Cell, vesicle transport	2
Development	5
Transport	12
No ontology	8
Unknown	19

In our study, we found by SSH screening and confirmed by qPCR analysis that H2B is up-regulated during PHOT1-mediated de-etiolation in tomato, indicating that it could play a role during the establishment of photomorphogenesis in tomato (Fig. 4a). Histones form the protein core of the nucleosome around which the DNA helix is wrapped. In this compact state, histones block the association of transcription factors to their binding sites, thus repressing transcription. Several post-translational modifications (acetylation, methylation or phosphorylation) of histone "tails" can influence nucleosome compaction and access to DNA. Moreover, their spatio-temporal regulation as well as their ability for cross-talk renders the regulation of gene expression even more complex [26]. In plants, chromatin remodeling plays an important role during plant growth and development, especially in response to light. Indeed, a large-scale reorganization of chromatin can be observed during the floral transition in Arabidopsis [27]. During de-etiolation, the perception of light induces a remarkable reprogramming of gene expression that leads the heterotrophic seedling to become an autotrophic organism which will be able to complete its life cycle. In darkness, the photomorphogenic repressor DET1 binds to the H2B tails of the nucleosomes surrounding the genes which are repressed in this condition. When light is perceived, the H2B acetylation concomitant with the release of DET1 enables the activation of genes involved in photomorphogenesis [28]. It would be interesting in the near future to validate the potential involvement of H2B in the control of de-etiolation in tomato and thereby to follow the relationship between gene expression and H2B enrichment during this process. Finally, we could identify and confirm that the subunit RPN10/PSMD4 of the 26S proteasome regulatory complex is up-regulated during de-etiolation (Fig. 4b). For this reason, it is tempt-ing to hypothesize that light-regulated histone expression/modification and ubiquitin-proteasome-mediated protein degradation might interact during tomato de-etiolation.

Cell wall modification

In our study, seven tomato EST encoding proteins involved in cell wall modification were found to be up-regulated in the etiolated hypocotyl of tomato seedlings exposed for 30 min to BL: pectin acetylesterase, pectin-esterase, xyloglucan endotransglucosylase-hydrolase 1 (XTH), or endoglucanase. This suggested that de-etiolation induced a strong modification of the cell wall structure and/or composition. Plant cell walls consist of a complex network of cellulose microfibrils embedded in a matrix of hemicelluloses (mainly xyloglucans), pectins, and glycoproteins [29]. During cell maturation, cell walls lose the ability to expand [30]. Growth cessation is accompanied by cell wall tightening [31]. Various modifications of cell wall structure during maturation have been proposed, including changes in hemicellulose. For example, the maturation of pea tissues is characterized by an increase in the total amount of xyloglucan [32]. Xyloglucan endotransglucosylase/hydrolases (XTH) are enzymes capable of modifying xyloglucan during cell expansion. They comprise a subgroup of the glycoside hydrolase family 16. XTH proteins characterized to date have endotransglycosylase (XET) or hydrolase (XEH) activities towards xyloglucans, or both. Their phylogenic study indicates that they are organized into three groups: I/II, III-a, and III-b. Only members of the III-a group are strict XEH [33]. Transgenic tomatoes with altered levels of XTH gene showed higher XET activity, lower hemicellulose depolymerization and reduced fruit softening during ripening. This suggests that XET could have a role in maintaining the structural integrity of the cell wall [34], [35]. Thus, whereas some XTH members are critical in promoting cell wall expansion, others are required for wall strengthening in cells that have completed the expansion process [36]. The analysis by qPCR of the XTH

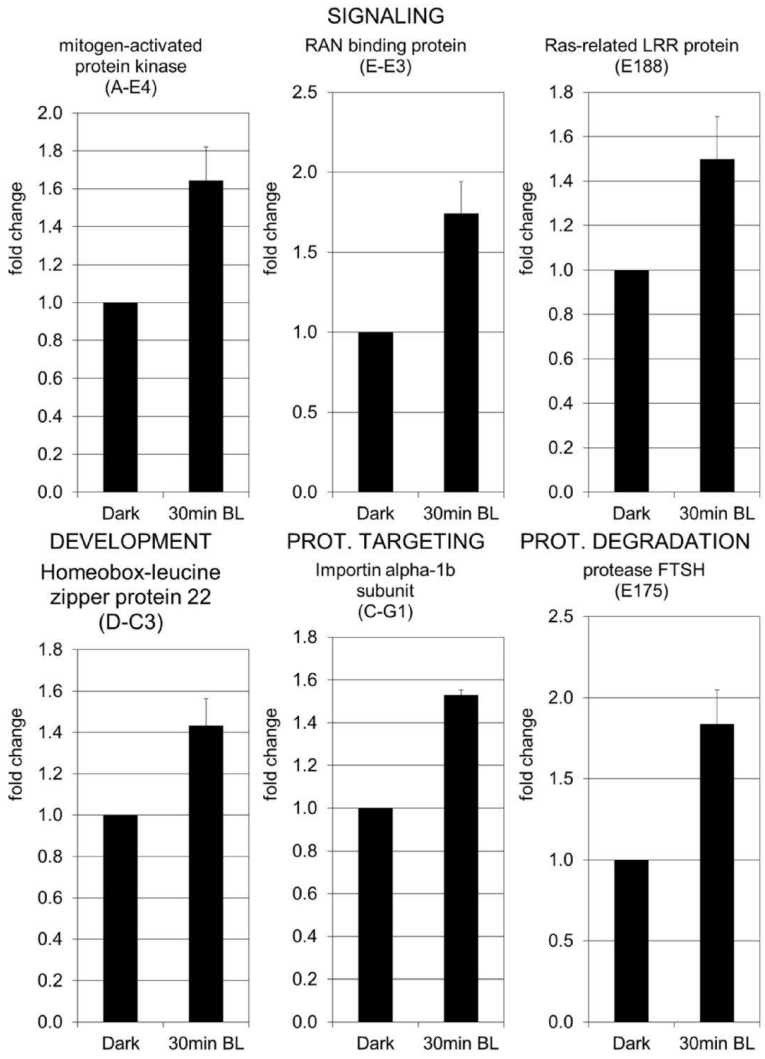


Fig. 3 Analysis by quantitative real-time PCR of expression of selected genes belonging to different functional categories. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the pp2ase gene as housekeeping gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results

identified by SSH screening confirmed that it is up-regulated by BL (Clone 12; Fig. 5a). Based on the aforemen-tioned literature, we can assume its role in cell wall strengthening during de-etiolation. Pectins, comprising an-other important cell wall component, are synthesized in the cis-Golgi, methyl-esterified in the medial-Golgi, substituted in the trans-Golgi, and then secreted into the cell wall. Zhao and co-authors [37] reported that de-esterification of methyl-esterified pectin may also be associated with growth cessation in both grasses and dicotyledons and may con-tribute to wall tightening by strengthening pectin–calcium networks. Pectin acetylation is another modification of pec-tins which probably occurs between the Golgi and the cell wall during pectin exocytosis. Its occurrence and function are poorly understood. The degree of O-acetylation of pectin changes during growth and differentiation of plant tissues, but also in response to environmental conditions. Pectin acetylesterases trigger the deacetylation of pectin. The overexpression of the black cottonwood (Populus tri-chocarpa) PAE1 gene in tobacco has been shown to impair the cellular elongation of floral organs. Thus, it appears that pectin acetylesterases function as an important regulator of pectin acetylation status to affect the physiochemical prop-erties of the cell wall's polysaccharides and consequently to affect cell extensibility [38]. The confirmation by qPCR that pectin acetylesterase (B-E2; Fig. 5b) is up-regulated by BL supports the hypothesis that they are actors of the inhib-ition of cell expansion which occurs during de-etiolation.

Based on our data and the analysis of the literature, we can hypothesize that exposure to BL rapidly induces

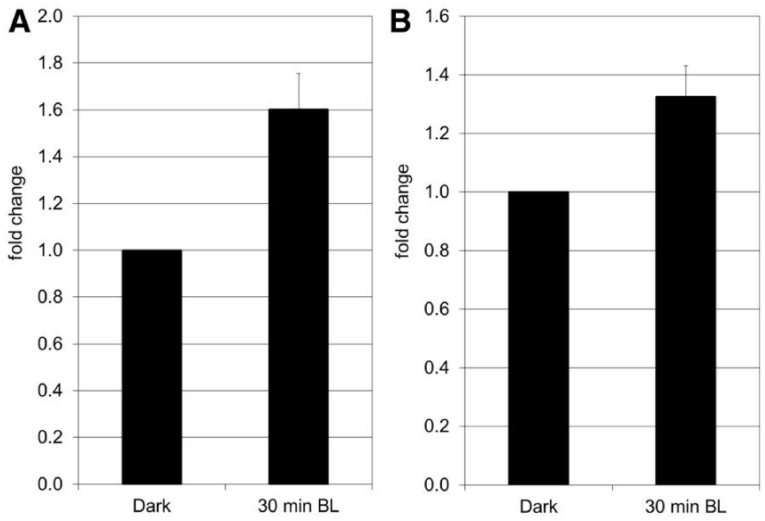


Fig. 4 Analysis by qPCR of the expression of two genes encoding proteins involved in translation and transcription: Histone 2B (a) and 26S proteasome regulatory complex, subunit RPN10/PSMD4 (b). The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the pp2ase gene as housekeeping gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results

changes in cell wall properties, namely extensibility. It would be thus interesting to validate this hypothesis through physico-chemical measurement of the cell wall of tomato seedlings' hypocotyl during BL-induced de-etiolation.

Role of vacuolar H^+-ATPase during de-etiolation

In tomato, three ESTs encoding vacuolar H + -ATPase (V-ATPase: 9, B-D5, E169) subunits were found to be up-regulated during PHOT1-mediated inhibition of hypocotyl growth. This was confirmed by qPCR for the V-type H^+-ATPase subunit c2 (B-D5; Fig. 6a). Considering the role of V-ATPase during de-etiolationis important if one considers that hypocotyl growth in darkness does not require the division of cortical or epidermal cells and cells elongate along an acropetal spatial and temporal gradient [39]. Cell expansion is achieved by: i) increase in cell ploidy via endoreduplication, and ii) os-motic water uptake into the vacuole, creating the turgor pressure necessary for the irreversible extension of the

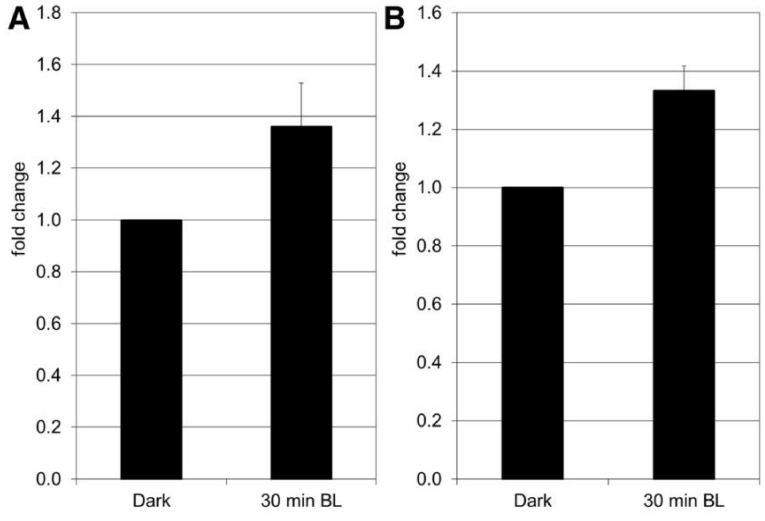


Fig. 5 Analysis by qPCR of the expression of two genes encoding proteins involved in cell wall modification: xyloglucan endotransglucosylase-hydrolase/XTH (a) and pectin acetylesterase (b). The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the pp2ase gene as housekeeping gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results

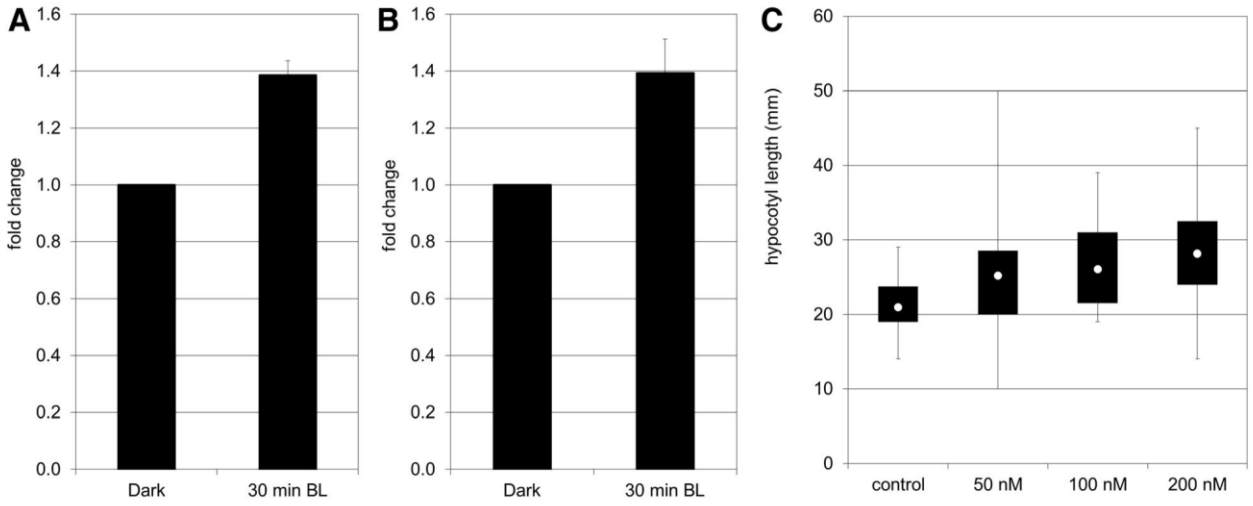


Fig. 6 Involvement of V-H^+-ATPase during de-etiolation of tomato seedlings. a Analysis by qPCR of V- ATPase subunit c2 (B-D5) during de-etiolation. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the pp2ase gene as housekeeping gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results. b Analysis by qPCR of VHA-A1 subunit during de-etiolation. The data represent the average fold change of 3 independent biological replicates ± SEM. Normalization was done using the pp2ase gene as housekeeping gene. Fold change was calculated compared to the value obtained for the dark control sample. The non-parametric Mann-Whitney U test (Statistica 12) was used to determine the significance of the results. c Effect of bafilomycin A1 on hypocotyl growth of tomato seedlings grown in BL . Germinated seeds were grown either in darkness or under constant BL on Murashige and Skoog medium containing varying concentrations of BafA1. After 5 days of growth, the length of hypocotyl was measured with a ruler to the nearest millimeter. The data are presented as boxes and whiskers. The whiskers represent the range of the data; the white dot within the box indicates the median value, while the boxes' lower and upper boundaries indicate the first and third quartiles, respectively. An average of 45 plantlets coming from independent replicates was measured. The non-parametric Kruskal-Wallis Anova with multiple comparison of mean rank was used for statistical significance of the data (Software: Statistica 12);
a: statistically different from the control condition with p-value ≤ 0.01

cell wall caused by the synthesis, incorporation, and cross-linking of new cell wall components. The cell ex-pansion is restricted by cell wall extensibility [40], [41]. V-ATPases are potentially involved in creating or regu-lating turgor pressure. They represent a major fraction of the total tonoplast proteins. V-ATPases also are present in the trans-Golgi network (TGN), where they are essential for its proper function [42]. Whereas inhib-ition of the tonoplast-localized V-ATPase does not affect cell expansion, inhibition of that which is TGN-localized is sufficient to restrict cell expansion [43]. Moreover, the det3 mutant, a possible negative regulator of photo-morphogenesis affected in V-ATPase function, was ori-ginally proposed to be impaired in vacuolar solute uptake resulting in adequate turgor pressure for cell ex-pansion [3]. Recent evidence has shown that a cell wall defect in the mutant is responsible for its reduced hypo-cotyl cell expansion [43]. Together, these data indicate that V-ATPase plays a role in cell wall integrity/synthesis through its function in the TGN-mediated secretory pathway, thereby participating in the restriction of cell expansion.

In eukaryotes, V-ATPase consists of at least 12 distinct subunits organized in two large subcomplexes: the cyto-solic V1 and membrane Vo subcomplexes. The cytosolic V1 complex is constituted of subunits A through H and catalyzes the hydrolysis of ATP which is associated with the pumping of protons into a compartment via the membrane-bound Vo complex. The Vo complex includes three integral proteins, named subunits a, c, c", and one hydrophilic subunit d [44]. In tomato, two isoforms of the subunits A (A1 and A2) were isolated. Whereas VHA-A2 isoform was found to be specifically expressed in roots, VHA-A1 isoform was ubiquitously expressed in all tissues and up-regulated by salinity stress [45]. The analysis of expression of the VHA-A1 isoform in the elongating zone of the tomato hypocotyl during BL-induced de-etioation revealed the accumulation of VHA-A1 transcripts during the time-course of the experiment (Fig. 6b). When tomato seedlings were grown in BL on a medium containing vary-ing concentrations of bafilomycin A1, a specific inhibitor of V-ATPases, the length of hypocotyl increased with in-creased concentration of bafilomycin A1 (Fig. 6c). These results indicated that in tomato, like in barley, BL induces accumulation of V-ATPase as well as its activation [12]. Both events appear to be required to trigger the restriction of cell expansion occurring during de-etiolation. To con-clude, we found a strong evidence that V-ATPases play a role during BL-mediated inhibition of hypocotyl growth. It nevertheless would be interesting to verify if V-ATPase

participates in elaborating the turgor pressure required for cell expansion or if it contributes to cell wall integrity.

Conclusion

BL-induced de-etiolation is a sequential process depending first on PHOT1 during the first 30–40 min of exposure to BL, with CRY1 being later responsible for the establishment of the steady-state growth rate. Whereas CRY1-mediated de-etiolation has been characterized at the molecular level [46], no information had been available concerning the PHOT1-mediated phase of de-etiolation.

Our analysis contributes to the understanding of PHOT1-mediated de-etiolation in plants and more particu-larly in important crop species. Using a subtracted cDNA library, we were able to identify 152 genes quickly up-regulated by BL. Their annotation revealed deep changes in chromatin modelling, transcription, and translation, but also in cellular processes and signaling such as cell wall in-tegrity/synthesis, cytoskeleton, and trafficking/secretion. By using a high-throughput RNAseq method we could obtain more precise information concerning genes differentially expressed during PHOT1-mediated de-etiolation. We are currently developing such analysis including also a tomato mutant depleted of PHOT1 generated in our laboratory by artificial microRNA. Nevertheless, the current study already opens the doors toward processes upon which to focus our attention, notably chromatin modelling and the potential role of histone 2B, as well as the involvement of V-ATPase in either generating appropriate turgor pressure or partici-pating in cell wall integrity/synthesis. It is also noteworthy that an array of sequences encodes for protein of unknown function and represents a pool of proteins with novel func-tions in PHOT1-mediated de-etiolation.

Availability of data and material

The dataset supporting the conclusions of this article is included within the article and its Additional files 1 and 2.

Additional files

Additional file 1: Table S1. Gene ontology and functional annotation of the expressed sequence tags (ESTs) up-regulated by 30 min of exposure to blue light. (XLSX 68 kb)

Additional file 2: Nucleic acid sequences of the 168 ESTs found to be putatively up-regulated 30 min after exposure to BL. (DOCX 45 kb)

Competing interests
The authors declare that they have no competing interests.

Author's contributions
P.H. performed the qPCR validation of the data, including the preparation of cDNA. V.B. designed the study, prepared and analyzed the SSH library, interpreted data, and prepared the manuscript. All authors have read and approved the manuscript.

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Affinity chromatography revealed 14-3-3 interactome of tomato (Solanum lycopersicum L.) during blue light-induced de-etiolation

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ABSTRACT

De-etiolation is the first developmental process under light control allowing the heterotrophic seedling to be-come autotrophic. The phytohormones cytokinins (CKs) largely contribute to this process. Reversible phos-phorylation is a key event of cell signaling, allowing proteins to become active or generating a binding site for specific protein interaction. 14-3-3 proteins regulate a variety of plant responses. The expression, hormonal regulation, and proteomic network under the control of 14-3-3s were addressed in tomato (Solanum lycopersicum L.) during blue light-induced photomorphogenesis. Two isoforms were specifically investigated due to their high expression during tomato de-etiolation. The multidisciplinary approach demonstrated that TFT9 expression, but not TFT6, was regulated by CKs and identified cis-regulating elements required for this response. Our study revealed > 130 potential TFT6/9 interactors. Their functional annotation predicted that TFTs might regulate the activity of proteins involved notably in cell wall strengthening or primary metabolism. Several potential in-teractors were also predicted to be CK-responsive. For the first time, the 14-3-3 interactome linked to de-etio-lation was investigated and evidenced that 14-3-3s might be involved in CK signaling pathway, cell expansion inhibition and steady-state growth rate establishment, and reprograming from heterotrophy to autotrophy.

Biological significance: Tomato (Solanum lycopersicum L.) is one of the most important vegetables consumed all around the world and represents probably the most preferred garden crop. Regulation of hypocotyl growth by light plays an important role in the early development of a seedling, and consequently the homogeneity of the culture. The present study focuses on the importance of tomato 14-3-3/TFT proteins in this process. We provide here the first report of 14-3-3 interactome in the regulation of light-induced de-etiolation and subsequent photomorphogenesis. Our data provide new insights into light-induced de-etiolation and open new horizons for dissecting the post-transcriptional regulations.

1. Introduction

Cell signaling mechanisms often involve posttranslational mod-ifications (PTMs) of proteins, affecting their conformation, activity, stability, and/or localization [1]. The reversible phosphorylation of the serine, threonine or tyrosine residue has been probably the most ex-tensively studied [2]. Very often, however, phosphorylation of the protein alone is not sufficient to modulate protein function. Rather, phosphorylation is required to ensure the binding of interactors which will themselves regulate protein function. Among the plant phosphopeptide-binding proteins, 14-3-3 proteins (14-3-3s) are the best characterized [3]. They have recently attracted attention due to their remarkable capacity to affect a wide array of physiological, develop-mental, and cellular processes such as primary metabolism, hormone signaling, response to light, cell growth and division, pathogen–plant interaction, and response to biotic and abiotic stresses [4–7]. The 14-3-3s are small, acidic proteins highly conserved, found in all eukaryotic organisms examined so far. Whereas yeast has two isoforms and mammals seven, most plant genomes contain a dozen 14-3-3 genes which can be divided into epsilon and non-epsilon groups, based on

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their sequence homology and intron/exon structure [8,9]. The epsilon group is considered as “ancestral” and to fulfill fundamental functions; in opposite, the non-epsilon group evolved later and members of this group might be related to organism-specific functions [10]. Due to re-dundancy of 14-3-3 function, it has been suggested that the function specificity is related rather to the spatiotemporal regulation of the ex-pression of the different isoforms. Nevertheless, the presence of phe-notypes for single and multiple 14-3-3 knock-out mutants and the dif-ferential subcellular localization of 14-3-3s within the cell support the view that function specificity varies for the different 14-3-3s [11–14].

