

Sucrose and Starch Content Negatively Correlates with PSII Maximum Quantum Efficiency in Tomato (*Solanum lycopersicum*) Exposed to Abnormal Light/Dark Cycles and Continuous Light

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Light is most important to plants as it fuels photosynthesis and provides clues about the environment. If provided in unnatural long photoperiods, however, it can be harmful and even lethal. Tomato (*Solanum lycopersicum*), for example, develops mottled chlorosis and necrosis when exposed to continuous light. Understanding the mechanism of these injuries is valuable, as important pathways regulating photosynthesis, such as circadian, retrograde and light signaling pathways are probably involved. Here, we use non-targeted metabolomics and transcriptomics analysis as well as hypothesis-driven experiments with continuous light-tolerant and -sensitive tomato lines to explore the long-standing proposed role of carbohydrate accumulation in 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 sucrose and starch content with the severity of continuous light-induced damage quantified as the maximum quantum efficiency of PSII (F_v/F_m) was found across several abnormal light/dark cycles, supporting the hypothesis that carbohydrates play an important role in the continuous light-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.

Keywords: Cytokinin • F_v/F_m • Photoperiod • Senescence • Sugar • Tomato (*Solanum lycopersicum*).

Abbreviations: ACC, 1-aminocyclopropane 1-carboxylate; ARR, Arabidopsis response regulator; BAP, 6-benzylaminopurine; CAB-13, a tomato type III light-harvesting Chl *a/b*-binding protein; CL, continuous light; ERF1, ethylene-responsive

transcription factor 1; ETR, ethylene receptor; F_v/F_m , maximum quantum efficiency of PSII; GC-TOF-MS, gas chromatography coupled with time-of-flight mass spectrometry; GO, Gene Ontology; G6P, glucose 6-phosphate; HPAEC, high-performance anion exchange chromatography; HPS, high pressure sodium; HXK1, hexokinase 1; IPT, isopentyl transferase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LHCB3, Type III light-harvesting Chl *a/b*-binding protein; LHClI, light-harvesting complex of PSII; PC, principal component; PCA, principal component analysis; PET, photosynthetic electron transport; *PhANG*, photosynthesis-associated nuclear gene; PSS, phytochrome photostationary state; RNaseq, RNA sequencing; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAG, senescence-associated gene; SnRK1, SNF1-related protein kinase 1; TOR, target of rapamycin.

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 reaches saturation, after which the excess 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. 2011, Velez-Ramirez et al. 2012, Velez-Ramirez et al. 2014). Although many studies suggested that carbohydrate accumulation is an important factor in inducing the injury under CL (Arthur et al. 1930, Dorais et al. 1996,

Demers et al. 1998, Matsuda et al. 2014), 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 relationship to carbohydrate metabolism—the *type III light harvesting Chl a/b-binding protein 13* (LHCB type III CAB-13 or CAB-13) (Velez-Ramirez et al. 2014). This gene encodes a protein of the light-harvesting complex of PSII (LHCII). Tomato CAB-13 is homologous to Arabidopsis (*Arabidopsis thaliana*) LHCB3. Nevertheless, a role for carbohydrate accumulation cannot be discarded. A transcriptomics study showed that the Gene Ontology (GO) term ‘Carbohydrate metabolic process’ is significantly enriched in 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).

Under many conditions, carbohydrate accumulation is associated with down-regulation of photosynthesis, including long-term exposure to high CO₂ (Stitt 1991), magnesium deficiency (Cakmak and Kirkby 2008), sugar feeding (Krapp et al. 1991) and mutations that affect carbohydrate metabolism (Baker and Braun 2007, Baker and Braun 2008). Photosynthetic control, which includes photosynthetic down-regulation and retrograde signaling processes, is a set of short- and long-term acclimation responses that regulate photosynthesis in such a way that ATP and NADPH production is co-ordinated 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 (Nott et al. 2006, Inaba 2010). It is proposed that the chlorophyll intermediate Mg-protoporphyrin IX (Mochizuki et al. 2001, Larkin et al. 2003, Nott et al. 2006), the phosphorylation status of LHCII (Pursiheimo et al. 2001), the redox state of PET components (Pfannschmidt et al. 2001, Fey et al. 2005, Nott et al. 2006) and plastid-derived singlet oxygen (¹O₂) (op den Camp et al. 2003, Wagner et al. 2004, Lee et al. 2007) 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 H₂O₂ and ¹O₂ 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 H₂O₂, ¹O₂, 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 (Ruckle et al. 2007, Lepistö and Rintamäki 2012, Ruckle et al. 2012). For instance, in dysfunctional chloroplasts, Ruckle et al. (2007) showed that plastid signals convert light signaling pathways from positive to negative regulators of some *PhANGs*. This might explain the effect of light quality (Arthur 1936, Globig

et al. 1997, Murage et al. 1997, Demers and Gosselin 2002) 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.

