CONTINUOUS LIGHT ON TOMATO

From Gene to Yield

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Aarón I. Vélez-Ramírez

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General Introduction

Continuous-light-induced injury in tomato: An old enigma

Aaron I. Velez-Ramirez

Why grow tomatoes under continuous light?

High-tech greenhouse horticulture is highly efficient in using resources like water and nutrients. In The Netherlands, in 2005, greenhouses occupied an area of 10,500 ha, and about 20% of this area was equipped with supplementary lighting. Supplementary lighting is used to increase both the light intensity during cloudy days and the daily light period during winter. By 2008, when this project was conceived, rose growers in The Netherlands and other Northwest European Countries were already using supplementary lighting for large periods of the day in order to increase yield. In cut and pot roses, continuous light (CL) increases the number of flowers by up to 12 or 34%, respectively, in comparison to an 18-h photoperiod regime (Mortensen & Gislerød, 1999, Pettersen *et al.*, 2007). For tomato (*Solanum lycopersicum*), more than 160 ha of greenhouse area were equipped with supplementary lighting in 2006 (original report in Dutch, cited by Heuvelink *et al.* (2006)). This inspired people in the Dutch horticultural industry to pursue the cultivation of tomato under CL in order to increase yield using the infrastructure already in place. Although innovative, this endeavor revived an old scientific enigma — Unlike roses, tomato plants develop potentially lethal injuries if cultivated under CL (Arthur *et al.*, 1930) — and highlighted a huge gap in our understanding of light signaling, photosynthesis and circadian rhythms in tomato. Here, we present the results of a 5-year effort to better understand the physiological basis of the CL-induced injuries in tomato and develop the tools (genetic and conceptual) to cultivate tomatoes under CL.

Continuous light induced injuries in tomato

Between 1924 and 1928, Arthur *et al.* (1930) extensively studied the effects of artificial climate on many plant species in an attempt to find the environmental conditions that allowed cultivating plants at their maximum capacity throughout their life cycle. By doing so, they discovered that, unlike most tested species, tomato plants develop a serious disorder when exposed to CL, which can even result in plant death. This seminal work inspired many others; along the decades, each attempt to understand this disorder took advantage of the knowledge and technology of the time. Although important and valuable discoveries were made, by the time this project started, a detailed and substantiated physiological explanation of this disorder was still missing.

Tomato is usually grown under natural photoperiods or, if supplementary light is used, under a maximum of 16 to 18h photoperiod. Figure 1.1a shows a healthy leaflet from a tomato plant grown under a 16-h photoperiod. When exposed to CL, tomato plants show a set of characteristic symptoms, of which interveinal mottled chlorosis starting at the leaf/leaflet basis is the most distinctive (Fig. 1.1b-d). Equally distinctive is the fact that such chlorosis gradually extends towards the leaf/leaflet tips/edges in younger leaves until it covers the complete leaf surface (Fig. 1.1f-h). Other symptoms include epinasty (curling of leaf blades) (Fig. 1.1j), necrotic spots (Fig. 1.1k), and smaller leaves/leaflets (Fig. 1.1e-h). Under conditions favoring the development of this disorder (*i.e.* high light intensity,

exclusion of sunlight from the CL regime and constant temperature), tomato plants eventually die if exposure to CL lasts long enough (Fig. 1.1i). In literature, this disorder has received several names, including photoperiodic chlorosis (Daskaloff & Ognjanova, 1965, Withrow & Withrow, 1949), light-injury (Hillman, 1956), continuous irradiation injury (Tibbitts *et al.*, 1990) and constant-light injury (Cushman & Tibbitts, 1998, Cushman *et al.*, 1995). In this study, we use the term *CL-induced injury*.

Brief chronological summary on key studies

As the knowledge in plant physiology increased and new technologies became available, the hypotheses and experiments aiming to explain the physiological mechanism of the CL-induced injuries evolved. One may say that a day in the library is worth years in the laboratory. In Chapter 2, therefore, we critically review the previous research using a modern understanding of plant physiology, and Supplementary Table 4.1 provides an extensive literature list on the topic. Here, therefore, I limit myself to a chronological review of the studies that contributed most to the hypotheses postulated and/or tested in this study.

Soon after Arthur *et al.* (1930) described the phenomenon of CL-induced injury in tomato, Darrow (1933) reported tomato plants growing, vigorously and without injury, under natural CL provided by the Arctic summer in Alaska, less than 2° from the Arctic circle. This posted serious doubts regarding the true nature of the factor inducing the disorder as the important differences between sunlight and artificial light could be the culprit instead of the CL itself. In order to reveal the true nature of the CL-induced injury, in Chapter 6, we used modern light sources, which mimic the spectral distribution of sunlight, to shed light on this concern. Withrow and Withrow (1949) reported that the CL-induced injury is higher at higher temperatures and old leaves developed under 15-h photoperiod did not show marked chlorosis when exposed to CL. Further studying these observations, the work of Hillman (1956) is probably the single most important contribution to the understanding of this disorder. By careful observation of tomato plants transferred to CL, and then back to non-injurious photoperiod regimes, he showed that when a healthy tomato plant is transferred to CL, the first leaves to show injury will show them at the leaf basis, and in progressively younger leaves the injury extends towards the tip. Likewise, when an injured plant is transferred back to a non-injurious photoperiod, the recovery follows an opposite pattern; that is, the leaf tip is injured and the leaf basis remains green. These observations suggest that only young leaves could develop into injured or healthy leaves, depending on the prevalent light regime during a critical developmental stage. Furthermore, he showed that a daily change in temperature prevents CL-induced injury in tomato without affecting plant weight. Then he showed that the light intensity and spectral distribution used to grow the plants under non-injurious photoperiods influenced the injury severity once transferred to CL. Finally, he showed that abnormal light/dark cycles, which are light/dark cycles with a periodicity substantially differing from the terrestrial 24-h periodicity (*e.g.* 4-h light/4-h dark cycles), induced the same kind of chlorosis in tomato as the one induced by CL. Throughout this dissertation, we are coming back to Hillman's observations as they offer key clues linking the CL-induced injury with

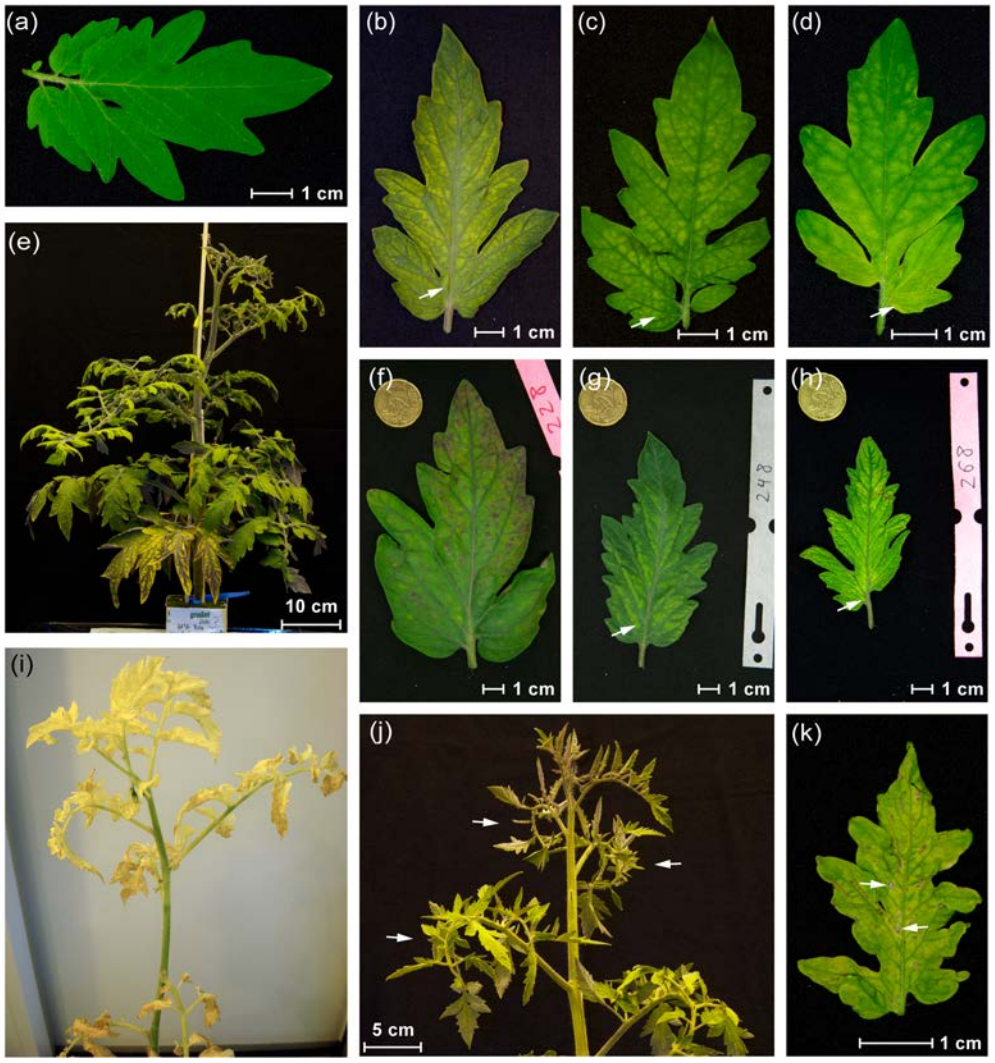

Figure 1.1 | Continuous-light-induced injuries in tomato. (**a**) Healthy tomato leaflet grown under 16-h photoperiod provided by a sulfur plasma lamp. (**b**–**d**) Tomato leaflets showing the characteristic, interveinal, mottled chlorosis induced by continuous light; notice that the injury severity is higher at the leaflet bases (white arrows). In **b**, **c** and **d,** light was provided by high-pressure sodium, red and blue light-emitting diodes and sulfur plasma lamps for 2, 3 and 2 weeks, respectively. (**e**) Tomato plant after three weeks of continuous light provided by high-pressure sodium lamps; notice that upper leaves (white arrows), which developed under continuous light, are smaller than lower leaves developed under 16-h photoperiod. (**f**–**h**) Leaflets from the second, sixth and seventh true leaves, respectively, from the same tomato plant after 3 weeks of continuous light provided by high-pressure sodium lamps. Leaflets **f**, **g** and **h** were fully expanded, appeared and not visible, respectively, at the time of transfer to continuous light; notice the absence of continuous-light-induced injury in **f**. In **g** and **h**, the injury severity is higher at the leaflet bases (white arrows). (**i**) Tomato plant killed by

continuous light provided by high-pressure sodium lamps (**j**) Tomato leaves showing epinasty (white arrows) after exposing the plant to continuous light provided by high-pressure sodium lamps for 24 days. (**k**) Tomato leaflet showing severe chlorosis and necrosis (white arrows) after exposing the plant to continuous light provided by a sulfur plasma lamp for 25 days.

the circadian clock (Chapter 6), light signalling (Chapter 7) and the coordination between nuclear and plastid developmental programs (Chapter 8).

After an exhaustive literature search, we found that Daskaloff and Ognjanova (1965) reported that wild tomato species are tolerant to CL. Unfortunately, this important finding was ignored by numerous studies done in the 1980's and 1990's, likely because it was published in German during the pre-internet era. We use the CL-tolerance found in wild tomatoes as a fundamental resource to breed CL-tolerant tomatoes and investigate the physiological mechanism inducing injury under CL.

Outside planet Earth, the familiar 24-h day/night periodicity is not the rule. Hence, studying the effects of artificial light sources and artificial light regimes on plants is most interesting for space exploration. In the 1980's and 1990's, the USA National Aeronautics and Space Administration (NASA) funded research on the effects of CL on tomato and potato. Among the research outcomes, Wheeler and Tibbitts (1986) showed that depending on the cultivar, potato plants are also CL-sensitive, and Cushman *et al.* (1995) reported that the CL-induced injury in potato appears to be a senescence-like event leading to a catastrophic loss of photosynthetic competence. Likewise, Cushman and Tibbitts (1998) showed that *Never ripe* tomato plants, an ethylene insensitive mutant, did not show CL-induced epinasty, yet they observed no difference in the CL-induced reduction of chlorophyll content compared with wild-type tomato. Furthermore, they found that transgenic tomato plants carrying an antisense copy of the *ACC-oxidase* gene, which encodes for the last enzyme required for ethylene biosynthesis, did show CL-induced epinasty, yet the chlorophyll content was higher than in wild-type plants and *Never ripe* mutant plants that were exposed to CL. All together, these studies suggest that the CL-induced injury in tomato might be accelerated senescence; a hypothesis explored in Chapter 8.

Although a role for carbohydrate accumulation in the induction of the CL-induced injury was suggested from the very beginning by Arthur *et al.* (1930), studies in the 1990's further investigated this hypothesis. For instance, Dorais *et al.* (1996) suggested that low sucrose phosphate synthase activity, leading to starch accumulation in the chloroplast, could explain why tomato plants do not yield more when exposed to CL. Likewise, Demers *et al.* (1998) showed that fruit pruning had no effect on the severity of the CL-induced injuries in tomato, suggesting that sugar accumulation in CL-exposed tomato leaves results from limitations in sugar export rather than sink limitations. In Chapter 8, therefore, we focus on the effects of carbohydrate accumulation and metabolism and their relation with the CL-induced injury.

Finally, Globig *et al.* (1997) reported that enrichment of far-red light reduced the injury symptoms, suggesting the involvement of the red/far-red photoreceptor, phytochrome. In Chapter 7, we used tomato mutants and transgenic lines lacking or

overexpressing phytochrome, respectively, to further study the role of phytochrome signaling in CL-induced injury. To prevent redundancy and enhance readability, discussion of additional studies is limited to Chapter 2 (a review paper on the topic) and when needed throughout the rest of this dissertation.

Objectives and general approach

The objectives of the present study were to (i) better understand the physiological basis of the CL-induced injuries in tomato, (ii) identify the gene(s) responsible for CL-tolerance in wild tomato species, (iii) supply the knowledge required to breed CL-tolerance into domesticated tomatoes and (iv) evaluate the effect of CL on greenhouse tomato yield when using a CL-tolerant genotype.

The complexity of the CL-induced injury and diversity of this thesis' objectives demanded the use of multiple strategies. First, researching the physiological mechanism behind the sensitivity of tomato plants to CL using hypothesis-driven experiments was an important and valuable strategy, yet it does not differ much from the classic approaches used in other studies. In addition, focus on the genetic and physiological basis of CL-tolerance as well as the efforts to develop the knowledge needed to use CL-tolerant tomato in horticulture mark important distinctions in respect to previous studies. Likewise, the use of data-driven approaches, such as transcriptomics and metabolomics, further contributes to study the topic from multiple perspectives. The different approaches, *e.g.* data- vs. hypothesis-driven experiments, present advantages and disadvantages, yet by combining them we aimed to take advantage of the best of both of them. Next, I will introduce these approaches and describe how they contribute to the different objectives.

Genetic mapping and molecular marker–assisted selection

Natural genetic variation is of great value for research and breeding purposes. In tomato, for instance, many traits of agronomic interest are present in wild tomato species, but they are absent in domesticated tomatoes, *e.g.* (Frary *et al.*, 2004, Fulton *et al.*, 2000). By using introgression line populations and quantitative trait loci (QTL) analysis, it is possible to identify genetic loci associated with a trait of interest; this not only facilitates further research on the physiological basis of the trait but also accelerates the introgression of the trait into modern cultivars (Eshed & Zamir, 1994, Tanksley & Nelson, 1996). This approach has been applied in the research of tomato resistance to biotic and abiotic stresses, fruit weight and composition as well as seed quality (Finkers *et al.*, 2007, Foolad, 2007, Khan *et al.*, 2012, Prudent *et al.*, 2009).

An important aspect of genetic mapping is the ability to identify and quantify genotypic and phenotypic variation. Multiple techniques for molecular marker identification (Agarwal *et al.*, 2008, Ganal *et al.*, 2009) and high-throughput phenotyping (Dhondt *et al.*, 2013) are available. Hence, the introgression line populations and molecular marker-assisted selection were chosen to investigate the genetic basis of CL-tolerance and to introgres this trait into modern breeding lines (Chapter 4). During the course of this study,

new advances like the identification of large numbers of single nucleotide polymorphism (SNP) markers in tomato (Sim *et al.*, 2012, Viquez-Zamora *et al.*, 2013) and the sequencing of multiple tomato genomes (150 tomato genome consortium, 2013, Tomato Genome Consortium, 2012) further enhanced the power of genetic mapping for future research and breeding efforts.

Data-driven science

Scientific advancement can be summarized as a cycle between ideas and data. In the hypothesis-driven science approach, the cycle starts with ideas/hypotheses; then consequences of such hypotheses are *deduced*, and an experiment is performed in order to obtain data that, hopefully, validates or rejects the hypothesis. Alternatively, the cycle could also start with data acquisition. Then ideas/hypotheses are *inferred* from data in order to explain what was observed; this is known as data-driven approach. Although some disciplines, or certain periods in history, might favour one approach over the other, they can also be considered as complementary to each other, both performing best if carried out iteratively; for further reading see Kell & Oliver (2004). In this study, we used both approaches in order to gain insights into the physiological mechanism underlying injury in CL-exposed tomato plants. By doing so, we aimed to benefit from modern knowledge on plant physiology, which suggested several hypotheses to test (see below), and to take advantage of the -omics technologies, which allowed us to not restrain to ideas deduced from a rather limited knowledge base regarding the CL-induced injury. Hence, in Chapters 4 and 8, transcriptomics and metabolomics are exploited to evaluate the effect of CL on tomato lines tolerant and sensitive to CL.

In this study, RNAseq and GC-TOF-MS analysis were used to evaluate the transcriptome and metabolome, respectively. Although these two techniques deliver large quantities of data, the bottleneck is the extraction of useful information/ideas from such large data sets. To solve this, specialized algorithms must be used. The algorithm to use depends on the nature of the data and the question to answer. For instance, the transcriptomics data of the present study were analyzed using multivariate analysis techniques specially developed for RNAseq data (Robinson & Oshlack, 2010, Robinson *et al.*, 2010), category enrichment analysis based on the gene ontology classification (Young *et al.*, 2010) and category mapping based on the KEGG classification (Luo & Brouwer, 2013). Once the proper analysis is performed, its outcome might be difficult to interpret. Hence, visualization techniques, *e.g.* Supek *et al.* (2011), are of great value to present the outcome in a human-friendly way. Considering that this is an area of very active research, the value of the data sets should increase with time as better algorithms are developed.

Hypothesis-driven science

An important section of this thesis is devoted to hypothesis-driven experiments (Chapters 5-8). Chapter 2 provides an overview of all plausible hypotheses aiming to explain CL-induced injury in tomato, either collected from the literature or postulated in this study.

Here, therefore, I focus on the four questions that received significant attention in this thesis, and of which the answer was pursued using a hypothesis-driven approach: Is CL-tolerance located in the shoot? Which factor or aspect in CL is triggering the injury? Is the light signalling pathway involved in this disorder? And is carbohydrate accumulation inducing or accelerating the development of the injury?

To investigate whether CL-tolerance is functionally located in the root or shoot, grafting was used (Chapter 5). Grafting is an unambiguous tool for diagnosing long-distance transport and action in plant research (Turnbull & Lopez-Cobollo, 2013). Besides, it is also extensively used to alter the scion phenotype in horticultural crops (Mudge *et al.*, 2009). In tomato, for example, grafting on tolerant rootstocks improves plant performance under salt (Albacete *et al.*, 2009), heat (Rivero *et al.*, 2003a, Rivero *et al.*, 2003b) and cold (Venema *et al.*, 2008) stress.

Regarding the second question, is should be considered that plants can not only use light as energy source, but also can extract valuable information regarding light quality, quantity, direction and photoperiod thanks to a set of photoreceptors and the circadian clock (Casal, 2013, Moglich *et al.*, 2010). Additionally, light can also be harmful to plants if provided in excess (Li *et al.*, 2009). Hence, if a non-injurious natural photoperiod is compared with a CL treatment provided by an artificial light source, several factors that differ between both conditions can be identified. Each of these factors is potentially responsible for triggering the injury in CL-grown tomatoes. In short, these factors include (i) differences in the light spectral distribution between sunlight and artificial light, (ii) continuous signalling to the photoreceptors, (iii) constant supply of light for photosynthesis, (iv) constant photo-oxidative pressure, and (v) a mismatch between the internal circadian clock frequency and the external light/dark cycle, a phenomenon known as circadian asynchrony. Concluding which factor induces the injury is not simple because, in most cases, CL treatments affect all factors at the same time. Despite this difficulty, testing which factor(s) is(are) responsible for inducing injury in CL-grown tomato plants is of great value as it can point to the pathway that is upstream of the physiological processes leading to the injury. Chapter 6 is completely devoted to answer this question.

A third important question to be answered with a hypothesis-driven approach was inspired from the report that enrichment of light with far-red reduced the injury symptoms in CL-exposed tomato plants (Globig *et al.*, 1997). Considering that phytochromes are the photoreceptors responsible for perceiving red/far-red light (Bae & Choi, 2008, Chen & Chory, 2011), the hypothesis that phytochrome signaling is involved in CL-induced injury in tomato is highly plausible, yet it has never been tested. In Chapter 7, we test this hypothesis.

Whether carbohydrate accumulation is responsible for inducing the injury in CL-exposed tomato is the fourth important question scrutinized in this thesis using a hypothesis-driven approach. Interestingly, the hypothesis that carbohydrate accumulation is responsible for inducing injury in CL-grown tomato plants has been suggested ever since the discovery of this disorder (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996). Under many conditions, carbohydrate accumulation in plants is associated with down-

regulation of photosynthesis and leaf chlorosis (Baker & Braun, 2007, Baker & Braun, 2008, Cakmak & Kirkby, 2008, Krapp *et al.*, 1991, Stitt, 1991). Hence, testing this hypothesis was a priority, and the results are reported in Chapter 8.

The questions discussed above deal either with the induction of the injury or the physiological process inducing the injury. An additional and important unanswered issue is the nature of the injury itself. Although no chapter in this dissertation is exclusively devoted to answer this question, the issue is discussed in most chapters. Most notably, Chapter 2 proposes three possibilities based on previous studies and Chapter 9 propose a unified model based on the results of the present study.

Crop modelling and ecology

Having in mind the original motivation for the present study — Cultivate a tomato crop under CL to increase yield — in this dissertation we also investigate and discuss the use of CL-tolerant tomato in greenhouse horticulture from a crop ecological perspective. Crop ecology deals with the application of ecological and physiological principles in crop production. Hence, the yield predictions and trial reported in Chapters 3 and 4, respectively, are intended to dissect the underpinning factors resulting in a particular yield outcome. Those factors can be classified into environmental factors, physiological factors and factors related with crop management. In the first category, temperature and light are the most important ones, and although they can be manipulated to some extent in greenhouse horticulture, a limit exists. Photosynthesis and respiration rates, canopy architecture and sink strength, which is the ability of the fruit to attract assimilated sugars, belong to the second category and depend on an interaction between the genotype and the environment. In the third category, fruit and leaf pruning, crop density as well as control of lamps, heaters and vents can greatly influence some of the factors in the two former categories. Hence, the interaction of all these factors ultimately determines tomato yield (Heuvelink & Dorais, 2005). As the change in one factor may affect multiple other factors via feedback and feed forward loops, predicting the outcome of any given alteration goes beyond human intuition. To solve this, crop models can aid in predicting the outcome by taking into account known interactions incorporated in a mechanistic way, *e.g.* Heuvelink (1999). However useful, the use of crop models has its limits as CL might alter several factors, yet the exact effects on each one of them are largely unknown. Hence, extensive crop ecology studies would be needed to collect this information experimentally. Although such a study goes beyond the scope of this dissertation, in Chapter 3 and 4 we present the conceptual framework, an experimental study as well as the genetic resources and tools to pursue this interesting approach.

Thesis outline

The objectives of the present study were to (i) better understand the physiological basis of the CL-induced injuries in tomato, (ii) identify the gene(s) responsible for CL-tolerance in wild tomato species, (iii) supply the knowledge required to breed CL-tolerance into

domesticated tomatoes and (iv) evaluate the effect of CL on greenhouse tomato yield when using a CL-tolerant genotype.

Chapter 1 describes how innovation efforts encounter the unsolved scientific enigma of the injuries that tomato plants develop when exposed to CL. The term *CL-induced injury* is defined, and a detailed description of the symptoms of this disorder given. Additionally, an overview of the studies that most influenced the hypotheses postulated and/or tested in this dissertation is presented. Finally, a description and motivation of the main questions that this dissertation pursued to answer is presented alongside a short description of the strategy chosen to answer them.

Chapter 2 reviews the literature, published over the last 80 years, on CL-induced injury using modern knowledge of plant physiology. By doing so, new hypotheses aiming to explain this disorder are postulated and summarized in addition to the ones collected from the literature. Additionally, we discuss that the requirement to use CL in circadian research could potentially mask processes that normally only occur when plants are exposed to 24-h day/night cycles.

Chapter 3 presents a simulation study with the aim to predict tomato yield if an ideal CL-tolerant genotype is cultivated under CL in greenhouses. After introducing some basics of greenhouse technology and greenhouse energy budgets, we outline the challenges that, in practice, could arise when cultivating greenhouse tomatoes under CL. By using concepts of crop ecology, we discuss the need of adjusting current crop management practices in accordance with the physiological alterations that are expected when exposing a CL-tolerant tomato crop to CL.

Chapter 4 presents the results of our search for the gene responsible for CL tolerance as well as the results from a yield trial using CL-tolerant lines. To achieve that, the CL-tolerance found in wild tomato species is mapped and introgressed into domesticated tomato. Sequence analysis, expression data and silencing experiments point to a single gene as responsible for the tolerance. After breeding the trait into modern F_1 hybrid lines, a yield trial was conducted to study the effect of CL on photosynthesis, development and yield.

Chapter 5 investigates whether CL-tolerance in tomato is systemic or is limited to a particular plant part. By grafting CL-tolerant shoots on CL-sensitive rootstocks, which in turn had their own shoots, we observe whether CL-tolerance is graft-transferable. The results are discussed in context of the several physiological mechanisms of long-distance signaling in plants.

Chapter 6 explores the factors that, according to our hypotheses, might be responsible for inducing the injury observed in CL-exposed tomato. First, using a plasma lamp that mimics the spectral distribution of sunlight, we investigate whether the CL-induced injury arises from the differences between sunlight and artificial light. Secondly, using light emitting diodes (LEDs), several treatments explore whether CL-induced injury in tomato arises from a continuous energy supply for photosynthesis or from continuous signaling to the photoreceptors. Thirdly, we explore whether circadian asynchrony is the factor inducing the CL-induced injury in tomato.

Chapter 7 tests the influence of phytochromes on CL-induced injury in tomato. Tomato mutants lacking functional phytochromes and transgenic lines over-expressing functional phytochromes are exposed to two CL treatments with contrasting far-red content. We find that phytochrome A, B1 and B2 all influence the severity of CL-induced injury, but to different levels and depending on the light spectral distribution. After further analyzing expression data from chapter 4, we show that CL induces a photosynthetic down-regulation in CL-sensitive tomato plants but not in the CL-tolerant line. The potential involvement of light signaling in this down-regulation is discussed.

Chapter 8 explores the role of carbohydrate accumulation in the CL-induced injury. Using metabolomics and targeted analysis of primary metabolites, we found evidence that carbohydrate accumulation correlates with CL-induced injury in tomato. Two hypotheses linking carbohydrate accumulation and CL-induced injury are considered: retrograde-signaling-dependent photosynthetic down-regulation and accelerated senescence. Complementary experiments and further analysis of gene expression data described in chapter 4 are used to evaluate both hypotheses.

Chapter 9 provides a synthesis of the most important findings and proposes a generic model of CL-induced injury in tomato. We also present perspectives on what future directions to take to further elucidate the physiological basis of CL-tolerance and successfully implement it in greenhouse horticulture.


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Plants under continuous light

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Abstract

Continuous light is an essential tool for understanding the plant circadian clock. Additionally, continuous light might increase greenhouse food production. However, using continuous light in research and practice has its challenges. For instance, most of the circadian-clock-oriented experiments were performed under continuous light; consequently, interactions between the circadian clock and the light signaling pathway were overlooked. Furthermore, in some plant species continuous light induces severe injury, which is only poorly understood so far. In this review we aim to combine the current knowledge with a modern conceptual framework. Modern genomic tools and rediscovered continuous-light-tolerant tomato species (*Solanum spp.*) could boost the understanding of the physiology of plants under continuous light.

Continuous light provides a framework for circadian clock and photosynthesis research

Natural day-night cycles expose plants to daily fluctuations in light (photoperiods) and temperature (thermoperiods). Technology, on the other hand, enables us to grow plants under almost constant environmental conditions. Although continuous light (CL) is achievable within a growth chamber, growing plants under CL does not represent the natural situation. Nevertheless, studying plant responses to CL is valuable for fundamental and applied sciences; by growing plants under CL, we can study the circadian clock, understand the photosynthetic machinery better, and increase productivity of photosynthetic organisms.

Here, we (i) discuss the strengths and weaknesses of using CL in circadian research, (ii) review the adverse effects of CL on some plant species and (iii) focus on hypotheses that aim to explain CL-induced injury. By combining previous hypotheses with the current understanding of the circadian machinery, and the light signaling pathways, we propose new hypotheses that aim to explain the poorly understood CL-induced injury.

With future research efforts in mind, we also explore the following three aspects: (i) we highlight points for interpreting and designing experiments using CL, (ii) we consider the scope for increasing plant productivity by using CL, and (iii) we rediscover CL-tolerant genotypes of tomato that have languished for almost half a century of research.

Continuous light helped to unravel the circadian clock machinery

Plants have a competitive advantage when the internal physiological activities match the external day/night cycle (Dodd *et al.*, 2005). During the day/night cycle, photoperiods, thermoperiods and/or the circadian clock differentially regulate large proportions of the genome (Covington *et al.*, 2008, Facella *et al.*, 2008, Michael *et al.*, 2008b). The circadian

clock coordinates plant physiological processes to specific times of the day or night (Graf *et al.*, 2010, Harmer, 2009, Más & Yanovsky, 2009, Michael *et al.*, 2008b, Poire *et al.*, 2010, Pruneda-Paz & Kay, 2010). The circadian clock consists of transcriptional interlocked feedback loops that control downstream targets, gates clock input signals and interacts with other signaling pathways (Harmer, 2009, Más & Yanovsky, 2009, Pruneda-Paz & Kay, 2010). The plant circadian clock is defined by three characteristics; (i) fluctuations in light and temperature set the pace of the clock (Gould *et al.*, 2006, Michael *et al.*, 2008b, Yamashino *et al.*, 2008), (ii) temperature compensation maintains accurate timing over a broad range of physiological temperatures (Gould *et al.*, 2006), and (iii) self-sustained oscillations show a periodicity of ~24h under constant conditions (e.g. CL) (Michael *et al.*, 2008b, Yamashino *et al.*, 2008).

Mutations in clock components cause free-running rhythms that diverge from the ~24h periodicity (Millar *et al.*, 1995). *Arabidopsis* (*Arabidopsis thaliana*) lines carrying a luciferase gene fused to the Cab2 promoter (a circadian-regulated gene) have been mutagenized; monitoring bioluminescence cycling allowed the identification of clock mutants (Millar *et al.*, 1995). CL aids studying the temperature compensation mechanism of the circadian clock. Under CL, characterized clock-mutants maintain accurate rhythms at certain temperatures, but at other temperatures, rhythms show altered amplitude and/or peak levels (Gould *et al.*, 2006). Furthermore, growing plants under CL showed that the circadian clock, rather than physical processes driven by temperature, largely accounts for the observed rhythmic variation in leaf growth of *Arabidopsis* and tobacco (*Nicotiana tabacum*) (Poire *et al.*, 2010). Moreover, CL can rescue the severe developmental defects of some circadian clock (Michael *et al.*, 2008a) and starch-biosynthesis mutants (Smith & Stitt, 2007). In summary, CL is a valuable tool for studying the plant circadian clock itself and clock-controlled processes. However, it is clear that CL does not occur in nature (see below, Future research). Most of the clock-oriented experiments were performed under CL (or continuous darkness), thus interactions between the circadian clock and the light signaling pathway were largely overlooked (Niwa *et al.*, 2009, Nozue *et al.*, 2007). The daily and the photoperiodic responses of hypocotyl elongation are examples of such overlooked interactions.

Under CL, *Arabidopsis* seedlings show a peak in the rate of hypocotyl elongation at the subjective dusk; under short days, however, this peak occurs at dawn (Michael *et al.*, 2008a, Nozue *et al.*, 2007). This "daily response" is light dependent because the hypocotyl elongation is rapid and arrhythmic under continuous darkness (Nozue *et al.*, 2007). Moreover, *Arabidopsis* seedlings show different hypocotyl lengths at different photoperiods; this is called the "photoperiodic (or seasonal) response of hypocotyl elongation" (Niwa *et al.*, 2009). A "coincidence mechanism", which originally was proposed by Nozue *et al.* (2007), explains these daily (Nozue *et al.*, 2007) and photoperiodic phenomena (Niwa *et al.*, 2009). Briefly, at a specific time during a 24h cycle, the clock up-regulates the transcription levels of *PIF4/5*, which positively regulate growth (Niwa *et al.*, 2009, Nozue *et al.*, 2007). In CL and long days, up-regulation of *PIF4/5* transcripts occurs during the day period, but because light-activated phytochrome B (PHYB) represses growth, hypocotyl growth does

not occur (Niwa *et al.*, 2009, Nozue *et al.*, 2007). Phytochrome-induced degradation of several PIFs (including PIF4) to lower steady-state levels has been reported (Monte *et al.*, 2007). In addition, it was proposed that PHYB indirectly represses the abundance of growth-inducing phytohormones transcripts (Michael *et al.*, 2008a). In short days, however, up-regulation of *PIF4/5* transcripts "coincides" with darkness at predawn (Nozue *et al.*, 2007); at this time, additionally, growth-inducing-phytohormone transcripts show peak expression (Michael *et al.*, 2008a). This explains why hypocotyl elongation peaks at dawn (daily response) and is inhibited under CL and long days (photoperiodic response) (Niwa *et al.*, 2009). In summary, CL and long day photoperiods do not have a period of darkness when the clock-gate opens (Niwa *et al.*, 2009), and thus do not allow the natural development of clock-controlled, dark-dependent processes. Therefore, CL should be used carefully in circadian research.

Continuous light induces injury in some species

Some CL-grown photosynthetic organisms show increased productivity (see below, Future research). However, CL also induces negative effects in several plant species. The most visible CL-induced negative effects are leaf chlorosis (Arthur *et al.*, 1930, Cao & Tibbitts, 1991, Cushman & Tibbitts, 1991, Cushman & Tibbitts, 1996, Cushman & Tibbitts, 1998, Cushman *et al.*, 1995, Demers *et al.*, 1998, Gestel *et al.*, 2005, Globig *et al.*, 1997, Hillman, 1956, Murage & Masuda, 1997, Murage *et al.*, 1996, Murage *et al.*, 1997, Pettersen *et al.*, 2010b, Tibbitts *et al.*, 1990, Wheeler & Tibbitts, 1986, Withrow & Withrow, 1949) and necrosis (Cushman & Tibbitts, 1991, Cushman & Tibbitts, 1996, Cushman & Tibbitts, 1998, Cushman *et al.*, 1995, Demers *et al.*, 1998, Hillman, 1956). CL also lowers photosynthetic parameters, including lower photosynthetic capacity at saturating light (Gestel *et al.*, 2005, Pettersen *et al.*, 2010a, Pettersen *et al.*, 2010b), lower quantum yield (Pettersen *et al.*, 2010b), lower maximum rate of Rubisco carboxylation (Gestel *et al.*, 2005, Pettersen *et al.*, 2010a), and lower maximum rate of electron transport (Gestel *et al.*, 2005, Pettersen *et al.*, 2010a, Rowell *et al.*, 1999). The CL-sensitive species include eggplant (*Solanum melongena*) (Murage & Masuda, 1997, Murage *et al.*, 1996, Murage *et al.*, 1997), geranium (*Geranium sp.*) (Arthur *et al.*, 1930), some onion species (*Allium fistulosum*) (Gestel *et al.*, 2005), peanut (*Arachis hypogaea*) (Rowell *et al.*, 1999), some cultivars of potato (*Solanum tuberosum*) (Cao & Tibbitts, 1991, Cushman & Tibbitts, 1991, Cushman & Tibbitts, 1996, Cushman & Tibbitts, 1998, Cushman *et al.*, 1995, Tibbitts *et al.*, 1990, Wheeler & Tibbitts, 1986), tomato (*Solanum lycopersicum*) (Arthur *et al.*, 1930, Cushman & Tibbitts, 1998, Demers *et al.*, 1998, Dorais *et al.*, 1996, Globig *et al.*, 1997, Withrow & Withrow, 1949), and even lichens (*Xanthoria parietina*) (Korhonen & Kallio, 1987) and mosses (*Pleurozium schreberi* and *Ceratodon purpureous*) (Aro & Valanne, 1979). Between species, however, different responses to CL exist (Cushman & Tibbitts, 1998, Dorais & Gosselin, 2002, Dorais *et al.*, 1996, Murage & Masuda, 1997).

Why has CL a positive effect on some species and a negative one in many others? In the past, several explanations have been proposed to explain various aspects of the CL

induced injury. However, a unifying hypothesis has not yet been presented. In the following sections, we will present hypotheses that aim to explain CL-induced injury.

Light intensity, light spectral distribution and air temperature affect CL–induced injury

Several environmental factors affect CL-induced injury (Fig. 2.1a). At a higher light intensity, CL-induced injury is more severe (Aro & Valanne, 1979, Arthur *et al.*, 1930, Korhonen & Kallio, 1987, Murage *et al.*, 1997). Although this is true for some CL-sensitive species (*e.g.* tomato), CL-tolerant *Arabidopsis* seedlings also showed decreasing chlorophyll content at an increasing intensity of CL (Ruckle *et al.*, 2007). This suggests a photo-inhibition component in the CL-induced injury. Light intensity as well as the spectral distribution of CL influences the degree of injury (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997). Different artificial light sources have different potential to injure plants under CL (Hakala *et al.*, 2005). The interactions between the spectral distribution of the different light sources and the CL-induced injury are complex. A higher percentage of blue light in the CL increased the injury (Demers & Gosselin, 2002). Continuous red light alone can also induce injury (Murage *et al.*, 1997), and addition of far-red light reduced the CL-induced injury (Globig *et al.*, 1997). Taking together the evidence, no conclusive links can yet be made between particular wavelengths and injury. The spectral distribution of solar light substantially differs from the spectral distribution of the more damaging artificial light sources (Hogewoning *et al.*, 2010a); interestingly, if solar light, partially or totally (see below, Future research), provides CL, the injury is reduced or even absent, respectively (Arthur *et al.*, 1930, Darrow, 1933, Demers & Gosselin, 2002). Hence the question arises if the CL-induced injury is caused by the continuity of light itself or by an interaction between photoperiod and light spectral distribution?

Higher temperatures also increase CL-induced injury (Withrow & Withrow, 1949). CL caused injury in tomato plants grown at 24°C but not at 12°C (Withrow & Withrow, 1949). Interestingly, diurnal fluctuations in air temperature (thermoperiods) prevent CL-induced injury in eggplant (Murage *et al.*, 1997), lichens (Korhonen & Kallio, 1987), potato (Cao & Tibbitts, 1992, Cushman & Tibbitts, 1991, Cushman *et al.*, 1995, Tibbitts *et al.*, 1990) and tomato (Demers & Gosselin, 2002, Hillman, 1956). However, thermoperiods did not prevent a CL-induced decrease in maximal photosynthesis rate in peanut plants (Rowell *et al.*, 1999). Why do temperature fluctuations prevent the CL-induced injury in some species? Under CL, thermoperiods can synchronize most of the *Arabidopsis* transcripts to the same time of the day as the circadian clock would do (Michael *et al.*, 2008b). This suggests that a circadian clock entrained by thermoperiods could prevent CL-induced injury. An entrained circadian clock is potentially also present in other non-injurious CL treatments. As stated above, using solar light, partially or totally, to achieve CL is less or not injurious, respectively (Arthur *et al.*, 1930, Darrow, 1933, Demers & Gosselin, 2002); these less or non-injurious treatments could train the clock because

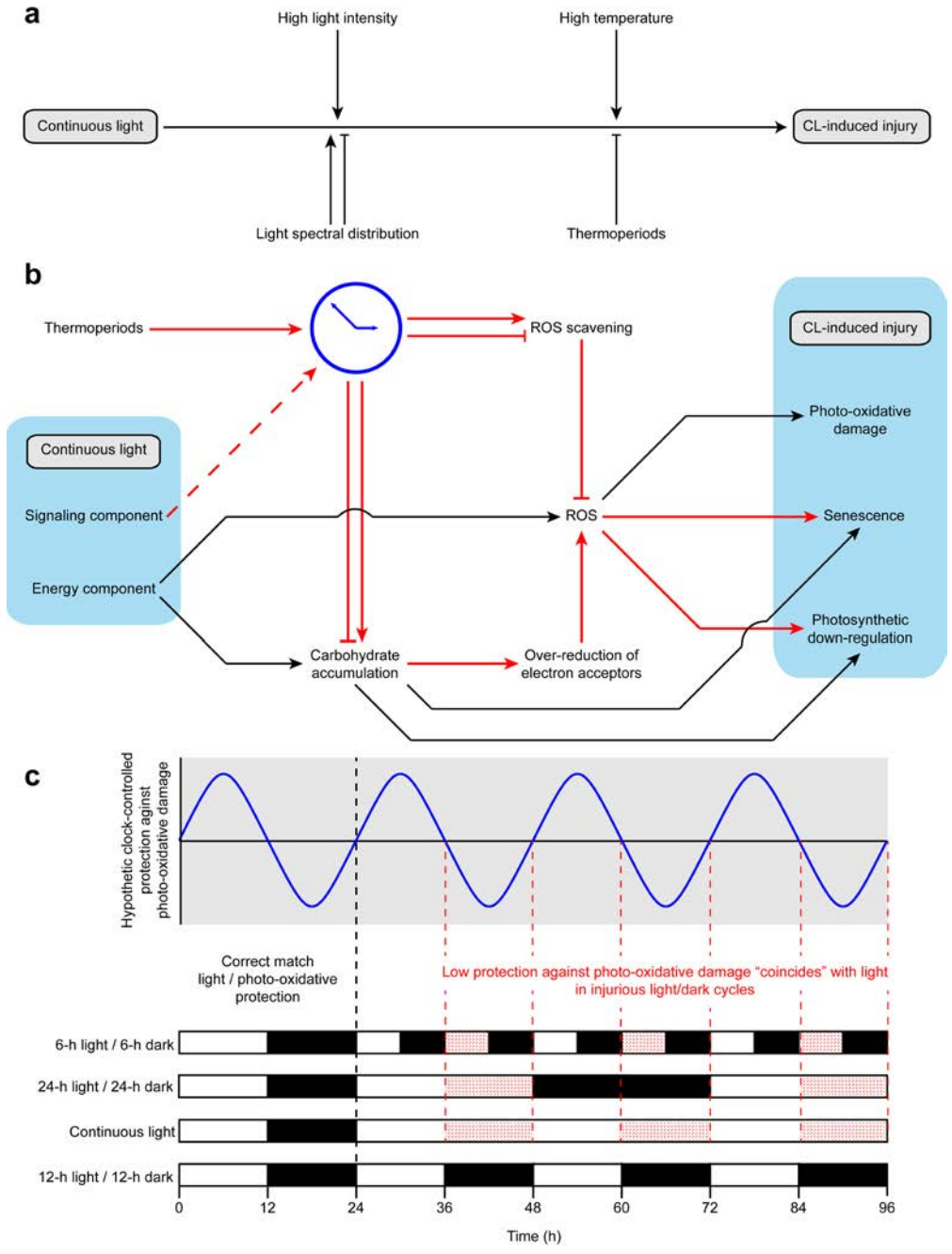

Figure 2.1 | Continuous-light-induced injury. (a) CL injures some plant species (Arthur *et al.*, 1930, Cao & Tibbitts, 1991, Cushman & Tibbitts, 1991, Cushman & Tibbitts, 1996, Cushman & Tibbitts, 1998, Cushman *et al.*, 1995, Demers *et al.*, 1998, Gestel *et al.*, 2005, Globig *et al.*, 1997, Hillman,

1956, Murage & Masuda, 1997, Murage *et al.*, 1996, Murage *et al.*, 1997, Pettersen *et al.*, 2010b, Tibbitts *et al.*, 1990, Wheeler & Tibbitts, 1986, Withrow & Withrow, 1949). At higher temperature (Withrow & Withrow, 1949) and higher light intensity (Aro & Valanne, 1979, Arthur *et al.*, 1930, Korhonen & Kallio, 1987, Murage *et al.*, 1997), the injury is higher. The light spectral distribution influences the degree of injury (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997); that is, some light sources injure plants more than others do. If CL is supplemented by fluctuating air temperature (thermoperiods), some CL-sensitive species do not show CL-induced injury (Cao & Tibbitts, 1992, Cushman & Tibbitts, 1991, Cushman *et al.*, 1995, Demers & Gosselin, 2002, Hillman, 1956, Korhonen & Kallio, 1987, Murage *et al.*, 1997, Tibbitts *et al.*, 1990). (**b**) Hypotheses showing potential mechanism of CL-induced injury. For plants, CL implies continuous energy supply for photosynthesis (energy component) and continuous signaling to the photoreceptors (signaling component) (Jiao *et al.*, 2007, Millenaar *et al.*, 2009, Moglich *et al.*, 2010); both could play a role inducing injury. The CL-induced injury could be photo-oxidative damage, early senescence and/or photosynthetic down-regulation (feedback inhibition). Black arrows depict previous suggested links between CL and CL-induced injury; red arrows show the new links proposed in this paper. Carbohydrate hyper-accumulates in leaves of CL-grown plants (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Gestel *et al.*, 2005). Such hyper-accumulation could induce senescence (Lim *et al.*, 2007) and/or photosynthetic down-regulation (Gestel *et al.*, 2005, Koussevitzky *et al.*, 2007). It has been suggested that CL-induced injury is a sign of accelerated leaf senescence (Cushman & Tibbitts, 1998, Cushman *et al.*, 1995). In the literature, however, the most discussed mechanism of injury under CL is photosynthetic down-regulation triggered by carbohydrate accumulation (Arthur *et al.*, 1930, Demers *et al.*, 1998, Demers & Gosselin, 2002, Dorais & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1996). Alternatively, carbohydrate accumulation could cause over-reduction of electron acceptors; then, the electron transport chain could donate electrons to O_2 generating ROS (Cakmak & Kirkby, 2008). In turn, ROS could down-regulate photosynthesis-associated genes (Moulin *et al.*, 2008), induce programmed cell death (PCD) (the cell death occurring in leaf senescence is a type of PCD (Lim *et al.*, 2007)) ((Danon *et al.*, 2006, Kim *et al.*, 2008, Triantaphylidès & Havaux, 2009) and/or cause oxidative damage (Kim *et al.*, 2008). Continuous signaling to the photoreceptors would not entrain the circadian clock (dashed red arrow). The circadian clock influences, according to the time of the day, carbohydrate metabolism (Graf *et al.*, 2010, Lu *et al.*, 2005, Weise *et al.*, 2006) and ROS scavenging genes (Facella *et al.*, 2008); it is unknown how the circadian clock and clock outputs would behave under long term CL. The relative relevance of each depicted pathway is unknown. (**c**) Circadian asynchrony in the CL-induced injury. If tomato plants are grown under light/dark cycles differing too greatly from 24h periodicity (e.g. 6h light / 6h dark or 24h light / 24h dark), a similar injury as observed under CL is present (Highkin & Hanson, 1954, Hillman, 1956). We propose that this is due to asynchrony between clock-controlled protection against photo-oxidative damage (blue line) and the light period. It is reasonable to assume that protection against photo-oxidative damage fluctuates in response to clock outputs (Facella *et al.*, 2008, Rikin *et al.*, 1993). Therefore, under some injurious photoperiods plants receive light while not properly protected against photo-oxidative damage (red-shaded areas). As a result, injury occurs. Figure 2.1c was modified from (Highkin & Hanson, 1954). See text for more detail.

they imply considerable diurnal changes in light intensity, light spectral distribution and temperature. However, the hypothesis that an entrained circadian system is advantageous under CL is debatable. As discussed before, plants have a competitive advantage when the internal physiological activities match the external day-night cycle; under prolonged CL (more than 48 h), arrhythmic *Arabidopsis* plants had higher CO_2 fixation rate than wild-type plants with an oscillating clock (Dodd *et al.*, 2005). Some transcripts cycle only in response to thermoperiods and not in response to photoperiods or the circadian clock (Michael *et al.*, 2008b); therefore, a circadian-independent explanation of the absence of

CL-induced injury under thermoperiods is still plausible. For instance, a continuous carbon supply might induce the injury (see below).

CL-induced carbon unbalance could down-regulate photosynthesis

As most physiological processes, carbon metabolism is influenced by the diurnal cycle. During the day, plants open their stomata, fix CO_2 and accumulate starch. At night, stomata close, carbon fixation stops and the accumulated starch supports plant metabolism until the next morning (Graf *et al.*, 2010). If all substrates for photosynthesis were supplied continuously, we would expect a continuous CO_2 fixation rate (see below, Future research). However, under short-term exposure to CL, the circadian clock oscillates freely; being under circadian control, stomata open and close according to the clock oscillations. During the phase in which stomata close, the CO_2 supply decreases with a parallel decrease in CO_2 fixation rate (Dodd *et al.*, 2005). Conversely, arrhythmic clock-mutant plants do not show rhythmic stomatal closure under CL; consequently, the CO_2 fixation rate is continuous (Dodd *et al.*, 2005).

CL-grown plants show substantial increase in starch and sugar concentrations (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Gestel *et al.*, 2005). High sugar concentrations could down-regulate photosynthesis (Gestel *et al.*, 2005) and/or induce early senescence (Lim *et al.*, 2007). An inability to export starch breakdown products could induce chlorosis and chloroplast degradation in *Arabidopsis* (Stettler *et al.*, 2009). Similarly, maize (*Zea mays*) *tie-dyed1* mutants, which do not have adequate carbohydrate export, showed a high-light-dependent chlorosis potentially attributable to carbohydrate accumulation (Baker & Braun, 2007, Baker & Braun, 2008, Braun *et al.*, 2006). In CL-grown plants, a continuous sucrose supply could antagonize the synchronization between the shoot- and root-circadian-clock (James *et al.*, 2008). Does this mean that such continuously high carbohydrate accumulation is responsible for the CL-induced injury? Many authors have suggested this (Arthur *et al.*, 1930, Demers *et al.*, 1998, Demers & Gosselin, 2002, Dorais & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1996) (Fig. 2.1b), but contradictory evidence exists (Arthur *et al.*, 1930).

Sugars are synthesized in photosynthetic active leaves (sources) and exported to non-photosynthetic tissues (sinks). A balance between source/sink strengths is crucial for adequate sugar translocation. Under CL, the source strength is higher than normal because CL provides a continuous sugar supply. Apparently, a parallel increase in sink strength (Gestel *et al.*, 2005) and/or sucrose export capacity (Demers & Gosselin, 2002, Dorais & Gosselin, 2002) does not occur under CL. Therefore, the available extra sugar cannot be allocated to sink tissues. Although decreasing the sink strength by pruning fruits did not increase the CL-induced injury (Demers *et al.*, 1998), high sink strength could decrease the injury. When grown under CL, bulb-forming onions (*Allium cepa*) did not show down regulation of photosynthesis, whereas non-bulbing onions (*A. fistulosum*) did (Gestel *et al.*, 2005). Similar conclusions were obtained on potato (Cushman & Tibbitts, 1996). In both cases, the good performance under CL was attributed to high sink strength from the bulbs and tubers, respectively (Cushman & Tibbitts, 1996, Gestel *et al.*, 2005). Although CL-

induced injury positively correlates with carbohydrate hyper-accumulation and negatively correlates with high sink strength, a causal relationship between carbohydrate hyper-accumulation and CL-induced injury is still missing. Instead of down-regulating photosynthesis, high sugar concentrations could trigger senescence in CL-exposed leaves.

Continuous light could accelerate leaf senescence

It has been suggested that CL induced injury is a sign of accelerated leaf senescence (Cushman & Tibbitts, 1998, Cushman *et al.*, 1995). High sugar and ethylene concentrations are potential triggers of the suggested CL-induced leaf senescence (Lim *et al.*, 2007, Wingler *et al.*, 2006). The high sugar concentrations in CL-grown plants suggest a high-sugar-induced senescence as the reason of CL-induced injury. However, the hypothesis of high sugar concentrations triggering leaf senescence is controversial (van Doorn, 2008).

Ethylene plays a role in leaf senescence (Trobacher, 2009), and likely also in CL-induced injury (Cushman & Tibbitts, 1998). Silver thiosulfate, an ethylene inhibitor, reduces CL-induced injury in CL-sensitive potatoes cultivars (Cushman & Tibbitts, 1998). Furthermore, a tomato containing an antisense transgene of ACC-oxidase, an ethylene biosynthesis enzyme, showed no decrease in chlorophyll when exposed to CL (Cushman & Tibbitts, 1998). However, the *Never ripe* tomato mutant, which is ethylene insensitive, was injured by CL as much as the wild-type (Cushman & Tibbitts, 1998). This last observation suggests that ethylene is not required to trigger the CL-induced injury, at least in tomato. Considering the presented evidence, the CL-induced injury might be due to accelerated leaf senescence (Fig. 2.1b), but the evidence is not convincing. Therefore, other hypotheses should be considered.

CL-induced injury could be due to photo-oxidative damage

Several lines of evidence suggest that CL significantly increases photo-oxidative pressure. For instance, compared to tomato, pepper plants show lower CL-induced injury which correlates with higher carotene and xanthophyll content (Demers & Gosselin, 2002). Similarly, compared to eggplant, pepper plants have higher reactive oxygen species (ROS) - detoxifying enzyme activities, correlating with the absence of CL-induced injury (Murage & Masuda, 1997). When grown under CL, tobacco plants showed higher ROS-detoxifying enzyme activity than under diurnal photoperiods (Peter *et al.*, 2010). In *Arabidopsis*, a knockout mutant with reduced content of 2-Cys peroxiredoxin, an antioxidant enzyme, showed lower CO_2 fixation rate under CL (Pulido *et al.*, 2010). Conversely, wild-type plants did not show differences in CO_2 fixation rate between CL and light/dark cycles (Pulido *et al.*, 2010). Similarly, the accumulation of ascorbic acid, an antioxidant, was 171% higher in CL-grown *Arabidopsis* plants than in control plants (Yabuta *et al.*, 2007). Interestingly, chlorosis and necrosis in CL-grown and Mg-deficient tomatoes are strikingly similar. In addition, photo-oxidative damage probably also plays an important role in inducing chlorosis and necrosis in leaves of Mg-deficient plants (Cakmak & Kirkby, 2008).

As in CL-grown plants, Mg-deficient plants show more chlorosis at higher light intensities (Cakmak & Kirkby, 2008) as well as greater sugar accumulation (Hermans & Verbruggen, 2005). Furthermore, Mg-deficiency also alters the expression of circadian clock genes (Hermans *et al.*, 2010a, Hermans *et al.*, 2010b). These observations suggest common disturbed pathways between Mg-deficient and CL-injured plants.

Although Mg is required for chlorophyll synthesis, the plain inability to synthesize functional chlorophyll as the reason of chlorosis in Mg-deficient plants is not sufficient to explain some observations. For instance, after removing Mg from the nutrient solution, chlorosis only developed in light-exposed leaves (Cakmak & Kirkby, 2008). It is suggested that photo-oxidative damage by ROS is responsible for chlorosis and necrosis in Mg-deficient plants (Cakmak & Kirkby, 2008). Enhancement of ROS-detoxification and photo-protection genes in Mg-deficient *Arabidopsis* plants supports this hypothesis (Hermans *et al.*, 2010b). Two possible sources of ROS exist in Mg-deficient plants. First, Mg-deficient plants accumulate carbohydrates (Hermans & Verbruggen, 2005). Such carbohydrate accumulation could cause over-reduction of electron acceptors; under these conditions, the electron transport chain could donate electrons to O_2 generating ROS (Cakmak & Kirkby, 2008). Second, Mg-deficient plants accumulate chlorophyll intermediates (Cakmak & Kirkby, 2008). If present in excess, chlorophyll intermediates can generate ROS (Tanaka & Tanaka, 2006). Remarkably, the first potential ROS source is common between Mg-deficient plants and CL-grown plants. ROS could down-regulate photosynthesis-associated genes (Moulin *et al.*, 2008), induce programmed cell death (PCD) (the cell death occurring in leaf senescence is a type of PCD (Lim *et al.*, 2007)) (Danon *et al.*, 2006, Kim *et al.*, 2008, Triantaphylidès & Havaux, 2009) and/or cause oxidative damage (Kim *et al.*, 2008) (Fig. 2.1b).

CL-induced injury could have a signaling and a photo-damaging component.

We have discussed above the effects of CL on carbohydrate metabolism, and their possible role in inducing injury. It is worth adding that the circadian clock influences starch metabolism (Graf *et al.*, 2010, Lu *et al.*, 2005, Weise *et al.*, 2006) and chlorophyll synthesis (Kato *et al.*, 2007, Matsumoto *et al.*, 2004, Mochizuki *et al.*, 2010). If tomato plants are grown under light/dark cycles differing too greatly from 24h periodicity (e.g. 6h light / 6h dark or 24h light / 24h dark), a similar injury as observed under CL is present (Highkin & Hanson, 1954, Hillman, 1956); this suggests an involvement of the circadian clock. Surprisingly, few papers have linked the circadian clock with the CL-induced injury (Dodd *et al.*, 2005, Highkin & Hanson, 1954). In the last section of this paper, therefore, we propose to integrate the circadian clock, light signaling, and CL-induced injury (Fig. 2.1).

For plants, light has two components, an energy component that drives photosynthesis and a signaling component perceived by several photoreceptors (Jiao *et al.*, 2007, Millenaar *et al.*, 2009, Moglich *et al.*, 2010). CL alters the physiology of plants by

supplying these two components continuously; this makes it difficult to identify the component that is responsible for the CL-induced injury. Some authors concluded that the energy component triggers the injury under CL (Wheeler & Tibbitts, 1986). Here, we propose that both components play a role.

As discussed earlier, the energy component could trigger injury by causing a carbon unbalance or by photo-oxidative damage. Previous papers support these hypotheses. For instance, higher sink strength correlates with lower CL-induced down-regulation of photosynthesis in some onion species (Gestel *et al.*, 2005); additionally, at higher light intensities the CL-induced injury increases (Aro & Valanne, 1979, Arthur *et al.*, 1930, Korhonen & Kallio, 1987, Murage *et al.*, 1997). However, a signaling component might still be crucial in the CL-induced injury. For instance, it is likely that light-labile proteins are effectively not present in CL-grown plants. The clock-controlled PIFs transcription factors (Monte *et al.*, 2007), and the photoreceptors phytochrome A (Bae & Choi, 2008, Mockler *et al.*, 2003) and cryptochrome 2 (Mockler *et al.*, 2003) are examples of light-labile proteins; therefore, their downstream targets should have altered expressions under CL. Nonetheless, the protective effect of thermoperiods (Cao & Tibbitts, 1992, Cushman & Tibbitts, 1991, Cushman *et al.*, 1995, Demers & Gosselin, 2002, Murage *et al.*, 1997, Tibbitts *et al.*, 1990) strongly suggests a role of the circadian clock in the CL-induced injury.

As previously discussed, accumulation of carbohydrates could injure CL-grown plants through ROS production. Alternatively, sugar accumulation could induce a plastid-to-nucleus signal that down-regulates photosynthesis-associated nuclear genes (Koussevitzky *et al.*, 2007). Such plastid signal could "rewire" the light signaling network in such a way that blue light represses photosynthesis-associated genes instead of inducing them (Larkin & Ruckle, 2008, Ruckle *et al.*, 2007). In addition, single high-fluence pulses of blue light destabilized transcripts of the light harvesting chlorophyll a/b binding protein (LHCB) (Folta & Kaufman, 2003). Furthermore, in seedlings with dysfunctional chloroplasts, bright light decreased the expression of *LHCB* (Ruckle *et al.*, 2007). Moreover, singlet oxygen can induce blue-light-dependent PCD (Danon *et al.*, 2006). Thus, the higher CL-induced injury with higher light intensity (Aro & Valanne, 1979, Arthur *et al.*, 1930, Korhonen & Kallio, 1987, Murage *et al.*, 1997) and higher percentage of blue (Demers & Gosselin, 2002) supports the hypothesis of blue-light signaling as crucial component inducing injury to CL-grown plants.

Overall, the evidence suggests that both energy and signaling components play a role in the CL-induced injury. Microarray experiments in tomato revealed that photosynthesis and stress-responsive genes, including ROS-scavenging genes, are up-regulated during daytime and down-regulated at night (Facella *et al.*, 2008). Under CL, cotton (*Gossypium hirsutum*) leaves showed clock-controlled rhythmic changes in chilling resistance (Rikin *et al.*, 1993). Are CL-grown plants subjected to clock-controlled cyclic periods of sensitivity to photo-oxidative damage? If that is the case, CL would photo-damage plants during every subjective night (Fig. 2.1c). Under long-term exposure to CL, circadian fluctuations could (i) damp, *i.e.* their amplitude would diminish over time, or (ii) continue without synchronization between cells. If the circadian fluctuations damp, would

the expression of clock-regulated, ROS-scavenging genes damp completely or would it continue at a constant rate? Increased sensitivity to photo-damage will be the result if the genes expressions damp absolutely. Alternatively, circadian fluctuations could continue under long term exposure to CL. Under constant conditions, circadian fluctuations of mammalian fibroblast cultures appeared to damp at the cell population level; when monitoring individual fibroblasts, however, it was clear that circadian fluctuations did continue for more than a week (Welsh *et al.*, 2004). It is unknown if individual plant cells can maintain circadian fluctuations under long term exposure to CL. Evidence suggest that little or no coupling exist between the circadian clock of cells located within different sections of the same leaf (Thain *et al.*, 2000). Within a single leaf, therefore, circadian clocks of individual cells could continue to cycle independently form each other under long term exposure to CL. If so, the previously described mechanism (Fig. 2.1c) still is a plausible explanation of CL-induced injury; individual cells (or regions) would be damaged independently because each cell/region would be at their subjective night at different times. Therefore, in the CL-induced injury, a signaling component could down-regulate ROS scavenging capacity while an energy component photo-damages the plant.

Future research

Continuous light does not exist in nature

In the strict sense, CL would mean a light with constant intensity and spectral distribution. However, such light does not naturally occur on earth, not even in arctic regions during the summer, because the polar day presents considerable variations in light intensity (Fig. 2.2) and spectral distribution (Krüll, 1976). These spectral fluctuations are enough to set the circadian clock of arctic birds (Krüll, 1976). Although artificial lighting allows us to achieve CL, the spectral distribution of most artificial light sources differs significantly from the solar one (Hogewoning *et al.*, 2010a). Temperature is an additional complication in CL treatments (Arthur, 1936). In natural conditions, temperature fluctuates during the day/night cycle. Setting temperature to a constant value, will affect temperature-dependent plant processes. Furthermore, under constant temperature, CL-grown plants are exposed to higher degree-hours than diurnal-grown plants. Exposure to higher degree-hours could mean a higher developmental rate under CL. However, a fluctuating temperature under CL would set the circadian clock (Gould *et al.*, 2006, Michael *et al.*, 2008b, Yamashino *et al.*, 2008). Thus, CL treatments can result in concomitant temperature and light quality treatments. Therefore, in order to benefit from CL experiments, we should take proper care interpreting past CL-experiments and designing new ones. Fluctuations in light intensity, light spectral distribution, and temperature are the main points requiring attention.

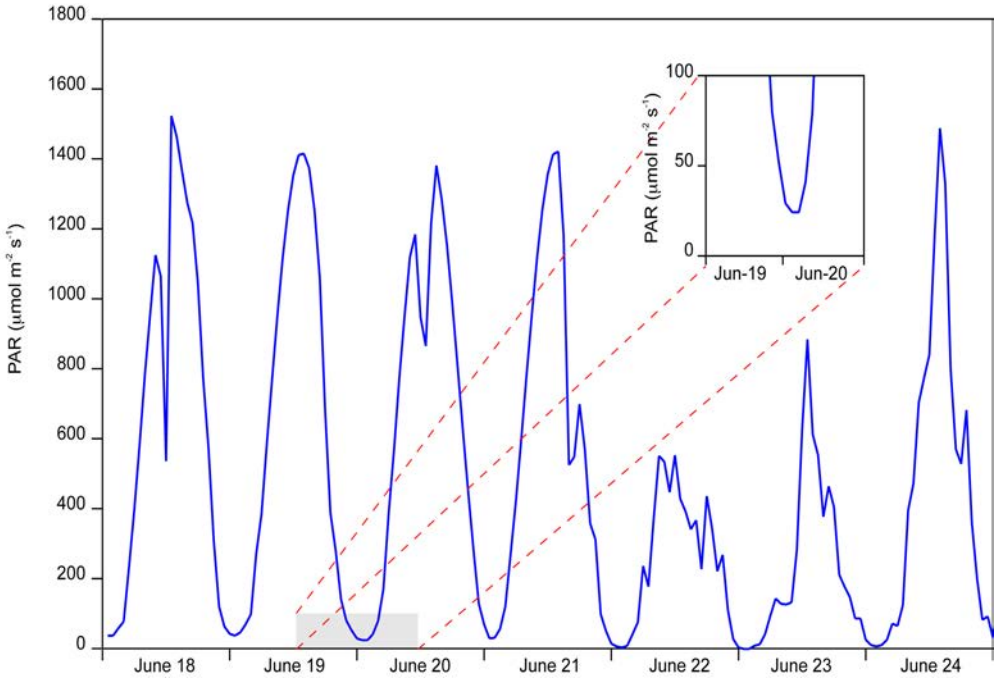

Figure 2.2 | Photosynthetic active radiation (PAR) in the Arctic summer. Data represents hourly PAR average measured around the 2007 summer solstice at the Toolik Field Station from the Institute of Arctic Biology, University of Alaska Fairbanks (68° 38' N, 149° 36' W) (see: http://toolik.alaska.edu/edc/index.php). Graph insert represents a magnification of the gray-shaded rectangle; notice that PAR at midnight is only 25 mmol m^{-2} s^{-2}.

