Inter-leaf rhythmicity in Solanum lycopersicum contrasts with neutrality in wild tomatoes

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Abstract

Cells, tissues, and organs harbour complex systems to allow communication between one another. The biological rhythms can be contrasting among organs, tissues, and cells, adjusting the physiology differently along the organism’s regions, while also synchronising flowering and metabolism. Here, we revealed that Solanum lycopersicum manifests more balanced rhythms across the whole plant than wild tomatoes. Accordingly, the leaf development program is more coordinated in this organism than in wild species, in that young S. lycopersicum leaves develop slowly in comparison to mature leaves. Young leaves from wild tomatoes display higher photosynthetic rate than mature leaves, while large metabolite accumulations occur across plant segments. Consequently, diel metabolite levels are rather similar between young and mature leaves in the wild tomato S. pennelli, whereas the expression patterns for circadian clock genes are widely contrasting between differently aged leaves. We further demonstrated that introduction of domestication alleles into the wild tomato S. pimpinellifolium appears to synchronize the development of young and mature leaves, rendering this similar to that observed for S. lycopersicum. Collectively, the strengthening of inter-organ relationships in S. lycopersicum indicates an increased synchronization of its biology, which is probably fundamental to explain its elevated yield.

RESEARCH ARTICLE

Running title: Inter-organ relationships in tomato species

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SUMMARY
Cells, tissues, and organs harbour complex systems to allow communication between one another. The biological rhythms can be contrasting among organs, tissues, and cells, adjusting the physiology differently along the organism’s regions, while also synchronising flowering and metabolism. Here, we revealed that Solanum lycopersicum manifests more balanced rhythms across the whole plant than wild tomatoes. Accordingly, the leaf development program is more coordinated in this organism than in wild species, in that young S. lycopersicum leaves develop slowly in comparison to mature leaves. Young leaves from wild tomatoes display higher photosynthetic rate than mature leaves, while large metabolite accumulations occur across plant segments. Consequently, diel metabolite levels are rather similar between young and mature leaves in the wild tomato S. pennellii, whereas the expression patterns for circadian clock genes are widely contrasting between differently aged leaves. We further demonstrated that introduction of domestication alleles into the wild tomato S. pimpinellifolium appears to synchronize the development of young and mature leaves, rendering this similar to that observed for S. lycopersicum. Collectively, the strengthening of inter-organ relationships in S. lycopersicum indicates an increased synchronization of its biology, which is probably fundamental to explain its elevated yield.

Key words: temporal synchronization; biological rhythms; metabolism; leaf ecophysiology; de novo domestication

INTRODUCTION
Historically, humans have been selecting high-yielding plants as a means to ensure their food security and support population growth, leading to drastic alterations in plant development. Most plant traits are affected by timing, thus necessitating that the plant itself has accurate mechanisms to record and respond to time. Accordingly, timekeeping mechanisms monitor geophysical time and record coincidences, which adjust the organism’s development and frequently promote the anticipation of future environmental conditions. A single organism may have diverse timekeepers working either independently or in synchronicity, which makes that organism has clocks associated with development, circadian rhythms, and metabolic oscillations. Thus, the developmental clock defines the precise timing to induce cell differentiation related to the growth of specific organs and subsequently shifting their morphology. The circadian clock, in turn, represents the timekeeper that synchronises the endogenous system of the organism following “solar time”, to regulate oscillating rhythms according to a period of 24 hours. Furthermore, autonomous metabolic oscillations may occur across diverse growth conditions independently from other oscillatory systems, characterising the so-called metabolic clock. Remarkably, it remains unclear why biological clocks in modern crops are characterized by triggering the phenomena of flowering synchronization, circadian clock deceleration, and broader metabolic adjustments than their wild species.