An alternative hypothesis is that carbohydrate accumulation results in early leaf senescence in CL-exposed tomatoes (Velez-Ramirez et al. 2011). 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 d 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 (Lim et al. 2007, Wingler et al. 2009, Fischer 2012, Thomas 2013). 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 use RNA sequencing (RNaseq) and metabolomics data to explore the role of carbohydrates in CL-induced injury in tomato. We postulate that the continuous light-induced injury in tomato shows 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.

Results

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, Dorais et al. 1996, Demers et al. 1998), as a result of photosynthetic down-regulation (Velez-Ramirez et al. 2011). 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 2 and 3 weeks. The level of CL-induced injury in these samples, evaluated with Chl fluorescence imaging after 3 weeks of treatment, has been previously reported (Velez Ramirez et al. 2017). **Table 1** shows how carbohydrate content was influenced by genotype (A131 or a CL-tolerant introgression line named CLT), time under light

Table 1 Effect of four light (L)/dark (D) cycles on leaf carbohydrate content of A131 and CLT plants on a fresh weight basis

Time of day	16 h L/8 h D (16 h 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 ($\mu\text{g mg}^{-1}$)								
End dark	10.97 \pm 1.58	18.31 \pm 0.48	4.41 \pm 0.82	9.26 \pm 0.92	10.58 \pm 2.49	13.48 \pm 2.46	15.02 \pm 2.24	29.82 \pm 2.59
End light	13.71 \pm 1.23	19.65 \pm 1.45			21.21 \pm 4.01	20.61 \pm 3.39	27.83 \pm 4.05	37.21 \pm 9.32
fructose ($\mu\text{g mg}^{-1}$)								
End dark	21.58 \pm 2.46	31.92 \pm 0.77	9.67 \pm 1.80	17.90 \pm 1.71	20.95 \pm 1.70	31.14 \pm 4.34	30.52 \pm 2.60	41.37 \pm 3.37
End light	26.71 \pm 1.26	33.16 \pm 0.96			29.67 \pm 3.36	34.80 \pm 3.19	36.87 \pm 5.40	51.05 \pm 12.13
Sucrose ($\mu\text{g mg}^{-1}$)								
End dark	6.32 \pm 0.35	6.23 \pm 0.21	11.08 \pm 0.38	10.33 \pm 0.45	3.63 \pm 1.28	6.13 \pm 0.70	1.95 \pm 0.66	0.60 \pm 0.36
End light	12.48 \pm 0.70	10.17 \pm 0.45			9.29 \pm 5.52	12.06 \pm 4.07	8.28 \pm 4.39	17.38 \pm 0.35
Starch ($\mu\text{g mg}^{-1}$)								
End dark	75.79 \pm 2.69	42.06 \pm 3.17	75.79 \pm 9.24	242.06 \pm 8.80	16.58 \pm 4.44	11.02 \pm 2.51	25.58 \pm 6.60	7.83 \pm 4.03
End light	83.37 \pm 4.69	41.77 \pm 3.86			25.10 \pm 9.59	11.76 \pm 2.41	59.68 \pm 6.34	32.66 \pm 4.41

Values represent mean of four replicates \pm SE.

treatment (1 or 3 weeks), time of sampling (end of the light or dark period) and 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 1 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. 1c, 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. In other words, the higher the sucrose and starch content after 1 week of treatment, the higher the injury after 3 weeks of treatment would be. No clear correlation was observed for glucose and fructose (Fig. 1a, b).

Matsuda et al. (2014) reported similar results. Although the levels of individual soluble sugars (glucose, fructose and sucrose) were not reported in that study, the degree of CL-induced injury in tomato showed a negative correlation with the turnover of total soluble sugars during night-time. In other words, CL injury developed in the treatments that showed no difference in soluble sugar content between day and night.

Girdling induces soluble sugar accumulation and accelerates senescence.

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 means other than CL. A way to achieve this is by girdling, i.e. the removal (or damaging) of the phloem in the leaflet petiole to inhibit carbohydrate export. Using A131 and CLT tomato plants grown entirely under 16 h light/8 h dark cycles, petioles of mature leaves were girdled with hot wax. Five days after girdling, F_v/F_m and carbohydrate content were assessed (Fig. 2). Control and girdled leaves of both genotypes showed signs of senescence, as evident from the lower F_v/F_m (Fig. 2a, b) in contrast to young, fully expanded leaves (Figs. 1, 3); nonetheless, girdled leaves showed significantly lower F_v/F_m values than the non-girdled leaves ($P < 0.05$). Additionally,

chlorosis in girdled leaflets was more severe at the tips than at the leaflet bases (Fig. 2a). This pattern resembles the chlorosis that tomato senescing leaves show when grown under a non-injurious 16 h photoperiod—CL-induced chlorosis is usually more evident at the leaf bases. Altogether, the results suggest that girdling accelerates senescence in tomato leaves regardless the genotype used. As expected, glucose, sucrose and fructose content were significantly higher in girdled leaflets than in the control leaflets ($P < 0.01$ or $P < 0.001$, see Fig. 2c–e), yet no difference in starch content was observed ($P > 0.05$) (Fig. 2f); indicating a co-occurrence of accelerated senescence and soluble sugar accumulation in tomato leaves.