Increasing plant productivity by using continuous light

Potentially, CL is a way to increase plant productivity. Prolonging the photoperiod increases the hours per day that photosynthetic organisms can fix CO_2. Therefore, prolonging the photoperiod towards CL could translate into more biomass (Demers *et al.*, 1998). In several experiments, CL has been shown to increase biomass production. For instance, cultivation under CL enhances to some extent the growth of cyanobacteria (*Cyanothece sp.*) (Min & Sherman, 2010), purple photosynthetic bacteria (*Rhodobacter sphaeroides*) (Eroglu *et al.*, 2010), microalgae (Du *et al.*, 2010), *Arabidopsis* (Handford & Carr, 2007, Lepisto *et al.*, 2009), lettuce (*Lactuca sativa*) (Arthur *et al.*, 1930, Gaudreau *et al.*, 1994), some potato cultivars (*Solanum tuberosum*) (Wheeler & Tibbitts, 1986) and roses (*Rosa x hybrida*) (Pettersen *et al.*, 2007, Suthaparan *et al.*, 2010). Using CL in production systems ultimately depends on the cost-to-benefit ratio. High value products such as horticultural commodities, *e.g.* tomatoes, and secondary metabolites, *e.g.* algae-produced carotenoids, could potentially benefit from cultivation under CL. The advantage of CL would be higher during winter than during summer (Dorais & Gosselin, 2002). For

full benefit, however, we need to understand the mechanism(s) by which photosynthesis is down regulated under CL.

Continuous-light-induced injury in tomato, an old enigma in a modern world

Experiments done in the 1920's discovered that CL injures tomato plants (Guthrie, 1929). Since then, several researchers have tried to unravel the mechanism of such injury (Arthur *et al.*, 1930, Cushman & Tibbitts, 1998, Demers *et al.*, 1998, Dorais *et al.*, 1996, Globig *et al.*, 1997, Withrow & Withrow, 1949). More than 80 years on, a unifying hypothesis has not yet been presented. However, a new opportunity to solve this old enigma exists. First, recent advances in the understanding of the circadian and photosynthetic machineries allow us to formulate new hypotheses; here, we present those hypotheses. Second, by carefully reviewing the literature, we rediscovered that some wild tomato species are tolerant to CL (Daskaloff & Ognjanova, 1965). It was claimed that *Solanum hirsutum* and *Solanum pimpinellifolium* grew well and showed no chlorosis under CL cultivation (Daskaloff & Ognjanova, 1965). Grafting experiments located the CL-tolerance (CLT) trait in the shoot (Daskaloff & Ognjanova, 1965). Interestingly, the CLT trait was inherited in the F_1 as dominant in an *S. lycopersicum* X *S. pimpinellifolium* cross (Daskaloff & Ognjanova, 1965), and the F_2 segregated in a proportion close to 3:1 (Daskaloff & Ognjanova, 1965). Altogether, these results suggest that CLT is a monogenic, dominant trait, functionally located in the shoot. Third, tools like quantitative trait loci (QTL) analysis, molecular marker assisted breeding and gene expression profiling are now available. Science and industry can benefit by combining those three elements. For science, by unraveling the mechanism of the CL-induced injury, our understanding of light and circadian control of plant processes will advance. For the horticultural industry, by breeding and cultivating a CL-tolerant tomato cultivar, greenhouse tomato production could increase substantially. In Northwest Europe and North America, modern glasshouses are ready to implement such an innovative cultivation system.

Conclusion

Using CL has many positive implications for fundamental and applied sciences. For instance, CL was crucial for understanding the plant circadian clock. In addition, studying the physiology of plants under CL helps to increase understanding of the photosynthetic machinery and regulation. Furthermore, CL potentially can increase greenhouse food production. However, using CL in research and practice has both pitfalls and challenges. For instance, most of the clock-oriented experiments were performed under CL; therefore, some possible interactions between the clock and light signaling were not realized. Moreover, CL-induced injury is still poorly understood. Nonetheless, accumulated evidence, a modern conceptual framework, modern genomic tools and the rediscovered CL-tolerant tomatoes will boost our understanding of the physiology of plants under CL. Cloning of

the gene(s) responsible for the CLT trait should be possible in the short term by using introgression lines, QTL analysis and the available genome sequences of *S. lycopersicum* (Mueller *et al.*, 2005) and *S. pimpinellifolium* (D. Ware*, W. R. McCombie and Z. B. Lippman,* Cold Spring Harbor Laboratory). Altogether, great opportunities exist to unravel whether CL induces injury in tomato by photo-damaging the plant and/or by disrupting circadian and light signaling pathways.

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Continuous light as a way to increase greenhouse tomato production: Expected challenges

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Abstract

Tomato plants need six hours of darkness per day for optimal growth; therefore, photosynthesis does not take place for 25% of the day. If tomatoes could be grown under continuous light, a substantial increase in production is expected. In practice, however, continuous light-grown tomato plants develop a potentially lethal mottled chlorosis. Such continuous-light-induced injury is only poorly understood so far. Recently, we proposed a number of hypotheses that aim to explain the continuous-light-induced injury, and rediscovered that wild-tomato species were reported as continuous-light-tolerant. Here, we (i) present a simulation study which shows that if an ideal continuous-light-tolerant tomato genotype is used and no crop adaptations to continuous light are assumed, greenhouse tomato production could be 26% higher when using supplementary lighting for 24 h day^{-1} in comparison with using supplementary lighting only for 18 h day^{-1} during day time, and (ii) discuss expected changes in greenhouse energy budgets and alterations in crop physiological responses that might arise from cultivating tomatoes under continuous light.

Introduction

In principle, cultivating greenhouse crops under continuous light (CL) should increase yield. This is because by prolonging the photoperiod, the hours per day that plants can fix CO_2 increases. This is a proven concept; for instance, cultivation under CL enhances, to some extent, the growth of lettuce (*Lactuca sativa*) (Arthur *et al.*, 1930, Gaudreau *et al.*, 1994), some potato cultivars (*Solanum tuberosum*) (Wheeler & Tibbitts, 1986) and roses (*Rosa x hybrida*) (Pettersen *et al.*, 2007, Suthaparan *et al.*, 2010). Using CL in production systems ultimately depends on the cost-to-benefit ratio. Considering the high cost of supplementary lighting, only high value horticultural commodities like tomatoes (*Solanum lycopersicum*) could potentially benefit from cultivation under CL.

Continuous light, however, induces negative effects in many plant species, reviewed by Velez-Ramirez *et al.* (2011) (Chapter 2). Among all species negatively affected by CL, tomato is particularly sensitive (Arthur *et al.*, 1930, Cushman & Tibbitts, 1998, Demers *et al.*, 1998, Dorais *et al.*, 1996, Globig *et al.*, 1997, Withrow & Withrow, 1949). The most visible CL-induced negative effects in tomato are leaf chlorosis (Arthur *et al.*, 1930, Cushman & Tibbitts, 1998, Demers *et al.*, 1998, Globig *et al.*, 1997, Hillman, 1956, Withrow & Withrow, 1949) and necrosis (Cushman & Tibbitts, 1998, Demers *et al.*, 1998, Hillman, 1956). The physiological reasons of the CL-induced injury in tomato remain unclear. Recently, by combining previous experimental evidence with the current understanding of plant physiology, we proposed a set of hypotheses that aim to explain the CL-induced injury, and re-discovered that wild-tomato species were reported as CL-tolerant more than 45 years ago (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Modern tools like quantitative trait loci analysis, molecular marker assisted breeding and gene expression profiling should allow the breeding of a CL-tolerant tomato. Although a CL-tolerant tomato genotype is an important achievement, it would not guarantee an increase in

greenhouse tomato yield by its own. For that, a better understanding of crop ecology and the mechanism by which CL injures tomato will be also needed. In this paper, we (i) calculate that, for Dutch winter season, CL could potentially increase greenhouse tomato production by 26% when using an ideal CL-tolerant tomato genotype (a genotype showing no detrimental effects of any kind when cultivated under CL), and (ii) discuss the expected challenges, regarding greenhouse technology and crop ecology, in cultivating tomatoes under CL.

Increasing greenhouse tomato production by using continuous light

The potential yield increase that could result from CL was quantified in a simulation study with the tomato crop growth simulation model TOMSIM (Heuvelink, 1999). In these calculations, CL-induced injury and possible physiological and/or morphological crop adaptations to CL are not considered. The model calculates potential production in a pest, disease and weed free environment with ample supply of water and nutrients. The model consists of modules for greenhouse radiation transmission (set at 71% for diffuse radiation), radiation interception by the crop, leaf and canopy photosynthesis, and dry matter production. Maintenance respiration was calculated based on dry mass of 190, 223, 324 and 120 g m^{-2} for leaves, stem, fruit and roots, respectively. In agreement with potential production calculations (Challa & Bakker, 1999), we assumed a constant leaf area index of 3 (90% light interception) and a fixed partitioning to the tomato fruits of 70%; therefore, theoretical maxima were obtained rather than yield predictions. Fruit dry matter content was assumed to be 6.5%. Representative global radiation data for De Bilt, located in the center of the Netherlands, was used as input to the model (Breuer & van de Braak, 1989). Inside temperature was 20°C, and a CO_2 concentration was constantly of 700 μmol mol^{-1}. Two supplementary light intensities (200 or 300 μmol m^{-2} s^{-1}) combined with two durations of supplementary light (18 h day^{-1} or CL) were considered; 18 h day^{-1} light implied a 6 h dark period. Lights were continuously ON during 18 h or 24 h day^{-1}(CL), also when outside light levels were high.

Compared to 18 h day^{-1} supplementary light (200 μmol m^{-2} s^{-1}), CL resulted in a potential yield increase of 22%. The yield increase was even higher (26%) at 300 μmol m^{-2} s^{-1} of supplementary light (Table 3.1). This substantial yield increase agrees very well with experimental data obtained by Dueck *et al.* (2007). These authors applied 162 μmol m^{-2} s^{-1}, between the 9th of December and 5th of April, and observed a yield increase from 13 to 16.7 kg m^{-2} when lights were used 18 h instead of 12 h day^{-1}; these results imply an increase of 3.7 kg m^{-2} as a result of 6 additional hours of supplementary light. Assuming no detrimental effects of CL, therefore, adding another 6 h of light (CL instead of 18 h day^{-1}) would mean a yield increase of another 3.7 kg m^{-2} (20.4 kg m^{-2} in total), which implies a 22% yield increase as found in this study at a similar light intensity (200 μmol m^{-2} s^{-1}).

Using an ideal CL-tolerant tomato genotype, therefore, this study shows that an increase of 26% in tomato production is plausible.

Expected challenges in using continuous light

In the Netherlands by 2005, for instance, the greenhouse industry covered an area of 10,500 ha; about 20% of the greenhouse area was equipped with supplementary lighting. For tomato production, more than 160 ha of greenhouse area were equipped with supplementary lighting (original report in Dutch, cited by Heuvelink *et al.* (2006)). For growing tomatoes under CL, therefore, the infrastructure is already in place. For commercial success of CL-grown tomatoes, however, CL-tolerant cultivars should be bred and the current cultivation practices should most likely be adjusted. Under CL, (i) supplementary lighting, (ii) greenhouse heating and ventilation, and (iii) crop management must be reconsidered. In all cases, plant physiological, crop ecological and greenhouse technological knowledge should guide the adjustments.

Table 3.1. Simulated tomato fresh yield ($kg.m^{-2}$) from the 1st of October till the 1st of April at two intensities of supplementary light and two durations of lighting (photoperiod).

Supplementary light intensity ($\mu mol.m^{-2}.s^{-1}$)	Photoperiod ($h.day^{-1}$)		Yield increase (%)
	18	24	
200	36.0	44.1	22
300	50.8	63.9	26

Continuous supplementary lighting in practice

Regarding supplementary lighting, which combination of light intensity, light spectral distribution and photoperiod is the best one to use? The advantage of supplementary lighting is higher during winter than during summer (Dorais & Gosselin, 2002); hence, should CL only be used during the winter months? Considering that photoperiods longer that 14 to 18 h day^{-1} induce, to a lesser extent, the same symptoms as CL does (Demers *et al.*, 1998, Dorais *et al.*, 1996, Withrow & Withrow, 1949), it is expected that CL-tolerant tomatoes will also grow well under photoperiods longer than 18 h day^{-1} yet shorter than 24 day^{-1} (CL). A photoperiod of, for example, 22 h of light day^{-1} should result in a higher yield since it is significantly longer than the current industry maximum of 18 h day^{-1}, yet a photoperiod of 22 h day^{-1} still gives two hours of darkness to the plants. Then, can higher

yields be achieved by cultivating tomatoes under 22 h day^{-1} without having to make too many adjustments in crop and greenhouse management?

The light spectral distribution influences the degree of CL-induced injury (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997). The light spectral distribution of high-pressure sodium (HPS) lamps, which are already installed in some greenhouses, is more or less fixed. However, the potential implementation of light-emitting diodes (LED) lamps in greenhouses implies the possibility of managing light spectral quality. Such implementation will pose huge challenges since the number of potential lighting regimes will significantly increase. This is simply because LED lighting would allow an independent control of light intensity, photoperiod and spectrum. Having this in mind, testing all the potential lighting regimes would not be feasible. Instead, a fundamental understanding of plant physiology (photosynthesis and photo-morphogenesis) should lead the design of potential light regimes to be used in practice.

Greenhouse heating and ventilation under continuous light

Nowadays, the increasing human population demands higher yields using fewer resources. Land, water, mineral nutrients and energy are becoming ever scarcer. Therefore, any increase in yield should come with, at least, no decrement in resource use efficiencies. In comparison with open-field agriculture, modern greenhouses are already highly efficient using land, water and mineral nutrients, yet their demand of energy is still large. In a greenhouse situated in northern latitudes, energy is mainly used for heating; in some cases, cooling and supplementary lighting are also used, which also implies energy use. At first glance, therefore, it seems that using continuous light in greenhouses would not only increase tomato yield but also energy consumption. However, it is debatable whether or not cultivating greenhouse tomatoes under CL will decrease energy use efficiency (kg of tomatoes produced per Joule consumed).

For a comprehensive explanation on physical principles of greenhouse climate, the reader is referred to Bakker *et al.* (1995). In short, the energy balance of a greenhouse depends on all energy inputs and outputs. In temperate climates, the main energy inputs are shortwave radiation and heating; the main energy outputs include latent heat flow, sensible heat flow and longwave radiation. If the energy outputs are high, then the greenhouse should be heated. At night, the vents are closed; therefore, the latent heat loss is negligible. Consequently, longwave radiation and sensible heat flow are the main energy outputs of the greenhouse at night. If the greenhouse is losing, for instance, 100 W m^{-2}, then the heating system should deliver 100 W m^{-2} as well. Usually, those 100 W m^{-2} would come from pipes containing hot water. If the HPS lamps are ON, however, an extra energy input would exist. The HPS lamps transform electricity into a combination of shortwave radiation, longwave radiation and sensible heat. The proportion of shortwave radiation that is ultimately converted into carbohydrates is very low (Hall *et al.*, 1999); according to crop and greenhouse simulations, only about 2% of the annual energy use (including solar radiation, heating systems and CO_2 enrichment) of a greenhouse ends as carbohydrates (Elings *et al.*, 2005). In practice, therefore, the electricity put into the HPS lamps is effectively an energy

input that will heat the greenhouse. Hence, when using CL in a greenhouse, the amount of W m^{-2} put into the lighting system would reduce, to almost the same extent, the demand of W m^{-2} that the heating system should deliver. This reasoning suggests that the energy demand of a greenhouse would be the same whether the HPS lamps are on or off; though, this reasoning is likely to be true only if there is no crop in the greenhouse. If a tomato crop is cultivated under CL, the energy demand of the greenhouse is difficult to predict since we are not sure how much a tomato crop would transpire under such abnormal growing conditions. Considering that light induces opening of stomata, a tomato crop grown under CL is expected to transpire more during the night; this would increase the relative humidity in the greenhouse during the night. If there is no active dehumidification, ventilation, with the consequent sensible and latent heat losses, would be the only way to reduce the expected higher air relative humidity. Hence, the energy use efficiency of CL-grown tomatoes would depend on (i) the extent of the expected increase in yield, (ii) the extent of the expected increase in transpiration, and (iii) the combination of greenhouse technologies used. The yield and transpiration of CL-grown tomatoes cannot be accurately predicted yet, as the crop models are not calibrated with CL-grown-crop data.

Greenhouse technologies to be used when cultivating tomatoes under CL are worth discussing. Several sources of heat, electricity and CO_2 exist. Each of those sources conveys different costs and operates at different efficiencies. Two common heat sources are the gas boiler, and the combined heat and power (CHP) generator; they deliver heat and CO_2 or heat, CO_2 and electricity, respectively. Usually during the day, in case of using a CHP generator, hot water is stored in a buffer tank, CO_2 is injected into the greenhouse, and electricity is both used to power the lamps and could be sold to the electricity network; during the night, the stored hot water is used to heat the greenhouse. In a greenhouse with a CL-grown crop, CO_2 and electricity would be also needed at night. Therefore, the CHP generator also should work at night. Additionally, the use of HPS lamps during the night could reduce the demand of hot water; therefore, the CHP generator could deliver too much heat. The possibilities of storing and/or selling extra heat and selling extra electricity could be particularly important determinants in the economic success of using CL in greenhouses. Similarly to the heat, CO_2 and electricity sources, the different ways of reducing air relative humidity, *i.e.* by ventilation in conventional greenhouses and active dehumidification in closed and semi-closed greenhouses, conveys different costs and energy efficiencies. Although it requires low investment, dehumidification by ventilation conveys large losses in latent heat, sensible heat and CO_2. In contrary, active dehumidification prevents losses of heat and CO_2 to the environment, but the initial investment is large. In addition to the investment and operational costs, regulations will also influence the greenhouse technologies to be used. In the Netherlands, for instance, regulations require screens to reduce light emission from the greenhouse facades to the environment by 95% between 20:00 and 24:00 hours in order to reduce light pollution (Minister van Volkshuisvesting Ruimtelijke Ordening en Milieubeheer, 2002). When screens are closed, heat and water vapor accumulates within the greenhouse; if CL is used, therefore, the greenhouse should be properly equipped to cope with such extra heat and water vapor that

cannot be easily ventilated to the environment between 20:00 and 24:00. For cultivating tomato under CL, which combination of greenhouse technologies is the best in terms of costs, energy efficiency and reduction in light pollution? Combining greenhouse climate models and crop models, calibrated with the proper data sets, will help in choosing a combination of technology and management that increases tomato yield without decreasing energy use efficiency.

Crop management under continuous light

To our knowledge, there is no ecological study of a tomato crop grown under CL. Therefore, the questions regarding crop management are numerous. Tomato yield is determined by assimilates availability (source strength) and the capacity of the tomato fruits to compete for those assimilates (generative sink strength) with the roots and young leaves (vegetative sink strength); in the long term, though, a good balance between vegetative and reproductive growth ensures maximum partitioning to the fruits without compromising future source strength (young leaves) (Heuvelink & Dorais, 2005). Environmental factors and cultural practices influence source and/or sink strengths; for maximum yield, therefore, an optimal combination of light intensity, CO_2 concentration, air relative humidity, water availability, leaf and fruit pruning, plant density and temperature is needed. Under CL, most likely, some of these factors will need adjustments from current optimal settings. CL will provide plants with extra assimilates at night (higher source strength); can the fruits import those extra assimilates, or should the generative sink strength be increased in CL-grown tomato crop? If so, increasing fruit load is a way to increase generative sink strength. If air temperature, air speed, water vapor pressure deficit and stomatal conductance are kept constant, plant temperature will be higher when the HPS lamps are on. If the thermal time concept holds under CL, faster development is expected in CL-grown tomatoes since the plant temperature would be higher during night. A consequence of faster development is higher leaf and truss appearance rates, which implies a shorter growing period for each fruit; this would result in lower average fruit weight. Hence, should the air temperature set point during the night be lower in order to compensate for higher thermal sum in a CL-grown tomato crop?

Diurnal fluctuations in air temperature (thermoperiods) prevent CL-induced injury in tomato plants (Demers & Gosselin, 2002, Hillman, 1956, Ohyama *et al.*, 2005). In principle, therefore, thermoperiods could be used to cultivate CL-sensitive tomato genotypes under CL; nonetheless, it is yet to be proven whether or not a CL-grown tomato crop would have a higher yield, in comparison with a photoperiod of 18 h day^{-1}, when using thermoperiods. As reported by Hillman (1956), fluctuating temperature from 26°C to 17°C prevented CL-induced injury; while thermoperiods of 26/20°C did not prevented injury in CL-grown tomato plants. Apparently, a difference of at least 9°C is needed to prevent CL-induced injury in tomato. The optimum temperature for cultivating a greenhouse tomato crop, at the productive stage, is between 19 and 22°C (Peet & Welles, 2005). Such temperature is high enough to achieve almost maximum photosynthesis, yet it is low enough to reduce as much as possible maintenance respiration, which consumes

assimilates without yielding tomatoes. Therefore, the thermoperiods previously found to protect tomato plants from CL-induced injury are too warm. In CL-sensitive potatoes, however, thermoperiods of 14/22°C prevented CL-induced injury (Cushman & Tibbitts, 1991, Tibbitts *et al.*, 1990). Hence, is reasonable to expect that thermoperiods of 20/11°C would prevent CL-induced injury in tomato plants; to our knowledge, nonetheless, there is no report of CL-grown tomato plants with thermoperods of 20/11°C. Even assuming that thermoperiods of 20/11°C prevent CL-induced injury in tomato, cultivating a tomato crop under CL using thermoperiods would not be extent of potential disadvantages. For instance, low temperatures (around 10°C) reduce specific leaf area (cm^2 leaf g $leaf^{-1}$), might cause split trusses and malformed flowers, reduce crop photosynthesis, reduce sink strength and might inhibit fruit set because of low pollen viability (Heuvelink & Dorais, 2005). In practice, therefore, would thermoperiods allow a higher tomato production of a CL-grown crop in comparison with current standard practices, while keeping economic and environmental costs low?

The questions continue; for instance, from which developmental stage can growers apply CL in the cultivation? In tomato, CL reduce leaf expansion (unpublished data); this will result in changes in crop canopy and, consequently, in light interception. Smaller leaves will allow deeper light penetration in the crop canopy, which could have a positive effect on overall crop photosynthesis; in order to prevent light reaching the ground, however, more leaves to get the same leaf area index could be needed. Potential effects of CL over tomato quality should not be overlooked. For instance, CL-grown peppers have increased capsaicin levels (Murakami *et al.*, 2006a); will CL change the quality of the produced tomatoes? Answering these questions by detailed crop ecology studies on CL-grown tomato is crucial.

Conclusion

Using modern breeding techniques and wild-tomato species, as a source of CL-tolerance, the breeding of a CL-tolerant tomato genotype is plausible. According to crop model simulations, a 26% increase in tomato yield could be achieved assuming no crop adaptations to CL. These results, however, only show the potential of cultivating an ideal CL-tolerant tomato genotype under CL. In practice, it is yet to prove that the CL-tolerance from wild-tomato species is enough to breed an ideal CL-tolerant tomato genotype. Additionally, the simulation study performed here did not considered physiological and/or morphological adaptations to CL, which are likely to occur. To achieve the potential yield increase by using CL, therefore, a detailed study on a CL-tolerant tomato crop is needed; the physiology of the plants, the ecology of the crop and energy-consumption of the greenhouse should be closely monitored. This knowledge is needed to guide the development of crop and greenhouse management techniques for cultivating CL-tolerant tomatoes under CL.

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A single locus confers tolerance to continuous light and allows substantial yield increase in tomato

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Abstract

An important constraint for plant biomass production is the natural day-length. Artificial light allows for longer photoperiods, but tomato plants develop a detrimental leaf injury when grown under continuous light — a still poorly understood phenomenon discovered in the 1920's. Here, we report a dominant locus on chromosome 7 of wild tomato species that confers continuous-light-tolerance. Genetic evidence, RNAseq data, silencing experiments and sequence analysis all point to the *type III Light-Harvesting Chlorophyll a/b Binding protein 13* (*CAB-13*) gene as a major factor responsible for the tolerance. In *Arabidopsis thaliana*, this protein is thought to have a regulatory role balancing light harvesting by photosystems I and II. Introgressing the tolerance into modern tomato hybrid lines, results in up to 20% yield increase, showing that limitations for crop productivity, caused by the adaptation of plants to the terrestrial 24-h day-night cycle, can be overcome.

Introduction

Experiments in the 1920's revealed that tomato (*Solanum lycopersicum*) plants, when grown under continuous light (CL), develop a potentially lethal injury characterized by mottled leaf chlorosis and necrosis (Arthur *et al.*, 1930). However, many other plant species, like the model plant Arabidopsis (*Arabidopsis thaliana*) (Handford & Carr, 2007, Lepisto *et al.*, 2009), pepper (*Capsicum annuum*) (Dorais *et al.*, 1995, Dorais *et al.*, 1996, Masaharu *et al.*, 2004, Murage & Masuda, 1997, Murakami *et al.*, 2006b), lettuce (*Lactuca sativa*) (Arthur *et al.*, 1930, Gaudreau *et al.*, 1994) and rose (*Rosa x hybrida*) (Mortensen & Gislerod, 2011, Pettersen *et al.*, 2007) are not injured by CL. Hence, the high sensitivity to CL of tomato, an emerging model organism for the Solanaceae family and fleshy-fruited plants (Kimura & Sinha, 2008), is intriguing and has motivated plenty of research efforts (see Supplementary Table 4.1 for complete literature list and for reviews see (Demers & Gosselin, 2002, Sysoeva *et al.*, 2010, Velez-Ramirez *et al.*, 2011 (Chapter 2))). More than eight decades after the original discovery, however, the physiological basis of this CL-induced injury remains poorly understood.

In contrast to natural day/night cycles, CL implies continuous energy supply for photosynthesis, continuous photo-oxidative pressure, continuous signaling to the photoreceptors, and a mismatch between the internal circadian clock frequency and the external light/dark cycle known as circadian asynchrony (Velez-Ramirez *et al.*, 2011 (Chapter 2)). The importance of these four components on the CL-induced injury has been previously investigated in several experimental setups. For instance, carbohydrate accumulation, which presumably results from continuous energy supply for photosynthesis, has been regarded for a long time as a potential trigger of CL-induced injury (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)). The photoinhibition and adaptation of photosystems I and II (PSI and PSII) have been studied in CL-grown tomatoes by Dorais *et al.* (1995). Addition of far-red light to the CL treatment reduced injury symptoms (Globig *et al.*, 1997), suggesting an involvement of

phytochromes. Finally, the role of circadian asynchrony on the CL-induced injury has been examined and/or suggested in several papers (Dodd *et al.*, 2005, Highkin & Hanson, 1954, Hillman, 1956, Kristoffersen, 1963, Velez-Ramirez *et al.*, 2011 (Chapter 2)). However, concluding which component induces the injury is not simple because, in most cases, CL affects all four components simultaneously. We have proposed that a combination of these components could induce the injury rather than a particular one on its own (Velez-Ramirez *et al.*, 2011 (Chapter 2)).

Understanding why CL injures tomato plants is not only valuable for fundamental research but also has potential applications, as tomato is the most important vegetable crop worldwide. Under greenhouse conditions, a tomato crop simulation model, TOMSIM (Heuvelink, 1999), predicted that a hypothetical CL-tolerant tomato genotype, would yield between 22 and 26% more fruits when using supplementary lighting for 24 h day^{-1} (CL) in comparison with using supplementary lighting only for 18 h day^{-1} during day time under Dutch greenhouse conditions (Velez-Ramirez *et al.*, 2012 (Chapter 3)).

In this study, we locate CL-tolerance, mapping to the lower arm of chromosome 7, in eight wild tomato accessions. Genetic evidence, RNAseq data, silencing experiments and sequence analysis all point to the *type III Light Harvesting Chlorophyll a/b Binding protein 13* (*CAB-13*) gene as a major factor responsible for the tolerance. By introgressing the tolerance into modern tomato F_1 hybrid lines, we achieve up to a 20% yield increase. This not only proves that limitations for crop productivity, caused by the adaptation of plants to the terrestrial 24-h day/night cycle, can be overcome but also opens new research lines in the most important process of photosynthesis.

Results

Continuous light tolerance across the Solanum genus

Looking for CL-tolerance, we exposed nine wild tomato accessions to CL. All but one wild tomato accessions (*Solanum pimpinellifolium* LA1589) were CL-tolerant (Supplementary Table 4.1). We also tested several *S. lycopersicum* genotypes including reference lines (Heinz, Moneyberg, Moneymaker and M82), a modern inbred line (A131), F_1 hybrid cultivars (*e.g.* Encore, Tourance and Westland), rootstocks (*e.g.* Maxifort) and an heirloom variety (Sub-Arctic Plenty); all genotypes but one (Sub-Arctic Plenty) were CL-sensitive (Supplementary Table 4.1). From literature, we collected phenotypic data of additional wild tomato species, inter-species hybrids, modern F_1 hybrid tomato cultivars, heirloom tomato varieties and related species like potato (*Solanum tuberosum*) and eggplant (*Solanum melongena*). The occurrence of CL-tolerance and -sensitivity across the Solanum genus facilitates physiological and genetic studies on this trait.

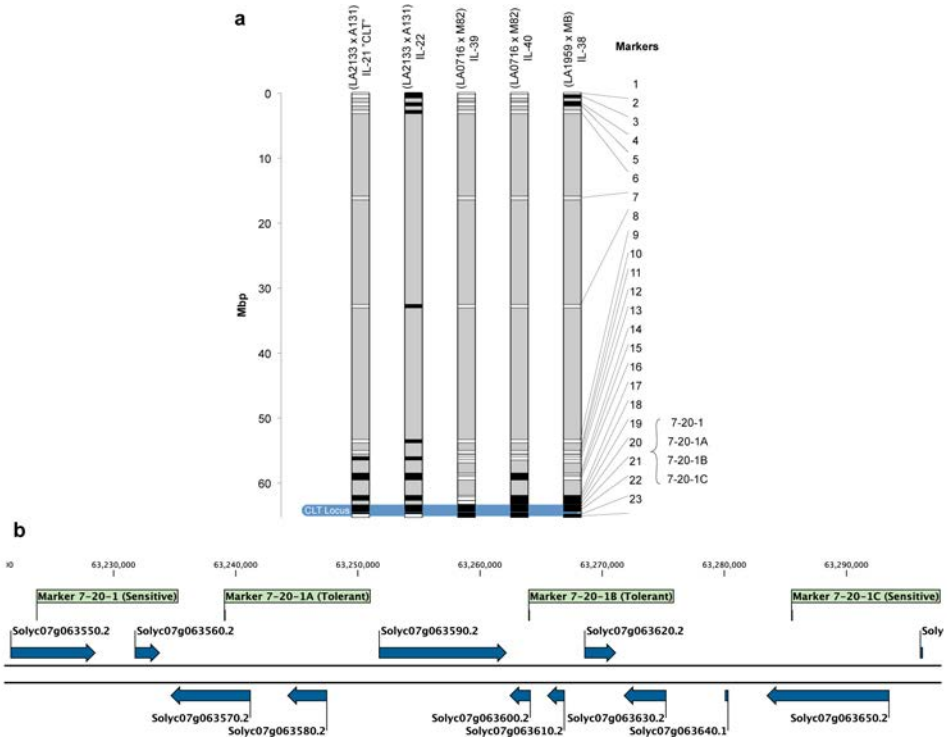

Figure 4.1 | Chromosomal localization of continuous light tolerance. (**a**) The continuous light tolerance locus is located in the lower arm of chromosome seven (grey columns). Based on the public tomato reference genome v2.40 (Tomato Genome Consortium, 2012), single nucleotide polymorphism markers positions are in Mpb. White horizontal bars represent *Solanum lycopersicum* allele, and black bars represent non-*S. lycopersicum* allele in several continuous-light-tolerant introgression lines (IL) (see accession number of each parent at the top). All alleles are homozygous for either parent. According to 361 additional markers, the other eleven chromosomes are homozygous for *S. lycopersicum* (with the exception of two markers in some lines; these exceptions have no relevance for the CL-tolerance localization). All non-*S. lycopersicum* parents are continuous-light-tolerant; hence, the continuous-light-tolerant (CLT) locus was initially mapped between markers 19 and 22 (1.2 Mbp) and represented by an horizontal blue bar. From the LA2133 x A131 population, IL-21 is named "CLT" and used for further research in this study. (**b**) After fine mapping continuous light tolerance in "CLT" F_3 families, the locus was further located between markers 7-20-1A and 7-20-1B, which segregated as tolerant in the F_3. Markers 7-20-1 and 7-20-1C segregated as sensitive in the F_3. The *CAB-13* gene (Solyc07g063600.2) contains the marker 7-20-1B, which is associated with CL-tolerance, and its role in the continuous light tolerance was further tested in this study.

Continuous light tolerance is a dominant trait

We used 96 introgression lines from three populations, genotyped with 384 single nucleotide polymorphism (SNP) markers, to map the CL-tolerance. All lines carrying an introgression in the lower arm of chromosome 7, derived from a CL-tolerant wild donor,

were CL-tolerant (Supplementary Table 4.1). CL-tolerance was initially mapped between SNP markers 19 and 22 (Fig. 4.1a). To investigate the inheritance of the trait, CL-tolerant introgression lines 21 (here after named "CLT") and 22, both from the LA2133 x A131 population, were crossed with their recurrent CL-sensitive parent (A131). F_1 progeny were all CL-tolerant, indicating that the trait is dominant. F_1 plants were self-fertilized to produce an F_2 generation; when 769 plants were exposed to CL, they segregated 2.95:1.05 tolerant:sensitive (568 tolerant:201 sensitive, X^2 [3:1] = 0.53; P=0.47). This strongly suggests that CL-tolerance is a monogenic, dominant trait. Using F_2 plants with a recombination between markers 19 and 22, 18 F_3 families were generated. After genotyping with additional SNP markers, the plants were exposed to CL, and the CL-tolerance locus was fine-mapped between markers 7-20-1A and 7-20-1B. The physical distance between these markers is ~25kb, and only 4 genes are located between them; when identifying the gene responsible for the CL-tolerance, additional genes in the vicinity were considered (Fig. 4.1b).

After pooling publicly available tomato and wild tomato genome sequences (150 tomato genome consortium, 2013, Tomato Genome Consortium, 2012) and RNAseq-derived transcriptomes (details below), we compared the sequence of all genes in the CL-tolerance locus vicinity belonging to 5 CL-tolerant and 5 CL-sensitive genotypes (Supplementary Table 4.2). Additionally, Supplementary Table 4.3 shows the expression of all genes in the CL-tolerance locus evaluated with RNAseq. From all genes in the locus, the *type III Light harvesting chlorophyll a/b binding protein 13* gene (*LHCB type III CAB-13* or *CAB-13, Solyc07g063600.2*) is the most likely candidate to confer tolerance to CL as it was down regulated when exposed to CL in A131 plants, a 9-base deletion was detected in the promoter region of all CL-sensitive lines and it is known to be involved in light harvesting for photosynthesis (Supplementary Tables 4.2 and 4.3).

CAB-13 silencing impairs continuous light tolerance

To confirm that CAB-13 is responsible for the CL-tolerance in tomato, we silenced its expression in a CL-tolerant line (CLT) using virus-induced gene silencing (VIGS). The chlorophyll fluorescence parameter F_v/F_m, which is widely used to assess biotic and abiotic stress (Baker, 2008), was selected to quantify the level of CL-induced injury. The parameter F_v/F_m represents the maximum quantum efficiency of PSII; therefore the lower the F_v/F_m value, the higher the injury is. The tomato *CAB-13* gene consists of three exons; a unique region from exon 3 was cloned into the VIGS vector *pTRV* (Liu *et al.*, 2002a, Liu *et al.*, 2002b) (Fig. 4.2a). The *pTRV::PDS* (Liu *et al.*, 2002a) and *pTRV::GUS* (Tameling & Baulcombe, 2007) constructs were used as positive and negative controls, respectively.

Regardless of the tomato genotype, all *pTRV::PDS*-infiltrated plants showed photo-bleaching symptoms (Supplementary Fig. 4.1), indicating that pTRV infection efficiency was close to, or even at, 100%. As expected (Liu *et al.*, 2002a), the occurrence of photo-bleached spots within these plants was patchy (Supplementary Fig. 4.1a). Therefore, the RT-qPCR data of all pTRV-infected plants (Supplementary Fig. 4.1b) should be taken with caution as, unlike chlorophyll fluorescence imaging, only a small leaf area was sampled.

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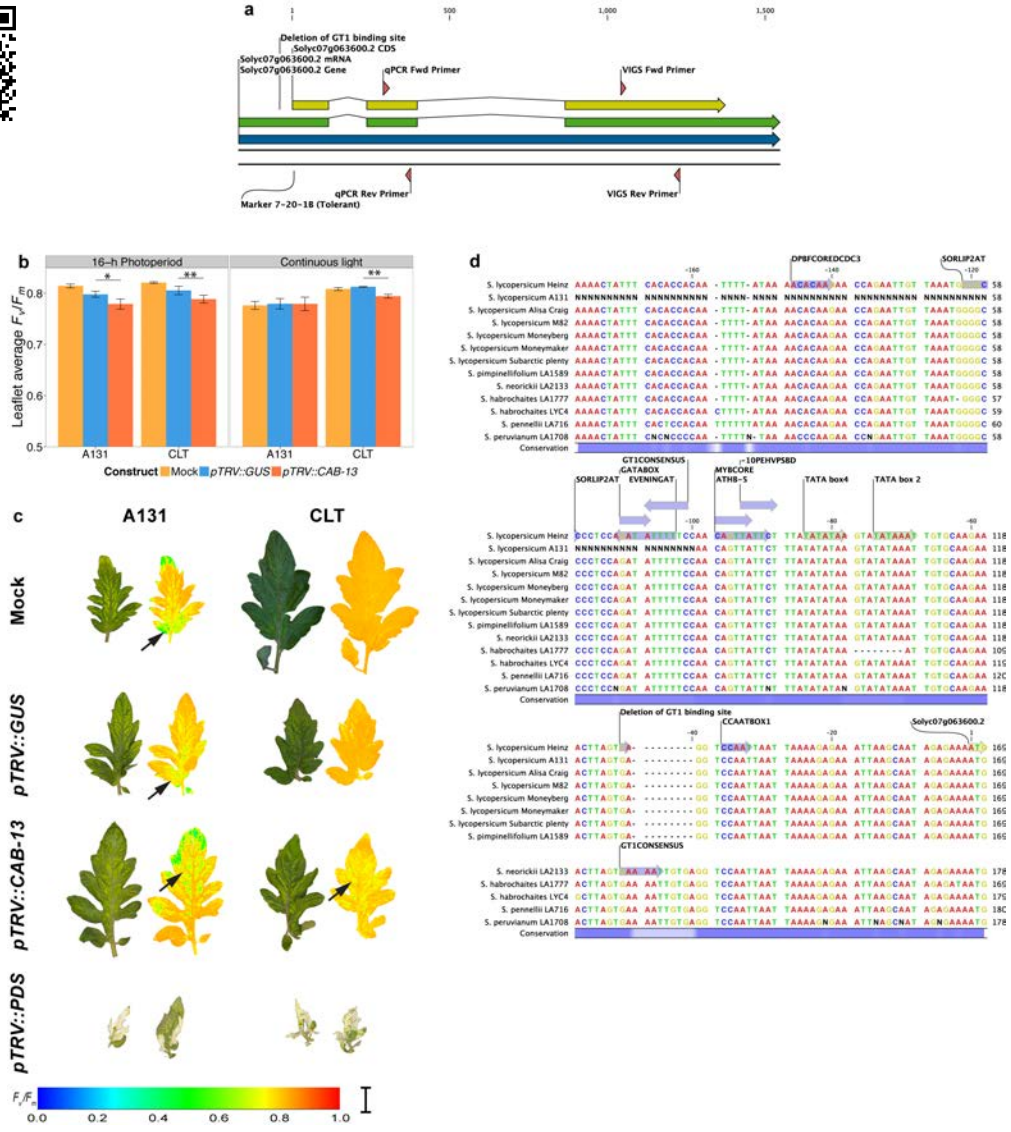

Figure 4.2 | *CAB-13* is involved in continuous light tolerance in tomato. (**a**) Structure of the tomato *CAB-13* gene on chromosome 7, consisting of three exons. The mRNA is depicted with green arrows and the coding region with yellow arrows. Red arrows indicate the primers used for VIGS and RT-qPCR. A molecular marker associated with continuous light tolerance is also indicated. (**b**) Maximum efficiency of PSII (F_v/F_m) of A131 and CLT plants treated with several *pTRV* constructs before (left panel, 16-h photoperiod) and after exposure to continuous light (right panel, continuous light). Values represent mean ± s.e.m., n=4 plants. Asterisks represent statistical difference between *pTRV::GUS* and *pTRV::CAB-13* (other contrasts are not presented); * P<0.05, ** P<0.01, Fisher's protected LSD test. Scale bar = 2 cm. (**c**) Continuous light induced injury in Mock, *pTRV::GUS*, *pTRV::CAB-13* and *pTRV::PDS* treated A131 and CLT plants as seen by RGB images (left) and

chlorophyll fluorescence images (right). Notice the characteristic mottled chlorosis induced by continuous light (indicated with arrows). (**d**) Promoter region of *CAB-13* of several continuous light-sensitive and -tolerant lines. Predicted regulatory elements are highlighted with blue and green arrows (see supplementary Table 4.4 for the complete list). Notice the 9 pb deletion of GT1 binding site shared by all continuous light sensitive lines (with the exception of Sub-Arctic Plenty).

Under 16-h photoperiod, CLT and control A131 plants infiltrated with *pTRV::CAB-13* showed slightly lower F_v/F_m than control plants infiltrated with *pTRV:GUS* (Fig. 4.2b), yet no chlorosis was observed. After three weeks of CL, the F_v/F_m was low in all A131 plants regardless the VIGS construct, but CLT plants kept a high F_v/F_m with the exception of *pTRV::CAB-13* infiltrated plants (Fig. 4.2b). In addition to the low F_v/F_m, these plants showed interveinal chlorosis characteristic of the CL-induced injury (Fig. 4.2c). Considering that (i) the leaf area imaged with the chlorophyll fluorescence camera was much larger than the leaf area harvested for qPCR and that (ii) the appearance (at the expected time and in the expected leaves) of the characteristic CL-induced interveinal chlorosis in CL-tolerant plants after exposure to CL unequivocally indicates that the tolerance has been broken, the results show that *CAB-13* plays a key role in the CL-tolerance in tomato.

A deletion in *CAB-13* is present in all sensitive lines

To further investigate the role of *CAB-13* in the CL-tolerance, the PlantPAN (Chang *et al.*, 2008) tool was used to identify regulatory elements in the promoter region. Among others, tomato CAB-13 promoter contains putative regulatory elements associated to light-responsive, photosynthetic and circadian clock-controlled genes like GT-1 consensus (Terzaghi & Cashmore, 1995), SORLIP2AT (Hudson & Quail, 2003), -10PEHVPSBD (Thum *et al.*, 2001), GATA box (Lam & Chua, 1989) and the EVENINGAT (Harmer *et al.*, 2000); all identified putative regulatory elements are summarized in Supplementary Table 4.4. Remarkably, one of these regulatory elements, the second copy of GT1 consensus, is deleted in all CL-sensitive lines (Fig. 4.2d), suggesting that GT-1 transcription factor is involved in the CL-tolerance/sensitivity in tomato.

Interestingly, Sub-Arctic Plenty cultivar also has a deletion of GT-1 consensus, comparable to sensitive cultivars (Fig. 4.2d), yet they are CL-tolerant (Supplementary Table 4.1). We were not able to find in literature a described cross with a CL-tolerant wild tomato in its lineage (Harris, 1972, Kemp, 1961). Therefore, a *CAB-13*-independent CL-tolerance in Sub-Arctic Plenty cannot be ruled out. Considering that Sub-Arctic Plenty tomato cultivar was bred in the most northern agricultural research establishment in Canada (The Beaverlodge Research Farm, http://www4.agr.gc.ca) (Harris, 1972), an unintended selection for CL-tolerance, driven by long summer days, is a possibility.

Disruption of carbohydrate metabolism and photosynthesis

To identify metabolic pathways altered by CL in uninjured leaves, we performed gene expression analysis and gene ontology (GO) enrichment analysis on RNAseq-derived data.

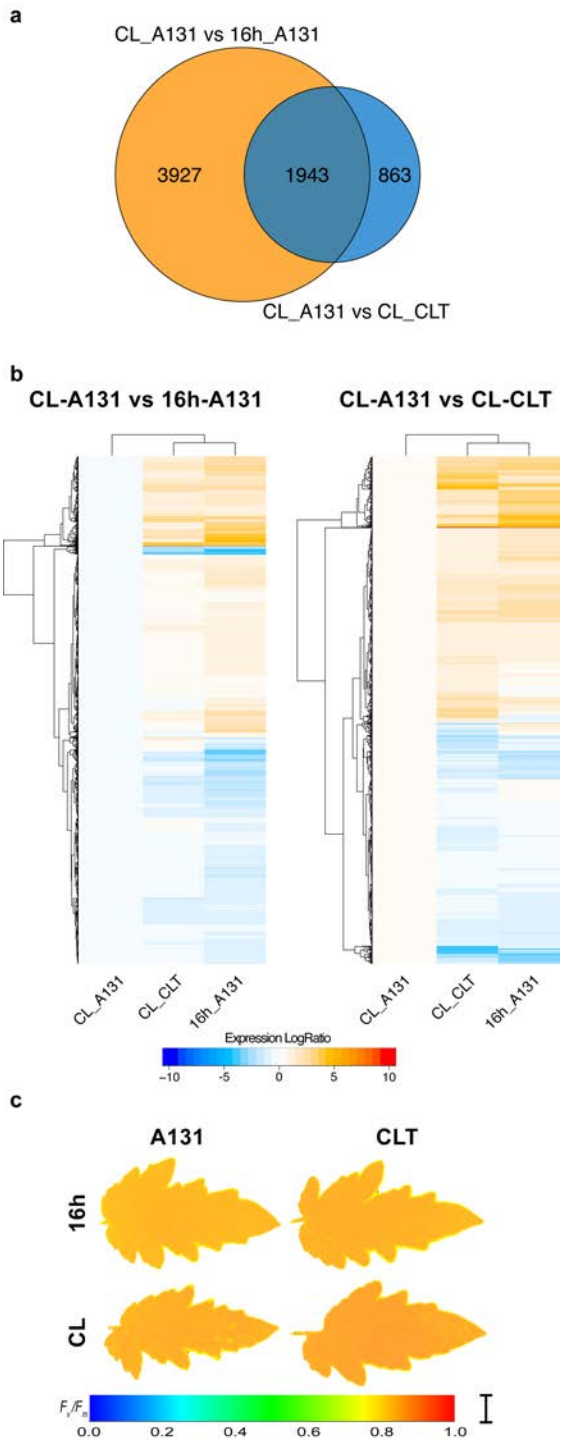

Figure 4.3 | RNAseq analysis. (**a**) Venn diagram showing the number of differentially regulated genes between A131 exposed to 16-h photoperiod and continuous light (left, orange) and between A131 and CLT exposed to continuous light (right, blue). (**b**) Hierarchical clustering of differentially regulated genes. A single colored horizontal line represents each gene. The colors represent the relative expression level of that gene, n=3. (**c**) Chlorophyll fluorescence images of representative leaves used in the experiment. Scale bar = 2 cm.

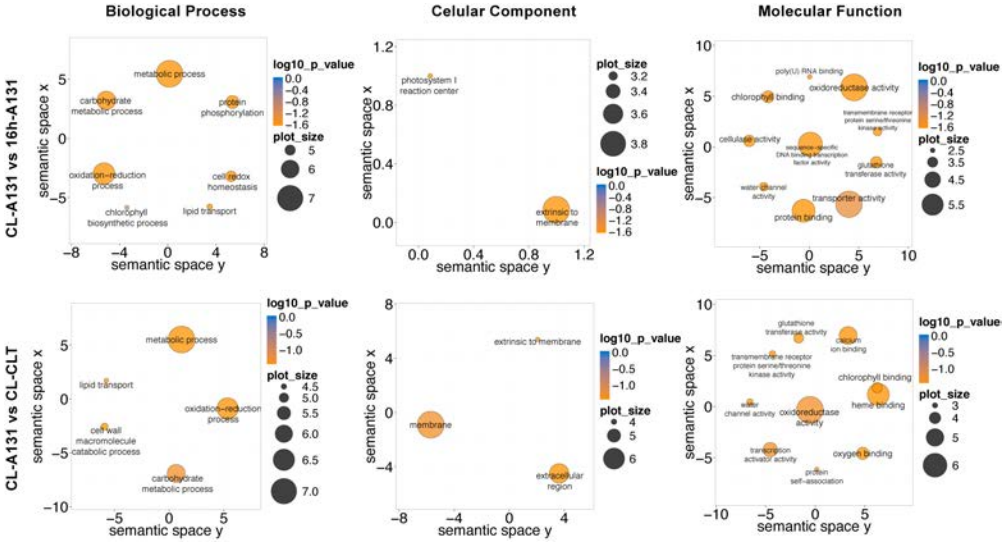

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Figure 4.4 | Gene Ontology enrichment analysis. Gene ontology (GO) terms significantly enriched for two contrast (see labels at the left) and three GO categories (see labels at the top). Each circle represents a GO term. Within the Cartesian coordinates (x,y), the closer the circles rest, the more related the GO terms are. The size of the circles is proportional to the number of child GO terms. The circle color represents the significance of the enrichment.

First, we identified differentially expressed genes in the contrasts CL A131 vs 16h A131 and CL A131 vs CL CLT (Fig. 4.3 and Supplementary Tables 4.5 and 4.6). The first contrast evaluates the effect of CL in A131 plants, and the second one evaluates the genotype effect under CL. From the 31350 genes that passed quality control, we found 5870 and 2806 differentially expressed genes in response to CL and genotype, respectively (Fig. 4.3a); Figure 4.3b shows cluster analyses for each contrast. The number of genes differentially regulated in both contrasts was 1943; hence the differentially regulated genes that responded exclusively to CL or genotype was 3927 and 863, respectively. In the GO enrichment analysis, 19 and 17 GO terms were found significantly enriched for each contrast. Data are summarized in Fig. 4.4; the closer two given terms lie in the semantic space (Supek *et al.*, 2011) (x and y coordinates), the more similar they are. Additionally, the size of each GO term indicates how general it is, and the color indicates the significance of enrichment (Fig. 4.4). Among others, the GO terms "carbohydrate metabolic process", "chlorophyll bio-synthetic process", "Photosystem I reaction center" and "chlorophyll binding" were enriched when A131 plants are exposed to CL. Similarly, the terms "carbohydrate metabolic process", "chlorophyll binding" and "heme binding" were enriched in CLT as compared to A131 when exposed to CL. These results indicate that photosynthesis and carbohydrate metabolism are strongly affected by CL at the transcriptional level, even in adult, uninjured leaves (Fig. 4.3c).

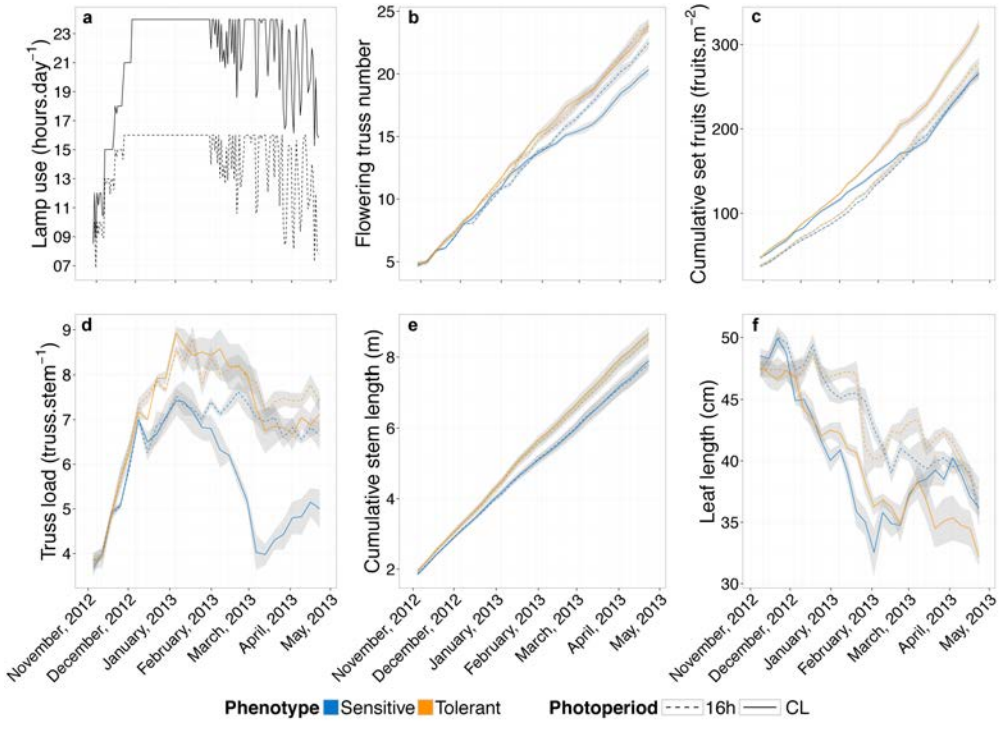

Figure 4.5 | Developmental measurements during the yield trial. Measurements were done on Idooll F_1 continuous light-tolerant and -sensitive homozygous lines; parent lines where backcrossed 4 times with a continuous light-tolerant introgression line, being *Solanum pennellii* the wild donor. (**a**) Hours a day that the lamps where on. Between November and December, the photoperiod set point was increased in 4 and 5 steps from 10 and 12 to 16 and 24 hours per day, respectively. During the dark winter months of December and January, the lamps are on during the complete "light" period. Later during the season, however, solar irradiance was, at times, high (>350 W.m^{-2}) so lamp use was not needed during some hours a day to achieve the set point of 16 and 24 hours of light per day. (**b**) Flowering truss number. (**c**) Cumulative set fruits. (**d**) Number of fruits per stem. Around February, the continuous-light-sensitive tomato plants exposed to continuous light became so sick, that we were forced to prune trusses in order to save the plants and keep the trial going. (**e**) Cumulative stem length. (**f**) Length of the topmost fully expanded leaf. (**b-f**) Measurements were done weekly, values are mean ± s.e.m. (grey shadow), n=4.

Development is not affected in tolerant lines

Parallel to our genetic and physiological work, we also developed and tested an application of the CL-tolerance. After years of breeding (up to six generations), F_1 hybrid lines, tolerant to CL, in the background of elite commercial cultivars (Idooll and Westland) were used in a yield trial in the 2012-2013 winter season. Pilot trials done in previous years showed that compensation measures were needed for the extra assimilates available under CL. Therefore, crop density was increased, CL was applied gradually only starting after the crop became generative (Fig. 4.5a) and the temperature was managed according to each treatment needs.

The resulting climate conditions within the greenhouse are reported in Supplementary Fig. 4.2.

To better understand how CL affects development and ecology of the crop in the long term, we took weekly measurements of key parameters that, if affected, could have an impact on yield (Fig. 4.5). No difference was found in flowering truss appearance rate between CL-tolerant plants cultivated under 16-h photoperiod and CL (Fig. 4.5b). Considering that average daily temperature was similar in both photoperiods (Supplementary Fig. 4.2c), these results indicate that CL does not have an effect on development rate in tomato. Similarly, we found no effect of CL on fruit set in CL-tolerant plants (Fig. 4.5c). In CL-sensitive plants, in contrast, CL injured the plants so badly that development and fruit set were severely reduced (Fig. 4.5b and c). Around February, actually, trusses from CL-sensitive plants cultivated under CL were pruned in order to rescue the plants and to proceed with the experiment – a dead plant within the crop would mean a disruption in the canopy structure that could compromise the validity of the trial. Figure 4.5d shows the resulting truss load. Regardless of the light treatment, flowers opened only during daytime and closed during the night or subjective night. This indicates that the circadian clock was still running in the plants and was probably reset by daily changes in temperature and light. Given that bumblebees were only allowed to exit the hive between sunrise and sunset, we encountered no problems with pollination. As seen in Fig. 4.5e, CL had no effect on stem elongation, but the CL-tolerant line was longer than the CL-sensitive line. To avoid differences in light interception by the different lines, we kept the top of each plant at the same height. CL-exposed tomato leaves tend to have smaller leaf area (Hillman, 1956). The weekly leaf length measurements (Fig. 4.5f) showed shorter leaves. The visibly open canopy observed in the CL-grown crop most likely resulted from a lower leaf area.

Photosynthesis during continuous light in tomato

To know whether CL-tolerant plants can use the artificial light provided during night to efficiently fix carbon, we measured photosynthetic gas exchange every minute during two days and nights using ambient light. Regardless of the photoperiod, photosynthesis rate closely correlated with light intensity (Supplementary Fig. 4.3a and b). We found no difference in the quantum efficiency of CO_2 fixation (ΦCO_2) between day and night in CL-cultivated plants (Supplementary Fig. 4.3c), indicating that CL-tolerant plants are able to use the artificial light provided at night to efficiently fix CO_2.

To assess the effects of CL on photosynthesis in more detail, we performed photosynthesis measurements using combined gas exchange and chlorophyll fluorescence techniques. In representative leaves of each genotype and light treatment, leaf absorbance across the photosynthetically active spectrum was measured (Fig. 4.6a) and later used in computation of several parameters. As expected, CL-injured leaves had a lower leaf absorbance as the result of chlorosis. Light and CO_2 response curves showed that photosynthesis greatly diminished in CL-sensitive tomatoes when grown under CL (Fig. 4.6b and c). In contrast, CL-tolerant leaves only showed a slight decrease in photosynthesis

rate across light and CO_2 levels. This decrease could not be attributed to a single aspect of the photosynthetic machinery as most of the estimated parameters of the Farquhar-von Caemmerer-Berry (FvCB) (Farquhar *et al.*, 1980) model were slightly diminished in CL-tolerant tomatoes when grown under CL (Table 4.1). Figure 4.6d shows the relation between the quantum efficiency of PSII e^- flow (ΦPSII) and the quantum efficiency of CO_2 assimilation (ΦCO_2). The data fitted well to the FvCB model as indicated by high r^2 values (>0.98); although this was not the case for data from CL-sensitive leaves exposed to CL (Table 4.1), photosynthetic parameters estimated from these leaves should be taken with caution as a large variation from leaf to leaf was observed in CL-injured leaves.

Continuous light can increase tomato yield

As expected, most CL-sensitive tomato lines yielded less kg of tomatoes per m^2 when grown under CL as compared to 16-h photoperiod (Fig. 4.7). In contrast, CL-tolerant lines produced the same or more tomatoes under CL than under 16-h photoperiod. The yield increase was up to 20% in the line with the highest number of backcrosses (CL-tolerant homozygous, Idooll_pen5940). Even though some CL-tolerant lines did not show significant yield increase at the end of the trial (April 2013, Fig. 4.7), they did show a trend to yield more under CL than 16-h photoperiod up to mid March 2013 (Supplementary Fig. 4.4). Considering that most of the lines showing significant yield increase under CL were heterozygous for CL-tolerance, a potential linkage drag could explain why no clear trend in tomato yield was observed when comparing the CL-sensitive lines cultivated under 16-h photoperiod with the CL-tolerant lines cultivated under CL. Remarkably, a heterozygous CL-tolerant line cultivated under CL achieved the highest yield (21.4 $kg.m^2$).

Discussion

It is well known that domesticated tomatoes are sensitive to CL; interestingly, *S. pimpinellifolium*, the closest relative of domesticated tomatoes (Tomato Genome Consortium, 2012), is CL-sensitive too (Supplementary Table 4.1). In contrast, all other tested wild tomatoes are tolerant to CL. This suggests that this trait was lost during domestication. Whether tolerance to CL provides an adaptive advantage in nature is not known since CL does not exist in nature as it is achieved with artificial light (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Here, nevertheless, for the first time since its discovery nearly half a century ago (Daskaloff & Ognjanova, 1965), the CL-tolerance present in wild tomato species was used for research and tested in practice. It was found that the *CAB-13* gene is a key component of the CL-tolerance found in wild tomato species. It was also shown that CL influences carbohydrate metabolism and photosynthesis, yet plant development and fruit set are unaffected. Finally, our results show that the concept of increasing tomato yield using this trait and CL is feasible.

Genetic (Fig. 4.1), gene expression (Supplementary Table 4.3), silencing (Fig. 4.2) and sequence data (Fig. 4.2 and Supplementary Table 4.2) all point to the *CAB-13* gene as

responsible for CL-tolerance in wild tomatoes, hypothetically, as a result of two copies of GT-1 consensus in its promoter. *CAB-13*, a type III LHCB, belongs to the LHC super-gene family. In Arabidopsis, this super-gene family encodes six very similar proteins (LHCB1-6) (Jansson, 1999). Tomato *CAB-13* gene is homologous to Arabidopsis *LHCB3*. In Arabidopsis, LHCB1-3 form the "major" trimeric LHCII antenna complexes, and LHCB4-6 form the monomeric "minor" antenna complexes (Caffarri *et al.*, 2009). The antenna complexes harvest light and transfer the energy to the PSII core; together they constitute the PSII-LHCII supercomplex, see Kouril *et al.* (2012) for a recent review. In Arabidopsis, absence of LHCB3 results in alterations of the composition, structure, stability and efficiency of PSII-LHCII supercomplexes (Caffarri *et al.*, 2009, Damkjær *et al.*, 2009, Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b). For instance, LHCB3 can be replaced by LHCB1 and/or LHCB2, but the resulting LHCII trimer binds to the PSII-LHCII supercomplex in a slightly altered position (Damkjær *et al.*, 2009). In knock-out plants lacking LHCB3 (ko*Lhcb3*), the PSII-LHCII supercomplex stability is compromised in such a way that it lacks some LHCII subunits (Caffarri *et al.*, 2009). Similarly, high-light-acclimated plants show a reduction in LHCB3, which leads to higher PSII quantum yield of charge separation and supercomplexes lacking the same subunits as in ko*Lhcb3* plants (Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b). Here, we have shown that CL induces down-regulation of tomato *CAB-13*, and that CL-exposure results in higher *CAB-13* expression in CL-tolerant plants than in CL-sensitive plants (Supplementary Table 4.3). This suggests that CL alters CAB-13 levels and PSII-LHCII supercomplex structure.

The absorption spectra and quantum efficiencies of PSI and PSII are different, yet they work in series. For maximum efficiency, balanced excitation of PSI and PSII is vital. In response to short term changes in light intensity and quality, LHCII trimers can move between PSI and PSII in order to keep both photosystems equally excited – a process that requires LHCII trimer phosphorylation and is known as state transitions (Kargul & Barber, 2008). After long-term acclimation to natural light conditions, "extra" LHCII trimers (composed by LHCB1-2) are associated with both PSII and PSI; the formation of PSI-LHCII complexes appears to be required for balanced excitation of both photosystems in the long term (Wientjes *et al.*, 2013a). Interestingly, in ko*Lhcb3* leaves, the rate of state transitions (LHCII from PSII to PSI direction) is enhanced and the level of LHCII trimer phosphorylation is higher, suggesting that LHCB3 modulates the rate of state transitions (Damkjær *et al.*, 2009). Considering that LHCII phosphorylation and *LHCB* expression are possibly co-regulated (Pursiheimo *et al.*, 2001), the true importance and function of LHCB3 might be more than what our current knowledge suggests. The GO terms "chlorophyll binding" and "PSI reaction center" were significantly enriched when A131 plants are exposed to CL. Thus suggesting that the CL-induced injury in tomato might be the result of unbalanced excitation of PSI and PSII.

Deletion of the GT-1 consensus binding site in CL-sensitive lines (Fig. 4.2) suggests a connection between the CL-induced injury and light signaling. GT-1 binding sites are present in many light-regulated genes like LHC proteins (also known as Cab), the

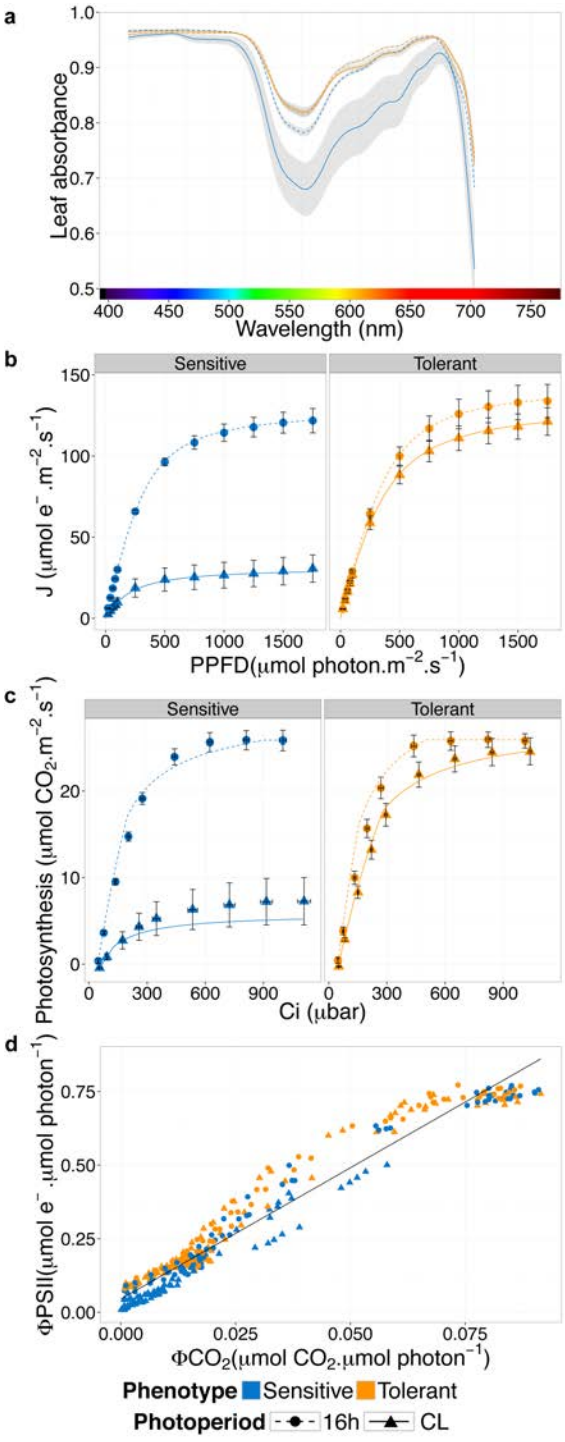

Figure 4.6 | Photosynthetic responses of tomato leaves to continuous light. (**a**) Absorbance of continuous light-tolerant and -sensitive leaves developed under 16-h photoperiod or continuous light. Mean ± s.e.m (grey shadow), n=3. (**b**) Response of J (electron transport) to light (PPFD). Mean ± s.e.m., n=4. Lines where were drawn by fitting the predicted parameters (Table 4.1) to the FvCB model. (**c**) Response of photosynthesis to intracellular CO_2 concentration (Ci). Mean ± s.e.m., n=4 leaves. Lines where were drawn by fitting the predicted parameters (Table 4.1) to the FvCB model. (**d**) Relationship between the quantum efficiency of PSII e^- flow on PSII-absorbed light basis (ΦPSII) and the quantum efficiency of CO_2 assimilation on the leaf PPFD-absorbed basis (ΦCO_2) in continuous light-tolerant and -sensitive leaves developed under 16-h photoperiod or continuous light.