The 14-3-3s are present in the cell as a combination of homodimers and heterodimers. Dimerization occurs through residues of the first four alpha helices in the N-terminal region of the protein [15]. This region is highly variable and could contribute to dimer specificity. Some iso-forms have preference to form homo- or heterodimers, as was shown for two human 14-3-3 isoforms [16]. It also has been hypothesized that homo- and heterodimers could serve different functions [17]. Although 14-3-3 isoform dimerization is not well understood in plants, it likely depends on the intrinsic affinities among 14-3-3s [18]. The formation of a 14-3-3 dimer is required for the binding of a client protein, thereby affecting its activity, stability, conformation, and localization [19]. Because different 14-3-3 s harbor different specificity toward different targets, heterodimers might act as adapters associating two proteins which otherwise would not interact together [20]. Nevertheless, such interaction has never been demonstrated until now. Whereas it is well characterized that the phosphorylation marks the protein for changes and the activation or inactivation is completed once 14-3-3 is bound [21], to date no specific enzymatic function has been ascertained for 14-3-3s. This has led to the idea that their function is solely due to physical interaction [22]. 14-3-3 proteins often interact with their binding partners through three well-defined binding motifs (mode I, II and III) [23,24]. In plants, the most commonly occurring motif is a variation of mode I [LX(R/K)SX(pS/pT)XP]. Phosphorylation of the target protein is often, but not always, a prerequisite for 14-3-3 binding to occur [25–27]. Like other proteins, 14-3-3 s can be subjected to post-translational modifications. The first event of 14-3-3 phosphorylation was reported for the Arabidopsis 14-3-3ω isoform. Since that time, large phosphoproteomic studies have revealed that phosphorylation of 14-3-3s is a common event. Further studies conducted with Arabidopsis have demonstrated that phosphorylation events occur in the highly variable C-terminal region of the isoform. Furthermore, a conserved serine residue in the N-terminal region of the 14-3-3 has been char-acterized to be involved in 14-3-3 dimerization [28,29]. Phosphory-lated 14-3-3s might have reduced affinity toward the interacting pro-tein through 14-3-3 dimer destabilization [14,18].

As sessile organisms, plants are exposed to environmental fluctua-tions and developed mechanisms to ensure optimal growth. Light is probably the most important environmental factor influencing plant growth and development throughout the plant cycle, from germination to flowering, and that includes the direction of growth [30]. This is especially true during the shift from skoto- to photomorphogenesis, called de-etiolation, when the seedling has to become autotrophic. Plants have evolved highly sophisticated, unique photoreceptors to perceive and respond to light. Several classes of photoreceptors may be described: phytochromes (PHYs), which sense mainly red/far-red light (RL); cryptochrome 1 and 2 (CRY1/2), phototropin 1 and 2 (PHOT1/2) and zeitlupe (ZTL) which perceive blue light (BL), and the recently identified UltraViolet B (UVB) photoreceptors [31,32]. BL inhibits hy-pocotyl growth quicker and to a greater extent than does RL [33]. In Arabidopsis, BL-mediated hypocotyl growth inhibition is a two-step process, mediated both by PHOT1 and CRY1 [34,35]. These photo-receptors trigger complex networks, including modulation of enzymatic activity, dynamic regulation of gene expression, and protein–protein interactions. BL perception by PHOT1 induces autophosphorylation of multiple serine residues of the receptor which are essential for further signal transduction. Isoforms of the non-epsilon 14-3-3 group have been characterized to bind PHOT1 [36]. Nevertheless, the role of this in-teraction is still unknown. We recently conducted a study to understand the molecular mechanisms underlying BL-mediated de-etiolation in tomato. As in Arabidopsis, we observed a two-phase evolution of the growth rate, with the rapid inhibition of the hypocotyl growth rate being correlated with inhibition of cell expansion, cytokinins (CKs) accumulation, and inhibition of endoreduplication [37]. Studies of 14-3-3 proteins have mostly focused on Arabidopsis; therefore almost no information is available for economically important crops, such as to-mato. The altered hypocotyl length of Arabidopsis 14-3-3 mutants grown in RL or in darkness suggests a role for 14-3-3 proteins in the control of hypocotyl elongation [38,39]. However, their possible in-volvement in plant response to BL is not yet clear. In tomato, 12 iso-forms have been identified and are referred to as Tomato Fourteen Three (TFTs). Earlier study of antisense transgenic tomato plants had demonstrated that at least two isoforms were involved in light-medi-ated plant development [40]. In the present study, we concentrated on events arising during establishment of the steady-state growth rate under BL exposure while giving special attention to the role of 14-3-3s in that process. We also describe here how two tomato 14-3-3s are in-volved in the BL-mediated response of tomato hypocotyl. Using im-mobilized recombinant tomato 14-3-3 proteins, > 130 proteins related to a large variety of cellular processes were identified as potential 14-3-3 interactors. This study yields a better understanding of networks regulated by 14-3-3s and contributing to light-induced growth rate limitation observed during de-etiolation.

2. Materials and methods

2.1. Cultivation

Seedlings of the wild-type (WT) tomato Solanum lycopersicum (L.) cv. Rutgers were grown as already described [37]. The Petri dishes (120 × 120 mm) were incubated vertically in darkness at 22 °C for 3 days (Weiss, Great Britain). Etiolated seedlings were irradiated by blue light (BL; TL-D 36 W/18-Blue, Philips; total photon fluence rate 10 μmol m^{-2} s^{-1}) for up to 120 min. For gene expression profiling, the elongation zone of the hypocotyl was rapidly harvested under BL or safety green light for BL-irradiated and darkness-grown seedlings, re-spectively, then frozen in liquid nitrogen and stored at −80 °C until proceeding.

For analysis of cytokinin response, germinated seedlings were transferred on Murashige and Skoog medium supplemented with 0, 1, 5 or 10 μM isopentenyladenine (iP, Olchemim, Czech Republic). The seedlings were further grown in darkness for 3 additional days. Afterwards, the elongation zone of the hypocotyl was excised under safety green light for RNA extraction and gene expression. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until proceeding.

Antisense TFT6 tomato lines were obtained by RNAi in the back-ground Ailsa Craig [41]. These silenced lines were characterized for reduced growth parameters, including height, leaf number and dry weight. Seeds were kindly provided by Prof. M. Roberts from Lancaster University (UK). No antisense lines were available for the isoform TFT9.

2.2. Quantitative reverse transcription polymerase chain reaction

For all experiments concerning gene expression profiling, total RNA was isolated using a ZR Plant RNA MiniPrep kit (Zymo Research, USA) according to the manufacturer's instructions. RNA was treated with 2 units of Turbo DNAse (Ambion, USA) for 45 min at 37 °C. Reverse transcription was performed from 2 μg of the total RNA using RevertAid H minus transcriptase (ThermoScientific, USA). cDNA was diluted 50 times and used in a reaction containing GreenMaster SYBR Premix with high ROX (Jena Biosciences, Germany) and 300 nM of forward and reverse primer. Primers were designed using IDT qPCR assay design and

OligoAnalyzer with default parameters taken from http://eu.idtdna.com/scitools/Applications/RealTimePCR/). Their specificities were checked by BLAST (http://blast.ncbi.nlm.nih.gov) against the tomato database. The primer sequences and efficiencies are presented in Table 2. qPCR reactions were carried out on a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) as follows: an initial denatura-tion for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, then 1 min at 60 °C. Melting curve analysis was performed after 40 cycles to verify primer specificity. Each sample was analyzed in three to five independent biological replicates and in technical triplicates. Each in-dependent biological replicate represented a pool of 25 explants. The cycle threshold value was determined for each sample. Three reference genes, PP2Acs, EF1α and Tip41like were used for expression normal-ization of genes of interest [42]; these genes were determined as being the most stable reference genes in our conditions (data not shown). Normalized relative quantities were obtained using the efficiency cor-rected 2^{-Ct} method [43,44]. In order to exclude the effect of biolo-gical (intra-assay) variation on the statistical significance of results, $\log_{10}$ transformation of normalized relative quantities, mean centering and autoscalling were performed [45]. The relative quantification was determined in comparison to the expression of TFT1 gene in dark control sample and/or untreated samples. Presented data show the mean ± standard error of the mean (SEM) of three to five independent biological replicates. Statistical significance was supported by non-parametric Kruskal-Wallis Anova & Median test analysis followed by a post-hoc multiple comparison of mean rank (Statistica 12, Statsoft).

2.3. Hypocotyl growth measurement

For growth measurement, seeds of the tft6-ko mutant and its cor-responding wild-type (WT) Ailsa Graig were surface sterilized and sown on MS medium as previously described [46]. Germinated seeds were grown in the dark for 2 days before expose to continuous blue light (BL; TL-D 36 W/18-Blue, Philips; total photon fluence rate 10 μmol m^{-2} s^{-1}). Dishes were regularly scanned and the length of hypocotyl was determined with ImageJ (https://imagej.nih.gov/ij/). Three independent biological replicates containing 30 seedlings were measured.

2.4. Protein extraction, recombinant TFT9 and TFT6, and pull-down assay

For the identification of putative interactors of TFT6 and TFT9, cytoplasmic protein fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific, USA) according to manufacturer's instructions. With the modification that buffers were supplemented with plant protease and phosphatase inhibitors (Sigma-Aldrich). Each sample corresponded to the pool of hypocotyl of 30 etiolated seedlings grown in the dark for 3 days and exposed or not for
2 h to BL (L-D 36 W/18-Blue, Philips; total photon fluence rate 10 μmol m^{-2} s^{-1}).
For pull-down assay, recombinant proteins were prepared for TFT9 and TFT6, both fused to histidine-tag. For this purpose, the coding re-gions of TFT9 (Solyc07g053260) and TFT6 (Solyc11g010200) were cloned into the C-terminal His-vector pQE60 (Qiagen, Germany) and N-terminal His-vector PET28b + (Novagen), respectively. The constructs were introduced into E. coli strain BL21 (DE). Expression of re-combinant His-tagged proteins was induced for 4 h at 37 °C by addition of 1 mM isopropylthio-β-galactoside to overnight-grown bacterial cul-ture. Cells were collected by centrifugation for 20 min at 4500 xg at 4 °C, then resuspended in His-binding buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 10 mM imidazole, 0.03% Triton X-100). Lysis of bacterial cells was performed by repeated freezing/thawing cycles in liquid nitrogen and at 42 °C, respectively. The cell lysate was clarified by centrifugation at 12,000 xg at 4 °C for 20 min. The supernatant containing expressed His-tagged TFT protein was immobilized on nickel-charged His-Affinity Gel using His-Spin Protein Miniprep (Zymo Research, USA) and incubated with cyto-plasmic proteins extract from excised elongating zones of etiolated hypocotyls or hypocotyls illuminated by BL for 2 h. Two controls were included: the cytoplasmic protein extract without immobilized bait, and the immobilized bait incubated with protein extraction buffer.
Cytoplasmic proteins extracted from elongation zone of etiolated seedling hypocotyls exposed or not to BL were incubated with im-mobilized TFT9 or TFT6 for 1 h at 4 °C and unbound fractions were washed with two volumes of wash buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 50 mM imidazole, 0.03% Triton X-100). The bound proteins were eluted with 150 μl of the elu-tion buffer containing 50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride and 250 mM imidazole), diluted with 1.5 ml digestion buffer (2 M urea, 50 mM ammonium bicarbonate), digested overnight with an immobilized trypsin (Promega), desalted by C18 SPE.

2.5. Liquid chromatography–mass spectrometry analysis

The identification of potential TFT-interactors was performed by liquid chromatography coupled to mass spectrometry. For this purpose, tryptic digests (obtained as described above) corresponding to 5 μg of protein extract were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted up to a 120 min with a 4% to 40% acetonitrile gradient; spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s. The measured spectra were ex-tracted by Bruker's Data Analysis 4.1 [47]. Recalibrated MGF files were searched against tomato protein sequence database (ITAG 2.4) by Se-quest HT and Mascot 2.4 with the following parameters: Enzyme – trypsin, max two missed cleavage sites; Mass tolerance – 35 ppm (MS) and 0.1 Da (MS/MS); Modifications – up to three dynamic modifica-tions including Met oxidation, Asn/Gln deamidation, Lys/His/Leu/Arg methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation. Results were integrated by Proteome Discoverer 2.0 (Thermo), and the quantitative differences were evaluated by calculating normalized numbers of peptide spectral matches [48], Putative BL-dependent TFT interactors (not identified in darkness-grown samples or significantly increased at BL) were targeted in experimental replicates via a selected-reaction monitoring-based analysis (TSQ Quantiva, Thermo). Only proteins having at least one detectable proteotypic peptide were con-sidered for the analysis, and the resulting data were evaluated in Sky-line 3.1 (MacCossLab Software, https://skyline.gs.washington.edu).

Two independent biological replicates were performed in order to validate the results. Statistical significance was validated by t-test ($p < 0.05$). The mass spectrometry proteomics data have been de-posited to the ProteomeXchange Consortium via the PRIDE [49] partner repository with the dataset identifier PXD006123 and https:// doi.org/10.6019/PXD006123.

2.6. Proteomic profiling of hypocotyl of seedlings grown under different light conditions

Plants for analysis of light-dependent changes in hypocotyl pro-teome were cultivated as described above with the following mod-ifications: seeds of cultivar Micro-Tom were stratified at 4 °C for 5 days and cultivated at 24 °C in a LED-equipped growth chamber (Polyklima, Germany) under (i) continuous blue light (20 μmol m^{-2} s^{-1}), (ii) white light (80 μmol m^{-2} s^{-1}), or (iii) darkness. Hypocotyls of 30 seedlings per replicate were excised and flash-frozen, and the total protein was extracted and processed as described above. For comparative analyses, peptide concentration was determined by a colorimetric peptide assay (ThermoScientific, USA) and then aliquots corresponding to 5 μg of peptide were analyzed. Identification of the proteins was done by liquid

chromatography–mass spectrometry as described above. Changes in protein abundance were deemed significant if the corresponding amounts of detected peptides were significantly ($p < 0.05$) changed at least two-fold in both biological replicates. The whole experiment was performed in two independent biological replicates.

2.7. Yeast two-hybrid assay

The ORFs of TFT6 (Solyc11g010200), TFT9 (Solyc07g053260), Phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690), V-ATPase subunit A (Solyc12g055800) and phototropin 1 (PHOT1; Solyc11g072710) were fused to the GAL4-activating domain (AD) or GAL4-binding domain (BD) via cloning into pGADT7 or pGBKT7 vectors (Clontech, USA), respectively. The Saccharomyces cerevisiae Y2H GOLD yeast strain (Clontech, USA) was co-transformed with bait and prey using the polyethylene glycol/lithium acetate method. Transformants were se-lected on synthetic defined (SD) media lacking leucine and tryptophan (SD-Leu-Trp). Four individual colonies were grown overnight in liquid culture (SD-Leu-Trp) at 30 °C and 10-fold dilutions were dropped on SD-Leu-Trp and selective media lacking leucine, tryptophan and histidine (SD-Leu-Trp-His) and SD-Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazol (3-AT).

2.8. Subcellular localization of TFTs

The ORFs of TFT6 and TFT9 genes were fused to yellow fluorescent protein in the pEarlyGate104 vector. Both constructs were inserted into Agrobacterium tumefaciens C58 cells. Transient expression in leaves of 4-week-old Nicotiana benthamiana was performed as described by [50] To prevent silencing, A. tumefaciens C58 carrying a construct that expresses the silencing suppressor P19 was added to the mixtures. The ratio of cells carrying P19:effector was 1:5. Mixtures were incubated for 3 h in darkness at room temperature before infiltration. Three days after in-filtration, leaf circle sections were collected and analyzed using a confocal Zeiss LSM 780 microscope.

2.9. Promoter sequence analysis and site-directed mutagenesis

The prediction of cis-acting element binding sites was performed by PlantPAN2.0 (http://plantpan2.itps.ncku.edu.tw/; [51]). The 1.4 kb sequences upstream of the start codon were retrieved from En-semblPlants (http://plants.ensembl.org/index.html). A Q5® Site-Di-rected Mutagenesis Kit (New England Biolabs, USA) was used to create specific, targeted changes in two-component response regulators sites in double-stranded plasmid DNA containing a truncated TFT9 promoter sequence.

2.10. Protoplast isolation and dual luciferase reporter assay

The sequence of TFT9 promotor and its truncated versions were cloned into pGL3 vector (Promega, USA) to obtain a Firefly luciferase reporter system. Leaves of tomato plants 4 weeks old were used for protoplasts transformation as described by [52]. In brief, 10^6 proto-plasts were transformed with 20 μg of plasmid DNA mixture consisting of TFT-reporter vector and vector driving the expression of Renilla lu-ciferase. This latter allowed normalizing the assay. Incubation was done in darkness at room temperature for 2 h. Subsequently, 10 μM iP were added to the reaction for 16 h. The protoplasts were lysed in passive lysis buffer before determination of Firefly and Renilla LUC activities (Dual-Glo Luciferase Assay System, Promega, USA) by measuring lu-minescence with a luminometer (BioTek, USA).

2.11. Prediction of tomato cytokinin-responsive proteins

Prediction of tomato cytokinin-responsive proteins based on orthology with Arabidopsis CK-responsive protein [53] was done as followed: tomato sequences were used as query in GreenPhyl v4 (http://www.greenphyl.org/cgi-bin/index.cgi) to retrieve Arabidopsis homolog sequences inferred from phylogeny and best blast mutual hit (BBMH) matching sequences [54]. Further the Arabidopsis accessions were searched into the database of CK-responsive proteins described by [53]. When an Arabidopsis accession was found to be CK-responsive, the tomato homolog sequence was annotated as CK-responsive as well.

2.12. Statistical analysis

Statistical significance of the results was supported either by the nonparametric Kruskal-Wallis ANOVA followed by multiple compar-isons of mean ranks, or by one-way ANOVA followed by post-hoc Bonferroni test (Statistica v.12, StatSoft).

3. Results

3.1. Expression of 14-3-3s in hypocotyl elongation zone during BL-induced de-etiolation

The phylogenic analysis of tomato 14-3-3s revealed their organi-zation into the epsilon group including TFT7, 8, 9 and 12 and the non-epsilon group encompassing TFT1, 2, 3, 4, 5, 6, 10 and 11 (Fig. S1). As in Arabidopsis, this was supported by the exon-intron structure: with structures 7–6 and 4–3 for the epsilon and non-epsilon members, re-spectively [8]. The non-epsilon group can be divided into three sub-groups. In tomato, like in Arabidopsis, the hypocotyl of seedlings grown in the dark elongates along an acropetal gradient, with the portion beneath the cotyledon and hook forming the elongation zone of hypo-cotyl [37]. The expression profile of the 12 tomato isoforms was de-termined in the elongation zone by quantitative real-time PCR (qPCR) during BL-mediated de-etiolation (Fig. 1). Unfortunately, the high homology between TFT4 and TFT11 did not allow us to design specific primers to distinguish these two isoforms. Interestingly, TFT12 was not detectable under our study conditions, which was consistent with a predicted expression restricted to pollen both in Arabidopsis and to-mato [55]. The non-epsilon TFT6 and the two epsilon TFT9 and TFT7 isoforms were the most abundant in the elongation zone of the hypo-cotyl. Our observation was supported by the proteomic analysis done on the hypocotyl of seedlings grown under different light regimes (Table S1). Indeed, both TFT6 and TFT9 proteins were found to be highly accumulated in the hypocotyl of tomato seedlings grown under continuous BL, with TFT6 being the most accumulated. Consequently, we focused our attention on TFT9 (epsilon isoform) and TFT6 (non-epsilon), whose expression was high and stimulated by exposure to BL, leading to the hypothesis that they could play a particular role in the process. Under our study conditions, we observed that tomato plants deficient in TFT6 expression were shorter than the control plants when grown under continuous BL (10 μmol m^{-2} s^{-1}; Fig. 2). This observation further supported the hypothesis that TFT6 plays a role in light-con-trolled growth, and might be required for cell expansion in light and establishment of the steady-state growth rate.

3.2. Identification of TFT6/9 interactors

The 14-3-3/TFT proteins are important actors of the phosphoryla-tion/dephosphorylation signaling pathway cascade. The accumulation of TFT6 and TFT9 transcripts in response to BL exposure in the elon-gation zone of etiolated hypocotyl led us to hypothesize that they are part of the regulatory network triggering de-etiolation. In the present study, we initiated the interactomic study of these two phylogenetically distinct tomato isoforms belonging to the non-epsilon group (TFT6) and the epsilon group (TFT9). For this purpose, the cytoplasmic proteins were extracted from the elongation zone of hypocotyl of etiolated seedlings exposed or not for 2 h to BL. Proteins not identified in dark-

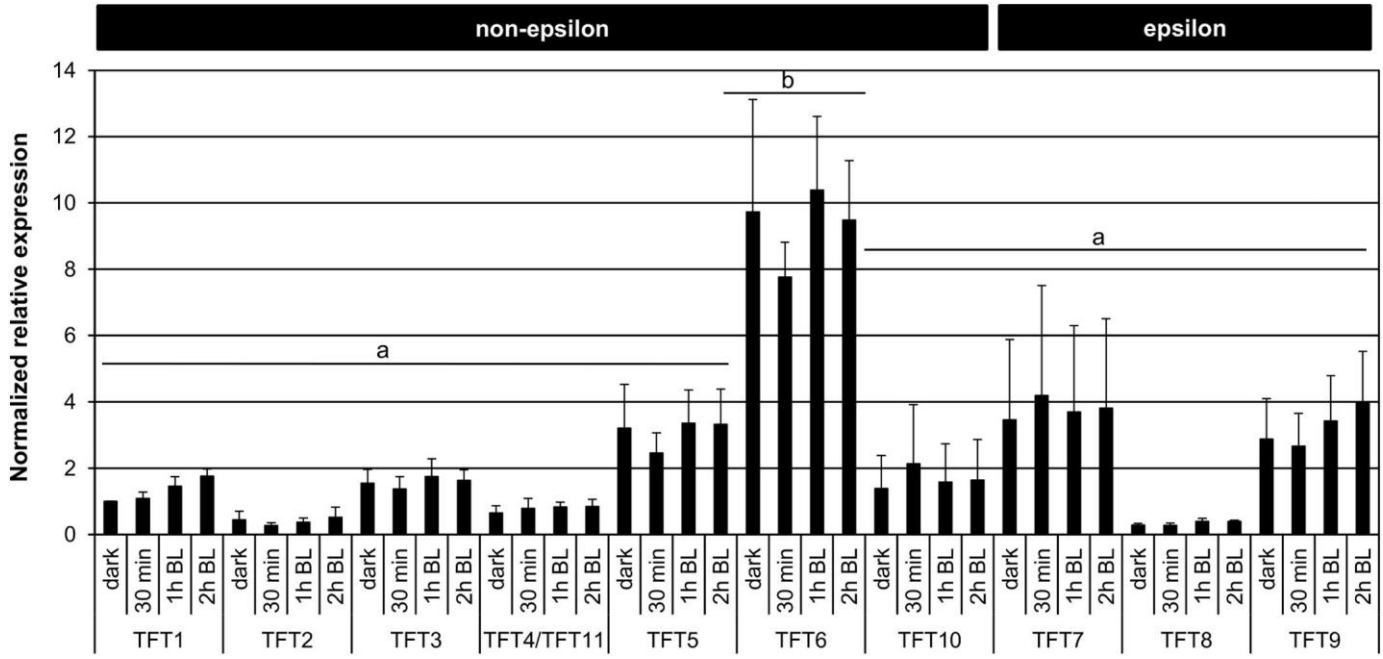

Fig. 1. Expression profile of the tomato TFT genes in the elongation zone of hypocotyl during BL-induced de-etiolation. The graph represents the average of three to five independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three reference genes: PP2Acs (Solyc05g006590), Tip41-like (Solyc10g049850) and EF1α (X14449). Log_{10} transformation, mean centering and autoscalling were performed as described by [45]. Results were expressed as fold change relative to the expression of the TFT1 gene in the elongation zone of seedlings grown in darkness. TFTs are grouped according to their relation to epsilon and non-epsilon group, as indicated by boxes. A non-parametric Kruskal-Wallis Anova & Median test analysis was performed followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft); identical letters indicate no significant difference.

grown seedlings or significantly accumulated in BL-exposed seedlings were determined as putative BL-dependent TFT interactors. Altogether, > 870 proteins were found in hypocotyl pull-down assays (false discovery rate, FDR = 1%), but only 133 were exclusive from BL-elongated hypocotyls or significantly increased compared to the dark-grown hypocotyl elongation zone (absolute ratio > 2.0, $p < 0.05$; Table 1 and Table S1). Most of these 133 proteins contained high-scoring 14-3-3 binding sites as determined by 14-3-3-Pred [56]. Of them, 18 were found to be nonspecific to one or the other isoform (representing nearly 50% of the putative TFT9 interactors), 96 were specific to the non-epsilon TFT6, and 19 were specific to the epsilon TFT9 (Fig. 3a). Thirty-three percent of the client proteins identified in the present study have already been reported as client proteins of 14-3-3s in other species (Table 1). The proteins were functionally annotated based on gene ontology and KEGG metabolite pathways (Fig. 3b).