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 (Lim et al. 2007, Fischer 2012, 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. 3). In CL-exposed plants, however, cytokinin treatment largely prevented the CL-induced injury. To evaluate further the effect of CL on cytokinin signaling, transcriptome contrasts between A131 and CLT (Velez-Ramirez et al. 2014) were mapped to the tomato Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for 'Plant hormone signal transduction' (sly04075). Supplementary Fig. S1 shows that tomato type-A Arabidopsis response regulator (ARR)-like genes, 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 and 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 (Supplementary

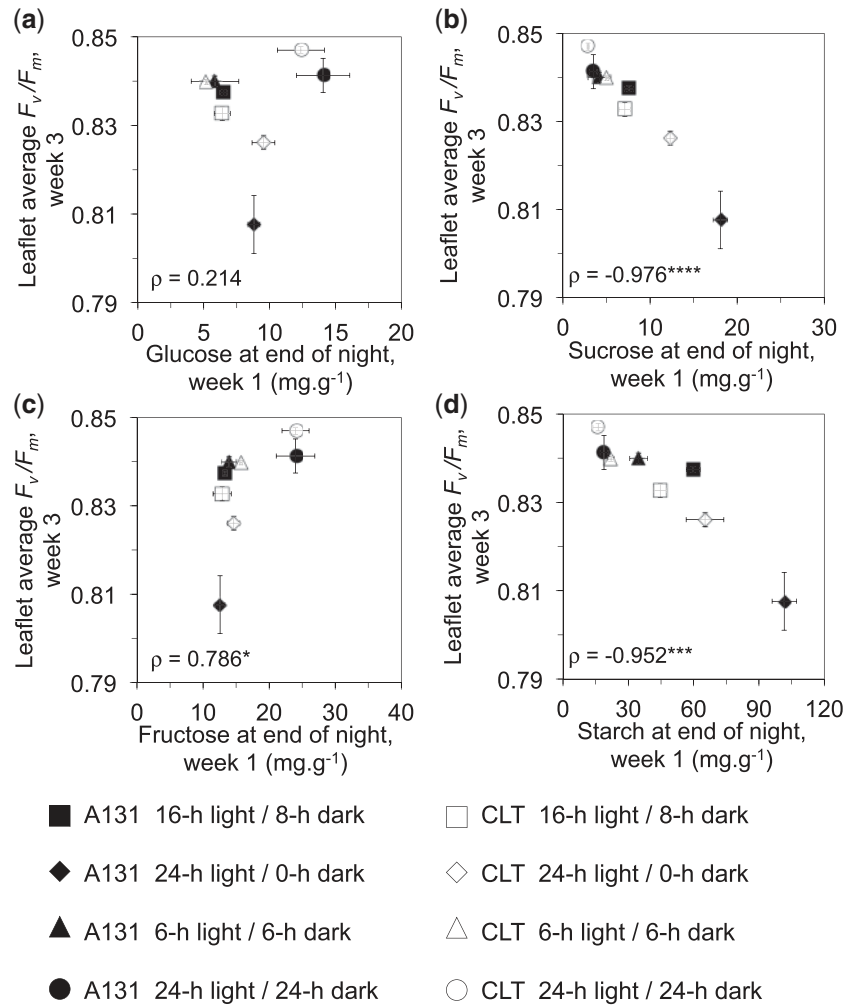


Fig. 1 Correlation between carbohydrate content at the end of the dark period and F_v/F_m in A131 and CLT tomato leaves exposed to four light/dark cycles. Carbohydrate content was measured in leaflets (sixth true leaf) 1 week after transfer to treatments. Previously reported, F_v/F_m was measured in leaflets 3 weeks after transfer to treatments (Velez Ramirez et al. 2017). Filled 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. (a) Glucose, (b) fructose, (c) sucrose and (d) starch. In all graphs, carbohydrate values ('x'-axes) represent the mean of four replicates, and F_v/F_m ('y'-axes) represent the mean of eight replicates; in both axes, error bars represent the SE. In the top right corner of all graphs, ρ values indicate the Spearman correlation coefficient between carbohydrate levels and chlorosis; asterisks indicate statistical significance; * $P < 0.05$; *** $P < 0.001$; and **** $P < 0.0001$.

Fig. S1a). Interestingly, ERF1 expression was also higher in A131 than in CLT plants when both genotypes were exposed to CL (Supplementary Fig. S1b).