Cultivated species are assumed to have mainly alterations at the developmental clock in comparison with wild species, shifting the biology of their shoot apical meristem (SAM), which shapes architecture and synchronizes the flowering time of crops. The SAM maturation clock of S. lycopersicum shows a more advanced maturation status than in tomato wild species, culminating in a faster transition from inflorescence meristem to floral meristem. Notably, variations in the cis -regulatory region of the gene SELF PRUNING 5G (SP5G) seems to be critical for flowering induction of S. lycopersicum. Clique ou toque aqui para inserir o texto. Beyond the regulation of the flowering time, the SP5G gene also plays an important role in the plant responses to the environment, orchestrating also the tolerance to abiotic stresses. Abiotic stresses responses are linked intimately to the circadian clock activity, whereas tomato domestication selected plants with a slower circadian clock. Clique ou toque aqui para inserir o texto. A key component connecting stress responses and circadian clocks appears to be carbon metabolism, as revealed by metabolic signatures which are assumed to orientate the origin and evolution of some species, including adzuki bean (Vigna angularis).
and barley (*Hordeum vulgare*). Thus, singularities in temporal biology are expected to occur across plant species, and as such temporal coincidences among developmental, circadian, and metabolic clocks must occur to enable the improved yield of cultivated tomato in comparison to wild tomatoes.

Communication across tissues of complex biological systems occurs due to cell-to-cell relationships, contributing to multicellularity structuration in both animals and plants. Accordingly, mice (*Mus musculus*) submitted to a high-fat diet had losses in the temporal coincidences of circadian metabolism among eight distinct tissues, promoting aberrant cell proliferation and growth of these tissues. Notably, mice liver exhibits an independent circadian clock oscillating autonomously from all other clocks, while this clock is dependent on the light/dark cycles to sustain the rhythmicity. In good agreement, each leaf tissue exhibits a particular clock that expresses differential contributions to the global leaf circadian clock, and the rhythmicity is asymmetrically coupled across tissues, ensuring correct leaf development in *Arabidopsis thaliana*. Recently, we suggested that domestication was an agent setting biological clocks on cultivated species, reducing the heterogeneity among distinct tissues. It seems reasonable to posit that this may have contributed to photoperiod adaptation and synchronized flowering in domesticated plants. To reveal potential synchrony among biological clocks in wild and domesticated tomatoes, we combined physiological, metabolic, and genetic assays to demonstrate differences among these species. Our results revealed that domesticated tomato has synchronized circadian rhythms in both cotyledons, a fact that is not observed for the wild tomato *S. pimpinellifolium*. We additionally demonstrated that metabolism and gene expression are more integrated between leaves of domesticated tomatoes, which appears to be a factor to help explaining their elevated yield in comparison with their wild relatives.

**RESULTS**

The similar circadian rhythms in cotyledons of *Solanum lycopersicum*

Decelerated circadian rhythms are observed in cultivated tomatoes while faster rhythms occur at wild tomato species. Otherwise, each tissue displays a specific circadian clock contributing to adjust singular processes in the leaf, and differently from the centralized circadian clock of mammals, the plant circadian clock seems not exhibit a clear centralization. Nevertheless, shoot apex clocks seem to have the capacity to synchronize circadian rhythms across plant structures, affecting distant organs such as roots. Despite certain imprecision regarding the centralization of circadian rhythms in plants, it remains the consensus that circadian misalignment is greatly deleterious for the fitness of both mammals and plants. Based on this, we decided to assess circadian rhythms in red fruit tomatoes, *S. lycopersicum* (cv. M82) and the wild tomato *S. pimpinellifolium*.

The exposure of the two *A. thaliana* cotyledons to constant light revealed contrasting circadian rhythms for both over several days, indicating the independence of each cotyledon clock. Therefore, we assessed circadian rhythms in cotyledon pairs of *S. lycopersicum* and *S. pimpinellifolium*, which revealed contrasting rhythms only for the wild species (Fig. 1A-B). Accordingly, the difference for the circadian period was 0.8 h between the cotyledons of *S. pimpinellifolium*. Thereafter, we turned our attention to identifying the developmental and physiological consequences of the absence of differences in circadian rhythms in the *S. lycopersicum*.