To a large extent, TFT6-interacting proteins belong to the functional category “metabolism” (35% of the sequences). Client proteins be-longing to the “cell organization” and “hormone metabolism and sig-naling” categories were found specifically to interact with TFT6. It can also be noted that seven hypothetical TFT6 interactors have a role in cell wall (CW) modification and synthesis. In contrast, no particular functional categories could be related to binding with TFT9. Nevertheless, up to 25% of the proteins interacting with TFT9 were related to “transport” and identified as subunits of the vacuolar H^+-ATPAse (V-ATPase).

The functional relevance of the putative TFT interactors identified in cv. Rutgers was supported by a proteomic profiling performed on Micro-Tom tomato seedlings grown for 7 days under various light (continuous BL, white light/WL, or in darkness/D). Altogether, > 900 proteins were identified in a liquid chromatography–mass spectrometry shotgun analysis of two biological replicates. In these conditions, only about half of the putative TFT6/9 interactors identified were detectable in the “light” proteome. Therefore, a more sensitive, selected-reaction monitoring-based protein quantitation was employed, allowing identi-fying that 98 of the 133 putative TFT6/9 interactor identified were

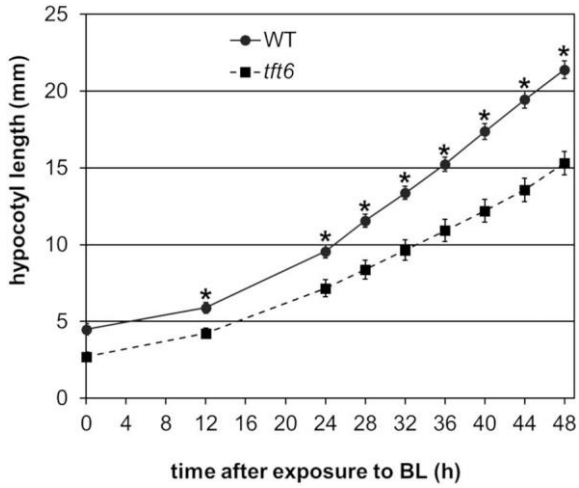

Fig. 2. Growth of etiolated hypocotyl of Ailsa Craig (WT) and tft6 mutant after exposure to BL. Germinated seedlings grown for 2 days in the dark where transferred to continuous BL (total photon fluence rate 10 μmol m^{-2} s^{-1}). The length of hypocotyl was determined by ImageJ after scanning of the dishes. Three independent biological replicates containing at least 30 seedlings were measured. *: statistically significant between the WT and tft6 ($p < 0.05$; Statistica 12, StatSoft).

present in the light-related proteomic profiling (Table S2). Consequently, these results confirmed that most of the putative TFT6/9 interactors are proteins involved in light-regulated growth. By com-paring their relative amounts to those from the D-grown hypocotyls, we found that 87 and 64 were significantly accumulated in BL- and WL-grown hypocotyls, respectively. Finally, 34 proteins were significantly more accumulated in BL-grown seedlings than in those grown in WL. Nevertheless, 17 of them – including TFT9 and TFT6 – did not meet the selected two-fold threshold criteria. With the exception of TFT10, all

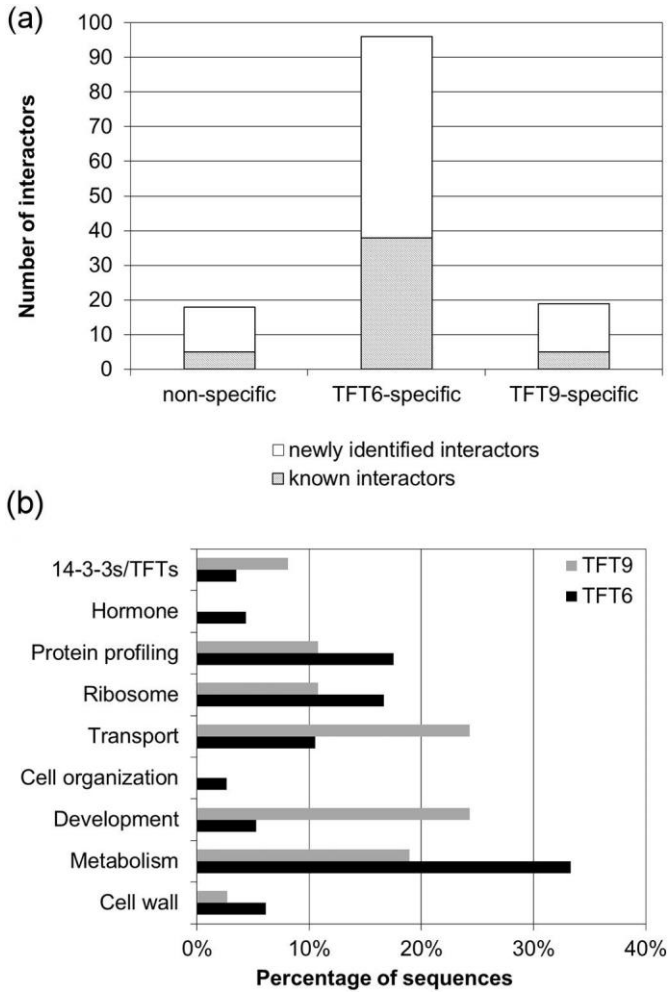

Fig. 3. Identification of 133 potential interactors of TFT6 and/or TFT9 (a) and their functional characterization (b). In (a), "non-specific" indicates interactors which were present in the interactome of both TFT6 and TFT9. The number of already known (gray shaded) and newly identified (white) interactors are also indicated. In (b), the data are expressed as percentages of the total number of proteins identified in the interactome of either TFT6 or TFT9.

detected 14-3-3 proteins were significantly accumulated in response to the BL and/or WL irradiation.

14-3-3s form homo- and heterodimers. In our study, we determined that both TFT isoforms possibly interacted with other TFT isoforms. Specifically, TFT6 and TFT4 were identified among the possible client proteins of TFT9; in turn, TFT9, TFT10 and TFT3 were identified in TFT6 interactome (Table 1). An yeast two-hybrid (Y2H) assay con-firmed the interactions between TFT9 and TFT6 and between TFT9 and TFT4. Nevertheless, no interaction between TFT6 and TFT4 could be observed, which is in agreement with the fact that the TFT6 inter-actome did not contain TFT4 (Fig. 4a). The subcellular localization of the three proteins indicated that whereas TFT6 and TFT9 are localized both in cytoplasm and the nucleus, TFT4 seemed to be restricted to cytoplasm (Fig. 4b–d).

3.3. Isopentenyladenine (iP) affects TFT6 and TFT9 expression

De-etiolation relays on a complex hormonal regulatory network. While auxin, gibberellins, ethylene and abscisic acid stimulate growth, CKs strongly limit cell expansion. In tomato, we demonstrated that iP, a free active form of CKs, accumulated in the elongation zone of etiolated seedlings in response to BL exposure [37]. Moreover, under the present study conditions, exogenously applied iP resulted in shortening of the hypocotyls in a dose-dependent manner (Fig. 5a), further supporting the role of CKs in hypocotyl growth inhibition [57]. Two Arabidopsis 14-3-3s (GF14ψ and GF14ε) were identified as early CK-response pro-teins [58]. Consequently, we addressed the question whether in tomato, TFT expression might be regulated by CKs. Therefore the accumulation of TFT6 and TFT9 transcripts was monitored in the hypocotyl elonga-tion zone of seedlings grown on medium supplemented with iP at dif-ferent concentrations (Fig. 5b). TFT6 expression was not significantly affected by exogenous iP, whatever the concentration used. In contrast, TFT9 transcripts significantly accumulated in the presence of exogenous iP in a dose-dependent manner. The Arabidopsis orthologue of TFT9 (14-3-3μ, At2g42590.2) was not predicted to be CK-responsive in ear-lier studies (Table 1), probably due to the difference of material ana-lyzed or different threshold applied [58,59]. Taken together, our data indicate that during BL-induced de-etiolation of tomato seedlings, TFT9 expression is regulated by CKs, whereas the regulation of TFT6 ex-pression is CK-independent.

3.4. TFT9 expression is stimulated by iP via binding of two-component response regulators (RRs)

We analyzed the upstream region (1.2 kb) of the TFT9 gene by PlantPAN2.0 using the Arabidopsis database as a reference. We could predict the presence of 21 putative cis-regulating element binding sites related to the type B-RR (RRB). A transcriptional activation assay was designed in order to confirm the iP-stimulated expression of TFT9 and identify the binding motifs responsible for this activation. For this purpose, the 1.2 kb native promoter sequence and different truncated versions were fused to the luciferase reporter gene and transfected into tomato protoplasts (Fig. 6a). The addition of iP increased the luciferase activity by 40% compared to mock-treated protoplasts (Fig. 6b). Truncation of the promoter between −1224 and −1158 resulted in a two-fold increase of the luciferase activity, indicating that this region might contain a repressor element. A further deletion of the region between −1158 and −929 resulted in a complete loss of CK stimula-tory effect. Two core CK response motifs were present at the positions −1106 and −1035, both localized on the same strand. To decipher their implication in iP-mediated regulation of TFT9 expression, these two sites were point-mutated (Fig. 6c): mutation in either or both of these binding sites abolished the CK stimulatory effect, suggesting that both sites are required for CK-mediated regulation of TFT9 expression.

To further validate the possible RRB-mediated activation of TFT9 expression, the promTFT9::luciferase reporter was co-transformed with the pEarlygate201:ARR1 vector driving the expression of ARR1, an Arabidopsis RRB (Fig. 6d). In the absence of exogenous iP, the co-ex-pression of ARR1 resulted in an increase in luciferase activity by 40%. Removal of the hypothetic "repressor" sequence did not significantly modify the ARR1-stimulated activation of TFT9 promoter. The co-transformation of ARR1 with promTFT9::luciferase having either one or two mutated RRB binding sites did not result in increased luciferase activity. The same experiment was performed with the constitutively active ARR1DDK, a truncated version of ARR1 from which the receiver domain was removed (Fig. 6e). No increase in luciferase assay could be observed compared to the native form of the ARR1. Based on our re-sults, it might be concluded that CK-regulated expression of TFT9 is mediated by RRB and requires the two binding sites situated 1106 and 1035 upstream from the start codon.

3.5. TFT6 and TFT9 integrate CK signaling pathway

Based on the phylogenetic relationship and the best blast mutual hit, 74 of the TFT6/9 interactors were identified as orthologs to Arabidopsis genes [54]. Of them, 21 were predicted to be functional orthologs of known Arabidopsis cytokinin-responsive proteins ([59]; Table 1). These putative CK-responsive TFT interactors are involved in processes such as cell wall modeling, metabolism, transport, and protein profiling.

Table 1

List of the 133 putative interactors of TFT6 and/or TFT9 identified by LC/MS analysis in the hypocotyl of etiolated tomato seedlings exposed to blue light (total photon fluence rate 10 μmol m^{-2} s^{-1}). Only proteins interacting with TFT6 or TFT9 in BL or showing 2 fold-change in abundance compared to dark-grown hypocotyl were considered. A complete table reporting the information of the identified peptides and proteins is provided as Supporting information Table S1.

Category/ Accession	Protein description[d]	Putative 14-3-3 binding motif with highest score[a]	Position of pS/pT	Interact with		Potential cytokinin responsive based on homology with Arabidopsis[b]	Arabidopsis homolog sequence[c]
				TFT6	TFT9		
Cell wall							
Solyc03g123630	Pectinesterase[d]	FLARDItFQNT	366	x		no	AT3G14310.1
Solyc02g088690	UDP-glucose 6-dehydrogenase[d]	TAWNSDtLPIY	46	x		–	not found
Solyc10g047030	Beta-xylosidase 4	RTYRFYtGPTV	621	x		no	AT5G64570.1
Solyc04g015560	Beta-D-glukosidase	NIVRSMsEMVP	196	x		–	not found
Solyc04g005340	Alpha-1,4-glucan protein synthase[d]	PFFQSAtLPKD	298	x		no	AT3G02230.1
Solyc01g097340	GDP-D-mannose-epimerase	LQTRSFtFIDE	245	x		yes	AT5G28840.2
Solyc07g006650	Xylose isomerase[d]	VRKRYQsFDSE	351	x	x	no	AT5G57655.2
Metabolism							
TCA or Krebs cycle							
Solyc03g115990	Malate dehydrogenase[d]	DVVRANtFVAQ	248	x		yes	AT3G47520.1
Solyc01g005560	Isocitrate dehydrogenase[d]	GLAHRAtLDNN	347	x		yes	AT1G65930.1
Solyc07g052350	Aconitase	EFGKFYsLPAL	124	x		–	not found
Solyc07g055840	Citrate synthase[d]	LRGRSPsTETD	487	x		–	not found
Solyc03g111140	Malate synthase	HFMRDYsDLLI	335	x	x	no	AT5G03860.2
Glycolysis							
Solyc05g006520	Pyruvate dehydrogenase E1[d]	PVERIRsLILA	323	x		no	AT1G59900.1
Solyc01g100360	Dihydrolipoyl dehydrogenase	IPAACFtHPEI	444	x		no	AT3G16950.2
Solyc05g008600	Fructose-bisphosphatase aldolase[d]	AGKRLAsIGLD	82	x		no	AT2G01140.1
Solyc09g009020	Enolase[d]	–	–	x		no	AT2G36530.1
Solyc10g085550	Enolase[d]	–	–	x		–	not found
Calcin cycle							
Solyc05g014470	Glyceraldehyde-3-phosphate dehydrogenase[d]	RGGRAAsFNII	207	x	x	–	not found
Solyc07g066610	Phosphoglycerate kinase[d]	YLVGAVsTPKR	263	x		no	AT1G56190.1
Solyc02g085950	RUBISCO[d]	GLKSTAsFPVS	34	x	x	–	not found
Solyc10g086580	RUBISCO activase 1	ITKNFLtLPNI	150	x		–	not found
Solyc02g070980	Chlorophyll a/b binding protein[d]	RKAVAKsAPSS	42	x		–	not found
Solyc09g014520	Chlorophyll a/b binding protein[d]	–	–	x		no	AT5G01530.1
Amino acids							
Solyc08g065220	Glycine dehydrogenase[d]	QQVRSIsVEAL	67	x		yes	AT4G33010.1
Solyc11g011380	Glutamine synthetase[d]	MRSKARtLPGP	39	x	x	no	AT5G37600.1
Solyc10g081510	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase[d]	MARGNAsVPAM	103	x		yes	AT5G17920.2
Solyc12g010040	Leucine aminopeptidase 2[d]	IFTKFQsSPIW	30	x		no	AT4G30920.1
Solyc01g007940	Glutamate-glyoxylate aminotransferase	TEGAMYsFPQI	391	x		–	not found
Solyc07g032740	Aspartate aminotransferase[d]	MADRIIsMRQQ	277	x		–	not found
Solyc09g092380	Adenosylhomocysteinase[d]	LYGCRHsLPDG	247	x		–	not found
Nucleotide metabolism							
Solyc10g086190	Adenosine kinase	–	–	x		–	not found
Solyc11g068830	Uridine 5′-monophosphate synthase	DLRLIVsYPSI	43	x		no	AT3G54470.1
Redox							
Solyc06g005150	Ascorbate peroxidase[d]	–	–	x		yes	AT1G07890.8
Solyc06g005260	Glutaredoxin	–	–	x		no	AT5G40370.1
Solyc03g096040	1-Cys peroxiredoxin	NKGHKVtYPII	95	x	x	no	AT1G48130.1
Solyc10g082030	Peroxiredoxin	HISQSIsVPSA	31	x		no	AT5G06290.1
Others							
Solyc07g005390	Aldehyde dehydrogenase[d]	PVIRINsVEEG	404	x		yes	AT2G24270.4
Solyc04g076880	Phosphoenolpyruvate carboxykinase[d]	IKVRIVsARAY	251		x	no	AT4G37870.1
Solyc02g080630	Lactoylglutathione lyase	CSNRFFtLPRL	36	x		no	AT1G11840.6
Solyc04g078920	Peroxisomal multifunctional enzyme 2	–	–	x		no	AT5G42890.1
Solyc06g071910	3-oxoacyl-reductase	YASRNItVNAI	252	x		–	not found
Solyc04g082780	Cinnamoyl CoA reductase-like	GTVRDPsDDKY	42	x		no	AT2G33590.1
Solyc01g098850	Glucose ribitol dehydrogenase	TTKRGRsYPPV	37	x		yes	AT1G54870.1
Solyc01g089970	Nucleoside diphosphate kinase	–	–	x		yes	AT4G09320.1
Solyc11g010960	Alcohol dehydrogenase[d]	NFSRRAtGEKD	35	x		–	not found
Solyc10g077010	Calmodulin	–	–	x		–	not found
Solyc05g008860	Leucine-rich repeat protein kinase	PSMRHIsDALD	704		x	no	AT2G01210.1
Storage proteins/Remobilization of resources							
Globulin							
Solyc09g072560	11S globulin seed storage protein 2	IRQNSLsLPNF	96	x	x	–	not found
Solyc09g025210	11S globulin CRU4	TTVNSLtLPIL	323	x	x	no	AT5G44120.3
Solyc11g072380	Vicilin	GVMRVVsKGGF	58	x		no	AT2G28490.1
Solyc09g090150	Legumin 11S-globulin B	RRGRAKtDCRI	45		x	–	not found
Solyc09g082340	Vicilin	CQRRCQsEQQG	105		x	no	AT3G22640.1
Solyc03g005580	Legumin 11S-globulin 3	RALRSKtECQI	48		x	–	not found

(continued on next page)

Table 1 (continued)

Category/ Accession	Protein description[d]	Putative 14-3-3 binding motif with highest score[a]	Position of pS/pT	Interact with		Potential cytokinin responsive based on homology with Arabidopsis[b]	Arabidopsis homolog sequence[c]
				TFT6	TFT9		
Lipidic catabolism							
Solyc12g010920	Oleosin	–	–	x	x	no	AT3G01570.1
Solyc02g086490	Oleosin	–	–		x	–	not found
Solyc02g071700	GDSL esterase_lipase	CCQISKtMPEG	293	x		–	not found
Solyc05g013690	GDSL esterase lipase	PKEKHFsEGLY	168		x	–	not found
Solyc08g078160	Oleosin	–	–		x	–	not found
Others							
Solyc09g008770	Embryonic protein DC-8	KERRDQsQRDV	34	x		no	AT2G36640.1
Cell cytoskeleton							
Solyc03g078400	Actin[d]	PPERKYsVWIG	340	x		no	AT5G09810.1
Solyc04g011500	Actin 4	PPERKYsVWIG	340	x		no	AT3G12110.1
Solyc03g025730	Tubulin beta chain 8[d]	QQYRALtVPEL	285	x		–	not found
Transport							
Solyc01g087120	ATP synthase delta subunit	AGRIRStLPHL	10	x		yes	AT5G13450.1
Solyc01g007320	ATP synthase beta subunit[d]	ALRRARtRVEA	600	x		yes	ATCG00480.1
Solyc11g039980	ATP synthase subunit 1	–	–	x		–	not found
Solyc04g018100	Sodium/hydrogen exchanger 7	GCLRDQsTEQL	642	x		–	not found
Solyc01g103220	Cytochrome c	–	–	x		no	AT4G10040.1
Solyc07g053830	ADP, ATP carrier protein 3[d]	ALWRGNtANVI	150	x		no	AT4G28390.1
Solyc11g067000	ATP-binding cassette transporter	LGARSQtMVEY	1104	x		–	not found
Solyc03g113400	Plasma membrane H^+-ATPase (LHA1)[d]	TRSRSWsFVER	786	x		–	not found
Solyc12g055800	V-type proton ATPase subunit A[d]	GYVRKVsGPVV	28		x	–	not found
Solyc01g111760	V-type proton ATPase subunit B[d]	MTRRDHsDVSN	385		x	no	AT4G38510.5
Solyc01g073690	V-type proton ATPase subunit D	SLKRGIsLGSA	226		x	no	AT3G58730.1
Solyc08g008210	V-type proton ATPase subunit E	–	–		x	–	not found
Solyc08g081910	V-type proton ATPase subunit E	–	–		x	no	AT1G64200.1
Solyc04g049330	V-type proton ATPase subunit G1	MLLRHVtTVKN	107	x	x	no	AT3G01390.2
Solyc12g056110	V-type proton ATPase subunit E	–	–	x	x	–	not found
Solyc03g097790	V-type proton ATPase subunit C	SRYWVVsLPVQ	9	x	x	no	AT1G12840.1
Solyc05g056020	V-type proton ATPase subunit G 2	MLLRQVtTVKN	107	x	x	–	not found
Ribosome							
Solyc01g096580	30S ribosomal protein S10	–	–	x	x	–	not found
Solyc09g005720	60S ribosomal protein L23	PKYPRVsAPGR	61	x	x	no	AT2G39460.2
Solyc05g009370	50S ribosomal protein L15	FKARAFsTSAK	216	x		no	AT3G25920.1
Solyc06g073370	40S ribosomal protein S18[d]	TGRRGKtVGVS	145	x		–	not found
Solyc02g082000	30S ribosomal protein S19	–	–	x		–	not found
Solyc08g006040	40S ribosomal protein S6	GERRRKsVRGC	98	x		–	not found
Solyc05g055230	40S ribosomal protein S17-like protein	VIERYYsKMTL	22	x		no	AT5G04800.4
Solyc03g119360	40S ribosomal protein S7-like protein	PKERNNtEYKL	163	x	x	–	not found
Solyc06g069090	40S ribosomal protein S7-like protein	PKERNNtEYKL	163	x		no	AT1G48830.2
Solyc06g072120	40S ribosomal protein SA	QLQTSYsEPRL	118	x		–	not found
Solyc06g082650	60S ribosomal protein L10	–	–	x		–	not found
Solyc12g005330	50S ribosomal protein L2	GPARFRsLDFG	31	x		–	not found
Solyc06g083820	60 ribosomal protein L14	VQKRRAsLNDF	101	x		yes	AT2G20450.1
Solyc01g104590	60S ribosomal protein L3	TKKRVVtLRQS	341	x		–	not found
Solyc06g073310	Ribosomal L9-like protein	FGSRKAtASIR	70	x		–	not found
Solyc11g067100	60s acidic ribosomal protein-like protein	GREKLAsVPSG	64	x		no	AT2G27710.4
Solyc11g065670	60S ribosomal protein L12[d]	ERDRKKtKNIK	96	x	x	–	not found
Solyc08g075090	60S ribosomal protein L7	GGHRMNtAEML	196	x		–	not found
Solyc02g021400	40S ribosomal protein S28	–	–	x		–	not found
Protein profiling							
Solyc08g082820	Heat shock protein 70[d]	CSRRGNsLVVL	10	x		no	AT5G28540.1
Solyc07g042250	Chaperonin Cpn10[d]	NNSRSFsRLVV	45	x		yes	AT5G20720.3
Solyc03g007890	Heat shock protein 90	DKIRFEsLTDK	50	x		yes	AT5G52640.1
Solyc04g081570	Chaperone protein htpG	LRERVVtDPED	729	x		yes	AT4G24190.1
Solyc06g083620	26S protease regulatory subunit 4	–	–	x		no	AT4G29040.1
Solyc01g105710	Peptidyl-prolyl cis-trans isomerase[d]	–	–	x		no	AT5G64350.1
Solyc04g072250	Heat shock protein Hsp20	PPSLSTtTPEG	211	x		–	not found
Solyc07g016150	Elongation factor 1-beta[d]	–	–	x		yes	AT1G30230.2
Solyc11g069790	RuBisCO large subunit-binding protein	GIARKVtITKD	370	x		yes	AT2G28000.1
Solyc11g072190	Elongation factor beta-1	–	–	x		no	AT5G19510.1
Solyc10g051380	Glycine-rich RNA-binding protein[d]	LDGRNItVNEA	79	x		–	not found
Solyc01g109660	Glycine-rich RNA-binding protein[d]	LDGRNItVNEA	78	x		yes	AT2G21660.2
Solyc01g096450	Aspartic proteinase nepenthesin-1	QPLRIKtTPLL	275	x		no	AT5G07030.1
Solyc03g119040	Guanine nucleotide-binding protein	VPRRRLtGHGH	61	x		yes	AT1G18080.1
Solyc12g005270	Histone H2A[d]	MAGRGKtLGSG	7	x		–	not found
Solyc09g059210	Heat shock protein Hsp20	–	–	x		–	not found
Solyc07g065840	Heat shock protein 90	DKIRFEsLTDK	50	x		yes	AT5G56030.1
Solyc08g079170	Heat shock protein 70	MIARALtRKGT	330	x		no	AT1G62740.1