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

To explore further the effects of CL at the metabolite level, two genotypes were used for non-targeted metabolomics analysis, the CL-sensitive reference line M82 and the CL-tolerant line IL-39 (Velez-Ramirez et al. 2014) from the *S. pennellii* (LA0716) \times *S. lycopersicum* M82 population, described by Eshed and Zamir (1994). Using gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF-MS), 98 metabolites were identified (Supplementary Table S1). 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. 4a, b). This suggests that continuous light alters carbohydrate metabolism in a similar way in CL-sensitive and -tolerant tomato lines. The first three principal components (PCs) explained approximately 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 relationship was found between the untransformed mean and SD with the metabolite rank (Fig. 4c); this indicates that the magnitude of metabolite concentration and variation did not have an impact on either the clustering or ranking. Sugars ranked high in their contribution to the first three PCs (Fig. 4d). This indicates that CL significantly alters carbohydrate metabolism,

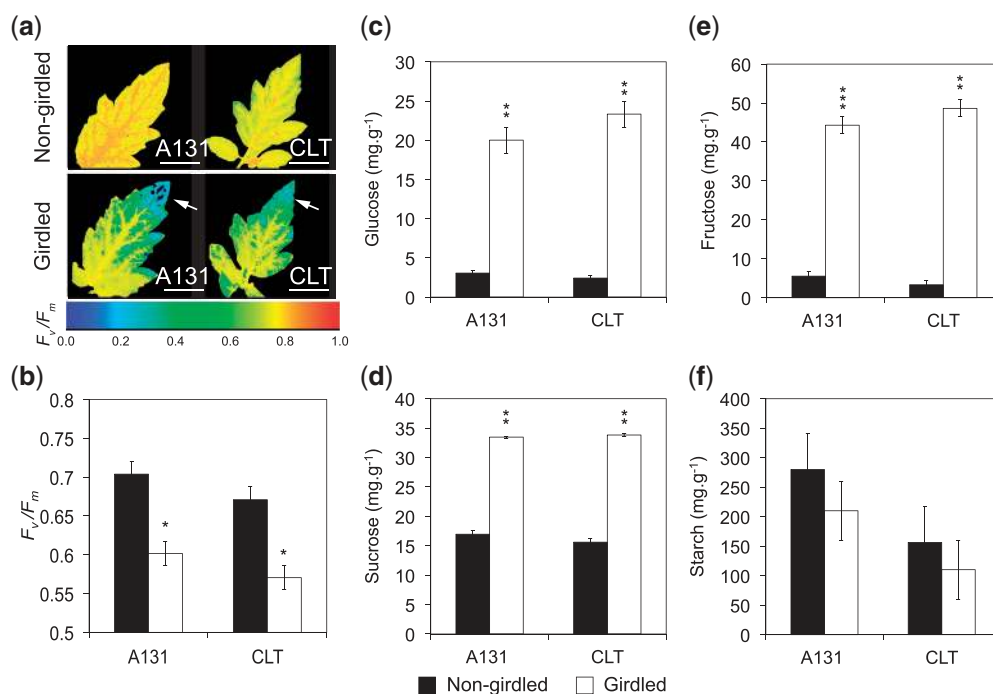


Fig. 2 Girdling-induced carbohydrate accumulation and chlorosis in tomato leaves. One terminal side leaflet (fourth true leaf) of A131 and CLT tomato plants grown under 16 h light/8 h dark cycles for 35 d was girdled with hot wax. Within the same leaf, the opposite leaflet was used as the non-girdled paired control. Five days after girdling, Chl fluorescence images were taken, and carbohydrate content was quantified; the same leaflets were used for both measurements. (a) Chl fluorescence images of girdled and non-girdled leaflets; maximum efficiency of PSII (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. (b) Average of F_v/F_m in girdled and non-girdled leaflets. (c–f) Carbohydrate content of girdled and non-girdled leaflets. (c) Glucose; (d) fructose; (e) sucrose; (f) starch. Carbohydrate content of non-girdled leaflets is represented in black, and carbohydrate content of girdled leaflets is represented in white. In all graphs, bars represent the mean of six replicates, and error bars represent the SE; asterisks indicate that the difference between girdled and non-girdled leaflets is statistically significant; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

but no clear differences between CL-tolerant and -sensitive genotypes were found.

Continuous light down-regulates key enzymes involved in carbon fixation

In order to assess the effects of CL on the expression of genes related to carbohydrate metabolism, we used a published transcriptome RNaseq data set (Velez-Ramirez et al. 2014) to inspect for alterations in carbon metabolic pathways. The expression data of > 31,000 genes were used to evaluate 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 the CL-tolerant introgression line named CLT (Velez-Ramirez et al. 2014). The expression data of both contrasts were mapped to tomato-specific KEGG pathways. Supplementary Fig. S2 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 Supplementary Fig. S2, for instance, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39) color-coded expression is the average fold change of the small and large subunits. For an overview of annotated tomato genes in the KEGG pathways, follow the links in the figure legends. From Supplementary Fig. 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 a 16 h photoperiod and CL-tolerant plants exposed to CL.