Table 4.1. Photosynthetic parameters of continuous light-tolerant and -sensitive tomato leaves developed under 16-h photoperiod or continuous light.

Parameter	Units	Sensitive		Tolerant		Significance (p-value)[b]		
		16-h photoperiod	Continuous light	16-h photoperiod	Continuous light	Genotype	Treatment	Interaction
Maximum quantum efficiency of PSII (F_v/F_m)	-	0.8±0.005	0.58±0.088	0.79±0.006	0.79±0.006	0.043	0.026	0.033
Quantum efficiency of PSII e^- flow at strictly limiting light ($\Phi_{2(LL)}$)	mol CO_2.mol photon^{-1}	0.76±0.006	0.33±0.089	0.76±0.007	0.74±0.009	0.001	0.000	0.001
Day respiration (R_d)	µmol CO_2.m^{-2}.s^{-1}	1.42±0.221	1.31±0.265	1.7±0.25	2.21±0.244	0.035	0.423	0.225
Lumped parameter s	-	0.42±0.008	0.4±0.031	0.4±0.015	0.39±0.026	0.386	0.449	0.734
Conversion efficiency of incident light into e^- flow at strictly limiting light ($\kappa_{2(LL)}$)	mol e^-.mol photon^{-1}	0.32±0.008	0.13±0.032	0.3±0.014	0.29±0.022	0.007	0.000	0.001
Convexity factor between e^- transport rate and incident light (θ)	-	0.78±0.027	0.37±0.139	0.79±0.011	0.7±0.028	0.034	0.005	0.043
Maximum e^- transport rate (J_{max})	µmol e^-.m^{-2}.s^{-1}	129.56±8.56	31.35±9.01	144.92±12.39	132.39±10.11	0.000	0.000	0.001
Maximum rate of Rubisco activity-limited carboxylation (V_{cmax})	µmol CO_2.m^{-2}.s^{-1}	81.59±6.53	24.52±7.35	104.89±16.84	72.55±6.68	0.005	0.001	0.253
Rate of triose phosphate export from the chloroplast (T_p)	µmol.m^{-2}.s^{-1}	9.08±0.382	2.85±0.969	9.18±0.225	8.9±0.585	0.000	0.000	0.000
Coefficient of determination for the complete FvCB model (r^2)[a]	-	0.98±0.004	0.71±0.08	0.99±0.002	0.99±0.002			

Values represent mean ± s.e.m., n=4.

[a] We used temperature corrected, generic values for the Michaelis-Menten constants of Rubisco for CO_2 and O_2 (K_{mC} and K_{mO}, respectively) as well as parameter Γ^*. At 25°C, K_{mC}=272.372mbar, K_{mO}=165.788mbar and Γ^*= 37.411mbar.

[b] Calculated using a multivariate analysis of variance (MANOVA)

small subunit of Rubisco (RbcS) and phytochrome A (Terzaghi & Cashmore, 1995). Upon Ca^{2+}-dependent phosphorylation, GT-1 transcription factor binds to the DNA sequence $^{G}_{T}{}^{A}_{T}$GTGPu$^{A}_{T}$AA$^{A}_{T}$Pu$^{A}_{T}$ (Maréchal *et al.*, 1999, Nagata *et al.*, 2010, Terzaghi & Cashmore, 1995). GT-1 binding sites are often found in tandem (Terzaghi & Cashmore, 1995). For instance, the pea *RbcS-3A* promoter contains six GT-1 binding sites (Gilmartin *et al.*, 1990), yet a -166 deletion shows that the presence of only two of them, named boxII and boxIII, is sufficient for transcription (Kuhlemeier *et al.*, 1987). Early studies suggested that both boxII (-150, positive strand) and boxIII (-124, negative strand) are needed for expression (Kuhlemeier *et al.*, 1988); however, it was later shown that instead of the core GT-1 binding site of boxIII, a GATA motif partially overlapping with boxIII is absolutely required for transcription, together with boxII (Sarokin & Chua, 1992). Interestingly, the tomato *CAB-13* promoter in CL-tolerant tomatoes shows some similarities with the pea *RbcS-3A* promoter; it contains a GT-1 binding site at -50 (positive strand) and another one, partially overlapped with a GATA motif, at -105 (negative strand) (Fig. 4.2). Hence, the missing GT-1 binding site at -50 in all CL-sensitive tomato lines could be responsible for the lower expression of *CAB-13* in CL-sensitive lines under CL (Supplementary Fig. 4.1 and Table 4.6). Although the GT-1 binding site of boxII and the GATA motif of boxIII are sufficient for pea *RbcS-3A* expression in mature leaves, sequences upstream of -170, which contain redundant GT-1 binding sites, are required for high-level expression in young developing leaves (Kuhlemeier *et al.*, 1988). Considering that only young tomato leaves are sensitive to CL (Hillman, 1956, Withrow & Withrow, 1949), *CAB-13* expression in developing tomato leaves exposed to CL might be affected by the absence of the GT-1 binding site. All together, we propose a hypothesis in which the absence of GT-1 binding site in the *CAB-13* promoter allows a CL-induced down-regulation of *CAB-13* in developing tomato leaves, resulting, as discussed above, in unbalanced PSI and PSII excitation.

Hyper-accumulation of carbohydrates, observed in CL-exposed leaves, has been proposed to induce injury in tomato plants (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)). Here, GO analysis showed that CL affects carbohydrate metabolism in uninjured leaves (Fig. 4.3 and 4.4). Leaf carbohydrate quantification shows that glucose and fructose concentrations are higher in CL-tolerant (CLT) than in CL-sensitive (A131) tomato plants when grown under 16-h photoperiod, and in both genotypes CL decreases glucose and fructose content. No interaction between genotype and photoperiod was observed. Interestingly, when grown under CL, sucrose content is constantly high in both genotypes (Supplementary Table 4.7); in other words, sucrose accumulation occurs in both CLT and CL-sensitive A131 plants. If sugars play a role in inducing injury and/or down-regulating photosynthesis under CL, these observations suggest that CL-tolerance in CLT tomatoes is downstream carbohydrate accumulation. A recent study suggested that the tomato SUGAR PARTITIONING AFFECTING (SPA) protein, a DnaJ chaperone related-protein, mediates sink-source relationships by increasing the leaf capacity to export sugars (Bermúdez *et al.*, 2014). In our RNAseq dataset, CL slightly down-regulated *SPA* (Solyc04g081320.2) expression

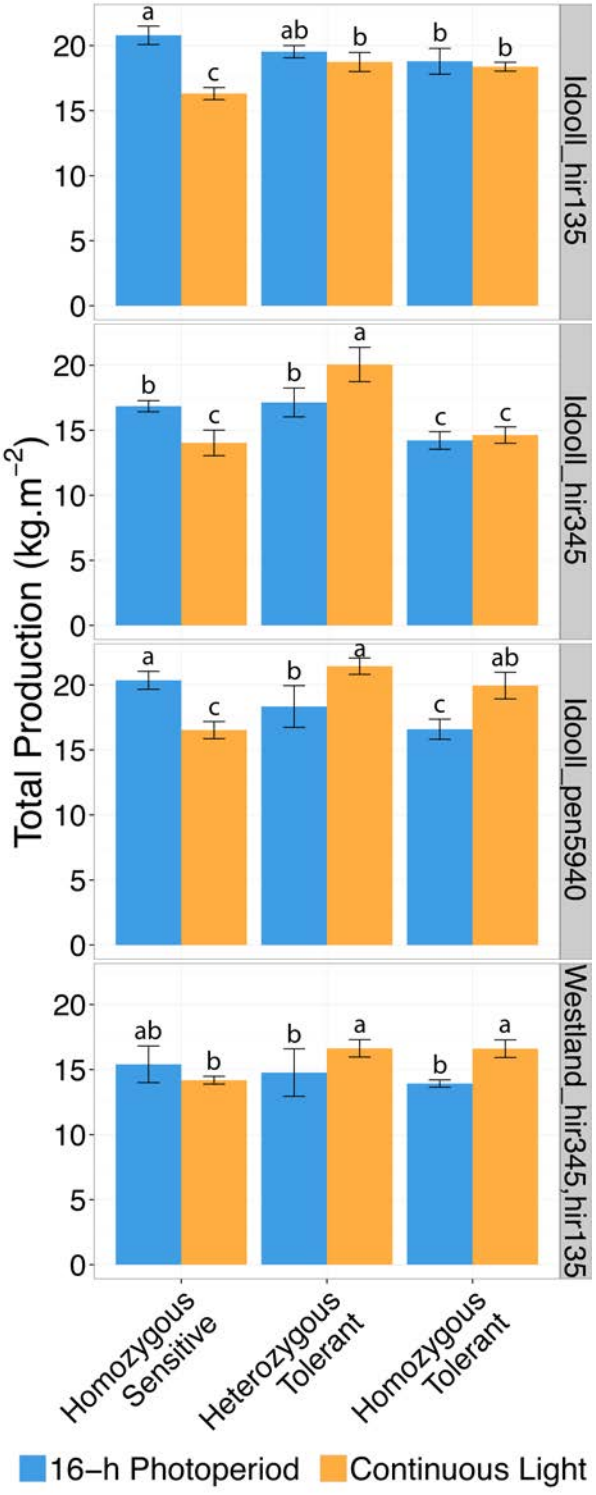

Figure 4.7 | Yield under 16-h photoperiod and continuous light. Each panel depicts the yield of F_1 hybrids grouped in four categories; in each category, several continuous light-tolerant introgression lines were backcrossed with the parents of "Idooll" or "Westland" F_1 commercial hybrids (see background and wild donor on the left). After 3 to 4 backcrosses, the newly bread parents, segregating as continuous light-tolerant or -sensitive, where used to generate homozygous or heterozygous and tolerant or sensitive Idooll and Westland F_1 hybrids (see labels at the bottom). Mean ± s.e.m., n=3 plots. Within each of the four groups, bars with different letter are statistically different ($P<0.05$), Fisher's protected LSD test.

(logFC=-1.057, FDR p-value=0.002) in A131 plants, yet no effect was found between A131 and CLT tomato plants under CL (FDR p-value=0.066) (see Supplementary Data at Nature Communications, doi:10.1038/ncomms5549).

Regarding a potential application of CL-tolerance, it was shown that the trait can easily be introgressed into domesticated tomato with various genetic backgrounds (A131, M82, Moneyberg, Idooll and Westland) using several wild donors (*S. neorickii*, *S. pennellii, S. habrochaites* and *S. chilense*). Additionally, the yield trial has shown that tomato yield per m^2 can be increased using CL-tolerant tomato lines, up to 20% (Fig. 4.7). Such yield increase agrees with the crop model computations, which predicted that the theoretical maximum yield increase achievable by cultivating tomatoes under CL is 22% when applying a similar photoperiod increase (Velez-Ramirez *et al.*, 2012 (Chapter 3)). Nonetheless, this simulation study assumed no crop adaptations to CL, but we have observed some adaptations that could have a significant impact on yield. For instance, day respiration was higher in the tolerant genotype (Table 4.1) and leaves were smaller when exposed to CL (Fig. 4.5f), which negatively influences light interception. Further increasing crop density could solve this and, predictable, could further increase yield under CL.

Understanding why CL increases yield is a question of much interest. However, the yield trial was not designed to answer this question, but rather to succeed in cultivating tomatoes under CL for the first time. Nonetheless, the results suggest that the observed yield increase was achieved by a combination of higher crop density, which was only made possible by the use of CL, and "night" photosynthesis (Supplementary Fig. 4.3a); however, unquantifiable, at least in this study, contributions of other factors, like altered canopy structure (Fig. 4.5), higher day respiration in CL-tolerant lines (Table 4.1) and unknown, yet potential, effects on assimilate partitioning and leaf senescence, prevent us from assigning a numeric contribution of each factor to the observed yield increase.

Although the involvement of other genes in the CLT locus (Fig. 4.1) cannot be completely discarded with the evidence presented here, multi-level evidence presented in this study supports the involvement of *CAB-13* in this trait. This implies that photosynthesis and the PSII antenna are involved in the CL-induced injury at a higher degree than previously thought. Nonetheless, the role of carbohydrate metabolism, light signaling and the circadian clock cannot be ruled out. The identification of wild tomato *CAB-13* as responsible for the CL-tolerance is a significant breakthrough, and it is the base of further research on photosynthesis and its interaction with carbohydrate metabolism, light signaling and the circadian clock.

Materials and Methods

Plant materials and genetic stocks

All tomato (*Solanum lycopersicum*) varieties as well as introgression line populations and wild tomato species used in this study were provided by Monsanto Holland B.V. (Bergschenhoek, The Netherlands) with the exception of Sub-Arctic Plenty, of which seeds

came from Thompson & Morgan (Ipswich, Suffolk, UK). Introgression lines (IL) from three populations were used to map the continuous light tolerance trait. The first IL population is a BC_3F_3 *S. neorickii* (LA2133) x *S. lycopersicum* A131 consisting of 43 lines. This population was originally developed and described by Fulton *et al.* (2000). The original BC_2F_5 *S. neorickii* (LA2133) x *S. lycopersicum* E6203 population was backcrossed with the inbred line A131 to obtain the BC_3F_3 population that was used in this study. In this IL population, the *S. neorickii* chromosomes are reasonable well represented; only the bottom of chromosome 1 and 8 and the top of chromosome 11 are not presented in the population. The "CLT" line used in the RNAseq experiment belongs to this population. The second population is a *S. chilense* (LA1959) x *S. lycopersicum* Moneyberg consisting of 49 lines. The population consists for 2/3 of BC_4F_3 and 1/3 of BC_4F_4 lines. The *S. chilense* chromosomes are well represented in the IL population except for the top of chromosome 5 and 6. The third IL population is a BC_3F_4 *S. pennellii* (LA0716) x *S. lycopersicum* M82 consisting of 49 lines of which only five lines with an introgression on chromosome 7 were used. This population was developed and described by Eshed & Zamir (1994).

For the yield trial, several continuous light-tolerant introgression lines were backcrossed with the parents of "Idooll" or "Westland" F_1 commercial hybrids (Monsanto Holland B.V.). The wild donors of CL-tolerance in those introgression lines were *S. habrochaites* (lines hir135 and hir345) and *S. pennellii* (line pen5940). After 3 to 4 backcrosses, the newly bred parents, segregating as CL-tolerant or –sensitive, where used to generate homozygous or heterozygous and tolerant or sensitive Idooll and Westland F_1 hybrids.

Genotyping introgression lines

Introgression lines were genotyped with 384 single nucleotide polymorphism (SNP) markers using a custom GoldenGate genotyping assay (Illumina, San Diego, California, USA). The sequences, alleles, accession numbers and positions of the 27 SNP markers that mapped to chromosome 7 can be found in Supplementary Table 4.8. Genomic marker positions were based on the public tomato reference genome v2.40 (Tomato Genome Consortium, 2012).

Growth conditions and treatments

For phenotyping CL-tolerance of tomato varieties, introgression line populations and wild-tomato species, plants were grown in climate cells at Monsanto Holland B.V. (Bergschenhoek, The Netherlands). Plants, at least four repetitions, were sown in rockwool, irrigated with hydroponic nutrient solution and grown at 21 °C and 70% RH. After growing the plants for two weeks under fluorescent tubes at an intensity of 120 $\mu mol.m^{-2}.s^{-1}$ under 16-h photoperiod, plants were exposed to CL at an intensity of 120 $\mu mol.m^{-2}.s^{-1}$ provided by high-pressure sodium (HPS) lamps. A genotype was called CL-sensitive if all replicates (n=6) showed interveinal, mottled chlorosis in young, fully

expanded leaves. A genotype was called CL-tolerant if the entire foliage of all replicates (n=6) showed neither signs of chlorosis nor necrosis after at least 6 weeks of exposure to CL.

Plants used for the RNAseq analysis and VIGS experiments were grown in climate cells at Wageningen University (Wageningen, The Netherlands). Plants were sown in rockwool, irrigated with hydroponic nutrient solution and grown at 21 °C and 70% RH. Light was provided by HPS lamps (Master SON-T Green Power 400W, Philips, Eindhoven, The Netherlands) and supplemented with incandescence lamps (Philinea T30 120W, Philips, Eindhoven, The Netherlands) at an irradiance of 350 $\mu mol.m^{-2}.s^{-1}$; red-to-far-red ratio was 2.873, and the phytochrome photostationary state (PSS) (Sager *et al.*, 1988) was 0.857. After growing the plants for four weeks under 16-h photoperiod, plants were exposed to CL by leaving the lamps (HPS and incandescent) continuously ON without changing any other setting.

RNAseq and gene ontology enrichment analyses

One month old A131 and CLT plants were transferred to CL. After one week of treatment, the leaflets of the top-most fully expanded leaf (5^{th} true leaf) were harvested for sequencing the transcriptome using SOLiD3 technology (Applied Biosystems, Foster City, California, USA). The raw reads were mapped to the public tomato genome v2.40 (Tomato Genome Consortium, 2012), using the ITAG gene annotations v2.3 and the CLC Genomics software v5.1.6 (CLCbio, Aarhus, Denmark). Further analysis was performed in R (R Core Team, 2013) exclusively using reads that mapped to a single place in the genome. To find differentially expressed genes the R package "edgeR" was used (Robinson *et al.*, 2010). Only genes with an expression higher than 0.01 counts per million (CPM) in at least three samples were used for further analysis. A trimmed mean of M-values normalization procedure accounted for library size (Robinson & Oshlack, 2010). A generalized linear model procedure of "edgeR" tested for differentially expressed genes in the contrasts A131 24h vs A131 16h and A131 24h vs CLT 24h. Genes were called differentially expressed when its FDR-corrected p-value was lower than 0.05.

The R package "GOseq" was used for gene ontology (GO) enrichment analysis (Young *et al.*, 2010). First, the GOseq procedure calculated the probability that a gene was called differentially expressed as a function of its length; this probability was then included into the statistical model. This prevents that a GO category is called significantly enriched if contains an above-average number of long or short genes. The most accurate "sampling method", included in GOseq, tested each GO category. GO categories containing less than three genes were filtered out. A GO category was called significantly enriched when its FDR-corrected p-value was lower than 0.05. The results were visualized using the R package "REVIGO" (Supek *et al.*, 2011).

Virus-induced gene silencing (VIGS)

RNA was extracted from a three-weeks old CLT tomato plant according to Schuurmans *et al.* (2003) and purified with silica membranes columns. In short, fifty mg of frozen

pulverized leaf tissue was homogenized with 500 ml of extraction buffer (350 mM glycine, 48 mM NaOH, 340 mM NaCl, 40 mM EDTA and 4% SDS) at 50 °C. Then the homogenate was extracted three times with phenol/chloroform/isoamylalcohol (25:24:1 v/v), and later RNA was precipitated with 120 µl of 8 M LiCl. After washing the RNA with 500 ml of ethanol 70% (v/v) at -20 °C, RNA was dissolved in 87.5 ml of RNA-free water. Finally, the RNA was purified with RNeasy spin columns (Qiagen, Venlo, Netherlands).

cDNA was synthetized with iScript (Bio-Rad, Hercules, California, USA) and PCR-amplified using high fidelity Polymerase (Invitrogen, Carlsbad, California, USA) with the primers 5'-ATTCTAGCAGTATTGGG-3' and 5'-TCTTCCGTTCTTGATTTCCT-3'. The resulting 189-pb *CAB-13* fragment was found to be unique after blasting its sequence to the reference tomato genome. This fragment was cloned into pCR4^{TM}4Blunt-TOPO plasmid (Invitrogene), and competent *E. coli* (DH5a^{TM}-T1^{R}) cells (Invitrogene) were transformed and grown on agar plates with kanamycin (10 mg.ml^{-1}). The plasmid was digested with EcoRI, and the fragment cloned into EcoRI-linearized pTRV2 VIGS vector (Liu *et al.*, 2002a, Liu *et al.*, 2002b); then DH5a cells were transformed by electroporation and selected with kanamycin. After confirming fragment and plasmid integrity by sequencing and restriction analysis (XcmI and EcoRI), respectively, the *pTRV2::CAB-13* plasmid was cloned into *Agrobacterium tumefaciens* strain pCH32-C58C1 by electroporation. Previously described *pTRV::PDS* (Liu *et al.*, 2002a) and *pTRV::GUS* (Tameling & Baulcombe, 2007) constructs were used as positive and negative controls, respectively. Agrobacterium culture containing pTRV1 was mixed with each of the pTRV2-derivate cultures at 1:1 ratio at a final $O.D._{600}$=0.8 and used to inoculate 10-days old A131 and CLT tomato plants grown under 16-h photoperiod following the procedure of Liebrand *et al.* (2012). Two and a half weeks after infiltration, pTRV2::PDS-treated plants showed photo-bleaching symptoms. At that point, chlorophyll fluorescence images were taken and the CL treatment started. After three weeks of treatment, chlorophyll fluorescence images were once more taken on the topmost fully expanded leaves.

Rt-qPCR

RNA and cDNA were extracted and synthetized, respectively, as described before, but treating the RNA with DNase (Sigma). The Rt-qPCR primers for *Lhcb3* were 5'-CTGCTCAAACTCCTTCATACTT-3' and 5'-AAAGGCCTCGGGATCAGC-3'. From the RNAseq data, five genes with the most stable expression across genotypes and photoperiod treatments were selected as reference genes. The used primers and genes were 5'-GCCACTTCTCCTATCAGTTTTT-3' and 5'-CCAAAGATGAACCCCAAAACA-3' for Solyc03g097870.2, 5'-TGGTGCTCCCCTTCCAGC-3' and 5'-TGGCTCTCCTCCTCCGTT-3' for Solyc06g048410.2, 5'-ATGCCTACTCGTTACACACT-3' and 5'-CCGGTCTTGAACCTCTCCT-3' for Solyc06g073300.1, 5'-TTCACTGCGTGTCTTCCT-3' and 5'-CATCTTGCTTCTCACCCTT-3' for Solyc09g010440.2, and 5'-AAACACCAAAGACGACCTCA-3' and 5'-CACAGACAGAACGAGATCC-3' for

Solyc12g010060.1. The geometric mean of all five genes was used to normalize *CAB-13* expression.

Sequencing and sequence alignment

The reference tomato genome sequence v2.4 (Heinz) and the draft *S. pimpinellifolium* genomic scaffold sequences are publicly available (Tomato Genome Consortium, 2012). The CLT locus sequence of tomato and wild tomato lines were obtained and aligned by the 150 tomato genome consortium in collaboration with the user community (150 tomato genome consortium, 2013). For A131 and CLT lines, transcriptome sequences were available from the RNAseq experiment. *CAB-13* promoter sequences from selected tomato and wild tomato lines were obtained by Sanger sequencing, using the primers 5'-TGGTGCGCATGAGTCTAAACA-3' and 5'-CCAGCAGTATCCCATCCGTAAT-3'. CLC Workbench software v6.8.4 (CLCbio, Aarhus, Denmark) was used for sequence alignment.

Chlorophyll fluorescence imaging

Intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences, Lincoln, USA). After 20 minutes of dark adaptation, leaflets were detached and immediately used for measurements in a chlorophyll fluorescence imaging system (FluorCam, Photon System Instruments, Brno, Czech Republic). FluorCam model 700MF was used in the RNAseq experiment; in the VIGS experiment, Fluorcam 800MF was used instead. Fluorcam v. 5.0 and 7.0 software were used to control and process the images in FluorCam 700MF and Fluorcam 800MF respectively. In both FluorCam models, maximum quantum efficiency of photosystem II (F_v/F_m) (Baker, 2008) was calculated following a custom-made protocol. During a period of 5 seconds, red-orange light-emitting diodes (LEDs) produced measuring light flashes (the average irradiance produced by the measuring light flashes was <0.5 $\mu mol.m^{-2}.s^{-1}$); then a halogen lamp (FluorCam 700MF) or red-orange LEDs (FluorCam 800MF) produced a one-second saturating light pulse. The saturating light pulses had an intensity of 2500 and 3500 $\mu mol.m^{-2}.s^{-1}$ in Fluorcam 700 MF and Fluorcam 800MF, respectively. In FluorCam 700MF, F_o and F_m images were recorded every 80 or 400 milliseconds, respectively; in Fluorcam 800MF, both F_o and F_m images were recorded every 100 ms. Fluorescence images recorded during the first 5 seconds were averaged to produce a single F_o image, and images recorded during the latter 1-second saturating light pulse were averaged to produce a single F_m image. Form these two images a spatially resolved F_v/F_m image was constructed using the expression $F_v=F_m-F_o$. Leaflet average F_v/F_m was calculated using ImageJ software version 1.44o (Schneider *et al.*, 2012).

Yield trial

The yield trial was performed on two contiguous, independent greenhouse compartments on GreenQ facilities (Bleiswijk, The Netherlands) (+52° 1' 49.05", +4° 31' 46.91"). Each

greenhouse compartments had six gutters, and the planted area was 124.71 and 107.76 m^2. All plants were grafted on Maxifort (Monsanto Holland B.V., Bergschenhoek, The Netherlands) and planted according to a completely randomized block design, consisting of four blocks. To account for a potential edge effect, plants too close to the greenhouse wall were not used for any measurement. Within each block, parcels had three plants, and each plant had two stems; the resulting densities of 2.10 and 2.45 stems.m^{-2} was achieved by adding extra plants on the edges when needed. The crop was trained according to the high-wire system and irrigated with hydroponic nutrient solution according to standard practices. CO_2 enrichment was used with a set point of 700 ppm. Bumblebees (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were used for pollination; hive exits were open only between sunrise and sunset – bumblebees cannot navigate without the sun, yet they exit the hive if lamps are on. Leaf and fruit pruning was applied when needed to keep the vegetative and generative strength equally balanced in all F_1 lines. Temperature was adjusted weekly accordingly to crop development. As Dutch law prohibits opening the greenhouse screens before midnight when the lamps are ON (Minister van Volkshuisvesting Ruimtelijke Ordening en Milieubeheer, 2002), the coldest period in the CL compartment was shifted to start just after midnight; the compartment at 16-h photoperiod was managed according to standard practices.

The yield trial started on the first of October 2012 with grafted plants bearing two stems and the first flower. Photoperiod was gradually increased to 16h and 24h of light.day^{-1}, reaching the final set points on the 22nd and 28th of November 2012, respectively. To achieve these photoperiods, HPS lamps, with an installed capacity of ±130 µmol.m^{-2}.s^{-1}, were used as many hours as needed; lamps were turned OFF when incoming solar irradiance was higher than 350 W.m^{-2}. On week 5 2013, stem density was increased to 3.15 and 3.68 stems.m^{-2}. The trial was finalized on the 22nd of April 2013.

Yield per F_1 hybrid line and per plot was recorded weekly. Additionally, two F_1 Idooll lines were selected for performing detailed developmental and photosynthetic measurements. These lines were selected because its parents were backcrossed the most (four times). Both lines had *S. pennellii* as wild donor (line pen5940); one line was homozygous CL-sensitive and the other one was homozygous CL-tolerant. Developmental measurements were performed weekly, and they consisted of measuring leaf and stem length, recording flowering, setting and harvested truss number, and keeping record of fruit set and truss load. Photosynthetic measurements were done on week 3 2013 on the top most fully expanded leaf. By this time, the complete canopy had developed under the 16-h photoperiod or CL.

Photosynthesis measurements

Photosynthesis measurements were done with a gas exchange system (LI-6400, Li-Cor Biosciences, Lincoln, USA). All measurements were performed with a block temperature of 21 °C. For continuously measuring photosynthesis during two day/night cycles, the standard 2x3 cm chamber with a clear window (Propafilm film) was used. Greenhouse air was first buffered in a 40 l container, and then used in the measurements without further

conditioning. Flow rate was set at 350 mmol air.s^{-1}. IRGAs where automatically matched every 30 minutes and data was logged every minute. Light intensity inside the leaf chamber was estimated using the readings from the quantum sensor placed outside the chamber (LI-190, Li-Cor Biosciences, Lincoln, USA) corrected with the measured light transmittance of the Propafilm film. After two days of measurements, the enclosed leaves expanded beyond the initial 6 cm^2. To correct for this change in leaf area, the leaf was detached, cut and photographed at the end of the measurements. The new area was calculated using ImageJ software version 1.44o (Schneider *et al.*, 2012). A linear change in leaf area was assumed and used to correct all gas exchange measurements.

For measuring light and CO_2 response curves, a 2-cm^2 leaf chamber equipped with a fluorometer was used (6400-40, Li-Cor Biosciences, Lincoln, USA). At every change in light or CO_2, the IRGAs where matched and chlorophyll fluorescence and gas exchange data was simultaneously logged. After dark-adapting the leaf for 20 minutes, F_v/F_m was measured. Then, waiting 5 minutes after each change, a 12-step light curve (20, 40, 60, 80, 100, 250, 500, 750, 1000, 1250, 1500 and 1750 μmol photon.m^{-2}.s^{-1}) was performed at a CO_2 concentration of 600 ppm; below 100 μmol photon.m^{-2}.s^{-1}, only red light was used (restriction of the apparatus), and from 250 μmol photon.m^{-2}.s^{-1} on, red light was supplemented with 10% blue light. Finally, waiting 3 minutes after each change, a 9-step CO_2 curve (50, 100, 200, 300, 400, 600, 800, 1000, 1200 ppm) was performed at a light intensity of 1750 μmol photon.m^{-2}.s^{-1}. When necessary, water vapor was scrubbed from the greenhouse air priory to the injection into the leaf chamber to prevent water condensation on the IRGAs lenses. Depending on the photosynthesis rate at each CO_2 and light level, flow rate was carefully set between 200 and 300 mmol air.s^{-1}; during all measurements the flow rate was low enough to achieve a good signal to noise ratio (ΔCO_2 > 0.2 μmol CO_2.mol^{-1} in 96.8% of the measurements) and, yet high enough to prevent CO_2 depletion in the leaf chamber (ΔCO_2 < 30 μmol CO_2.mol^{-1}) and also to reduce diffusion leaks at the minimum. In addition, all measurements were corrected for diffusion leaks using a leak rate constant calculated according to manufacturer instructions. The parameters of the FvCB model (Farquhar *et al.*, 1980), where estimated combining gas exchange and chlorophyll fluorescence data according to Yin *et al.* (2009), with some modifications (see Supplementary Methods for details).

Statistical analysis

The observed against expected proportion in the segregating F_2 population was tested with a X^2 test. Statistical significance of the leaflet average F_v/F_m and yield data was determined with an ANOVA test. For the F_v/F_m data, a power transformation was used to achieve homogeneous variances. ANOVA and X^2 tests were performed with IBM SPSS Statistics software version 19 (IBM, Somers, USA). The FvCB model parameters were estimated in Microsoft Excel using the GRG nonlinear iteration procedure.

End Notes

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Author Contributions

F.F.M. conceived the project; F.F.M., D.V., E.H. and W.v I. designed the general strategy. F.F.M., P.M.J.A.v P. and A.I.V.-R. genotyped and phenotyped all tomato lines and performed the genetic mapping and fine mapping; P.M.J.A.v P. and A.I.V.-R. aligned and analyzed CAB-13 sequences. A.I.V.-R. and D.V. analyzed the RNAseq data and performed the GO analysis; A.I.V.-R., F.F.M., W.v I. and D.V. designed, performed and analyzed the VIGS experiment. A.I.V.-R., F.F.M., E.H. and W.v I. designed and supervised the yield trial. A.I.V.-R. and W.v I. performed and analyzed all photosynthesis measurements. A.I.V.-R. wrote the paper with input from all co-authors. F.F.M. acted as coordinator/project leader.

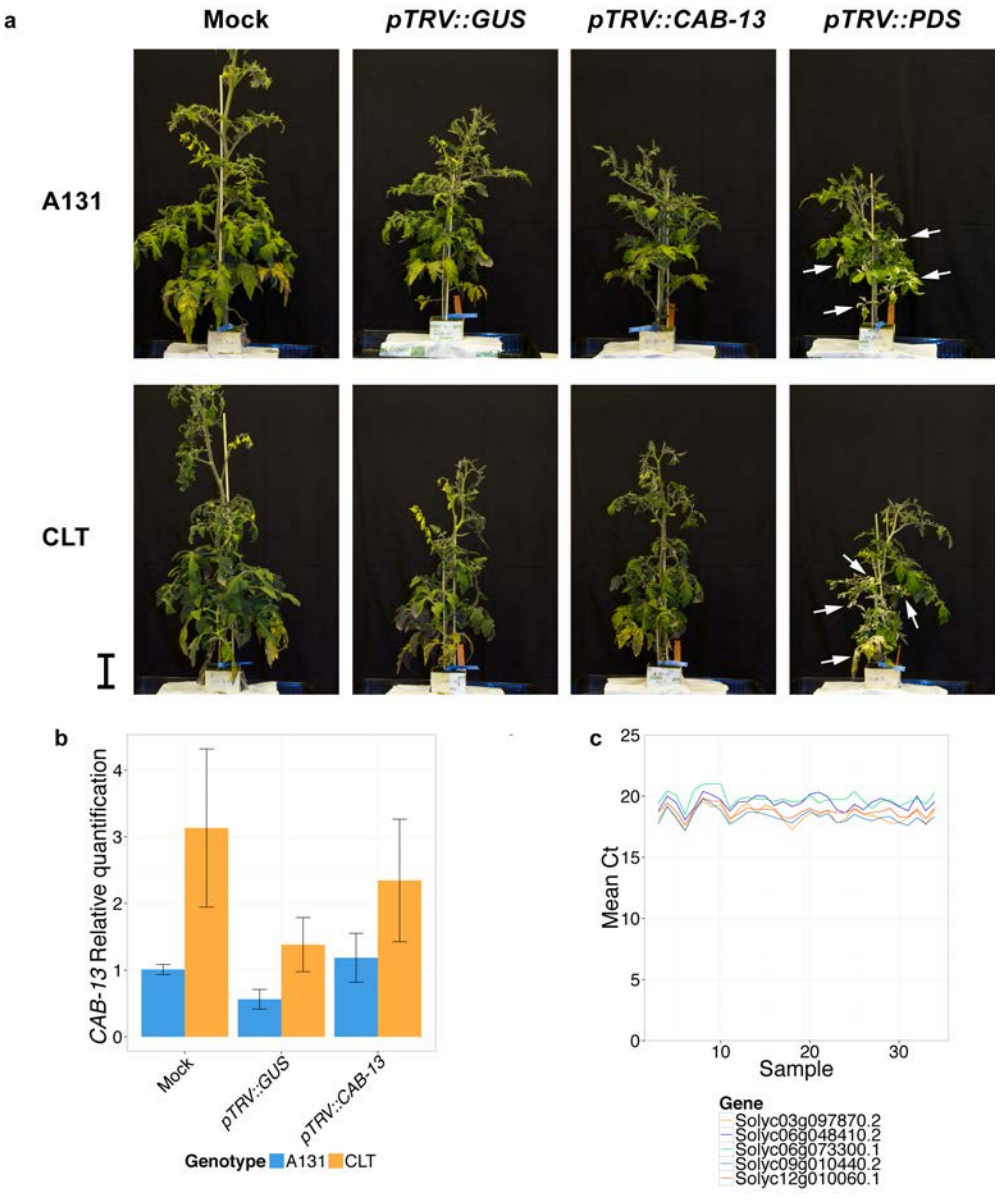
Competing financial interests

Monsanto Invest N.V. has filed a patent (WO 2011/028121 A1) related to the research presented in this manuscript. F.F.M and P.M.J.A.v P. are named inventors in this patent application. The remaining authors declare no competing financial interests.

Reference accessions

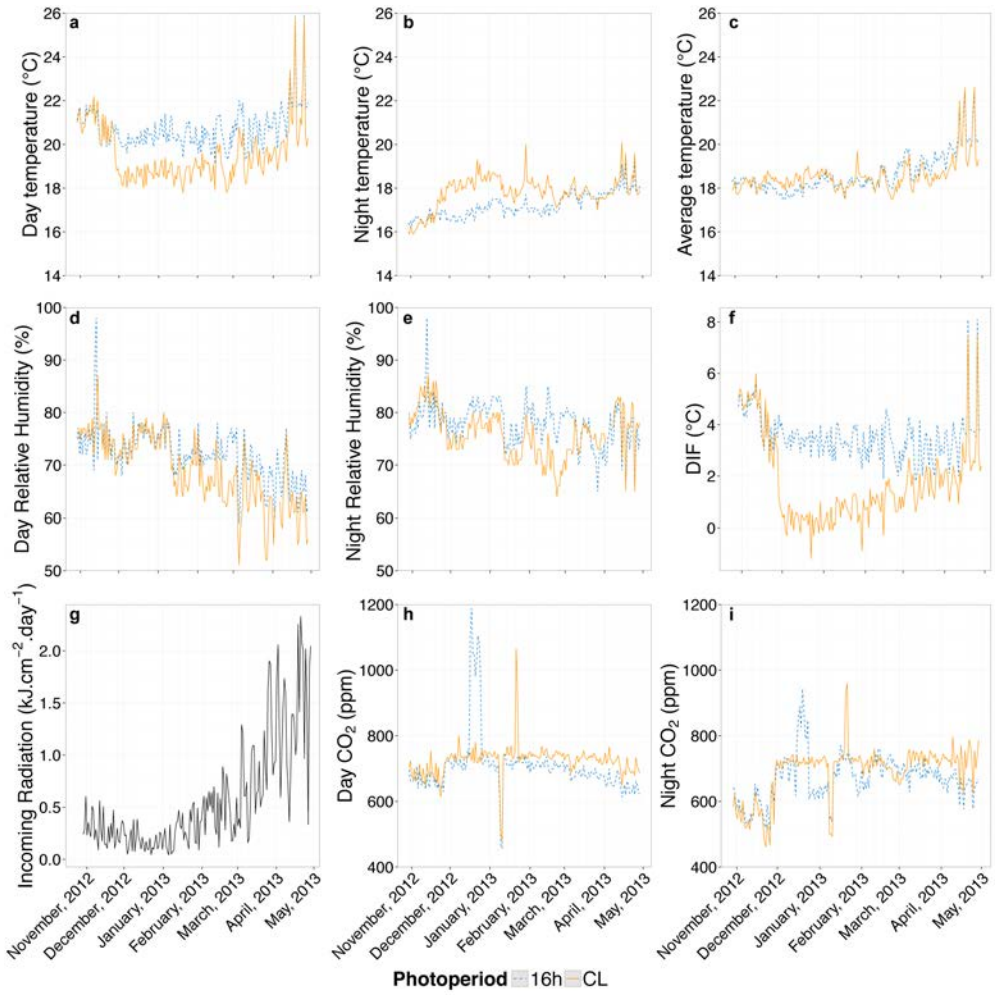
Sequence read archive (SRA): SRP041241

Supplementary Information

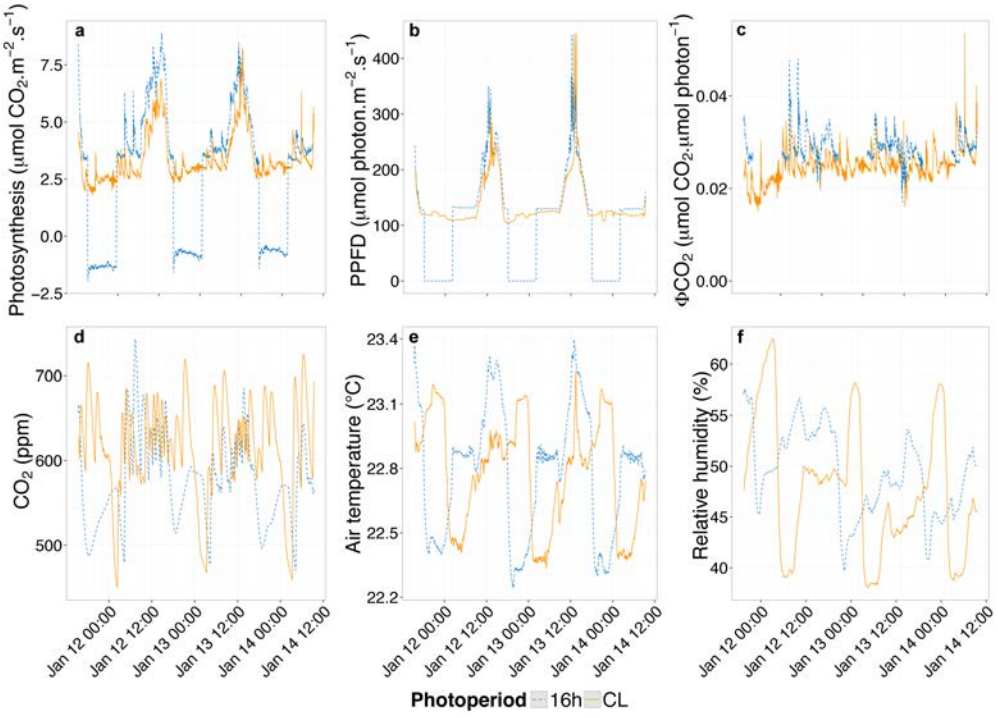


Supplementary Figure 4.1 | Virus-induced gene silencing in tomato is patchy. (**a**) Ten-days old A131 and CLT plants were infiltrated with several *pTRV* constructs (see construct name at the top). Two and a half weeks later, *pTRV::PDS* plants showed photo bleaching symptoms; at this point, all plants were transferred to continuous light. After three weeks of treatment, RGB images of

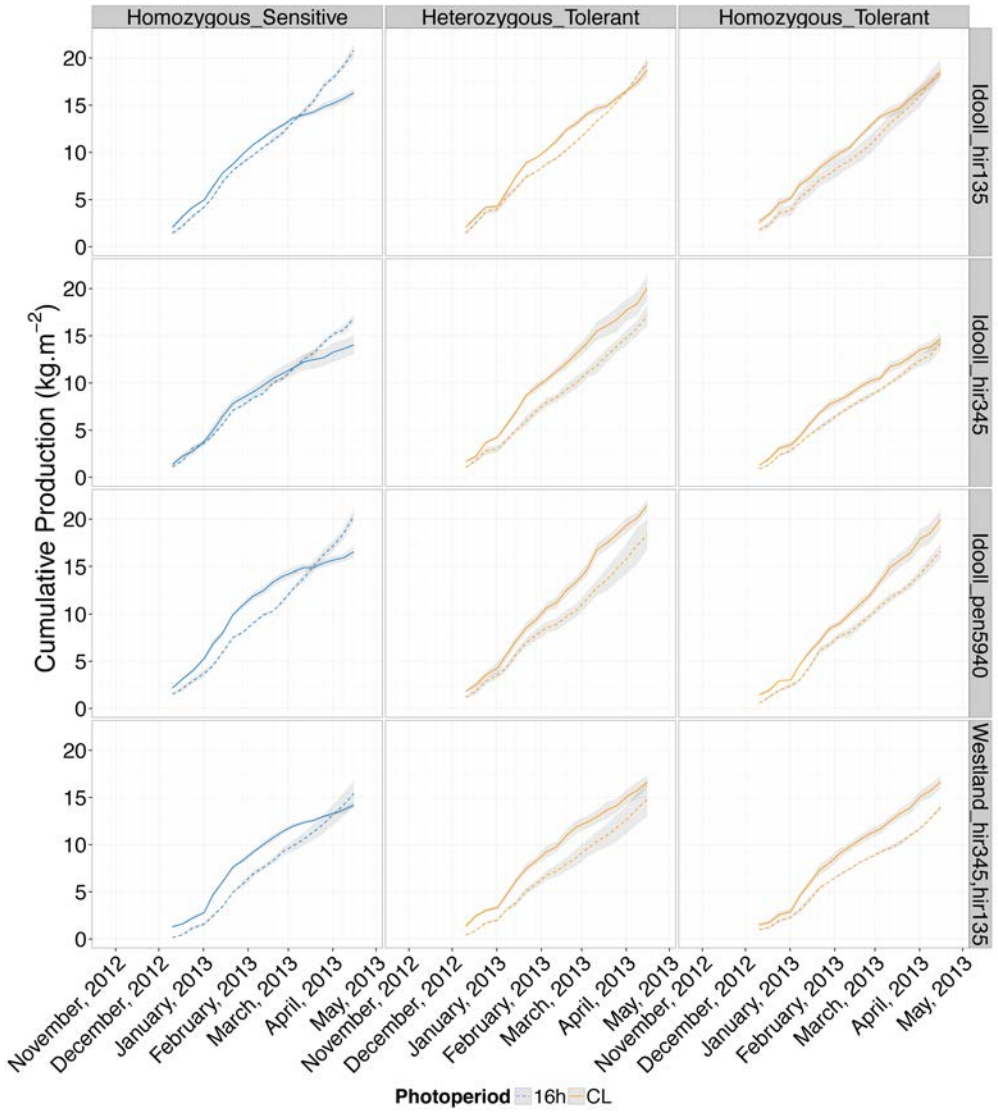
representative plants were taken. Notice the patchiness of the pTRV-induced silencing, which becomes evident by the photo-bleached spots on *pTRV::PDS* treated plants (indicated with white arrows). Scale bar = 10 cm. (**b**) *CAB-13* relative expression assessed by RT-qPCR. *CAB-13* expression is relative to Mock-treated A131 plants. Samples were collected after three weeks on exposure to continuous light (5.5 weeks after infiltration). Values represent mean ± s.e.m., n=4 independent replicates. (**c**) Mean Ct value of reference genes used to normalize *CAB-13* expression in (**b**), technical replicates were done.



Supplementary Figure 4.2 | Greenhouse climate data during yield trial. Measurements were registered daily from October 2012 to April 2013. (**a**) Average greenhouse air temperature during daytime. (**b**) Average greenhouse air temperature during nighttime. (**c**) Average greenhouse air temperature. (**d**) Average greenhouse air Relative humidity during daytime. (**e**) Average greenhouse air Relative humidity during nighttime. (**f**) Differential day-night greenhouse air temperature. (**g**) Incoming solar radiation. (**h**) Average CO_2 concentration in the greenhouse air during daytime. (**i**) Average CO_2 concentration in the greenhouse air during nighttime.



Supplementary Figure 4.3 | Gas exchange measurements during two days of continuous light. Using a gas exchange system and logging every minute, photosynthetic measurements where taken during a couple of clear sky days on continuous-light-tolerant tomatoes. Each line represents measurements on a single leaf. Such leaves, the topmost fully expanded leaf, developed completely under continuous light. (**a**) Photosynthesis rate. (**b**) Photosynthetic photon flux density (PPFD). (**c**) Quantum efficiency of CO_2 assimilation on the basis of absorbed PPDF. (**d**) CO_2 concentration in the reference IRGA after buffering the greenhouse CO_2 concentration with a 40-liter container. (**e**) Air temperature inside the leaf chamber. (**f**) Relative humidity inside the leaf chamber.



Supplementary Figure 4.4 | Cumulative Production under 16-h photoperiod and continuous ligth. Each panel depicts the yield of F_1 hybrids grouped in four categories; in each category, several continuous light-tolerant introgression lines were backcrossed with the parents of "Idooll" or "Westland" F_1 commercial hybrids (see background and wild donor on the left). After 3 to 4 backcrosses, the newly bread parents, segregating as continuous light-tolerant or -sensitive, where used to generate homozygous or heterozygous and tolerant or sensitive Idoll and Westalnd F_1 hybrids (see labels at the top). Yield per line was registered weekly from December 2012 to April 2013. Mean ± s.e.m. (grey shadow), n=3 plots.

Supplementary Table 4.1. Continuous light tolerance phenotype of 147 *Solanum* genotypes (mainly tomato).

Species	Details	Continuous light tolerance phenotype[a]	Reference
	Domesticated tomato		
S. lycopersicum	A131	–	This paper
S. lycopersicum	Alisa Craig	–	(Cushman & Tibbitts, 1998, Kristoffersen, 1963)
S. lycopersicum	Alisa Craig ACC-oxidase Antisense	*	(Cushman & Tibbitts, 1998)
S. lycopersicum	Alisa Craig *Never ripe* mutant	*	(Cushman & Tibbitts, 1998)
S. lycopersicum	Campari	–	This paper
S. lycopersicum	DRS540	–	This paper
S. lycopersicum	Encore	–	This paper
S. lycopersicum	Extra Early	–	(Kristoffersen, 1963)
S. lycopersicum	Extra Early Canner	–	(Hillman, 1956)
S. lycopersicum	Heinz[e]	–	This paper
S. lycopersicum	Indiana Baltimore	–	(Withrow & Withrow, 1949)
S. lycopersicum	Kecskemet 363	–	(Daskaloff & Ognjanova, 1965)
S. lycopersicum	Komeett	–	(Daskaloff & Ognjanova, 1965)
S. lycopersicum	Laura	–	(Globig *et al.*, 1997)
S. lycopersicum	M82	–	This paper
S. lycopersicum	Marmande	–	(Descomps & Deroche, 1973)
S. lycopersicum	Maxifort	–	This paper
S. lycopersicum	Momotaro Fight	–	(Matsuda *et al.*, 2012)
S. lycopersicum	Moneyberg	–	This paper
S. lycopersicum	Moneymaker	–	This paper
S. lycopersicum	Potentate	–	(Kristoffersen, 1963)
S. lycopersicum	Red Cherry	–	(Hillman, 1956)
S. lycopersicum	Selandia	–	(Kristoffersen, 1963)
S. lycopersicum	Sub-Arctic Plenty	+	This paper
S. lycopersicum	Trend	–	(Demers *et al.*, 1998, Dorais *et al.*, 1995, Dorais *et al.*, 1996)
S. lycopersicum	Tourance	–	This paper
S. lycopersicum	Vedettos	–	(Vézina *et al.*, 1991)
S. lycopersicum	Vendor	–	(Bradley & Janes, 1985, Globig *et al.*, 1997)
S. lycopersicum	Verlioka	–	(Sysoeva *et al.*, 2012)
S. lycopersicum	Westland	–	This paper
S. lycopersicum	Unknown cultivar[b]	–	(Arthur *et al.*, 1930)
	Wild tomato species		
S. chilense	LA1959	+	This paper
S. chmielewskii	LA1840	+	This paper
S. habrochaites	G1560	+	This paper
S. habrochaites	LA1777	+	This paper
S. habrochaites	Lyc4	+	This paper
S. habrochaites[3]	Unknown accession	+	(Daskaloff & Ognjanova, 1965)
S. pennelli	LA0716	+	This paper
S. neorickii	LA2133	+	This paper
S. peruvianum	LA1708	+	This paper
S. pimpinellifolium	LA1589	–	This paper
S. pimpinellifolium[4]	Unknown accession	+	(Daskaloff & Ognjanova, 1965)
	Inter-species hybrids		
S. lycopersicum x S.	"Triumph" (originally reported as *L. esculentum* cv. Kecskemet	+	(Daskaloff & Ognjanova, 1965)

S. lycopersicum x S. Pimpinellifolium[c] $-F_1$	"Triumph" (originally reported as *L. esculentum* cv. Kecskemet 363 x L. *racemigerum*)	+	(Daskaloff & Ognjanova, 1965)
S. lycopersicum x S. Pimpinellifolium[c]	"No. 10" (originally reported as inter-species crossing with *L. racemigerum*)	+	(Daskaloff & Ognjanova, 1965)
S. lycopersicum x S. Pimpinellifolium[c]	"Plovdivska konserva" (originally reported as inter-species crossing with *L. racemigerum*)	+	(Daskaloff & Ognjanova, 1965)
S. lycopersicum x S. Pimpinellifolium[c]	"XXIV-13" (originally reported as inter-species crossing with *L. racemigerum*)	+	(Daskaloff & Ognjanova, 1965)
S. lycopersicum x S. habrochaites[d] $-F_1$	(originally reported as *L. esculentum* cv. Komet x *L. hirsutum*)	-	(Daskaloff & Ognjanova, 1965)
Introgression line populations			
S. lycopersicum x *S. neorickii* population (42 Lines tested)	*S. neorickii* LA2133 in the genetic background of *S. lycopersicum* breeding line "A131"	- (without introgression on chromosome 7) + (with introgression on chromosome 7)	This paper
S. lycopersicum x *S. chilense* population (46 Lines tested)	*S. chilense* LA1959 in the genetic background of *S. lycopersicum* cv. "Moneyberg"	- (without introgression on chromosome 7) + (with introgression on chromosome 7)	This paper
S. lycopersicum x *S. pennellii* population (4 Lines tested)	*S. pennellii* LA0716 in the genetic background of *S. lycopersicum* cv. "M82"	- (without introgression on chromosome 7) + (with introgression on chromosome 7)	This paper
Potato and eggplant			
S. tuberosum	Kennebac	-	(Cao & Tibbitts, 1991, Cushman & Tibbitts, 1991, Cushman & Tibbitts, 1998, Cushman *et al.*, 1995, Tibbitts *et al.*, 1990, Wheeler & Tibbitts, 1986)
S. tuberosum	Superior	-	(Cao & Tibbitts, 1991, Cushman & Tibbitts, 1998, Tibbitts *et al.*, 1990, Wheeler & Tibbitts, 1986)
S. tuberosum	Denali	+	(Cao & Tibbitts, 1991, Cushman & Tibbitts, 1998)
S. tuberosum	Norland	+	(Cushman & Tibbitts, 1998, Wheeler & Tibbitts, 1986)
S. tuberosum	Haig	+	(Cao & Tibbitts, 1991)
S. tuberosum	Norchip	+	(Wheeler & Tibbitts, 1986)
S. tuberosum	Russet Burbank	+	(Wheeler & Tibbitts, 1986)
S. melongena	Senryo	-	(Murage & Masuda, 1997, Murage *et al.*, 1996, Murage *et al.*, 1997)

[a] After exposing the plants to continuous light, a given genotype was called sensitive to continuous light (-) if interveinal mottled chlorosis in young, fully expanded leaves was observed in our trials or reported in literature. Likewise, a given genotype was called tolerant to continuous light (+) if the complete foliage remained healthy, showing neither signs of chlorosis nor necrosis, after, at least, six weeks of exposure to continuous light. Lines marked with an asterisk (*) were reported to be slightly less sensitive that their respective controls, yet they still showed mottled chlorosis after exposure to continuous light (Cushman & Tibbitts, 1998).

[b] First report of CL-induced injury (Arthur *et al.*, 1930).

[c] Originally reported as *Lycopersicon racemigerum,* unknown accession number (Daskaloff & Ognjanova, 1965).

Supplementary Table 4.2. Mutations in Exon and promoter regions of all genes in the continuous-light-tolerance locus.

Gene	Description	Mutation	Position[a]	Heinz[b]	Alisa Craig[b]	Moneymaker[b]	A131[b]	LA1589[b]	LA1777[c]	LYC4[c]	LA716[c]	LA2133[c]	CLT[c]	Details/Relevance
Solyc07g06 3550.2	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1 (AHRD V1 *--- AGAP1_XENLA); contains Interpro domain(s) IPR001164 Arf GTPase activating protein	C>>T	6490	C	C	C	C	C	T	T	T	T	T	Silent
Solyc07g06 3560.2	Cotton fiber expressed protein 1 (AHRD V1 ***- O81373_GOSHI); contains Interpro domain(s) IPR008480 Protein of unknown function DUF761, plant	A>>T	348	A	A	A	?	A	T	T	T	T	T	Silent
		T>>C	552	T	T	T	?	T	C	C	C	C	?	Silent
		A>>G	1345	A	A	A	?	A	G	G	G	G	G	Silent
		C>>A	1479	C	C	C	C	C	A	A	A	A	A	Proline>>Glutamine (Nonpolar>>Polar)[d]
		C>>T	1684	C	C	C	C	C	T	T	T	T	T	Silent
Solyc07g06 3570.2	Cytochrome c biogenesis protein family (AHRD V1 ***-D7MU78_ARALY); contains Interpro domain(s) IPR003834 Cytochrome c assembly protein, transmembrane region	G>>A	598	G	G	G	G	G	A	A	A	A	A	Glycine>>Serine (Nonpolar>>Polar), in a low complexity region[d]
		ACA>>TCA/TCC	607-609	AC A	AC A	AC A	AC A	AC A	TC C	TC C	TC C	TC C	TC A	Threonine>>Serine/Serine (Both Polar), in a low complexity region[d]
		C>>T	655	C	C	C	C	C	T	T	T	T	T	Proline>>Serine (Nonpolar>>Polar)[d]
		A>>G	3917	A	A	A	A	A	G	G	G	G	G	Silent
Solyc07g06 3580.2	Unknown Protein (AHRD V1)	A>>G	-196	A	A	A	?	?	G	G	G	G	?	?
		T>>C	-151	T	T	T	?	?	C	C	C	C	?	?
		C>>T	-85	C	C	C	?	?	T	T	T	T	?	?
Solyc07g06 3590.2	Myosin-like protein (AHRD V1 ***-Q9LHE9_ARATH); contains Interpro domain(s) IPR001609 Myosin head, motor region	G>>A	384	G	G	G	G	G	A	A	A	A	?	Silent
		C>>G	438	C	C	C	?	C	G	G	G	G	G	Isoleucine>>Methionine (Both Nonpolar)[d]
		GCG>>GCA/GTA	1134-1135	GC G	GC G	GC G	GC G	GC G	GC A	GC A	GT A	GC A	GC A	Alanine>>Alanine/Valine (All Nonpolar)
		A>>T	1254	A	A	A	?	A	T	T	T	T	T	Silent
		C>>A	1934	C	C	C	C	C	A	A	A	A	A	Proline>>Threonine (Nonpolar>>Polar) Inside predicted domain (myosin motor domain). Out side aTP-binding site
		A>>G	3414	A	A	A	A	A	G	G	G	G	G	Silent
		T>>A	5619	T	T	T	T	T	A	A	A	A	A	Silent
		T>>C	5688	T	T	T	?	T	C	C	C	C	C	Silent
		C>>T	6720	C	C	C	C	C	T	T	T	T	T	Threonine>>Isoleucine (Polar>>Nonpolar)

														Inside predicted domain (myosin motor domain). Out side aTP-binding site
		G>>A	7269	G	G	G	?	G	A	A	A	A	?	Serine>>Asparagine (Both Nonpolar) Inside predicted domain (myosin motor domain). Out side aTP-binding site
		C>>A	7354	C	C	C	C	C	A	A	A	A	A	Silent
		C>>T	9502	C	C	C	C	C	T	T	T	T	?	Threonine>>Isoleucine (Polar>>Nonpolar), in a low complexity region[d]
		A>>G	10044	A	A	A	?	A	G	G	G	G	G	Silent
Solyc07g063600.2	Chlorophyll a-b binding protein 13, chloroplastic (AHRD V1 ***-CB23_SOLLC); contains Interpro domain(s) IPR001344 Chlorophyll A-B binding protein	9bp deletion	-39	NO	NO	NO	NO	NO	YES	YES	YES	YES	YES	See Figure 1 for details
Solyc07g063610.2	Dynein light chain 2 cytoplasmic (AHRD V1 ***- C3KIM6_ANOFI); contains Interpro domain(s) IPR001372 Dynein light chain, type 1 and 2	No Mutations												
Solyc07g063620.2	Uncharacterized secreted protein (AHRD V1 **-- B6TUX2_MAIZE); contains Interpro domain(s) IPR010634 Protein of unknown function DUF1223	A>>T	-89	A	A	A	A	A	T	T	T	T	T	?
		G>>T	-87	G	G	G	G	G	T	T	T	T	T	?
		G>>A	67	G	G	G	?	G	A	A	A	A	A	Glycine>>Serine (Nonpolar>>Polar), in a low complexity region[d]
		G>>C	81	G	G	G	?	G	C	C	C	C	C	Lysine>>Asparagine (Basic>>Polar)[2]
Solyc07g063630.2	Vesicle-associated membrane family protein (AHRD V1 ***-D7MET3_ARALY); contains Interpro domain(s) IPR008962 PapD-like	A>>G	1385	A	A	A	A	A	G	G	G	G	G	Isoleucine>>Methionine (Both Nonpolar) Inside predicted domain (inositol metabolism)
		T>>A	2724	T	T	T	T	T	A	A	A	A	A	Aspartic acid>>Glutamic acid (Both Acidic)[d]
		G>>A	2733	G	G	G	G	G	A	A	A	A	A	Silent
Solyc07g063640.1	Wound induced protein (AHRD V1 ***- B6SKC8_MAIZE)	No Mutations												
Solyc07g063650.2	Ubiquitin carboxyl-terminal hydrolase (AHRD V1 **** B9N564_POPTR); contains Interpro domain(s) IPR016652 Ubiquitinyl hydrolase	C>>T	6515	C	C	C	C	C	T	T	T	T	T	Silent
		C>>T	8136	C	C	C	C	C	T	T	T	T	T	Silent
		C>>T	9586	C	C	C	C	C	T	T	T	T	C	Silent

[a] Relative to the start codon.
[b] Continuous light sensitive.
[c] Continuous light tolerant.
[d] Outside predicted domains in NCBI.

Supplementary Table 4.3. Expression ratio of all genes in the continuous light tolerant locus between A131 16h vs A131 24h and A131 24h vs CLT 24h.

		A131 24h – A131 16h				A131 24h – CLT 24h			
Gene	Description	logFC	logCPM	p-value	FDR-corrected p-value	logFC	logCPM	p-value	FDR-corrected p-value
Solyc07g063550.2	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1 (AHRD V1 *--- AGAP1_XENLA); contains Interpro domain(s) IPR001164 Arf GTPase activating protein	-0.01	5.39	9.76E-01	9.87E-01	0.11	5.39	5.30E-01	8.22E-01
Solyc07g063560.2	Cotton fiber expressed protein 1 (AHRD V1 ***- O81373_GOSHI); contains Interpro domain(s) IPR008480 Protein of unknown function DUF761, plant	1.27	0.44	2.15E-02	9.55E-02	0.81	0.44	1.23E-01	4.15E-01
Solyc07g063570.2	Cytochrome c biogenesis protein family (AHRD V1 ***- D7MU78_ARALY); contains Interpro domain(s) IPR003834 Cytochrome c assembly protein, transmembrane region	-0.21	5.51	2.46E-01	5.02E-01	0.02	5.51	9.25E-01	9.82E-01
Solyc07g063580.2	Unknown Protein (AHRD V1)	0.46	2.68	1.16E-01	3.16E-01	-1.01	2.68	2.13E-04	5.60E-03
Solyc07g063590.2	Myosin-like protein (AHRD V1 ***- Q9LHE9_ARATH); contains Interpro domain(s) IPR001609 Myosin head, motor region	0.63	3.26	1.67E-02	7.91E-02	-0.60	3.26	1.74E-02	1.27E-01
Solyc07g063600.2	Chlorophyll a-b binding protein 13, chloroplastic (AHRD V1 ***- CB23_SOLLC); contains Interpro domain(s) IPR001344 Chlorophyll A-B binding protein	-3.43	11.61	1.83E-12	1.17E-10	-2.10	11.61	5.27E-06	3.21E-04
Solyc07g063610.2	Dynein light chain 2 cytoplasmic (AHRD V1 ***- C3KIM6_ANOFI); contains Interpro domain(s) IPR001372 Dynein light chain, type 1 and 2	0.44	6.58	5.89E-02	1.97E-01	0.02	6.58	9.39E-01	9.82E-01
Solyc07g063620.2	Uncharacterized secreted protein (AHRD V1 **-- B6TUX2_MAIZE); contains Interpro domain(s) IPR010634 Protein of unknown function DUF1223	0.51	1.96	1.06E-01	2.97E-01	0.34	1.96	2.63E-01	6.14E-01
Solyc07g063630.2	Vesicle-associated membrane family protein (AHRD V1 ***- D7MET3_ARALY); contains Interpro domain(s) IPR008962 PapD-like	0.27	5.71	1.58E-01	3.89E-01	0.21	5.71	2.83E-01	6.31E-01
Solyc07g063640.1	Wound induced protein (AHRD V1 ***- B6SKC8_MAIZE)	-0.61	-0.03	2.28E-01	4.78E-01	-0.67	-0.03	1.70E-01	4.96E-01
Solyc07g063650.2	Ubiquitin carboxyl-terminal hydrolase (AHRD V1 **** B9N564_POPTR); contains Interpro domain(s) IPR016652 Ubiquitinyl hydrolase	0.13	5.22	4.70E-01	7.13E-01	0.48	5.22	7.89E-03	7.44E-02

Supplementary Table 4.4. Transcription factor binding sites in the *S. neorickii* LA2133 *CAB-13* promoter (chromosome 7).