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Table 1 (continued)

Category/ Accession	Protein description[d]	Putative 14-3-3 binding motif with highest score[a]	Position of pS/pT	Interact with		Potential cytokinin responsive based on homology with Arabidopsis[b]	Arabidopsis homolog sequence[c]
				TFT6	TFT9		
Solyc06g005060	Elongation factor 1- alpha[d]	ERERGItIDIA	72		x	–	not found
Solyc01g111170	Peptidyl-prolyl cis-trans isomerase[d]	NFVKKHtGPGI	100		x	no	AT2G21130.1
Solyc06g036290	Heat shock protein 90	DKIRFEsLTDK	50		x	–	not found
Solyc01g067590	Sucrose non-fermenting 4-like protein	PRRLVWsGPDN	264		x	–	not found
Solyc11g008420	SKP1-like protein	–	–	x		–	not found
Solyc03g005220	Histone H2A[d]	–	–	x		–	not found
Hormone biosynthesis and signaling							
Solyc06g073580	1-aminocyclopropane-1-carboxylate oxidase[d]	PKYKSFsYTEY	329	x		–	not found
Solyc12g099380	Two-component response regulator [d]	MEERSDtTTLK	186	x		–	not found
Solyc11g008560	AP2-like ethylene-responsive TF	ASLRRKsSGFS	359	x		–	not found
Solyc07g043450	CK glukosidase	FACRISsFGLP	32	x		–	not found
Solyc06g081980	Pyridoxal biosynthesis protein	GGVARMsDPQL	79	x		no	AT5G01410.1
14-3-3s/TFTs							
Solyc11g010200	TFT6				x	no	AT1G78300.1
Solyc03g034180	14-3-3 like			x	x	yes	AT5G38480.1
Solyc07g053260	TFT9			x		no	AT2G42590.2
Solyc02g063070	TFT4				x	yes	AT5G38480.1
Solyc04g076060	TFT10			x		yes	AT5G10450.1
Solyc04g074510	TFT3			x		no	AT1G35160.2

Each identified TFTs binding partner was tested for the presence of 14-3-3 binding motive using 14-3-3Pred (Madeira et al., 2015): the putative binding motif as well as the position of putative core phospo-serine (pS) or phosho- threonine (pT) is indicated.

[a] Only the 14-3-3 binding motif with the highest score as determined by 14-3-3-Pred is indicated here.
b Identification of cytokinin responsive tomato proteins based on their homology with Arabidopsis CK-responsive proteins [53].
c Arabidopsis homolog sequences were predicted by GreenPhyl V4 based on phylogeny and best blast mutual hit (BBMH) matching sequences [54].
d identified as 14-3-3 s interactor in different species [90–92,100,111–116].

Table 2
Primers used for analysis of expression profile by qRT-PCR.

Gene	Accession[a]	Primer sequence
PP2ACS	Solyc05g006590	F: 5′-CGATGTGTGATCTCCTATGGTC R: 5′-AAGCTGATGGGCTCTAGAAATC
Tip41like	Solyc10g049850	F: 5′-GGTTCCTATTGCTGCGTT R: 5′-CGAAGACAAGGCCTGAAA
EF1a	X14449	F: 5′-CCCAAGAGGCCATCAGACAA R: 5′-CAACAGGGACAGTTCCAATACCA
TFT1	Solyc11g010470	F: 5′-AACACATCCGATACGACTTGG R: 5′-CCCATAGTGTCCAGTTCAGC
TFT2	Solyc12g057110	F: 5′-TGGCTGAATTTAAGACCGGAG R: 5′-AAGTTGAAGCCAGTCCAAG
TFT3	Solyc04g074510	F: 5′-CTGGCTCTCAACTTCTCTGTG R: 5′-TCCCAATGTATCCAACTCAGC
TFT4/TFT11	Solyc02g063070	F: 5′-GACTTGCCCTTAACTTTTCCG R: 5′-CCTTGTAAGATTCCTCACCCAG
TFT5	Solyc04g012120	F: 5′-GTGCTTGTAATCTCGCCAAAC R: 5′-GCATATCCGAGGTCCACAAAG
TFT6	Solyc11g010200	F: 5′-GCTCCTACACATCCAATCCG R: 5′-GATTACAGGCACGATCAGGAG
TFT7	Solyc04g074230	F: 5′-AATGGTTGAAGCAATGAAGGC R: 5′-AGACAAGATCCGCCATGAAG
TFT8	Solyc12g010860	F: 5′-GGCTTCATCCAAAGAACGTG R: 5′-ATCAACCATCTCATCGTAGCG
TFT9	Solyc07g053260	F: 5′-TGAATTGACTGTGGAGGAAAG R: 5′-AATAGTCTCCCTTCATCTTGTG
TFT10	Solyc04g076060	F: 5′-GAAAATCTCAGCCGTGAACAG R: 5′-AGATTCCGTTCCTCAACAGTG
TFT12	Solyc05g012420	F: 5′-TTTCGTTACCTCGCTGAGTTC R: 5′-GTTCAAAGCAAGACCAAGACG
V-ATPaseA	Solyc12g055800	F: 5′-CGGACATGACAACCTCATTG R: 5′-GGCCTCTGAATACCATCGAA
GDSL	Solyc05g013690	F: 5″-TTGTGCTGCTCCTTTCAATG R: 5′-CCGTGCTTGTTAGCGTGATA
PEPCK	Solyc04g076880	F: 5′-GGCTGCAATATGGGCCCCGA R: 5′-AAAACCACGTTTTCCAGCAC

[a] Accession numbers were obtained from Sol Genomics Network (https://solgenomics.net/).

Furthermore, TFT6 might interact with Solyc12g099380, a two-com-ponent response regulator homologous to the Arabidopsis type-B ARR2. Because TFT6 was found to be involved in hypocotyl growth in light conditions, one might assume that TFT6 is involved in a feedback regulation of the CK signaling pathway.

3.6. Validation of interaction between TFT9 and three putative interactors

Three of the putative TFT9 interactors were further investigated: phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690) and V-ATPase subunit A (V-ATPaseA; Solyc12g055800) (Fig. 7). The analysis of their expression by qPCR indicated that they were all induced by exposure to BL, reinfor-cing the hypothesis that they might take part in BL-induced de-etiola-tion (Fig. 7a). The transcription of PEPCK and GDSL was also stimulated in the hypocotyl of seedling growing in the dark in the presence of 1 μM iP, suggesting that they might be a down-stream target of CK signaling. Such regulation was not observed for the V-ATPaseA, suggesting that different mechanism(s) might be responsible for its regulation during BL-induced de-etiolation. Finally, the Y2H assay confirmed that all 3 proteins physically interact with TFT9, confirming and validating thus our interactome data obtained by pull-down assay.

4. Discussion

In a previous study, we evidenced that the first and rapid growth inhibition of tomato seedling occurring during the first 30–45 min of exposure to BL is controlled by PHOT1 and involves a local accumu-lation of the cytokinins in the elongation zone of hypocotyl [37]. In the present study, we focused on understanding the role that 14-3-3/TFT might have in this process, determining the interactome of two phylo-genetically distinct isoforms: the non-epsilon TFT6 and the epsilon TFT9 isoforms.

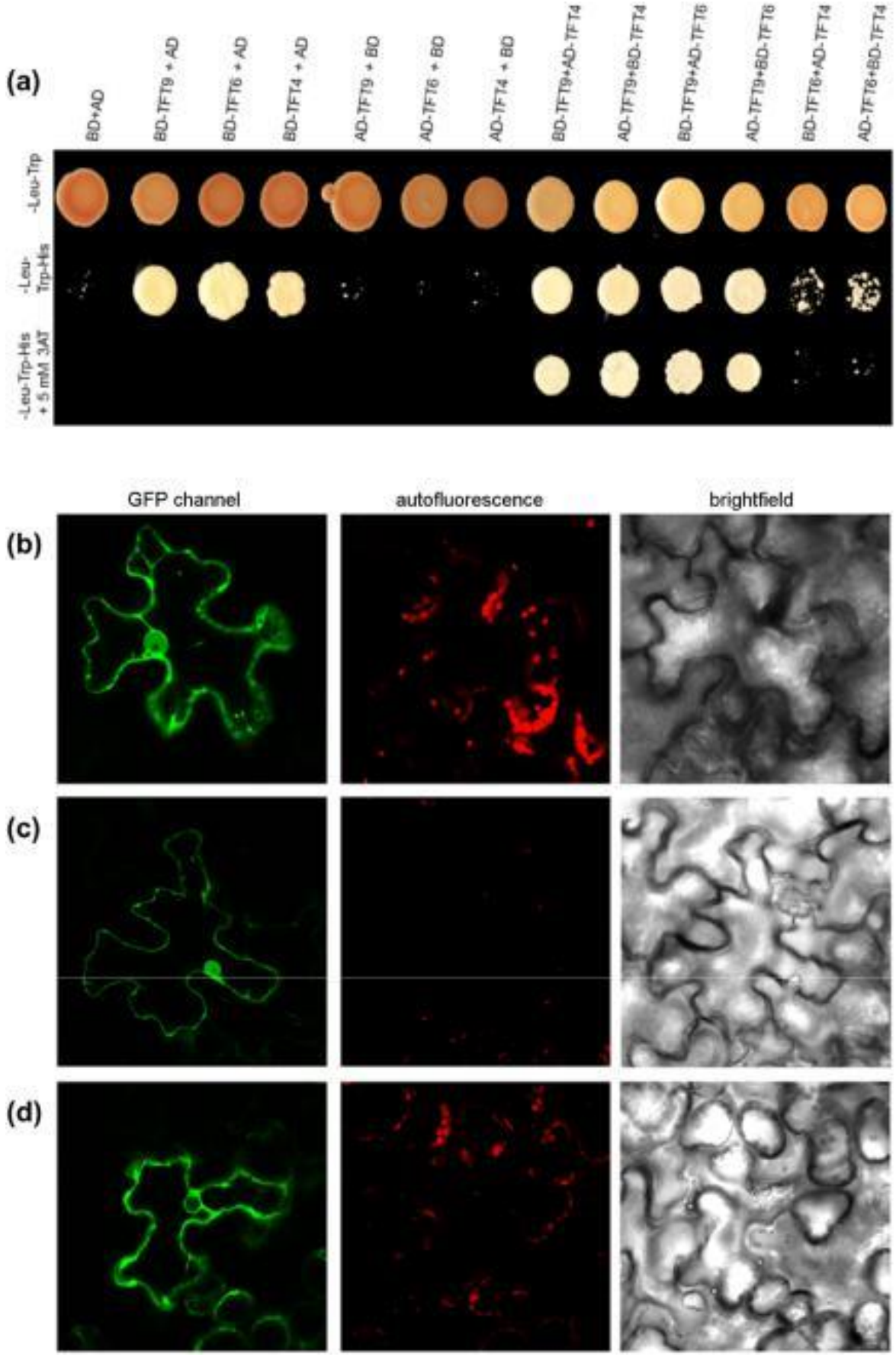

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Fig. 4. TFT interaction (a) and subcellular localization (b–d). (a) Interaction between TFT9 and TFT6/TFT4 as shown by yeast two-hybrid assays. Representative images are presented and include the respective controls and reciprocal tests. Yeasts transformed with plasmids expressing TFTs fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (−Leu-Trp), leucine, tryptophan and histidine (−Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control. Confocal laser-scanning subcellular localization of TFT9 (b), TFT6 (c), and TFT4 (d) in tobacco leaves. The same cell was observed with the GFP channel, autofluorescence and brightfield.

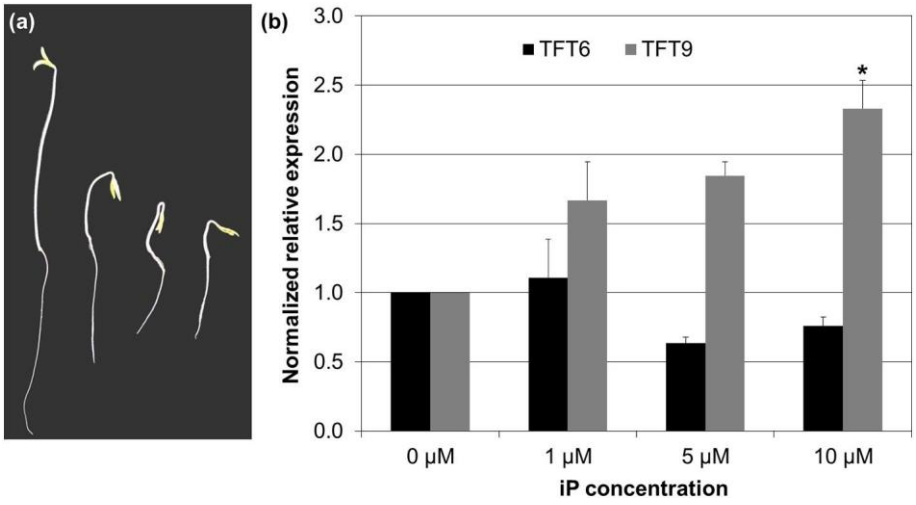

Fig. 5. Effect of exogenous isopentenyladenine (iP) on the darkness-grown seedlings of tomato (a), and the expression of TFT6 and TFT9 genes in the elon-gation zone of hypocotyl of seedlings grown in darkness (b). In (a), the seedlings were grown in darkness on Murashige and Skoog medium con-taining different concentrations of iP. From left to right: control, 1 µM, 5 µM and 10 µM. In (b), three independent biological replicates were analyzed and each replicate contained a pool of hypocotyl elon-gation of 25 seedlings; the bars represent the stan-dard errors of the means. Normalization was done in relation to three reference genes: PP2Acs (Solyc05g006590), Tip41-like (Solyc10g049850) and EF1α (X14449). Log_{10} transformation, mean cen-tering and autoscalling were performed as described by [45]. Results were expressed as fold change re-lative to the expression observed in the control sample. A non-parametric Kruskal-Wallis Anova & Median test analysis was performed followed by a post-hoc multiple comparison of mean rank (*: sta-tistically significant from the control sample with $p < 0.05$; Statistica 12, StatSoft).

4.1. Tomato 14-3-3s are regulated by and act downstream from CKs

The tomato genome contains 12 TFT isoforms, which are differen-tially expressed among diverse tissues and have isoform-specific func-tions [41]. Except of TFT12 which is predicted to be specifically ex-pressed in flower and pollen, all tomato TFTs were detected in the elongation zone of hypocotyl. The abundance of the non-epsilon members was much more important than that of the epsilon members, which was in agreement with the expression of 14-3-3 genes in different species [55]. Nevertheless, in the present study, we evidenced both at the transcriptional and protein level that the non-epsilon TFT6 and the epsilon TFT9 were the most abundant in the elongation zone of hypo-cotyl of etiolated seedlings during BL-induced de-etiolation. It is ac-cepted for a long time that CKs are mediators of photomorphogenesis and that iP, one of the three active CKs, specifically accumulated in the elongation zone of tomato hypocotyl during de-etiolation [60]. The hormonal regulation of 14-3-3 proteins has previously been reported in Arabidopsis [58,59,61]. In tomato, whereas the expression of the non-epsilon TFT6 gene was insensitive to CK, the hormone strongly regu-lated the expression of the epsilon TFT9 gene. The in silico analysis of the TFT9 promoter predicted an enrichment in type-B RR binding sites; two of them were determined to be essential for the CK-mediated reg-ulation of TFT9 expression. This is in agreement with the activation of CK-responsive genes requiring the combination of two RR binding sites in Arabidopsis [62]. The activation of TFT9 promoter in the presence of the Arabidopsis ARR1 in tomato protoplasts grown in absence of exo-genous CK demonstrated that TFT9 is a primary target of CK signaling pathway leading to the restriction of hypocotyl growth. Surprisingly, the use of the constitutively active truncated ARR1 protein [63] did not significantly increase the response. This might be due to the poly-morphism between Arabidopsis and tomato. Indeed, the Arabidopsis ARR1 recognizes a specific extended motif [62] which is different from the two binding sites essential for the activation of the tomato TFT9 promoter. Consequently, this might result in an imperfect or limited activation of the tomato promotor by ARR1. The in silico analysis of TFT6 promoter also revealed the presence of two RR binding sites in the same orientation and in close vicinity, suggesting a potential regulation by CKs. Nevertheless, an enrichment in RR binding sites in the sur-roundings of the active sites has been reported as a common trait of CK-regulated genes [62]. Whereas this was the case for the TFT9 promoter, this was not observed for the TFT6 promoter and could explain why TFT6 expression is not regulated by CKs.

Not only TFTs are regulated by CK during BL-induced de-etiolation and photomorphogenesis, but they also are involved in the down-stream events of CK signaling pathway. Indeed, our proteomic data revealed that a set of putative TFT6/9 interactors are orthologues of known Arabidopsis CK-responsive proteins playing a role in cell wall modeling, metabolism, or protein profiling [59]. Interestingly, a tomato orthologue of Arabidopsis ARR2, a type-B response regulator, was found to be a potential target of TFT6. In Arabidopsis, in the presence of CKs, in contrast to ARR1, ARR2 is phosphorylated leading to reduced stability and degradation via the 26S proteasome [64,65]. Whether the binding of tomato orthologue of ARR2 with TFT6 leads to its stabili-zation or degradation is not known. Nevertheless, this suggests a role of TFTs in feedback regulation of CK signaling pathway to desensitize the plant to CKs, keeping the response within a certain range and allowing the establishment of the steady-state growth rate (Fig. 8). This is sup-ported by the analysis of tomato tft6-ko plants revealing that TFT6 is required to ensure proper growth in the light. In a different context, the role of TFT6 in promoting growth was also demonstrated during low phosphorus stress [66].

4.2. Tomato 14-3-3s form homo- and heterodimers

It is well known that 14-3-3s form hetero- and homodimers. In our study, we identified different TFTs among the potential TFT6/9 binding partners, indicating that TFT6 and TFT9 form heterodimers. Although it is increasingly apparent that 14-3-3s are important regulators of plant growth and development, the question of which specific biological roles are associated with the distinct isoforms remains unresolved, especially during BL-induced de-etiolation and photomorphogenesis.

All 14-3-3s examined so far exhibit a highly conserved nuclear ex-port signal (I/LxxxLxxxLxL) in their C-terminal region [10]. Both TFT6 and TFT9 were found to localize both in nucleus and cytoplasm.

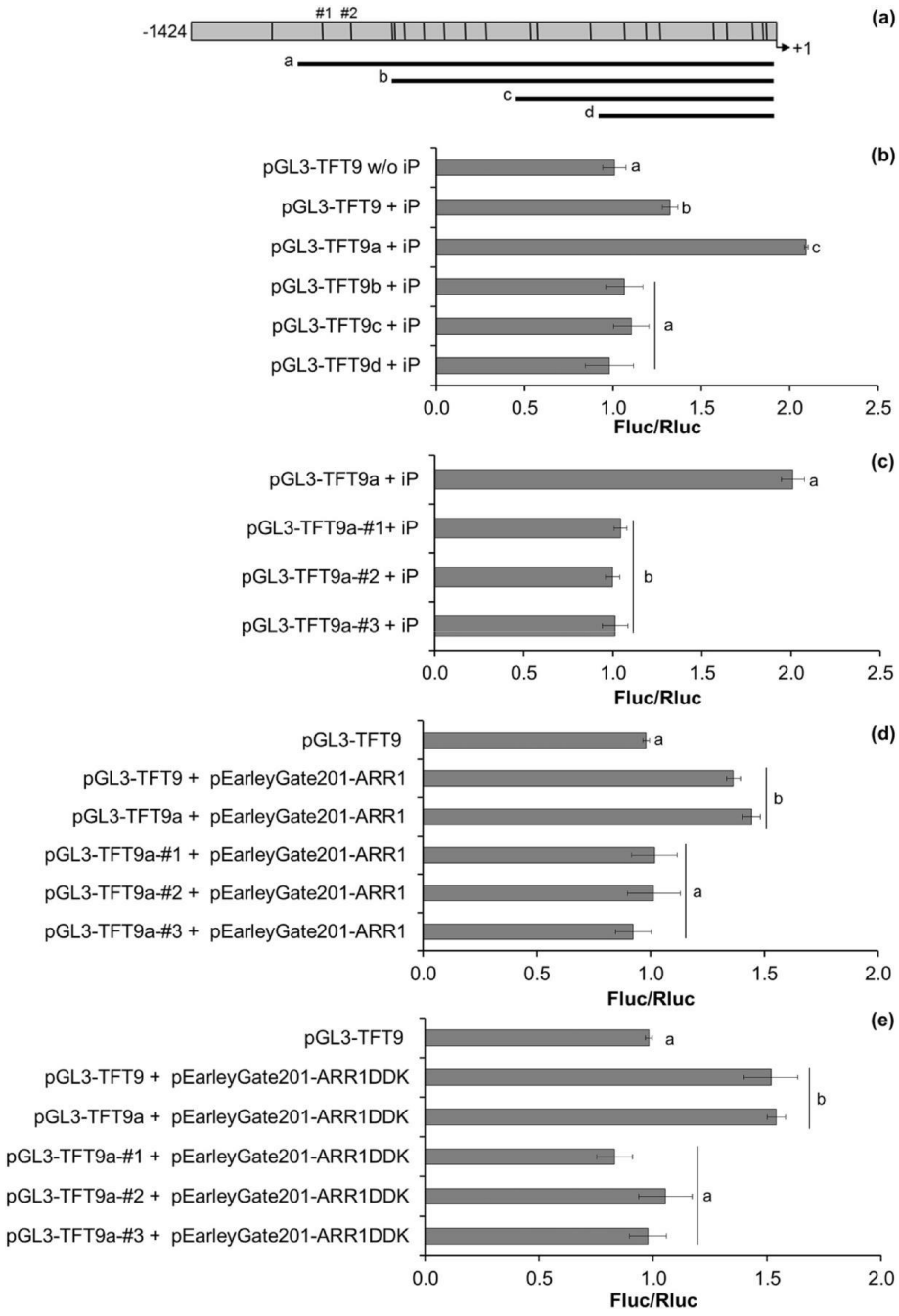

Fig. 6. Identification of cytokinin-response region in the TFT9 promoter by dual luciferase reporter assay.