To assess further the effect of CL on carbohydrate metabolism at the transcriptional level, two more tomato KEGG pathways were evaluated. Supplementary Figs. S3 and S4 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 (Supplementary Fig. S4). 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

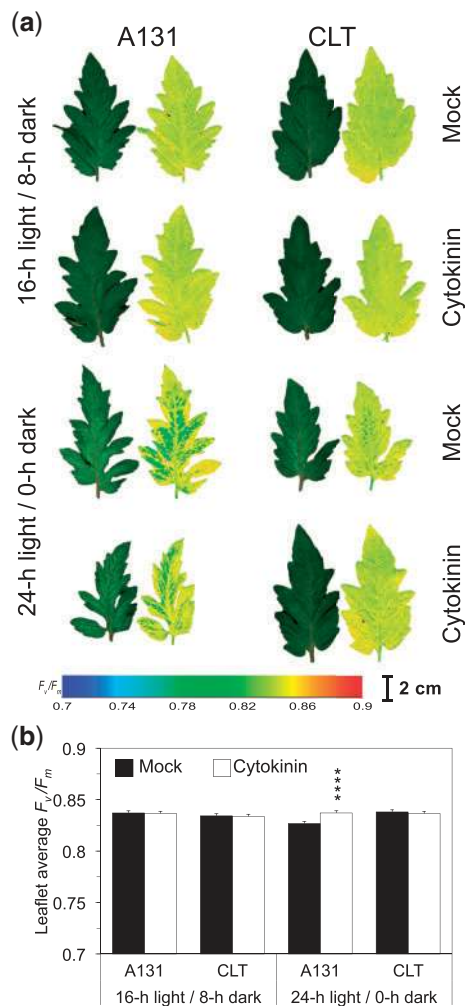


Fig. 3 Cytokinin (BAP) treatment diminishes the continuous light-induced injury in tomato. (a) Leaflets (sixth true leaf) of A131 and CLT tomato plants exposed to 24 h light/0 h dark cycles (continuous light). At the start of the treatment, plants were sprayed, every 3 d, with cytokinin or mock solution for 2 weeks. 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 PSII (dark-adapted F_v/F_m). (b) Injury quantification in leaflets of A131 and CLT tomato plants in (a); bars represent the mean of six replicates, and error bars represent the SE. In all graphs, Mock-treated plants are represented in black, cytokinin-sprayed plants in white; asterisks indicate that the value is significantly different from the mock control; **** $p < 0.0001$.

degradation of starch to maltose and dextrin, are up-regulated by CL and there is no difference between genotypes when exposed to CL (Supplementary Fig. S3). Overall, it seems that CL down-regulates carbon-fixing enzymes and up-regulates the starch-degrading capacity.

Discussion

In this study, we have shown that CL alters carbohydrate metabolism at the transcriptional and metabolite level (Fig. 4; Supplementary Figs. S2, S3). Sucrose and starch content

negatively correlate with the maximum quantum efficiency of PSII (F_v/F_m) under four light treatments (Fig. 1), and cytokinin treatment prevents the CL-induced injury in tomato (Fig. 3). These results suggest that altered carbohydrate metabolism plays an important role in inducing injury in CL-grown tomato plants as has been proposed (Arthur et al. 1930, Dorais et al. 1996, Demers et al. 1998, Velez-Ramirez et al. 2011, Matsuda et al. 2014).

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-Benson cycle, require only ATP and not NADPH (Foyer et al. 2012). Consumption of ATP without spending NADPH results in short- and long-term acclimations (including retrograde signaling) that regulate photosynthesis in such a way that ATP and NADPH production is co-ordinated 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; in plant cells, several regulatory systems that control growth and development receive input from sucrose, glucose, glucose 6-phosphate (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, such as the hexokinase (HXK1) glucose sensor, the SNF1-related protein kinase1 (SnRK1) and the target of rapamycin (TOR) kinase system (Thomas 2013).