Factor	Site[a]	Strand	Seq	Species	Source
ATHB-5	-97	+	cagTTATTc	Arabidopsis	TRANSFAC
RAV1	-103	+	ttcCAACAgtta	Arabidopsis	TRANSFAC
Dof1	-92	-	attCTTTAtat	Maize	TRANSFAC
PBF	-29	+	attAAAAGaga	Maize	TRANSFAC
MYB.Ph3	-175	-	aaAACTAtttcac	Petunia	TRANSFAC
-10PEHVPSBD	-93	+	TATTCT	Barley	PLACE
ARR10	-111	+	AGATATTT	Arabidopsis	JASPER
CAATBOX1	-69	-	ATTG	pea	PLACE
CAATBOX1	-46	-	ATTG	pea	PLACE
CAATBOX1	-35	+	CAAT	pea	PLACE
CAATBOX1	-11	+	CAAT	pea	PLACE
CAATBOX1	-158	+	CAAT	pea	PLACE
CAATBOX1	-132	-	ATTG	pea	PLACE
CCAATBOX1	-36	+	CCAAT	Soybean	PLACE
Core	-33	+	ATTA	Arabidopsis	AGRIS
Core	-31	-	TAAT	Arabidopsis	AGRIS
Core	-29	+	ATTA	Arabidopsis	AGRIS
Core	-17	+	ATTA	Arabidopsis	AGRIS
DOFCOREZM	-25	+	AAAG	maize	PLACE
DOFCOREZM	-89	-	CTTT	maize	PLACE
DPBFCOREDCDC3	-146	+	ACACAAG	carrot/Arabidopsis	PLACE
EVENINGAT	-111	-	AGATATTTT	Arabidopsis/eggplant	PLACE
GATABOX	-110	+	GATA	petunia/Arabidopsis/rice	PLACE
GATABOX	-110	+	GATA	petunia/Arabidopsis/rice	PLACE
GATABOX	-110	+	GATA	petunia/Arabidopsis/rice	PLACE
GT1CONSENSUS	-50	+	GAAAAT	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-50	+	GAAAAT	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-50	+	GAAAAT	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-50	+	GAAAAT	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-106	-	TTTTTC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-106	-	TTTTTC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-106	-	TTTTTC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-106	-	TTTTTC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-105	-	TTTTCC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-105	-	TTTTCC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-105	-	TTTTCC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1CONSENSUS	-105	-	TTTTCC	pea/oat/rice/tobacco/Arabidopsis	PLACE
GT1GMSCAM4	-106	-	TTTTTC	soybean	PLACE
HMG-1	-30	-	AATTAAAAG	Pea	JASPER
HMG-1	-148	-	AAACACAAG	Pea	JASPER
MNB1A	-25	+	AAAGA	Maize	JASPER
MNB1A	-90	-	TCTTT	Maize	JASPER
MYB.ph3	-174	+	AAACTATTT	Petunia	JASPER
MYB2AT	-97	-	CAGTTA	Arabidopsis	PLACE
MYB2CONSENSUSAT	-97	-	CAGTTA	Arabidopsis	PLACE
MYBCORE	-100	-	CAACAG	Arabidopsis/petunia	PLACE
MYBCORE	-100	-	CAACAG	Arabidopsis/petunia	PLACE
MYBCORE	-97	+	CAGTTA	Arabidopsis/petunia	PLACE
MYBCORE	-97	+	CAGTTA	Arabidopsis/petunia	PLACE
NODCON2GM	-24	-	AAGAG	soybean	PLACE
OSE2ROOTNODULE	-24	-	AAGAG	bean/Medicago/soybean/Sesbania	PLACE
OSE2ROOTNODULE	-24	-	AAGAG	bean/Medicago/soybean/Sesbania	PLACE
OSE2ROOTNODULE	-24	-	AAGAG	bean/Medicago/soybean/Sesbania	PLACE
POLASIG2	-30	+	AATTAAA	rice	PLACE
POLLEN1LELAT52	-61	+	AGAAA	tomato	PLACE
POLLEN1LELAT52	-21	+	AGAAA	tomato	PLACE
POLLEN1LELAT52	-5	+	AGAAA	tomato	PLACE
RAV1-A	-100	+	CAACA	Arabidopsis	AGRIS
RAV1AAT	-100	+	CAACA	Arabidopsis	PLACE
SORLIP2AT	-121	+	GGGCC	Arabidopsis	PLACE
SORLIP2AT	-120	-	GGCCC	Arabidopsis	PLACE
TAAAGSTKST1	-89	-	CTTTA	potato	PLACE
TATABOX2	-74	+	TATAAAT	pea/tobacco/bean	PLACE
TATABOX2	-74	+	TATAAAT	pea/tobacco/bean	PLACE
TATABOX2	-74	+	TATAAAT	pea/tobacco/bean	PLACE
TATABOX4	-76	+	TATATAA	bean/sweet	potato
TATABOX4	-87	-	TTATATA	bean/sweet	potato
TATABOX4	-84	+	TATATAA	bean/sweet	potato
TATAPVTRNALEU	-76	-	TATATAAA	bean/maize	PLACE
TATAPVTRNALEU	-76	-	TATATAAA	bean/maize	PLACE
TATAPVTRNALEU	-88	+	TTTATATA	bean/maize	PLACE
TATAPVTRNALEU	-88	+	TTTATATA	bean/maize	PLACE

Produced by "PlantPAN" tool[25]. [a] **Relative to the start codon.**

Supplementary Table 4.5. Top 20 differentially expressed genes (by FDR-corrected p-value) of the contrast CL A131 – 16h A131.

Gene	Description	logFC	logCPM	p-value	FDR-corrected p-value
Solyc08g063090.2	Delta-6-desaturase (AHRD V1 **** D3YN48_9ERIC); contains Interpro domain(s) IPR012171 Fatty acid/sphingolipid desaturase	-3.99	5.49	1.76E-83	5.52E-79
Solyc06g071820.2	Speckle-type poz protein (AHRD V1 *--- Q179U2_AEDAE); contains Interpro domain(s) IPR000197 Zinc finger, TAZ-type	-3.72	5.69	2.91E-61	4.56E-57
Solyc08g007130.2	Beta-amylase 8 (AHRD V1 **** D7MC27_ARALY); contains Interpro domain(s) IPR001554 Glycoside hydrolase, family 14	4.28	8.08	8.42E-56	8.80E-52
Solyc08g075490.2	Carotenoid cleavage dioxygenase 4B	-3.61	6.46	1.69E-52	1.32E-48
Solyc01g010480.2	Potassium voltage-gated channel subfamily H member 8 (AHRD V1 *--- KCNH8_HUMAN); contains Interpro domain(s) IPR013767 PAS fold	-3.86	6.71	2.38E-51	1.49E-47
Solyc08g005960.1	Cortical cell-delineating protein (AHRD V1 *--- B6U436_MAIZE); contains Interpro domain(s) IPR013770 Plant lipid transfer protein and hydrophobic protein, helical	-4.43	8.92	2.98E-50	1.56E-46
Solyc03g120020.2	AE family transporter anion exchange (AHRD V1 *--* A4RY02_OSTLU); contains Interpro domain(s) IPR003020 Bicarbonate transporter, eukaryotic	-4.27	3.30	4.41E-45	1.98E-41
Solyc04g050620.2	Cytochrome P450	-3.53	4.47	1.62E-44	6.36E-41
Solyc01g006830.2	Os02g0200800 protein (Fragment) (AHRD V1 *-*- Q0E303_ORYSJ); contains Interpro domain(s) IPR009675 Targeting for Xklp2	-2.78	6.15	1.69E-42	5.87E-39
Solyc05g009420.1	Unknown Protein (AHRD V1)	2.99	5.42	2.62E-42	8.22E-39
Solyc03g115770.2	Timing of CAB expression-like	2.99	5.57	1.22E-40	3.48E-37
Solyc10g078510.1	Glucan endo-1 3-beta-glucosidase 5 (AHRD V1 ***- B6SXV6_MAIZE); contains Interpro domain(s) IPR013781 Glycoside hydrolase, subgroup, catalytic core	-4.01	2.54	4.90E-39	1.28E-35
Solyc04g079050.1	UDP-glucosyltransferase (AHRD V1 **** Q8H6A4_STERE); contains Interpro domain(s) IPR002213 UDP-glucuronosyl/UDP-glucosyltransferase	-3.07	5.36	1.11E-38	2.68E-35
Solyc06g068620.2	Unknown Protein (AHRD V1)	-3.54	4.04	4.23E-37	9.48E-34
Solyc06g069150.1	Genomic DNA chromosome 3 P1 clone MPE11 (AHRD V1 *-*- Q9LUA5_ARATH)	-3.31	4.46	1.72E-36	3.58E-33
Solyc08g029000.2	Lipoxygenase (AHRD V1 **** Q43191_SOLTU); contains Interpro domain(s) IPR001246 Lipoxygenase, plant	4.25	3.09	1.95E-34	3.83E-31
Solyc09g092260.2	Chaperone protein dnaJ 20 (AHRD V1 ***- B6U349_MAIZE); contains Interpro domain(s) IPR001623 Heat shock protein DnaJ, N-terminal	3.20	4.93	1.24E-33	2.28E-30
Solyc04g071800.2	Cytochrome P450	-3.30	8.25	1.52E-33	2.64E-30
Solyc11g010380.1	Mate efflux family protein (AHRD V1 **-- D7MN36_ARALY); contains Interpro domain(s) IPR002528 Multi antimicrobial extrusion protein MatE	2.71	7.09	3.13E-33	5.17E-30
Solyc06g082190.2	Serine/threonine/tyrosine kinase (AHRD V1 *-** Q9AWA6_ARAHY); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase	-3.20	2.60	1.07E-32	1.68E-29

Supplementary Table 4.6. Top 20 differentially expressed genes (by FDR-corrected p-value) of the contrast CL A131 - CL CLT.

Gene	Description	logFC	logCPM	p-value	FDR-corrected p-value
Solyc07g053530.1	Fasciclin-like arabinogalactan protein 4 (AHRD V1 ***- A9XTK9_GOSHI); contains Interpro domain(s) IPR000782 FAS1 domain	-3.25	4.96	5.53E-44	1.73E-39
Solyc07g052790.1	Nbs-lrr, resistance protein	4.75	2.20	2.38E-41	3.73E-37
Solyc12g038650.1	Unknown Protein (AHRD V1)	-7.47	1.32	8.86E-33	9.26E-29
Solyc07g056510.2	Glutathione S-transferase (AHRD V1 **** D3Y4H6_9ROSI); contains Interpro domain(s) IPR004046 Glutathione S-transferase, C-terminal	5.60	2.77	1.06E-31	8.34E-28
Solyc07g063270.2	Nucleolar GTP-binding protein 2 (AHRD V1 **** B6TMG1_MAIZE); contains Interpro domain(s) IPR012971 NGP1, N-terminal	-2.26	7.40	1.05E-28	6.59E-25
Solyc03g006700.2	Peroxidase (AHRD V1 **** Q50LG4_TOBAC); contains Interpro domain(s) IPR002016 Haem peroxidase, plant/fungal/bacterial	3.00	3.47	5.31E-27	2.77E-23
Solyc07g052470.2	Syntaxin (AHRD V1 ***- Q6X9V9_HORVD); contains Interpro domain(s) IPR010989 t-SNARE	3.62	1.78	1.32E-26	5.90E-23
Solyc07g062500.2	Cytochrome P450	3.48	5.44	1.78E-25	6.97E-22
Solyc07g062700.2	Sodium/calcium exchanger family protein (AHRD V1 ***- D7KKF3_ARALY); contains Interpro domain(s) IPR004837 Sodium/calcium exchanger membrane region	2.71	8.51	3.04E-25	1.02E-21
Solyc07g055530.2	Cytochrome P450	3.97	1.87	3.25E-25	1.02E-21
Solyc10g083690.2	Cytochrome P450	3.11	2.41	1.33E-23	3.78E-20
Solyc06g011350.2	Aquaporin 2 (AHRD V1 ***- O65357_SAMSA); contains Interpro domain(s) IPR012269 Aquaporin	3.71	3.99	7.89E-23	2.06E-19
Solyc02g092860.2	Cytochrome P450	2.35	3.42	3.23E-22	7.80E-19
Solyc07g065410.1	Unknown Protein (AHRD V1)	2.77	3.44	2.21E-20	4.94E-17
Solyc08g029000.2	Lipoxygenase (AHRD V1 **** Q43191_SOLTU); contains Interpro domain(s) IPR001246 Lipoxygenase, plant	2.76	3.09	6.21E-20	1.30E-16
Solyc07g056320.2	ER glycerol-phosphate acyltransferase (AHRD V1 **** B9T753_RICCO); contains Interpro domain(s) IPR002123 Phospholipid/glycerol acyltransferase	1.90	3.99	1.17E-18	2.30E-15
Solyc09g007520.2	Peroxidase (AHRD V1 **** C1KA92_POPTR); contains Interpro domain(s) IPR000823 Plant peroxidase	4.34	1.60	2.56E-17	4.66E-14
Solyc07g056450.2	Glutathione S-transferase-like protein (AHRD V1 **** Q8GVD1_SOLLC); contains Interpro domain(s) IPR004045 Glutathione S-transferase, N-terminal	-3.87	0.60	2.68E-17	4.66E-14
Solyc07g062810.2	Branched-chain amino acid aminotransferase-like (AHRD V1 **** Q5NAM3_ORYSJ); contains Interpro domain(s) IPR001544 Aminotransferase, class IV	3.03	1.94	3.62E-17	5.98E-14
Solyc08g068440.2	Lipoyl synthase (AHRD V1 **** B8J937_ANAD2); contains Interpro domain(s) IPR013785 Aldolase-type TIM barrel	-4.23	0.48	4.32E-17	6.78E-14

Supplementary Table 4.7. Effect of continuous light on leaf carbohydrate content of A131 and CLT plants.

Time of day[a]	16h photoperiod		Continuous light[a]		Significance (p-value)[b]		
	A131	CLT	A131	CLT	Genotype	Photoperiod	Interaction
			glucose (µg.mg^{-1})				
End of Dark	10.97±1.58	18.31±0.48	3.99±0.65	8.2±0.52	0.000	0.000	0.149
End of Light	13.71±1.23	19.65±1.45	4.82±1.04	10.32±1.15	0.002	0.000	0.882
			fructose (µg.mg^{-1})				
End of Dark	21.58±2.46	31.92±0.77	8.57±1.45	15.06±1.21	0.001	0.000	0.319
End of Light	26.71±1.26	33.16±0.96	10.76±2.26	20.74±1.64	0.002	0.000	0.423
			sucrose (µg.mg^{-1})				
End of Dark	6.32±0.35	6.23±0.21	11.41±0.47	9.75±0.49	0.121	0.000	0.159
End of Light	12.48±0.70	10.17±0.45	10.74±0.27	10.90±0.36	0.062	0.353	0.036

One month-old tomato plants, grown under 16-h photoperiod, were exposed to continuous light or kept under 16-h photoperiod for three weeks. The top-most fully expanded leaf was used for carbohydrate determination (see Supplementary Methods). Values represent mean ± s.e.m., n=4.
[a] In the continuous light treatment, two sets of samples were taken. Although the lamps were always ON, samples were taken at the same time as in 16-h photoperiod; in other words, samples were taken at the subjective "end of dark" and subjective "end of light" times.
[b] Calculated using analysis of variance (ANOVA)

Supplementary Table 4.8. SNP markers mapping to chromosome 7.

Marker	Marker	SNP position on chromosome 7 (bp)[a]	Sequence	*S. lycopersicum* allele
1		139841	GATGAGATTGTTACATGATTTTGTGCAGATCTAGCACAAGCCTTAGCCAAATTAAAGGAG[T/G]CCTCTCCATATTCGGTCCCTCTGGTCATTTTCGGTCACATGCACAAACAGCTTGCTTCTG	T
2	C2_At4g30580	462817	TTNGAGAATCTTCCTTCTCCAGATACTCCTGCAGTATATGTGTCCAACCATCAGAGCTTT[T/C]TGGACATATATACATTACTTACTCTTGGGAGAAACTTCAAGTTCATC	T
3	C2_At5g57655	1519534	TTTTCATTTTGCGTTTTCTGCAGGAGAGTTCAAATTGAACATCGAGTGCAACCATGCCAC[T/G]TTGGCTGGTCACAGGTTTGTGTGTTTTCAGTGCAAATTAACTATGTCCGTCTTTTGCACC	G
4		1691515	GTCATTACTATGTCCACAGATGCCACAGATCTAGATCA[A/T]CCGTTACTATAGCCACAGATGCCACAGATCTAGATCATATTATCCTTCTTGCATTTTGTG	A
5	C2_At1g14850	2880982	TAGATTGCGTCTCCTGCGCTCCGTACTGGCTTTACTTCGTGAATGGGCACTTTCTGTCTT[T/C]GCACAGGGAATGGGTACAAGTGTTACTGGAGCCTCTCTGATTCTTGGTGGAACTTTATCA	T
6		16129772	TTATGACTTAGCCAACGGGAAAACATTTAAANTGGATGTTTTTGTATATTTACCTCCAAT[A/T]AAACCCTAAATGAATTTCATGACTAATAAATGGTTCAAANCAATGATATTTTCATCTTTA	T
7		32765743	TGTTATACCGTGATTTATANCAAATGATTGGACAATAAGGCCTTTCACCATTCTTGCTCT[T/C]GATGTCAGTGTCACTGAAAGTAACAGTAGTTGGAGTACCCATAATATTTGATCGGATGGA	C
8	C2_At3g13050	53550100	TATTTTAGCAGCTATAATGGTGGATACAATTGGTCGAAAATTCAGTGTGGCACTTATGTG[T/C]GGTTTAAGCTTCCTGTTCCTTTTACCGCTTCTTGCACCTCAACTCCCTGCTTTNACTACT	C
9		55245506	AGGTCCTCATTGTCTGTGTTGCTCCGCCCTGCAATTGTCCTAAATTTAACTGAACTAATG[T/C]ATGGGGAAAAATGAAATGGGGATTCGTTTGTGCAGGGAGGATTTGAATCTGGTGCAGTGG	C
10		55835456	AAAGGTCAGGCTATTGTTTCTTTTTTGTAGTTTGTAGTAGCACAATTAGGAACCAAATGC[A/G]TTGTGCTACAGTAGTCTTAGTACAAGCTGGTGCAAATATACCTGCTACCGTTATGGTCCC	G
11	C2_At4g26680	56184660	CGAGATGAAGAGAACCGGTGTGGCTCCTAATGTTGTCACTTATAATACGCTGATAAACGC[A/G]TATAGTCAGGTTGGTAATTCTGAAATGGGAAGTAGGCTTTTCGAGGAGATGGCAAACAAT	A
12	T1726	56600584	TTTCAAGAACCTTAGTGATGTTAGCCCTATTAATACCTTGGCTGGAGGNAACTTATACTC[T/G]TTGAACTTCACCGATGACTCTGGGACCGTTCATCTTAACTCAGGATGGTCTAGGACTAAA	T
13	C2_At4g03210	58725585	ACTTGTGTTTAAATTGCAGATGTCATCAGATGGACCAACCCACAATGAGTTTGATTTTGA[A/G]TTTTTAGGCAATACAACTGGTGAACCATATACAGTACAAACAAATGTGTATGTCAATGGT	A
14		58987587	TCAACTAAAGAAAGAACTGCAACTGAACTCCCTTCAAACTGACACGATTAAATGCCTGCT[A/T]AANAAAGTTCACAAAGACGAAACAGGTATAAAATGTTTTGTTATGTGAGAATTCCTATAC	T
15		59245967	CAGCATGGGAAGGACTAGAATGTGTCTGACATGTCCTTCAAGGTATTTTTTCAGTTGTTC[A/T]TTGCAGGTTTTATTACTGTACAGAGCATGATGATACATAATTAGAAGATTGGTTNTATCT	A
16		62151010	ATTCATAGCGTAATCTGTTAATCCGTATGCCCATCAATGTTGTTTGTAGTTCAGGATGTT[T/C]GGATGGATACTCACAGTCTTTGGCCTATTTGNTATTATTGTACTTGGAAGCTTGTTTATA	T
17		62403930	AGAACTATTGAAGAAATCAGTACATCTCCACATCCCTTTTTTGAAGAAAAAAAATACACC[T/G]AACAGTTTAAAAATGAAAATGGAAGGCATGGAGGAATTCCTAGCTAAAATCTTTAGTTAT	G
18		62977648	TTTGTCGTTTGATACTTGTATGCAATCAGCTTCAGTAGCCAGCTTCGTATATCTTTCAGA[A/T]GAAAAANATCCTGATTTGTTACATAGTATTTCGGTATGCACTTGTGACTGATCAGTTTAC	A
19	T1738	63597060	TGTCCTNTGATTTCTATCTCCCTGNTTTGTGTAATAAAGAAGTTTGTGTATTTGGCAGAT[T/C]GAGGCAGTAGTGACTAAGGCTGAGCTCAAGTATCTTGCTTTCTTGTGCAAGTCTGAGGTT	C
20		63802071	GAAGCTGAAGAAGATTGGCTTTGCATGCTAAGGAAGAGTTTCTGATTATTACCAGGTACA[A/G]CAACTTCTTTCTTCATGAGTTTCCTCTTGCGTAAAGATACAGAAATTGGAATATTGTCAT	A
7-20-1		63223672	GAATTGGCTTTATGTATTTGGAATCCTTGTATTTACTGTATTTTTCTTTT[T/G]CTCCAATGCAGGGGTCCTAGATGG	T
7-20-1A		63239042	TCTGCTGTTGTTATTTCAGTATATGTTATCTCATAAAGAAAAAAAACAGAACAGAGTAGT[A/G]AGAAAGGAACCATTTCTTTAATTCATGGGAAACAGGCCATACAAAAATATACATGCACAT	A
7-20-1B		63263971	TCCCTTAGGGGGTTAGCATATTTGGTCTGGCCCAAAAATGGAGTTGCTCTAACAACTGTGGCTGAGCTACCTGTTG[C\T]TGCCATTTTCTCTATTGCTTAATTTCTCTTTTAATTAATTGGAC	C
7-20-1C		63285480	CCAATCCATTGCTGCCTCTACTCCACTGTTGGAAGTATTGATAGCAGCCTTCTGACAATG[A/G]AGTAGATTAAATCCCATTGAAACAAGTTGTGCAACAATATCATCGTCAGCCAGAAGCTTT	A
21		64122769	GGAAAGCCTACTGTTGTGGAATTCTATGCCGATTGGTGTGAAGTTTGTCGAGAATTAGCT[T/C]CAGATGTCTATAAAGTTGAACAGCAGTACAAGTAAATCTCTTTTTTGTTATTGACTCCTG	T
22		64803574	ATCAAGGGTTTATAACAATAAAAAGGTAATCATCAGAAAATGATGTATAGTTGGAAAAAA[A/G]AACCTTCCAAGATGGTGAATCAAAAGCATAAAAAATAGCTTCCTTGAATTCGTCTTTGTA	G
23		65015001	TATTACTGGTTANTCGCCATCTATTTGGTTTCAAATGCTTGGCTGCTTAGTTATTACTAA[T/C]TTTTGGTTTTGATTACACATAGTGGACTAACTCCTACATGAAGATGAATAGAACAGTTAT	C

[a] Based on the public tomato genome sequence v2.4 **(Tomato Genome Consortium, 2012)**.

Supplementary Methods

Photosynthesis parameter estimation

The parameters of the FvCB model (Farquhar *et al.*, 1980), where estimated combining gas exchange and chlorophyll fluorescence data according to Yin *et al.* (2009), with some modifications. When needed, measured leaf absorbance values where used to calculate absorbed light. Day respiration (R_d) and the lumped parameter s were estimated as the intercept and slope of the linear regression of photosynthesis against ($I_{inc}\Phi_2/4$) at limiting light (<100 μmol photon.m^{-2}.s^{-1}) according to (Yin *et al.*, 2009, Yin *et al.*, 2011). Where I_{inc} is the incident light and Φ_2 is the measured $\Delta F_v/F_m'$ at each light intensity. Then, the quantum efficiency of PSII e^- flow at strictly limiting light ($\Phi_{2(LL)}$) was estimated according to Yin *et al.* (2009) using the measured $\Delta F_v/F_m'$ as input. The conversion efficiency of incident light into e^- flow at strictly limiting light ($\kappa_{2(LL)}$), the convexity factor between e^- transport rate and incident light (θ) and the maximum e^- transport rate (J_{max}) where estimated according to Yin *et al.* (2009) using previously calculated parameters s and $\Phi_{2(LL)}$ as well as measured I_{inc} and $\Delta F_v/F_m'$. As technical difficulties in the greenhouse prevented us to measure photosynthesis at low O_2 concentration, calculating the kinetic properties of Rubisco as suggested by Yin *et al.* (2009) was not possible. Therefore a widely used alternative was adopted (Sharkey *et al.*, 2007), which consisted in using temperature corrected, generic values for the Michaelis-Menten constants of Rubisco for CO_2 and O_2 (K_{mC} and K_{mO}, respectively) as well as parameter Γ^*. At 25°C, K_{mC}=272.372mbar, K_{mO}=165.788mbar and Γ^*= 37.411mbar (Bernacchi *et al.*, 2002). Assuming a variable mesophyll conductance, the maximum rate of Rubisco activity-limited carboxylation (V_{cmax}) and the rate of triose phosphate export from the chloroplast (T_p) were estimated by using all equations of the FvCB model, all data points from the light and CO_2 curves and all previously estimated parameters in a single iteration procedure according to Yin *et al.* (2009). Photosynthesis under the two lowest and highest CO_2 concentrations were manually set to be Rubisco-limited and triose phosphate utilization-limited, respectively. The model automatically estimated the limitations under all other CO_2 concentrations and all light intensities.

Carbohydrate quantification

For carbohydrates content quantification, 15 mg of freeze-dried leaf material were extracted in 5 ml of ethanol 80% (v/v) at 80 °C for 20 minutes. After centrifuging the mixture for 5 min at 7000 RCF, the supernatant was recovered and evaporated using a rotavapor apparatus. The reduced supernatant residue was re-suspended in a final volume of 1 ml distilled water, placed in an ultrasonic bath for 10 minutes and centrifuged 15 min at 25000 RCF to remove the insoluble particles. Finally, the samples were diluted and analyzed for soluble sugars (Glucose, Sucrose and Fructose) by a high performance anion exchange chromatography (HPAEC) system equipped with a GS50 pump, a PED detector

and a CarboPac PA1 (4x250mm) column (Dionex, Sunnyvale, USA). Samples were eluted with 100 mM NaOH.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Continuous-light-tolerance in tomato is graft-transferable

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Under revision

Abstract

Continuous light induces a potentially lethal injury in domesticated tomato (*Solanum lycopersicum*) plants. Recently, continuous-light tolerance was reported in several wild tomato species, yet the molecular mechanisms underpinning tolerance/sensitivity are still elusive. Here, we investigated from which part of the plant continuous-light tolerance originates and whether this trait acts systemically within the plant. By exposing grafted plants bearing both tolerant and sensitive shoots, the trait was functionally located in the shoot rather than the roots. Additionally, an increase in continuous-light tolerance was observed in sensitive plants when a continuous-light-tolerant shoot was grafted on it. Cultivation of greenhouse tomatoes under continuous light promises high yield increases. Our results show that in order to pursuit this, the trait should be bred into scion rather than rootstock lines. In addition, identifying the nature of the signal/molecule(s) and/or the mechanism of graft-induced, continuous-light tolerance can potentially result in a better understanding of important physiological processes like long distance signaling.

Introduction

After the seminal work of Arthur *et al.* (1930) showing that continuous light (CL) injures domesticated tomato (*Solanum lycopersicum*) plants, many studies confirmed and further investigated this phenomenon; see Velez-Ramirez *et al.* (2014)(Chapter 4). Despite the great interest and extensive research on the topic during several decades already, a physiological explanation of the CL-induced injury is still missing. In recent years, nonetheless, a renewed interest in the topic has resulted in a number of reviews (Sysoeva *et al.*, 2010, Velez-Ramirez *et al.*, 2011 (Chapter 2)) and several research papers (Matsuda *et al.*, 2012, Sysoeva *et al.*, 2012, Velez-Ramirez *et al.*, 2012 (Chapter 3), Velez-Ramirez *et al.*, 2014 (Chapter 4)).

We recently showed that (i) several wild tomato species are tolerant to CL, (ii) the trait can be mapped to the *type III Light harvesting chlorophyll a/b binding protein* (*CAB-13*) gene on chromosome seven, and (iii) when introgressed into elite F_1 hybrid lines, tomato yield was up to 20% higher under CL than under a 16-h photoperiod (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This not only confirms the predictions of the potential yield increase when using CL (Velez-Ramirez *et al.*, 2012 (Chapter 3)) but also provides a research model to study the phenomenon. For instance, by using a CL-tolerant introgression line and its CL-sensitive control, we showed that the gene ontology terms carbohydrate metabolism, chlorophyll biosynthesis, chlorophyll binding and photosystem I reaction center are significantly enriched in differentially-regulated genes in tomato plants exposed to CL (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

The CL-induced injury appears to act locally as no increase in sensitivity to CL was observed in healthy sensitive plants when CL-injured tomato plants were approach-grafted with those healthy plants and then exposed to CL (Hillman, 1956). This evidence

suggests that CL-induced injury is not the result of an "injurious transferable substance", but rather the result of a process taking place locally in each leaf (Hillman, 1956). In this study, we further investigated whether CL-tolerance acts systemically or locally. Grafted plants, having one CL-tolerant and one CL-sensitive shoot, were exposed to CL, and the CL-induced injury was assessed using chlorophyll fluorescence imaging. The results not only confirms that CL-tolerance is located in the shoot, but also shows that it is graft-transferable as a reduced sensitivity to CL was observed in CL-sensitive plants when CL-tolerant shoots were grafted on them.

Results and Discussion

Grafting above the rootstock cotyledons and decapitating the scion a few days after grafting resulted in two equal shoots of different genotypes on one plant. The rootstock shoot emerged from a cotyledon axillary bud, and the scion shoot emerged (at the same time) from an axillary bud in the scions first true leaf axil (Fig. 5.1). CLT and A131 tomato lines are CL-tolerant and –sensitive, respectively; all possible A131 / CLT grafting combinations were created. Hereafter, the rootstock genotype is always given before the scion genotype; hence, the "A131 / CLT" plant represented in Fig. 5.1 is a plant with an A131 rootstock (roots and one shoot) and a CLT scion (one shoot).

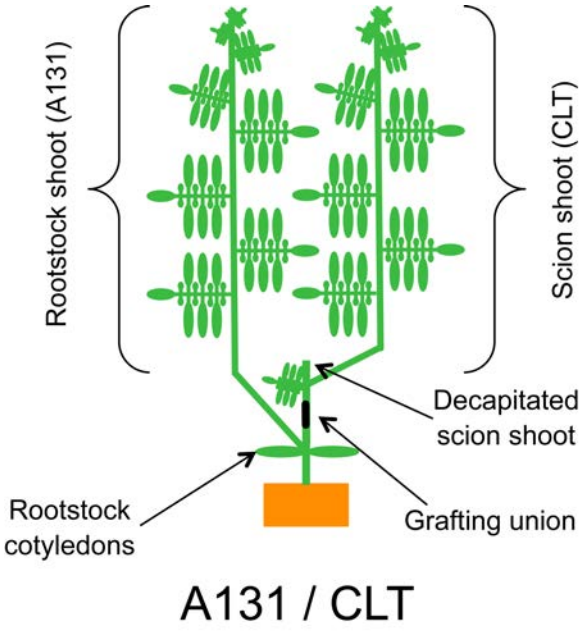

Figure 5.1 | Schematic representation of a grafted tomato plant used in this study. By grafting above the rootstock cotyledons and decapitating the scion few days after grafting, two equal shoots of different genotypes were obtained in one plant. The rootstock shoot emerged from a bud at the rootstock cotyledons base, and the scion shoot emerged (at the same time) from a bud at the scion first true leaf base. In this paper, the rootstock genotype is always written before the scion genotype; in this example, therefore, "A131 / CLT" is a plant with a A131 rootstock (roots and shoot) and a CLT scion (shoot).

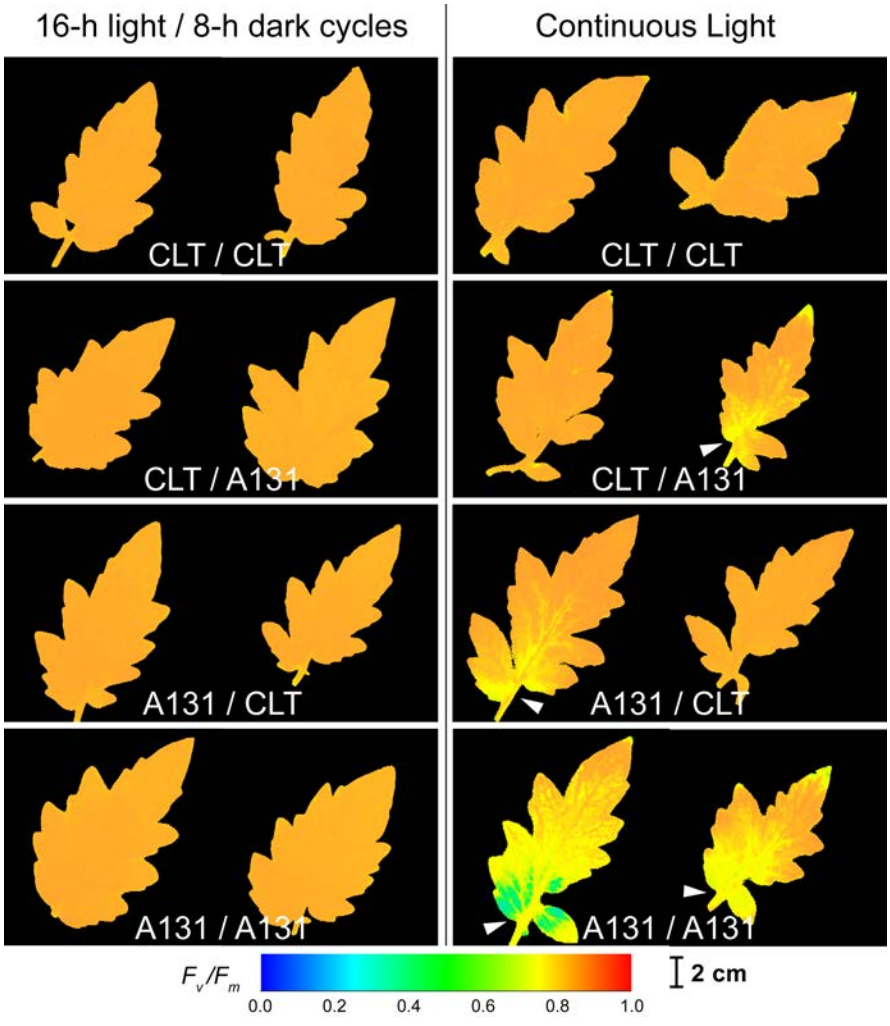

Figure 5.2 | Continuous-light-induced injury in leaflets of grafted tomato plants. Tomato plants having two equally sized shoots, each growing from the rootstock and scion respectively, were exposed to continuous light. All possible A131 / CLT combinations were used. The rootstock genotype is always written before the scion genotype; for instance, "A131 / CLT" is labeling a plant with a A131 rootstock (roots and shoot) and a CLT scion (shoot) (see Figure 1). After two weeks of exposure to continuous light, chlorophyll fluorescence images of the top-most, fully expanded leaves of each shoot were taken. Control plants were kept under 16-h light / 8-h dark cycles. Images represent, in a false color scale (see color scale), maximum efficiency of photosystem II (Fv /Fm). Within each panel, left image comes from the rootstock shoot and right image from the scion shoot. White triangles point to continuous-light-induced injury in continuous-light-exposed A131 leaflets from plants lacking a companion CLT shoot.

A companion CLT shoot diminishes CL-induced injury in A131 shoots.

When grafted tomato plants were kept under 16-h photoperiod, regardless of the grafting combination, both shoots in each plant developed normally as evidenced by the chlorophyll fluorescence images (Fig. 5.2). When exposed to CL, however, all A131 shoots developed CL-induced injury in young leaves characterized by mottled chlorosis at the leaves/leaflets bases, while CLT shoots presented no CL-induced injury at all, confirming previous studies that the CL-tolerance is functionally located in the aerial part of the plant (Daskaloff & Ognjanova, 1965, Velez-Ramirez *et al.*, 2014 (Chapter 4)). From the chlorophyll fluorescence images, it was evident that the severity and extent of the CL-induced injury in A131 leaflets was less when a companion CLT shoot was present (Fig. 5.2). In Fig. 5.3, these differences are quantified by averaging the leaflet F_v/F_m of each biological replicate. The chlorophyll fluorescence parameter F_v/F_m has has been used to quantify the level of CL-induced injury in tomato (Velez-Ramirez *et al.*, 2014 (Chapter 4)). The parameter F_v/F_m represents the maximum quantum efficiency of PSII (Baker, 2008); therefore the lower the F_v/F_m value, the higher the injury is. When a CLT scion shoot accompanied an A131 rootstock shoot, A131 leaflet F_v/F_m was on average 0.048 higher than the F_v/F_m value of comparable A131 rootstock shoots without a CLT scion shoot companion ($P<0.05$) (Fig. 5.3). Similarly, when a CLT rootstock shoot accompanied an A131 scion shoot, A131 leaflet F_v/F_m was on average 0.024 higher than the F_v/F_m value of comparable A131 scion shoots without a CLT rootstock shoot companion, yet this difference was not significant ($P>0.05$). No differences between CLT shoots were observed ($P>0.05$).

As higher light intensity enhances the CL-induced injury in tomato (Arthur *et al.*, 1930, Withrow & Withrow, 1949) and eggplant (Murage *et al.*, 1997) care was taken to keep each shoot as far apart from its companion as possible. Although some shading was unavoidable, shading cannot explain the decreased CL-induced injury in A131 shoots when a CLT companion shoot was present because a companion A131 shoot shaded its companion shoot to the same extent, but it did not protect the companion A131 shoot from CL. Two hypotheses can be considered to explain the lower sensitivity to CL in A131 shoots when a CLT companion shoot is present. First, a "transferable injurious substance" could be transferred from A131 shoots but is inactivated in CLT shoots. Alternatively, the CL-tolerance itself, *e.g.* a signal/molecule, could be transferred from CLT shoots to A131 shoots.

Previous evidence does not support the hypothesis of a transferable injurious substance in the CL-induced injury.

Hyper-accumulation of carbohydrates, observed in CL-exposed tomato leaves, has been proposed to induce injury in tomato plants (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)). Hence, sucrose might act as such hypothetical "injurious transferable substance". However, only circumstantial evidence supports this hypothesis, and some earlier experimental evidence even contradicts it since

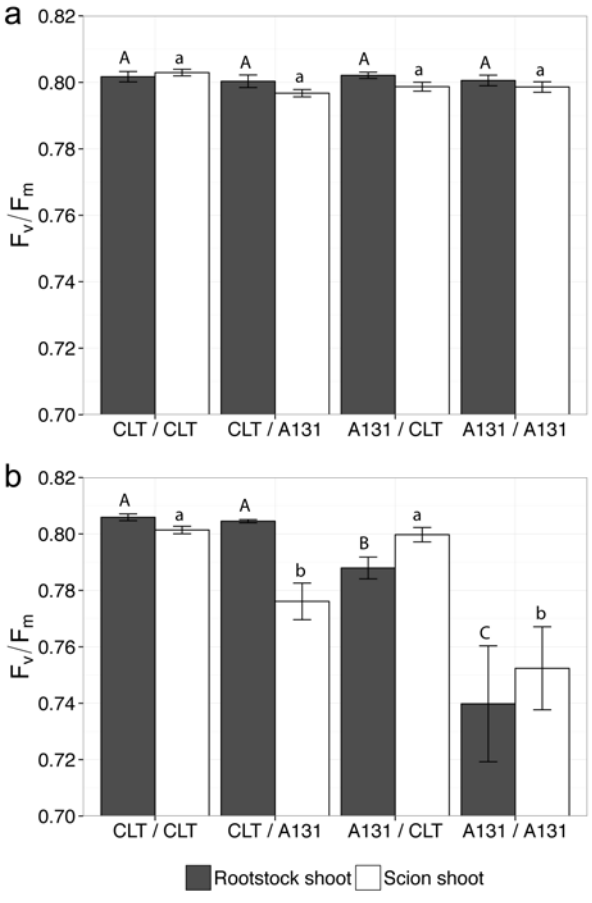

Figure 5.3 | Effect of continuous light on A131 and CLT grafted plants. Grafted tomato plants having two equally sized shoots, each growing from the rootstock and scion respectively, were exposed to continuous light. All possible A131 / CLT combinations were used; the rootstock genotype is always written before the scion genotype; for instance, "A131 / CLT" is a plant with a A131 rootstock (roots and shoot) and a CLT scion (shoot) (see Figure 1). After two weeks of exposure to continuous light, chlorophyll fluorescence images of the top-most, fully expanded leaves of each shoot were taken. (**a**), Leaflet average dark-adapted Fv /Fm of control plants kept under 16-h light / 8-h dark cycles. (**b**), Leaflet average dark-adapted Fv /Fm of plants exposed to continuous light. Rootstock shoots are represented in black, and scion shoots are represented in white. In all graphs, bars represent mean of four replicates, and error bars represent SE. Rootstock shoot Fv /Fm means sharing the same capital letter are not significantly different ($p>0.05$), and scion shoot Fv /Fm means sharing the same lower case letter are not significantly different ($p>0.05$).

CL-induced injury can occur even in the absence of carbohydrate accumulation (Arthur *et al.*, 1930). In addition, fruit pruning did not affect carbohydrate accumulation in CL-exposed tomato plants suggesting that sink limitations are not responsible for the observed carbohydrate accumulation under CL (Demers *et al.*, 1998). Hence, it has been suggested that CL-induced carbohydrate accumulation in tomato is the result of decreased export capacity rather than decreased sink strength (Demers *et al.*, 1998, Dorais *et al.*, 1996). All together, the evidence does not support the hypothesis that sucrose from A131 is being exported to and metabolized in the companion CLT shoot acting as a sink.

Furthermore, when CL-injured plants were approach-grafted to intact sensitive plants, no increase in sensitivity to CL was observed (Hillman, 1956). This experiment cannot discard that CL-induced injury results from the accumulation of an unidentified

injurious substance, but it shows that such hypothetic substance is not graft-transferable. Hence, experiments described in the literature do not support the hypothesis of a "transferable injurious substance" from CL-sensitive shoots that is metabolized or inactivated in CLT shoots.

Continuous-light-tolerance in tomato is graft-transferable.

The better performance observed in A131 shoots whenever a CLT companion shoot was present must be the result of CL-tolerance from the CLT shoot acting systemically in A131 shoots. Grafting is not only used as an unambiguous tool for diagnosing long-distance transport and action in plant research (Turnbull & Lopez-Cobollo, 2013) but also extensively used to alter the scion phenotype in horticultural crops (Mudge *et al.*, 2009). In tomato, for instance, grafting on tolerant rootstocks improves plant performance under salt (Albacete *et al.*, 2009), heat (Rivero *et al.*, 2003a, Rivero *et al.*, 2003b) and cold (Venema *et al.*, 2008) stress. Here, grafting of companion CLT scion shoots improved tolerance of A131 rootstock shoots under CL. Considering the directionality of xylem and phloem connections (Turnbull & Lopez-Cobollo, 2013), we postulate that a graft-transferable signal or molecule from CLT translocates to A131 shoots via the phloem and increases CL-tolerance in A131 shoots.

Tomato phloem sap contains sugars, amino acids and nutrient ions (Alfocea *et al.*, 2000, Valle *et al.*, 1998). In addition, phloem sap also contains macromolecules like proteins and RNA (Turnbull & Lopez-Cobollo, 2013). Future efforts to identify the nature of the transmissable signal/molecule(s) conferring CL-tolerance should not only focus on small-molecule compounds like hormones, but proteins and RNA should be considered as well. It is known that small RNAs (like micro RNAs and short-interfering RNAs) and proteins (like the FLOWERING LOCUS T protein) are long distance signals able to cause gene silencing and flowering, respectively, in other parts of the plant than where they are produced; see Turnbull and Lopez-Cobollo (2013) and references therein. Small-interfering RNAs can induce DNA methylation in a sequence-specific manner; DNA methylation strongly influences chromatin structure and gene expression (Saze *et al.*, 2012). Recently, Wu *et al.* (2013) reported that heritable, alterations of DNA methylation occur in tomato scions when grafted on eggplant (*Solanum melongena*). Identification of the nature of the signal/molecule(s) and the mechanism of action of the graft-induced CL-tolerance should shed light on the mechanism of a new example of the important physiological process of long distance signaling.

Materials and Methods

Plant materials and growing conditions

Two tomato lines were used: A131 is a CL-sensitive inbred line, and CLT is a CL-tolerant introgression line derived from a *S. neorickii* (LA2133) x *S. lycopersicum* A131 population.

A131 and CLT have been previously described (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Seeds were provided by Monsanto Vegetable Seed Division (Bergschenhoek, The Netherlands).

Plants were grown in rockwool blocks at 21 °C and 70% RH. Commercial hydroponic nutrient solution for tomato was used (Yara Benelux B.V., Vlaardingen, The Netherlands); after combining and diluting premixed liquid fertilizers, the solution contained 12.42 mM NO_3, 7.2 mM K, 4.1 mM Ca, 3.34 mM SO_4, 1.82 mM Mg, 1.2 mM NH_4, 1.14 mM P, 30 mM B, 25 mM Fe, 10 mM Mn, 5 mM Zn, 0.75 mM Cu and 0.5 mM Mo (EC = 2.00 $dS.m^{-1}$ and pH = 5.0-5.5). Light was provided by high-pressure sodium (HPS) lamps (Master SON-T Green Power 400W, Philips, Eindhoven, The Netherlands) and supplemented with incandescence lamps (Philinea T30 120W, Philips, Eindhoven, The Netherlands). The light intensity was 350 $\mu mol.m^{-2}.s^{-1}$; red-to-far-red ratio was 2.873, and the phytochrome photostationary state (PSS) (Sager *et al.*, 1988) was 0.857. Two weeks after sowing, A131 and CLT seedlings were grafted above the cotyledons, in all possible combinations, using plastic grafting clips. To protect the seedlings from desiccation, during the first three days after grafting, seedlings were covered with a transparent plastic, and sonic humidifiers were installed inside the chamber. Four days after grafting, the scion shoots were decapitated leaving only one true leaf in the scion. This promoted the emergence of side shoots from buds located at the rootstock cotyledons and scion leaf bases; only one shoot from the rootstock and one from the scion, growing in opposite directions, were allowed to grow; any extra shoot was pruned. This resulted in plants having two equally sized shoots, each of them growing from the rootstock and scion respectively (Fig. 5.1). To avoid mutual shading from shoots within the same plant as much as possible, each shoot was clamped to wooden sticks held at a slight divergent angle (not represented in Fig. 5.1); so the larger the shoots grew the larger the space between them. In that way, plenty of light fell on the complete span of each shoot.

Chlorophyll fluorescence imaging

Intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences, Lincoln, USA). After 20 minutes of dark adaptation, leaflets were detached and immediately placed inside a commercial chlorophyll fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic). Fluorcam v. 5.0 software were used to control and process the images. Maximum quantum efficiency of photosysmtem II [dark adapted F_v/F_m, see Baker (2008)] was calculated as described previously (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

Statistical analysis

As the two shoots in each plant are not independent of each other, we performed two independent analyses of variance (ANOVA) on rootstock and scion shoots. The analysis considered two factors (grafting combination and photoperiod). Regardless of the shoot genotype, therefore, the effect of photoperiod and grafting combination on F_v/F_m was

analyzed within the same type of shoot (rootstock or scion shoot). To achieve equal variances, a power transformation was used on the F_v/F_m values. All statistical analyses were performed with R (R Core Team, 2013).

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leaflet
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CLT

Circadian asynchrony triggers continuous-light-induced injury in tomato

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Abstract

Use of artificial light in horticulture and plant research is widespread, yet several artificial-light-induced disorders remain unexplained. A remarkable example is the continuous-light-induced injury in tomato. A better understanding of the mechanism of these disorders would allow a better implementation of artificial light in horticulture and research but would also advance the understanding of the physiology of plants. Here, in an effort to understand why continuous light is injurious to tomato, we formulated a list of factors that differ between injurious and non-injurious light regimes; each of these factors is potentially responsible for triggering the injury in CL-grown tomato and were experimentally tested. These factors include (I) differences in the light spectral distribution between sunlight and artificial light, (II) continuous light signal, (III) constant supply of light for photosynthesis, (IV) constant photo-oxidative pressure and (V) circadian asynchrony — a mismatch between the internal circadian clock frequency and the external light/dark cycles. The evidence presented here suggests that continuous-light-induced injury does not result from the unnatural spectral distribution of artificial light or the continuity of the light *per se.* Instead, circadian asynchrony seems to be the main factor inducing the injury. However, circadian asynchrony does not directly induce injury via photoinhibition as the discovered diurnal fluctuations in photoinhibition sensitivity of tomato seedlings are not under circadian control.

Introduction

Sunlight sustains essentially all life on the earth's surface. Plants use light for photosynthesis, but light intensity, spectral distribution and direction are also perceived by photoreceptors, enabling plants to modulate their growth and development (Bae & Choi, 2008, Casal, 2013, Christie, 2007, Jiao *et al.*, 2007). With the introduction of electric artificial light in the 19th century, scientist like Siemens (1880) questioned if light provided by an electric arc is enough to promote plant grow as sunlight does. Today, we take for granted that artificial light is not only good enough for plant growth but also for its use in plant research. Considering the plethora of plant studies done under artificial light, the questions raised by Siemens (1880) seem pointless nowadays. Recently, however, Hogewoning *et al.* (2010a) showed that plant development is significantly altered by fluorescent and high pressure sodium lamps, which are widely used in plant research, as compared with artificial solar light, though light intensity and photoperiod were similar. Hereafter, we use the terms *sunlight* to refer to the natural light coming from the sun and *artificial solar (AS) light* to refer to man-made light with a spectral distribution that closely matches the one of sunlight. With the development and implementation of new lighting technologies, like light-emitting diodes (LEDs), new poorly understood disorders arise. For instance, exposure of cucumber plants to pure red LED light results in dysfunctional leaves showing poor photosynthetic performance (Hogewoning *et al.*, 2010b). Furthermore, some of the questions raised during

the early days of plant research under artificial light still remain unanswered. A remarkable question, first raised by Arthur *et al.* (1930), is why continuous light (CL) injures tomato while other plant species grow well under 24h of light per day? Understanding the mechanism of this and other artificial-light-induced disorders not only promises better implementation of artificial light in horticulture and research but may also advance our knowledge of plant physiology.

The recent re-discovery that wild tomato species are tolerant to CL (Velez-Ramirez *et al.*, 2011 (Chapter 2)), and that such tolerance is linked to the *type III light harvesting chlorophyll a/b binding protein 13* (*LHCB type III CAB-13* or *CAB-13*) advances our understanding of this disorder (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Tomato *CAB-13* is homologous to Arabidopsis (*Arabidopsis thaliana) LHCB3*. As LHCB3 is part of the light harvesting complex (LHCII) of photosystem II (PSII), several studies have investigated its function in the composition, structure, stability and efficiency of Arabidopsis PSII-LHCII supercomplexes (Caffarri *et al.*, 2009, Damkjær *et al.*, 2009, Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b). However, a clear mechanism linking *CAB-13* and CL-tolerance in tomato is still missing. The current inability to fully understand why *CAB-13* confers CL-tolerance highlights the poor knowledge of the function and regulation of the most important process of light harvesting by LHCII and the role of type III LHCB proteins. The association of *CAB-13* with CL-tolerance in tomato suggests that its importance for the photosynthetic machinery is even bigger than our current understanding suggests.

Mapping of CL-tolerance in wild tomato species to *CAB-13*, although important for breeding and future research on photosynthesis, does not answer the question why CL is injurious to domesticated tomato. To answer this question, we compared an injurious CL treatment, provided by artificial light, with a non-injurious natural day/night cycle and identified a number of factors that differ between these conditions; each of these factors is potentially responsible for triggering the injury in CL-grown tomatoes. In short, these factors include (I) differences in the light spectral distribution between sunlight and artificial light, (II) continuous light signal, (III) constant supply of light for photosynthesis, (IV) constant photo-oxidative pressure, and (V) a mismatch between the internal circadian clock frequency and the external light/dark cycle, a phenomenon known as circadian asynchrony (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Concluding which factor induces the injury is not simple because, in most cases, CL treatments affect all factors at the same time.

Regarding the first factor, the spectral differences between artificial light and sunlight, tomato plants are reported to grow vigorously and without injury under natural CL provided by the Arctic summer as far north as 65° 30' (the Arctic circle is at 66° 33' 44'') (Darrow, 1933). Similarly, Arthur *et al.* (1930) reported that injury in tomato was less severe if CL was supplied by sunlight during the day and artificial light during the night in contrast to using artificial light during the whole day. Additionally, the spectral distribution of artificial light emitted by lamps influences the injury severity (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997). These observations suggest that the spectral distribution of artificial light is the triggering factor in CL-induced injury in tomato. However, growing tomatoes during the arctic summer or in a greenhouse, with

lamps turned on during the night, does not provide a constant environment in terms of temperature and light (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Diurnal fluctuations in air temperature (thermo-periods) diminish CL-induced injury in tomato (Hillman, 1956, Kristoffersen, 1963, Matsuda *et al.*, 2012, Sysoeva *et al.*, 2012). Therefore, the possibility that temperature and light intensity fluctuations prevent injury induction in tomato plants when grown under polar days cannot yet be discarded. Here, we test whether the CL-induced injury results from the discrepancy between the spectral distribution of sunlight and artificial light sources or from the continuity of light *per se* by exposing tomato plants to continuous AS light, using a plasma lamp that closely mimics the spectral distribution of sunlight.

A continuous light signal, perceived by the plant photoreceptors, is the second factor. Phytochromes perceive red/far-red light, while blue/UV light is perceived by cryptochromes and phototropins (Casal, 2013, Jiao *et al.*, 2007). Under non-injurious natural day/night cycles, light is perceived during the light period, and signals are transduced to downstream components. During the dark period, light is lacking; hence these photoreceptors cannot be activated. In contrast, CL causes continuous activation of photoreceptors. In such a case, the signal would depend solely on the gating of the circadian clock and the light stability of the photoreceptors and not on the presence/absence of light. Several studies show that light signaling during times of the day that the circadian clock up regulates dark-dependent processes results in different growth patterns between plants grown under CL or short days (Niwa *et al.*, 2009, Nozue *et al.*, 2007). Hence, it is reasonable to hypothesize that continuous signaling to the photoreceptors is a candidate factor to trigger CL-induced injury.

Regarding factor III, constant supply of light for photosynthesis, under natural day/night cycles, the light reactions of photosynthesis only takes place during the day. Under CL, however, photosynthetic fixation of CO_2 is continuous (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In principle, this could be the cause of the observed carbohydrate accumulation in CL-exposed tomato leaves. Considering that such accumulation is proposed to play a role in the injuries that tomato plants develop under CL (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)), the constant photosynthesis facilitated by CL should be considered as a potential triggering factor. Likewise, photo-oxidative pressure (factor IV) also considers the energy content of light, provided during the subjective night, as a potential factor triggering injury in CL-grown tomatoes. Light can damage chloroplast components if light absorption surpasses quenching processes like photosynthesis and non-photochemical quenching (Li *et al.*, 2009). In the visible spectrum, the potential of light to damage isolated chloroplast depends on the number of photons absorbed; hence the photoinhibition action spectrum closely matches leaf absorbance (Jones & Kok, 1966). Considering that photosynthesis is also a process driven by the number of absorbed photons, here, we used red and blue light-emitting diodes (LEDs) to construct CL treatments that provide a constant photosynthetically active photon flux density (PPFD) with diurnal fluctuations in the spectral distribution, *i.e.* light color, as well as treatments with diurnal PPFD fluctuations with constant dim blue of red

light, which should saturate most photoreceptor responses. Hence, these treatments were intended to dissect the effects of factors II, III and IV on CL-induced injury.

The last factor (V) is circadian asynchrony, which is the mismatch between the internal circadian clock frequency and the external light/dark cycles. Previously, it has been proposed that circadian asynchrony may trigger CL-induced injury in tomato (Velez-Ramirez *et al.*, 2011 (Chapter 2)). The reasoning behind this hypothesis is that if tomato plants are grown under light/dark cycles differing sufficiently from 24h periodicity (*e.g.* 6h light / 6h dark or 24h light / 24h dark), which implies circadian asynchrony, a similar injury as observed under CL will develop (Highkin & Hanson, 1954, Hillman, 1956). Considering that photosynthesis and stress-responsive genes, including reactive oxygen species (ROS)-scavenging genes, are up-regulated during daytime and down-regulated at night (Facella *et al.*, 2008), it is reasonable to assume that protection against photo-oxidative damage, or photoinhibition, fluctuates in response to clock outputs. Hence, asynchrony between clock-controlled protection against photoinhibition and the presence of light during the subjective night might trigger the CL-induced injury (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Subjecting, at intervals of one hour, CL-exposed tomato seedlings to a short period of very high light intensity and measuring the resulting degree of damage test this hypothesis. In all experiments, CL-tolerant tomato lines serve as a positive control that will not develop CL-injury.

Results

Factor I. Differences between natural and artificial light

In order to test whether the large differences in spectral distribution between sunlight and artificial light sources are responsible for continuous light (CL)-induced injury in tomato, we exposed CL-tolerant (CLT) and CL–sensitive (A131) tomato plants to CL provided by red/blue LED or artificial solar (AS) light. When given at a photosynthetically active photon flux density (PPFD) of 100 $\mu mol.m^{-2}.s^{-1}$, the intensity used in most of the light treatments in the present study (Table 6.1), the difference between the spectral distributions of AS light (Fig. 6.1a) and natural sunlight (Fig. 6.1b) is less than 1 $\mu mol.m^{-2}.s^{-1}$ at any given wavelength (Fig. 6.1c). A131 and CLT tomato plants exposed to red/blue LED light or AS light for 16 hours per day developed normally without any signs of injury (Fig. 6.2). Tomato plants exposed to "16h AS" light showed the highest leaflet average F_v/F_m, indicating that the AS is not injurious. As expected, CL-sensitive A131 tomato plants developed interveinal chlorosis (most evident in the chlorophyll fluorescence images) after exposure to "24h red/blue" LED light (Fig. 6.2); the decrease in leaflet average F_v/F_m was evident and significant ($P<0.05$) compared with plants subjected to "16h red/blue" treatment. Interestingly, continuous AS light ("24h AS") not only injured A131 plants as "24h red/blue" treatment did, but also injured CL-tolerant (CLT) plants, which showed clear signs of CL-induced injury (Fig. 6.2). Nonetheless, CLT plants showed a significantly ($P<0.05$) higher leaflet average F_v/F_m than A131 plants under "24h AS". This indicates that

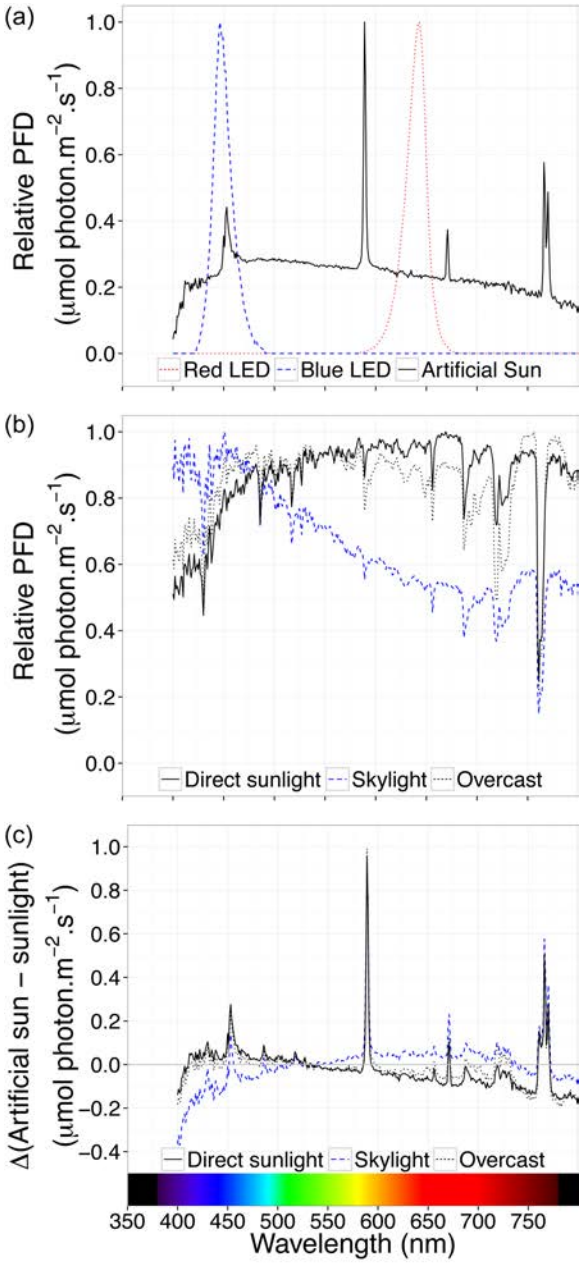

Figure 6.1 | Spectral distributions of artificial light, emitted by the lamps used in this study, and natural sunlight. In panel (**a**), relative photon flux density (PFD) of red light-emitting diodes (LEDs) (dotted red line), blue LEDs (dashed blue line) and artificial sunlight emitted by a plasma lamp (solid black line). In panel (**b**), Relative PFD of natural light measured around the autumn equinox at noon in Wageningen, The Netherlands (~52°59' N 5°39' E) as reported by (Hogewoning *et al.*, 2010a); direct sunlight (solid black line), skylight during a clear day (dashed blue line) and overcast light (dotted black line). Panel (**c**) shows the absolute difference between artificial solar and natural sunlight if both are given at 100 µmol.m^{-2}.s^{-1} PPFD; artificial solar vs. direct sunlight (solid black line), artificial solar vs. skylight (dashed blue line) and artificial solar vs. overcast light (dotted black line).

the CL-induced injury in tomato is not the result of large spectral differences between artificial light sources (like LED light) and sunlight and continuous AS light is remarkably injurious for tomato plants.

Factor II. Continuous light signal

To test if a diurnal change in light quality, without varying the PPFD, diminishes or even prevents the CL-induced injury, A131 and CLT tomato plants were exposed to two LED-based light treatments, *i.e.* "red night" and "blue night". A131 plants exposed to "red night" treatment, which implies a diurnal cycle in blue light, showed CL-induced injury with a leaflet average F_v/F_m not significantly different from injured plants exposed to "24h red/blue" light (Fig. 6.2). A131 plants exposed to "blue night" treatment, which implies a diurnal fluctuation in red light, developed CL-induced injury, but the severity was higher than in A131 plants exposed to 24h red/blue LED light as a significant lower F_v/F_m shows ($P<0.05$) (Fig. 6.2). Leaflets of CLT plants exposed to "red night" and "blue night" showed some spots with lower F_v/F_m, yet the leaflet average F_v/F_m was not significantly different from uninjured control plants ($P>0.05$). Altogether, a diurnal cycle in red or blue light did not prevent continuous light induced injury.

Factors III and IV. Constant high photosynthetically active photon flux density

Factors III and IV deal with constant photosynthesis and photo-oxidative pressure, respectively, which are both driven by the number of absorbed photons. Hence, as diurnal fluctuation in light quality at constant PPFD did not prevent CL-induced injury, we wondered if a continuous signal, in the background of normal diurnal fluctuations in PPFD, would induce the injury. This was achieved by keeping a dim red or blue light continuously on in the background of a 16h photoperiod provided by AS light. Regardless of the genotype, tomato plants exposed to "AS-dim blue" light treatment developed normally without any signs of injury (Fig. 6.2). In contrast, A131 plants exposed to "AS-dim red" treatment showed clear symptoms of CL-induced injury, like interveinal chlorosis; the F_v/F_m average is halfway between uninjured control plants grown under "16h AS" and injured A131 plants exposed to "24h red/blue" treatments (Fig. 6.2). In contrast, CLT plants hardly showed any injury symptoms (Fig. 6.2). This shows that constant high PPFD is not required for causing CL-induced injury in tomato; instead, dim red light during night fulfills the requirements for a weak injury induction.

Factor V. Circadian asynchrony

Previous research showed that abnormal light/dark cycles (cycles with a periodicity sufficiently longer or shorter than 24h) induce a mottled chlorosis similar to the one induced by CL (Highkin & Hanson, 1954, Hillman, 1956, Kristoffersen, 1963). Such abnormal light/dark cycles will cause circadian asynchrony, which is the fifth factor considered here to potentially cause the CL-induced injury; hence, we performed an adapted version of the experiments of Hillman (1956), Highkin and Hanson (1954) and Kristoffersen (1963) with the important addition of CL-tolerant tomato plants as control.