The developmental hierarchy at leaves of *Solanum species*

Most of the food plant selections over history were performed based on reproductive yield, given that humans most likely selected plants according to desirable fruits and grains. Even though leaf shape was recently demonstrated to be an excellent predictor for fruit production and quality in tomatoes, the role of leaves to ensure elevated crop productivities was rather neglected. In agreement with this observation, latitude is closed associated with tomato production, affecting genotypes distribution across earth globe, which may have differently affected tomato organs. We thus decided to investigate the major tomato traits affected by latitude using the data from the germplasm bank of Universidade Federal de Viçosa, Brazil (www.bgh.ufv.br). For this purpose, we mapped the relationships among latitude with growth traits of distinct organs in *S. lycopersicum* genotypes. Our *in silico* assays revealed that leaf traits are correlated with latitude in approximately 100 tomato genotypes from diverse regions of the Earth (Supplementary Fig. S1-2).
We next decided to turn our attention to leaf development and investigate whether *S. lycopersicum* could have a synchronized development of leaves. Although canopies of cultivated plants have homogenous leaves, they capture light heterogeneously. Indeed, analyses of the leaf developmental trajectories in *S. lycopersicum* revealed an uneven photosynthetic competence, which spreads heterogeneously across leaf zones. Investigating the leaf development in wild tomatoes, a complex behaviour in leaf series was revealed, in which shade avoidance is ephemeral whereas leaf length and area are widely variable. These traits tend to negatively correlate with the shade avoidance index during early development while have a positive correlation at later stages of leaf development. Thus, wild tomatoes exhibit an unequal shade avoidance along the leaf developmental series, whereby each leaf might exhibit a distinct degree of shade avoidance. *S. lycopersicum* and wild tomatoes are characterized by an ephemeral shade avoidance that is not continuous over leaves. Accordingly, complex developmental patterns were visualised in leaves of a single plant, which occurs due to a differential light and promotes the differential shade avoidance across plant regions. By analysing canopy and leaf patterns, we observed that the developmental plasticity seems to be higher in wild tomatoes in comparison with *S. lycopersicum* (Supplementary Fig. S3), indicating a leaf shape homogenization in the last species.

Thus, we postulated that a potential developmental overlap may exist covering the third and fourth leaves of wild (*S. pimpinellifolium*, *S. habrochaites*, *S. neorickii*, and *S. pennellii*) tomatoes which does not occur in the *S. lycopersicum*. To reduce the impacts of variations in leaf shape and length, we performed analyses mainly on older leaflets from third (L3) and fourth (L4) leaves, which allowed us to assess both developmental and physiological competence from 15 to 35 days after sowing (DAS). Intriguingly, the same developmental pattern was observed for all wild tomatoes, wherein the L3 had a higher leaf area than L4 only at the first observations, in general around 15 or 20 DAS (Figure 2A). In sharp contrast, *S. lycopersicum* was always characterized with L3 displaying a larger leaf area than L4 across all experimental observations (Figure 2A), suggesting a leaf developmental coordination in *S. lycopersicum*.

Specific leaf area (SLA) is an important trait influencing the plant’s ability to intercept photosynthetic irradiation. Commonly, older leaves exhibit a lower SLA than young leaves, denoting a better photosynthetic ability and a more advanced development due to cell differentiation status. Differences at SLA not occurred between L3 and L4 over the early development of wild tomatoes, whereas it was essentially different between L3 and L4 during the 15 to 30 DAS in *S. lycopersicum* (Figure 2B). The physiological maturity of a leaf occurs when it becomes a source of photoassimilates for other plant organs. Accordingly, chlorophyll content is an excellent indicator of physiological maturity revealing when a leaf is no longer a sink but rather a source of sugars. We thus measured chlorophyll levels over leaf development, finding a similar pattern to that observed for leaflet area and SLA, with differences practically absent between L3 and L4 even in the early developmental stages of wild tomatoes (Figure 2C). Only *S. lycopersicum* displayed clear differences in chlorophyll levels and chlorophyll $a/b$ ratio over development (Figure 2C-D), suggesting a more defined transition sink to source in leaves of this species.

Decreases in chlorophyll levels exert a direct role in the reduction of the number of lateral branches, which seems to modulate shoot branching and alter energy balance. Likewise, we next determined the levels of sugars related to energy homeostasis, founding higher sugar levels in L3 than in L4 during the early stages of the leaf development exclusively in *S. lycopersicum* (Supplementary Fig. S4). Over development of M82, the glucose and fructose levels were increasing in L4, wherein higher levels of these sugars were found in later developmental stages for L4 in comparison to L3, a fact that was not observed for sucrose (Supplementary Fig. S4). Meanwhile, we did not observe a clear pattern for sugar levels in wild tomatoes, and as such L3 and L4 frequently had similar levels of glucose, fructose, and sucrose regardless of the developmental stage. Altogether, these findings suggest that development and metabolism are likely more coordinated in *S. lycopersicum* than in wild tomatoes.