Caution should be exercised 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 both natural processes in response to age and an ever-changing natural environment, respectively. However, CL, as achieved in a growth chamber, is far from being natural (Velez-Ramirez et al. 2011). When tomatoes are exposed to CL, nevertheless, the existing signaling network must be responsible for induction of the injury, yet it is unlikely that they would follow a canonical senescence or retrograde signaling pathway. 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 from old leaves to young organs (Lim et al. 2007, Fischer 2012, Thomas 2013). The earliest structural changes during leaf senescence-associated cell death occur in the chloroplast; hence a decrease in Chl content and maximum photochemical efficiency (i.e. F_v/F_m) are well-established senescence markers (Lim et al. 2007). Additionally, our results show that cytokinins, which are well-known senescence inhibitors (Lim et al. 2007, Fischer 2012, Thomas 2013) can prevent a

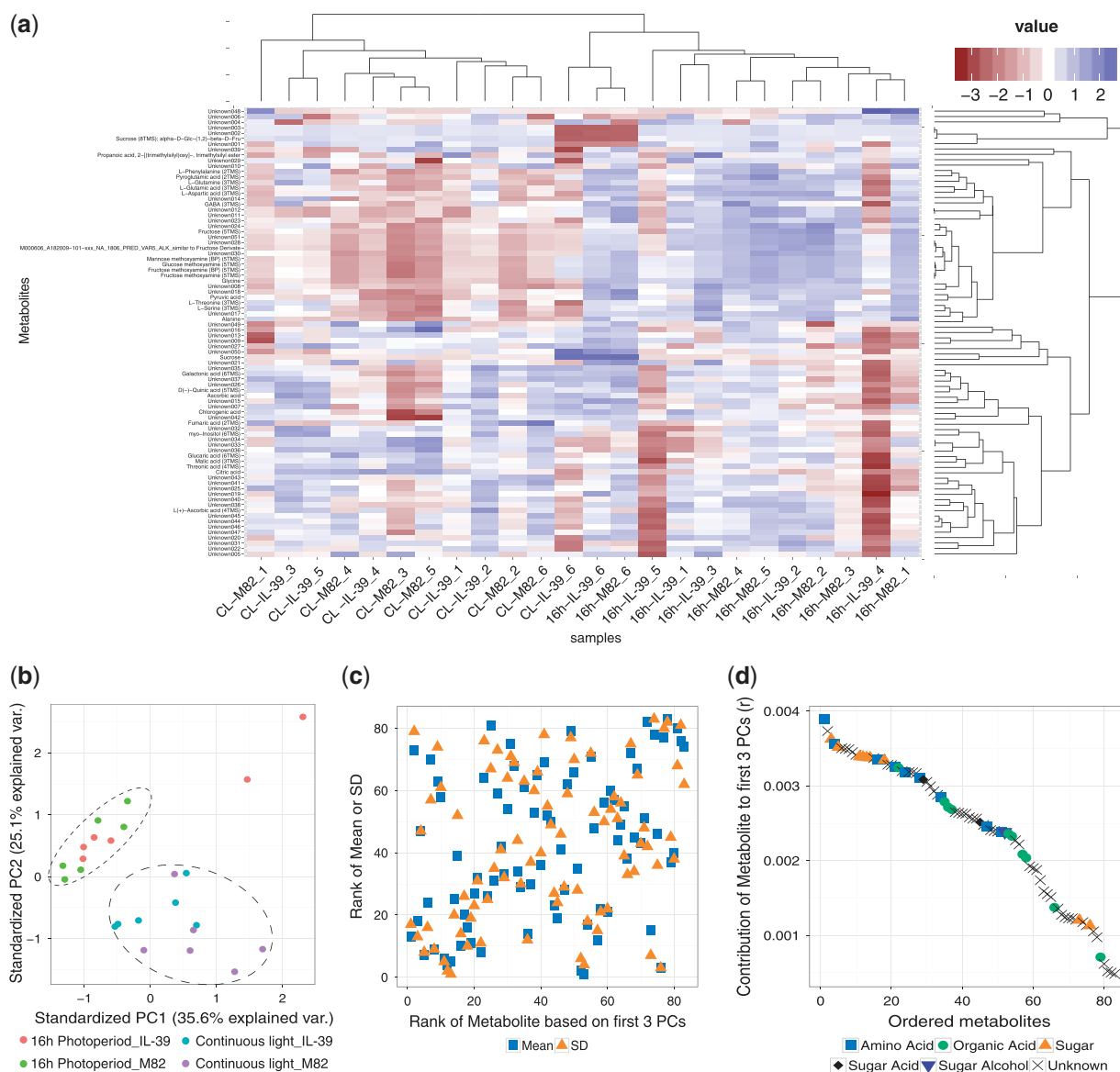


Fig. 4 Hierarchical cluster analysis and principal component analysis (PCA) on metabolomics data. Continuous light (CL)-sensitive M82 and CL-tolerant IL-39 tomato plants ($n = 6$) were exposed to CL or kept under a 16 h photoperiod. Polar metabolites were extracted and analyzed with GC-TOF-MS. (a) Hierarchical cluster analysis of the 83 putative metabolites. (b) PCA; again, samples clustered according to light treatment. (c) Relationship between the contribution of each metabolite to the first three principal components (van den Berg et al. 2006) and the untransformed mean and SD. (d) Metabolites ordered by their contribution to the first three principal components; symbols represent each metabolite type (see key); note that sugars ranked high in their contribution to the first three principal components.