(a) Schematic representation of the different version of the promoter used for the reporter assay. The upper box represents the initial promoter sequence (1424 bp); the arrow followed by "+1" shows the start of the coding sequence of the TFT9 gene. The lines inside the box indicate the positions of the CK cis-regulating elements as identified by PlantPAN2.0. #1 and #2 show the two cis-regulating elements which were mutated by point-mutation PCR. The lines beneath the promoter, annotated a–d, depict the four truncated versions of the promoter. (b) Dual luciferase assay showing the activity of different truncated versions of TFT9 promoter. Tomato leaf protoplasts were polyethylene glycol-transformed with different truncated versions of the TFT9 pro-moter cloned into the pGL3 vector; the luciferase activity was measured after 16 h of incubation with 10 μM iP in darkness. The letters refer to the different truncated promoters as described in (a). (c) Dual luciferase assay showing the activity of point-mu-tated TFT9 promoter. Two cis-regulating elements were point-mutated alone (#1 at position −1106 and #2 at position −1035) or in combination (#3). The luciferase assay was performed as described in (b). Dual luciferase assay showing the activity of point-mutated TFT9 promoter in the presence of the Arabidopsis ARR1 in its native (ARR1; d) or con-stitutively active (ARR1DDK; e) forms. The luciferase assay was performed as described in (b). Statistical significance was supported by one-way ANOVA and Tukey post-hoc (Statistica 12, StatSoft); different letters indicate that the difference between samples was statistically significant.

Nevertheless, we cannot ascertain that this subcellular localization re-flects their specific localization or their localization after interaction with putative endogenous tobacco binding proteins as this was already suggested [67].

4.3. Tomato 14-3-3s integrate hormone metabolism and signaling pathways

Plant hormones form a complex network regulating a plethora of physiological processes, including hypocotyl growth [68]. The 14-3-3 interactome currently available suggests 14-3-3s to participate in pro-cesses mediated by almost any phytohormone [7]. In tomato, we de-termined that TFT6 is involved in CKs and ethylene biosynthesis and signaling. Ethylene has long been reported to play a role during hy-pocotyl growth, being an inhibitor in the dark and promoter in the light, especially BL [69,70]. The 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, catalyzing the production of ACC, is stabilized by 14-3-3s [71]. In the present study, we identified an ACC oxidase (ACO) as a putative interactor of 14-3-3/TFT. ACO catalyzes the last step of ethylene synthesis; its binding to 14-3-3 might contribute to ethylene production and accumulation during light exposure. It is noteworthy that in Arabidopsis the aforementioned ARR2 is a target of both CKs and ethylene [72]. If such a dual targeting exists in tomato, this might contribute to the establishment of the steady-state growth rate observed during de-etiolation and photomorphogenesis.

4.4. Tomato 14-3-3s regulate H^+-ATPases

By generating a proton gradient across plasma membranes (PM), PM H^+-ATPase (PM-ATPase) provides the driving force for nutrient uptake, phloem loading, water movements, stomatal closure and opening [73]. In darkness-grown seedlings, the accumulation of auxin induces activation of the PM-ATPase [74], leading to acidification of

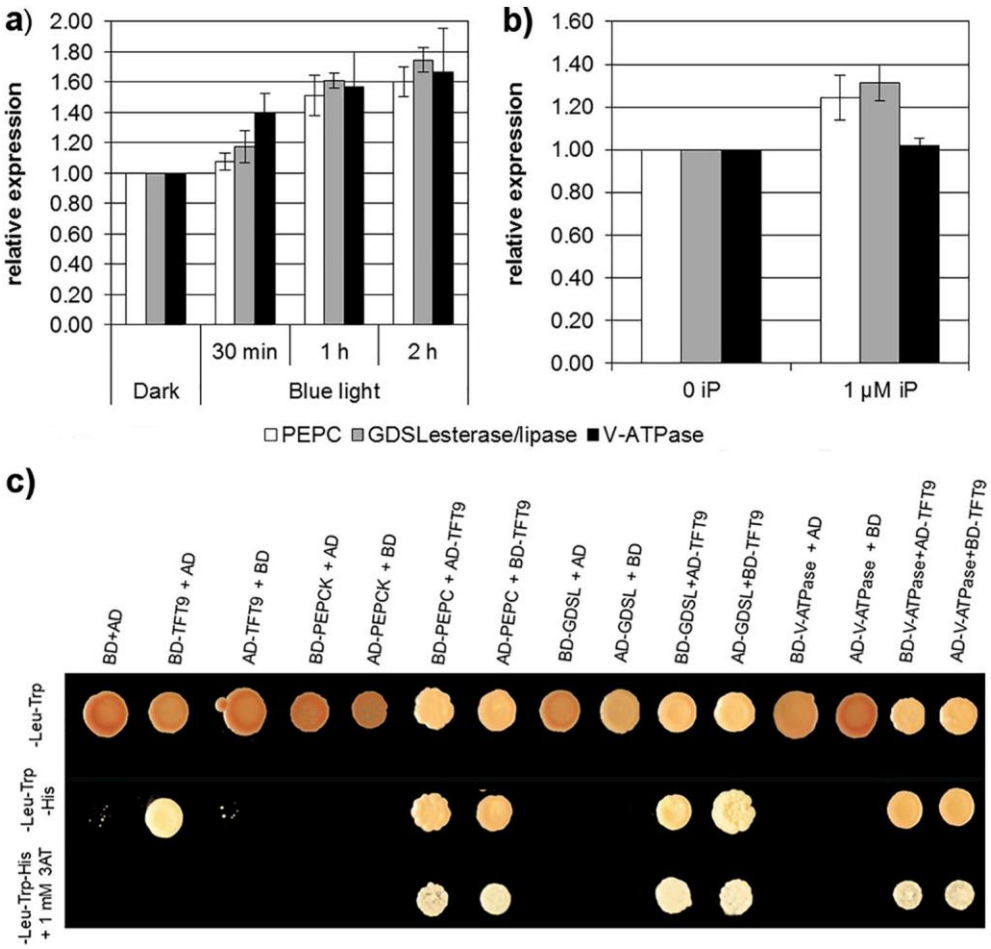

Fig. 7. Characterization of 3 putative in-teractors of TFT9 during BL-induced de-etiolation: phosphoenolpyruvate carbox-ykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690) and V-ATPase subunit A (Solyc12g055800).

(a) Analysis of expression during BL-in-duced de-etiolation. The graph represents the average of three independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three reference genes: PP2Acs (Solyc05g006590), Tip41-like (Solyc10g049850) and EF1α(X14449). Log_{10} transformation, mean centering and autoscalling were performed as described by [45]. Results were expressed as fold change relative to the expression of the same gene in the elongation zone of seed-lings grown in darkness. (b) Effect of exo-genous cytokinin (1 µM iP) on gene ex-pression in the elongation zone of hypocotyl of dark-grown seedlings. The graph re-presents the average of three independent biological replicates, with bars showing the standard errors of the means. Normalization and quantification were performed as in a). Results were expressed as fold change re-lative to the expression of the same gene in the elongation zone of seedlings grown in the absence of iP. (c) Yeast-two-hybrid assay showing the interaction between TFT9 and different putative interactors. A representative image is presented and in-cludes the respective controls and re-ciprocal tests. Yeasts transformed with plasmids expressing TFT9 and a putative interactor (PEPCK, GDSL or V-ATPAse) fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (−Leu-Trp), leucine, tryptophan and histidine (−Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control.

the cell wall (CW) and activation of enzymes responsible for CW loos-ening. Moreover, the activation of the pump leads to hyperpolarization of the PM, activation of K^+-inward rectifying channels, water uptake, and subsequent cell expansion. The activity of PM-ATPase varies from an auto-inhibited state to an up-regulated state [75]. Up-regulation of the PM-ATPase requires the phosphorylation of its penultimate residue and subsequent binding of 14-3-3 protein that stabilizes the pump in this state [9,76–78]. The phosphorylation of two other sites inactivates the pump, but whether or not this is related to 14-3-3 binding remains unclear [78]. Upon exposure to light, one might expect the inactivation of the proton pump. In our study, the PM-ATPase subunit LHA1 was identified as a TFT6 interactor. It is hardly conceivable that the binding of TFT6 on PM-ATPase results in the complete inactivation of the en-zyme. In fact, when the tft6-knockout plants were grown in the light, they had shorter hypocotyls compared to wild-type plant, suggesting that the functional TFT6 contribute to hypocotyl elongation. For this reason, the binding of TFT6 to the PM-ATPase might contribute to a low activity of the enzyme, thus ensuring the establishment of the steady-state growth rate of the hypocotyl whose growth is not inhibited but only restricted during de-etiolation and photomorphogenesis.

In our study, V-ATPases were found also to interact with TFT6/9. In plants, they are active both at the vacuole and trans-Golgi network (TGN) [79]. They are multisubunit complexes organized into two do-mains [80]. We previously demonstrated that transcripts encoding different V-ATPase subunits accumulated during de-etiolation in to-mato [81]. In the present study, nine subunits of the V-ATPase were found in the interactome of the two tomato 14-3-3s. Because the pull-down assay used does not lead only to the identification of 14-3-3 in-teractors, but also of protein complexes, it cannot be excluded that some of these subunits might indeed not directly interact with the TFTs under focus. To our knowledge, only the barley V-ATPaseA was found specifically to interact with 14-3-3. In the present study, we confirmed by Y2H assay that the tomato V-ATPaseA also binds 14-3-3 during de-etiolation, and more specifically the epsilon TFT9 isoform. Whereas exposure to light induced the expression of V-ATPaseA, no regulation by iP could be observed, suggesting a CK-independent regulation of the abundance of this transcript. The role of V-ATPase in cell expansion is rather controversial. Initially identified as a negative regulator of photomorphogenesis, BL has been shown to induce the activation of V-ATPase and its binding to 14-3-3 [82]. Inhibition of the TGN-localized V-ATPase restricted cell expansion, making V-ATPase essential for trafficking, transport, and deposition of cellulose required for cell wall strengthening [83]. It also has been proposed that V-ATPase might bind to actin in order to constrain cell size [84].

4.5. Tomato 14-3-3s regulate cell wall (CW) remodeling enzymes

Plant CW is composed of cellulose, which forms microfibrils em-bedded in the hemicellulose/pectin matrix. Pectins contribute greatly to CW strengthening [85]. In the present study, several TFT interactors were related to CW remodeling, such as pectinesterase (PME), GDP-D-mannose-3,5-epimerase (GME), UDP-glucose dehydrogenase (UGD), and alpha-1,4-glucan-protein synthase (GPS). PME catalyzes hydrolysis of the methyl ester group of pectin chain; highly methyl-esterified

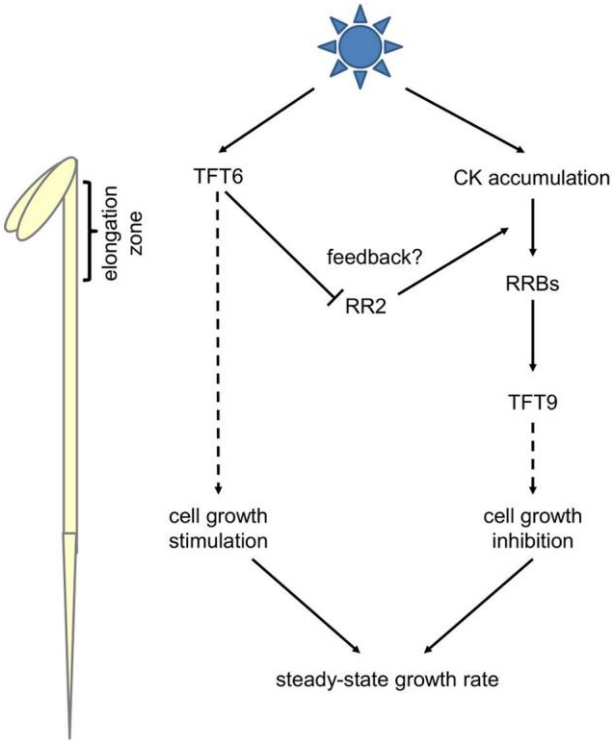

Fig. 8. Schematic representation of the interaction between TFTs and cytoki-nins (CK) during BL-induced de-etiolation and photomorphogenesis of etiolated tomato seedling.

pectins are very plastic, whereas low methyl-esterification results in stiffer CW. In Arabidopsis, the high activity of PME has been shown to restrict cell elongation [86]. GME participates in biosynthesis of such CW polysaccharides as glucomannans and pectin. Its partial inactiva-tion causes structural alterations of pectin network and results in growth defects [87]. UGD catalyzes the formation of UDP-glucuronic acid, the main precursor for the synthesis of hemicelluloses and pectic polymers [88]. Finally, GPS is highly similar to the reversibly glyco-sylated polypeptide proteins observed in Arabidopsis and involved in the synthesis of CW components [89]. Consequently, it appears that TFTs participate in the inhibition of cell expansion through the reg-ulation of different enzymes involved in synthesis and modification of the CW constituents.

4.6. Tomato 14-3-3s regulate protein folding

The switch from skoto- to photomorphogenesis requires a deep re-programming of the cell, marked by the activation of transcription and synthesis of new proteins. In this context, we identified a huge number of ribosomal proteins as TFT interactors. Whereas ribosomal proteins are highly abundant and constitute a common artifact in proteome analyses, all those identified here contained a high-scoring TFT-binding motif and their orthologues had been reported to associate with 14-3-3 proteins [90–92]. Therefore, in tomato 14-3-3s might regulate protein translation while associating with ribosomes. Similarly, several cha-perones of the heat shock protein (Hsp) family were identified as po-tential TFT interactors. Hsp proteins are responsible for protein folding, assembly, translocation, and degradation; their involvement during photomorphogenesis has been demonstrated [93,94].

4.7. Tomato 14-3-3s are involved in reserve mobilization and primary metabolism

De-etiolation and the subsequent photomorphogenesis are marked by the switch from heterotrophy to autotrophy. In our conditions, several proteins related to storage were identified as TFT-interacting proteins. These proteins belong to oleosins and globulin family (vicilin and legumin). Oleosins are proteins involved in stabilization of lipid droplets, composed essentially of triacylglycerols. The phosphorylation of oleosin induces their degradation via the 26S proteasome signaling pathway, allowing the mobilization of oil storage to provide carbon and energy for growth of the seedling [95,96]. Vicilin and legumin accu-mulate in protein bodies during seed maturation. They are mobilized during germination and early seedling development to provide amino acids required for protein synthesis [97]. Globulins are phosphorylated during germination. Like for oleosins, their phosphorylation might address them for degradation and subsequent resource mobilization [98,99].

Several enzymes putatively regulated by 14-3-3/TFTs were found to be involved in the primary metabolism which includes the Calvin cycle, glycolysis, Krebs/TCA cycle, amino acid metabolism, chloroplast elec-tron transport chain, and sucrose and starch metabolism. The role of 14-3-3s in the primary metabolism has been demonstrated in Arabidopsis [100]. Our study on tomato hypocotyl evidenced that TFT6 and TFT9 might contribute to mobilization of resources from storages and in-itiation of the primary metabolism, which is characteristic for the transition from heterotrophy to autotrophy.

4.8. Phosphoenolpyruvate carboxykinase (PEPCK) and GDSL-esterase/lipase (GDSL): Two interesting putative interactors of the epsilon TFT9 isoform

We identified and confirmed by Y2H assay the interaction of TFT9 with a PEPCK (Solyc04g076880) and GDSL (Solyc05g013690). PEPCK are ubiquitous in flowering plants catalyzing the reversible decarbox-ylation of oxaloacetate to produce phosphoenolpyruvate (PEP) and CO_2. During the gluconeogenesis, PEPCK is responsible for the pro-duction of sugars from storage lipids and proteins [101]. The resulting sugars constitute both a source of energy for the growing plants and a source of material for new cell wall component deposition [101,102]. The activity of PEPCK is regulated by many factors, including light and phosphorylation. In many plant species, PEPCK activity was found to be higher in tissue grown in the dark compared to tissue illuminated by light [103]. Interestingly, in maize, the activity was found to be related to the presence of the non-phosphorylated form of the PEPCK [104]. Among the four phosphorylated residues in the N-terminal region of the PEPCK, the Ser^{55} was found to play an important role in this reversible phosphorylation. Interestingly, it is localized inside a putative 14-3-3 binding site. Therefore, the light-induced reduction of PEPCK activity might be related to the inhibitory role of 14-3-3 as it was demonstrated for nitrate reductase, sucrose phosphate synthase and glutamine syn-thetase [105].

GDSL represent a class of newly identified lipolytic enzymes. They function in plant development, morphogenesis, synthesis of secondary metabolites and defense response [106]. In the tomato genome, we identified 224 putative GDSL which are organized into 3 major clades: I, II and III. One of the TFT9 putative interactors (Solyc05g013690) was identified to belong to the clade Id and to present a high homology with rice and arabidopsis fucosidases which release the fucosyl moiety from xyloglucan-derived oligosaccharides [106,107]. During etiolation, CW loosening correlates with the increase in the xyloglucan cleavage driven by cellulases with low pH optimum. The resulting fucose-containing nonasaccharide inhibits the growth induced by auxin, protons, gib-berellins and fusicoccin that stabilizes the interaction between 14-3-3 and its partner [108–110]. In pea, α-fucosidase is expressed only in elongating tissues [110]; in arabidopsis, AtFXG1 is detectable in leaves of different age, with higher activity in younger, developing leaves [107]. Both examples pinpoint the relation with cell elongation. In the present study, we confirmed that the GDSL encoded by So-lyc05g013690 interact with TFT9. Its expression was stimulated both

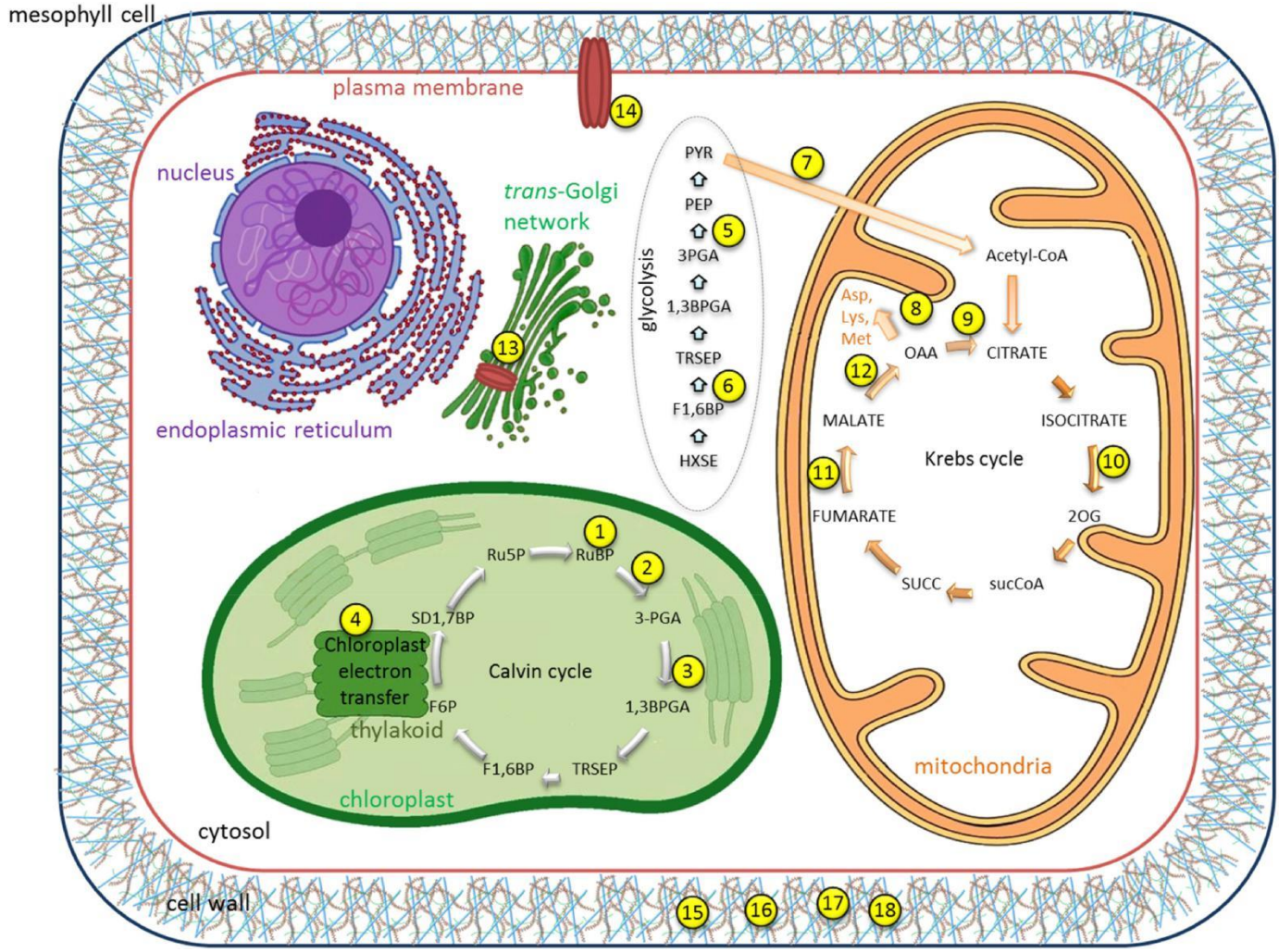

Fig. 9. Tomato 14-3-3 s/TFTs during blue light-mediated photomorphogenesis. For the sake of simplifying the figure, substrates and some reaction steps have been omitted, and the size of organelles is not respected. Only enzymes which were identified as putative interactors of 14-3-3/TFT are indicated by numbers. 1, Rubisco activase 1 (Solyc10g086580); 2, Rubisco (Solyc02g085950); 3, Phosphoglycerate kinase (Solyc07g066610); 4, chlorophyl a/b binding protein (Solyc02g070980, Solyc09g014520); 5, Enolase (Solyc09g009020, Solyc10g085550); 6, Fructose-bisphosphatase aldolase (Solyc05g008600); 7, Pyruvate dehydrogenase E1 (Solyc05g006520); 8, Aspartate aminotransferase (Solyc07g032740); 9, Citrate synthase (Solyc07g052350); 10, Isocitrate dehydrogenase (Solyc01g005560); 11, Malate synthase (Solyc03g111140); 12, Malate dehydrogenase (Solyc03g115990); 13, V-type proton ATPase (Solyc12g055800, Solyc01g111760, Solyc01g073690, Solyc08g008210, Solyc08g081910, Solyc04g049330, Solyc12g056110, Solyc03g097790, Solyc05g056020); 14, H + -ATPase (Solyc03g113400); 15, Pectinesterase (Solyc03g123630); 16, UDP-glucose 6-dehydrogenase (Solyc02g088690); 17, Alpha-1,4-glucan protein synthase (Solyc04g005340.2.1); 18, GDP-D-mannose-3,5-epimerase (Solyc01g097340).

Abbreviations (listed alphabetically): 1,3BPGA, 1,3-bisphosphoglycerate; 2OG, 2-oxoglutarate; 3PGA, 3-phosphoglycerate; acetyl-CoA, acetyl coenzyme A; Asp, aspartic acid; F1,6BP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; HXSE, hexose (glucose and/or fructose); Met, methionine; Lys, lysine; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; SD1,7BP, sedoheptulose 1,7-bisphosphate; SUCC, succinate; succCoA, succinyl coenzyme A; TRSEP, triose phosphate, collectively dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by exposure to BL and exogenous CK. Further investigation will be re-quired to: 1) determine whether the GDSL has a fucosidase activity, 2) the function of fucosylated oligosaccharides in the control of hypocotyl elongation, 3) the functional significance of the binding to 14-3-3.