We further performed CO$_2$ curves using *S. lycopersicum*, *S. pimpinellifolium*, and *S. pennellii* to compare gas exchange responsiveness between L3 and L4. Net photosynthesis ($A_N$) is homogenous in leaflets of M82 and *S. pimpinellifolium*, whereas *S. pennellii* exhibited differences in $A_N$ between L3 and L4 for most levels.
of CO\textsubscript{2} assessed (Figure 3). Attempting to understand the differences observed, we split seven segments from L3 to L4 of M82 and \textit{S. pennellii} to map metabolic alterations across these segments. We observed that M82 was metabolically invariant along the segments, while \textit{S. pennellii} exhibited remarkable metabolite accumulation specifically in metabolites related to sink function including sucrose, citrate, and GABA (Figure 3). Collectively, these results indicated that wild tomato leaflets reach maturity faster, which may mean that they can be self-sufficient earlier, in contrast with the observations for the \textit{S. lycopersicum}.

Physiological traits seemingly play a more important role in determining high rates of leaf growth than morphological features, being that \textit{S. lycopersicum} is more impacted for variations in these traits than wild species. Thus, we investigated leaflet physiology in M82, \textit{S. pennellii}, and introgression lines (ILs) harbouring \textit{S. pennellii} genomic fragments. These ILs allowed the identification of several quantitative trait loci (QTLs), including the identification of regions intimately associated with tomato domestication, branching, flowering, and circadian clocks. In this sense, IL5-4 harbours a wild allele of \textit{SP5G}, a gene that regulates architecture and flowering, whereas IL9-2-6 and IL9-3 harbour the genes promoting the circadian clock deceleration. By investigating the L3 and L4 development of M82, IL5-4, IL9-2-6, IL9-3, and \textit{S. pennellii}, we observed a similar developmental window for these leaflets in ILs and \textit{S. pennellii} (Supplementary Table 1). Thus, around 35 DAS, the area and mass of leaflets were similar between L3 and L4 of the same plant in the ILs and in wild species, wherein this relationship does not occur in \textit{S. lycopersicum} being that L3 displays a higher area, SLA and mass than L4 (Supplementary Table 1). By contrast, ILs and wild tomatoes showed similar SLA for the leaflets L3 and L4, describing a similar ability to intercept radiation (Supplementary Table 1). This finding suggested a similar developmental trajectory between third and fourth leaves, which prompted us in whether ILs and \textit{S. pennellii} would exhibit contrasting physiologies for each leaflet.

**Heterogeneous physiology but similar metabolism between leaves of \textit{S. pennellii}**

Remarkable variations in photosynthesis-related genes can be observed between \textit{S. lycopersicum} and wild tomatoes, occurring an inverse correlation between transcripts related to leaf development and photosynthesis. Intriguingly, despite \textit{S. pennellii} leaflets exhibiting a similar SLA, a higher \textit{A\textsubscript{N}} for L4 in comparison with L3 was observed (Supplementary Figure S6). Meanwhile, our results revealed that \textit{S. pennellii} exhibited a differential \textit{V\textsubscript{c, max}} for different leaves of the same plant, with the highest values for L4 than L3 (Supplementary Figure S6). In agreement with these data, a similar pattern for ILs and \textit{S. pennellii} was observed for \textit{A\textsubscript{N}}, \textit{R\textsubscript{d}}, \textit{P\textsubscript{R}}, \textit{g\textsubscript{m}}, \textit{V\textsubscript{c, max}}, and biochemical limitations, supporting the notion of weak source-to-sink relationships in these plants (Supplementary Fig. S6-7). Taken together, our findings suggested that these genomic regions might regulate the metabolic synchronization between L3 and L4. To further elucidate this, we decided to perform metabolite analyses on these leaflets in an attempt to identify the source-to-sink patterns.

Despite the higher \textit{A\textsubscript{N}} at L4, the diel metabolism of glucose, fructose and sucrose revealed few differences between L3 and L4 for ILs and \textit{S. pennellii} (Figure 4 and Supplementary Figure S7). Interestingly, the coupling between organism size and metabolic rates displays few relationships with the selection of plants over domestication, which may be due to the weak inter-tissue metabolic relationships. To summarize, the absence of differences for sucrose/starch metabolism between L3 and L4 on ILs and \textit{S. pennellii} seems to describe a rather weak inter-leaf relationship. Metabolite diversity in wild and domesticated \textit{Phaseolus vulgaris} revealed that tissue specificity is the major factor affecting the metabolite dataset, yet wild genotypes display more metabolite diversity. Meanwhile, considering a single tomato plant, metabolite specialization across different tissues seems to result from a differential gene expression in mature and young organs. Taking the findings of these two studies together, it seems reasonable to anticipate that gene expression might explain the contrasting metabolic regulation at L3 and L4 of M82 and \textit{S. Pennellii}.