CL-induced decrease of F_v/F_m in A131 tomato plants (Fig. 3). This is consistent with the hypothesis of accelerated senescence induced by CL.

The inhibitory effect of leaf senescence by cytokinin in tomato is well documented. The expression of *isopentenyl transferase* (*IPT*), encoding the rate-limiting step in cytokinin biosynthesis, under the control of Arabidopsis senescence-associated genes promoters, *SAG12* or *SAG13*, suppresses leaf senescence in tomato (Swartzberg et al. 2006, Swartzberg et al. 2011). In addition, 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 with A131 plants under a 16 h photoperiod, yet no difference was found between CL-exposed A131 and CLT plants (Velez-Ramirez et al. 2014). 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 probably not dependent on the glucose *HXK1* sensor, which is consistent with the lack of correlation between glucose and chlorosis (Fig. 1a).

In contrast to cytokinin, ethylene is involved in the induction of leaf senescence (Lim et al. 2007, Fischer 2012). 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. Supplementary Fig. S1 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 a 16 h photoperiod and CLT plants exposed to CL. This suggests that 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. 3) and knocking down of *ACC oxidase* expression (Cushman and Tibbitts 1998) suggest that CL-induced injury is a type 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 base (Arthur et al. 1930, Withrow and Withrow 1949, Hillman 1956, Velez Ramirez et al. 2015). 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. 2). This indicates that CL-tolerant CLT tomato plants are not tolerant to girdled-induced senescence. Usually, CL-induced chlorosis is most severe at the leaf/leaflet basis, but this pattern is not a rule; mottled chlorosis with irregular distribution across tomato leaflets is also seen in CL-exposed tomato plants (Fig. 3; see also Velez-Ramirez et al. 2014). The chlorosis pattern in CL-exposed tomato leaves might result from the developmental state at which the tissue was exposed to CL. For instance, 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 a 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 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 co-ordination 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) shows that CL down-regulates photosynthesis genes associated with the Calvin–Benson cycle (Supplementary Fig. S2), PSI and PSII, and the light-harvesting complexes I and II (Velez-Ramirez et al. 2014). As discussed above, excessive synthesis of starch and sucrose results in a decreased ATP to NADPH ratio, favoring over-reduction of PET components because 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 need to be down-regulated via retrograde signals. Interestingly, evidence shows that retrograde signals heavily interact with the light signaling network in Arabidopsis (Ruckle et al. 2007, Lepistö and Rintamäki 2012, Ruckle et al. 2012). Considering that the light spectral distribution has an effect on the severity of the CL-induced injury (Arthur 1936, Globig et al. 1997, Murage et al. 1997, Demers and Gosselin 2002), the hypothesis that light 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 (Hillman 1956, Kristoffersen 1963, Velez Ramirez et al. 2017). With the exception of the 16 h photoperiod control treatment, all light treatments in Fig. 1 imply circadian asynchrony because light may be present at times when a free-running circadian cycle would be at its night-time phase. Interestingly, all these abnormal light/dark cycles result in CL injury with an intensity that correlates with the sucrose and starch content at the end of the dark period (Fig. 1). Considering that the circadian clock influences, according to the time of the day, carbohydrate metabolism (Lu et al. 2005, Weise et al. 2006, Graf et al. 2010), it is reasonable to link hypothetically the circadian clock, sugar metabolism and the CL-induced injury. Similar conclusions were reached by Matsuda et al. (2014) who suggested that the disturbance of the diurnal regular fluctuation of soluble sugars may promote CL-induced injury with a potential role for the circadian clock.

Altogether, the evidence suggests that the CL-induced injury might be a kind of photosynthetic down-regulation that shows characteristics of both cytokinin-regulated senescence and light-modulated retrograde signaling. Such down-regulation seems to be triggered by an inadequate integration of sugar metabolism/signaling, light signaling/presence and the circadian clock phase. Ethylene and cytokinin signaling are most probably 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, senescence-like pathways or a combination of both.

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 (Velez-Ramirez et al. 2014). Line IL-39 was developed from line IL-7-2 (LA4065) (Eshed and Zamir 1994). Seeds were provided by Monsanto Vegetable Seed Division. Plants were grown in rockwool blocks at 21°C and 70% relative humidity. Commercial hydroponic nutrient solution for tomato was used (Yara Benelux BV); after combining and diluting pre-mixed liquid fertilizers, the solution contained 12.42 mM NO₃⁻, 7.2 mM K, 4.1 mM Ca, 3.34 mM SO₄²⁻, 1.82 mM Mg, 1.2 mM NH₄⁺, 1.14 mM P, 30 μM B, 25 μM Fe, 10 μM Mn, 5 μM Zn, 0.75 μM Cu and 0.5 μM 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 400 W, Philips) and supplemented with incandescent lamps (Philinea T30 120 W, Philips). The light intensity was 350 μmol.m⁻² s⁻¹; the red-to-far-red ratio was 2.873, and the phytochrome photostationary state (PSS) (Sager et al. 1988) was 0.857. CL treatment consisted of just leaving the lamps (HPS and incandescent) continuously on without changing any other setting. Plants were grown at 21°C, 70% relative humidity and ambient CO₂ levels.