5. Conclusion

To our knowledge, this is the first report concerning the role of 14-3-3 proteins during BL-induced photomorphogenesis. We provided evi-dence that tomato 14-3-3s participate in BL-mediated early seedling development, starting with the regulation of the BL-photoreceptor PHOT1 (Fig. S2). The interactome of two isoforms, TFT6 and TFT9, predicted that 14-3-3s might affect an array of client proteins involved in cell elongation, metabolism, protein synthesis, and hormone pathways. The functional significance of these interactions needs to be further addressed in the future. Fig. 9 depicts the different processes potentially regulated by 14-3-3s during tomato de-etiolation. We have demonstrated that 14-3-3s are potential components of the CK signaling pathway, which in turn regulates their expression. Moreover, the in-teractomic data provide highly valuable, novel targets for detailed mechanistic studies that will deal with understanding the role or roles of 14-3-3s during photomorphogenesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.12.017.

Conflict of interest

The authors declare that no conflict of interest exists in the

submission of this manuscript. All authors approved the manuscript for publication.

Author contributions

Conceptualization: P.H. and V.B.; Methodology: P.H., V.B. and M.Č.; Investigation: P.H., N.K., M.L., M.Č, E.G.M.; Writing – Original Draft: P.H. and V.B.; Writing – Review & Editing: M.Č., E.G.M. and V.B.; Funding acquisition: B.B. and P.G.; Resources: B.B. and P.G.; Supervision: B.B., P.G. and V.B.

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Curriculum vitae

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Education

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Employment

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Research intership

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Teaching experience

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Conferences

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Hloušková, P., Kokáš, F., Galuszka, P., Majeská Čudejková, M: Study of the root systém in four winter wheat cultivars developing two contrasting phenotypes. (Root Research International Symposium, Jerusalem, Israel)

Hloušková, P., (oral presentation): *phot1* mutant line in tomato (Molecules of Life, Brno)

2015 **Hloušková, P**., Černý, M., Bergougnoux, V.:Role of TFT9 in blue light-induced de-etiolation in tomato. (New Methods in Agriculture Research, Brno, CZ)

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Study of blue light-induced de-etiolation in tomato: role of TFT proteins, PHOT1 and cytokinins

Summary of Ph.D. Thesis

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SOUHRN

De-etiolace je první vývojový proces pod kontrolou světla, který umožňuje heterotrofnímu semenáčku stát se autotrofním. Rostlinné hormony cytokininy (CK) velmi přispívají k tomuto procesu. Reversibilní fosforylace je klíčovou událostí buněčné signalizace, která umožňuje aktivovat proteiny nebo vytvářet vazebná místa pro specifickou interakci proteinů. 14-3-3 proteiny (TFT) regulují různé reakce rostlin. Po ozáření modrým světlem dochází ke snížení rychlosti růstu hypokotylů ve dvou fázích. Po rychlé inhibici růstu zprostředkované fototropinem 1 (PHOT1) během prvních 30 minut po osvětlení následuje ustálený růst, který je řízen kryptochromem 1 (CRY1). Zatímco de-etiolace zprostředkovaná CRY1 je prozkoumána, menší pozornost se věnuje fázi de-etiolace pod kontrolou PHOT1.

Pomocí subtraktivní cDNA knihovny byly identifikovány důležité regulační mechanismy, potenciálně regulované PHOT1 u rajčete (*Solanum lycopersicum* L.). Indukce transkripce a translace, stejně tak jako modifikace struktury chromatinu je důležitá z hlediska skutečnosti, že zahájení fotomorfogeneze je založeno na výrazném reprogramování buňky. Modré světlo také blokuje expanzi buněk rychlou modifikací buněčné stěny.

Dále byla studována exprese, hormonální regulace a proteomická síť pod kontrolou 14-3-3 proteinů při fotomorfogenezi indukované modrým světlem. TFT6 a TFT9 isoformy byly specificky zkoumány kvůli jejich vysoké expresi během de-etiolace u rajčete. Multidisciplinární přístup ukázal, že exprese TFT9, ale ne TFT6, byla regulována CK a byly identifikovány cis-regulační elementy potřebné pro tuto reakci. Následně byl zkoumán interaktom TFT6/9 spojený s de-etiolací a bylo dokázáno, že 14-3-3 proteiny by mohly být zapojeny do signalizační dráhy CK, inhibice expanze buněk, ustáleného stavu růstu a reprogramování z heterotrofie.

Nakonec byla použita amiRNA interference k přípravě mutantních rostlin *phot1* u rajčete. Snížená exprese *PHOT1* výrazně ovlivnila fenotyp rostlin, především výšku a počet trichomů na povrchu listu i stonku. Rovněž vroubkování okraje listů a jejich zploštění bylo rozdílné, což by mohlo být spojeno se změněnou úrovní transpirace a účinností fotosyntézy. Plody mutantní rostliny byly tmavě zelené s nižším počtem semen, vykazujících zpožděné klíčení. Proteomická analýza perikarpu plodů ukázala změny v proteinech spojených s organoleptickými vlastnostmi a syntézou karotenoidů, což je v souladu s vyšším obsahem karotenoidů, důležitých složek potravy.

SUMMARY

De-etiolation is the first developmental process under light control allowing the heterotrophic seedling to become autotrophic. The phytohormones cytokinins (CKs) largely contribute to this process. Reversible phosphorylation is a key event of cell signaling, allowing proteins to become active or generating a binding site for specific protein interaction. 14-3-3 proteins regulate a variety of plant responses. Upon exposure to blue light (BL), reduction of hypocotyl growth rate occurs in two phases: a rapid inhibition mediated by phototropin 1 (PHOT1) within the first 30 min of illumination, followed by the cryptochrome 1 (CRY1)-controlled establishment of the steady-state growth rate. Although some information is available for CRY1-mediated de-etiolation, less attention has been given to the PHOT1 phase of de-etiolation.

Using subtractive cDNA library, we were able to identify important regulatory mechanisms, potentially regulated by PHOT1 in tomato (*Solanum lycopersicum* L.). The profound induction of transcription/translation, as well as modification of chromatin structure, is relevant in regard to the fact that the entry into photomorphogenesis is based on a deep reprograming of the cell. Also, BL restrains the cell expansion by the rapid modification of the cell wall.

Furthermore, the expression, hormonal regulation, and proteomic network under the control of 14-3-3s were addressed during blue light-induced photomorphogenesis. TFT9 and TFT6 isoforms were specifically investigated due to their high expression during tomato de-etiolation. The multidisciplinary approach demonstrated that *TFT9* expression, but not *TFT6*, was regulated by CKs and identified *cis*-regulating elements required for this response. Subsequently, the TFT6/9 interactome linked to de-etiolation was investigated and evidenced that 14-3-3s might be involved in CK signaling pathway, cell expansion inhibition, steady-state growth rate establishment, and reprograming from heterotrophy.

Finally, amiRNA interference was used to generate tomato *phot1* plants. Downregulation of *PHOT1* caused distinct phenotype in plant height and number of trichomes on the leaf and stem surface. Also, the serrations on the leaf margine and leaf flattening was altered which may be associated with changed level of transpiration and efficiency of photosynthesis. The fruits of mutant plant were darker green with lower number of seeds showing delayed germination. Proteomic analysis of fruit pericarp showed changes in proteins connected with organoleptic properties of fruit and carotenoid biosynthesis pathway which is in correlation with higher content of carotenoids, important components in human diet.

CONTENT

AIMS OF WORK

The overall objectives of present PhD thesis were to gain insights in understanding the blue-light (BL)-induced de-etiolation in tomato (*Solanum lycopersicum* L.), more precisely to uncover how 14-3-3 proteins, phototropin 1 (PHOT1) and cytokinins are involved in BL-mediated hypocotyl elongation. For this purpose, the work was divided into three main sections:

i) Study of PHOT1-mediated phase of BL-induced hypocotyl growth

ii) Elucidation of the role of 14-3-3 proteins and isopentenyladenine in BL-mediated hypocotyl growth

iii) Preparation and characterization of *phot1* tomato plants

INTRODUCTION

Light is one of the crucial environmental factors affecting plant from the early development. Apart from the acting as a source of energy, it is also important information carrier and regulates various aspects of plant development, most notably seedlings de-etiolation – the transition from a pale heterotrophic seedling to a green photosynthetically competent one. The seedlings kept in the dark are characterized by the apical hook formation, unexpanded cotyledons and fast elongating hypocotyl. In opposite, the light treatment of dark-grown seedlings causes the opening of apical hook, expansion of cotyledons, and reduction in hypocotyl growth rate (Neff et al., 2000).

Consistent with the remarkable changes in morphology and development, light induces the extensive reprogramming of transcriptome that is a consequence of the differential expression of at least 20% of the whole genome leading to the coordinate regulation of the major biochemical pathways upon transition from dark to light (Jiao et al., 2005).

At the beginning of de-etiolation, the photons of incoming light are absorbed by plants via specific chromoproteins – photoreceptors, working individually or in combination. Plants have evolved the sophisticated system of photoreceptors able to absorb light to perceive changes in light wavelengths, intensity, duration or direction adjusting their growth, reproduction and survival. They possess three main classes of photoreceptors: phytochromes (PHYs), cryptochromes (CRYs), and phototropins (PHOTs), capable of absorbing red/far-red, blue, and blue light, respectively (Quail, 2002).

Studying de-etiolation, the attention is focused on the process in which light inhibits hypocotyl elongation of seedlings of higher plants because it is easily measured and mutants of this response are available. The genetic screening of mutants altered in hypocotyl growth revealed these that are mostly impaired in specific photoreceptors or their downstream targets. Whereas *cry1* mutants exhibit longer hypocotyls under BL, no differences in hypocotyl length can be observed in *phot1* mutant (Kang et al., 2008). Nevertheless, a fine-tune analysis of the growth rate of the different mutants demonstrated that de-etiolation under blue light is a two-phase process involving PHOT1 during the first 30-40 min after illumination and requiring CRY1 to establish the stable steady-state growth (Parks et al., 2001; Bergougnoux et al., 2012). Both red light photoreceptors, PHYA and PHYB, are also important for de-etiolation respose as mutants affected in these genes display etiolated phenotype when grown in RL/FR (Khanna et al., 2006).The first three hours of RL illumination, the hypocotyl growth is regulated by PHYA. After that, the effect of PHYB is implicated. Under far-red light, the hypocotyl elongation is solely influenced by PHYA (Parks et al., 2001).

Nevertheless, the modulation of the light quality alone does not induce a specific response of the plant. Not surprisingly, plant hormones are believed to interact with light perception and signaling during regulation of plant de-etiolation. Cytokinin (CK) treatment of dark-grown seedlings mimics the de-etiolated phenotype of light-grown seedlings (Chory et al., 1991). Similar phenotype was observed with the dark-grown *altered meristem program* (*amp*) mutant with high endogenous CK (Chin-Atkins et al., 1996). The *cytokinin-insensitive 4* (*cin4*) mutant, allelic to the *cop10* mutant, links CK and light (Vogel et al., 1998). Mechanisms of CK-mediated de-etiolation are not fully understood, despite the importance of CK histidine kinase receptors (Riefler et al., 2006). Overexpression of *ARABIDOPSIS RESPONSE REGULATOR 2* (*ARR2*) gene, component of CK signaling, resulted in dark-grown seedlings with short hypocotyls (Hass et al., 2004).

Cell signaling mechanisms often involve posttranslational modifications (PTMs) of proteins, affecting their conformation, activity, stability, and/or localization (Wold, 1981). Very often, however, phosphorylation of the protein alone is not sufficient to modulate protein function. Rather, phosphorylation is required to ensure the binding of interactors which will themselves regulate protein function. Among the plant phosphopeptide-binding proteins, 14-3-3 proteins (14-3-3s) are the best characterized (Chevalier et al., 2009). They have recently attracted attention due to their remarkable

capacity to affect a wide array of physiological, developmental, and cellular processes such as primary metabolism, hormone signaling, response to light, cell growth and division, pathogen–plant interaction, and response to biotic and abiotic stresses (Denison et al., 2011; Cotelle and Leonhardt, 2015; Lozano-Durán and Robatzek, 2015; Camoni et al., 2018).

The 14-3-3s are small, acidic proteins highly conserved, found in all eukaryotic organisms examined so far. Whereas yeast has two isoforms and mammals seven, most plant genomes contain a dozen 14-3-3 genes which can be divided into epsilon and non-epsilon groups, based on their sequence homology and intron/exon structure (DeLille et al., 2001; Rosenquist et al., 2001). The epsilon group is considered as "ancestral" and to fulfill fundamental functions; in opposite, the non-epsilon group evolved later and members of this group might be related to organism-specific functions (Ferl et al., 2002; Jaspert et al., 2011). Due to redundancy of 14-3-3 function, it has been suggested that the function specificity is related rather to the spatiotemporal regulation of the expression of the different isoforms. Nevertheless, the presence of phenotypes for single and multiple 14-3-3 knock-out mutants and the differential subcellular localization of 14-3-3s within the cell support a view that specificity of function varies for the different 14-3-3s (Rosenquist et al., 2001; Paul et al., 2009; Wilson et al., 2016; Keicher et al., 2017).

The 14-3-3s are present in the cell as a combination of homodimers and heterodimers. Some isoforms have preference to form homo- or heterodimers, as was shown for two human 14-3-3 isoforms (Wilker et al., 2005). It also has been hypothesized that homo- and heterodimers could serve different functions (Aitken, 2002). Although 14-3-3 isoform dimerization is not well understood in plants, it likely depends on the intrinsic affinities among 14-3-3s (Paul et al., 2012). The formation of a 14-3-3 dimer is required for the binding of a client protein, thereby affecting its activity, stability, conformation, and localization (Gökirmak et al., 2010). Because different 14-3-3s harbor different specificity towards different targets, heterodimers might act as adapters associating two proteins which otherwise would not interact together (Jones et al., 1995). Nevertheless, such interaction has never been demonstrated until now. Whereas it is well characterized that the phosphorylation marks the protein for changes and the activation or inactivation is completed once 14-3-3 is bound (Paul et al., 2008), to date no specific enzymatic function has been ascertained for 14-3-3s. This has led to the idea that their function is solely due to physical interaction (de Boer et al., 2013). 14-3-3 proteins interact with their binding partners through three well-defined binding motifs (mode I, II and III) (Yaffe et al., 1997; Coblitz et al., 2005). In plants, the most commonly occurring motif is a variation of mode I [LX(R/K)SX(pS/pT)XP]. Phosphorylation of the target protein is often, but not always, a prerequisite for 14-3-3 binding to occur (Ottmann et al., 2007; Johnson et al., 2010; Taoka et al., 2011).

BL perception by PHOT1 induces autophosphorylation of multiple serine residues of the receptor which are essential for further signal transduction. Isoforms of the non-epsilon 14-3-3 group have been characterized to bind PHOT1 (Sullivan et al., 2009). Nevertheless, the role of this interaction is still unknown. A relationship between light and 14-3-3s in the control of hypocotyl growth and overall plant growth has been reported for several Arabidopsis 14-3-3 mutants grown in RL and in darkness (Mayfield et al., 2007; Zhao et al., 2015). A recent study demonstrated that 14-3-3s participate in light signaling by binding with PHYTOCHROME INTERACTING FACTORs (PIFs; (Adams et al., 2014). PIFs are repressors of photomorphogenesis in darkness, and their degradation is mediated by the RL-activated PHYs. Two of them, PIF4 and PIF5, were demonstrated to also act downstream from both CRYs and PHOTs (Sun et al., 2013; Pedmale et al., 2016). Finally, the CK response regulator - ARR2 was found to interact with 14-3-3υ (Dortay et al., 2008; Braun et al., 2011).

MATERIAL AND METHODS

PLANT MATERIAL

Experiments were conducted on wild-type (wt) *Solanum lycopersicum* (L.) seedlings of the Rutgers and Ailsa Craig cultivars. Antisense *tft6* tomato lines were obtained by RNAi in the background Ailsa Craig. The seeds were kindly provided by Prof. M. Roberts from Lancaster University (UK). Antisense *phot1* tomato lines were generated by amiRNA interference in the cv. Rutgers background in this study.

CULTIVATION

The seeds were sown and cultivated as described in (Bergougnoux et al., 2009). The hypocotyl growth measurement was done according to Bergougnoux et al. 2009. For germination assay, the seed germination, defined as radicle protrusion, was scored for nine days in the dark and under BL illumination. Four independent biological replicates containing 30 seeds were counted

For library preparation and gene expression, germinated seeds were transferred to fresh MS medium in the dark for 3 additional days to a culture chamber maintained at 23 °C. For BL-induced de-etiolation, dishes containing 3-day-old etiolated seedlings were transferred for 30, 60 or 120 min under BL provided by fluorescent lamps (BL; TL-D 36W/Blue, Phillips; total photon fluence rate 10 $\mu mol.m^{-2}.s^{-1}$). For analysis of cytokinin response, germinated seedlings were transferred on Murashige and Skoog medium supplemented with 0–10 μM isopentenyladenine (iP, Olchemim, Czech Republic). The seedlings were further grown in darkness for 3 additional days. Afterwards, the elongation zone of the hypocotyl was excised for RNA extraction and gene expression.

RNA EXTRACTION AND SUBTRACTIVE LIBRARY CONSTRUCTION

For all experiments, the elongating zone of the hypocotyl of 3-day-old etiolated seedlings was excised from the rest of the seedling, either under green safety light (dark control) or under BL after 30 min of exposure to BL. The elongating zone corresponding to the portion of hypocotyl situated beneath the hook and cotyledons was limited to the upper third of the hypocotyl as described in (Bergougnoux et al., 2012). Samples were immediately frozen in liquid nitrogen and stored at −80 °C before RNA isolation. Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). Remaining traces of DNA were removed with a recombinant DNAseI (Takara) and RNA were subsequently purified by a phenol:chloroform:isoamyl alcohol (25:24:1) step. $PolyA^+$ mRNA were purified using the Straight A's mRNA isolation system (Novagen). Quantity and quality of mRNA were checked by spectrophotometer and electrophoresis. The suppression subtractive hybridization (SSH) library was constructed according to the instructions of the PCR-Select cDNA Subtraction Kit (Clontech). The principle of SSH library is illustrated by the figure 1. In order to identify genes up-regulated by the exposure to BL, subtractive hybridization was performed using cDNA from hypocotyls exposed for 30 min to BL as tester against cDNA from control hypocotyls (not exposed to BL) as driver. Thesubtraction efficiency was evaluated by a PCR reaction amplifying a region of the tomato EF1α gene and the PCR product was analyzed after 15, 20, 25, 30, and 35 cycles.

CLONING, SCREENING FOR DIFFERENTIAL EXPRESSION, SEQUENCING AND ANALYSIS

Secondary SSH-PCR products were inserted into pGEM-T Easy Vector (Promega) and cloned into *Escherichia coli* DH5α strain. A blue–white screening was performed in order to obtain a bank of subtracted ESTs. White colonies were picked and grown in 96-well microtiter plates in a lysogeny broth medium containing ampicillin ($100\ mg.L^{-1}$). Screen for differentially expressed ESTs was performed by dot blot hybridization as described in the PCR-select cDNA subtraction kit (Clontech). For this purpose, plasmids were isolated, quantified and transferred to Hybond-N+ nylon membranes. Membranes were prepared in duplicates with equal amounts of plasmids and were hybridized either with the BL-specific tester probe or the dark-specific driver probe, both DIG-labelled. Detection was performed with an anti-digoxigenin antibody coupled with a horse radish peroxidase.

Cold detection was performed by enhanced chemiluminescent detection and exposure to X-ray films. Autoradiographies were scanned and the intensity of the dots was determined using ImageJ software. The dot intensity of a specific clone obtained with the BL-specific probe was compared to that obtained with the dark-specific probe. All clones showing higher intensity with BL-specific probe compared to dark-specific probe were selected and sequenced by Macrogen (Korea). A total of 168 ESTs were found to be differentially expressed. Their sizes ranged from 128 to 1387 bp, with an average size of 447 bp. Because during the process of library preparation the cDNA were restricted by RsaI, the first step of the analysis was to retrieve the full-length of the gene and the corresponding protein for downstream analyses by BLAST against the tomato database at SolGenomics Network. The gene ontology annotation was performed with Blast2GO according to plant-specific Gene Ontology terms

(Conesa et al., 2005). Concurrently, the functional annotation was performed by Mercator/MapMan which allows attributing DEGs a functional pathway (Klie and Nikoloski, 2012; Lohse et al., 2014).

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

To confirm the differential expression of the selected ESTs, total RNA was extracted from the elongating zone of 3 day-old etiolated seedlings exposed or not to BL as previously described. Reverse transcription was performed from 1 µg of the total RNA according to the instructions of the PrimeScript kit (Takara), followed by subsequent RNaseH treatment (Takara) and purification on a Macherey-Nagel column to remove any compounds which could have an inhibitory activity during subsequent steps. For quantitative real-time PCR, cDNA samples were diluted by 5-fold and used in a reaction containing SYBR Premix ExTaq (Takara) PCR Master Mix and 200 nM of each primer. Three technical repeats were run for each sample on the Mx3000P thermocycler (Stratagene) in a two-step amplification program. The initial denaturation at 94 °C for 10 s was followed by 40 cycles of 94 °C for 5 s and 60 °C for 20 s. A dissociation curve was obtained for each sample. Each sample was analyzed in three to five independent biological replicates and in technical triplicates. Each independent biological replicate represented a pool of 25 explants. The cycle threshold value was determined for each sample. Three housekeeping genes, *PP2Acs*, *EF1α* and *Tip41like* were used for expression normalization of genes of interest (Dekkers et al., 2008); these genes were determined as being the most stable housekeeping genes in our conditions. Normalized relative quantities were obtained using the efficiency corrected $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). In order to exclude the effect of biological (intra-assay) variation on the statistical significance of results, $\log_{10}$ transformation of normalized relative quantities, mean centering and autoscalling were performed (Willems et al., 2008). The relative quantification was determined in comparison to the expression of *TFT1* gene in dark control sample and/or untreated samples or sample in wt. Presented data show the mean ± standard error of the mean (SEM) of three to five independent biological replicates. Statistical significance was supported by non-parametric Kruskal-Wallis Anova & Median test analysis followed by a post-hoc multiple comparison of mean rank (Statistica 12, Statsoft).

PROTEOMICS AND LC-MS/MS ANALYSIS

Total proteins of leaves and fruits were extracted with trichloroacetic acid (TCA)/acetone precipitation followed by phenol extraction, diluted with 1.5 ml digestion buffer (2 M urea, 50 mM ammonium bicarbonate) and digested with an immobilized trypsin (Promega) overnight. Tryptic digests corresponding to 5 µg of protein extract were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted up to a 120 min witha 4% to 40% acetonitrile gradient; spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s. The measured spectra were extracted by Bruker's Data Analysis 4.1 (Cerna et al., 2017). Recalibrated MGF files were searched against tomato protein sequence database (ITAG 2.4) by Sequest HT and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys/His/Leu/Arg methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation. Results were integrated by Proteome Discoverer 2.0 (Thermo), and the quantitative differences were evaluated by calculating normalized numbers of peptide spectral matches (PSM) (Černý et al., 2013). Differently expressed proteins were targeted in experimental replicates via an SRM-based analysis (TSQ Quantiva, Thermo). Only proteins with at least three detectable proteotypic peptide were considered for the analysis and the resulting data were evaluated in Skyline 3.1 (MacCossLab Software, https://skyline.gs.washington.edu) and the statistical significance was validated by t-test ($p<0.05$).