The contrasting gene expression of \textit{S. pennellii} leaflets

Transcriptional variation has been widely used to monitor circadian rhythms over the past years. For this reason, we next assessed the gene expression of M82 and \textit{S. pennellii} growing under natural light/dark cycles to identify potential synchronicity between L3 and L4. **TIMING OF CAB EXPRESSION1 (TOC1)** is a pivotal component in circadian clock regulation since it represses and is repressed by **LATE ELONGATED**
Under natural conditions, the expression of Circadian clock genes in plant leaves is influenced by developmental and environmental factors. For example, *Lycopersicon esculentum* (tomato) and *Brassica rapa* (rape) have different expressions of *CAB13 (Light Harvesting Chlorophyll a/b Binding protein 13)* in their leaves. In tomatoes, *CAB13* expression is higher in the upper leaves, whereas in rapes, it is higher in the lower leaves. This difference is likely due to the environment and the developmental stage of the leaves.

The expression of *CAB13* in wild tomatoes is lower than in cultivated tomatoes, suggesting a difference in the regulation of the circadian clock. In wild tomatoes, *CAB13* expression is higher in the upper leaves, whereas in cultivated tomatoes, it is lower. This difference is likely due to the developmental stage of the leaves.

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Our study sheds light on the differential regulation of circadian clocks in leaves of wild and cultivated *Solanum lycopersicum*. We observed that the expression of *CAB13* in cultivated tomatoes is higher in the upper leaves, whereas in wild tomatoes, it is lower. This difference is likely due to the developmental stage of the leaves.

Discussion

Our study sheds light on the differential regulation of circadian clocks in leaves of wild and cultivated *Solanum lycopersicum*. We observed that the expression of *CAB13* in cultivated tomatoes is higher in the upper leaves, whereas in wild tomatoes, it is lower. This difference is likely due to the developmental stage of the leaves.

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and stomatal conductance only in oilseed varieties. Furthermore, oilseed has a shorter circadian period coupled with a higher \( V_{\text{c max}} \) than leafy and turnip morphotypes, whereas it was not investigated whether a temporal regulation across leaves of oilseed may exist. Cultivated tomato in turn displays photosynthetic differences according to leaf age, wherein leaves with elevated age had much lower \( A_g \) capacity and \( R_d \) than younger leaves. Thus, Rubisco activity (per g protein) is invariant across young and mature leaves of \( S. lycopersicum \), whereas Rubisco total activity expressed per unit leaf area was lower in older than younger leaves (Xu et al., 1997), which seems to contribute to creating a photosynthetic hierarchy across leaves.

Autonomy to regulate genetic and metabolic traits is fundamental for organ development and thus synchronize tissues of whole organism with environmental variations. The misalignment of circadian relationships among tissues causes a desynchronized development prejudicing organism health. Similarly, each \( A. thaliana \) leaf tissue manifest specific circadian programs, while mesophyll and vascular clocks are the major drivers of the global leaf clock. This asymmetry among clocks may be synchronized and shoot apex clocks can couple circadian rhythms of other organs, sending proteins to roots that increase or decrease the circadian period according to with temperature. The synchronization level among cells decreases with developmental progression and cell-to-cell communication probably enhances desynchronization, while individual plant cells under constant light manifest autonomous circadian oscillations. Circadian rhythms heterogeneity in intact plants are seemingly corrected by light/dark cycles, thereby these cycles are extremely important to generate systemic responses to environmental variations. Shifts in photoperiod responsiveness were obtained over the tomato domestication process, whereas the expression of genes related to this process is intimately dependent on light/dark cycles. A single plant of wild tomatoes have many more cells than cultivated tomato due to higher branching index. As consequence, these wild species are classical examples that constant light does not greatly disrupt their phenotype, in contrast to the situation in cultivated tomato, suggesting a lower synchronization between environment and clocks of wild species.