Table 6.1. Light treatment characteristics

Light Treatment	PFD (µmol photons.m^{-2}.s^{-1})		PPFD (µmol photons.m^{-2}.s^{-1})		PSS (Sager *et al.*, 1988)		R:Fr ratio		Irradiance (W.m^{-2})		Photoinhibitory activity $\sum_{nm=400}^{800} \alpha\phi$ (Jones & Kok, 1966)	
	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night
16h red/blue	100	0	100	0	0.88	a	b		20.3	0.0	8.2	0.0
24h red/blue	100		100		0.88		b		20.3		8.2	
Red night	100		100		0.88	0.89	b		20.3	18.7	8.2	6.7
Blue night	100		100		0.88	0.48	b		20.3	26.6	8.2	14
16h artificial solar	126	0	100	0	0.72	a	1.11	a	26.3	0.0	6.4	0.0
24h artificial solar	126		100		0.72		1.11		26.3		6.4	
Artificial solar–dim red	123	10	100	10	0.75	0.89	1.33	a	25.6	1.9	6.4	0.7
Artificial solar–dim blue	123	10	100	10	0.72	0.48	1.11	a	26.4	2.7	7.2	1.4
High–pressure sodium + Philinea[c]	368	0	345	0	0.86	a	2.89	a	74.3	0.0	15.4	0.0

a Not applicable in darkness

b Not applicable due to the complete absence of FR

c Applicable to all abnormal light / dark cycles treatments

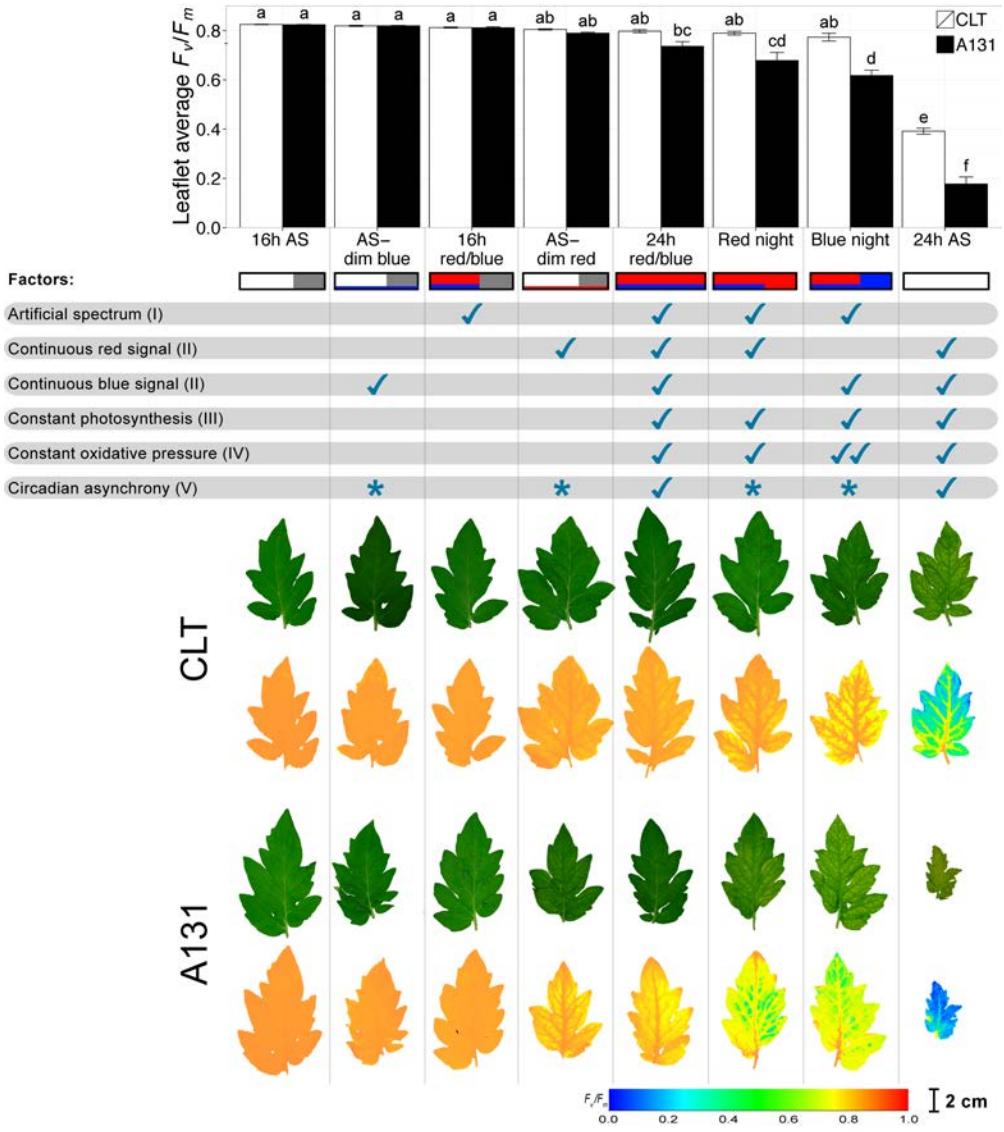

Figure 6.2 | Effect of eight light treatments on the appearance and severity of continuous light-induced injury in tomato leaves. A131 and CLT tomato plants were grown under 16-h light / 8-h dark cycles for two weeks and then transfer to various light treatments. After 25 days, the level of continuous light-induced injury was assessed by chlorophyll fluorescence imaging. Leaflet average F_v/F_m is shown at the top; values represent mean ± SE (n=5 to 8); bars not sharing the same letter are statistically different (P<0.05). Light treatments are ordered form the least injurious (left side) to the most injurious (right side); treatment names and schemes graphically illustrating each light treatment are shown at below the graph and are valid for the whole figure. AS stands for Artificial Solar light, which is emitted by a plasma lamp. Blue and red stands for light emitted by light-emitting diodes (LEDs). At the center of the figure, all the factors that differ between CL and non-injurious natural

day/night cycles are summarized alongside their relation with the light treatments. A check mark (✓) indicates that a given factor is present in that treatment; the number of check marks being proportional to the incidence of that factor in a given treatment. An asterisk (*) indicates special cases where the light treatment contains a diurnal fluctuation in light quality and/or intensity; this could result in a circadian asynchrony that is not as strong compared with light treatments with constant light quality and intensity. Further details can be found in Table 6.1 text and in the text. Photographs and chlorophyll fluorescence images of representative leaflets are shown at the bottom.

Four weeks old tomato plants were exposed to various abnormal light/dark cycles for three weeks. A131 young leaves developed CL-induced injury at the bases, clearly evident in the chlorophyll fluorescence images, when exposed to CL and abnormal light / dark cycles (Fig. 6.3). In all A131 plants exposed to 24-h light / 24-h dark cycles and 6-h light /6-h dark cycles, chlorosis at the leaflet bases was evident from the 6th and 7th leaf onwards, respectively (Fig. 6.3a). Notice that A131 plants exposed to 6-h light /6-h dark cycles did not show clear injury symptoms on the 6th leaf (images not shown), but the 7th leaf was injured (Fig. 6.3a), which is consistent with the report that the CL-induced injury gradually becomes more severe in younger leaves (Hillman, 1956). As the chlorophyll fluorescence images of the 7th leaf from all other treatments are not available, the images in Fig. 6.3 should be considered as purely qualitative evidence showing that abnormal light/dark cycles injure CL-sensitive tomato plants but not CL-tolerant CLT plants. To confirm this, the experiment was repeated, with similar results (data not shown); in both occasions A131 plants showed clear signs of interveinal chlorosis at the bases of young leaves and CLT plant were completely uninjured. Figure 6.3b shows the F_v/F_m for the 6th leaf (Fig. 6.3b). In contrast to A131 plants, CLT young leaves did not show any signs of injury (Fig. 6.3a,b), suggesting that the injury induced by CL and abnormal light / dark cycles have a similar origin.

Young tomato leaves show non-circadian controlled, diurnal changes in sensitivity to photoinhibition

A previously proposed hypothesis that would explain a common origin between the injury induced by CL and abnormal light/dark cycles considers the possibility of a circadian-controlled sensitivity to photoinhibition (Velez-Ramirez *et al.*, 2011 (Chapter 2)). To test this hypothesis, we exposed young tomato leaves to a very high light intensity for 5 minutes at different times during the photoperiod. The sensitivity to photoinhibition was quantified as the difference in F_v/F_m before and after the 5-minutes exposure. Using a different plant each time, the sensitivity to photoinhibition was assessed every hour during the course of a 12-h day / 12-h dark cycle and during 48h of CL (Fig. 6.4). A131 and CLT young leaves showed diurnal changes in their sensitivity to photoinhibition. At dawn, the sensitivity was high, and it gradually decreased until dusk; during the night, the sensitivity increased again until dawn. When plants where transferred to CL, the sensitivity to photoinhibition gradually decreased during the first 12 hours as in a normal day/night cycle. During the first subjective night, however, the sensitivity to photoinhibition did not increase. Actually, from 12h after plants were transferred to CL, the sensitivity to photoinhibition remained at

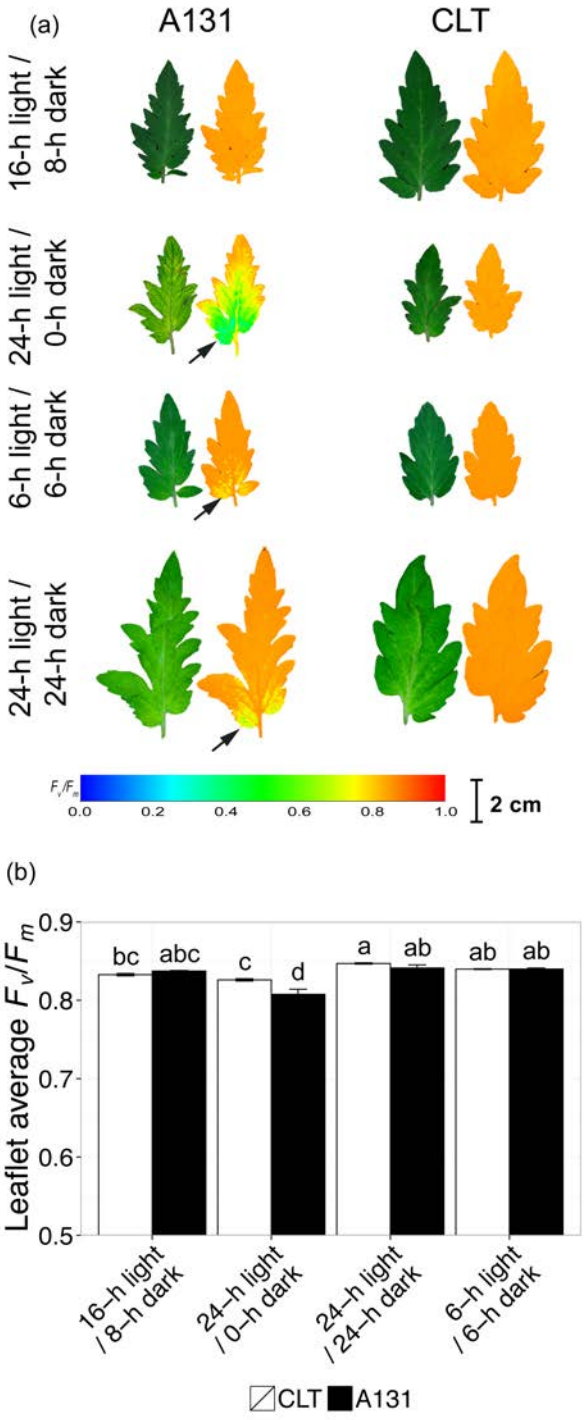

Figure 6.3 | Continuous light-induced injury as a result of abnormal light/dark cycles. A131 and CLT tomato plants were grown under 16-h light / 8-h dark cycles for four weeks and then transferred to three other light/dark cycles for three additional weeks (24-h light / 0-h dark, 12-h light / 12-h dark and 24-h light / 24-h dark cycles). Control plants were left at 16-h light / 8-h dark cycles. The level of continuous light-induced injury was quantified by the chlorophyll fluorescence parameter F_v/F_m. In panel (**a**), representative photographs (left side) and chlorophyll fluorescence images (right side) showing the appearance of injury (arrows) in continuous light-sensitive A131 leaves after exposure to continuous light and abnormal light / dark cycles; all leaflets come from the 6th true leaf, with the exception of leaflets from 6-h light / 6-h dark cycles, which come from the 7th true leaf instead. In panel (a), values represent leaflet average F_v/F_m ± SE (n=8); bars not sharing the same letter are statistically different (P<0.05); all averaged leaflets come form the 6th true leaf.

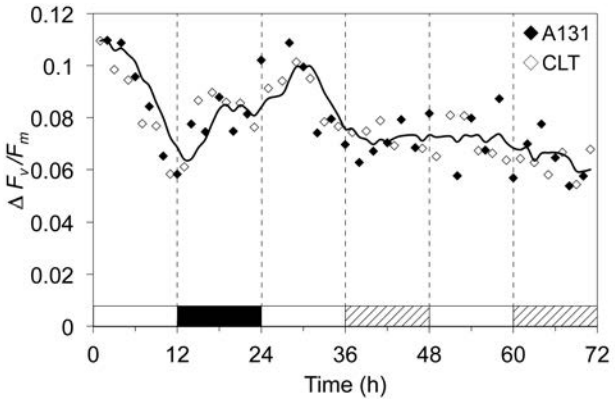

Figure 6.4 | Diurnal changes in sensitivity to photoinhibition of A131 and CLT tomato young leaves. A131 and CLT tomato seedlings were grown under 12-h light / 12-h dark cycles for two weeks and then transferred to continuous light. At intervals of one hour, during the last light / dark cycle and two subsequent days under continuous light, seedlings were exposed to very high light (~3500 mmol.m-2.s-1) for 5 minutes. Chlorophyll fluorescence images of the first true leaf were taken before and after exposure to high light; the reduction of maximum efficiency of photosystem II (D F_v /F_m) represents the seedlings sensitivity to photoinhibition at different times during the light / dark cycle and continuous light.; hence, the higher D F_v /F_m the sensitivity to photoinhibition. Closed symbols represent A131 leaves, and open symbols represent CLT leaves. The black line is a moving average (n=5) of both A131 and CLT leaves. The horizontal bars at the bottom of the graph represent the light status at the time of measurement; open and closed bars represent day- and nighttime respectively, while the pattern-filled bars represent the subjective nights. All seedlings had the same age at the time of exposure to high light, and each seedling was used at only one time point (see material and methods for details).

the level it would have at dusk. In other words, the leaf sensitivity to photoinhibition decreases during the light period and increases during the dark period towards a maximum at dawn, but this process is not under circadian control.

Discussion

Since the discovery of CL-induced injury in tomato by Arthur *et al.* (1930), many kinds of artificial light sources have become common in plant research, fluorescent tubes and high-pressure sodium lamps being remarkable examples. Recently, light emitting diodes (LED) and plasma-based artificial solar light (AS) further extend the light sources available for plant research, see for instance Hogewoning *et al.* (2010a). Here, LED and AS light allowed us to test hypotheses related to the CL-induced injury in tomato that would not otherwise have been possible to test. We previously described five factors (I-V) that differ between non-injurious day/night cycles and CL; here we will discuss the evidence for the involvement of each of these factors in the induction of injury in CL-exposed tomatoes.

The first factor deals with the artificial spectral distribution of artificial light that is used to achieve CL. Exposing tomato plants to sunlight with constant intensity and spectral

distribution at constant temperature would be needed to test if the CL-induced injury truly results from CL *per se* or from the unnatural spectral distribution of artificial light. As no place on earth would provide the needed environmental conditions, this issue has remained unanswered so far. Here, by means of a plasma lamp supplying AS light and growth chambers, we have achieved an environment with constant temperature and constant PPFD with a spectral distribution remarkably similar to natural sunlight (Fig. 6.1c). Tomato plants developed severe injuries when exposed to continuous artificial sunlight ("24h AS") (Fig. 6.2), while tomato plants grown under "16h AS" performed the best, showing the highest F_v/F_m, indicating that plants develop normally under plasma-based AS. Also cucumber plants were reported to grow well under AS light (Hogewoning *et al.*, 2010a). Interestingly, CLT tomato plants also developed injuries under "24h AS" (Fig. 6.2). Minor, detrimental effects of CL in CL-tolerant tomato have been reported before. For instance, detailed photosynthesis measurements on CL-tolerant F_1 hybrid tomato plants cultivated in greenhouses under natural day/night cycles, supplemented with HPS light for 24h day^{-1}, showed that despite the CL-tolerance, a slight CL-induced decrease in photosynthesis was observed (Velez-Ramirez *et al.*, 2014 (Chapter 4)), indicating that the CL-tolerance in tomato is not absolute. The data presented here, indicate that "24h AS" treatment is so injurious that even CL-tolerant plants get affected. Nonetheless, CLT tomato plants are consistently CL-tolerant if red/blue LED or HPS lamps are used to compose the CL treatment (Fig. 6.2, Fig. 6.3 and (Velez-Ramirez *et al.*, 2014 (Chapter 4))). Differences between these two light sources and AS include the presence of far-red (Table 6.1 and Fig. 6.1) and small amounts of UV light (data not shown) in AS but not in the former two. Elucidating why continuous AS is so injurious to tomato plants remains for future studies. For now, we have shown that domesticated tomatoes are sensitive to CL even if the light spectral distribution between 400 and 800 nm is virtually the same as sunlight. Hence, the main triggering factor inducing injury in CL-exposed tomatoes is most probably not the extensive differences between sunlight and commonly used artificial light sources such as fluorescent tubes, LED and HPS lamps.

Continuous light signals would be the second factor considered as potential trigger of injury in CL-grown tomato. Considering that addition of far-red light to the CL treatment is reported to reduce injury in tomato (Globig *et al.*, 1997), the red/far-red phytochrome photoreceptors must be considered (Bae & Choi, 2008, Rockwell *et al.*, 2006). Before discussing further, it is argued that the concept of phytochrome photostationary state (PSS) (Holmes & Fukshansky, 1979) should be used rather than red-to-far red ratio as the latter fails to characterize, for instance, blue LED light (Table 6.1). In short, a PSS of 1 means that all phytochrome is in the activated state (far-red-absorbing form, Pfr), while a PSS of 0 means that all phytochrome is in the deactivated state (red-absorbing form, Pr). Table 6.1 shows the calculated PSS for all light treatments. Considering that, in the absence of red light, blue light can lower the PSS, the non-injurious "AS-dim blue" treatment has a lower PSS; notice that at night the active phytochrome is deactivated or degraded. In contrast, the weakly injurious "AS-dim red" and the injurious "24h AS" treatments set the subjective night PSS at 0.89 and 0.72,

respectively, which implies a larger pool of active phytochrome than during a normal night. The low injury severity in "AS-dim red" treatment (Fig. 6.2) could be prompted by the low light intensity during the subjective night (Table 6.1) as several previous experiments demonstrated that the higher the light intensity the higher the CL-induced injury (Arthur *et al.*, 1930, Murage *et al.*, 1997). These results suggest that activation of phytochrome during the subjective night and higher light intensity favors the development of CL-induced injury. However, when comparing "24h AS" and "24h red/blue", which are two light treatments with constant spectral distribution and PPFD, no correlation between PSS and injury severity is found. Furthermore, if phytochrome activation during the subjective night triggers the CL-induced injury, then we would expect the "Blue night" treatment to be less injurious than the "Red night" and "24h red/blue" treatments. As seen in Fig. 6.2, however, this was not the case. Altogether, there is no clear correlation between PSS and the severity of the injury; nonetheless, we should consider that phytochrome signaling is complex. Phytochrome-mediated responses are classified into three categories, namely very-low-fluence response (VLFR), low-fluence response (LFR) and high-irradiance response (HIR) (Casal *et al.*, 1998). Each of these three responses is mediated by different phytochrome types, saturates at different light intensities and responds differently to red and far-red light (Bae & Choi, 2008, Casal *et al.*, 1998). For example, inter conversion between active and inactive forms, which is driven by both red and far-red light, is essential for PHYA-mediated HIR (Possart *et al.*, 2014, Rausenberger *et al.*, 2011). Therefore, the puzzling effects of the various light treatments on the CL-induced injury (Fig. 6.3) could arise from such complex nature of phytochrome responses.

In addition to phytochrome signaling, light signaling triggered by blue light should also be considered. For instance, plastid signals could "rewire" the light-signaling network in such a way that blue light represses photosynthesis-associated genes instead of inducing them (Larkin & Ruckle, 2008, Ruckle *et al.*, 2007). Additionally, single high-fluence pulses of blue light destabilized *LHCB* transcripts (Folta & Kaufman, 2003), and in seedlings with dysfunctional chloroplasts, bright light decreased the expression of *LHCB* (Ruckle *et al.*, 2007). Considering that the expression of *CAB-13*, a type III LHCB, is down-regulated by CL in sensitive tomato and was found to be responsible for CL-tolerance in wild tomato (Velez-Ramirez *et al.*, 2014 (Chapter 4)), it is plausible that blue light plays a role in regulating *CAB-13* and the expression of other photosynthetic genes under CL. Considering the effect of the "blue night" and "red night" treatments, it seems that neither constant blue nor red light is needed to induce injury in CL-exposed tomatoes. Additionally, the injurious abnormal light/dark cycles (Fig. 6.3) do not provide a continuous light signal. Although some evidence suggests that light signaling could ultimately influence injury severity (Globig *et al.*, 1997), a continuous light signal is not required to induce injury in CL-tomatoes. Hence, factor II cannot be the triggering factor.

As factor III and IV, which are constant photosynthesis and photo-oxidative pressure, depend both on the number of photons absorbed, it is difficult to disentangle them. Striving for equal photosynthesis across treatments, all the light treatments used in this study had the same PPFD (Table 6.1). Although small differences in photosynthesis

rate between plants grown under different light sources at the same PPFD are reported (Hogewoning *et al.*, 2010a), those small differences in photosynthetic rate are unlikely to result in the large observed differences in injury between "24h AS" and "24h red/blue" treatments (Fig. 6.2). Regarding photo-oxidative pressure, there is no clear trend linking the CL-induced injury with the calculated photoinhibitory activity of each treatment (Table 6.1); such photoinhibitory activity is an estimation of how much a given light source could damage chloroplast components based on its spectral distribution and the action spectra of photoinhibition measured by Jones and Kok (1966). Considering that carbohydrate accumulation is suggested to be responsible for the CL-induced injury (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)) and that CAB-13, which is linked to CL-tolerance in wild tomato species, is suggested to play a role balancing the light harvested by PSI and PSII (Velez-Ramirez *et al.*, 2014 (Chapter 4)), it is plausible that a constant PPFD contributes to the development of injury in CL-grown tomato by promoting photosynthesis and/or photo-oxidative pressure. As shown in Fig. 6.3, however, constant PPFD is not an absolute requirement for injury induction as abnormal light/dark cycles also induce the same kind of injury, yet they do not provide constant PPFD.

Although factors II to IV might play a role in inducing injury in CL-grown tomato plants, its absence in the injurious abnormal light/dark cycles strongly suggests that they are not the triggering factor that we are looking for. Instead, circadian asynchrony, factor V, seems to be the main triggering factor in CL-induced injury. Tomato plants show free-running cycles with a periodicity of ~24h under constant conditions like CL (Facella *et al.*, 2008, Giuliano *et al.*, 1988). The 6-h light / 6-h dark, 16-h light / 8-h dark and 24-h light / 24-h dark cycles used in this study imply a periodicity of 12, 24 and 48h respectively; hence, exposing tomato plants to the first and last light/dark cycle causes circadian asynchrony. Continuous light (24-h light / 0-h dark cycles) would imply an infinite periodicity or no periodicity; in any case, it also implies circadian asynchrony. Here, we showed that no matter whether the PSS or PPFD were constant or presented in a diurnal cycle, mottled chlorosis always appeared in young A131 leaves if plants were exposed to a light regime that implied circadian asynchrony. In addition, despite any circadian asynchrony imposed on the plants, CLT leaves never showed CL-induced injury when exposed to abnormal light/dark cycles (Fig. 6.3). Previous experiments showed that the stronger the circadian asynchrony (larger deviation from a 24-h periodicity) the worse the injury (Hillman, 1956, Kristoffersen, 1963). For instance, light / dark cycles with a periodicity of 8, 10, 12, 15 and 16h induced injury in tomato plants, but cycles with a periodicity of 18h only induced very slight injury; while cycles with a periodicity of 20, 24 and 30h did not induce injury (Hillman, 1956). In the other extreme, light / dark cycles with a periodicity of 48 and 72h also induced injury, being more severe under 72-h cycles (Kristoffersen, 1963). In addition to the injury induced by the abnormal cycles themselves, Hillman (1956) showed that exposing tomato plants to 8, 10, 12, 15 or 16-h cycles for five days previous to the exposure to CL increased the plant sensitivity to develop CL-induced injury.

Previously, we proposed that circadian asynchrony could induce the CL-induced injury by a mismatch between a fluctuating, circadian-controlled protection against photoinhibition and a continuous photo-oxidative stress imposed by light (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Here, however, experimental evidence contradicts this hypothesis. When A131 and CLT seedlings were exposed to very high light for 5 minutes at different times during the first 48h of CL, no circadian-controlled fluctuations in sensitivity to photoinhibition were observed, yet a diurnal fluctuation was observed under 12-h light / 12-h dark cycles (Fig. 6.4). This suggests that light itself up-regulates protection to photoinhibition in a circadian-independent way. Dorais *et al.* (1995) showed lower photoinhibition in thylakoid membranes isolated from CL-grown tomatoes than in those isolated from 12-h light / 12-h dark cycles after a 30 minute exposure to high light (3500 $\mu mol.m^{-2}.s^{-1}$), suggesting that CL-exposed tomatoes have a higher protection against photoinhibition.

In summary, the CL-induced injury does not result from the unnatural spectral distribution of artificial lamps, yet CL *per se* is also not absolutely needed to induce the injury as abnormal light / dark cycles induce an injury equivalent to the CL-induced injury. Although circadian asynchrony might be the triggering factor in the CL-induced injury, deregulation of normal, daily changes in sensitivity to photoinhibition do not play a role, at least not directly, as they do not show a circadian controlled rhythm. For historical and practical reasons, we suggest to keep using the term CL-induced injury to refer to this disorder. How light intensity influences the severity of the CL-induced injury remains to be uncovered, yet photosynthetic down regulation triggered by carbohydrate accumulation, blue light signaling or long-term photoinhibition are worth considering.

Materials and Methods

Plant materials and growth conditions

Two tomato lines were used in this study. A131 is an inbred line sensitive to continuous light (CL), and CLT is a CL-tolerant introgression line in the background of A131. The wild donor of CL-tolerance in CLT was *S. neorickii* (LA2133). Both lines have been previously described (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Seeds were provided by Monsanto Vegetable Seed Division (Bergschenhoek, The Netherlands). Seeds were sown in rockwool blocks at 21 °C and 70% RH. Commercial hydroponic nutrient solution for tomato was used (Yara Benelux B.V., Vlaardingen, The Netherlands); after combining and diluting premixed liquid fertilizers, the solution contained 12.42 mM NO_3, 7.2 mM K, 4.1 mM Ca, 3.34 mM SO_4, 1.82 mM Mg, 1.2 mM NH_4, 1.14 mM P, 30 mM B, 25 mM Fe, 10 mM Mn, 5 mM Zn, 0.75 mM Cu and 0.5 mM Mo (EC = 2.00 $dS.m^{-1}$ and pH = 5.0-5.5). Seedlings used for light treatments experiments were initially grown under high-pressure sodium (HPS) lamps (Master SON-T Green Power 400W, Philips, Eindhoven, The Netherlands) supplemented with incandescence lamps (Philinea T30 120W, Philips, Eindhoven, The Netherlands) at a photosynthetically active photon flux density (PPFD) of

345 µmol.m^{-2}.s^{-1}; red-to-far-red ratio was 2.89, and the phytochrome photostationary state (PSS) (Sager *et al.*, 1988) was 0.858. After growing the plants for two to four weeks under 16-h light / 8-h dark cycles, plants were transferred to the various light treatments.

For measuring diurnal changes in sensitivity to photoinhibition, CLT and A131 tomato seedlings were grown under 12-h light / 12-h dark cycles; light was provided by a mixture of blue and red light emitting diodes (LEDs) (Royal Blue and Red Luxeon K2, Lumileds Lighting Company, San Jose, USA). Blue and red LEDs provided a PPFD of 20 and 100 µmol.m^{-2}.s^{-1} respectively; the PSS was 0.885. After 14 days, sensitivity to photoinhibition was measured.

Light treatments

In addition to the abovementioned red and blue LEDs as well as HPS lamps, a plasma lamp was used. This lamp (Triple A class Solar Simulator Lamp (AAA), Plasma International GmbH, Offenbach am Main, Germany) closely mimics the spectral distribution of sunlight (Fig. 6.1). For LED and plasma lamp-based treatments, two week-old plants were used. "16h red/blue" and "24h red/blue" treatments consisted in providing a mixture of red (80 µmol.m^{-2}.s^{-1}) and blue (20 µmol.m^{-2}.s^{-1}) LED light for 16 or 24 hours per day, respectively. "Red night" and "blue night" treatments consisted in providing a mixture of red (80 µmol.m^{-2}.s^{-1}) and blue (20 µmol.m^{-2}.s^{-1}) LED light for 16 hours a day plus only red (100 µmol.m^{-2}.s^{-1}) or blue (100 µmol.m^{-2}.s^{-1}) light, respectively, for the remaining 8 hours per day. Using the plasma lamp to provide artificial solar (AS) light, "16h AS" and "24h AS" treatments consisted in providing 100 µmol.m^{-2}.s^{-1} of PPFD for 16 or 24 hours per day, respectively. "AS-dim red" and "AS-dim blue" treatments consisted in providing AS light (90 µmol.m^{-2}.s^{-1}) for 16 hours per day plus red (10 µmol.m^{-2}.s^{-1}) or blue (10 µmol.m^{-2}.s^{-1}), respectively, for 24 hours a day. Figure 6.1 shows the spectral distribution of all light sources, and Table 6.1 summarizes all LED and plasma-based light treatments. For the "abnormal light / dark cycles" treatments, four week-old plants were kept under the abovementioned HPS light supplemented with incandescence lamps, but the light / dark cycles were changed to 24-h light / 0-h dark, 6-h light / 6-h dark or 24-h light / 24-h dark.

Chlorophyll fluorescence imaging

Imaging of the maximum quantum efficiency of photosysmtem II (F_v/F_m) (Baker, 2008) was performed as previously described (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In summary, intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences, Lincoln, USA). After 20 minutes of dark adaptation, leaflets were detached and immediately used for measurements in a chlorophyll fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic). In the abnormal light / dark cycles and photoinhibition experiments, a Fluorcam 800MF was used instead. Leaflet average F_v/F_m was calculated using ImageJ software version 1.44o (Schneider *et al.*, 2012).

Photoinhibition experiment

Sensitivity to photoinhibition was measured every hour in 14 days-old seedlings. Three sets of plants were used; seedlings in the first set were kept always under 12-h light / 12-h dark cycles; while seedlings in the second and third set were transferred to continuous light at day 12 and 13, respectively. In that way, each set received zero, one or two days of continuous light, yet they had the same age at the time of measurement.

To measure sensitivity to photoinhibition, seedlings were first placed inside the FluorCam 800MF, and the first true leaf was carefully immobilized in a fixed position facing the camera lens. After dark-adapting the seedlings for 20 minutes, F_v/F_m was determined. Then, the FluorCam red-orange LED panels were turned on to maximum power for 5 minutes. The resulting PPFD on the first true leaf was 3684±57 $\mu mol.m^{-2}.s^{-1}$. To prevent a concomitant temperature shock, a 12V fan cooled the seedlings when the LED panes were set at maximum power; leaf temperature did not raise more than 3 °C (monitored with a K-type thermocouple touching the abaxial leaf surface). After the 5-minute exposure to high light, the seedlings were dark adapted again for 20 minutes, and the F_v/F_m was determined for a second time. Sensitivity to photoinhibition was calculated as the difference in leaflet average F_v/F_m between the first and the second measurement.

Statistical analysis

Statistical significance of the leaflet average F_v/F_m was determined with an ANOVA test performed with R (R Core Team, 2013).

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Phytochrome A protects tomato plants from injuries induced by continuous light

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Abstract

Plants perceive and transduce information about light quantity, quality, direction and photoperiod via several photoreceptors and use it to adjust their growth and development. A role for photoreceptors has been hypothesized in the injuries that tomato plants develop when exposed to continuous light as the light spectral distribution influences the injury severity. Up to now, however, only indirect clues suggested that phytochromes (PHY), red/far-red photoreceptors, are involved in the continuous-light-induced injuries in tomato. In this study, mutant and transgenic tomato plants lacking or over-expressing phytochromes were exposed to continuous light, with and without far-red light enrichment, to test the role of individual phytochromes on the induction and/or prevention of injury. *PHYA* over-expression confers complete tolerance to continuous light regardless the light spectrum. Under continuous light with low far-red content, PHYB1 and PHYB2 diminished and enhanced the injury, respectively, yet the effects were small. These results confirm that phytochrome signaling networks are involved in the injury induction under continuous light. The link between type III light harvesting chlorophyll a/b-binding protein 13 (CAB-13), which was previously shown to be important in determining tolerance/sensitivity to continuous light, and PHYA is discussed.

Introduction

Sunlight is essential for life on earth. Plants not only have developed a photosynthetic machinery to harvest the energy from the sun but also a set of photoreceptors that can sense light quality, quantity, direction and photoperiod, allowing them to adjust their growth and development according to the light environment (Bae & Choi, 2008, Christie, 2007, Jiao *et al.*, 2007, Moglich *et al.*, 2010). When artificial light is used, however, physiological disorders can arise, yet it is not always clear whether the cause resides in the photosynthetic or light sensing processes. A remarkable example, first reported by Arthur *et al.* (1930), are the injuries that domesticated tomatoes develop when exposed to continuous light (CL), which include mottled chlorosis and poor photosynthetic performance. It is still unclear if the altered photosynthetic performance is a cause or consequence, and which of the light signalling mechanisms plays a role, if any. Based on evidence accumulated over the years and a modern understanding of plant physiology, we previously proposed that both, photosynthesis and light signalling play a role in the development of the injury in CL-grown tomato plants (Velez-Ramirez *et al.*, 2011 (Chapter 2)).

Recently, the languished CL-tolerance found in wild tomato species (Daskaloff & Ognjanova, 1965) was mapped to the *type III Light harvesting chlorophyll a/b binding protein* 13 gene (*LHCB type III CAB-13* or *CAB-13*) on chromosome seven (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In addition to genetic mapping, expression data showed a positive correlation between *CAB-13* expression and CL-tolerance. For instance, in CL-sensitive tomatoes, CL down regulated *CAB-13* expression, but in a *Solanum lycopersicum* CL-

tolerant introgression line, known as "CLT", *CAB-13* expression was high under CL. Furthermore, when *CAB-13* expression was silenced in the CLT line, using virus-induced gene silencing (VIGS), plants lost their tolerance to CL (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This evidence strongly suggests the involvement of the photosynthetic machinery in the CL-induced injury. Previous studies also reported that the light spectral distribution influences the severity of the injury (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997). Therefore it is still unclear if the differences in CL-induced severity of the injury associated with different light sources are attributable to a photosynthetic, photo-oxidative or light signalling process (Chapter 6). Hence, the potential involvement of light signalling pathways must not be discarded. In tomato, a reduction in CL-induced injury was reported when the light was enriched with far-red light (Globig *et al.*, 1997). Hence, from all currently known photorecepetors (Moglich *et al.*, 2010), phytochromes are the most likely candidates to be involved, yet direct evidence is missing.

Phytochromes (PHY) translate red and far-red light into biological signals thanks to covalently attached chromophores that enable photo-conversion between two forms: the Pr form and the biologically active Pfr form. With a peak absorbance for red light, Pr is converted to Pfr upon light absorption. In turn, far-red light most effectively transforms back the Pfr to the biologically inactive Pr form (Bae & Choi, 2008, Rockwell *et al.*, 2006). Light colors other than red and far-red also drive Pr/Pfr inter-conversion, yet the absorbance and quantum yield are different for different wavelengths; in photoequilibrium, therefore, the ratio of Pr/Pfr depends on the light spectral distribution (Sager *et al.*, 1988). Upon photoisomerization, Pfr translocates to the nucleus where it activates the degradation of phytochrome-interacting factors (PIFs) and inhibits two E3 ubiquitin ligases (CUL4-DDB1-COP1-SPA and CUL4-DDB1-DET1-COP10); this results in light responses within the plant as PIF transcription factors promote dark and shade responses, and several positive light regulators, like Elongated Hypocotyl 5 (HY5), are degraded by COP1- and DET1-containing E3 ubiquitin ligases (Bae & Choi, 2008, Chen & Chory, 2011, Lau & Deng, 2012, Leivar & Quail, 2011).

Arabidopsis has five phytochromes, PHYA to PHYE (Sharrock & Clack, 2002). The tomato genome also encodes 5 phyochromes known as PHYA, PHYB1, PHYB2, PHYE and PHYF (Hauser *et al.*, 1995). Phytochromes are classified in two types, the PHYA and PHYB branch, yet this dichotomy does not directly correlates with their molecular properties and functions across species (Bae & Choi, 2008). Unlike PHYB, PHYA can only translocate to the nucleus with the help of Far-red Elongated Hypocotyl 1 (FHY1) and FHY1-like (FHL) (Casal, 2013, Chen & Chory, 2011). Phytochrome-mediated, light-induced responses are classified into three categories, namely very-low-fluence response (VLFR), low-fluence response (LFR) and high-irradiance response (HIR) (Casal *et al.*, 1998).

Little is known about PHY functions in adult tomato plants. Most of the studies have focused on germination, anthocyanin biosynthesis and seedling de-etiolation. Anthocyanin biosynthesis in tomato is regulated by a PHYB1/PHYB2-dependent red-HIR

component as well as PHYA-dependent far-red-HIR and a non-far-red-reversible red-LFR component (Kerckhoffs *et al.*, 1997, Weller *et al.*, 2000); however, PHYA antagonizes the effect of PHYB1, and PHYB2 cannot compensate the loss of PHYB1 as most of the red-HIR depends on PHYB1 (Weller *et al.*, 2000). In addition, unlike other species, in tomato such PHYA-dependent red-LFR of anthocyanin biosynthesis is strongly reduced in the *phyB1phyB2* double mutant (Weller *et al.*, 2000). Regarding other light-regulated process, mutant studies show that the red-HIR component of tomato seedling de-etiolation depends, redundantly, on PHYB1 and PHYB2, yet, as with anthocyanin biosynthesis, only PHYB1 can compensate for the loss of PHYB2 (Weller *et al.*, 2000). However, transgenic over-expression of *PHYB2* not only fully compensates but enhances the red-HIR component of anthocyanin biosynthesis even in the *phyB1phyB2* double mutant background (Husaineid *et al.*, 2007). Over-expression of PHYB1 had little or no effect on red-HIR responses (Husaineid *et al.*, 2007). Unlike anthocyanin biosynthesis, seedling de-etiolation is not affected by PHYA (Weller *et al.*, 2000). All together, phytochromes in tomato, as in other species, not only interact differently to control various traits but also in response to different light wavelengths and fluence rates. This makes it crucial to test the effect of each PHY under different light environments for every trait of interest.

In this study, in order to test whether phytochromes play a role in the CL-induced injuries in tomato, we exposed several phytochrome mutants and over-expressing lines to CL with two contrasting far-red light contents. The results show that *PHYA* over-expression confers complete CL-tolerance regardless the light spectral distribution. The roles of PHYB1 and PHYB2 appear to be less dominant than PHYA and depend on the light spectral distribution. These results not only confirm that phytochrome signaling networks are involved in the injury induction under CL but also provide further clues in understanding why CAB-13 is so important in determining tolerance/sensitivity to CL.

Results

Two-week-old plants of tomato wild-type, *phy mutants* and *PHYOE* lines were exposed to continuous light (CL) provided by high-pressure sodium (HPS) light, with and without addition of far-red light, see details in Fig. 7.1 and Table 7.1. Control plants were kept under 16-h photoperiod provided by HPS without supplemental far-red light. The presence and severity of CL-induced injury was assessed by the chlorophyll fluorescence parameter F_v/F_m (Chapter 6, Velez-Ramirez *et al.*, 2014 (Chapter 4)). The parameter F_v/F_m represents the maximum quantum efficiency of PSII, and a value of 0.83 is remarkably constant in non-stressed plants across species (Baker, 2008); therefore the lower the F_v/F_m value, the higher the injury is. After two weeks of treatment, all tomato lines grown under 16-h photoperiod showed F_v/F_m values that were not significantly different from the wild-type Moneymaker plants ($P>0.05$), and were similar as those reported in the literature for non-stressed healthy leaves (Baker, 2008). Under non-injurious conditions, leaflets of *phyB1* and *phyB1phyB2* single and double mutants had slightly lower F_v/F_m values than the wild-type (Fig. 7.2). For clarity and to account for these pleiotropic effects, results are also expressed

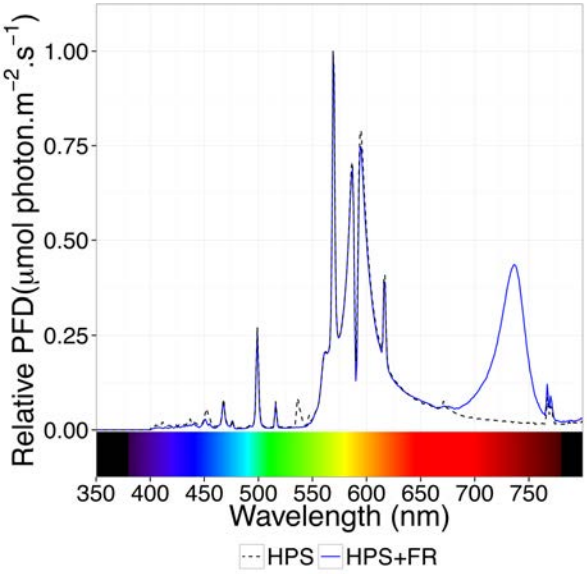

Figure 7.1 | Relative spectral distribution of the light treatments. The black line represents the spectral distribution of high-pressure sodium lamps (HPS). The dashed blue line represents the spectral distribution HPS light supplemented with far-red light-emitting diodes. See Table 7.1 for extra details.

as $\Delta\Delta F_v/F_m$, which is the difference between F_v/F_m of wild-type Moneymaker and the mutant/over-expressing lines under CL, after correction for the F_v/F_m value observed under 16-h photoperiod in the corresponding line (Fig. 7.3). A positive $\Delta\Delta F_v/F_m$ value represents a positive effect of the phytochrome mutation or over-expression on the CL-induced injury, *i.e.* a reduction in CL injury. Under CL, Moneymaker had lower leaflet F_v/F_m value ($P<0.05$) than under 16-h light (Fig. 7.2) and displayed clear interveinal chlorosis (Fig. 7.4).

PHYTOCHROME A over-expression protects tomato plants from continuous light

Interestingly, over-expression of *PHYA* completely prevented CL-induced injury regardless of the light spectrum and the F_v/F_m values under CL did not significantly differ from that of plants grown under 16-h photoperiod ($P>0.05$) (Fig. 7.2). Figure 7.4 shows that *PHYAOE* leaflets exposed to CL for two weeks showed no signs of chlorosis, while wild-type Moneymaker leaflets did. Supportive of a protective effect of PHYA, *phyA* mutant plants had slightly lower F_v/F_m values than Moneymaker under CL (Fig. 7.2). Overall, the results suggest that PHYA signaling diminishes the injuries that CL induces in tomato plants.

Double mutant *phyB2phyB2* shows mottled chlorosis even under 16h photoperiod

Under CL without far-red enrichment, *phyB1* and *PHYB1OE* lines had lower and higher F_v/F_m values, respectively, than wild-type Moneymaker (Fig. 7.2). These results suggest that PHYB1 signaling might diminish the CL-induced injury in tomato (Fig. 7.3). In contrast, *phyB2** and *PHYB2OE** lines had higher and lower F_v/F_m values than wild-type

Table 7.1. Light treatments characteristics

Light Treatment	PFD (µmol photons.m^{-2}.s^{-1})	PPFD (µmol photons.m^{-2}.s^{-1})	PSS (Sager *et al.*, 1988)	R:Fr ratio	Irradiance (W.m^{-2})	Photoinhibitory activity $\sum_{nm=400}^{800} \alpha\phi$ (Jones & Kok, 1966)
High pressure sodium + Philinea (HPS)	368	345	0.86	2.89	74	15.4
High pressure sodium + Philinea + Far Red (HPS+FR)	504	344	0.66	0.18	96	14.9

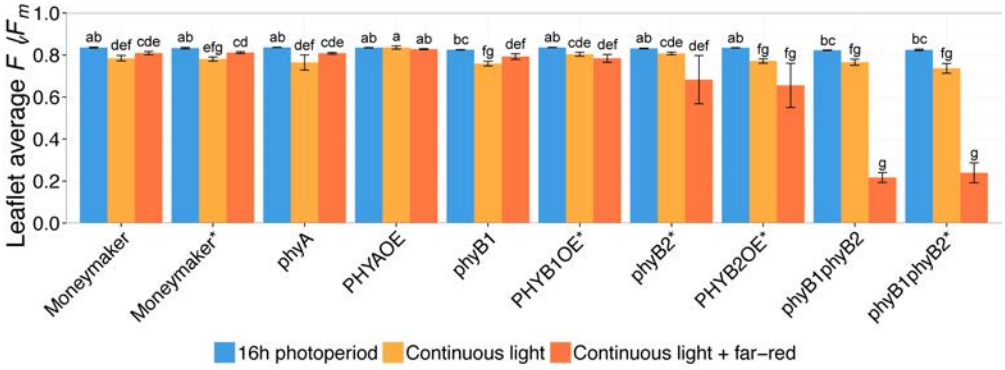

Figure 7.2 | Effect of two continuous light treatments on phytochrome mutants and over-expressing tomato lines. Tomato plants were grown under 16-h photoperiod for two weeks and then transferred to continuous light provided by high-pressure sodium (HPS) lamps (Continuous light, orange bars) or HPS lamps supplemented with far-red light (Continuous light + far-red, red bars). Control plants were kept at 16-h photoperiod (16h photoperiod, blue bars). After two weeks, chlorophyll fluorescence imaging assessed the level of continuous light-induced injury. Leaflet average F_v/F_m values represent mean ± SE (n=4); bars not sharing the same letter are statistically different (P<0.05). Line names marked with an * carry a circadian clock reporter construct (*Cab::Luciferase*).

Moneymaker*, respectively, suggesting that PHYB2 slightly enhances the CL-induced injury in tomato, at least when far-red light is not enriched.

Interestingly, double mutant lines *phyB1phyB2* and *phyB1phyB2** showed signs of chlorosis under 16-h photoperiod (Fig. 7.5). The mottled, interveinal chlorosis (Fig. 7.5b) resembles the characteristic mottled chlorosis that CL-exposed tomato leaves develop. Under CL, *phyB1phyB2* and *phyB1phyB2** double mutant lines presented severe CL-induced injuries (Fig. 7.2 and 5) (P<0.05). Furthermore, when CL treatment was enriched with far-red light, the CL-induced injury in both *phyB1phyB2* double mutant lines severely increased (Fig. 7.2, 7.3 and 7.5).

Enrichment of far-red light diminishes continuous-light-induced injury in tomato

In contrast to the *phyB1phyB2* double mutant lines, Moneymaker leaves showed less CL-induced injury symptoms when exposed to CL enriched with far-red light, compared with plants exposed to continuous HPS light without far-red enrichment, reflected in higher F_v/F_m values (Fig. 7.2). This is consistent with previous reports (Globig *et al.*, 1997). Although the differences are not significant, this trend is also visible in the chlorophyll fluorescence images (Fig. 7.5). Interestingly, *phyA* mutants showed the same trend as the wild type; that is less CL-induced injury when CL is enriched with far-red light (Fig. 7.2 and 7.4), suggesting that the protective effect of far-red light does not depend on PHYA.

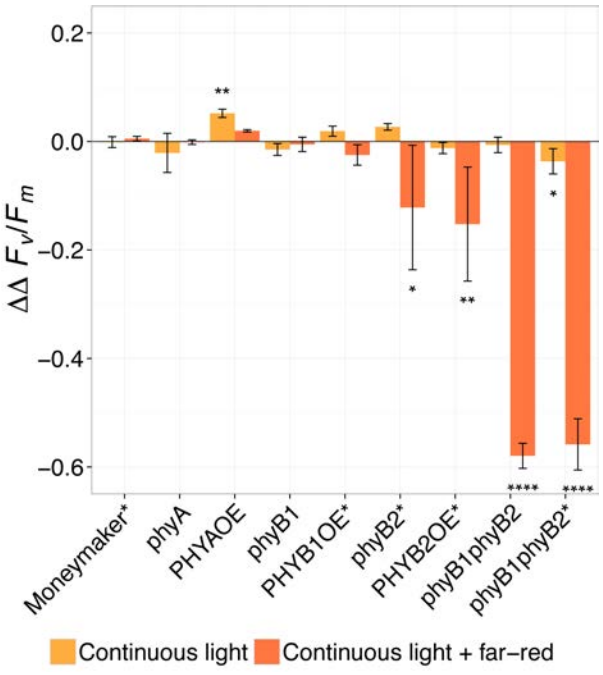

Figure 7.3 | Effect of continuous light on tomato, taking into account the pleiotropic effect of phytochrome mutations observed under 16-h photoperiod. Tomato plants were grown under 16-h photoperiod for two weeks and then transferred to continuous light provided by high-pressure sodium (HPS) lamps with and without far-red light enrichment (orange and red bars, respectively). The $\Delta\Delta F_v/F_m$ values represent the response of several phytochrome mutants and over-expressing lines to the light treatments taking the average continuous-light-induced decrease of F_v/F_m in the wild type (Moneymaker, MM) as a reference and correcting for the average slight decrease in F_v/F_m observed under 16-h photoperiod. That is, $\Delta\Delta F_v/F_m = -([\overline{MM_{CL}} - \{\overline{mutant_{CL}} \text{ or } \overline{OE_{CL}}\}] - [\overline{MM_{16h}} - \{mutant_{16h} \text{ or } \overline{OE_{16h}}\}]$. Where $\overline{MM_{CL}}$ and $\overline{MM_{16h}}$ are the average F_v/F_m in the wild-type Moneymaker under 16-h photoperiod and continuous light, respectively; $\overline{mutant_{16h}}$, $\overline{OE_{16h}}$, $\overline{mutant_{CL}}$ or $\overline{OE_{CL}}$are the average F_v/F_m in each mutant or overexpressing line under 16-h photoperiod or continuous light. The minus at the beginning of the equation is just for clarity; a positive value represents a positive effect of each phytochrome mutation or overexpression. Values represent mean ± SE (n=4). Asterisk on top of error bars indicate that the $\Delta\Delta F_v/F_m$ value is statistically different from zero; * P<0.05, ** P<0.01 and **** P<0.0001. See original data on Figure 7.2. Line names marked with an * carry a circadian clock reporter construct (*Cab::Luciferase*).

Expression of PHYB is lower in continuous-light-sensitive plants than in tolerant ones

In order to assess the effects of CL on tomato *PHY* expression, we mined a published whole transcriptome RNAseq data set derived from tomato plants exposed to CL at the same PPFD and spectral distribution (HPS) as in the preset study (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This data set contains differential expression levels for two contrasts; the first

contrast compares the effect of CL on the CL-sensitive tomato inbred line A131, and the second one evaluates the differences under CL between A131 and a CL-tolerant introgression line named CLT (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In A131 plants, CL exposure resulted in a significant up-regulation of *PHYE* expression, while the differential expression of all other *PHYs* is not significant (Table 7.2). Under CL, the expression of *PHYB1* and *PHYE* is higher in CL-tolerant CLT than in CL-sensitive A131 plants. Table 7.2 also shows the differential expression of *HY5*, a transcription factor downstream of the photoreceptors (Jiao *et al.*, 2007, Lee *et al.*, 2007a). Interestingly, *HY5* is significantly up-regulated by CL in A131 tomato plants, but there is no difference in expression between A131 and CLT plants when both are exposed to CL.

Table 7.2 Expression ratio of tomato *phytochrome* (*PHY*) genes and *HY5* transcription factor between A131 16h vs A131 24h and A131 24h vs CLT 24h.[§]

		A131 24h – A131 16h				A131 24h – CLT 24h			
Gene	Name	logFC	logCPM	p-value	FDR-corrected p-value	logFC	logCPM	p-value	FDR-corrected p-value
Solyc10g044670.1	*Phytochrome A* (*PHYA*)	0.40	5.14	0.06	0.19	0.24	5.14	0.25	0.60
Solyc01g059870.2	*Phytochrome B1* (*PHYB1*)	-0.11	5.45	0.59	0.80	-0.65	5.45	0.00	0.03
Solyc05g053410.2	*Phytochrome B2* (*PHYB2*)	-0.50	2.49	0.16	0.39	-0.67	2.49	0.05	0.26
Solyc02g071260.2	*Phytochrome E* (*PHYE*)	-1.21	4.90	0.00	0.00	-0.68	4.90	0.00	0.03
Solyc07g045480.2	*Phytochrome F* (*PHYF*)	0.23	3.57	0.31	0.59	-0.19	3.57	0.41	0.75
Solyc08g061130.2	*Elongated Hypocotyl 5* (*HY5*)	0.90	1.55	0.01	0.03	0.52	1.55	0.09	0.36

[§] A131 and CLT are sensitive and tolerant to continuous light (CL), respectiveley. Data taken from (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

Continuous light induces photosynthetic down-regulation in tomato plants

Considering that the CL-induced injury is proposed to be photosynthetic down-regulation (Velez-Ramirez *et al.*, 2014 (Chapter 4)), and phytochromes regulate the expression of several photosynthetic genes in tomato (Peters *et al.*, 1998), we also evaluated the effect of CL on photosynthesis at the transcriptional level. Therefore, the gene expression level of both contrasts was mapped to tomato-specific KEGG pathways (Fig. 7.6 and 7.7). As species-specific KEGG pathways are drawn over standard KEGG maps, care should be taken with the interpretation of white nodes. Some nodes contain no expression information because (i) that specific node does not exist in tomato (*e.g.* a bacterial antenna proteins in Fig. 7.7), (ii) the node is not yet annotated in tomato (only ~25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the gene(s) associated to that node were not detected in the data set (only ~14,000 genes with KEGG annotation

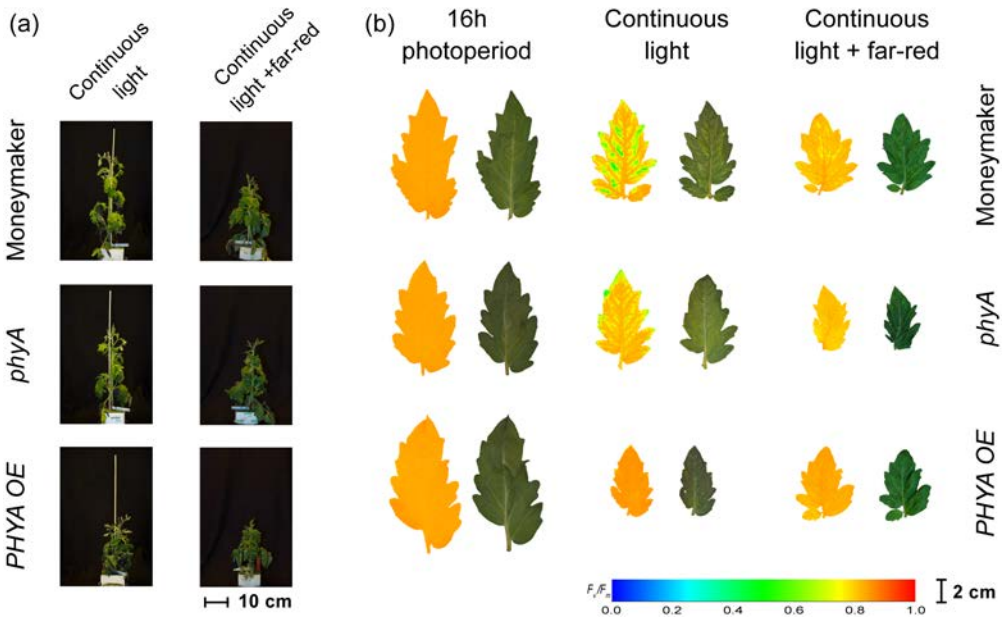

Figure 7.4 | Phenotypes of phytochrome A mutant (*phyA*) and over-expressing line (*PHYA OE*) under continuous light. Both lines are in the moneymaker background. In panel (**a**), tomato plants were grown under 16-h photoperiod for two weeks and then exposed to continuous light or continuous light + far-red for two weeks. In panel (**b**), representative leaflets (topmost fully expanded leaf) of plants in previous panel. Photographs (right side) and chlorophyll fluorescence images (left side) show the appearance of chlorosis on some lines. Across leaflet surface, a false color scale indicates F_v/F_m value (see guide at the bottom-left corner).

were detected in this data set). Additionally, the expression level of each node might be the mean of several genes; in Fig. 7.7, for example, *LHCB1* color-coded expression is the average fold change of the six type I LHCB proteins annotated in the KEGG database. For an overview of annotated tomato genes in KEGG pathways, follow the links in the figure legends.

Figure 7.6 shows the tomato KEGG pathway for "photosynthesis" (sly00195) as affected by CL. Interestingly, CL down regulates most of the annotated genes in A131 tomato plants. When both A131 and CLT plants were exposed to CL, most of the annotated genes showed lower expression in the CL-sensitive A131 plants than in CLT, which is CL-tolerant. Similarly, Fig. 7.7 shows the tomato KEGG pathway for "photosynthesis antenna proteins" (sly00196) as affected by CL. In A131 plants, CL represses the expression of all antenna proteins of PSII (*LHCB*) and PSI (*LHCA*) (Fig. 7.7). Additionally, CLT plants showed higher expression of all *LHCB* and *LHCA* proteins than A131 plants when both are exposed to CL. In other words, most of the photosynthesis

genes in CL-sensitive tomato plants exposed to CL are expressed at lower levels than in control plants under 16-h photoperiod and CL-tolerant plants exposed to CL. Furthermore, the same trend is observed in the tomato KEGG pathway for "porphyrin and chlorophyll metabolism" (sly00860) (Supplementary Fig. 7.1 and 7.2). That is, all enzymes in the chlorophyll biosynthesis pathway are expressed at lower levels in CL-exposed A131 plants compared with A131 plants under 16-h photoperiod and CLT plants exposed to CL. Altogether, CL down regulates photosynthesis in CL-sensitive but not in CL-tolerant tomato plants.

Discussion

At an irradiance of 345 $\mu mol.m^{-2}.s^{-1}$, the *PHYAOE* line was clearly tolerant to continuous light (CL) regardless of the light spectral distribution (Fig. 7.2, 7.3 and 7.4). Considering the classic VLFR and far-red HIR attributed to PHYA, such PHYA-dependent CL-tolerance in tomato is intriguing. As light induces the degradation of the PHYA Pfr form and attenuates PHYA signaling by repressing Far-red elongated hypocotyl 1 (FHY1) and FHY1-like (FHL), which are needed to shuttle PHY to the nucleus (Casal, 2013, Chen & Chory, 2011). Tomato PHYA is not an exception; after exposing wild-type and *PHYAOE* tomato seedlings to red light at 3 $\mu mol.m^{-2}.s^{-1}$, PHYA was greatly reduced as quantified with antibodies, yet trace amounts were still detectable after 4 hours (Husaineid *et al.*, 2007). Light-grown *phyA* and *PHYAOE* tomato plants show phenotypes different from wild-type plants (Husaineid *et al.*, 2007, van Tuinen *et al.*, 1995b), in most cases, however, this can by hypothetically attributed to PHYA accumulated during the daily dark period, which is subsequently activated to the Pfr form at dawn. Supporting the classic role of PHYA in the VLFR, *PHYAOE* tomato seedlings showed the strongest phenotypic differences from wild-type under continuous red light at ~0.01 $\mu mol.m^{-2}.s^{-1}$ as observed in fluence rate response curves of anthocyanin biosynthesis (Husaineid *et al.*, 2007). In Arabidopsis, however, a PHYA-dependent red HIR has been suggested as the use of *phy* mutant combinations showed that PHYA, PHYB and PHYD redundantly regulate mature plant architecture under continuous red light at an intensity of 160 $\mu mol.m^{-2}.s^{-1}$. At 100 and 180 $\mu mol.m^{-2}.s^{-1}$ of continuous red light, *phyB* mutants displayed inhibition of hypocotyl elongation, yet *phyAphyB* double mutants were remarkably insensitive to red light (Franklin *et al.*, 2007). An irradiance-dependent photoprotection of nuclear PHYA is proposed to explain such PHYA-dependent red HIR as PHYA was rapidly degraded after 2 hours but was still detectable for up to at least 8 hours of red light exposure at 1 or 180 $\mu mol.m^{-2}.s^{-1}$, respectively, and nuclear-localized PHYA:YFP epifluorescence was still detectable after 90 minutes of red light at 200 and not 1 $\mu mol.m^{-2}.s^{-1}$ (Franklin *et al.*, 2007). In tomato, *phyA* mutants showed anthocyanin accumulation similar to wild type under continuous red light (l_{max} 680 nm) at ~20 $\mu mol.m^{-2}.s^{-1}$, but under ~200 $\mu mol.m^{-2}.s^{-1}$, anthocyanin content in *phyA* mutants was approximately 38% lower than wild type (Husaineid *et al.*, 2007), suggesting the existence of a PHYA-dependent red HIR also in

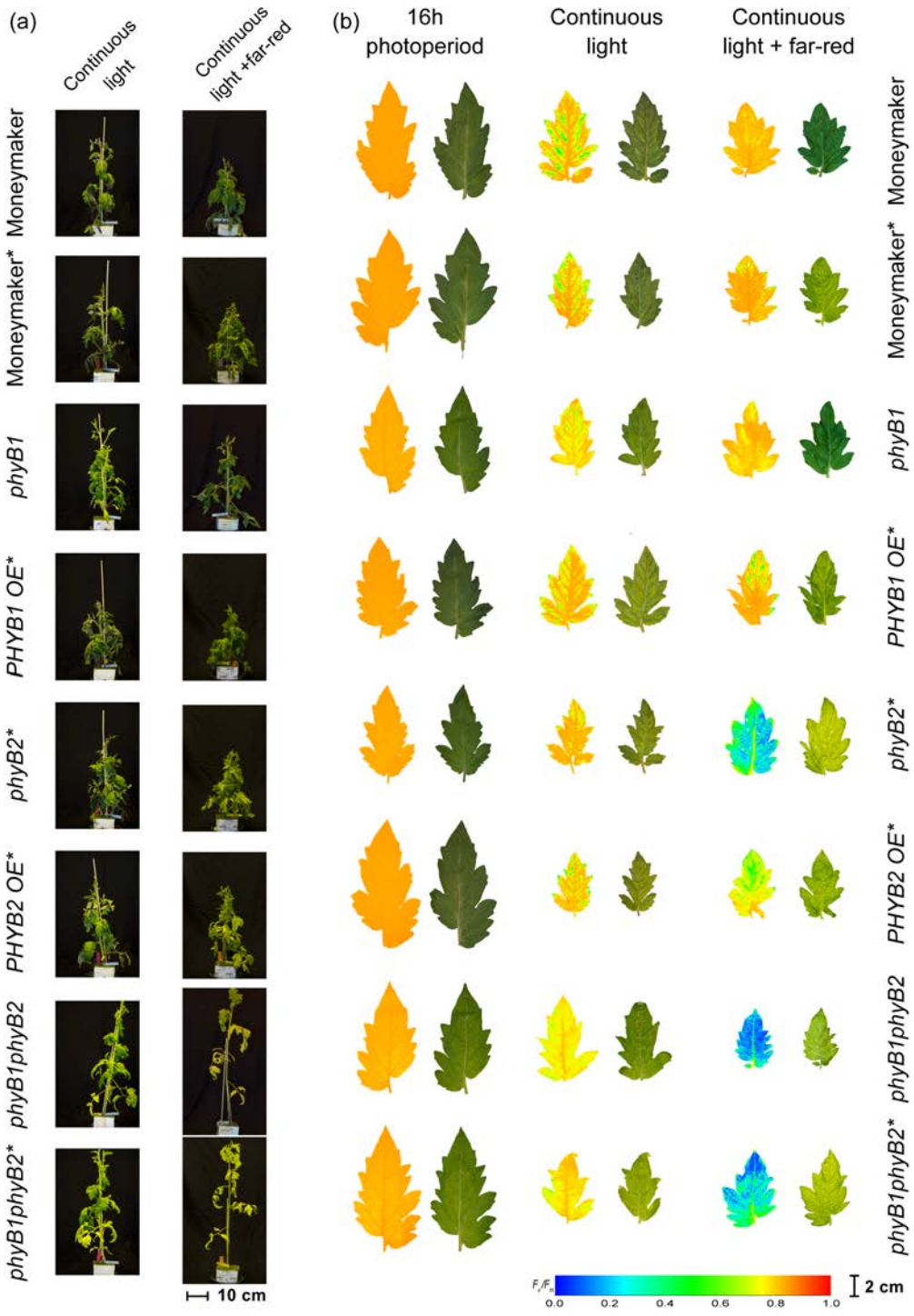
(a)
Continuous light
Continuous light +far-red
Moneymaker
Moneymaker*
phyB1
PHYB1 OE*
phyB2*
PHYB2 OE*
phyB1phyB2
phyB1phyB2*
10 cm
(b)
16h photoperiod
Continuous light
Continuous light + far-red
Moneymaker
Moneymaker*
phyB1
PHYB1 OE*
phyB2*
PHYB2 OE*
phyB1phyB2
phyB1phyB2*
Fv/Fm
0.0
0.2
0.4
0.6
0.8
1.0
2 cm

Figure 7.5 | Phenotypes of phytochrome B mutants and over-expressing lines under continuous light. Phytochrome B1 and B2 mutants (*phyB1* and *phyB2*), over-expressing lines (*PHYB1 OE* and PHYB2 OE) and double B1B2 mutant (*phyB1phyB2*). All lines are in the moneymaker background. In panel (**a**), tomato plants were grown under 16-h photoperiod for two weeks and then exposed to continuous light or continuous light + far-red for two weeks. In panel (**b**), representative leaflets (topmost fully expanded leaf) of plants in previous panel. Photographs (right side) and chlorophyll fluorescence images (left side) show the appearance of chlorosis on some lines. Across leaflet surface, a false color scale indicates F_v/F_m value (see guide at the bottom-left corner). In both panels, lines marked with an * carry a circadian clock reporter construct (*Cab::Luciferase*).

tomato. Hence, we propose that the protective effect of *PHYA* overexpression against CL at an irradiance of 100 $\mu mol.m^{-2}.s^{-1}$ truly depends on PHYA signaling.

PHYA signaling mediates VLFRs, which are extremely sensitive to Pfr as even pulses of far-red or "green safe" light can elicit enough Pfr to saturate VLFR; PHYA also mediates light signaling during dark-to-light transitions and far-red HIR (Bae & Choi, 2008, Casal *et al.*, 1998). This PHYA-dependent far-red HIR is unique as no other photoreceptor can mediate responses induced by far-red light (Possart *et al.*, 2014). Although red light is most efficient eliciting Pfr, *i.e.* increasing the PSS, far-red light is most efficient triggering PHYA-dependent responses. An antagonistic photoconversion cycling model explains the shift towards far-red in PHYA action spectrum (Possart *et al.*, 2014). Evidence shows that, similar to other phytochromes, PHYA Pfr is the active form in signaling, yet photocycling between Pr and Pfr forms is needed to both bind and, once inside the nucleus, release the FHY1/FHL transporters (Rausenberger *et al.*, 2011). A recent study shows that PHYA directly targets numerous genes related to photosynthesis, respond to light, stress and hormones in multiple far-red-modulated processes (Chen *et al.*, 2014). Thus, investigating the PHYA-dependent mechanism protecting tomato from CL must consider the unique properties of PHYA signaling.