HYPOCOTYL PROTEIN EXTRACTION, RECOMBINANT TFT9 AND TFT6, AND PULL-DOWN ASSAY

For the identification of putative interactors of TFT6 and TFT9, cytoplasmic protein fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific, USA) according to manufacturer's instructions. With the modification that buffers were supplemented with plant protease and phosphatase inhibitors (Sigma-Aldrich). Each sample corresponded to the pool of hypocotyl of 30 etiolated seedlings grown in the dark for 3 days and exposed or not for 2h to blue light (BL; L-D 36W/18-Blue, Philips; total photon fluence rate 10 µmol m^{-2} s^{-1}). For pull-down assay, recombinant proteins were prepared for TFT9 and TFT6, both fused to histidine-tag. For this purpose, the coding regions of TFT9 (Solyc07g053260) and TFT6 (Solyc11g010200) were cloned into the C-terminal His-vector PQE60 (Qiagen, Germany) and N and the C-terminal His-vector PET28b+ (Novagen), respectively. The constructs were introduced into *E. coli* strain BL21 (DE). Expression of recombinant His-tagged proteins was induced for 4h at 37°C by addition of 1 mM isopropylthio-*β*-galactoside to overnight-

grown bacterial culture. Cells were collected by centrifugation for 20 minutes at 4500 x*g* at 4°C, then resuspended in His-binding buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 10 mM imidazole, 0.03% Triton X-100). Lysis of bacterial cells was performed by repeated freezing/thawing cycles in liquid nitrogen and at 42°C, respectively. The cell lysate was clarified by centrifugation at 12,000 x*g* at 4°C for 20 minutes. The supernatant containing expressed His-tagged TFT protein was immobilized on nickel-charged His-Affinity Gel using His-Spin Protein Miniprep (Zymo Research, USA) and incubated with cytoplasmic proteins extract from excised elongating zones of etiolated hypocotyls or hypocotyls illuminated by BL for 2 hours. Two controls were included: the cytoplasmic protein extract without immobilized bait, and the immobilized bait incubated with protein extraction buffer. Cytoplasmic proteins extracted from elongation zone of etiolated seedling hypocotyls exposed or not to BL were incubated with immobilized TFT9 or TFT6 for 1 h at 4°C and unbound fractions were washed with two volumes of wash buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride, 50 mM imidazole, 0.03% Triton X-100). The bound proteins were eluted with 150 µl of the elution buffer containing 50 mM sodium phosphate buffer pH 7.7, 300 mM sodium chloride and 250 mM imidazole), diluted with 1.5 ml digestion buffer (2 M urea, 50 mM ammonium bicarbonate), digested overnight with an immobilized trypsin (Promega) for further analysis on LC-MS/MS (as described above).

YEAST TWO-HYBRID ASSAY

The ORFs of TFT6 (Solyc11g010200), TFT9 (Solyc07g053260), Phosphoenolpyruvate carboxykinase (PEPCK; Solyc04g076880), GDSL-esterase/lipase (GDSL; Solyc05g013690), V-ATPase subunit A (Solyc12g055800) and phototropin 1 (PHOT1; Solyc11g072710) were fused to the GAL4-activating domain (AD) or GAL4-binding domain (BD) via cloning into pGADT7 or pGBKT7 vectors (Clontech, USA), respectively. The *Saccharomyces cerevisiae* Y2H GOLD yeast strain (Clontech, USA) was co-transformed with bait and prey using the polyethylene glycol/lithium acetate method. Transformants were selected on synthetic defined (SD) media lacking leucine and tryptophan (SD-Leu-Trp). Four individual colonies were grown overnight in liquid cultures (SD-Leu-Trp) at 30°C and 10-fold dilutions were dropped on SD-Leu-Trp and selective media lacking leucine, tryptophan and histidine (SD-Leu-Trp-His) and SD-Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazol (3-AT).

PROTOPLAST ISOLATION AND DUAL LUCIFERASE REPORTER ASSAY

The sequence of TFT9 promotor and its truncated versions were cloned into PGL3 vector (Promega, USA) to obtain a *Firefly* luciferase reporter system. Leaves of tomato plants 4 weeks old were used for protoplasts transformation as described by (Mishra et al., 2002). In brief, 10^6 protoplasts were transformed with 20 µg of plasmid DNA mixture consisting of TFT-reporter vector and vector driving the expression of *Renilla* luciferase. This latter allowed normalizing the assay. Incubation was in darkness at room temperature for 2 h. Subsequently, 10 µM iP was added to the reaction for 16 h. The protoplasts were lysed in passive lysis buffer before determination of *Firefly* and *Renilla* LUC activities (Dual-Glo Luciferase Assay System, Promega, USA) by measuring luminescence with a luminometer (BioTek, USA).The prediction of *cis*-acting element binding sites was performed by PlantPAN2.0 (http://plantpan2.itps.ncku.edu.tw/; Chow et al., 2015). The 1.4 kb sequences upstream of the start codon were retrieved from EnsemblPlants (http://plants.ensembl.org/index.html). A Q5® Site-Directed Mutagenesis Kit (New England Biolabs, USA) was used to create specific, targeted changes in two-component response regulators sites in double-stranded plasmid DNA containing a truncated TFT9 promoter sequence.

amiRNA CONSTRUCT DESIGN AND TOMATO FRUIT TRANSFORMATION

The amiRNA construct, pRSphot1, was engineered from pRS300, replacing the 21 bases of the natural Arabidopsis miR319a as well as the partially complementary region of the miRNA* in a pBSK vector using overlapping PCRs as described by Schwab et al. (2006). WMD3 was used to design amiRNA sequences (21mers) for *SlPHOT1* based on the ITAG2.3 tomato genome annotation. WMD3 suggested several suitable amiRNA candidates that were further validated by BLAST on the ITAG2.3 tomato genome for their plant specificity and more particularly for their specificity towards *SlPHOT1*. Only one candidate was further considered to target *PHOT1* mRNA. The plasmid information for pRS300 was selected, and all appropriate primer sequences, needed for customization of pRSPHOT1, were retrieved using the primer design function of WMD3. Four oligonucleotide sequences required for engineering pRSPHOT1 into the Arabidopsis endogenous miR319a precursor by site-directed mutagenesis were thus obtained. Two more oligonucleotides, primers A and B, were based on the template plasmid sequence, located at each extremity of miR319a precursor and had *Asc*I and *Not*I restriction sites, respectively, to allow cloning into the binary vector pSK36. All PCRs were performed with high fidelity *Pfu* DNA Polymerase (Thermo Fisher Scientific) according Schwab et al. (2006). Three fragments were PCR amplified from the template clone pRS300 using the six primers in combination: A+II (272 bp), I+IV (206 bp) and III+B (340 bp). The three resulting fragments were gel purified (Promega) and then fused by one PCR with the two flanking primers A and B on a mixture of 1 µl of each previous PCR as template. The fusion product of 448 bp was again gel purified (Promega),

cloned into pGEM®-T Easy Vector after A-tailing (Promega), sequence verified, excised with *Asc*I/*Not*I and transferred into the binary vector pSK36 (Ikeda et al., 2006). In pSK36, the expression of the transgene is driven by the promoter of the Cauliflower mosaic virus 35S. The amiRNA plant expression vector was transformed into *Agrobacterium tumefaciens* strains LBA4404 and use to transform immature tomato fruits as described by Yasmeen et al. (2009). Mutant plants were selected based on their phenotype expected to be affected in their growth response to light. The presence of the transgene was validated by PCR.

PROTEIN ISOLATION AND WESTERN BLOT

Hypocotyls of 4-d-old etiolated seedlings were homogenized in buffer containing 0.1 M Tris-HCl, pH 8.5, 4% SDS, 2 mM phenylmethylsulfonyl fluoride, and 1:100 protease inhibitor cocktail (Sigma, USA) in a ration 1:1,5 (tissue:buffer). The homogenate was then heated at 80°C for 3 minutes and centrifuged at 14,000 rpm for 15 min at 4°C. Total protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, USA). 50 µg of protein extract was mixed with 4x sample buffer and reducing reagent (Thermo Scientific, USA) and boiled for loading on 4%-12% BisTris Plus gel (Thermo Scientific, USA) to separate the proteins in MOPS running buffer supplemented with antioxidant reagent (Thermo Scientific, USA). Proteins were transferred to polyvinylidene fluoride membranes (Millipore) following standard procedures described by Towbin et al. (1979). Antigen and antibodies directed against tomato PHOT1 were synthesized and produced in rabbit, respectively, by Eurogentec (Belgium). The antigen (H_2N-GKTETSRRDSGNSGRC-$CONH_2$) was selected in the N-terminal of the protein, before the LOV1 domain, and for its specificity to PHOT1. Anti-PHOT1 antibodies were diluted 1:500 in TBST buffer (20mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20) and incubated with membrane with gentle shaking overnight at 4°C. The goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma-Aldrich) was used as secondary antibody and diluted 1:5000 in TBST buffer for 1 hour at room temperature. Detection was done according instruction of the Pierce™ Fast Western Blot Kit, ECL Substrate (Thermo Fischer Scientific) and Gel Doc™ EZ System (Biorad).

MAIN RESULTS

PHOT1-MEDIATED EARLY STEP OF HYPOCOTYL GROWTH INHIBITION

In order to study the molecular events of the rapid inhibition of tomato hypocotyl growth observed within the first 30 min following exposure to BL, a SSH library was constructed and screened for genes whose expression is stimulated by BL and thus potentially regulated by phot1. We determined that 152 ESTs were potentially differentially expressed. Computational analysis using the software Blast2GO enabled annotation of the expressed sequences according to the terms of the three main Gene Ontology vocabularies (i.e., cellular compartment, molecular function and biological process; Fig. 1). Concerning molecular function, the most represented categories were those of binding and catalytic activities (Fig. 1A). Regarding cellular compartments, the most represented were ribosome, plastid, and nucleus, together accounting for more than 50% of total annotations (Fig. 1B). When taking into consideration the most relevant level of distribution for the biological process more than 40 categories were found for the biological process vocabulary. The number of categories was therefore simplified. The functional annotation was performed with Mercator, using the last updated version of the tomato annotation (ITAG2.4) (Lohse et al., 2014). Identical description of the ESTs was obtained when the annotation was performed by Blast2Go, KOG attribution or Blast against the specific annotated tomato genome ITAG2.4 (Fig. 1C). qPCR confirmed the differential expression detected by the screening of the cDNA library, meaning the up-regulation of the expression of ESTs as soon as 30 min after exposure to BL.

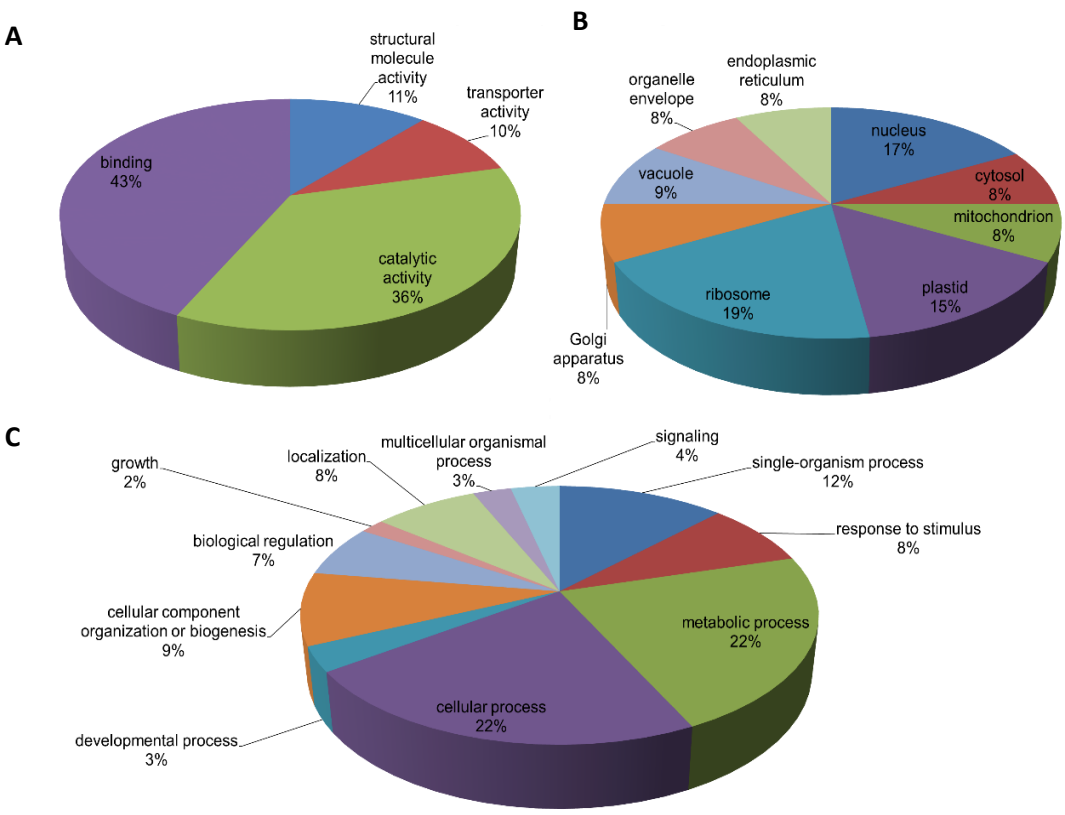

Figure 1. Gene Ontology terms distribution. Gene Ontology terms distribution by molecular functions (A), cellular components (B), and biological processes (C) vocabularies.

The functional annotations "Protein: synthesis, targeting, postranslation modification, degradation" and "RNA: processing, transcription, regulation of transcription" were the most represented, including 33 and 12 sequences, respectively. Sixteen differentially expressed sequences were predicted to encode proteins involved in translation, RNA processing and modification (ribosomal proteins), transcription (eukaryotic initiation factors), and chromatin structure and dynamics (Histone 2B – H2B, Histone H2A). H2B is involved in de-etiolation. In darkness, the photomorphogenic repressor DET1 binds to the H2B tails of the nucleosomes surrounding the genes which are repressed in this condition. When light is perceived, the H2B acetylation concomitant with the release of DET1 enables the activation of genes involved in photomorphogenesis (Benvenuto et al., 2002). Finally, we could identify and confirm that the subunit RPN10/PSMD4 of the 26S proteasome regulatory complex is up-regulated during de-etiolation. For this reason, it is tempting to hypothesize that light-regulated histone expression/modification and ubiquitin-proteasome-mediated protein degradation might interact during tomato de-etiolation.

In our study, seven tomato EST encoding proteins involved in cell wall modification were found to be up-regulated in the etiolated hypocotyl of tomato seedlings exposed for 30 min to BL: pectin acetylesterase, pectinesterase, xyloglucan endotransglucosylase-hydrolase 1 (XTH), or endoglucanase. This suggested that de-etiolation induced a strong modification of the cell wall structure and/or composition, namely extensibility.

Three ESTs encoding vacuolar H+-ATPase (V-ATPase) subunits were found to be up-regulated during PHOT1-mediated inhibition of hypocotyl growth. V-ATPases are potentially involved in creating or regulating turgor pressure. Whereas inhibition of the tonoplast-localized V-ATPase does not affect cell expansion, inhibition of that which is TGN-localized is sufficient to restrict cell expansion (Brüx et al., 2008). Moreover, the *det3* mutant, a possible negative regulator of photomorphogenesis affected in V-ATPase function, was originally proposed to be impaired in vacuolar solute uptake resulting in adequate turgor pressure for cell expansion (Schumacher et al., 1999). Recent evidence has shown that a cell wall defect in the mutant is responsible for its reduced hypocotyl cell expansion (Brüx et al., 2008). Together, these data indicate that V-ATPase plays a role in cell wall integrity/synthesis through its function in the TGN-mediated secretory pathway, thereby participating in the restriction of cell expansion. These results indicated that in tomato, like in barley, BL induces accumulation of V-ATPase as well as its activation (Klychnikov et al., 2007). Both events appear to be required to trigger the restriction of cell expansion occurring during de-etiolation.

14-3-3 PROTEINS AND CYTOKININS INVOLVEMENT IN HYPOCOTYL GROWTH INHIBITION UNDER BLUE LIGHT

It is known that the 14-3-3/TFT proteins are important actors of the phosphorylation/dephosphorylation signaling pathway cascade. We evidenced both at the transcriptional and protein level that the *TFT6* and *TFT9* were the most abundantly accumulated in the elongation zone of hypocotyl of etiolated seedlings during BL-induced de-etiolation (Fig. 2).

It is accepted for a long time that cytokinins (CKs) are mediators of photomorphogenesis and that iP, one of the three active CKs, specifically accumulated in the elongation zone of tomato hypocotyl during de-etiolation (Chory et al., 1989; Bergougnoux et al., 2012). In tomato, whereas the expression of the non-epsilon *TFT6* gene was insensitive to CK, the hormone strongly regulated the expression of the epsilon *TFT9* gene. The *in silico* analysis of the *TFT9* promoter predicted an enrichment in type-B RR binding sites; two of them at the positions -1035 and -1106 were determined to be essential for the CK-mediated regulation of *TFT9* expression. This was in agreement with the activation of CK-responsive genes requiring the combination of two RBS in Arabidopsis (Ramireddy et al., 2013). The activation of *TFT9* promoter in the presence of the Arabidopsis *ARR1* in tomato protoplasts grown in absence of exogenous CK demonstrated that TFT9 is a primary target of the CK signaling pathway leading to the restriction of hypocotyl growth.

We also initiated the interactomic study of these two tomato isoforms in hypocotyl during BL-induced de-etiolation.The 133 proteins were identified exclusive for BL-elongated hypocotyls or significantly increased compared to the darkness-grown hypocotyl elongation zone (absolute ratio > 2.0, $p < 0.05$). Most of the proteins identified as potential TFT interactors contained high-scoring 14-3-3 binding sites as determined by 14-3-3-Pred (Madeira et al., 2015).

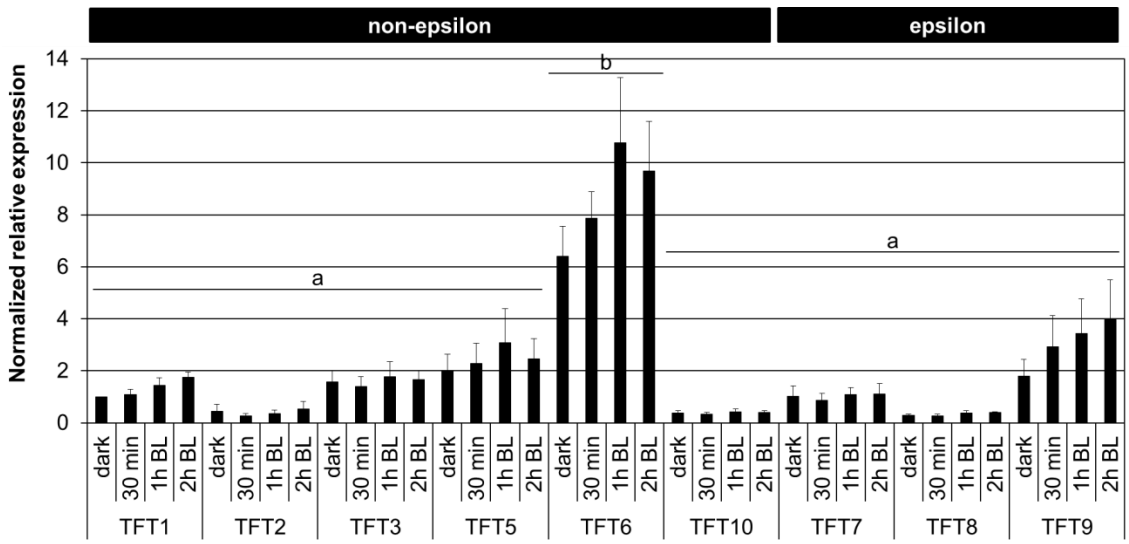

Figure 2. Expression profile of the tomato *14-3-3/TFT* genes in the elongation zone of hypocotyl during BL-induced de-etiolation. The graph represents the average of three to five independent biological replicates, with bars showing the standard errors of the means. Normalization was done in relation to three housekeeping genes: *PP2Acs*, *Tip41-like* and *EF1α*). Results were expressed as fold change relative to the expression of the *TFT1* gene in the elongation zone of seedlings grown in darkness. TFTs are grouped according to their relation to epsilon and non-epsilon group, as indicated by boxes. A non-parametric Kruskal-Wallis Anova & Median test analysis was performed followed by a post-hoc multiple comparison of mean rank (Statistica 12, StatSoft); identical letters indicate no significant difference.

Among them, the tomato ortholog of Arabidopsis ARR2, a type-B response regulator, was found to be a potential target of TFT6. Our observation is consistent with the Arabidopsis 14-3-3 interactom (Braun et al., 2011). In Arabidopsis, in the presence of CKs, ARR2 is phosphorylated and its stability is decreased, driving it to degradation via the 26S proteasome (Kim et al., 2012; Adams et al., 2014; Shull et al., 2016). Whether the binding with TFT6 leads to stabilization or degradation is not known. Nevertheless, this suggests a role in feedback regulation of CK signaling pathway to desensitize the plant to CKs, keeping the response within a certain range and allowing the establishment of the steady-state growth rate (Fig. 3B). Indeed, the analysis of tomato *tft6-ko* plants revealed that TFT6 is required to ensure proper hypocotyl growth in the light (Fig.3A). In a different context, the role of TFT6 in promoting growth was also demonstrated during low phosphorus stress (Xu et al., 2012).

It is well known that 14-3-3s/TFTs form hetero- and homodimers. In our interactomic study, we were able to identify different TFTs among the potential TFT6/9 binding partners, indicating that TFT6 and TFT9 form heterodimers. A yeast two-hybrid assay confirmed the interactions between TFT9 and TFT6 and between TFT9 and TFT4.

We determined that TFT6 might interact with both CKs and ethylene biosynthesis and signaling. Ethylene has long been reported to play a role during hypocotyl growth, being an inhibitor in the dark and promoter in the light, and especially under blue light (Ecker, 1995; Smalle et al., 1997; Vandenbussche et al., 2007; Yu et al., 2013). The 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, catalyzing the production of ACC, is stabilized by 14-3-3s (Yoon and Kieber, 2013). In the present study, we identified ACC oxidase as a putative interactor of 14-3-3/TFT. ACC oxidase catalyzes

the last step of ethylene synthesis. Its binding to 14-3-3 might contribute to ethylene production and accumulation during light exposure. Finally, exogenous CKs increased ethylene biosynthesis in hypocotyl, suggesting a crosstalk between the two hormones (Cary et al., 1995). It is noteworthy that in Arabidopsis the aforementioned ARR2 is a target of both CKs and ethylene (Hass et al., 2004). If such a dual targeting exists in tomato, this might contribute to establishment of the steady-state growth rate observed during de-etiolation and photomorphogenesis.