For the metabolomic experiment, M82 and IL-39 tomato lines were used. Plants were sown directly under a 16 h photoperiod or CL treatments. After 3 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 (Velez Ramirez et al. 2017); briefly, 4-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, 4-week-old A131 and CLT tomato plants, grown under a 16 h photoperiod, were also used. Girdling treatment consisted of enclosing a section (1.5–3 cm in length) of a leaflet petiole (fourth leaf) with modeling dough, and pouring hot wax (85°C) into the enclosed petiole section (Goldschmidt and Huber 1992). Cytokinin treatment consisted of spraying the plants till run-off with BAP solution [0.1 mM 6-benzylaminopurine in 1 N KOH pH 7.8 with 0.02% (v/v) Tween-20] every 3 d (Zavaleta-Mancera et al. 2007).

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

Relative metabolite contents were determined as described by Liseć et al. (2006) with modifications specific to tomato leaves (Etalo et al. 2013). Briefly, polar metabolite fractions were extracted from approximately 50 mg FW of frozen leaf tissue. The ground leaf tissue was homogenized in 700 μl of pre-cooled (-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 tube, 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 tube and aliquots (200 μl) were dried by vacuum centrifugation without heating.

The dried samples were derivatized online as described in Liseć et al. (2006) using a Combi PAL autosampler (CTC Analytics AG). First, 12.5 μl of *O*-methylhydroxylamine hydrochloride (20 mg ml⁻¹ 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 of 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 GC-TOF-MS consisting of an Optic 3 high-performance injector (ATAS GL Int.) and an Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments). A 2 μl aliquot of each sample was injected at 70°C using a split flow of 19 ml min⁻¹. The injector was rapidly heated at 6°C s⁻¹ to 240°C. The chromatographic separation was performed using a VF-5 ms capillary column (Varian; 30 m × 0.25 mm × 0.25 μm) including a 10 m 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 ionized by electron impact at 70 eV. Mass spectra were acquired at 20 scans s⁻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 1,400 V.

Data were processed using ChromaTOF 2.0 (Leco instruments) and MassLynx (Waters Inc.) and further analyzed using MetAlign (Lommen 2009) to extract and align the mass signals (signal to noise ratio ≥ 2). Mass signals that were present in < 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; <http://www.nist.gov/srd/mslist.htm>) and Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>) spectral libraries and by comparison with retention indices calculated using a series of alkanes. 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) 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 31,350 genes in the original data set, 14,219 had a unique mapping between all IDs. The R package 'Pathview' (Luo and 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 80% (v/v) ethanol at 80°C for 20 min. After centrifuging the mixture for 5 min at 7,000 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 of distilled water, placed in an ultrasonic bath for 10 min and centrifuged for 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 (4 × 250 mm) column (Dionex). Samples were eluted with 100 mM NaOH.

For starch quantification, the pellets obtained as described above were re-suspended in 3 ml of 80% (v/v) ethanol. After centrifuging the sample for 5 min at 7,000 RCF, the supernatants were discarded, and the pellets were washed twice more. Then, the pellets were dried using a vacuum concentrator, and 2 ml of α-amylase/rohalase solution (1 mg ml⁻¹ in H₂O) were added (Serva). After incubating the samples for 30 min in a shaking water bath at 90°C, 1 ml of amyloglucosidase solution (0.5 mg ml⁻¹ in 50 mM citrate buffer, pH 4.6) was added (Sigma-Aldrich), and samples were incubated for 15 min in a shaking water bath at 60°C. Finally the samples were centrifuged for 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.

Chl fluorescence imaging

Imaging of the maximum quantum efficiency of PSII (F_v/F_m) (Baker 2008) was performed as previously described (Velez-Ramirez et al. 2014). In summary, intact leaflets (attached to the plant) were dark-adapted using dark adapting clips (Li-Cor Biosciences). After 20 min of dark adaptation, leaflets were detached and immediately used for measurements in a Chl fluorescence imaging system (FluorCam 800MF, Photon System Instruments). 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 Chl 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 PCA using R (R Core Team 2013). Pearson's correlation coefficient, for computing the distance matrix, and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) were used for hierarchical clustering. The contribution of each metabolite to the first three PCs was calculated according to van den Berg et al. (2006). Statistical significance of the leaflet average F_v/F_m was determined with an analysis of variance (ANOVA) test performed with IBM SPSS Statistics software version 19.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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