In Arabidopsis, the red/far-red reversible LFR is mediated by phytochromes other than PHYA (Bae & Choi, 2008), and in tomato PHYB1 and PHYB2 mediate red HIR (Weller *et al.*, 2000). As HPS light is rich in wavelengths in the orange-red spectrum (Fig. 7.1), resulting in a distinctively high PSS value of 0.858 (Table 7.1), PHYB1 and PHYB2 signaling effects were expected under HPS light without far-red enrichment. Under such light treatment, PHYB1 and PHYB2 showed opposite roles, *phyB1* and *phyB2* mutations increased and decreased the CL-induced injury, respectively; accordingly, *PHYB1* and *PHYB2* overexpression decreased and increased the injury, respectively (Fig. 7.2, 3 and 5). Interestingly, *PHYB1* expression is not affected by CL in A131 plants, but CL-tolerant CLT plants show higher *PHYB1* expression than A131 plants when both are exposed to CL (Table 7.2). All together, the evidence suggests that PHYB1 signaling protects tomato plants from CL-induced injury, and PHYB2 enhances the injury.

When HPS light was enriched with far-red, the *phyB2** mutant performed worse than *phyB1*, yet the *phyB1phyB2*$^{(*)}$ double mutant lines performed the worst (Fig. 7.2 and 7.3), suggesting that, under this light condition, PHYB1 and PHYB2 redundantly protect tomato plants from CL, yet PHYB2 seems to have a dominant role. This is contrary to

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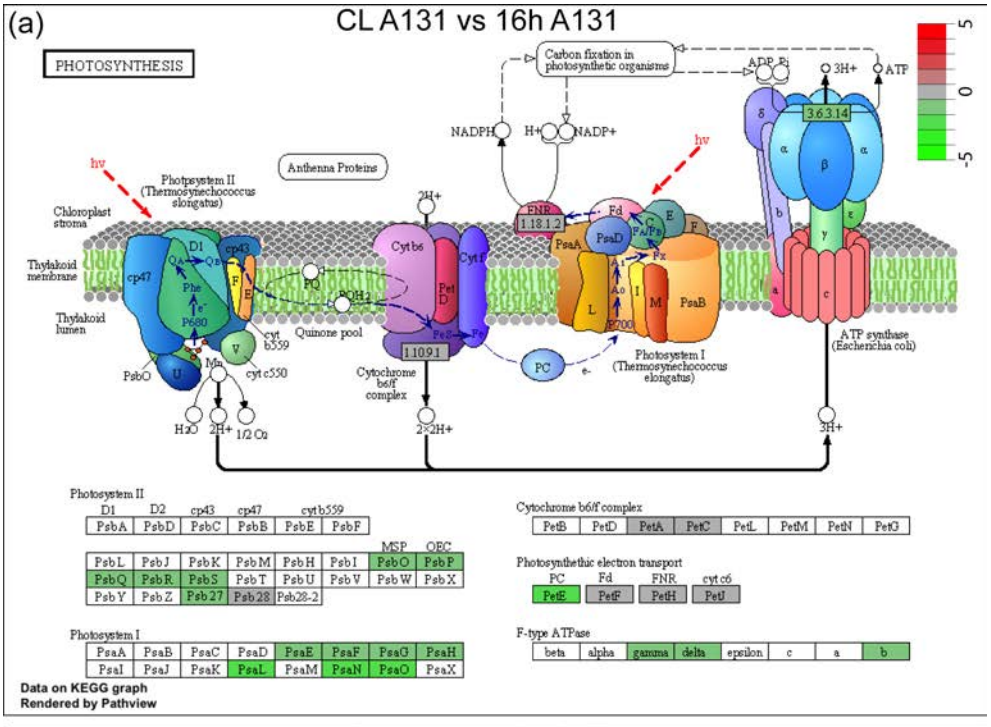

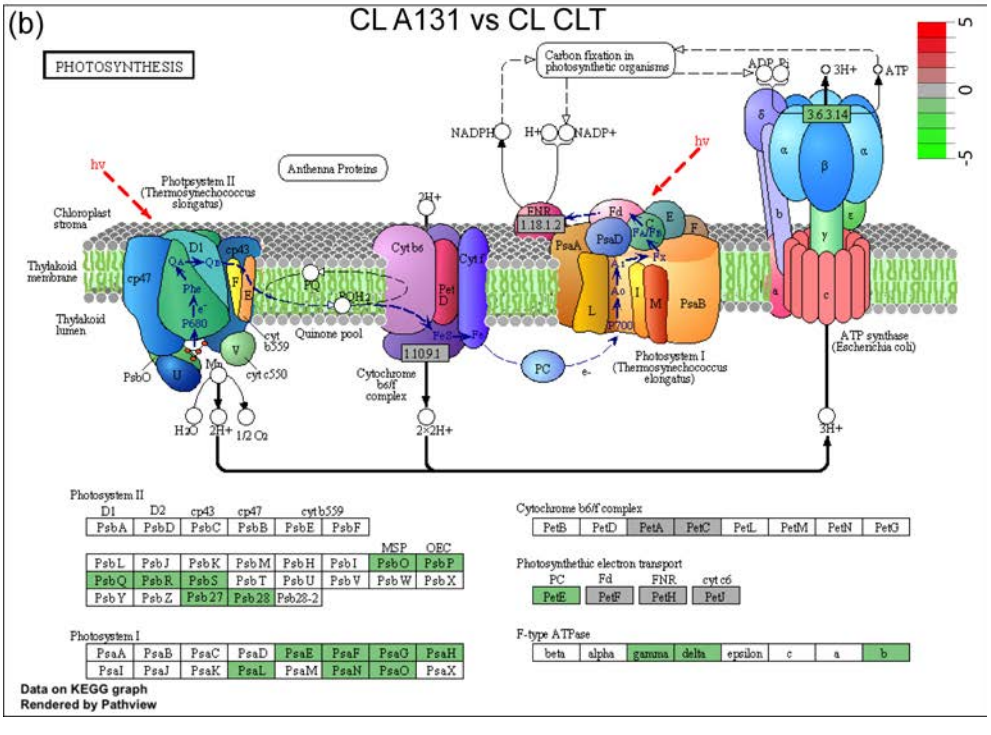

Figure 7.6 | Tomato "Photosynthesis" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for photosynthesis. In panel (**a**), contrast between A131 tomato plants exposed to 16-h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00195

what was observed in anthocyanin biosynthesis and seedling de-etiolation, where PHYB1 dominates over a redundant PHYB2 (Weller *et al.*, 2000). Intriguingly, *PHYB1OE** and *PHYB2OE** lines showed increased sensitivity to CL as *phyB1* and *phyB2* mutants did (Fig. 7.3), the effect being more severe in the case of *PHYB2*. Such apparent discrepancy between the PHYB2 role inferred from *phyB2** mutant and *PHYB2OE** line should be interpreted carefully as we observed large variation from plant to plant and discrepancies in inferring the role of tomato PHYB2 have been observed before. For instance, Weller *et al.* (2000) deduced from *phyB1*, *phyB2* and *phyB1phyB2* mutant lines that PHYB2 has a negligible effect on the red HIR in anthocyanin biosynthesis as the null mutation of *PHYB2* is only seen in the *phyB1* mutant background, yet Husaineid *et al.* (2007) showed that *PHYB2* overexpression not only can compensate for the loss of *PHYB1* but can even increase the wild-type red HIR to anthocyanin biosynthesis even in the *phyB1phyB2* double mutant background.

Far-red light enrichment should result in similar phenotypes between *phyB1*/*phyB2* mutant and *PHYB1*/*PHYB2* overexpression lines as far-red light enrichment should decrease the PHYB1/PHYB2 active Pfr pool. Considering that the PHYA-dependent red-LFR on anthocyanin biosynthesis in tomato depends on either PHYB1 or PHYB2 (Weller *et al.*, 2000), it should also be considered that the strong protective effect of PHYA overexpression against CL-induced injury depends, at least in part, on the supportive role of PHYB1/PHYB2. In this scenario, PHYA would be similarly active under either light treatment, but under far-red light enrichment PHYB1 and PHYB2 inactivation would be accountable for the slight loss in the ability of *PHYA* overexpression to protect tomato plants from CL (Fig. 7.3). Hence, testing whether the protective effect observed in the *PHYAOE* line remains in the *phyB1phyB2* double mutant background is of great interest for future experiments.

Phytochrome signaling might explain the observed effect of the light spectral distribution on the severity of the CL-induced injuries in tomato (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997). This effect is complex and interacts with the light intensity (Chapter 6). For instance, when a 16-h photoperiod of artificial solar light at 90 $\mu mol.m^{-2}.s^{-1}$ was extended to CL by superimposing dim red or

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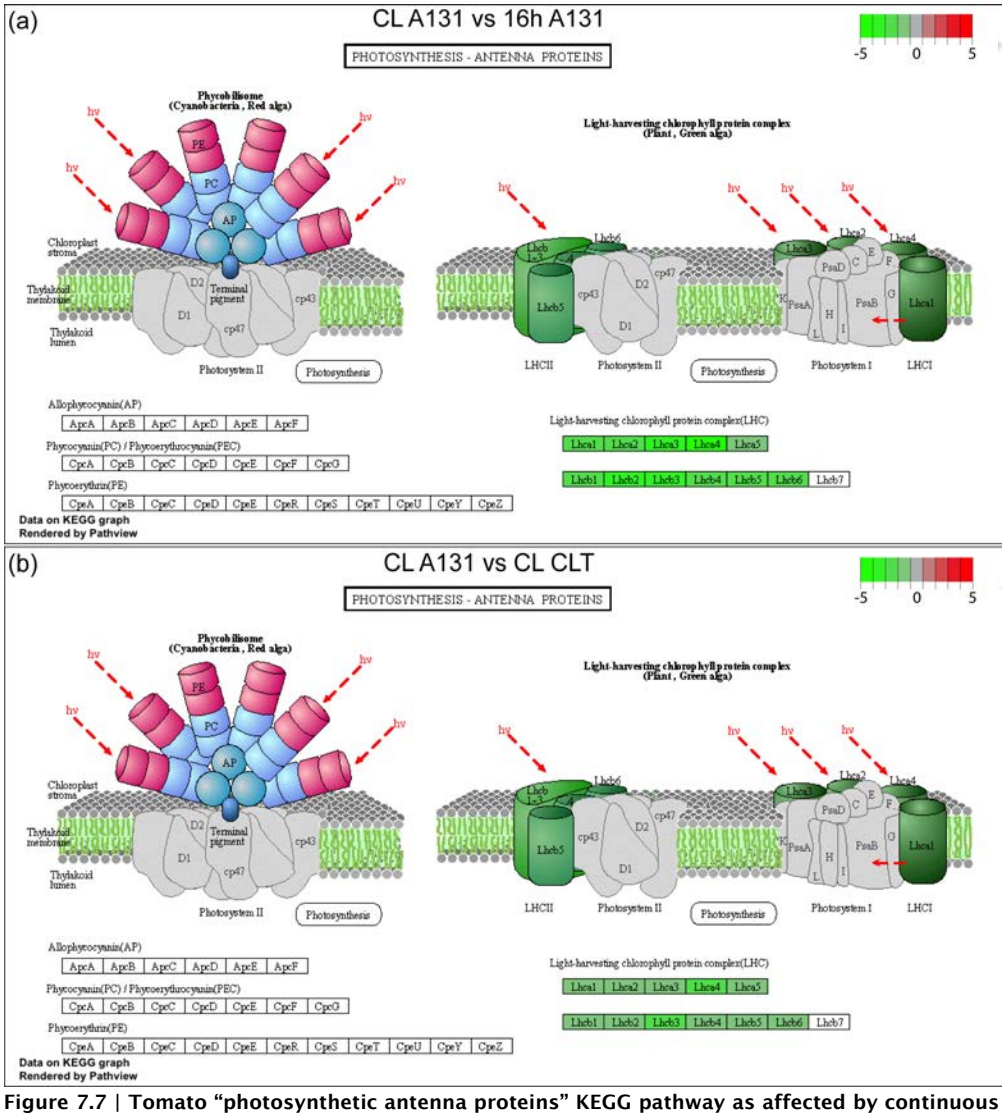


Figure 7.7 | Tomato "photosynthetic antenna proteins" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for photosynthetic antenna proteins. In panel (**a**), contrast between A131 tomato plants exposed to 16-h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00196

dim blue light for 24 h.day^{-1} at 10 µmol.m^{-2}.s^{-1}, no increase in CL-injury was observed in the "dim blue" treatment, yet plants exposed to the "dim red" treatment showed a slightly more CL-induced injury. However, when a 16-h photoperiod of red and blue light (at 80 and 20 µmol.m^{-2}.s^{-1}, respectively) was extended to CL with either red or blue light at 100 µmol.m^{-2}.s^{-1}, the "blue night" treatment was slightly more injurious that the "red night" treatment (Chapter 6). In other words, blue light during night was more injurious than red light, or *vice versa*, depending on the light intensity during the subjective night and/or the spectral distribution during the subjective day. Considering that tomato PHYA can mediate blue LFR in anthocyanin biosynthesis (Weller *et al.*, 2001) and phototropism (Srinivas *et al.*, 2004), it might be that PHYA signaling protects plants in the non-injurious "dim blue" treatment, but other factors potentially involved in the induction of injury under CL outweigh the positive effect of PHYA at higher irradiances of blue light in the "blue night" treatment; such other factors include photoinhibition, signaling from other photoreceptors and/or higher carbohydrate accumulation (Chapter 6, Velez-Ramirez *et al.*, 2011 (Chapter 2)).

Recently, the CL-tolerance found in wild tomato species was mapped to *CAB-13* on chromosome seven, yet the tolerance mechanism is still unclear (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In this study, the discovered PHYA-dependent CL-tolerance provides additional clues as tomato *CAB-13* expression is most probably under PHYA control. This hypothesis is based on knowledge of a closely related, better-studied tomato CAB protein: CAB-1, located on chromosome two (Pichersky *et al.*, 1985). *CAB-1* expression in tomato is up regulated by ultra violet, blue, red and far-red light (Wehmeyer *et al.*, 1990), suggesting the involvement of several photoreceptors in its regulation. Interestingly, at least PHYA and PHYB1 regulate *CAB-1* expression in tomato (Peters *et al.*, 1998). This suggests a link between the PHYA-mediated and CAB-13-mediated CL-tolerance in tomato, PHYA signaling might also be upstream of CAB-13 as is the case for CAB-1. In addition, RNAseq data support this hypothesis since CAB-13-mediated CL-tolerance is associated with higher expression of all tomato CAB proteins. Actually, expression of most photosynthesis genes in CL-sensitive tomato plants is repressed by CL, while CL-tolerant plants show higher expression than sensitive plants when both are exposed to CL (Fig. 7.6 and 7.7; Supplementary Fig. 7.1 and 7.2). Interestingly, CL up regulates *HY5* expression in CL-sensitive tomatoes, but there is no difference between CL-tolerant and -sensitive plants (Table 7.2). Considering that HY5 is a positive regulator of light-responsive genes, including photosynthesis genes, it is remarkable that photosynthesis genes are down regulated while at the same time *HY5* is up regulated.

In Arabidopsis seedlings, when chloroplast development is blocked with lincomycin, HY5 is converted from a positive to a negative regulator of *LHCB1*1* (Ruckle *et al.*, 2007). Interestingly, the evidence suggests that cryptochrome 1 (CRY1) and PHYB contribute to the repression of *LHCB* when chloroplast biogenesis is blocked, yet PHYA remains as positive regulator of *LHCB* expression regardless of the chloroplast state (Ruckle *et al.*, 2007). If a similar process occurs in tomato, it would suggest that the CL-induced

injury is the results of photosynthetic down-regulation enhanced by PHYB2 and prevented by PHYB1 and PHYA.

Materials and Methods

Plant materials and light treatments

Tomato phytochrome mutants (*phy*) and over expressing (*PHYOE*) lines are all in the *Solanum lycopersicum* cv. Moneymaker background, which is continuous light (CL) sensitive (Velez-Ramirez *et al.*, 2014 (Chapter 4)). All lines used here have been described previously: *phyA*-null mutant (*phyA-1* [fri^{1}]) (van Tuinen *et al.*, 1995b); *phyB1*-null mutant (*phyB-1* [tri^{1}]) (van Tuinen *et al.*, 1995a); *phyB2*-null mutant (*phyB2-1* [70F]) and *phyB1phyB2* double mutant (Weller *et al.*, 2000); and *PHYAOE* (A/3), *PHYB1OE* (B1/4) and *PHYB2OE* (B2/9) transgenic lines (Husaineid *et al.*, 2007). Some lines carried a circadian-clock reporter construct (VQ2) consisting of the *Luciferase* gene behind the *Cab* promoter (*CAB::Luciferase*) (Personal communication, van der Krol, 2014). Lines marked with an * carry the *Cab::Luciferase* construct. This construct had no effect on the phenotype of tomato plants grown under all light treatments. In addition, the presence, pattern and severity of chlorosis were not affected by the circadian-clock reporter construct. Although mutant *phyB2** and over-expressing lines *PHYB1OE** and *PHYB2OE** were only available in our seed bank with the *Cab::Luciferase* construct, the results showed that these lines are comparable to lines lacking the construct.

Plants were grown in rockwool blocks at 21 °C and 70% RH. Commercial hydroponic nutrient solution for tomato was used (Yara Benelux B.V., Vlaardingen, The Netherlands); after combining and diluting premixed liquid fertilizers, the solution contained 12.42 mM NO_3, 7.2 mM K, 4.1 mM Ca, 3.34 mM SO_4, 1.82 mM Mg, 1.2 mM NH_4, 1.14 mM P, 30 mM B, 25 mM Fe, 10 mM Mn, 5 mM Zn, 0.75 mM Cu and 0.5 mM Mo (EC = 2.00 $dS.m^{-1}$ and pH = 5.0-5.5). Supplemented with incandescence lamps (Philinea T30 120W, Philips, Eindhoven, The Netherlands), high-pressure sodium (HPS) lamps (Master SON-T Green Power 400W, Philips, Eindhoven, The Netherlands) were installed above a double celling. The photosynthetically active photon flux density (PPFD) was 345 $\mu mol.m^{-2}.s^{-1}$. Red-to-far-red ratio was 2.89, and the phytochrome photostationary state (PSS) (Sager *et al.*, 1988) was 0.858. After growing the plants for two weeks under 16h photoperiod, plants were transferred to continuous light with and without the addition of far-red (FR) light. For the HPS+FR light, Philinea incandescent lamps were also used, yet double number of HPS lamps was installed and a neutral density filter (Filter 209 0.3ND, LEE Filters, Hampshire, UK) filtered ~50% of the visible light. Above ~700 nm, nonetheless, filter transmittance was slightly higher, faintly enriching FR light. To increase even further the FR light, two types of far-red light-emitting diodes (LEDs) were placed below the ND filter, namely (GreenPower LED production module FR 120, Philips, Eindhoven, The Netherlands) and (Orean Retrofit Far-red LED, Lemnis Lighting B.V., Barneveld, The Netherlands). Finally, after placing the plants 15 cm closer to the lamp, a

homogeneous PPFD of 344 µmol.m^{-2}.s^{-1} was reached; the red-to-far-red ratio and PSS were 0.18 and 0.662, respectively. Fig. 7.1 shows the resulting spectral distribution of HPS and HPS+FR light; see Table 7.1 for further details.

Chlorophyll fluorescence imaging

Imaging of the maximum quantum efficiency of photosysmtem II (F_v/F_m) (Baker, 2008) was performed as previously described (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In summary, intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences, Lincoln, USA). After 20 minutes of dark adaptation, leaflets were detached and immediately used for measurements in a chlorophyll fluorescence imaging system (FluorCam 800MF, Photon System Instruments, Brno, Czech Republic). Leaflet average F_v/F_m was calculated using ImageJ software version 1.44o (Schneider *et al.*, 2012). The F_v/F_m parameter assessed the presence and severity of CL-induced injury as previously reported (Chapter 6, Velez-Ramirez *et al.*, 2014 (Chapter 4)). For clarity, nonetheless, results are also expressed as $\Delta\Delta F_v/F_m$. The $\Delta\Delta F_v/F_m$ values represent the response of phytochrome mutants and over-expressing lines to the light treatments taking the average CL-induced decrease of F_v/F_m in the wild type (Moneymaker, MM) as a reference and correcting for the average slight decrease in F_v/F_m observed under 16-h photoperiod. That is, $\Delta\Delta\, F_v/F_m = -([\overline{MM_{CL}} - \{\overline{mutant_{CL}}\ or\ \overline{OE_{CL}}\}] - [\overline{MM_{16h}} - \{\overline{mutant_{16h}}\ or\ \overline{OE_{16h}}\}]$. Where $\overline{MM_{CL}}$ and $\overline{MM_{16h}}$ are the average F_v/F_m in the wild-type Moneymaker under 16-h photoperiod and CL, respectively; $\overline{mutant_{16h}}$, $\overline{OE_{16h}}$, $\overline{mutant_{CL}}$ or $\overline{OE_{CL}}$ are the average F_v/F_m in each mutant or overexpressing line under 16-h photoperiod or CL.

Mapping of RNAseq data to tomato-specific KEGG pathways

Previously published expression data of CL-sensitive A131 and CL-tolerant CLT tomato plants exposed to CL (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to tomato-specific KEGG pathways. The Sol Genomic Network/Ensembl gene identifiers (*e.g.* Solyc07g063600.2) of the original data set were mapped to UniProt accessions (*e.g.* K4CH43) and then to the KEGG/GENEID/Entrez IDs (*e.g.* 101268123) using the UniProt ID mapping tool (www.uniprot.org/help/mapping); only genes mapping to unique IDs were used. From the 31350 genes in the original data set, 14219 had a unique mapping between all IDs. The R package "Pathview" (Luo & Brouwer, 2013) was used to map the originally reported LogFold change to the following tomato-specific KEGG pathways: "Photosynthesis" (sly00195), "Photosynthesis antenna proteins" (sly00196) and "Porphyrin and chlorophyll metabolism" (sly00860). For nodes containing more than one gene, mean LogFold change was used.

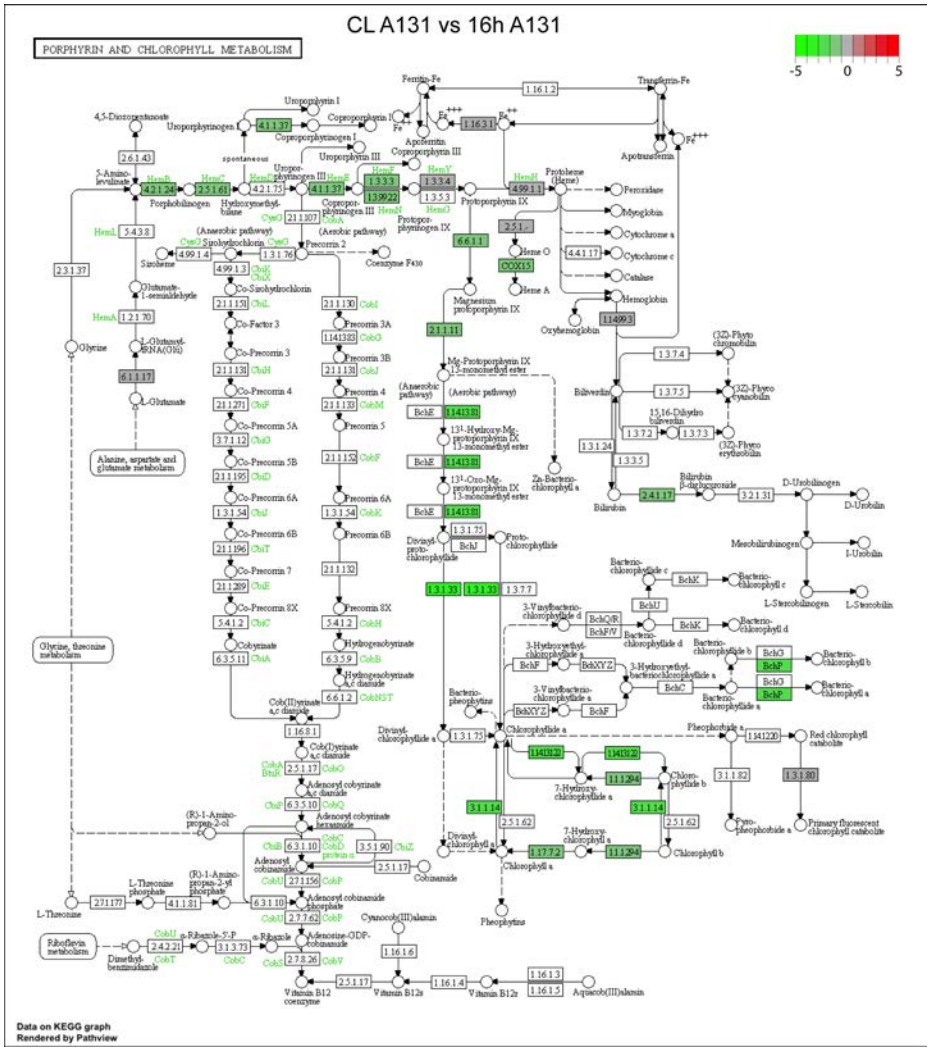
Statistical analysis

Statistical significance of the leaflet average F_v/F_m was determined with an ANOVA test performed with IBM SPSS Statistics software version 19 (IBM, Somers, USA).

Acknowledgements

We are most thankful to Sander Hogewoning (Plant Lighting B.V.) for the fruitful discussions and lending us the far-red LEDs. Alexander van del Krol kindly provided mutant and over-expressing phytochrome tomato lines. We also thank Alise Senberga, Taede Stoker and Gerrit Stunnenberg (Wageningen University) for the support in setting up and performing the experiment. This project was financially supported by the Technological Top Institutes-Green Genetics (TTI-GG) (Project 2CFD020RP).

Supplementary Information



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Supplementary Figure 7.1 | Tomato "porphyrin and chlorophyll metabolism" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for porphyrin and chlorophyll metabolism. Each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00860

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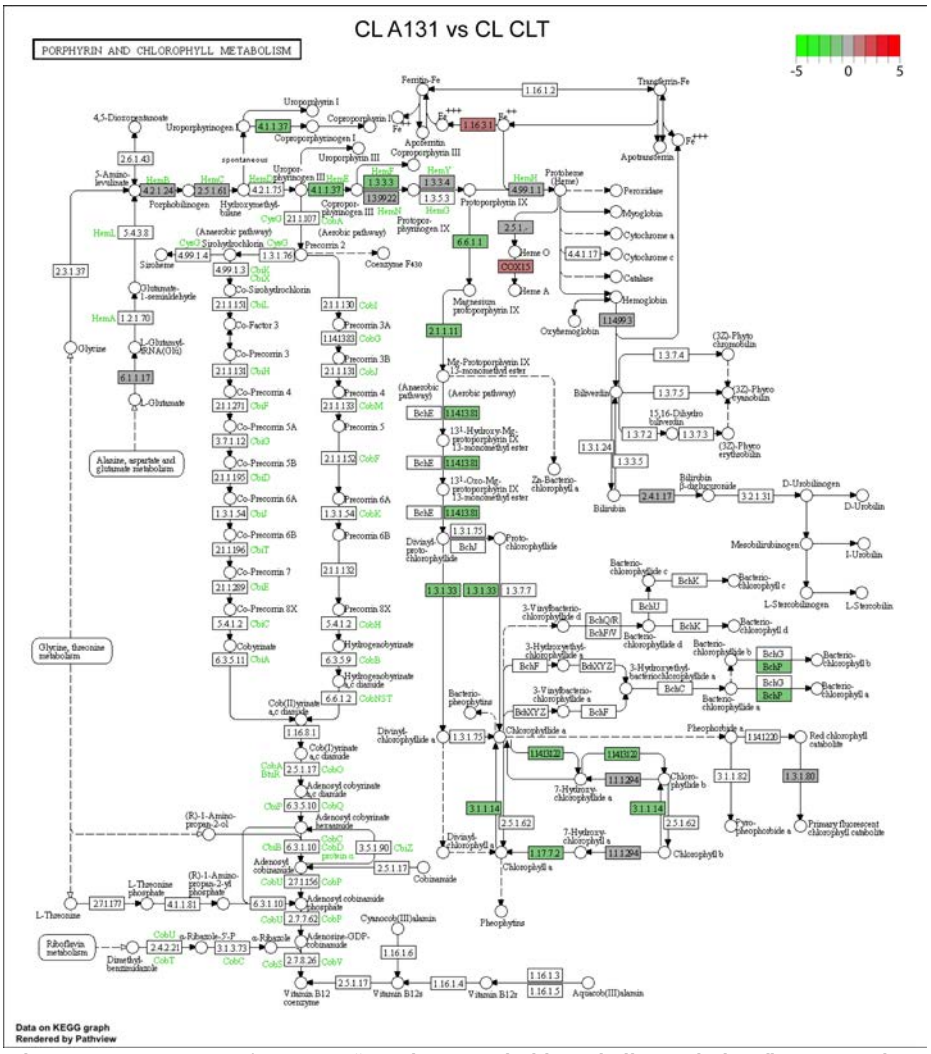

Supplementary Figure 7.2 | Tomato "porphyrin and chlorophyll metabolism" KEGG pathway as affected by continuous-light-tolerance. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for porphyrin and chlorophyll metabolism. Both lines were exposed to CL. Each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00860


tomato
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KEGG
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girdled
tolerant
chlorosis
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leaves
cytokinin
sucrose
data
cycles
dark
glucose
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represent
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circadian
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experiments
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Sucrose and starch content negatively correlates with PSII maximum quantum efficiency in tomato plants exposed to injurious light/dark cycles, including continuous light

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In preparation

Abstract

Light is most important to plants as it fuels photosynthesis and provides clues about the ever-changing environment. If provided in unnatural long photoperiods, however, it can be harmful and even lethal. Tomato plants, for example, develop mottled chlorosis and necrosis when exposed to continuous light. Understanding the mechanism of these injuries is most valuable as important pathways regulating photosynthesis, like circadian, retrograde and light signalling pathways, are likely involved. Here, with the added value of using tomato introgression lines tolerant to continuous light, we use untargeted metabolomics and transcriptomic data as well as hypothesis-driven experiments to explore the long ago proposed role of carbohydrate accumulation in the induction of this disorder. Analysis of metabolomics and transcriptomics data reveals a clear effect of continuous light on sugar metabolism and photosynthesis. A strong negative correlation between the level of sucrose and starch with the maximum quantum efficiency of photosystem II (F_v/F_m) was found across several abnormal light/dark cycles, supporting the hypothesis that carbohydrates play an important role in the CL-induced injury. We postulate that the continuous-light-induced injury in tomato is caused by down-regulation of photosynthesis showing characteristics of both cytokinin-regulated senescence and light-modulated retrograde signaling. Molecular mechanisms linking carbohydrate accumulation with down-regulation of carbon fixing enzymes are discussed.

Introduction

Light is of utmost importance to plants as it provides energy and clues about the environment. If provided in excess, however, it can be harmful. For example, the higher rate of photosynthesis with higher irradiance saturates at some level, after which the excess of light can cause photo-oxidative damage (Li *et al.*, 2009). In addition to high irradiance, long photoperiods can also be harmful. It has been known for many decades that tomato (*Solanum lycopersicum*) develops a characteristic, and potentially lethal, leaf mottled chlorosis and necrosis when exposed to continuous light (CL) (Arthur *et al.*, 1930). Understanding why CL is injurious to tomato has important implications for basic scientific research and practical applications (Velez-Ramirez *et al.*, 2012 (Chapter 3), Velez-Ramirez *et al.*, 2011 (Chapter 2), Velez-Ramirez *et al.*, 2014 (Chapter 4)). Only recently our understanding of the CL-induced injury is broadening. For instance, the plenty, and sometimes languished, studies done throughout the twentieth century were recently re-discussed in the light of the current knowledge on plant physiology (Velez-Ramirez *et al.*, 2011 (Chapter 2)). Although many papers suggested that carbohydrate accumulation is an important factor in inducing the injury under CL (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996), the mechanism that would explain this is not known and other hypotheses giving carbohydrate accumulation a secondary role or even not involving carbohydrates are also plausible. For instance, CL-tolerance in wild tomato species was

mapped to a photosynthetic gene with no known relation to carbohydrate metabolism – the *type III light harvesting chlorophyll a/b binding protein 13* (*LHCB type III CAB-13* or *CAB-13*) (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This gene is located on the lower arm of chromosome 7 and the encoded protein is part of the light harvesting complex of photosystem II (LHCII). Tomato *CAB-13* is homologous to Arabidopsis (*Arabidopsis thaliana) LHCB3*. Secondly, circadian asynchrony, which is the mismatch between the internal circadian rhythm and the external light/dark cycle, has been suggested to be the causal factor as light/dark cycles deviating from a 24-h period induce mottled chlorosis, similar to the induced by CL, in CL-sensitive tomato but not in a CL-tolerant introgresion line (Chapter 6). Third, using phytochrome mutants and transgenic phytochrome over-expressing lines, a recent study showed that tomato phytochrome A (PHYA) signalling reduces CL-induced injury, and phytochromes B1 and B2 seem to be also involved (Chapter 7). Despite these studies, a role for carbohydrate accumulation cannot be discarded. A transcriptomics study showed that the GO term "Carbohydrate metabolic process" is significantly enriched of differentially-regulated genes in CL-sensitive tomato plants exposed to CL (compared with a 16-h photoperiod) and in CL-tolerant plants under CL (compared with CL-sensitive plants) (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

Under many conditions, carbohydrate accumulation is associated with down-regulation of photosynthesis, including long-term exposure to high CO_2 (Stitt, 1991), magnesium deficiency (Cakmak & Kirkby, 2008), sugar feeding (Krapp *et al.*, 1991) and mutations that affect carbohydrate metabolism (Baker & Braun, 2007, Baker & Braun, 2008). Photosynthetic control, which includes photosynthetic down-regulation and retrograde signaling processes, is a set of short- and long-term acclimations that regulate photosynthesis in such a way that ATP and NADPH production is coordinated with the rate of their utilization in metabolism, preventing over-reduction of photosynthetic electron transport (PET) components (Foyer *et al.*, 2012). Plastid-to-nucleus retrograde signals tune the expression of *photosynthesis-associated nuclear genes* (*PhANGs*) to match the needs and status in the chloroplasts (Inaba, 2010, Nott *et al.*, 2006). It is proposed that the chlorophyll intermediate Mg-protoporphyrin IX (Larkin *et al.*, 2003, Mochizuki *et al.*, 2001, Nott *et al.*, 2006), the phosphorylation status of LHCII (Pursiheimo *et al.*, 2001), the redox state of PET components (Fey *et al.*, 2005, Nott *et al.*, 2006, Pfannschmidt *et al.*, 2001) and plastid-derived singlet oxygen ($^1O_2$) (Lee *et al.*, 2007b, op den Camp *et al.*, 2003, Wagner *et al.*, 2004) relay information to the nucleus about the chloroplast state in a particular environment. Interestingly, in Arabidopsis lacking *LHCB3*, the level of LHCII trimer phosphorylation is higher (Damkjær *et al.*, 2009), suggesting that LHCB3 could indirectly modulate retrograde signaling. CL-induced carbohydrate accumulation may favor over-reduction of PET components; this may not only increase H_2O_2 and $^1O_2$ release from PSI and PSII, respectively (Asada, 2006), but also causes an imbalance in the ATP:NADPH ratio as sucrose and starch synthesis require only ATP (Foyer *et al.*, 2012). With altered levels of H_2O_2, $^1O_2$, ATP and NADPH in CL-exposed plants, photosynthesis would have to be down regulated. In addition, recent evidence shows that retrograde signals heavily interact with the light-signaling network (Lepistö & Rintamäki, 2012, Ruckle *et al.*, 2012,

Ruckle *et al.*, 2007). For instance, in dysfunctional chloroplasts, Ruckle *et al.* (2007) showed that plastid signals convert light signalling pathways from positive to negative regulators of some *PhANGs*. This might explain the effect of light quality (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997) and PHYA signaling (Chapter 7) on the severity of CL-induced injury. Hence, it is reasonable to hypothesize that CL can alter the redox state of PET components, as a consequence of carbohydrate accumulation and over-reduction of electron acceptors, resulting in photosynthetic down regulation; PHYA and CAB-13 might tune this response.

An alternative hypothesis is that carbohydrate accumulation results in early leaf senescence in CL-exposed tomatoes (Velez-Ramirez *et al.*, 2011 (Chapter 2)). In the closely related potato (*Solanum tuberosum*), which depending on the cultivar can be CL-sensitive or –tolerant, Cushman *et al.* (1995) showed that the chloroplast ultrastructure of CL-sensitive potato cultivars displayed a senescence-like appearance after 7 days of CL. An understanding of regulatory mechanisms linking sugar status with plant growth and development is emerging (Smeekens *et al.*, 2010). Although van Doorn (2008) critically reviewed the role of sugar status in the induction of leaf senescence and posed some doubts, growing evidence supports an involvement of sugars in the initiation and/or acceleration of leaf senescence (Fischer, 2012, Lim *et al.*, 2007, Thomas, 2013, Wingler *et al.*, 2009). Considering that the well-known leaf yellowing associated with senescence reflects mainly chloroplast senescence in mesophyll cells (Lim *et al.*, 2007), it is reasonable to expect some overlap between the signaling networks controlling down/up regulation of *PhANGs* during leaf senescence in old leaves, chloroplast biogenesis in young leaves and quotidian photosynthetic control in mature leaves. Therefore, whether the CL-induced chlorosis is accelerated senescence or photosynthetic down-regulation might only be a matter of definition as both are hypothetically induced by carbohydrate accumulation and both might use a very similar signaling network to down-regulate *PhANGs*. In this study, therefore, we mine RNAseq and metabolomics data to explore the role of carbohydrates in CL-induced injury in tomato. The results show that (i) regardless of the CL-tolerance phenotype, CL significantly alters carbohydrate metabolism, (ii) CL down regulates photosynthesis genes in CL-sensitive but not in CL-tolerant tomato, (iii) cytokinin treatment (a well-known leaf senescence inhibitor) diminishes the severity of CL-induced injury in a CL-sensitive line and (iv) the level of chlorosis in tomato leaves negatively correlates with sucrose and starch content in several environmental conditions in both CL-sensitive and -tolerant lines.

Results

The carbohydrate metabolome is affected by continuous light regardless of the tomato genotype

Two genotypes were used for untargeted metabolomics, the CL-sensitive reference line M82 and the CL-tolerant line IL-39 (Velez-Ramirez *et al.*, 2014 (Chapter 4)) from the *S.*

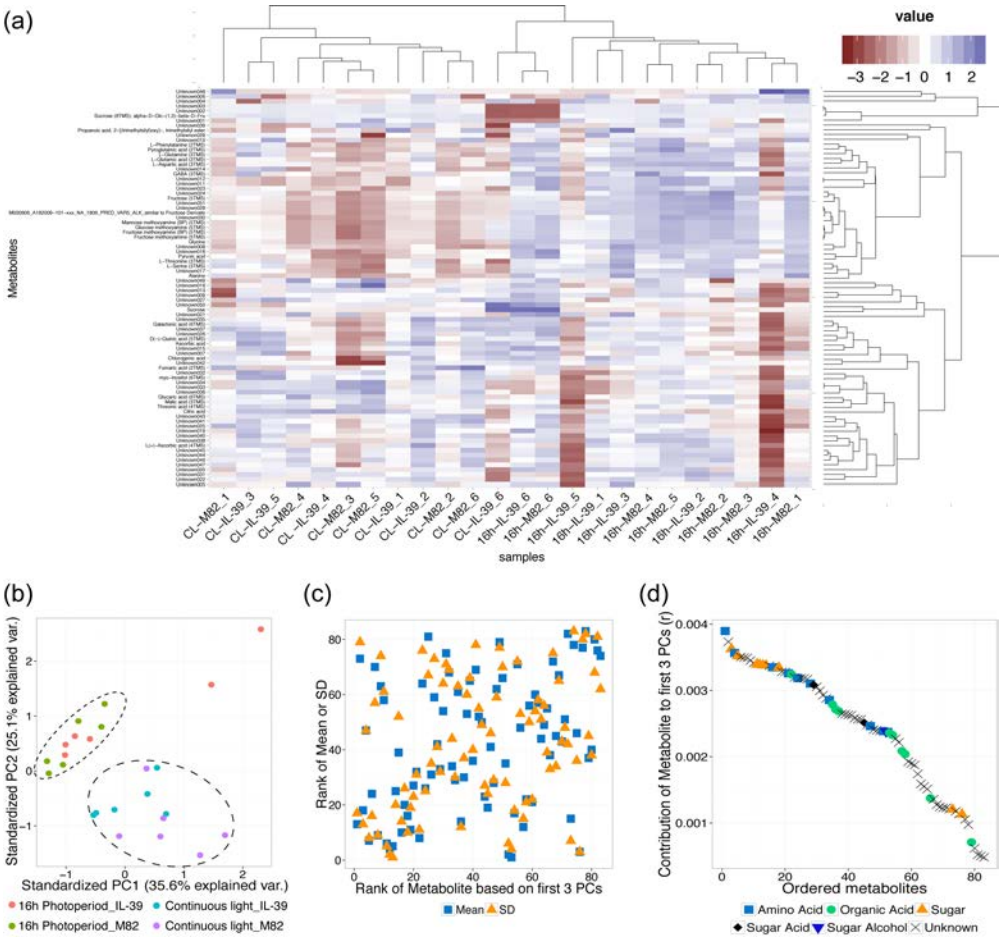

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Figure 8.1 | Hierarchical cluster analysis and principal component analysis on metabolomics data. Continuous light (CL)-sensitive M82 and CL-tolerant IL-39 tomato plants (n=6) were exposed to CL of kept under 16h photoperiod. Polar metabolites were extracted and analyzed with GC-TOFMS. In panel (**a**), hierarchical cluster analysis of the 83 putative metabolites. Panel (**b**), principal component analysis (PCA); again, samples clustered according to light treatment. Panel (**c**) relation between the contribution of each metabolite to the first three principal components (van den Berg *et al.*, 2006) and the untransformed mean and SD. Panel (**d**) metabolites ordered by its contribution to the first three principal components; symbols represent each metabolite type (see legend); notice that sugars ranked high in their contribution to the first three PC.

pennellii (LA0716) x *S. lycopersicum* M82 population, described by Eshed & Zamir (1994). Using GC-TOF-MS, 98 putative metabolites were identified (Supplementary Table 8.1). Hierarchical cluster analysis and principal component analysis (PCA) show that the samples clustered into two groups according to the light treatment and regardless of the genotype (Fig. 8.1a,b), suggesting that continuous light alters carbohydrate metabolism in a similar

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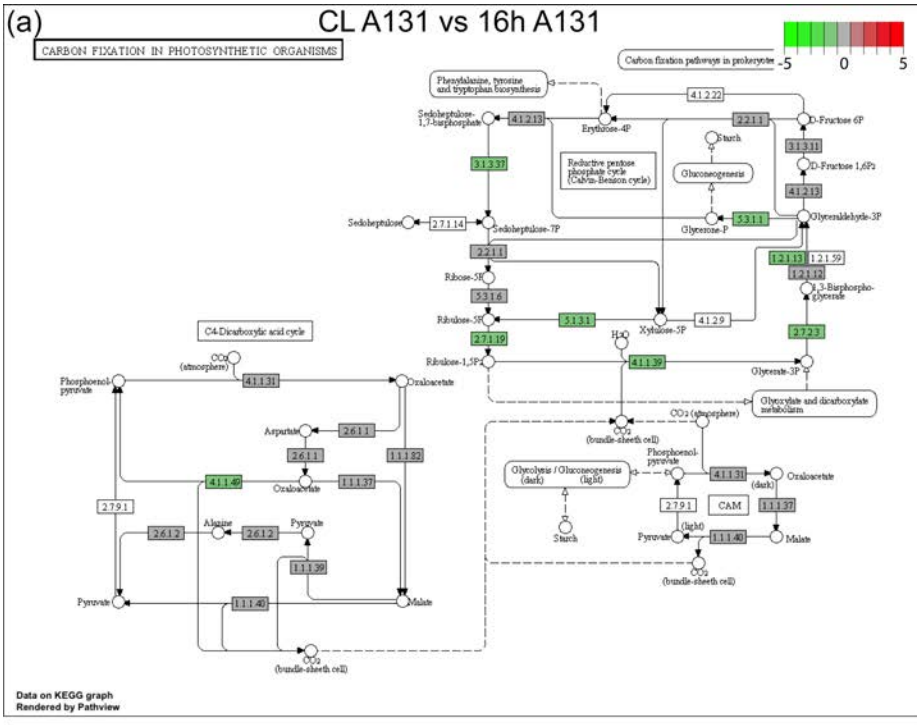

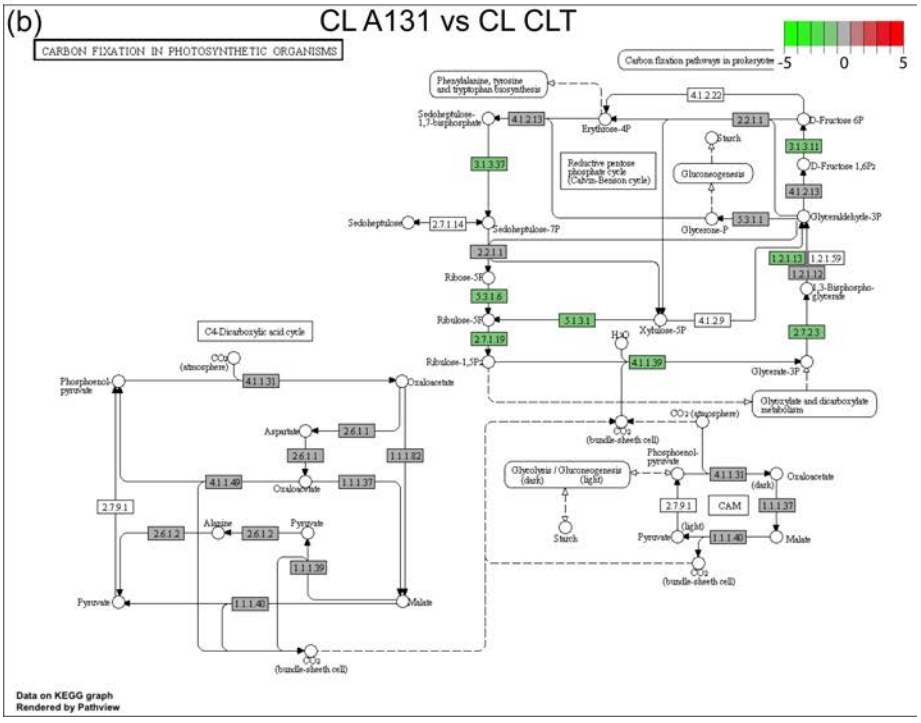

Figure 8.2 | Tomato "carbon fixation of photosynthetic organism" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)–sensitive A131 and CL–tolerant CLT tomato plants (Velez–Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for carbon fixation of photosynthetic organism. In panel (**a**), contrast between A131 tomato plants exposed to 16–h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria–specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg–bin/show_pathway?sly00710

way in CL-sensitive and –tolerant tomato lines. The first three principal components explained ~72% of the variation in the data set. When the contribution of each metabolite to the first three principal components (PCs) was calculated according to van den Berg *et al.* (2006), no relation was found between the untransformed mean and standard deviation with the metabolite rank (Fig. 8.1c); indicating that the magnitude of metabolite concentration and variation did not have impact on either the clustering or ranking. Sugars ranked high in their contribution to the first three PCs (Fig. 8.1d). Although this indicates that CL significantly alters carbohydrate metabolism, no clear differences between CL-tolerant and –sensitive genotypes were found.

Continuous light down regulates key enzymes involved in carbon fixation

In order to further assess the effects of CL on carbohydrate metabolism, we mined a previously published whole transcriptome RNAseq data set (Velez-Ramirez *et al.*, 2014 (Chapter 4)) and looked for alterations in carbon metabolic pathways. The expression data of more than 31,000 genes were used to evaluate two contrasts; the first contrast compares the effect of CL on the CL-sensitive tomato inbreed line A131, and the second one evaluates the differences under CL between A131 and a CL-tolerant introgression line named CLT (Velez-Ramirez *et al.*, 2014 (Chapter 4)). The expression data of both contrasts were mapped to tomato-specific KEGG pathways. Figure 8.2 shows the tomato KEGG pathway for "carbon fixation in photosynthetic organisms" (sly00710) as affected by CL. Notice that species-specific KEGG pathways are drawn on standard KEGG maps. Hence, care should be taken in the interpretation of white nodes as these contain no expression information because (i) that specific node does not exist in tomato (*e.g.* a bacteria-specific enzyme), (ii) the node is not yet annotated in tomato (only ~25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the gene(s) associated with that node were not detected in the transcriptomics data (only ~14,000 genes with KEGG annotation were detected). Furthermore, the expression level of each node might be the mean of several genes; in Fig. 8.2, for instance, RuBisCO (enzyme 4.1.1.39) color-coded expression is the average fold change of the small and large subunits. For an

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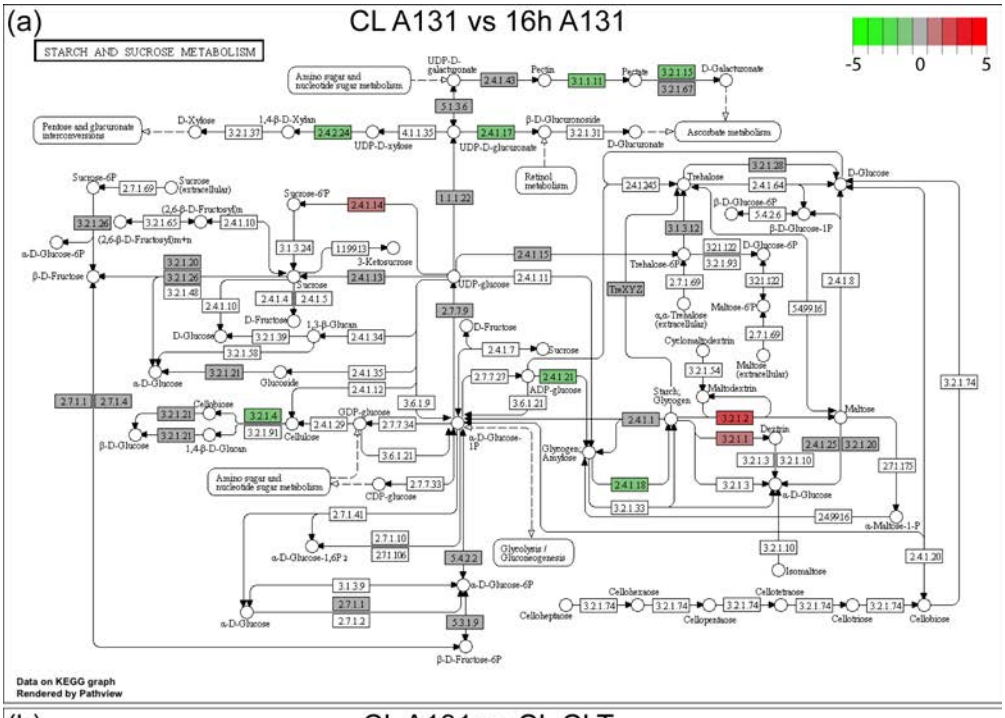

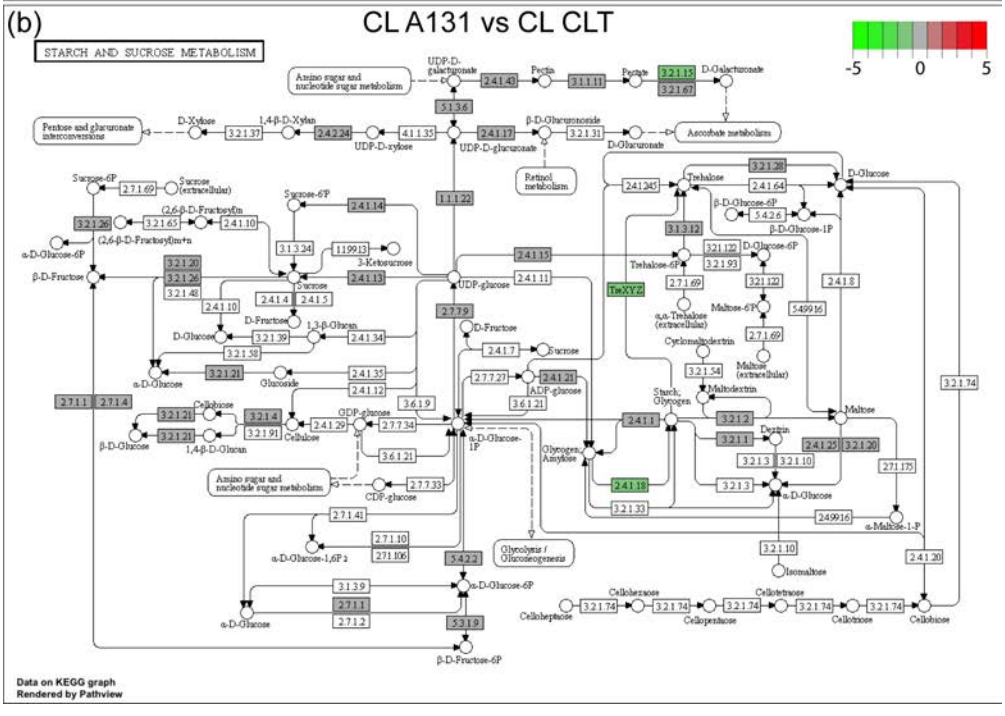

Figure 8.3 | Tomato "starch and sucrose metabolism" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for starch and sucrose metabolism. In panel (**a**), contrast between A131 tomato plants exposed to 16-h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00500

overview of annotated tomato genes in the KEGG pathways, follow the links in the figure legends. From Fig. 8.2, it is clear that several enzymes of the reductive pentose phosphate cycle (Calvin-Benson cycle), including RuBisCO, are down regulated in both contrasts, indicating that carbon fixation is significantly down-regulated at the transcriptional level in CL-sensitive tomato plants when exposed to CL compared with both sensitive plants kept at 16-h photoperiod and CL-tolerant plants exposed to CL.

To further assess the effect of CL on carbohydrate metabolism at the transcriptional level, two more tomato KEGG pathways were evaluated. Figure 8.3 and Supplementary Fig. 8.1 show tomato "starch and sucrose metabolism" (sly00500) and "glycolysis/gluconeogenesis" (sly00010) KEGG pathways, respectively. Enzymes in the glycolysis and gluconeogenesis pathway remained relatively unchanged in both contrasts with no clear effect of CL or genotype (Supplemental Fig. 8.1). Most of the starch and sucrose pathway also remained relatively unchanged, yet it is worth highlighting that the nodes representing the enzymes 3.2.1.1 and 3.2.1.2, which catalyze the degradation of starch to maltose and dextrin, are up-regulated by CL and there is no difference between genotypes when exposed to CL (Fig. 8.3). Overall, CL down regulates carbon-fixing enzymes and up regulates the starch-degrading capacity.

Carbohydrate accumulation correlates with injury in tomatoes exposed to continuous light

It has been proposed that carbohydrate accumulation could result in the characteristic CL-induced chlorosis in tomato (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996), as a result of photosynthetic down-regulation (Velez-Ramirez *et al.*, 2011 (Chapter 2)). To explore this possibility, we measured glucose, fructose, sucrose and starch content in tomato leaves exposed to CL and other abnormal light/dark cycles for one and three weeks. The level of CL-induced injury in these samples, evaluated with chlorophyll fluorescence imaging after three weeks of treatment, has been previously reported (Chapter 6). Table 8.1 shows that carbohydrate content was influenced by genotype (A131 or CLT), time under light treatment (one or three weeks), time of sampling (end of the light or dark period), and

Table 8.1 Effect of four light (L) / dark (D) cycles on leaf carbohydrate content of A131 and CLT plants. Values represent mean of 4 replicates ± SE.

Time of day	16-h L/ 8-h D (16h photoperiod)		24-h L/ 0-h D (continuous light)		6-h L/ 6-h D		24-h L/ 24-h D	
	A131	CLT	A131	CLT	A131	CLT	A131	CLT
				glucose (µg.mg^{-1})				
End Dark	10.97±1.58	18.31±0.48	4.41±0.82	9.26±0.92	10.58±2.49	13.48±2.46	15.02±2.24	29.82±2.59
End Light	13.71±1.23	19.65±1.45			21.21±4.01	20.61±3.39	27.83±4.05	37.21±9.32
				fructose (µg.mg^{-1})				
End Dark	21.58±2.46	31.92±0.77	9.67±1.80	17.90±1.71	20.95±1.70	31.14±4.34	30.52±2.60	41.37±3.37
End Light	26.71±1.26	33.16±0.96			29.67±3.36	34.80±3.19	36.87±5.40	51.05±12.13
				sucrose (µg.mg^{-1})				
End Dark	6.32±0.35	6.23±0.21	11.08±0.38	10.33±0.45	3.63±1.28	6.13±0.70	1.95±0.66	0.60±0.36
End Light	12.48±0.70	10.17±0.45			9.29±5.52	12.06±4.07	8.28±4.39	17.38±0.35
				starch (µg.mg^{-1})				
End Dark	75.79±2.69	42.06±3.17	75.79±9.24	42.06±8.80	16.58±4.44	11.02±2.51	25.58±6.60	7.83±4.03
End Light	83.37±4.69	41.77±3.86			25.10±9.59	11.76±2.41	59.68±6.34	32.66±4.41

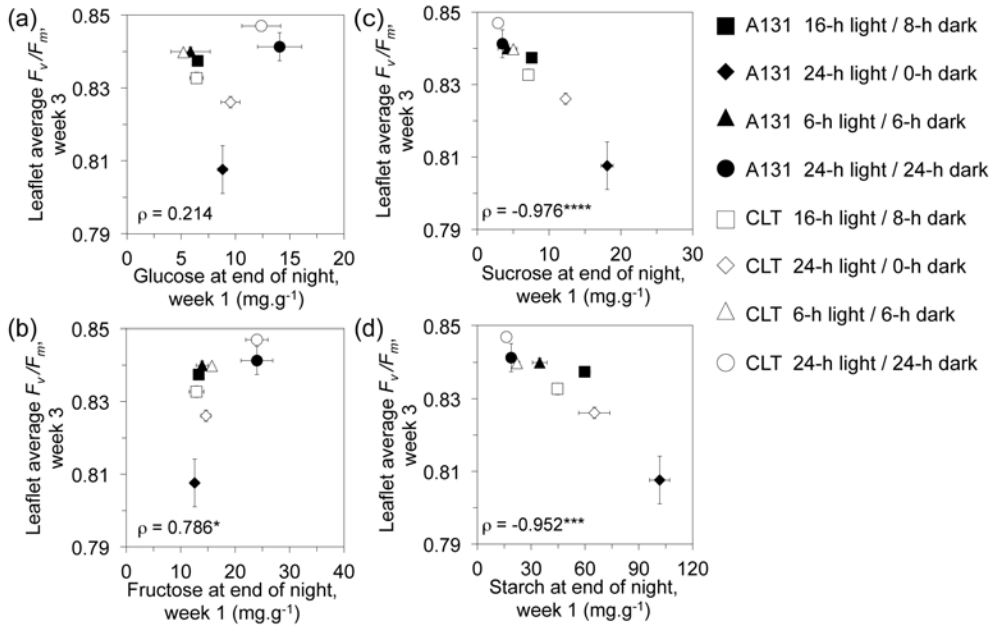

Figure 8.4 | Correlation between carbohydrate content and F_v /F_m in A131 and CLT tomato leaves exposed to four light / dark cycles. A131 and CLT tomato plants were grown under 16-h light / 8-h dark cycles for four weeks and then transferred to three other day/night cycles for three additional weeks; control plants were left at 16-h light / 8-h dark cycles. Carbohydrate content was measured in leaflets (sixth true leaf) one week after transfer to treatments. Previously reported, F_v /F_m was measured in leaflets three weeks after transfer to treatments (Chapter 6). Closed and open symbols represent A131 and CLT leaves respectively. Square symbols represent control plants grown at 16-h light / 8-h dark cycles, while diamond symbols represent leaves exposed to 24-h light / 0-h dark cycles (continuous light). Triangles represent leaves exposed to 6-h light / 6-h dark cycles, and circles represent leaves exposed to 24-h light / 24-h dark cycles. Panel (**a**) glucose, (**b**) fructose, (**c**) sucrose and (**d**) starch. In all graphs, carbohydrate values ("x" axes) represent mean of four replicates, and Fv /Fm ("y" axes) represent mean of eight replicates; in both axes, error bars represent SE. In the top-right corner of all graphs, ρ values indicate Spearman correlation coefficient between carbohydrate levels and chlorosis; asterisks indicates statistical significance; * P<0.05, ***P<0.001 and **** P<0.0001.

the light/dark cycle itself. If carbohydrate accumulation is the causal factor of CL-induced injury, then carbohydrate accumulation should occur before the onset of chlorosis. Indeed, after one week of exposure to CL, sucrose and starch content correlated with the severity of CL-induced injury (as analyzed after 3 weeks of treatment) (Fig. 8.4c,d). For instance, the correlation between sucrose and starch contents and leaflet average F_v/F_m was -0.976 and -0.952 (P<0.0001 and P<0.001) respectively. The higher the sucrose and starch content after one week of treatment, the higher the injury after three weeks of treatment. No clear correlation was observed for glucose and fructose (Fig. 8.4a,b).

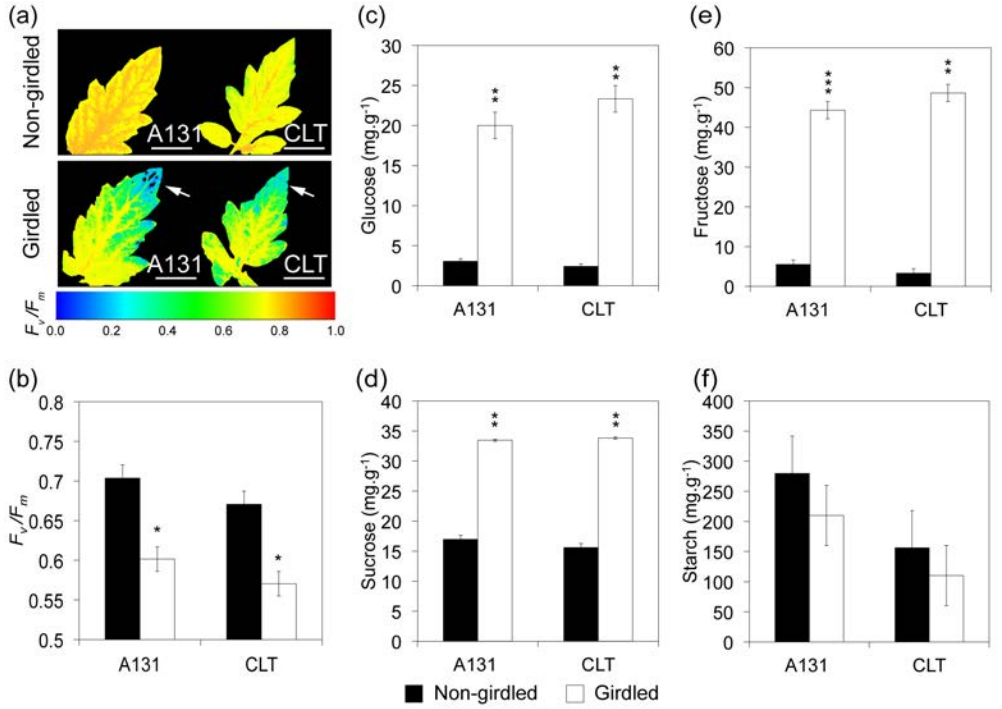

Figure 8.5 | Girdling-induced carbohydrate accumulation and chlorosis in tomato leaves. One terminal side leaflet (fourth true leaf) of A131 and CLT tomato plants grown under 16-h light / 8-h dark cycles for 35 days was girdled with hot wax. Within the same leaf, the opposite leaflet was used as non-girdled paired control. Five days after girdling, chlorophyll fluorescence images were taken, and carbohydrate content was quantified; the same leaflets were used for both measurements. In panel (**a**), chlorophyll fluorescence images of girdled and non-girdled leaflets; maximum efficiency of photosystem II (dark-adapted F_v /F_m) is represented in a false color scale (see color scale at the bottom). White arrows point to chlorosis. Scale bars = 5 cm. Panel (**b**), average of F_v /F_m in girdled and non-girdled leaflets. Panels c to f, carbohydrate content of girdled and non-girdled leaflets. (**c**), Glucose; (**d**), Fructose; (**e**), Sucrose; (**f**), Starch. Carbohydrate content of non-girdled leflets is represented in black, and carbohydrate content of girdled leaflets is represented in white. In all graphs, bars represent mean of six replicates, and error bars represent SE; asterisks indicate that the difference between girdled and non-girdled leaflets is statistically significant; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Girdling-induced carbohydrate accumulation co-occurs with chlorosis

If the CL-induced injury in tomato plants is caused by high carbohydrate content as our results suggest, then the same type of injury should occur when high carbohydrate content is induced by other means than CL. A way to achieve this is by girdling; that is the removal (or damaging) of the phloem in the leaflet petiole to inhibit carbohydrate export. The petioles were girdled with hot wax in leaflets of A131 and CLT tomato plants grown

entirely under 16-h light / 8-h dark cycles. Five days after girdling, F_v/F_m and carbohydrate content were assessed (Fig. 8.5). As expected, glucose, sucrose and fructose content were higher in girdled leaflets than in the control, non-girdled, leaflets (Fig. 8.5c-e), yet no difference in starch content was observed ($P>0.05$) (Fig. 8.5f). Parallel to the higher glucose, sucrose and fructose content, girdled leaflets had lower F_v/F_m than non-girdled leaflets (Fig. 8.5a,b). Chlorosis in girdled leaflets was more severe at the tips than at the leaflet bases (Fig. 8.5a). This pattern resembles the chlorosis that tomato senescing leaves show when grown under non-injurious 16-h photoperiod. In CL-exposed plants, however, chlorosis is more severe at the leaf/leaflet basis.