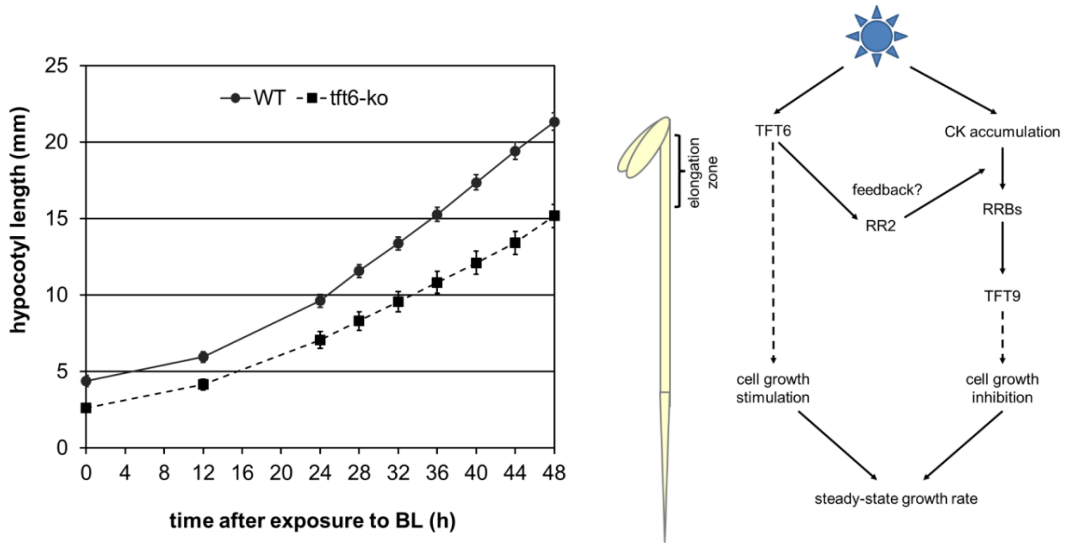

Figure 3. Left-Growth of etiolated hypocotyl of Alisa Craig and *tft6-ko* mutant after exposure to BL. Right-Schematic representation of the interaction between TFTs and cytokinins (CK) during BL-induced deetiolation and photomorphogenesis of etiolated tomato seedling

In darkness-grown seedlings, the accumulation of auxin induces activation of the PM-ATPase (Takahashi et al., 2012), leading to acidification of the cell wall (CW) and activation of enzymes responsible for CW loosening. Activation of the pump leads to hyperpolarization of the PM, activation of K^+-inward rectifying channels, water uptake, and subsequent cell expansion (Perrot-Rechenmann, 2010; Chapman et al., 2012). The activity of PM-ATPase varies from an auto-inhibited state where ATP hydrolysis is poorly coupled to H^+ transport and an up-regulated state with a close relationship between hydrolysis and transport (Falhof et al., 2016). Up-regulation of the PM-ATPase requires phosphorylation of its penultimate residue and subsequent binding of 14-3-3 protein that stabilizes the pump in this state (Fuglsang et al., 1999; Svennelid et al., 1999; Rosenquist et al., 2001; Cosgrove, 2014). The phosphorylation of two other sites inactivates the pump, but whether or not this is related to 14-3-3 binding remains unclear (Falhof et al., 2016). Upon exposure to light, one might expect inactivation of the proton pump. In our study, the PM-ATPase subunit LHA1 was identified as a TFT6 interactor. It is hardly conceivable that the binding of TFT6 on PM-ATPase results in the complete inactivation of the enzyme. In fact, when the *tft6*-knockout plants were grown in the light, they had shorter hypocotyls compared to wild-type plant, suggesting that the functional TFT6 contribute to hypocotyl elongation (Fig.3A). For this reason, the binding TFT6 to the PM-ATPase might contribute to a low activity of the enzyme, thus ensuring the establishment of the steady-state growth rate of the hypocotyl whose growth is not inhibited but only restricted during de-etiolation and photomorphogenesis.

In our study, V-ATPases, were found also to interact with TFT6/9. We previously had demonstrated that transcripts encoding different V-ATPase subunits accumulated during de-etiolation in tomato and that V-ATPase is important in de-etolation (Hlouškova and Bergougnoux, 2016). In the present study, nine subunits of the V-ATPase were found in the interactome of the two tomato 14-3-3s. As they form a complex, the binding of one subunit to the 14-3-3 can result in co-precipitation of the full complex.

Several TFT interactors were related to CW remodeling, such as pectinesterase (PME), GDP-D-mannose-3,5-epimerase (GME), UDP-glucose dehydrogenase (UGD), and alpha-1,4-glucan-protein synthase (GPS). In Arabidopsis, the high activity of PME has been shown to restrict cell elongation (Derbyshire et al., 2007). GME participates in biosynthesis of such CW polysaccharides as glucomannans and pectin (Reiter and Vanzin, 2001). Its partial inactivation causes structural alterations of pectin network and results in growth defects (Gilbert et al., 2009; Voxeur et al., 2011). UGD catalyzes the formation of UDP-glucuronic acid, the main precursor for the synthesis of hemicelluloses and pectic polymers (Lunn et al., 2013). Finally, GPS is highly similar to the reversibly glycosylated polypeptide proteins observed in Arabidopsis and involved in the synthesis of CW components (Rautengarten et al., 2011). Consequently, it appears that 14-3-3/TFTs participate in the inhibition of cell expansion through the regulation of different enzymes involved in synthesis and modification of the CW constituents.

The switch from skoto- to photomorphogenesis requires a deep reprogramming of the cell, marked by the activation of transcription and synthesis of new proteins. In this context, we identified a huge number of ribosomal proteins as TFT interactors.

De-etiolation and the subsequent photomorphogenesis are marked by the switch from heterotrophy to autotrophy. In our conditions, several proteins related to storage were identified as TFT-interacting proteins. These proteins belong to oleosins and globulin family (vicilin and legumin). The phosphorylation of oleosin induces their degradation via the 26S proteasome signaling pathway, allowing the mobilization of oil storage to provide carbon and energy for growth of the seedling (Parthibane et al., 2012; Deruyffelaere et al., 2015). Vicilin and legumin accumulate in protein bodies during seed maturation. They are mobilized during germination and early seedling development to provide amino acides required for protein synthesis (Tiedemann et al., 2000).

Several enzymes putatively regulated by 14-3-3/TFTs were found to be involved in the primary metabolism which includes the Calvin cycle, glycolysis, Krebs/TCA cycle, amino acid metabolism, chloroplast electron transport chain, and sucrose and starch metabolism. The role of 14-3-3s in the primary metabolism has been reviewed by (Diaz et al., 2011). Our study on tomato hypocotyl demonstrated that TFT6 and TFT9 contribute to mobilization of resources from storages and initiation of the primary metabolism, which is characteristic for the transition from heterotrophy to autotrophy.

We also identified and confirmed by Y2H assay the interaction of TFT9 with a PEPCK and GDSL.PEPCK activity was found to be higher in tissue grown in the dark compared to tissue illuminated by light. Among the four phosphorylated residues in the N-terminal region of the PEPCK, the Ser^{55} was found to play an important in this reversible phosphorylation. Interestingly, it is localized inside a putative 14-3-3 binding site. Therefore, the light-induced reduction of PEPCK activity might be related to the inhibitory role of 14-3-3 as it was demonstrated for nitrate reductase, sucrose phosphate synthase and glutamine synthetase (Lee et al., 2001). GDSL represent a class of newly identified lipolytic enzymes. Identified GDSL showed a high homology with rice and Arabidopsis fucosidases which release the fucosyl moiety from xyloglucan-derived oligosaccharides (Chepyshko et al., 2012). During etiolation, CW loosening correlates with the increase in the xyloglucan cleavage driven by cellulases with low pH optimum. The resulting fucose-containing nonasaccharide inhibits the growth induced by auxin, protons, gibberellins and fusicoccin that stabilizes the interaction between 14-3-3 and its partner (Fry et al., 1993; Augur et al., 1993).

We provided evidence that tomato 14-3-3s participate in BL-mediated early seedling development, starting with the regulation of the BL-photoreceptor PHOT1 (Fig. 4). The interactome of two isoforms, TFT6 and TFT9, predicted that 14-3-3s might affect an array of client proteins involved in cell elongation, metabolism, protein synthesis, and hormone pathways. The functional significance of these interactions needs to be further addressed in the future.

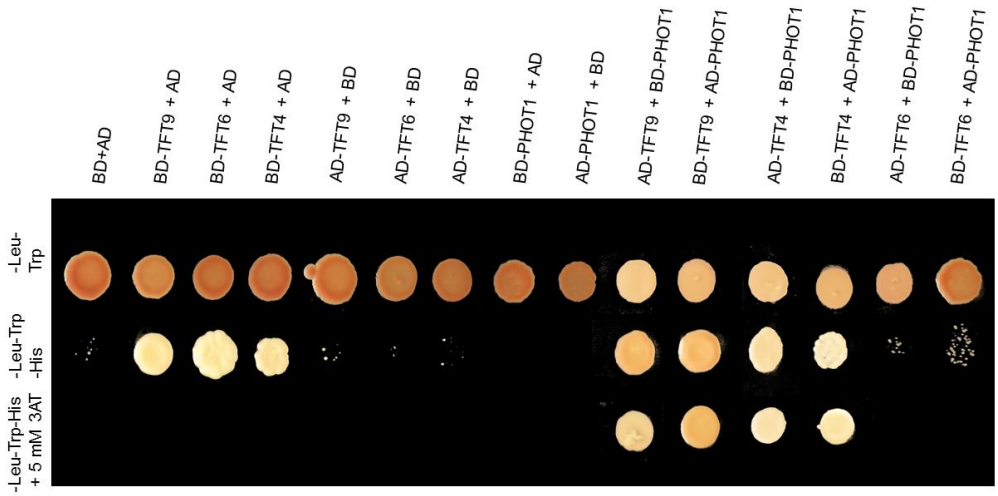

Figure 4. Yeast-two-hybrid assay showing the interaction between TFT9, TFT4 or TFT6 and PHOT1. Representative image is presented and includes the respective controls and reciprocal tests. Yeasts transformed with plasmids expressing TFT9, TFT4, TFT6 and PHOT1 fused to GAL4 activating (GAL-AD) or binding (GAL-BD) domain were selected on synthetic defined dropout medium lacking leucine and tryptophan (-Leu-Trp), leucine, tryptophan and histidine (-Leu-Trp-His), or Leu-Trp-His supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Empty vectors were used as a negative control.

MUTANT LINES OF *PHOT1*

Finally, using amiRNA interference, we generated plants with downregulated *PHOT1* gene, which was confirmed both at the RNA and protein level. Apparently, the hypocotyl growth rate was higher in seedling with reduced level of PHOT1 compared to wt, supporting the role of PHOT1 in hypocotyl growth inhibition (Parks et al., 2001). The amiRNA-*phot1* plants harbored a higher stature compared to the wt cv. Rutgers throughout the plant life. Such effect of modulation of BL photoreceptors on the overall growth of the plants has already been reported for different species. Whereas tomato antisense *cry1* plants were taller than the WT, the overexpression of *cry2* resulted in smaller plants (Ninu et al., 1999;Giliberto et al., 2005). Similarly, the fresh weight of Arabidopsis *phot1-5* plants grown under natural conditions (70 μmol $m^{-2}s^{-1}$) was higher (Takemiya et al., 2005). Two-fold increase in the growth and biomass was observed in *phot1* mutants in algae (patent n. WO 2016/197136-A2). The altered serration of the leaf margin was already reported in *nps1* mutant in tomato generated by ethyl methansulfonate-mutagenesis (EMS), showing amino acid transition in a highly conserved α helix prior to LOV2 domain of PHOT1 (Sharma et al., 2014). Under monochromatic red light, the leaves curl downwards, whereas flat leaves develop under monochromatic blue light. Previously, it was reported that phototropins mediate the leaf flattening under BL (Inoue et al., 2008; Kozuka et al., 2013). Accordingly, a phototropin-deficient mutants in Arabidopsis showed a curled-leaf phenotype as well as the leaves of our amiRNA-*phot1* plants generated in current study (Sakai et al., 2001; Sakamoto and Briggs, 2002; de Carbonnel et al., 2010).

The role of all photoreceptors in shoot branching was studied in the model *Physcomitrella*. Authors found out that CRYs and PHYs initiate the process of branching, whereas the position of branching is determined by PHOTs and PHYs (Uenaka et al., 2005). The changes in PHOT1 abundance in the tomato amiRNA-*phot1* lines might be responsible for the limited branching in comparison with wt plants.

The tomato amiRNA-*phot1* mutant also showed a hairy phenotype characterized by a huge number trichomes both on leaf and stem. Such hairy phenotype could represent an adaptation mechanism to better tolerate water deprivation. Indeed, the important population on trichomes on aerial parts of the plant might limit the water loss by transpiration, prevent leaf overheating or protect leaf

from UV-related photoinhibition either by reflection of UV radiation or absorption by pigments (Palliotti et al., 1994; Guerfel et al., 2009; Mo et al, 2016; Galmez et al., 2007; Holmes and Keller et al., 2002; Ehleringer and Mooney et al., 1978). Our proteomic study of leaves support the hypothesis that the tomato amiRNA-*phot1* plants could be more tolerant to drought stress. Indeed, proteins with role in drought tolerance such as aquaporins, protein disulfide isomerase or catalase were more abundant in the transgenic tomato (Devi et al., 2012;Zargar et al., 2017;Zhang et al., 2018). In Arabidopis, the defect in *PHOT1* stimulated the foraging behavior of the root to avoid the soil surface, constituting a mechanism of adaptation to drought stress (Galen et al., 2007). To confirm the role of PHOT1 in drought stress tolerance, a deeper phenotypic analysis will be required, involving different watering regimes and measurement of several parameters such as the temperature of leaves, chlorophyll fluorescence, gasometric parameters and antioxidant enzymatic activity.

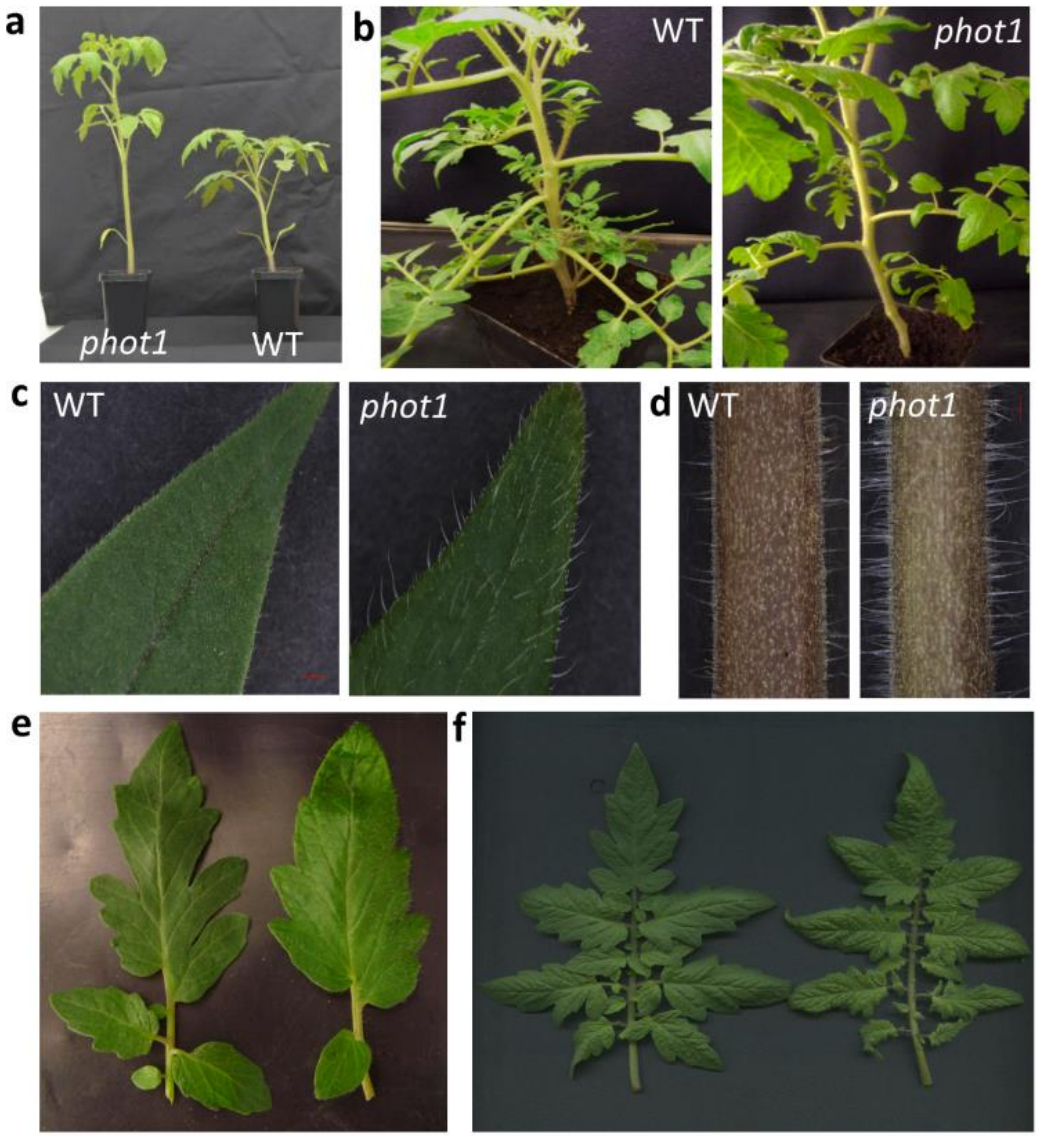

Figure 5. Phenotypic characterization of the cv. Rutgers and amiRNA-*phot* line. a) plant height – 20-day-old plant watered to 80% field capacity, b) stem branching, c) leaf trichomes, d) stem trichomes, e) representative leaflet, f) representative leaf.

Changes in physical properties of the fruits of tomato amiRNA-*phot1* plants were not surprising. Indeed, tomato mutants affected in light signaling pathways showed similar feature. Whereas mutants in negative regulators of photomorphogenesis *det1*, *ddb1*, *cop1* and *cry2* exhibits darker fruits, the positive regulator *hy5* mutant had pale green fruits. The change in pigmentation coincides with the nutritional quality of fruits. The darker green phenotype means the higher content of chlorophyll and carotenoids (Mustilli et al., 1999;Liu et al., 2004;Giliberto et al., 2005). Carotenoids are antioxidants which help to prevent some type of cancers and degenerative diseases (Bartley and Scolnik, 1995). Proteomic analysis was done to investigate the protein differences between amiRNA-*phot1* and wt plants. The fruit of the amiRNA-*phot1* plants were characterized by the presence in hight amount of the 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), enzyme of the MEP pathway responsible for the synthesis of the carotenoid precursors. It was already demonstrated that the upregulation of HDR leads to the increased level of carotenoids both during tomato fruit ripening and Arabidopis seedling de-etiolation (Botella-Pavía et al., 2004). Caseinolytic protease (Clp) was shown to be downregulated in the fruit of the amiRNA-*phot1* mutant. Interestingly, the gene encoding this enzyme was found to be accumulated in the hypocotyl of tomato illuminated by blue light. Despite the different organs, and the different level (transcriptional vs. traductional), these results evidenced that this enzyme is regulated by blue light in a PHOT1-dependent manner. CLP is involved in the regulation of the MEP pathway, probably through the degradation of HDR (Flores-Pérez et al., 2008;Moreno et al., 2018). The dehydroascorbate reductase (DHAR) is another interesting enzyme identified in our proteomic study whose abundance was increased in the fruit of amiRNA-phot1 plants. This enzyme is involved in ascorbate production. Ascorbic acid (Asc) is a major antioxidant in plants that detoxifies reactive oxygen species (ROS) and maintains photosynthetic function. Expression of dehydroascorbate reductase (DHAR), responsible for regenerating Asc from an oxidized state, regulates the cellular Asc redox state, which in turn affects cell responsiveness and tolerance to environmental ROS. Supression of DHAR resulted in the loss of chlorophyll a, lower rate of CO2 assimilation and premature leaf aging (Chen and Gallie, 2006). Despite its functions in photoprotection, Asc acts as a cofactor for enzymes such as violaxanthin deepoxidase, ethylene-forming enzyme, 2-oxoacid-dependent dioxygenases required for the synthesis of abscisic acid (ABA) and gibberellic acid (Chen and Gallie, 2006; Gest et al., 2013). The HPLC analysis of chlorophyll and carotenoid content confirmed the higher level of both pigments in mutant compared to wt predicting better nutritional quality of the fruit.

CONCLUSION

Light is an important environmental factor affecting plants alltrough their life spam. In the frame of the current study, we focused our interest on the blue part of the light spectrum and on the specific blue light (BL) photoreceptor PHOTOTROPIN 1 (PHOT1). PHOT1 has been described to play an important role in plethora of physiological responses, including phototropism, chloroplast movement, leaf bending, hypocotyl growth. Most of the studies related to BL and PHOT1 are conducted on the plant model Arabidopsis and only a minor amount of knowledge is available for plants species of economical importance, such as tomato (*Solanum lycopersicum* L.). Understanding how BL regulates tomato responses to environment and the specific role of PHOT1 will bring about tools for bioingeneering of tomato for better adaptation to environment pressure.

The current study started with simple theoretical questions: How is BL-induced de-etiolation regulated in tomato? What is the role of PHOT1 in this process? In tomato, like in Arabidopsis, BL-induced de-etiolation is a sequential process depending first on PHOT1 during the first 30–40 min of exposure to BL, with CRY1 being later responsible for the establishment of the steady-state growth rate (Bergougnoux et al., 2012; Folta and Spalding, 2001). Whereas CRY1-mediated de-etiolation has been characterized at the molecular level in Arabidopsis (Folta et al., 2003), no information is available concerning the PHOT1-mediated phase of de-etiolation. Therefore, we developed a suppression subtractive hydridization assay to revealed genes that were up-regulated by BL during the PHOT1-mediated phase of de-etiolation. We identified 152 genes quickly up-regulated by BL. Their annotation revealed deep changes in chromatin modelling, transcription, and translation, but also in cellular processes and signaling such as cell wall integrity/synthesis, cytoskeleton, and trafficking/secretion.

In general, cell signaling mechanisms often involve posttranslational modifications (PTMs) of proteins, affecting their conformation, activity, stability, and/or localization (Wold, 1981). The reversible phosphorylation of the serine, threonine or tyrosine residue has been probably the most extensively studied (Olsen et al., 2006). Very often, however, phosphorylation of the protein alone is not enough to modulate protein function. Rather, phosphorylation is required to ensure the binding of interactors which will themselves regulate protein function. Among the plant phosphopeptide-binding proteins, 14-3-3 proteins (14-3-3s) are the best characterized (Chevalier et al., 2009). They have recently attracted attention due to their remarkable capacity to affect a wide array of physiological, developmental, and cellular processes such as primary metabolism, hormone signaling, response to light, cell growth and division, pathogen–plant interaction, and response to biotic and abiotic stresses (Cotelle et al., 2015; Denison et al., 2011; Lozano-Durán and Robatzek, 2015; Camoni et al., 2018). The altered hypocotyl length of Arabidopsis 14-3-3 mutants grown in RL or in darkness suggests a role for 14-3-3 proteins in the control of hypocotyl elongation (Mayfield et al., 2007; Zhao et al., 2015). However, their possible involvement in plant response to BL is not yet clear. In tomato, 12 isoforms have been identified and are referred to as Tomato Fourteen Three (TFTs). Earlier study of antisense transgenic tomato plants had demonstrated that at least two isoforms were involved in light-mediated plant development (Roberts et al., 2002). In the present study, we focused on events arising during establishment of the steady-state growth rate under BL exposure while giving special attention to the role of 14-3-3/TFTs in that process. We also described how two tomato TFTs are involved in the BL-mediated response of tomato hypocotyl, and their regulation by cytokinins. Using immobilized recombinant tomato TFTs, more than 130 proteins related to a large variety of cellular processes were identified as potential TFT interactors. Their functional annotation predicted that TFTs might regulate the activity of proteins involved notably in cell wall strengthening or primary metabolism. Several potential interactors were also predicted to be CK-responsive. For the first time, the 14-3-3 interactome linked to de-etiolation was investigated and evidenced that 14-3-3s might be involved in CK signaling pathway, cell expansion inhibition and steady-state growth rate establishment, and reprograming from heterotrophy to autotrophy.

In the meantime, we generated tomato transgenic plants with knocked-down expression of *PHOT1* via the amiRNA methodology and the *Agrobaterium tumefaciens*-mediated transfornation of immature tomato fruits. From the phenotypic characterization of the amiRNA-*phot1* mutant we evidenced roles of tomato PHOT1 in leaf development, response to water deprivation, fruit development and maturation. Deeper analysis of the mutant is required in order to get a more precise insights concerning the role of PHOT1 in these processes.

In the frame of the present thesis, a huge amount of data has been collected to understand how BL modulates tomato development throught PHOT1. High-throughput RNA-sequencing, advanced phenotyping, generation of transgenic plants via CRISPR-Cas9 guided RNA methodology for specific candidates: these are all aspects that have to be developed in the future to complete this work.

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