Different organs of wild species can display variations in circadian rhythms, highlighting differences observed in cotyledon-to-cotyledon, leaf-to-leaf and shoot-to-root relations which extend to the control of the organ development. This may contribute to a biological advantage once that impairments in single organs would result in reduced impacts for underlying organs. It seems reasonable to anticipate that, when submitted to stressful conditions, the wild species most likely suffer less with the loss of leaves with a reduced physiological efficiency. A very interesting example of synchronization may be observed in \( Echinacea angustifolia \), a wild species that is perennial and displays the flowering time synchronized by fire. In cultivated tomato, biological synchronization seems to occur through enhanced coordination between circadian rhythms and leaf development. It would be interesting to determine whether, and to what extent, environmental factors manifested over the tomato diversification process may have boosted inter-organ relationships in plants.

The results obtained here also point to the developmental temporisation of cultivated tomato, which may homogenize photosynthesis across leaves and establish stronger source-to-sink relationships. By contrast, \( S. pennellii \) seems to show an atemporal regulation of traits programming source-to-sink relationships, with each leaf working rather independently. It can contribute to explaining the slow growth and reduced fruit mass of \( S. pennellii \), which exhibits a positive relationship with its higher ability to tolerate environmental adversities. Notably, metabolism and organism size were largely uncoupled by plant domestication, whereas circadian rhythms were also shaped with this process. The elevated diurnal expression of \( TOC1 \) leads ultimately to a higher starch turnover, improving biomass gain in \( A. thaliana \) polyploids when compared with their parental diploids. Consistently, \( TOC1 \) expression and starch levels demonstrated similar patterns in cultivated tomato, whilst higher \( TOC1 \) expression in L4 than L3 upon noon seems to be correlated with higher starch turnover and superior biomass gain of the young leaflet in wild relatives. In agreement, the circadian clock is particularly sensitive to sucrose in the darkness, being that \( GI \) plays the major role in sucrose sensing mediating shifts in rhythmicity of shoot circadian. Thus, sucrose is likely able to reset the circadian clock based on metabolic status acting as both signal and metabolite at the same time. \( GI \) expression was highly homogenous in M82 leaflets whereas sucrose levels were widely divergent between L3 and L4 over the diel period, indicating a lower interaction sucrose versus \( GI \) expression at these leaflets. The opposite was observed for \( S. pennellii \) that showed a contrasting \( GI \) expression between L3 and L4, despite almost
similar sucrose levels for both leaflets. It seems, therefore, that the contrasting development, physiology, and metabolism between older and younger leaves of *S. lycopersicum* likely program the source-sink relationships, which seems to be a remarkable contrast between cultivated and wild tomatoes.

Over the past decades our understanding of circadian clocks, development and metabolism experienced significant advances, wherein the function of thousands of genes, proteins, and metabolites have been demonstrated. As consequence, complex genetic features are associated with the tomato domestication syndrome linking, for example, the fruit flavour with productivity. Since wild species endure hostile environments, their biological clocks integrate a plethora of abiotic stresses responses, and thus *de novo* domestication describes the introduction of domestication genes and/or re-domestication into wild species, representing a promising strategy to develop crop ideotypes. To exemplify the potential of this new concept, increased fruit size and number, as well as a higher nutritional value, were observed in engineered *S. pimpinellifolium* lines. Notwithstanding these facts, the structure of circadian, developmental, and metabolic clocks along plant species evolution is far from being fully understood. We posit that distinct plant tissues may exhibit variations in inter-relationships over plant development. Domesticated tomato is an interesting case that may contribute in starting to solve how biological clocks are structured in plants, while *de novo* domestication provides an outstanding opportunity to re-structure these clocks to engineer ideal crops. The results described here provide compelling evidence for a higher coherence among tissues from domesticated tomato, which seemingly does not occur in their wild relatives. Future studies must explore the temporal (in)coherences among organs and tissues to generate ideal crops that are more productive under fluctuating environments, ensuring food security even in hostile environments.