Cytokinin diminishes continuous-light-induced injury in tomato

To explore the hypothesis that CL-induced injury is the result of sugar-induced senescence, we applied cytokinin, a well-known leaf senescence inhibitor (Fischer, 2012, Lim *et al.*, 2007, Thomas, 2013), to tomato plants exposed to CL. In plants grown entirely under 16-h light / 8-h dark cycles, cytokinin treatment did not have any effect in either tomato genotype (Fig. 8.6). In CL-exposed plants, however, cytokinin treatment prevented to a large extent the CL-induced injury. To further evaluate the effect of CL on cytokinin signaling, transcriptome contrasts between A131 and CLT (Velez-Ramirez *et al.*, 2014 (Chapter 4)) were mapped to the tomato KEGG pathway for "Plant hormone signal transduction" (sly04075). Figure 8.7 show that tomato type-A Arabidopsis response regulators (ARRs)-like genes, which are involved in cytokinin signaling, are down regulated by CL in A131 tomato plants. Induced by cytokinin, type-A ARRs can elicit positive or negative responses to abiotic stress in Arabidopsis (Ha *et al.*, 2012). Considering that ethylene is another hormone probably involved in CL-induced injury (Cushman & Tibbitts, 1998), it is worth highlighting that ethylene receptor (ETR) and the ethylene-responsive transcription factor 1 (ERF1) were up regulated in A131 tomato plants in response to CL (Fig. 8.7a). Interestingly, ERF1 expression was also higher in A131 than CLT plants when both genotypes were exposed to CL (Fig. 8.7b).

Discussion

In this study, we have shown that continuous light (CL) alters carbohydrate metabolism at the transcriptional level as well as the metabolite content (Figs. 8.1-3). Sucrose and starch content negatively correlate with the maximum quantum efficiency of photosysmtem II (F_v/F_m) under four light treatments (Fig. 8.4), and cytokinin treatment prevents the CL-induced injury in tomato (Fig. 8.6). These results suggest that altered carbohydrate metabolism might play an important role in inducing injury in CL-grown tomato plants as it has been proposed (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)). Altered carbohydrate metabolism could result in molecular and cellular adjustments via two pathways. The first one is based on the fact that sucrose and starch synthesis from glyceraldehyde 3-phosphate, derived from the Calvin

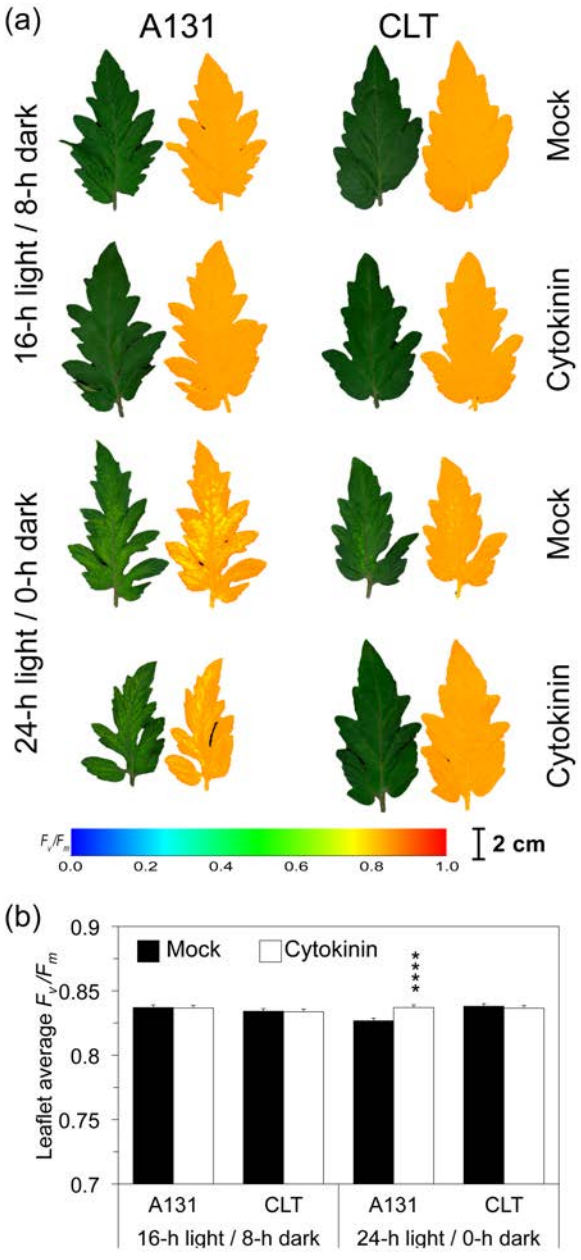

Figure 8.6 | Cytokinin (BAP) treatment diminishes the continuous-light-induced injury in tomato. In panel (**a**), Leaflets (sixth true leaf) of A131 and CLT tomato plants grown under 16-h light / 8-h dark cycles for four weeks and then sprayed, every three days, with cytokinin or Mock solution for two weeks; at the same time as spraying started, half of the plants were transfer to 24-h light / 0-h dark cycles (continuous light) (see treatment labels on the sides). Left-side leaflet images are photographs; right-side images represent, in a false color scale (see color scale in at the bottom), maximum efficiency of photosystem II (Dark adapted F_v/F_m). Panel (**b**), injury quantification in leaflets of A131 and CLT tomato plants in panel (a) ; bars represent mean of six replicates, and error bars represent SE. In all graphs, Mock-treated plants are represented in black, cytokinin-sprayed plants in white; asterisks indicate that the value is significantly different form the mock control; **** P<0.0001.

Benson cycle, require only ATP and not NADPH (Foyer *et al.*, 2012). Imbalances in ATP and NADPH utilization result in short- and long-term acclimations (including retrograde signaling) that regulate photosynthesis in such a way that ATP and NADPH production is coordinated with the rate of their utilization in metabolism, preventing over-reduction of

PET components (Foyer *et al.*, 2012). The second pathway involves direct sensing of sugar levels as in plant cells several regulatory systems that control growth and development receive input from sucrose, glucose, glucose 6-phospate (G6P) and trehalose 6-phosphate levels (Smeekens *et al.*, 2010). For instance, leaf senescence is thought to be responsive to sucrose, hexoses (glucose and fructose) and G6P through some of these regulatory systems, like the hexokinase (HXK1) glucose sensor, the SNF1-related protein kinase1 (SnRK1) and the target of rapamycin (TOR) kinase system (Thomas, 2013).

Caution should be taken when trying to conclude that CL-induced injury is the consequence of either retrograde-signaling-dependent photosynthetic down-regulation or accelerated leaf senescence. Leaf senescence or quotidian adjustments are natural process in response to an ever-changing natural environment, but CL, as achieved in a growth chamber, is far from being natural (Velez-Ramirez *et al.*, 2011 (Chapter 2)). When tomatoes are exposed to CL, nevertheless, the existing signaling network must be responsible for the injury induction, yet it is unlikely that they would follow canonical senescence or retrograde signaling pathways. Instead, we propose that the CL-induced injury in tomato shows features of both, accelerated senescence and retrograde-signaling-dependent photosynthetic down-regulation.

Features of accelerated senescence in the CL–induced injury in tomato

Leaf senescence is a genetically controlled process, influenced by internal and environmental factors, which is intended to recycle nutrients form old leaves to young organs (Fischer, 2012, Lim *et al.*, 2007, Thomas, 2013). The earliest structural changes during leaf senescence-associated cell death occur in the chloroplast; hence a decrease in chlorophyll content and maximum photochemical efficiency (*e.g.* F_v/F_m) are well-established senescence markers (Lim *et al.*, 2007). Considering, additionally, that cytokinins are well-known senescence-inhibitors (Fischer, 2012, Lim *et al.*, 2007, Thomas, 2013), the ability of cytokinin treatment to prevent a CL-induced decrease of F_v/F_m in A131 tomato plants (Fig. 8.6) is consistent with accelerated senescence induced by CL.

The inhibitory effect of leaf senescence by cytokinin in tomato is well documented; for instance, expression of *isopentenyl transferase* (*IPT*), encoding the rate-limiting step in cytokinin biosynthesis, under the control of the promoter of the Arabidopsis senescence-associated genes, *SAG12* or *SAG13,* suppresses leaf senescence (Swartzberg *et al.*, 2006, Swartzberg *et al.*, 2011). Also, the cross talk between sugar signaling and cytokinin should not be overlooked. HXK1 is thought to promote senescence by repressing cytokinin signaling (Thomas, 2013). In tomato, for instance, double-transgenic plants expressing both *AtHXK1* and either P_{SAG12}*::IPT* or P_{SAG13}*::IPT* displayed accelerated senescence despite the demonstrated senescence inhibition effect of P_{SAG12}*::IPT* or P_{SAG13}*::IPT* alone (Swartzberg *et al.*, 2011). In mature, uninjured A131 tomato leaves exposed to CL, the expression of *HXK1* (Solyc03g121070.2) was significantly down regulated as compared to A131 plants under a 16-h photoperiod, yet no difference was found between CL-exposed A131 and CLT plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

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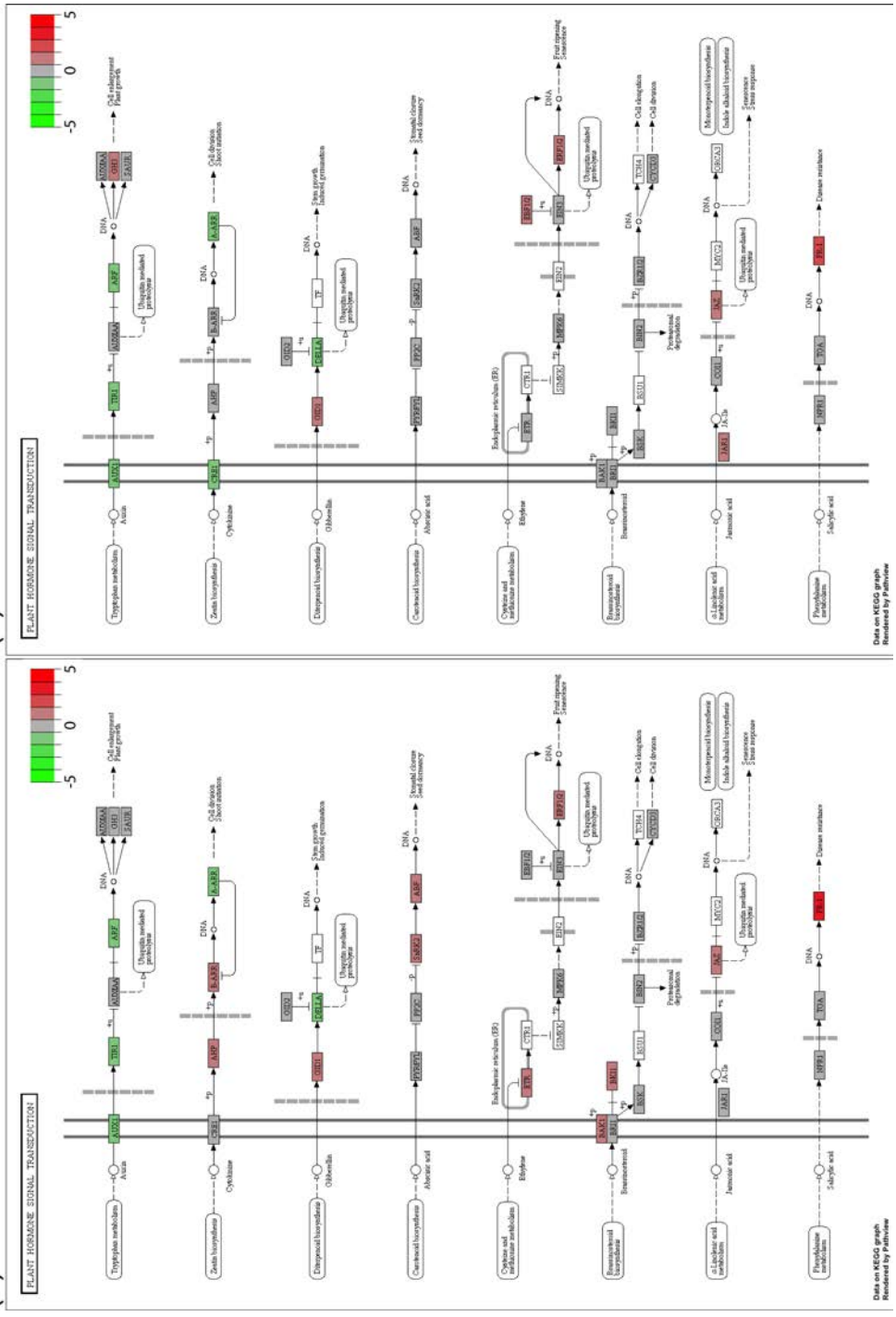

Figure 8.7 | Tomato "plant hormone signal transduction" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for plant hormone signal transduction. In panel (**a**), contrast between A131 tomato plants exposed to 16-h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly04075

This suggests that cytokinin signaling is, in principle, able to prevent senescence in tomato leaves under CL and that the hypothetical CL-induced senescence is likely not dependent on the glucose HXK1 sensor, which is consistent with the lack of correlation between glucose and chlorosis (Fig. 8.4a).

In contrast to cytokinin, ethylene is involved in the induction of leaf senescence (Fischer, 2012, Lim *et al.*, 2007). Interestingly, Cushman and Tibbitts (1998) showed that young tomato leaflets produced more ethylene when grown under CL and transgenic tomato plants containing an antisense gene of *1-aminocyclopropane 1-carboxylate (ACC) oxidase*, encoding the last enzyme required for ethylene biosynthesis, showed less CL-induced symptoms than wild-type plants. Figure 8.7 shows that the *ethylene-responsive transcription factor 1* (*ERF1*) was up-regulated in CL-exposed A131 tomato plants compared with both A131 plants kept at 16-h photoperiod and CLT plants exposed to CL. This suggests that, similar to ethylene production, ethylene signaling is also up regulated by CL.

Features of retrograde-signaling-dependent photosynthetic down-regulation in the CL-induced injury in tomato

Although the inhibition of CL-induced injury by cytokinin (Fig. 8.6) and knocking down of *ACC oxidase* expression (Cushman & Tibbitts, 1998) suggest that CL-induced injury is a kind of accelerated senescence, other observations are not consistent with this hypothesis. For instance, in naturally senescing leaves, chlorosis usually starts from the leaf tips or margins and progresses towards the leaf base (Lim *et al.*, 2007), while in CL-exposed tomato leaves chlorosis usually is more severe at the leaf basis (Arthur *et al.*, 1930, Chapter 6, Hillman, 1956, Withrow & Withrow, 1949). Girdling induced accumulation of glucose, fructose and sucrose as well as a decrease in F_v/F_m — with the same spatial pattern as observed in senescing tomato leaves — not only in A131 but, surprisingly, also in CLT (Fig. 8.5). This indicates that CL-tolerant CLT tomato plants are not tolerant to girdled-induced senescence. Although common, CL-induced chlorosis being most severe at the leaf/leaflet basis, is not a rule; mottled chlorosis with irregular distribution across tomato leaflets is also

seen in CL-exposed tomato plants (Fig. 8.6; see also (Chapter 7, Velez-Ramirez *et al.*, 2014 (Chapter 4)). The chlorosis pattern in CL-exposed tomato leaves results, at least in part, from the developmental state at which the tissue was exposed to CL. Hillman (1956) showed that (i) only young tomato leaves were sensitive to CL, (ii) leaves developed under CL could only recover if transferred back to 16-h photoperiod when still young, (iii) CL-induced chlorosis increasingly covered more area towards the leaf margins in increasingly younger leaves, and (iv) when injured plants were transferred back to non injurious photoperiods, healthy leaf tissue increasingly expanded towards leaf margins and apices in increasingly younger leaves. These observations imply that tomato leaf/leaflet bases develop later than the margins and apices, and, more importantly, young tomato leaf tissue has the full potential to develop into injured or healthy leaf tissue depending on the prevalent photoperiod at that critical developmental stage. This is not consistent with the idea that CL-induced injury is accelerated senescence. Instead, it seems that CL disrupts the delicate coordination between chloroplast development and the basic developmental program of the cell; a process that is most sensitive to plastid translation inhibitors or norfluorazon, which are known to trigger a retrograde signal (Pfannschmidt, 2010).

Analysis of RNAseq transcriptome data (Velez-Ramirez *et al.*, 2014 (Chapter 4)) clearly shows that CL down-regulates photosynthesis genes associated with the Calvin Benson cycle (Fig. 8.2), the photosystem I and II, and the light harvesting complexes I and II (Chapter 7). As discussed above, excessive synthesis of starch and sucrose results in a decreased ATP to NADPH ratio, favoring over-reduction of PET components as a result of the over-reduced NADPH pool. This increases H_2O_2 and $^1O_2$ release from PSI and PSII respectively (Asada, 2006). With altered levels of H_2O_2, $^1O_2$, ATP and NADPH in CL-exposed plants, photosynthesis may require to be down regulated via retrograde signals. Interestingly, recent evidence shows that these retrograde signals heavily interact with the light-signaling network in Arabidopsis (Lepistö & Rintamäki, 2012, Ruckle *et al.*, 2012, Ruckle *et al.*, 2007). Considering that over-expression of *PHYA* resulted in complete tolerance to CL in tomato (Chapter 7), the hypothesis that phytochrome signaling modulates retrograde signaling in CL-exposed tomatoes is most interesting for future research.

Several studies suggested that circadian asynchrony is the triggering factor in CL-induced injury in tomato (Chapter 6, Hillman, 1956, Kristoffersen, 1963). Although it has been suggested that circadian-controlled fluctuations in sensitivity to photoinhibition could explain why CL and other abnormal light/dark cycles are injurious to tomato plants (Velez-Ramirez *et al.*, 2011 (Chapter 2)), recent experiments showed that the daily fluctuations in sensitivity to photoinhibition observed in tomato seedlings are not under circadian control (Chapter 6). These observations strongly suggest that photoinhibition is not directly responsible for the CL-induced injury; nonetheless, a circadian-modulation of retrograde signals in tomato is still a possibility, and it would suggest that the CL-induced injury is a photosynthetic down-regulation triggered by signals and "metabolic signatures" at inappropriate times. For instance ATP and NADPH production, H_2O_2 and $^1O_2$ emission and carbohydrate content are higher during daytime than during nighttime. Under CL or

abnormal light/dark cycles, however, conflictive light, retrograde and circadian signals may arise; during a subjective night, the circadian clock is signaling "night", but in the presence of light the chloroplast status would be signaling "day". Under CL, natural development of dark-dependent processes cannot occur, even if the circadian clock up-regulates such processes. In Arabidopsis seedlings, for example, the circadian clock up regulates hypocotyl growth at a specific time, yet growth can only occur if this clock-controlled up regulation coincides with darkness (Niwa *et al.*, 2009, Nozue *et al.*, 2007). As discussed above, plastid maturation is coordinated with the nuclear developmental program and is sensitive to retrograde signals (Pfannschmidt, 2010). The observations that CL only induces injury in young developing leaves suggest that immature chloroplasts are particularly sensitive to a retrograde signals triggered by CL.

Altogether, we postulate that the CL-induced injury in tomato results from down-regulation of photosynthesis by an inadequate integration of retrograde, light and circadian signals. Ethylene and cytokinin signaling are most likely also involved, and future experiments should explore whether these hormone signaling pathways influence the severity of CL-induced injury via a pathway overlapping with retrograde signaling or via a parallel and more independent senescence-like pathway.

Materials and Methods

Plant materials and treatments

Four tomato lines were used: M82 and A131 are inbred lines sensitive to continuous light (CL), while IL-39 and CLT are CL-tolerant introgression lines in the background of M82 and A131, respectively. The wild donor of CL-tolerance in IL-39 and CLT were *S. neorickii* (LA2133) and *S. pennellii* (LA0716), respectively. All four lines have been previously described (Eshed & Zamir, 1994, Velez-Ramirez *et al.*, 2014 (Chapter 4)). Seeds were provided by Monsanto Vegetable Seed Division (Bergschenhoek, The Netherlands). Plants were grown in rockwool blocks at 21 °C and 70% RH. Commercial hydroponic nutrient solution for tomato was used (Yara Benelux B.V., Vlaardingen, The Netherlands); after combining and diluting premixed liquid fertilizers, the solution contained 12.42 mM NO_3, 7.2 mM K, 4.1 mM Ca, 3.34 mM SO_4, 1.82 mM Mg, 1.2 mM NH_4, 1.14 mM P, 30 mM B, 25 mM Fe, 10 mM Mn, 5 mM Zn, 0.75 mM Cu and 0.5 mM Mo (EC = 2.00 $dS.m^{-1}$ and pH = 5.0-5.5). Light was provided by high-pressure sodium (HPS) lamps (Master SON-T Green Power 400W, Philips, Eindhoven, The Netherlands) and supplemented with incandescence lamps (Philinea T30 120W, Philips, Eindhoven, The Netherlands). The light intensity was 350 $\mu mol.m^{-2}.s^{-1}$; red-to-far-red ratio was 2.873, and the phytochrome photostationary state (PSS) (Sager *et al.*, 1988) was 0.857. CL treatment consisted in just leaving the lamps (HPS and incandescent) continuously on without changing any other setting.

For the metabolomic experiment, M82 and IL-39 tomato lines were used. Plants were sown directly under 16-h photoperiod or CL treatments. After three weeks, leaf

samples (second true leaf) were collected, frozen with liquid nitrogen and kept at -80 °C until analysis. Unprocessed samples of A131 and CLT from a previously reported experiment on "abnormal light / dark cycles" were used (Chapter 6); briefly, four week-old A131 and CLT plants were used, and a computer-controlled timer set the HPS lamps ON/OFF to 24-h light / 0-h dark, 6-h light / 6-h dark or 24-h light / 24-h dark cycles. For the girdling and cytokinin experiments, also four-weeks-old A131 and CLT tomato plants, grown under 16h photoperiod, were used. Girdling treatment consisted in enclosing a section (1.5 to 3 cm in length) of a leaflet petiole (4^{th} leaf) with modeling dough, and pouring hot wax (85 °C) into the enclosed petiole section (Goldschmidt & Huber, 1992). Cytokinin treatment consisted in spraying the plants till runoff with BAP solution (0.1 mM 6-benzylaminopurine in 1 N KOH pH=7.8 with Tween20 0.02% (v/v)) every three days (Zavaleta-Mancera *et al.*, 2007).

Extraction and GC–TOF–MS analysis of tomato primary metabolites

Relative metabolite contents were determined as described by Lisec *et al.* (2006) with modifications specific to tomato leaves (Etalo *et al.*, 2013). Briefly, polar metabolite fractions were extracted from ~50 mg fresh weight (FW) of frozen leaf tissue. The ground leaf tissue was homogenized in 700 µl of precooled (-20 °C) methanol (100%), spiked with ribitol (0.2 mg.ml^{-1}) as an internal standard. The samples were incubated for 10 min at 70 °C and subsequently centrifuged at 21,000 RCF for 10 min. The supernatant was transferred into a new Eppendorf vial, and 375 µl of chloroform and 750 µl of water were added. The mixture was centrifuged at 21,000 RCF for 10 min. The methanol/water supernatant (polar phase) was carefully transferred into a new Eppendorf vial, and aliquots (200 μl) were dried by vacuum centrifugation without heating.

The dried samples were derivatized on-line as described by Lisec *et al.* (2006) using a Combi PAL autosampler (CTC Analytics AG). First, 12.5µL O-methlhydroxylamine hydrochloride (20 mg/ml pyridine) was added to the samples, which were then incubated for 30 min at 40 °C with agitation. Then the samples were derivatized with 17.5 µL MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) for 60 min. An alkane mixture (C10-C34) was added to determine retention indices of metabolites. The derivatized samples were analysed by gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS) consisting of an Optic 3 high performance injector (ATAS GL Int.) and an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments, St. Joseph, MI, USA). Two µl of each sample was introduced into the injector at 70 °C using a split flow of 19 ml.min^{-1}. The injector was rapidly heated with 6 °C/s to 240 °C. The chromomatographic separation was performed using a VF-5ms capillary column (Varian; 30m x 0.25 mm x 0.25 µm) including a 10m guard column with helium as carrier gas at a column flow rate of 1 ml.min^{-1}. The temperature was isothermal for 2 min at 70 °C, followed by a 10 °C min^{-1} ramp to 310 °C, and was held at this temperature for 5 min. The transfer line temperature was set at 270 °C. The column effluent was ionised by electron impact at 70eV. Mass spectra were acquired at 20 scans.sec^{-1} within a mass range of *m/z* 50 – 600, at a source

temperature of 200 °C. A solvent delay of 295 s was used. The detector voltage was set to 1400V.

Data were processed using ChromaTOF 2.0 (Leco instruments) and MassLynx (Waters Inc.) and further analysed using MetAlign (Lommen, 2009) to extract and align the mass signals (signal to noise ratio ≥ 2). Mass signals that were present in less than 2 samples were discarded. Signal redundancy per metabolite was removed by means of clustering, and mass spectra were reconstructed (Tikunov *et al.*, 2005). The mass spectra were subjected to tentative identification by matching to the NIST08 (National Institute of Standards and Technology, Gaithersburg, MD, USA; http://www.nist.gov/srd/mslist.htm) and Golm Metabolite (http://gmd.mpimp-golm.mpg.de/) spectral libraries and by comparison with rentetion indices calculated using a series of alkanes and fitted with a second order polynomial function (Strehmel *et al.*, 2008). Library hits were manually curated. Compound identification is limited to the availability of spectra in the libraries used.

Mapping of RNAseq data to tomato-specific KEGG pathways

Previously published expression data of A131 and CLT tomato plants exposed to continuous light (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to tomato-specific KEGG pathways. The Sol Genomic Network/Ensembl gene identifiers (*e.g.* Solyc07g063600.2) of the original data set were mapped to UniProt accessions (*e.g.* K4CH43) and then to the KEGG/GENEID/Entrez IDs (*e.g.* 101268123) using the UniProt ID mapping tool (www.uniprot.org/help/mapping); only genes mapping to unique IDs were used. From the 31350 genes in the original data set, 14219 had a unique mapping between all IDs. The R package "Pathview" (Luo & Brouwer, 2013) was used to map the originally reported LogFold change to the following tomato-specific KEGG pathways: "Carbon fixation of photosynthetic organism" (sly00710), "Starch and sucrose metabolism" (sly00500), "Plant hormone signal transduction" (sly04075) and "Glycolysis and Gluconeogenesis" (sly00010). For nodes containing more than one gene, mean LogFold change was used.

Carbohydrate quantification

For carbohydrate content quantification, 15 mg of freeze-dried leaf material were extracted in 5 ml of ethanol 80% (v/v) at 80 °C for 20 minutes. After centrifuging the mixture for 5 min at 7000 RCF, the pellet was stored at -20 °C and used for starch quantification while the supernatant was evaporated using a vacuum concentrator. The residue was re-suspended in a final volume of 1 ml distilled water, placed in an ultrasonic bath for 10 minutes and centrifuged 15 min at 25,000 RCF to remove any insoluble particles. Finally, the samples were diluted and analyzed for soluble sugars (glucose, sucrose and fructose) using high performance anion exchange chromatography (HPAEC), consisting of a GS50 pump, a

PED detector and a CarboPac PA1 (4x250mm) column (Dionex, Sunnyvale, USA). Samples were eluted with 100 mM NaOH.

For starch quantification, the pellets obtained as described above were re-suspended in three ml of ethanol 80% (v/v). After centrifuging the sample for 5 min at 7,000 RCF, the supernatants were discarded, and the pellets were washed two more times. Then, the pellets were dried using a vacuum concentrator, and two ml of α-amylase/rohalase solution (1 $mg.ml^{-1}$ in H_2O) were added (Serva, Heidelberg, Germany). After incubating the samples for 30 minutes in shaking water bath at 90 °C, one ml of amyloglucosidase solution (0.5 $mg.ml^{-1}$ in 50 mM citrate buffer, pH-4.6) was added (Sigma-Aldrich, St. Louis, USA), and samples were incubated for 15 minutes in a shaking water bath at 60 °C. Finally the samples were centrifuged 15 min at 25,000 RCF to remove any insoluble particles, diluted and analyzed using HPAEC as described above. As samples contained only glucose, however, a shorter run protocol was used and the samples were eluted with 100 mM NaOH containing 12.5 mM sodium acetate.

Chlorophyll fluorescence imaging

Imaging of the maximum quantum efficiency of photosysmtem II (F_v/F_m) (Baker, 2008) was performed as previously described (Velez-Ramirez *et al.*, 2014 (Chapter 4)). In summary, intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences, Lincoln, USA). After 20 minutes of dark adaptation, leaflets were detached and immediately used for measurements in a chlorophyll fluorescence imaging system (FluorCam 800MF, Photon System Instruments, Brno, Czech Republic). Leaflet average F_v/F_m was calculated using ImageJ software version 1.44o (Schneider *et al.*, 2012).

Multivariate and statistical analyses of metabolomics and chlorophyll fluorescence data

Mass intensity values of the representative mass were normalized using the fresh weight of each sample. Normalized values were log2-transformed and subsequently autoscaled (van den Berg *et al.*, 2006). Transformed data were used for cluster analysis and principal component analysis (PCA) using R (R Core Team, 2013). Pearson's correlation coefficient, for computing the distance matrix, and Unweithed Pair Group Method with Aritmetic Mean (UPGMA) were used for hierarchical clustering. The contribution of each metabolite to the first three principal components was calculated according to van den Berg *et al.* (2006). Statistical significance of the leaflet average F_v/F_m was determined with an ANOVA test performed with IBM SPSS Statistics software version 19 (IBM, Somers, USA).

Acknowledgements

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growth chambers. This project was financially supported by the Technological Top Institute-Green Genetics (TTI-GG) (Project 2CFD020RP).

Supplementary Information

Supplementary Table 1. Metabolites detected in tomato leaf tissue by GC-TOF-MS analysis. The table columns represent the following information: *m/z*: fragment ion chosen by MMSR as representative ion fragment from a cluster; Hit: NIST library matching hit; RI (retention index): retention index was calculated using alkanes retention time values; and Level of identification: see footnote.

m/z	Hit	RI	Level of identification[a]	*m/z*	Hit	RI	Level of identification[a]
59	Unknown001	990.5797633	3	73	Ascorbic acid	1849.364619	1
59	Unknown002	1016.144099	3	73	D(-)-Quinic acid (5TMS)	1852.010109	1
59	Unknown003	1025.083315	3	73	Fructose methoxyamine (5TMS)	1864.532891	2
73	Unknown004	998.1768372	3	73	Fructose methoxyamine {BP} (5TMS)	1874.530547	2
73	Unknown005	1012.607047	3	73	Unknown024	1882.558159	3
73	Pyruvic acid	1053.545621	1	73	Glucose methoxyamine (5TMS)	1890.863806	1
73	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	1060.089755	2	73	Mannose methoxyamine {BP} (5TMS)	1911.282918	1
73	Alanine	1097.637675	1	73	Unknown025	1921.724575	3
73	Unknown006	1109.138845	3	73	Unknown026	1930.757562	3
73	Unknown007	1132.675635	3	73	Unknown027	1943.250937	3
73	Unknown008	1201.615254	3	73	L(+)-Ascorbic acid (4TMS)	1949.348355	2
73	Unknown009	1243.922337	3	73	Unknown028	1975.182091	3
73	Unknown010	1251.00572	3	73	Galactonic acid (6TMS)	1991.118009	2
73	Unknown011	1255.004561	3	73	Unkwnon029	2002.408395	3
73	Unknown012	1255.004561	3	73	Glucaric acid (6TMS)	2008.20316	2
73	Unknown013	1280.113151	3	73	Unknown030	2025.660861	3
73	Glycine	1290.97601	1	73	Unknown031	2063.955434	3
73	Unknown014	1312.113829	3	73	myo-Inositol (6TMS)	2091.163148	1
73	Fumaric acid (2TMS)	1337.348468	1	73	Unknown032	2104.863508	3
73	L-Serine (3TMS)	1342.879486	1	73	Unknown033	2168.380295	3
73	Unknown015	1363.048732	3	73	Unknown034	2179.6415	3
73	L-Threonine (3TMS)	1368.552403	1	73	Unknown035	2192.535317	3

73	Unknown016	1414.258926	3	73	Unknown036	2287.864738	3
73	Unknown017	1434.7519	2	73	Unknown037	2314.595738	3
73	Malic acid (3TMS)	1476.541595	1	73	Unknown038	2368.703049	3
73	L-Aspartic acid (3TMS)	1508.727354	1	73	Unknown039	2415.451065	3
73	Pyroglutamic acid (2TMS)	1517.079908	1	73	Unknown040	2486.130326	3
73	GABA (3TMS)	1520.95388	1	73	Sucrose	2611.214364	1
73	Threonic acid (4TMS)	1545.091403	2	73	Sucrose (8TMS); alpha-D-Glc-(1,2)-beta-D-Fru	2618.712376	1
73	Unknown018	1568.903828	3	73	Unknown041	2901.062901	3
73	L-Glutamic acid (3TMS)	1612.560823	1	73	Unknown042	2955.857038	3
73	L-Phenylalanine (2TMS)	1624.302542	1	73	Unknown043	2959.518114	3
73	Unknown019	1665.421395	3	73	Unknown044	3042.772403	3
73	Unknown020	1743.150905	3	73	Chlorogenic acid	3073.037643	1
73	Unknown021	1754.543737	3	73	Unknown045	3085.948976	3
73	Unknown022	1762.808316	3	73	Unknown046	3107.032201	3
73	L-Glutamine (3TMS)	1774.141951	1	73	Unknown047	3150.184829	3
73	Fructose (5TMS)	1795.202948	2	77	Unknown048	1017.988141	3
73	Fructose (5TMS)	1804.10313	2	84	Unknown049	1525.896609	3
73	M000606_A182009-101-xxx_NA_1806_PRED_VAR5_ALK_similar to Fructose Derivate	1809.947251	2	119	Unknown050	1005.991792	3
73	Citric acid	1814.99133	1	204	Unknown051	1888.26001	3
73	Unknown023	1826.830448	3				

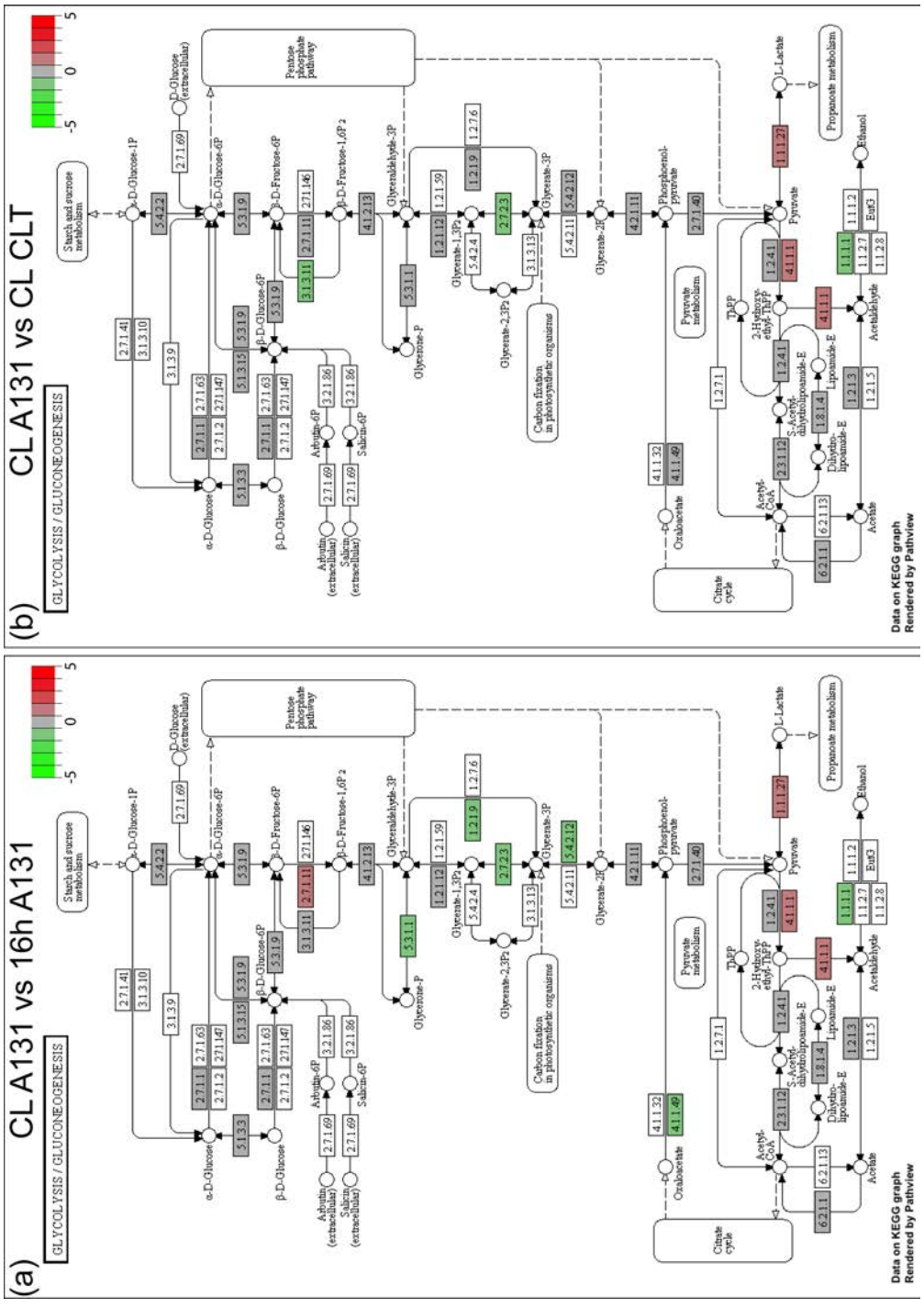
[a] Metabolites were identified partly following the metabolomics reporting standards (Sumner *et al*., 2007):

1. Identified compounds: Based upon similarity of mass spectra and retention time (retention index) of authentic reference standards.

2. Putatively annotated compounds: Based upon similarity of mass spectra with Golm Metabolome Database and NIST spectral database and the retention index published in literature.

3. Unknown compounds: Unidentified compounds.

Scan for Hi-Res image



Supplementary Figure 8.1 | Tomato "glycolysis/gluconeogenesis" KEGG pathway as affected by continuous light. Differential expression of tomato genes in continuous light (CL)-sensitive A131 and CL-tolerant CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)) was mapped to the tomato KEGG pathway for glycolysis/gluconeogenesis. In panel (**a**), contrast between A131 tomato plants exposed to 16-h photoperiod and CL. In panel (**b**), contrast between A131 and CLT exposed to continuous light. In both panels, each colored node represents the average Log fold change of all genes contained in that node. No expression information is available for the nodes in white because of (i) that specific node does not exist in tomato (e.g. a bacteria-specific enzyme), (ii) the node do exist in tomato, but it is not yet annotated (only ±25,000 tomato genes are currently annotated in the KEGG database) and/or (iii) the node do exist and is annotated in tomato, yet the gene(s) associated to that node were not detected in the data set (only ±14,000 genes with KEGG annotation were detected in this data set). For detailed information on each node, visit the KEGG website on the following link: www.genome.jp/kegg-bin/show_pathway?sly00010


tomato
tolerance
light
injury
plants
signals
CAB
retrograde
exposed
proposed
use
study
circadian
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LHCII
Arabidopsis
energy
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accumulation
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photosynthesis
greenhouse
gene
transgenic
leaves
higher
support
severity
PHYB
influencing
continuous
state
carbohydrate
developmental
plastid
ethylene
red
probably

General Discussion

On the continuous-light-induced injury in tomato

Aaron I. Velez-Ramirez

Tomato plants are injured by continuous light (CL) (Arthur *et al.*, 1930). In this thesis, I describe a series of experiments that aimed to unravel the physiological mechanism responsible for the induction of this injury, understand the genetic basis of CL-tolerance found in wild tomato species and breed the tolerance into domesticated tomatoes with the objective to increase greenhouse tomato yield.

The first part of this thesis reviews the plentiful, and sometimes languished, literature on the topic (Chapter 1) using modern concepts of plant physiology to formulate new hypotheses about the physiological basis of CL-induced injury (Chapter 2). Likewise, using model simulations and concepts of crop ecology and greenhouse technology, predictions on the expected yield increase were generated and challenges when cultivating CL-tolerant tomatoes under CL identified (Chapter 3). Next, this thesis focuses on studying diverse aspects of the CL-tolerance, including its genetic basis and practical applications (Chapter 4) and its localization and mobility within the plant (Chapter 5). Then, the last part of this study focuses on the physiological mechanism of the CL-induced injury in tomato, with emphasis on the factors responsible for triggering the injury (Chapter 6), the effect of far-red light and phytochromes (Chapter 7) and the role of carbohydrate accumulation (Chapter 8).

In this general discussion, I discuss the major findings and propose a generic model of CL-induced injury in tomato. In addition, I discuss the perspectives for growing tomato under CL and propose novel directions on how to continue the study of this disorder.

Environmental factors influencing the development of the injury

Several environmental factors increase or decrease the severity of CL-induced injury in tomato (Fig. 9.1a), which aids in the unraveling of the physiological mechanisms underlying the injury. These environmental factors have been previously described (Arthur, 1936, Arthur *et al.*, 1930, Demers & Gosselin, 2002, Globig *et al.*, 1997, Hillman, 1956, Kristoffersen, 1963, Matsuda *et al.*, 2012, Murage *et al.*, 1997, Sysoeva *et al.*, 2012) and discussed (Velez-Ramirez *et al.*, 2011 (Chapter 2)); in this chapter I focus on discussing them in relation to the latest understanding of CL-induced injury.

As many physiological processes, the CL-induced injury is enhanced at higher temperatures (Withrow & Withrow, 1949). Interestingly, daily temperature fluctuations (*i.e.* thermoperiods) diminish and even prevent the CL-induced injury in tomato (Hillman, 1956, Kristoffersen, 1963, Matsuda *et al.*, 2012, Sysoeva *et al.*, 2012). Considering that thermoperiods can entrain the circadian clock (Yamashino *et al.*, 2008), it is suggested that the protective effect of thermoperiods depends on its ability to reset the circadian clock (Velez-Ramirez *et al.*, 2011 (Chapter 2)). As explained next, however, some evidence does not fit with this hypothesis, and a simpler explanation is also plausible. Temperature fluctuation entrains the Arabidopsis (*Arabidopsis thaliana*) circadian clock (Yamashino *et al.*, 2008), and this process is robust even at different temperatures thanks to a phenomenon known as temperature compensation (Gould *et al.*, 2006). In tomato, however, not all thermoperiods can protect tomato plants from CL; for instance, Hillman (1956) reported that thermoperiods of 26°/17° C prevent the injury, but 26°/20° C does not. Additionally,

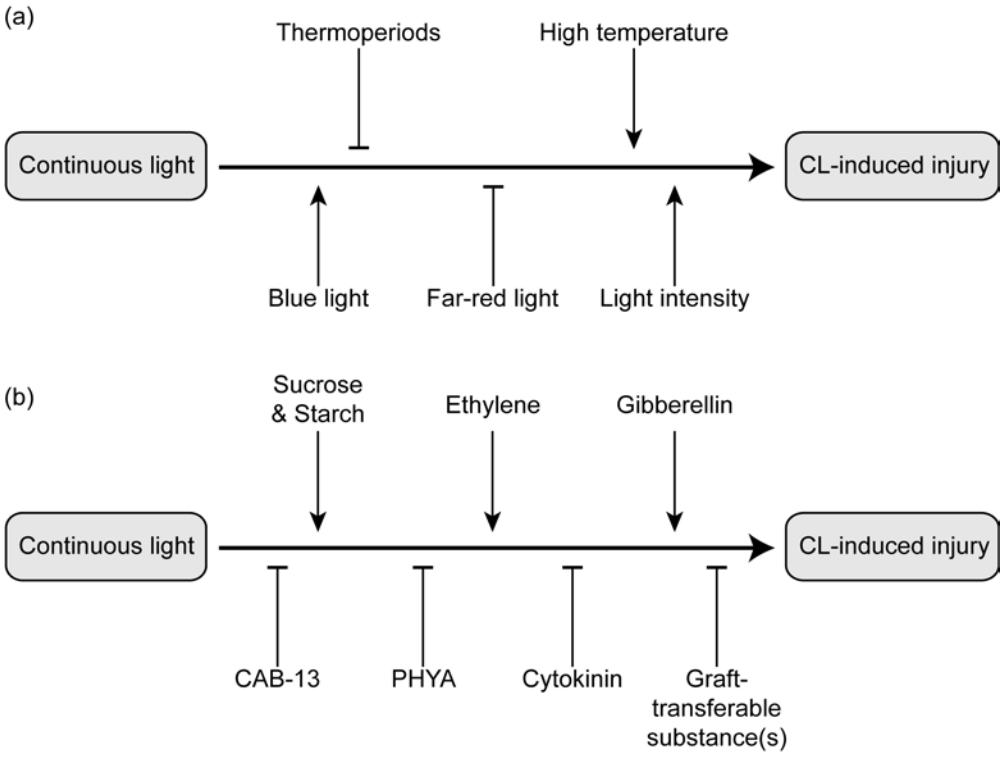

Figure 9.1 | Environmental and internal factors influencing the severity of continuous-light-induced injury in tomato. (**a**) Environmental factors. Diurnal fluctuations in air temperature (thermoperiods) diminish CL-induced injury in tomato (Hillman, 1956, Kristoffersen, 1963, Matsuda *et al.*, 2012, Sysoeva *et al.*, 2012). At a higher temperature, the injury is more severe (Withrow & Withrow, 1949). The light spectral distribution has an influence on the severity of the injury (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997); light sources with higher percentage of blue and far-red light increase (Demers & Gosselin, 2002) and decrease (Chapter 7, Globig *et al.*, 1997) the injury, respectively. The higher the light intensity the higher the CL-induced injury is (Arthur *et al.*, 1930, Murage *et al.*, 1997). (**b**) Internal factors. Sucrose and starch concentrations correlate with the continuous-light-induced injury in tomato plants exposed to several injurious light/dark cycles (Chapter 8). Transgenic tomato plants containing an antisense gene of *1-aminocyclopropane 1-carboxylate (ACC) oxidase*, which encodes the last enzymatic step in ethylene biosynthesis, show less CL-induced symptoms than wild-type plants (Cushman & Tibbitts, 1998). Spraying the plants daily with gibberellic acid for two weeks increases the chlorosis in tomato plants exposed to continuous light (Kristoffersen, 1963). The *type III CAB-13* gene (Solyc07g063600.2) is associated with tolerance to continuous light in wild tomato species (Velez-Ramirez *et al.*, 2014 (Chapter 4)). A mutation in *Phytochrome A* (*PHYA*) increases continuous-light-induced injury in tomato while overexpression of *PHYA decreases it* (Chapter 7). Cytokinin treatment greatly diminishes the injury symptoms in tomato plants exposed to continuous light (Chapter 8). Finally, grafting a continuous-light-tolerant tomato shoot on a sensitive tomato plant decreases the sensitivity of the latter, suggesting the transfer of a protective substance(s) via the graft union (Chapter 5). In both panels, the order of the arrows is arbitrary.

in this study tomato plants were cultivated under greenhouse conditions and exposed to CL; these plants were exposed to a thermoperiod of ~20.5°/~17° C and showed circadian rhythms in flower opening. Nevertheless, strong CL-injury developed (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This suggests that the protective effect of thermoperiods might not be related to the entrainment of the circadian clock but to a decreased metabolic activity during the daily cold period, resulting in slower development of the CL-induced injury.

Several studies report differences in the severity of CL-induced injury in tomato depending on the light intensity (Arthur *et al.*, 1930, Murage *et al.*, 1997) as well as on the type of lamps used (Demers & Gosselin, 2002). While the effect of light intensity is unambiguous, the differences in the spectral distribution among the lamps used are so large that linking a particular wavelength to the injury severity is not possible. Therefore, in my work, I have exposed tomato plants to CL with contrasting far-red content while keeping all other wavelengths constant (Chapter 7). By doing this, a protective effect of far-red light against the CL-induced injury could be unambiguously assigned.

Internal factors influencing the development of the injury

While previous studies investigated the role of ethylene and gibberellin in CL-induced injury in tomato (Kristoffersen, 1963), most of the other internal factors influencing the injury severity summarized in Fig. 9.1b were investigated in the present study. Spraying CL-exposed tomato plants with cytokinin (Chapter 8) or gibberellin (Kristoffersen, 1963) has a protective and enhancing effect on the disorder, respectively. However, the effect of ethylene, the other hormone reported in Fig. 9.1, is not as clear as the cytokinin and gibberellin effects. In potato, for instance, silver thiosulfate, which blocks ethylene receptors, reduces injury symptoms in CL-sensitive cultivars (Cushman & Tibbitts, 1998), while in tomato, transgenic plants carrying an antisense transgene of *ACC-oxidase*, an ethylene biosynthesis enzyme, showed no decrease in chlorophyll when exposed to CL (Cushman & Tibbitts, 1998). However, the *Never ripe* tomato mutant, which is ethylene insensitive, was injured by CL as much as the wild-type, although it did not show CL-induced epinasty (Cushman & Tibbitts, 1998). This suggests that ethylene is not required to trigger CL-induced injury, yet it is probably responsible for enhancing it and for the CL-induced epinasty.

Since the discovery that CL injures tomato plants, an important role of carbohydrates has been hypothesized (Arthur *et al.*, 1930, Demers *et al.*, 1998, Dorais *et al.*, 1996, Velez-Ramirez *et al.*, 2011 (Chapter 2)). This hypothesis, although plausible, was only supported by circumstantial evidence. That is, a correlation does not necessary arise from a causal relation. While carbohydrate accumulation occurs in CL-grown tomato plants, it is too simple to conclude that, hence, carbohydrate accumulation triggers CL-induced injury. Here, nevertheless, we have shown that the severity of the injuries in tomato plants exposed to CL, and other injurious abnormal light/dark cycles, correlates with the sucrose and starch content that independent plants showed two weeks before (Chapter 8). In our experimental setup, clear injury symptoms took about two weeks to develop; therefore, these results support the involvement of carbohydrate accumulation. Although a proven

physiological mechanism linking sucrose and/or starch accumulation with the CL-induced injury is still needed, we have proposed that such mechanism could be accelerated senescence or photosynthetic down-regulation (see further discussion below).

The protective effect of grafting a CL-tolerant shoot on a CL-sensitive plant is remarkable (Chapter 5). While control experiments support the hypothesis that this phenomenon depends on a mobile substance(s), further elucidating the physiological basis of this phenomenon goes beyond the scope of this thesis. For breeding purposes, nonetheless, this result is most relevant as it shows that the CL-tolerance is functionally located in the shoot, and for future studies, it is a powerful tool that can aid in testing hypotheses.

In my thesis, I studied transgenic and non-transgenic approaches to confer CL-tolerance to domesticated tomato. Some of the most promising are over-expression of PHYA (Chapter 7) and introgression of the wild allele of the locus containing *CAB-13* on chromosome seven (Velez-Ramirez *et al.*, 2014 (Chapter 4)). The role of these two proteins in light signaling and light harvesting, respectively, fully agrees with the nature of the disorder. Due to its importance, a critical discussion of the evidence and hypothesis linking these two proteins with CL-induced injury is provided next.

The role of CAB-13 in continuous-light-tolerance

Tolerance to CL in wild tomato was reported by Daskaloff and Ognjanova (1965). To our knowledge, however, the present study is the first one ever since to use this trait for research and breeding purposes. Genetic, RNAseq and sequence data points to the *type III light-harvesting chlorophyll a/b binding protein 13* gene (*LHCB type III CAB-13* or *CAB-13*) as responsible for the CL-tolerance found in wild tomato species (Velez-Ramirez *et al.*, 2014 (Chapter 4)). This gene encodes a protein member of the LHC super gene family. A search for LHC proteins in the tomato and Arabidopsis genomes resulted in 31 and 21 *CAB/LHC* genes, respectively (Kersey *et al.*, 2014). After retrieving and aligning all these protein sequences, a dendrogram was constructed in order to illustrate the diversity and classification of tomato CAB proteins (Fig. 9.2). LHC proteins are classified as A or B (also I or II) depending on which light harvesting complex (LHC) belong to; LHCI is composed from LHCA proteins, which harvest light for PSI, while LHCII is composed from LHCB proteins, which harvest light for PSII. LHCII is composed of 6 types of LHCB proteins (*i.e.* LHCB1-6) (Caffarri *et al.*, 2009). In Arabidopsis, another two rarely expressed LHCB types exist (*i.e.* LHCB7-8); notice that LHCB4.3 is now reclassified as LHCB8 (Klimmek *et al.*, 2006). In contrast to Arabidopsis, which contains only one copy of a type III LHCB (LHCB3), tomato has two copies, located on chromosome 7 and 12 (Fig. 9.2 and 9.3).

The *CAB-13* copy on chromosome 7 (Solyc07g063600.2) is located in the CL-tolerance locus and evidence presented in this thesis points to this gene as an important factor conferring CL-tolerance in wild tomato species (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Although the protein sequence of both copies show a 95% identity, enough differences at the nucleotide sequence on exon 3 allowed us to design a VIGS probe that specifically targets the *CAB-13* copy on chromosome 7 (Fig. 9.2b). For future studies, nonetheless, a

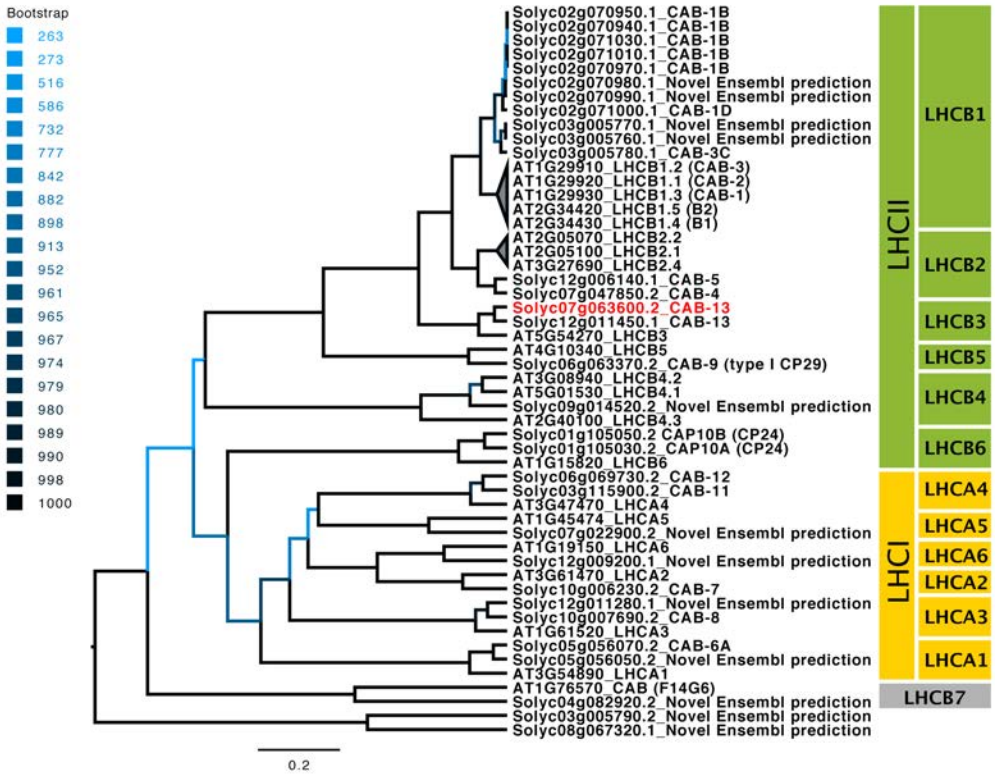

Figure 9.2 | The LHC gene family in tomato and Arabidopsis. Protein sequences of all homologous genes of CAB-13 in tomato (*Solanum lycopersicum*) and Arabidopsis (*Arabidopsis thaliana*) were collected and aligned. Using an unweighted pair group method with arithmetic mean (UPGMA) procedure a dendrogram was constructed to illustrate the diversity and classification of LHC proteins. Proteins associated with the antenna of photosystem I (LHCI) are highlighted in yellow and are known as LHCA proteins. Likewise, proteins associated with the antenna of photosystem II (LHCII) are highlighted with green and are known as LHCB proteins. CAB stands for chlorophyll a/b binding protein, and LHC stands for light-harvesting chlorophyll a/b binding protein; hence CAB and LHCA/B are synonyms. The type III CAB-13 protein, highlighted in red (Solyc07g063600.2), is encoded by the gene associated with continuous-light-tolerance mapping to chromosome 7. Notice that the tomato genome contains another *type III CAB-13* gene on chromosome 12 (Solyc12g011450.1).

redundant role of the *CAB-12* copy on chromosome 12 (Solyc12g011450.1) should be considered as silencing of both *CAB-13* copies could further support their involvement on the CL-tolerance and it might even explain the CL-tolerance in Sub-Arctic Plenty tomatoes, which is probably not associated with chromosome 7 *CAB-13* (Velez-Ramirez *et al.*, 2014 (Chapter 4)).

With the exception of this thesis, no other study has investigated tomato CAB-13. Therefore, in order to discuss hypotheses aiming to explain how CAB-13 could confer CL-tolerance in tomato, we use knowledge on Arabidopsis LHCB proteins. Notice that Arabidopsis is tolerant to CL (Handford & Carr, 2007, Lepisto *et al.*, 2009); hence, a relevant difference between tomato and Arabidopsis must exist and be responsible for the different CL-tolerance phenotype. In Arabidopsis, LHCB proteins form the LHCII antenna, which is closely associated with PSII and harvests light for it. As part of the PSII-LHCII supercomplex, LHC3 is exclusively found in the M trimer (Fig. 9.4a) (Caffarri *et al.*, 2009). Knockout or down-regulation of Arabidopsis *LHCB3* has several effects (Caffarri *et al.*, 2009, Damkjær *et al.*, 2009, Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b); these can be classified into effects on the PSII-LHCII supercomplex composition and efficiency (Fig. 9.4b), effects on state transitions (Fig. 9.4c), and effects on LHCII phosphorylation and LHCB expression (Fig. 9.4d). These effects provide clues on the possible role of tomato CAB-13 in conferring CL-tolerance.

In the Arabidopsis *Lhcb3* mutant, PSII-LHCII supercomplexes lack the M trimer and the monomeric CP24 subunits (Fig. 9.4b), which suggests that LHCB3 is the unit facing CP24 and mediating the association of the M trimer and CP24 subunits to LHCII (Caffarri *et al.*, 2009). Similarly, high-light-acclimated plants show a down-regulation of *LHCB3*, which leads to supercomplexes without M trimer and CP24 as well as higher PSII quantum yield of charge separation and shorter trapping time of PSII (Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b). Longer trapping times increases the probability of chlorophyll in the triplet exited state (^{3}Chl*); in turn, ^{3}Chl* can react with molecular oxygen to form the highly reactive singlet oxygen ($^1O_2$), which damages the photosynthetic membrane (Triantaphylidès & Havaux, 2009). In principle, therefore, loss of CAB-13 should decrease the LHCII antenna size and, consequently, the trapping time, leading to lower probability of photoinhibition by $^1O_2$ production. This suggests that CL-induced injury is not the result of photoinhibition. Indeed, Dorais *et al.* (1995) reported lower photoinhibition in thylakoid membranes isolated from CL-grown tomatoes than in those isolated from 12-h photoperiod after a 30 minute exposure to high light (3500 $\mu mol.m^{-2}.s^{-1}$). Likewise, this study shows that the sensitivity to photoinhibition presents a non-circadian diurnal cycle, which is maintained at its minimum in both CL-sensitive and –tolerant tomato seedlings when exposed to CL (Chapter 6). Although interesting, this does not explains how CAB-13 could confer CL-tolerance.

Another process to be considered when trying to understand the role of CAB-13 in CL-tolerance is the process of state transitions. In response to short-term changes in the light environment, state transitions are defined as the movement of LHCII trimers back and forth between PSI and PSII, a process that requires LHCII trimer phosphorylation (Kargul & Barber, 2008). A recent study reported that LHCII trimers are associated with PSI after long-term acclimation to most light conditions, including low, moderate and high light as well as sunlight; only upon sudden exposure to high light or far-red light, LHCII trimers move back to PSII (Wientjes *et al.*, 2013a). High light over-excites LHCII and far-red light is preferentially absorbed by PSI-LHCI; by moving LHCII back to PSII, therefore,

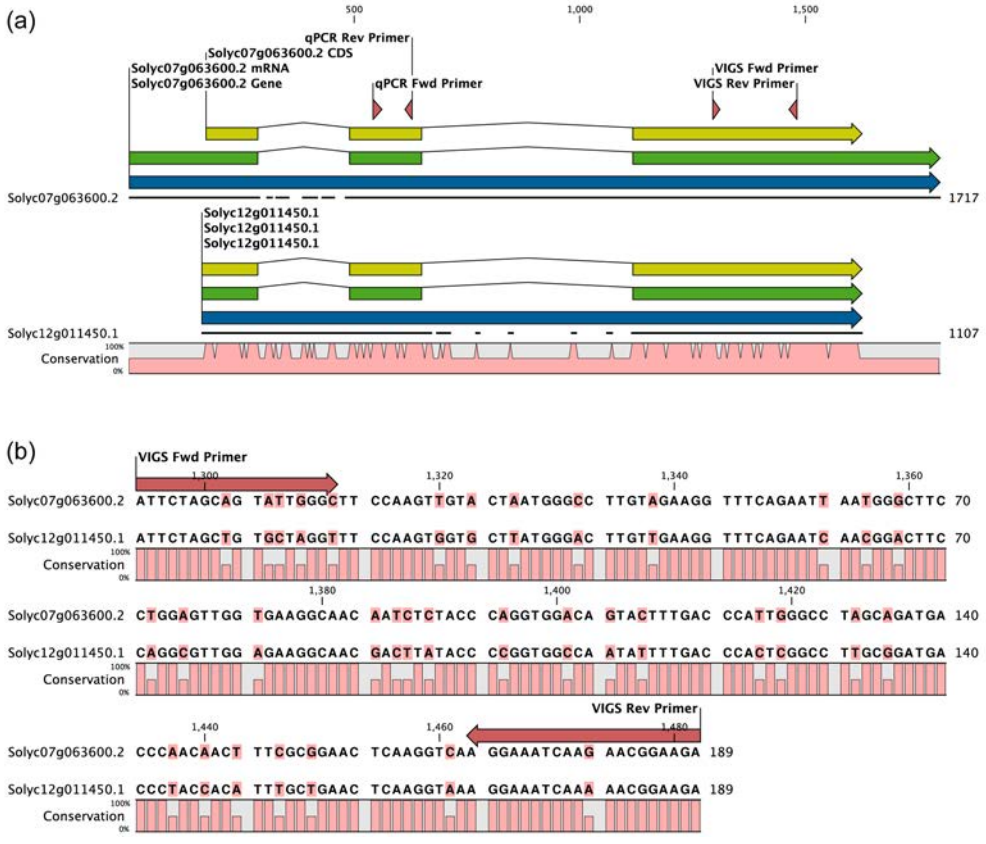

Figure 9.3 | Comparison between the two copies of *CAB-13* present in the tomato genome. Panel (**a**) shows the nucleotide sequence alignment of Solyc07g063600.2 and Solyc12g011450.1 genes located on chromosomes 7 and 12, respectively, and both encoding a type III CAB-13 protein. Solyc07g063600.2 is associated with continuous-light-tolerance. (**b**) Detail of fragment cloned into the *pTRV* VIGS vector used in Chapter 4 is shown.

those LHCII trimers can be quenched and the PSII antenna size increases, which balances the over-excitation of PSI. Remarkably, in the Arabidopsis *Lhcb3* mutant, the rate of LHCII trimer movement form PSII to PSI is higher (Fig. 9.4c) (Damkjær *et al.*, 2009). Considering that CL down-regulates *CAB-13* expression in CL-sensitive tomato plants but not in tolerant ones (Velez-Ramirez *et al.*, 2014 (Chapter 4)), it is of great interest to evaluate the rate of state transitions in CL-exposed tomatoes as an interaction effect of photoperiod and CL-tolerance genotype is expected. As discussed above, however, state transitions are only relevant in adjusting to short-term changes in the light environment; after long exposure to the new light environment, *LHCB* expression is adjusted accordingly. As the injurious CL treatment provided by lamps in a growth chamber is quite constant,

differences in state transition rates between CL-sensitive and –tolerant tomatoes would not explain how CAB-13 confers CL-tolerance.

In Arabidopsis *Lhcb3* leaves, the level of LHCII trimer phosphorylation is higher (Damkjær *et al.*, 2009). As discussed above, state transitions requires LHCII phosphorylation (Kargul & Barber, 2008). Additionally, altered LHCII phosphorylation might influence *LHCB* expression. This is because LHCII phosphorylation and *LHCB* expression are correlated with each other, suggesting that they are co-regulated by the activation state of the LHCII kinase (Pursiheimo *et al.*, 2001). The model presented in Fig. 9.4d was deduced from this evidence, and suggests that *LHCB3* down-regulation results in higher *LHCB* expression. This would suggest that LHCB3 indirectly regulates retrograde signals, which are plastid-derived signals that regulate nuclear gene-expression (Jarvis & López-Juez, 2013). As no study has investigated *LHCB* expression in *Lhcb3* leaves in detail, we cannot confirm or discard this model. If proven to be true, however, this reasoning cannot explain how tomato *CAB-13* could confer tolerance to CL, yet it is probably the most promising research line to follow. In addition to the phosphorylation state of LHCII, the chlorophyll intermediate Mg-protoporphyrin IX (Larkin *et al.*, 2003, Mochizuki *et al.*, 2001, Nott *et al.*, 2006), the redox state of photosynthetic electron transport (PET) components (Fey *et al.*, 2005, Nott *et al.*, 2006, Pfannschmidt *et al.*, 2001) and plastid-derived $^1O_2$ (Lee *et al.*, 2007b, op den Camp *et al.*, 2003, Wagner *et al.*, 2004) are also proposed to relay information to the nucleus about chloroplast state in a particular environment, resulting in adjusted expression of *photosynthesis-associated nuclear genes* (*PhANGs*). In Arabidopsis, however, the regulation of *PhANGs* by retrograde signals is not yet fully understood and even less is known in tomato. Other lines of evidence suggest that down-regulation of *PhANGs* by a retrograde signal play an important role in the CL-induced injury (see discussion below). Hence, the potential role of LHCB3/CAB-13 in regulating retrograde signals is worth investigating.

The role of PHYA in continuous-light-tolerance

My thesis presents strong evidence that phytochrome (PHY) signaling is involved in the regulation of CL-induced injury in tomato. First, I confirmed that enrichment of continuous light with far-red light reduces the severity of injuries in CL-exposed tomatoes and over-expression of PHYA confers complete tolerance to CL (Chapter 7). Positive and negative effects of *PHYB1* and *PHYB2* mutation and over-expression were also observed when tomato plants were exposed to CL with different far-red content. This further indicates that the phytochrome signaling network influences the injury severity in CL-exposed tomato plants in a light-quality-dependent manner.

As in other species, light induces the degradation of tomato PHYA at irradiances as low as 3 $\mu mol.m^{-2}.s^{-1}$ (Husaineid *et al.*, 2007). Hence, it is most interesting to test how PHYA over-expression results in CL-tolerance in tomato plants exposed to CL at 345 $\mu mol.m^{-2}.s^{-1}$ (Chapter 7). In Arabidopsis, an irradiance-dependent photoprotection of PHYA has been proposed (Franklin *et al.*, 2007). In tomato, *phyA* mutants showed anthocyanin accumulation similar to wild type under continuous red light at ~20

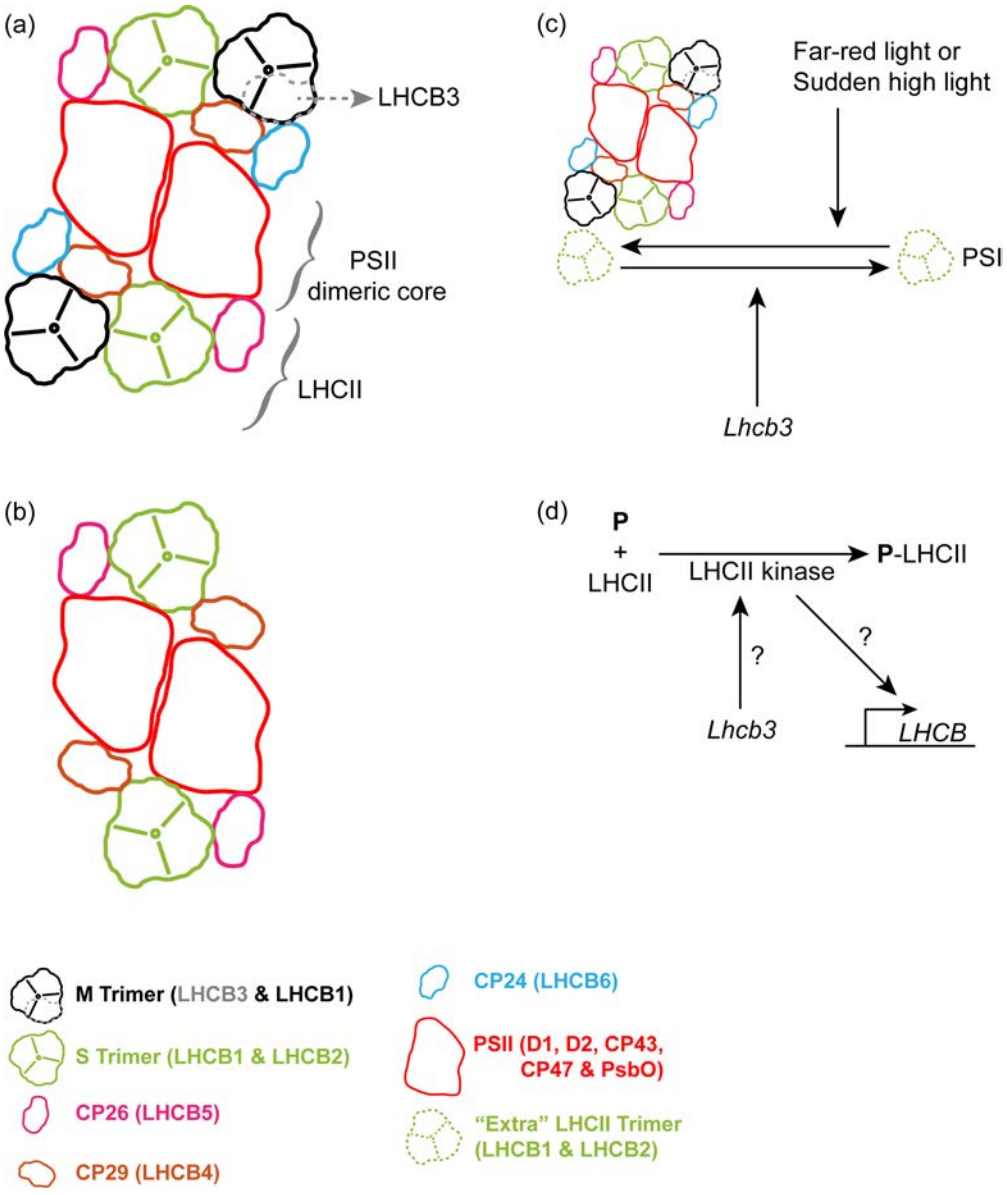

Figure 9.4 | Function of *Arabidopsis thaliana* LHCB3. (**a**) A schematic representation of the light harvesting complex II (LHCII) associated with the photosystem II (PSII) core, known as $C_2S_2M_2$ PSII–LHCII supercomplex as described by Caffarri *et al.* (2009). $C_2S_2M_2$ supercomplexes contain two units of PSII, S trimers and M trimers. The LHCII antenna is composed of monomeric and trimeric subunits (see legend). LHCB3 is only found in the M trimer and is the subunit facing CP24 (highlighted with a grey dashed line) (Caffarri *et al.*, 2009). (**b**) In Arabidopsis plants without or with low levels of LHCB3, as a result of knockout or exposure to high light, the PSII–LHCII supercomplexes lack the M trimers and the monomeric CP24 subunits (Caffarri *et al.*, 2009, Kouřil *et al.*, 2013, Wientjes *et al.*, 2013b). (**c**)

Under normal conditions, "extra" LHCII trimers, composed of LHCB1 and LHCB2, are associated with PSI; in response to stimuli, these "extra" trimers can move back and forward between PSII and PSI (Wientjes *et al.*, 2013a). In plants with *LHCB3* knocked out (ko*Lhcb3*), the rate of LHCII trimer movement from PSII to PSI is higher (Damkjær *et al.*, 2009). (**d**) In *Lhcb3* leaves, the level of LHCII trimer phosphorylation is higher (Damkjær *et al.*, 2009). In turn, LHCII phosphorylation and *LHCB* expression are correlated with each other, suggesting that they are co-regulated by the activation state of the LHCII kinase (Pursiheimo *et al.*, 2001). This suggests that LHCB3 might indirectly regulate *LHCB* expression.

$\mu mol.m^{-2}.s^{-1}$, but under ~200 $\mu mol.m^{-2}.s^{-1}$, anthocyanin content in *phyA* mutants was approximately 38% lower than wild type (Husaineid *et al.*, 2007); this suggests the existence of PHYA-dependent responses at high irradiances regardless of the reported induction of tomato PHYA degradation at low irradiance. Investigating an irradiance-dependent photoprotection of tomato PHYA is an exciting research line to follow.

Expression of *PhANGs* is light-induced via phytochrome and cryptochrome photoreceptors (McCormac & Terry, 2002, Ruckle *et al.*, 2007), which perceive blue, red and far-red light (Jiao *et al.*, 2007). When retrograde signals are triggered by chemical treatment, however, light signals are converted from positive to negative regulators of Arabidopsis *LHCB1*; interestingly, cryptochrome 1 and PHYB contribute to this *LHCB* repression, yet PHYA remains a positive regulator regardless of the state of the chloroplast (Ruckle *et al.*, 2007). This suggests a model where tomato PHYA and PHYB prevent and enhance, respectively, down-regulation of *PhANGs* in CL-exposed tomato. As discussed next, retrograde signals down-regulating *PhANGs* is a central concept in the proposed mechanism leading to CL-induced injury in tomato.

Model for the continuous-light induced injury in tomato

We propose that the CL-induced injury in tomato arises from retrograde signals halting chloroplast development that counteract signals derived from the cellular developmental program, which promote chloroplast development, resulting in the chlorotic phenotype. The first observation supporting this hypothesis is that only young tomato leaf tissue is sensitive to CL (Hillman, 1956, Withrow & Withrow, 1949). Extensive and elegant experiments by Hillman (1956) showed that when a healthy tomato plant is transferred to continuous light, the first leaves to show injury symptoms will show them at the leaf basis, and in progressively younger leaves the injury extends towards the tip. Likewise, when an injured plant is transferred back to a non-injurious photoperiod, the recovery follows a pattern opposite to injury; that is the leaf tip is injured and the leaf basis is green. Furthermore, in Arabidopsis developing leaves, cells at the tip are the first to cease dividing and start expanding, and this developmental front progresses down the leaf towards the base (Andriankaja *et al.*, 2012). Taking all these observations together, it can be deduced that leaf development in tomato follows a basipetal direction, and that the characteristic pattern of the CL -induced injury in tomato is the result of the developmental stage at which the cells were exposed to CL (Fig. 9.5a).

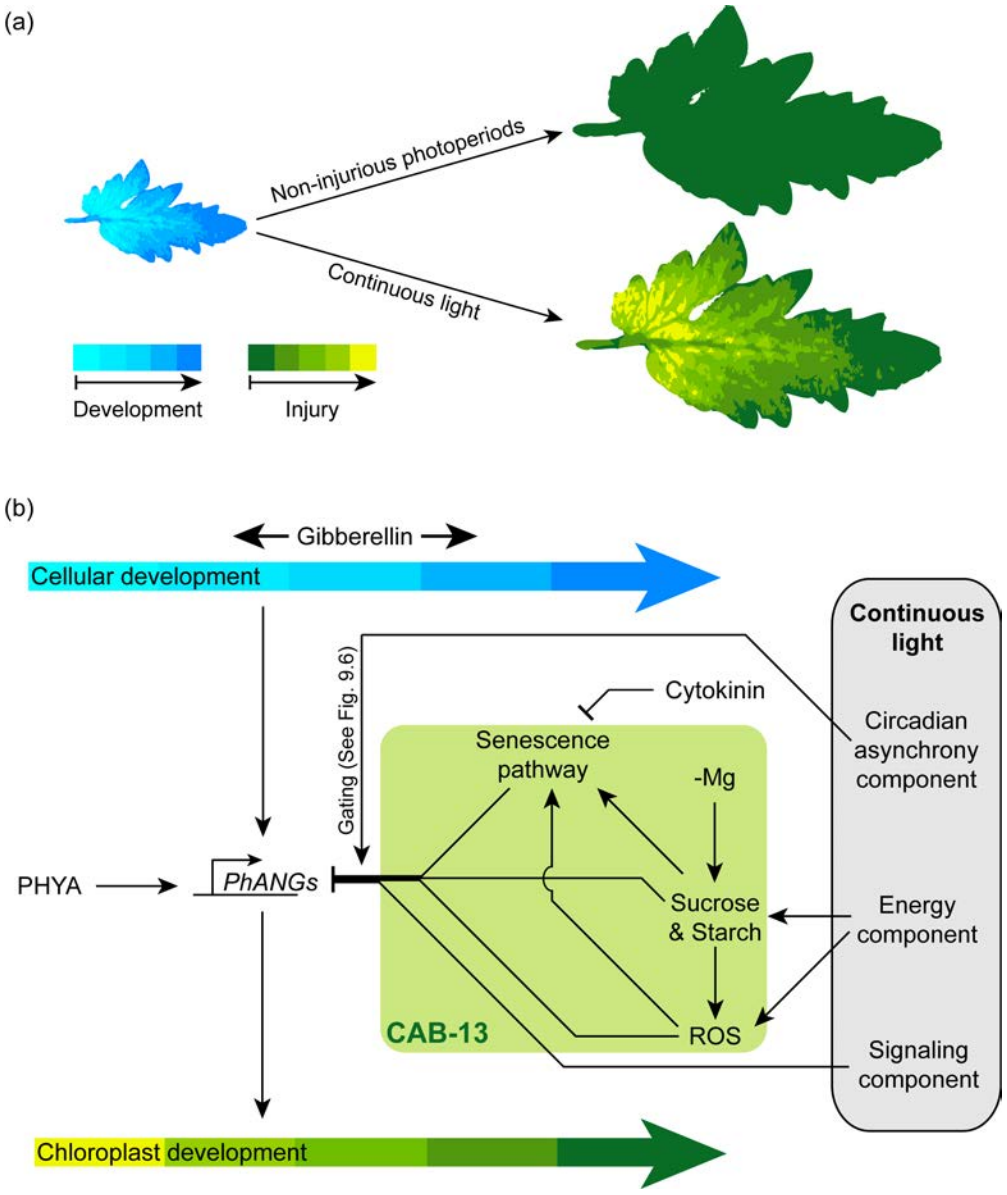

Figure 9.5 | Generic model for continuous-light-induced injury in tomato. Panel (**a**) illustrates how a young tomato leaf (left) develops into a healthy adult leaf or an injured leaf (right), depending on the light regime that young cells experienced before maturing. Considering that (i) in Arabidopsis developing leaves, cells at the tip are the first to cease dividing and start expanding, and this developmental front progresses down the leaf towards the base (Andriankaja *et al.*, 2012). Then, (ii) only young tomato leaves are sensitive to continuous light; (iii) when a healthy tomato plant is transferred to continuous light, the first leaves to show injury symptoms will show them at the leaf bases, and in progressively younger leaves the injury extends towards the tip. Furthermore, (iv) when

injured plants are transferred back to a non-injurious photoperiod, the recovery follows a pattern opposite to the injury one; that is the leaf tips are injured and the leaf bases are green (Hillman, 1956). All four observations together, it can be deduced that leaf development in tomato follows a basipetal direction (see scale in blue tones), and that the characteristic pattern of the continuous-light-induced injury (scale in green/yellow) in tomato is the result of the developmental stage at which the cells were exposed to continuous light. (**b**) Proposed underlying mechanism of continuous-light-induced injury in tomato. We propose that the continuous-light-induced injury in tomato arises from retrograde signals that counteract signals derived from the cellular developmental program that promote chloroplast development, like the expression of photosynthesis-associated nuclear genes (*PhANGs*). Once that the cellular developmental program progresses, chloroplast development cannot be completed, a phenomenon observed in Arabidopsis mutants or plants treated with chemicals that trigger such retrograde signaling, for reviews see (Jarvis & López-Juez, 2013, Pfannschmidt, 2010). Considering that gibberellin gradient concentrations define the boundaries between dividing and expanding cells in developing maize leaves (Nelissen *et al.*, 2012), the effect of gibberellin increasing the continuous-light-induced injury in tomato might be the result of altered cellular developmental program. In this model, a continuous light treatment is dissected into three components, see (Chapter 6); the circadian asynchrony component is the one triggering the injury (see Figure 9.6), and the energy and signaling components contribute to the injury development by enhancing retrograde signals as depicted here. The constant energy supply results in constant production of reactive oxygen species (ROS) and accumulation of sucrose and starch (Chapter 8). In turn, carbohydrate accumulation could cause over-reduction of electron acceptors, resulting in the electron transport chain donating electrons to O_2 generating ROS (Cakmak & Kirkby, 2008). ROS could down-regulate photosynthesis-associated genes (Moulin *et al.*, 2008) or induce programmed cell death (PCD) (Danon *et al.*, 2006, Kim *et al.*, 2008, Triantaphylidès & Havaux, 2009). As the cell death occurring in leaf senescence is a type of PCD (Lim *et al.*, 2007), we have connected ROS with the senescence pathway. In a wide range of situations carbohydrate accumulation is associated with down-regulation of photosynthesis (Baker & Braun, 2007, Baker & Braun, 2008, Cakmak & Kirkby, 2008, Krapp *et al.*, 1991, Stitt, 1991). Likewise, growing evidence supports an involvement of sugars in the initiation and/or acceleration of leaf senescence (Fischer, 2012, Lim *et al.*, 2007, Thomas, 2013, Wingler *et al.*, 2009). Leaf senescence is accompanied by decreased expression of photosynthesis genes (Lim *et al.*, 2007). Finally, considering that in dysfunctional chloroplasts, plastid signals convert light signalling pathways from positive to negative regulators of some *PhANGs* (Ruckle *et al.*, 2007), we propose that the signaling component of continuous light also contributes to the injury by interacting with retrograde signaling pathways. Cytokinin and PHYA protect tomato plants from the continuous-light-induced injury (Chapter 7, Chapter 8); the effect of cytokinin is most probably due to its ability to block tomato leaf senescence (Swartzberg *et al.*, 2006, Swartzberg *et al.*, 2011). Considering that in tomato, PHYA regulates CAB-1 expression (Peters *et al.*, 1998), it is reasonable to expect that PHYA also regulates the expression of other *PhANGs*. Finally, *CAB-13* is associated with continuous-light-tolerance in CLT tomato plants (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Considering that CLT tomatoes show a lower decrease in F_v/F_m in response to Mg deficiency than control A131 plants (data not shown) and Mg deficiency is associated with massive accumulation of carbohydrates (Cakmak & Kirkby, 2008, Cushman & Tibbitts, 1998), the protective effect of CAB-13 is probably downstream sucrose and starch accumulation. The green area highlights the potential site of CAB-13 action.

Studies on Arabidopsis offer clues to understand why developing tomato leaves are so sensitive to CL. When Arabidopsis seedlings are treated with plastid translation inhibitors or the carotenoid biosynthesis inhibitor norflurazon, both of which trigger retrograde signals, chloroplast development is impaired resulting in a chlorotic, pale phenotype. It is proposed that retrograde signals inform the nucleus about the progress of the plastid developmental program, but if mutations or chemical treatment interfere with

this coordination, the cellular developmental program progresses, resulting in incomplete plastid development (Jarvis & López-Juez, 2013, Pfannschmidt, 2010). — Notice that a plastid-derived haem signal reporting to the nucleus the chloroplast ability to receive photosynthetic gene products during plastid biogenesis has been described, yet the existence of a chloroplast development driver is still hypothetical; nonetheless, nuclear control of mitochondrial development in mammals suggests that this is feasible (Jarvis & López-Juez, 2013). — Therefore, we propose that something similar happens in CL-exposed tomato; that is, CL-induced retrograde signals counteract plastid development, including *PhAnGs* expression, as controlled by the cellular developmental program (Fig. 9.5b). This means that once chloroplast development is completed, CL would not be injurious to tomato plants any more, as is indeed observed. This hypothesis also offers clues to understand why gibberellin treatment increases the severity of the CL-induced injury in tomato (Kristoffersen, 1963). A gradient in the gibberellin concentrations defines the boundaries between dividing and expanding cells in developing maize leaves (Nelissen *et al.*, 2012); in Arabidopsis, additionally, this boundary between dividing and expanding cells coincides sharply with chloroplast differentiation and photosynthetic gene expression (Jarvis & López-Juez, 2013). Hence, the increase by gibberellin of CL-induced injury might be the result of an altered cellular developmental program.

An important aspect of the proposed model is how CL triggers a retrograde signal in the first place. In this thesis, we dissected a CL treatment into several factors and tested them for their involvement in triggering the injury (Chapter 6). These factors include continuous light signaling, continuous energy supply and circadian asynchrony. We show evidence that circadian asynchrony is the only factor present in all injurious, abnormal light/dark cycles, making it the likely factor that triggers CL-induced injury (see further discussion below). However, evidence presented here (Chapter 6, Chapter 7, Chapter 8) and elsewhere (Arthur, 1936, Demers & Gosselin, 2002, Globig *et al.*, 1997, Murage *et al.*, 1997) indicates that the other two factors implicated in CL, continuous energy and signaling, can influence the injury severity.

Figure 9.5b depicts the proposed model that links photosynthetic down-regulation with the CL energy and signaling components. The constant energy supply results in constant production of reactive oxygen species (ROS); likewise, constant energy for photosynthesis results in accumulation of sucrose and starch (Chapter 8). In turn, carbohydrate accumulation could cause over-reduction of electron acceptors, resulting in the electron transport chain donating electrons to O_2 generating ROS (Cakmak & Kirkby, 2008). ROS could down-regulate photosynthesis-associated genes (Moulin *et al.*, 2008) or induce programmed cell death (PCD) (Danon *et al.*, 2006, Kim *et al.*, 2008, Triantaphylidès & Havaux, 2009). As the cell death occurring in leaf senescence is a type of PCD (Lim *et al.*, 2007), we have connected ROS with the senescence pathway. In a wide range of situations carbohydrate accumulation is associated with down-regulation of photosynthesis (Baker & Braun, 2007, Baker & Braun, 2008, Cakmak & Kirkby, 2008, Krapp *et al.*, 1991, Stitt, 1991). Likewise, growing evidence supports an involvement of sugars in the initiation and/or acceleration of leaf senescence (Fischer, 2012, Lim *et al.*,

2007, Thomas, 2013, Wingler *et al.*, 2009). Leaf senescence is accompanied by decreased expression of photosynthesis genes (Lim *et al.*, 2007). The positive effect of cytokinin in preventing the CL-induced injury (Chapter 8) is most probably due to its ability to block tomato leaf senescence (Swartzberg *et al.*, 2006, Swartzberg *et al.*, 2011).

In my thesis, I also present compelling evidence that phytochrome signaling influences the injury severity in CL-exposed tomato plants (Chapter 7). Involvement of other photoreceptors, like phototropins or crytochromes is also plausible. In dysfunctional chloroplasts, plastid signals convert light signalling pathways from positive to negative regulators of some *PhANGs* (Ruckle *et al.*, 2007); as discussed earlier, cryptochrome 1 and type B phytochromes are responsible for this light-dependent repression of *PhANGs*. Interestingly, tomato PHYB1 over-expression (Chapter 7) and lamps with a higher percentage of blue light increase injury severity. Furthermore, in Arabidopsis, single high-fluence pulses of blue light destabilized *LHCB* transcripts in a phototropin-dependent manner (Folta & Kaufman, 2003). Considering that in tomato, PHYA regulates CAB-1 expression (Peters *et al.*, 1998), it is reasonable to expect that PHYA also regulates the expression of other *PhANGs*. Hence, we propose that, depending on the photoreceptor and chloroplast status, the signaling component of CL increases or decreases the injury severity by interacting with retrograde signaling pathways.

As discussed in the previous section and in Fig. 9.4, CAB-13 might influence retrograde signaling, which would explain how the wild tomato species allele of *CAB-13* confers CL-tolerance (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Interestingly, CLT tomato plants show a lower decrease in F_v/F_m in response to Mg deficiency than control A131 plants (data not shown); this suggests that CL-tolerant tomatoes are also tolerant to Mg deficiency. Interestingly, tomato leaves damaged by CL or by Mg deficiency look remarkably similar. Hence, testing CL-tolerant lines with a smaller introgression than CLT tomatoes is most interesting as it would confirm that the same locus confers tolerance to both CL and Mg deficiency. For now, considering that Mg deficiency is associated with massive accumulation of carbohydrates (Cakmak & Kirkby, 2008), the protective effect of CAB-13 might be hypothetically placed downstream of sucrose and starch accumulation (Fig. 9.5b).

This hypothetic model, so far, integrates two CL components — light as energy and signal. We propose that the third, and probably most important, element for triggering the CL-induced injury in tomato is circadian asynchrony. The experimental results presented here, show that no matter whether the light quality or intensity is constant or presented in a diurnal cycle, mottled chlorosis always appeared in young CL-sensitive tomato leaves if plants were exposed to a light regime that implied circadian asynchrony (Chapter 6). Tomato *LHCB* expression is under circadian control, being up-regulated during day and down-regulated at night (Giuliano *et al.*, 1988). As discussed earlier, light signals can up- and down-regulate LHCB expression, depending on the chloroplast status (Larkin & Ruckle, 2008, Ruckle *et al.*, 2007). Under non-injurious photoperiods, it is expected that the circadian down-regulation of LHCB, the absence of light and the inactivity of chloroplasts match with each other. Under CL or abnormal light / dark cycles,

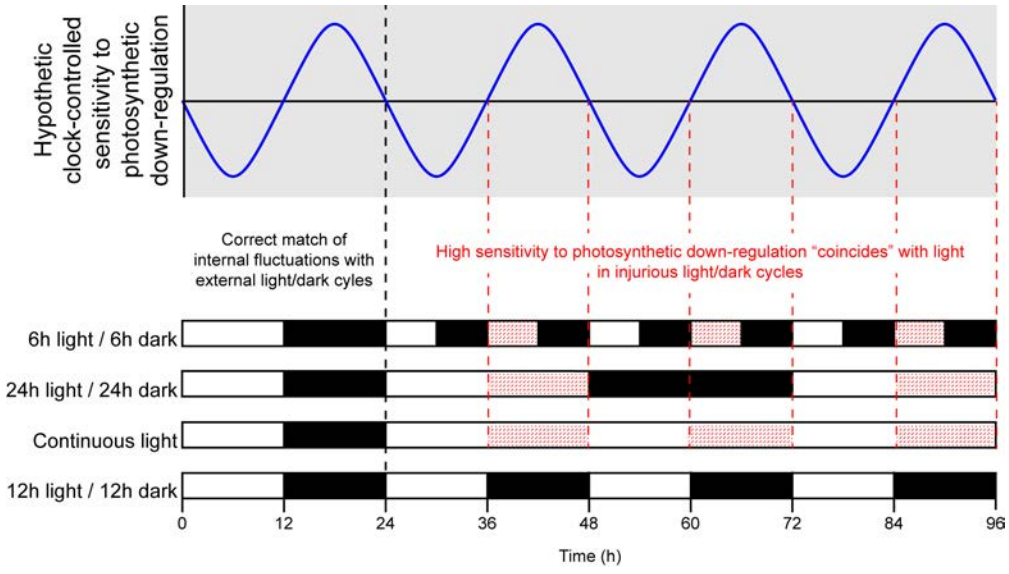

Figure 9.6 | Circadian asynchrony in the continuous-light-induced injury in tomato. Abnormal light/dark cycles (e.g. 6h light / 6h dark or 24h light / 24h dark) induce a similar injury as observed in plants exposed to continuous light (Chapter 6, Highkin & Hanson, 1954, Hillman, 1956). Tomato *LHCB* expression is under circadian control, being up-regulated during day and down-regulated at night (Giuliano *et al.*, 1988), and retrograde signals heavily interact with the light-signaling network in Arabidopsis (Lepistö & Rintamäki, 2012, Ruckle *et al.*, 2012, Ruckle *et al.*, 2007); hence, it is reasonable to suspect that light signals during the subjective night could enhance down-regulation of the expression of *PhANGs*. We propose that an asynchrony between a clock-gated sensitivity to photosynthetic down-regulation (blue line) and the light period is responsible for the continuous-light-induced injury in tomato. In this model, therefore, under injurious photoperiods plants receive light signals at incorrect times resulting in enhancement of photosynthetic down-regulation (red-shaded areas).

however, conflictive light, retrograde and circadian signals may arise. For instance, during a subjective night, the circadian clock is signaling "night", but the light and the chloroplast status would be signaling "day". Considering that retrograde signals heavily interact with the light-signaling network in Arabidopsis (Lepistö & Rintamäki, 2012, Ruckle *et al.*, 2012, Ruckle *et al.*, 2007), it is reasonable to suspect that light signals during the subjective night could enhance the clock-controlled down-regulation of *PhANGs* (Fig. 9.6). We propose that an asynchrony between a clock-gated sensitivity to photosynthetic down-regulation and the light period is responsible for the CL-induced injury in tomato. In this model, therefore, under injurious photoperiods plants receive light signals at incorrect times resulting in enhancement of photosynthetic down-regulation.

Practical implications for greenhouse horticulture

In addition to the physiological insights into the CL-induced injury and the genetic basis of CL-tolerance, this thesis also explores a practical application of CL-tolerance in greenhouse horticulture. The trait was bred into modern F_1 hybrid lines, and a yield trial was performed (Velez-Ramirez *et al.*, 2014 (Chapter 4)). Multiple lines were used in order to learn how the trait behaves under real production conditions. Lines segregating as CL-tolerant or –sensitive and homozygous or heterozygous were obtained in to two genetic backgrounds; this resulted in 9 and 3 lines in the Idooll and Westland backgrounds, respectively. All CL-sensitive lines yielded the same or less when exposed to CL in comparison to 16-h photoperiod. Likewise, all CL-tolerant lines yielded the same or more under CL. The lines that yielded the most (21.4 $kg.m^2$) and showed the highest CL-driven yield increase (20%) were in the Idooll background (Velez-Ramirez *et al.*, 2014 (Chapter 4)), which is in agreement with the yield increase predicted by the crop model (Velez-Ramirez *et al.*, 2012 (Chapter 3)). Considering that some variation from plant to plant was observed, further breeding is advised to obtain a line suitable for performing further studies.

For future studies, it is not only important to evaluate the CL-driven yield increase but most importantly to understand why CL increases yield. The results presented here suggest that "night" photosynthesis and the possibility to use a higher crop density are accountable for the observed yield increase (Velez-Ramirez *et al.*, 2014 (Chapter 4)); however, it is not possible to accurately quantify, in this study, the effect of these two factors nor others that could have an impact on yield, like light interception, assimilate partitioning and respiration. Therefore, future crop ecology studies on this matter should use a homogeneous canopy and a bigger cultivated area in order to accurately estimate the effect of CL on these parameters and their contribution to the yield increase. Regardless of the crop density, light interception by the CL-cultivated and control crops should be as equal as possible in order to facilitate comparisons. It also would facilitate the management of a CL-grown tomato crop as it helps to balance the increased assimilate availability provided by CL. This is because increasing crop density, when light interception is high, increases the number of flowers and fruits while keeping almost constant the total light interception by the crop and, consequently, assimilate availability. Previous studies (Demers *et al.*, 1998) and our experience in the yield trial suggest that CL increases source strength but not sink strength. For successfully cultivating a CL-tolerant crop under CL, therefore, it is of great importance to (i) start the CL treatment gradually and only when the crop becomes generative, (ii) increase crop density and (iii) weekly adjust day and night temperature as needed.

Use of CL in the greenhouse industry should also consider light pollution and the energy use efficiency of the new cultivating system. Regarding light pollution, the yield trial performed in this study complied with Dutch regulations (Minister van Volkshuisvesting Ruimtelijke Ordening en Milieubeheer, 2002), which require screens to reduce light emission from the greenhouse facades to the environment between 20:00 and 24:00 hours. As discussed in (Velez-Ramirez *et al.*, 2012 (Chapter 3)), the energy put into the lighting system also heats the greenhouse, reducing almost to the same extent the energy that the

heating system must deliver. This should result in similar energy requirements whether CL or a 16-h photoperiod is used in the greenhouse. Unfortunately, the configuration of the greenhouse compartments used in this study did not allow making proper comparisons in terms of energy use. Furthermore, ventilating the water vapor transpired by the crop during the subjective night is expected to be a considerable latent heat loss. For future trials, therefore, recording energy use and crop transpiration along with yield is most valuable as it would allow calculating energy use efficiency of the present and CL systems.

Perspectives

A major future challenge is to better understand how plastid development and activity is coordinated with the expression of *PhANGs* in the nucleus. This not only is a promising research line to better understand CL-induced injury in tomato but also is interesting in its own right. Although this thesis provides compelling evidence that tomato CAB-13 confers CL-tolerance (Velez-Ramirez *et al.*, 2014 (Chapter 4)), the exact mechanism underlying this is still missing. From the model presented in this chapter it can be inferred that plastid translation inhibitors, like lincomicin or chloramphenicol, should elicit injuries similar to the CL-induced injuries in CL-sensitive plants but not in CL-tolerant tomatoes. Furthermore, evaluating the composition, structure and efficiency of tomato LHCII as affected by CL, while keeping *PhANGs* expression monitored, should provide valuable information on how photosynthesis is regulated to adjust to the environment.

For future studies in light signaling, understanding the role of PHYA in conferring tolerance to CL is most interesting. An important question in this research line is: If light induces PHYA degradation, how can it be explained that tomato lines over-expressing PHYA are tolerant to CL? I suggest that the irradiance-dependent photo protection of Arabidopsis PHYA (Franklin *et al.*, 2007) also exists in tomato. The puzzling effect of diverse light treatments on the CL-induced injury severity (Chapter 7) is most probably the result of the complex light signaling networks in plants. Evaluating the contribution of cryptochromes and studying the expression pattern of tomato phytochromes during leaf development could shed light on the effect of type B phytochromes on the CL-induced injury. Expression of *PHYB1* and *PHYB2* under the control of their own promoters or the CMV promoter used in the transgenic over-expressing lines is most probably different during the CL-sensitive leaf developmental stage, and it can potentially identify the true role of PHYB1 and PHYB2 on the CL-induced injury in tomato.

From a crop ecology perspective, the outcome of this thesis leaves an important issue to be answered: From the observed 20% yield increase, how much is attributable to "night" photosynthesis, increased crop density, altered canopy structure, etc.? Answering this question would result in better implementation of the trait in practice. For the greenhouse industry, this implementation is promising. Despite the success in cultivating a tomato crop under CL for the complete season, the use of this trait offers more possibilities. In principle, CL-tolerance offers the possibility of cultivating a tomato crop anywhere between 16-h photoperiod and CL. This offers an additional management option for growers as the photoperiod can be increased to, for example, 20h of light per day during

cloudy winter periods, and be returned to a 16-h or 18-h photoperiod when and if needed. If temperature and truss load are correctly managed, it is expected that photoperiod can be adjusted in response to periods with lower or higher solar radiation, outside temperature as well as energy and product prices. Another promising application of CL is in the production of young tomato plants rather than tomato production in adult crops. That is, growing young CL-tolerant tomato plants during their first weeks of life under CL should result not only in faster production but also provides an additional management option to speed up the growth of small plants in order to obtain, at the end, homogeneous plants. In conclusion, CL-tolerance has the potential of radically changing the way greenhouse tomatoes are cultivated during winter.

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Summary

Light essentially sustains all life on planet earth surface. Plants transform light energy into chemical energy through photosynthesis. Hence, it can be anticipated that extending the daily photoperiod, using artificial light, results in increased plant productivity. Although this premise is true for many plant species, a limit exists. For instance, the seminal work of Arthur *et al.* (1930) showed that tomato plants develop leaf injuries if exposed to continuous light (CL). Many studies have investigated the physiological mechanism inducing such CL-induced injury. Although important and valuable discoveries were done over the decades, by the time the present project started, a detailed and proven physiological explanation of this disorder was still missing. Here, I present the results of a 5-year effort to better understand the physiological basis of the CL-induced injury in tomato and develop the tools (genetic and conceptual) to cultivate tomatoes under CL.

After an exhaustive literature search, it was found that Daskaloff and Ognjanova (1965) reported that wild tomato species are tolerant to CL. Unfortunately, this important finding was ignored by numerous studies done after its publication. Here, we used the CL-tolerance found in wild tomatoes as a fundamental resource. Hence, the specific objectives of this thesis were to (i) better understand the physiological basis of the CL-induced injuries in tomato, (ii) identify the gene(s) responsible for CL-tolerance in wild tomato species, (iii) breed a CL-tolerant tomato line and (iv) use it to cultivate a greenhouse tomato crop under CL.

Chapter 1 describes how innovation efforts encountered the unsolved scientific enigma of the injuries that tomato plants develop when exposed to CL. The term *CL-induced injury* is defined, and a detailed description of the symptoms observed in this disorder is shown. Additionally, an overview of the most important studies, influencing the hypotheses postulated and/or tested in this dissertation, is presented. Finally, a description and motivation of the main questions that this dissertation pursued to answer is presented alongside a short description of the strategy chosen to answer them.

Chapter 2 reviews the literature, published over the last 80 years, on CL-induced injury using modern knowledge of plant physiology. By doing so, new hypotheses aiming to explain this disorder are postulated in addition to the ones collected from literature. Additionally, we highlight that CL is an essential tool for understanding the plant circadian clock, but using CL in research has its challenges. For instance, most of the circadian-clock-oriented experiments are performed under CL; consequently, interactions between the circadian clock and the light signalling pathway are overlooked.

Chapter 3 explores the benefits and challenges of cultivating CL-tolerant tomato under CL. Considering that current commercial tomato varieties need six hours of darkness per day for optimal growth, photosynthesis does not take place during a quarter of the day.

Hence, if tomatoes could be grown under CL, a substantial increase in production is anticipated. A simulation study is presented, which shows that if an ideal continuous-light-tolerant tomato genotype is used and no crop adaptations to CL are assumed, greenhouse tomato production could be 26% higher when supplementing light to 24 h day^{-1} in comparison with a photoperiod (including supplementary lighting) of only 18 h day^{-1}. In addition, the expected changes in greenhouse energy budgets and alterations in crop physiological responses that might arise from cultivating tomatoes under continuous light are discussed.

Chapter 4 maps the locus conferring CL-tolerance in wild tomatoes to chromosome seven, and shows that its introgression into modern tomato cultivars enhances yield by 20%, when grown under CL. In addition, genetic evidence, RNAseq data, silencing experiments and sequence analysis all point to the type III Light-Harvesting Chlorophyll a/b Binding protein 13 (CAB-13) gene as a major factor responsible for the tolerance. In *Arabidopsis thaliana* this protein is thought to have a regulatory role in balancing light harvesting by photosystems I and II. The likely mechanisms that link CAB-13 with CL-tolerance are discussed.

Chapter 5 investigates from which part of the plant CL-tolerance originates and whether this trait acts systemically. By exposing grafted plants bearing both tolerant and sensitive shoots to CL, the trait was functionally located to the shoot rather than the roots. Additionally, an increase in continuous-light tolerance was observed in sensitive plants when a continuous-light-tolerant shoot was grafted on it. Our results show that in order to increase yield in greenhouse tomato production by using CL, the trait should be bred into scion rather than rootstock lines.

Chapter 6 discusses the factors that differ between injurious and non-injurious light regimes. Each of these factors may potentially be responsible for triggering the injury in CL-grown tomato and was experimentally tested here. In short, these factors include (i) differences in the light spectral distribution between sunlight and artificial light, (ii) continuous signalling to the photoreceptors, (iii) constant supply of light for photosynthesis, (iv) constant photo-oxidative pressure, and (v) circadian asynchrony — a mismatch between the internal circadian clock frequency and the external light/dark cycles. The evidence presented here suggests that the continuous-light-induced injury does not result from the unnatural spectral distribution of artificial light or the continuity of the light per se. Instead, circadian asynchrony seems to be the factor inducing the injury. As the discovered diurnal fluctuations in photoinhibition sensitivity of tomato seedlings are not under circadian control, it seems that circadian asynchrony does not directly induce injury via photoinhibition as it has been proposed.

Chapter 7 investigates a possible role for phytochromes (PHY) in CL-induced injury in tomato. Mutant and transgenic tomato plants lacking or over-expressing phytochromes were exposed to CL, with and without far-red light enrichment, to test the role of individual phytochromes on the induction and/or prevention of injury. *PHYA* over-expression confers complete tolerance to CL regardless the light spectrum. Under CL with low far-red content, PHYB1 and PHYB2 diminished and enhanced the injury, respectively,

yet the effects were small. These results confirm that phytochrome signaling networks are involved in the injury induction under CL. The link between CAB-13 and PHYA is discussed.

Chapter 8 investigates the role of carbohydrate accumulation in the induction of CL-induced injury in tomato by using untargeted metabolomics and transcriptomics data. These data reveal a clear effect of CL on sugar metabolism and photosynthesis. A strong negative correlation between sucrose and starch with the maximum quantum efficiency of photosystem II (Fv /Fm) was found across several abnormal light/dark cycles, supporting the hypothesis that carbohydrates play an important role in CL-induced injury. I suggest that CL-induced injury in tomato is caused by a photosynthetic down-regulation showing characteristics of both cytokinin-regulated senescence and light-modulated retrograde signaling. Molecular mechanisms linking carbohydrate accumulation with photosynthetic down-regulation are discussed.

Chapter 9 provides a synthesis of the most important findings and proposes a generic model of CL-induced injury in tomato. I propose that CL-induced injury in tomato arises from retrograde signals that counteract signals derived from the cellular developmental program that promote chloroplast development, such that chloroplast development cannot be completed, resulting in the chlorotic phenotype. Finally, perspectives on what future directions to take to further elucidate the physiological basis of this trait and successfully implement it in greenhouses are presented.

Samenvatting

Vrijwel al het leven op aarde is afhankelijk van licht. Via fotosynthese zijn planten in staat om de energie uit licht om te zetten in chemische energie. Wanneer de dagelijkse lichtperiode door middel van kunstmatig licht verlengd zou worden, valt een toename in de groei van planten te verwachten. Hoewel dit voor veel plantensoorten het geval is, zijn er ook beperkingen. Uit het werk van Arthur *et al.* (1930) blijkt dat bij tomatenplanten bladbeschadigingen optreden onder continue licht (CL). Er is veel onderzoek gedaan naar mogelijke fysiologische verklaringen voor deze CL-geïnduceerde schade. Ondanks alle eerdere inspanningen ontbrak er echter bij de start van dit project nog steeds een goede gedetailleerde en fysiologische verklaring voor het ontstaan van die schade. In dit proefschrift rapporteer ik de resultaten van 5 jaar onderzoek naar de fysiologische achtergrond van CL-geïnduceerde schade bij tomaat en naar manieren (genetisch en conceptueel) om tomaten toch onder CL te kunnen produceren.

Een uitgebreide literatuurstudie leverde op dat Daskaloff en Ognjanova al in 1965 hadden beschreven dat wilde tomatensoorten wel bestand zijn tegen CL. In vervolgstudies zijn deze resultaten helaas niet opgepikt en verder gebruikt. Voor het onderzoek in dit proefschrift is deze CL-tolerantie van wilde tomatensoorten een belangrijk startpunt geweest bij het behalen van de volgende doelstellingen: 1. een beter begrip van de fysiologische achtergrond van CL-schade bij tomaten; 2. het vinden van genen die betrokken zijn bij CL-tolerantie; 3. het creëren van een CL-tolerant tomatenras, en 4. het produceren van tomaten met dit ras in de kas onder CL.

In **hoofdstuk 1** wordt de term *CL-geïnduceerde schade* gedefinieerd en een gedetailleerde beschrijving van de symptomen gegeven. Vervolgens wordt een overzicht gegeven van de belangrijkste studies die ten grondslag liggen aan het huidige onderzoek en de hypotheses die zijn opgesteld en getest in dit proefschrift. Tenslotte worden de belangrijkste onderzoeksvragen vermeld, de reden waarom deze zijn gekozen, en wordt een korte beschrijving gegeven van de gevolgde strategie om deze vragen te beantwoorden.

Hoofdstuk 2 geeft een overzicht van de resultaten op dit gebied zoals die zijn gepubliceerd gedurende de laatste 80 jaar, opnieuw geïnterpreteerd vanuit moderne fysiologische inzichten. Naast oude hypotheses worden nieuwe opgesteld om de CL-geïnduceerde afwijkingen te verklaren. Daarnaast wordt beargumenteerd dat hoewel CL een belangrijke manier is om onderzoek te doen aan de circadiaanse klok in planten deze benadering ook zijn beperkingen kent: in het meeste onderzoek aan de circadiaanse klok wordt gebruik gemaakt van experimenten onder CL en worden interacties tussen de circadiaanse klok en andere lichtsignalering dus over het hoofd gezien.

In **hoofdstuk 3** worden de voor- en nadelen van het kweken van tomaten onder CL besproken. Voor optimale groei hebben de huidige commerciële tomatenrassen zes uur duisternis per dag nodig, en dus kan er gedurende een kwart van de dag geen fotosynthese

plaatsvinden. Wanneer tomaten onder CL geproduceerd kunnen worden, valt een substantiële toename in de productie te verwachten. Uit een simulatiemodel blijkt dat een ideaal CL-tolerant tomatenras een 26% hogere opbrengst zou kunnen hebben wanneer het wordt geteeld onder CL in plaats van 18 uur licht per dag. De verwachte gevolgen voor de energiehuishouding van de kas worden bediscussieerd en ook de mogelijk fysiologische aanpassingen van het gewas aan de veranderde omstandigheden.

Hoofdstuk 4 beschrijft dat CL-tolerantie in wilde tomaten is gelokaliseerd op chromosoom 7. Als deze eigenschap wordt ingekruist in een commercieel ras, neemt de opbrengst onder CL met 20% toe. Op basis van genetisch onderzoek, RNA-sequencing, gene-silencing en vergelijking van DNA sequenties, wordt geconcludeerd dat het gen CAB-13 (type III light-harvesting Chlorophyll a/b binding protein 13) een cruciale rol speelt in de CL-tolerantie. In Arabidopsis is aangetoond dat dit eiwit een rol speelt in de verdeling van de lichtopvang tussen fotosysteem I en II. Het mogelijke mechanisme van de werking van CAB-13 bij CL-tolerantie wordt besproken.

In **hoofdstuk 5** wordt onderzocht in welk deel van de plant CL-tolerantie een rol speelt, door het combineren van CL-tolerante en -gevoelige scheuten door middel van enten. Het blijkt dat CL-tolerantie in de scheut is gelokaliseerd, en niet in de wortel. Ook werd een toename in CL-tolerantie van een gevoelige scheut gevonden, als er een CL-tolerante scheut op de zelfde plant werd geënt. Hieruit wordt geconcludeerd dat voor commerciële productie onder CL de veredeling zich moet richten op de ent, en niet op de onderstam.

Hoofdstuk 6 beschrijft de factoren die verschillen tussen lichtcondities die wel of geen schade opleveren. Voor al deze factoren werd nagegaan of ze een rol spelen bij de CL-geïnduceerde schade. De onderzochte factoren zijn: 1. verschillen in spectrale verdeling tussen zonlicht en kunstmatig licht; 2. continue lichtsignalering via fotoreceptoren; 3. continue licht-energie beschikbaarheid voor fotosynthese; 4. een constante foto-oxidatieve druk; 5. circadiane asynchroniciteit - een ongelijkheid tussen de interne circadiaanse klok van de plant en de externe dag-nacht cyclus. Deze laatste factor blijkt de belangrijkste rol te spelen bij het veroorzaken van de schade. Ook blijkt de dagelijkse fluctuatie in gevoeligheid voor foto-inhibitie in tomatenkiemplanten niet gestuurd te worden door de circadiaanse klok. Dit speelt dus geen directe rol bij het ontstaan van schade, zoals eerder wel werd gesuggereerd.

In **hoofdstuk 7** wordt de mogelijke rol van fytochromen bestudeerd. Mutanten en transgene lijnen, met hogere of lagere expressie van fytochromen, werden blootgesteld aan CL, met en zonder extra verrood licht, om de rol van fytochromen te testen. Lijnen met over-expressie van fytochroom A blijken volledig CL-tolerant te zijn, onafhankelijk van het lichtspectrum. Bij CL met lage intensiteit verrood, hebben fytochroom B1 en B2 een klein, en tegengesteld effect. Deze resultaten bevestigen dat fytochromen een rol spelen bij het optreden van schade onder CL. De relatie tussen CAB-13 en fytochromen wordt bediscussieerd.

In **hoofdstuk 8** wordt ingegaan op de mogelijke rol van de ophoping van koolhydraten bij het ontstaan van CL-geïnduceerde schade. Hierbij wordt gebruik gemaakt van metabolomics en transcriptomics. De resultaten laten zien dat er een duidelijk effect is

van CL op het suiker metabolisme en op de fotosynthese. Een sterke negatieve correlatie tussen suiker- en zetmeelophoping enerzijds, en de maximale quantum-efficiëntie van fotosysteem II (Fv /Fm) anderzijds, duiden er op dat het koolhydraatmetabolisme een rol speelt bij het ontstaan van schade. Deze correlatie werd onder verschillende licht/donker cycli waargenomen. Ik veronderstel dat CL-geïnduceerde schade in tomaten veroorzaakt wordt door verlaging van de fotosynthese, enerzijds als gevolg van cytokinine-gereguleerde veroudering, anderzijds door licht-gemoduleerde retrograde signalering. De moleculaire mechanismes die een schakel vormen tussen koolhydraatmetabolisme en verlaging van de fotosynthese worden besproken.

Hoofdstuk 9 geeft een synthese van de belangrijkste resultaten van dit onderzoek en beschrijft een algemeen model ter verklaring van CL-geïnduceerde schade bij tomaat, waarin ik voorstel dat CL-schade wordt veroorzaakt door retrograde signalen die tegengesteld werken aan signalen voor de cellulaire ontwikkeling, waardoor de chloroplast ontwikkeling wordt verstoord. Dit resulteert in de zichtbare vergeling van het blad. Tenslotte wordt besproken wat de volgende stappen in het onderzoek naar de fysiologische achtergrond van CL-geïnduceerde schade zou kunnen zijn, en hoe de resultaten van het onderzoek gebruikt kunnen worden bij de teelt van tomaten in kassen onder CL.

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I am also in debt with my friends, colleagues and PhD fellows at the Horticulture and Product Physiology Group as well as at the Laboratory of Plant Physiology. Elias Kaiser, thanks for everything Mr! For the friendship, the technical support, the company inside and outside the lab, the discussions and, specially, for your relaxed and unique way of enjoying live. Mr Dimitrios Fanourakis thanks for your friendship, the time spent inside and outside Radix as well as for the scientific and non-scientific advice that made me understand so many things. To Bas Dekkers, thanks for the time spent on climbing, biking or just chatting and for being a living example of hard work and perseverance. Robert Okello, thanks for the long-lasting friendship and for being such a great listener, which is an outstanding achievement when I am the speaker. Izabela Witkowska, thanks for the company during early morning hours and for your expert advice on non-scientific issues. Sander Hogewoning, thanks for all the scientific and technical advice as well as for your pleasant company. Dalia Carvalho, thanks for your great friendship and for sharing with me your enthusiasm for life. Nikos, Didi, Tao, Vaia, Brian, Sassan, Elisa, Aina, Pavlos, Rene

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To all the people that I met at Monsanto and GreenQ, thanks for making this project not only successful but also enjoyable. Rolf, Pieter, Jeroen, Toon, Arjan, Peter, Piet Hein and Len thanks for your effort, ideas and input. To Harm Schreuders, I am very grateful for your invaluable help in the most tedious tasks that we needed to tackle, and for your unbreakable positive mood while getting the job done.

A special thanks goes to William Mensink. Mr, thanks for offering me your help in the lab, and far more importantly, for your friendship in the world beyond the lab. Thanks for the great time over the years in Bergschenhoek, Utrecht, Wageningen and Rotterdam.

I extend my gratitude to all those great persons that gave me their friendship and company outside work. I owe much more than my gratitude to Arturo Menchaca, Iemke Bisshops and Iliana Hidalgo. You helped me when I most needed it, and from there, extended your unconditional friendship over the years. You three made me feel that I was never alone in this lovely country that you helped me to understand and appreciate, mil gracias a los tres. Arturo and Iemke, thanks for being a living example of modesty and for letting me being a guest in your lovely home, life and family. Iliana, thanks for offering me your friendship, help, company and so many other things during all these years, thanks! Dear Niovi, I did not forget you. Thanks a lot for your friendship all these years and for reminding me in countless occasions of what is really important.

To the salsa team, Melina, Gabriela, Alexis and Andries, thanks for all the great moments on the dancing floor and any other random place. I truly enjoyed your company and complicity in countless dinners, parties and any other gathering that you can imagine, thanks. To the Mexican crew, Diana, Edgar, Andrea, Oswaldo, Miriam, Jennifer, Diego, Annie, Alvinn, Omar, Adriana, Martha, Daylan, Yadira, Victor, Vicente, Armando and Sugued, thanks for making me feel that our homeland came within us to Wageningen and not letting me forget what a great country Mexico is. To the Antwerp group, Gerardo, Ana Laura, Dirk, Teresita and Estela, thanks for welcoming me in a new foreign country and for your friendship during the most difficult part of this endeavor — the infamous writing period.

To the countless people that I met in Wageningen, thanks for making this little, lovely city so unique, so international and diverse, so Wageningen.

Como diría un amigo: “para el amigo mas cercano que vive mas lejos”. Don Polo, no tengo palabras para agradecer todo lo que me has dado, todo lo que me has hecho crecer vamos. Te respeto, escucho y admiro profundamente. A pesar de tu reticencia por la tecnología, mil gracias por haber mantenido el contacto y la amistad a distancia por ya tanto

tiempo que vivimos en lados opuestos del charco. Tal vez, cuando crezca, llegue a ser como tu — solo tal vez. Además, gracias por la espectacular foto de la portada!

A la fundación Velez-Ramirez — integrada por Aidé, Aarón, Daniel y Sarai — gracias por ser la familia mas maravillosa que uno pueda imaginar. A pesar de la distancia, agradezco de todo corazón los pocos, pero valiosísimos momentos que pasamos juntos a ambos lados del Atlántico y por los muchos momentos en los que la tecnología nos mantuvo unidos como familia. No puedo estar mas orgulloso de mi apellido y eso se debe a que me permite compararme con tan grandes seres humanos como lo son ustedes. Finalmente, y no por eso menos importante, quisiera extender el mas amoroso de los agradecimientos a esa gran persona que llego a mi vida a ponerla de cabeza, a llenarla de luz y felicidad, a abrirme los ojos a todo un mundo de nuevas aventuras y posibilidades. Ilane, gracias por todo el amor y apoyo incondicional durante la etapa mas difícil de este proyecto y por ayudarme a darme cuenta de lo importante que era abandonar mi burbuja de confort en Wageningen y hacer ese viaje a Amberes que significa para nosotros mucho mas que un mero viaje en tren. De aquí a lo que nos depare el futuro, solo puedo estar feliz y listo para enfrentar lo que venga con una sonrisa.

Curriculum vitae

Aaron I. Velez Ramirez was born on April 29th 1985 in Mexico City, Mexico. From an early age he showed a profound interest for nature and plants. During his high school years, he had the great opportunity to participate in the program "Youth towards Research", which allowed him to do an internship at a microbiology laboratory of the National Autonomous University of Mexico (UNAM). This experience pushed him to purse a scientific career; after completing high school in 2002, therefore, he studied Biology at the Iztacala Faculty of Higher Studies of the same university. During his BSc. studies he did several internships in research laboratories at the UNAM and at the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN). He graduated with honors as first in his class in 2007; for this achievement, he was granted the Gabino Barreda Medal in University Merit. In the same year, the Mexican National Council of Science and Technology (CONACyT) granted him a postgraduate study scholarship to follow a MSc. in Plant Sciences at Wageningen University in The Netherlands. During his MSc. thesis he researched the malfunctioning of stomata after exposure to high relative humidity under the supervision of Dr Uulke van Meeteren and Dr Jeremy Harbinson. By encouragement of Dr van Meeteren and Dr Wim van Ieperen, he pursued a PhD degree at the same laboratory. To finish his MSc. studies, he first did an internship at Monsanto Vegetable Seeds at Bergschenhoek, The Netherlands. During this period, under the supervision of Dr Frank F. Millenaar, he started working on the project that resulted in his PhD thesis. He obtained his MSc. degree with a specialization in Greenhouse Horticulture in 2009. In the same year he officially started his PhD under the auspice of the Graduate School of Experimental Plant Sciences at Wageningen University and the funding of the Technological Top Institute Green Genetics (TTI-GG). He is currently developing new projects in collaboration with Prof. Gerrit Beemster at the University of Antwerp, Belgium.

List of publications

Papers published in referred journals

Velez-Ramirez A.I., van Ieperen W., Vreugdenhil D. & Millenaar F.F. (2011) Plants under continuous light. *Trends in Plant Science*, **16**, 310-318.

Velez-Ramirez A.I., van Ieperen W., Vreugdenhil D., van Poppel P.M.J.A., Heuvelink E. & Millenaar F.F. (2014) A single locus confers tolerance to continuous light and allows substantial yield increase in tomato. *Nature Communications*, **5**, 4549.

Papers to be published in referred journals

Velez Ramirez A.I., Carreno-Quintero N., van Ieperen W., Millenaar F.F. & Vreugdenhil D. Sucrose and starch content negatively correlates with PSII maximum quantum efficiency in tomato plants exposed to injurious light/dark cycles, including continuous light. *In preparation*.

Velez Ramirez A.I., Dünner-Planella G., Vreugdenhil D., Millenaar F.F. & van Ieperen W. Circadian asynchrony triggers continuous-light-induced injury tomato. *In Preparation*.

Velez Ramirez A.I., van Ieperen W., Vreugdenhil D. & Millenaar F.F. Continuous-light-tolerance in tomato is graft-transferable. *Under revision*.

Velez Ramirez A.I., Vreugdenhil D., Millenaar F.F. & van Ieperen W. Phytochrome A protects tomato plants from injuries induced by continuous light. *In preparation*.

Conference proceeding

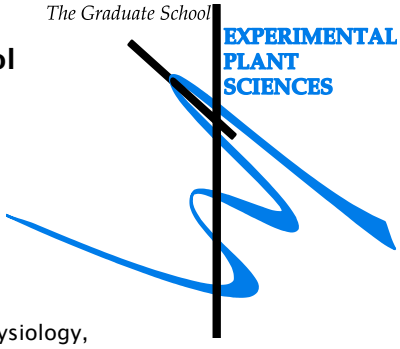
Velez-Ramirez A.I., Heuvelink E., van Ieperen W., Vreugdenhil D. & Millenaar F.F. (2012) Continuous Light as a Way to Increase Greenhouse Tomato Production: Expected Challenges. *Acta Horticulturae*, **956**, 51-58.

Paper published in a professional journal

Velez Ramirez A.I., Heuvelink E. & Kierkels T. (2012) Op zoek naar gewassen die tegen continue belichting kunnen : perspectieven bij tomaat. *Onder Glas*, **9**, 32-33.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Aaron Ivan Velez Ramirez

Date: 19 September 2014

Group: Plant Physiology & Horticulture and Product Physiology, Wageningen UR

1) Start-up phase	*date*
▶ **First presentation of your project**	
"Continuous Light on Tomato"	Oct 05, 2009
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
Review paper "Plants under Continuous Light", Trends in Plant Science, June 2011, Vol 16, No.6, pp 310 - 318	Sep 2009 - Feb 2011
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
2nd European Retreat of PhD Students in Plant Sciences, Cologne, Germany	Apr 15-17, 2010
EPS PhD student day, Utrecht University, NL	Jun 01, 2010
EPS PhD student day, Wageningen University, NL	May 20, 2011
3th European Retreat of PhD Students in Plant Sciences, Orsay, France	Jul 05-08, 2011
4th European Retreat of PhD Students in Plant Sciences, Norwich, UK	Aug 15-17, 2012
▶ **EPS theme symposia**	
EPS Theme 3 Symposium 'Metabolism and Adaptation, Leiden University, NL	Feb 19, 2010
EPS Theme 3 Symposium 'Metabolism and Adaptation, Wageningen University, NL	Feb 10, 2011
EPS Theme 3 Symposium 'Metabolism and Adaptation, Utrecht University, NL	Apr 26, 2012
▶ **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 04-05,

	2011
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 02-03, 2012
Seminars (series), workshops and symposia	
Seminar series Plant sciences, "The interaction of plants with their environment" by Harro Bouwmester	Sep 08, 2009
Seminar series Plant sciences, "Molecular Biology" by Ton Bisseling	Sep 08, 2009
Li-cor Webinar (online Seminar), "The importance of measuring chlorophyll fluorescence" by Shannon Loriaux	Sep 29, 2009
Seminar series Plant sciences, "Does plant physiology relate to consumer satisfaction?" by Olaf van Kooten	Oct 13, 2009
Seminar series Plant sciences, "Bioinformatics" by Jack Leunissen	Oct 13, 2009
Symposium "Photosynthesis: from femto to Peta and from nano to Global"	Nov 05, 2009
Seminar series Plant sciences, "The molecular dialogue between pathogens and plants" by Pierre de Wit	Nov 10, 2009
Seminar series Plant sciences, "Statistical modeling of genotype relations" by Fred van Eeuwijk	Nov 10, 2009
Seminar series Plant sciences, "System analysis for integrated assessment of trade-offs within agricultural systems: Building on the legacy of CT de Wit" by Ken Giller	Dec 08, 2009
Seminar series Plant sciences, "From bridging to closing the gap between phenotype and genotype" by Richard Visser	Dec 08, 2009
Agriculture Symposium "Discover. Validate. Sreeen. A new evolution in agriculture analysis"	Mar 03, 2010
Seminar series Plant sciences, "The challenges and focus of the endowed chair Organic Plant Breeding at Wageningen UR" by Edith Lammerts van Bueren	Mar 16, 2010
Seminar series Plant sciences, "Biosystematics tomorrow: understanding evolutionary processes by studying patterns of diversity" by Bert Visser	Jun 08, 2010
Seminar series Plant sciences, "Survival of the aligned: order in the cortical microtubule array" by Bela Mulder	Jun 08, 2010
Invited seminar, "Mobile RNA Silencing in Plants" by David Baulcombe	Sep 27, 2010
Science Webinar (online Seminar) "The Future of qPCR: Best Practices, Standarization, and the MIQE Guidelines" by Stephen A. Bustin, Gregory L. Shipley and Manju R. Sethi	Sep 30, 2010
Invited seminar, "The SOL Genomics Network: Genome Databases in the Post-Genome World" by Lukas Mueller	Oct 04, 2010
PE&RC Day 2010 "Selling Science: Why and how scientist sell science"	Oct 28, 2010
"CBSG Technology Symposium"	Nov 25, 2010
Photosynthesis Seminar Series, "Chlorophyll fluorescence as a powerful sensor of photosynthetic performance" by Wim Vredenberg	Mar 01, 2011
Photosynthesis Seminar Series, "Probing photosynthesis in vivo" by Jeremy Harbinson	Mar 15, 2011
Seminar series Plant sciences, "Career perspectives for young PSG colleagues" by several speakers	Jun 14, 2011
Invited seminar, "Biogenesis and function of microRNAs" by Javier Palatnik	Aug 25, 2011
Photosynthesis Seminar Series, "Plant phenomics, photosynthesis and the global food security challenge" by Robert Furbank	Sep 02, 2011

Mini-Symposium "Plant Breeding in the Genomic Era"	Nov 25, 2011
Photosynthesis Seminar Series, "Evaluation of the role of the water-water cycle as a mechanism of protecting the photosynthetic aparatus from high light" by Neil Baker	Dec 06, 2011
Invited seminar, "The Tomato Genome: From Genes to QTL and Networks" by Graham Seymour	Jan 24, 2012
Seminar series Plant sciences, "Debate on: How realistic is our "Two tmes more with two times less"-ambition?" by Luisa Trindade & Ken Giller	Jan 31, 2012
Photosynthesis Seminar Series, "Lessons from photosynthetic analyses in three widely used Arabidopsis ecotypes" by Cornelia Spetea Wiklund	Feb 21, 2012
Invited seminar, "Form and function of plant leaves"by Danny Tholen	Jan 22, 2013
Photosynthesis Seminar Series, "Integrating photosynthetic carbon assimilation from the leaf to the canopy" by Graham Farquhar	Mar 13, 2013
Workshop “How to write a convincing research proposal” by Cheryl Glenn	Mar 29, 2013
WEES Seminar Series, "Anticipating critical transitions" by Marten Scheffer	May 23, 2013
▶ **Seminar plus**	
▶ **International symposia and congresses**	
Symposium "Hacking the Biological Clock: Circadian Rhythm and Photosynthesis", Leiden, NL	Apr 10-13, 2012
7th International symposium light in horticulture	Oct 15-18, 2012
▶ **Presentations**	
Poster: Continuous Light on Tomato Plants	Apr 16, 2010
Presentation: Continuous Light on Tomato (update to Monsanto)	Jul 27, 2010
Presentation: Continuous Light on Tomato (TTI-GG Networking Event)	Sep 22, 2010
Poster: Unlike cultivated tomatoes, wild tomatoes are tolerant to continuous light	Apr 04, 2011
Poster: Circadian asynchrony could be responsible for the continuous-light-induced injury in tomato	Apr 02, 2012
Presenation: Hacking the “continuous light enigma”; is the circadian clock involved in the continuous-light induced injury?	Apr 12, 2012
Presentation: "¿Por qué las plantas de jitomate necesitan. “dormir”? Un Antiguo Enigma en un Mundo Moderno" (at National Autonomous University of Mexico)	Jun 27, 2012
Presentation: Continuous light on Tomato Plants (At Julich)	Sep 07, 2012
▶ **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Nov 14, 2012
▶ **Excursions**	
Subtotal Scientific Exposure	*21.1 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
PhD course 'Innovation for sustainability: Bringing theory into practice'	Nov 01-05, 2010
Postgraduate course 'System biology: Statistical analysis of ~omics data'	Dec 13-17,

	2010
One-day tutorial 'Basics of parameter estimation'	Feb 10, 2012
One-day tutorial 'Kick start R'	Apr 26, 2013
▶ **Journal club**	
Plant Physiology Laboratory Journal Club & Horticultural Production Chains FLOP meetings	2009-2013
▶ **Individual research training**	
Subtotal In-Depth Studies	*8.1 credits**

4) Personal development	*date*
▶ **Skill training courses**	
PhD Competence Assessment	Mar 16 & Apr 13, 2010
Scientific Writing	Mar 18-May 06, 2010
Teaching and Supervising Thesis Students	Jun 17 & Jun 18, 2010
Mini-Symposium "How to Write a World-Class Paper"	Oct 26, 2010
TTI-GG Networking Event	Sep 22, 2010
TTI-GG Networking Event	Sep 19, 2012
ExPectationS Day (EPS Career Event)	Feb 01, 2013
▶ **Organization of PhD students day, course or conference**	
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.2**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
** A credit represents a normative study load of 28 hours of study.*	

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Colophon

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