**Experimental Procedures**

**Plant material and growth conditions**

Tomato seeds were provided by Professor Dr. Lazaro E. P. Peres (Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo (ESALQ/USP), Brazil). Seeds from *Solanum lycopersicum* (cv. M82), *S. pimpinellifolium* (LA1589), *S. habrochaites* (LA1777), *S. neorickii* (LA2133), and *S. pennelli* (LA716). We additionally selected introgression lines (IL5-4, IL9-2-6, and IL9-3) harbouring genome fragments of *S. pennelli* in the genetic background M82 (*S. lycopersicum*), which were obtained from . In addition, we performed assays with *S. pimpinellifolium* lines harbouring loss-of-function mutations in six loci related to tomato domestication, as described by Zsögön et al. (2018). Seeds were surface-sterilized with 5% sodium hypochlorite for 10 min and then washed with running distilled water and subsequently sowed in a tray with a commercial substrate (Tropstrato HT©). Two weeks after the sowing, seedlings were transferred to 400 mL pots (one seedling per pot) containing the same substrate used for seedling production but supplemented with 1 g of NPK 10–10–10 and 4 g dolomitic limestone (Ca + Mg) per L of the substrate. Plants were grown in a controlled greenhouse located in Vícosa (20deg45'S, 42deg50'W, 650 m above sea level), Brazil, with a mean temperature of 28 degC, 12.0 hours (winter/spring/summer) of photoperiod, and a minimum of 600 μmol photons m⁻² s⁻¹.

Plants were watered regularly and throughout the entire growth period were maintained under naturally fluctuating conditions of light intensity, temperature, and relative air humidity. All physiological, and biochemical parameters analysed in the experiments were performed on the third and fourth leaves when source leaves were completely expanded, which occurred for 4-week-old plants. Additionally, each experiment was repeated at least three times (even in different growth facilities) with similar phenotypes observed each time. Throughout the experiment, the plants were grown under naturally fluctuating conditions of temperature and air relative humidity and were fertilized, as necessary. The pots were randomized periodically to minimize any variation within each light environment. For biochemical and molecular analyses, leaf samples were collected at different times during the day, before flash freezing in liquid nitrogen (N₂) and subsequent storage at -80 °C, where it remained until analysis. The means presented in the tables and figures were obtained from at least three independent replicates per treatment of single plant experimental plots (one plant per pot). The experiments were repeated at least three times with similar phenotypes observed each time.
Cotyledon movements and circadian rhythm determination

Seedlings were grown in controlled conditions of a growth chamber for four days under cool white fluorescent tubes (~100 μmol m⁻² s⁻¹) under 12h light/12h dark and 20:18 °C temperature, which was transferred subsequently to constant light and temperature (25 °C). A polystyrene ball was attached to the tip of the cotyledons of each seedling by using petroleum jelly according to Müller et al. (2015).

Growth analyses

Growth parameters were determined in 4-week-old plants by measuring leaf area, length, and mass as well as the specific leaf area (SLA). Leaf area was measured using a scanner (Hewlett Packard Scanjet G2410, Palo Alto, California, USA) and processing the resulting images on ImageJ. SLA was measured as described previously. At the end of the experiment, plants were harvested by cutting the segments above ground level in the sense of the third leaf for the fourth leaf, thus separating leaflets, petioles, and stem.

Measurements of gas exchange and chlorophyll fluorescence

Gas exchange parameters were determined simultaneously with chlorophyll a (Chl a) fluorescence measurements by using an open-flow infrared gas exchange analyser system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Instantaneous gas exchanges were measured after 1-hr illumination during the light period under 1,000 μmol m⁻² s⁻¹ at the leaf level (light saturation) of photosynthetically active photon flux density (PPFD). The reference CO₂ concentration was set at 400-μmol CO₂ mol⁻¹ air. All measurements were performed using the 2-cm2 leaf chamber at 25°C, as well as a 0.5 stomatal ratio (amphistomatic leaves), and leaf-to-air vapour pressure deficit was kept at 1.2 kPa, and the amount of blue light was set to 10% PPFD to optimize stomatal aperture. Briefly, the initial fluorescence emission (F₀) was by illuminating dark-adapted leaves (1 h) with weak modulated measuring beams (0.03 μmol m⁻² s⁻¹). A saturating white light pulse (8,000 μmol m⁻² s⁻¹) was applied for 0.8 s to obtain the maximum fluorescence. In light-adapted leaves, the steady-state fluorescence yield was measured with the application of a saturating white light pulse (8,000 μmol m⁻² s⁻¹) to achieve the light-adapted maximum fluorescence (Fm’) according to . ΦPSII represents the number of electrons transferred per photon absorbed in the PSII, and the electron transport rate (Jₘₜ) was calculated. Dark respiration (R_d) was measured after 2 hours in the dark period (at night), using the same gas exchange system described above, and it was divided by two (R_d/2) to estimate the mitochondrial respiration rate in the light (RL). Determination of mesophyll conductance (gₘ), maximum rate of carboxylation (Vₗₘₜ), maximum rate of photosynthetic limitations. The CO₂ concentration in the carboxylation sites (C_c) was calculated according to . Briefly, this method uses the values of A₊, gₛ, gₘ, Vₗₘₜ and C_c, and permits the partitioning into the functional components of photosynthetic constraints related to stomatal (Lₛ), mesophyll (Lₘ), and biochemical (L_b) limitations.

Determination of metabolite levels

Tomato segments were harvested and immediately frozen in liquid N₂ and stored at -80°C until further analysis. For leaves, all sampling procedures were carried out on the same leaf used for the analysis of gas exchange and fluorescence. Leaf samples were harvested at different time points along the light-to-dark cycle (6:00, 12:00; 18:00, 00:00, and 6:00). The extraction of metabolite extraction was executed by rapid grinding in liquid N₂ and immediate addition of the specific extraction buffer. Chlorophyll levels were determined according to, whereas glucose, fructose, sucrose, and starch were determined as described previously (Fernie et al., 2001). Total amino acids and soluble proteins were determined as previously described by Yemm et al. (1955) and, respectively.

Other metabolites were quantified according to gas chromatography associated with the mass spectrometry (GCMS) protocol. We used approximately 50 mg fresh weight to perform the extraction procedure, by using 1 mL of methanol and shaking (800 rpm) at 70 °C for 15 min; being that 60 μL of ribitol (0.2 mg mL⁻¹) was added as an internal standard. Subsequently, derivatization and sample injection were performed as previously described by Lisc et al. (2006). Chromatograms and generated mass spectra were evaluated
using the software TagFinder, using a reference library from the Golm Metabolome Database, and the identification and annotation of the detected peaks followed the recommendations for reporting metabolite data described in.

**Gene expression**

Total RNA was isolated from 50 mg of leaves using the TRIzol reagent (Invitrogen®), according to the manufacturer’s instructions. The integrity and amount of RNA were checked on 1% (w/v) agarose gel, and the concentration was measured before and after DNase I digestion (RNase free DNase I, Promega®) using spectrophotometry (OD260). DNase-treated RNA (2 μg) was used for cDNA synthesis using the SuperscriptTM III reverse transcriptase (Invitrogen®, Darmstadt, Germany) according to the manufacturer’s recommendations. Gene expression was accessed using real-time PCR (qRT-PCR) (Step One PlusTMReal Time PCR System, Applied Biosystems, CA, USA) with the SYBR green fluorescence detection (Applied Biosystems®), using the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX kit. qRT-PCR was performed according to the following temperature alternations: denaturation of double-stranded DNA and enzyme activation (95 °C, 30 s, 1x); denaturation of double-stranded DNA (95 °C, 3 s, 40x), and finally, primer’s annealing and extension of fragment by polymerase enzyme (58 °C, 30 s, 40x). Melt curve reaction: 95 °C, 15 s, 60 °C for 1 min, and 95 °C for 15 s (1x). The average CT of the ACTIN gene (reference control) was used for the relative expression analyses. The analyses were performed in duplicate in each PCR run, using 4 replicates for each genotype, and their mean cycle threshold was used for relative normalized expression analyses.

**Statistical analyses**

The experiments were randomized and designed with a minimum of three biological replicates of each treatment. Furthermore, experiments to describe phenotypes were repeated at least three times. Data were statistically tested for normality and subsequently examined using ANOVA (P < 0.05). Differences in the means (P < 0.05) displayed in figures and tables were examined by Student’s t-test. All statistical analyses were performed using R statistical software (www.r-project.org).

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**Competing Interest Statement**

The authors declare that they have no competing interests.

**Author Contributions**

J.A.S., and W.L.A. designed the research; J.A.S. performed most of the research with the support of A.O.M., T.W., M.F.S., W.B-S. and F.L.; A. Z., A.R.F., and A.N-N. contributed new reagents/analytic tools; J.A.S., A.R.F., A.N-N., and W.L.A. analyzed the data; and J.A.S., and W.L.A. wrote the article with input from all the others.

**References**

Figure 1. The circadian rhythms of cultivated tomatoes operate synchronously. Images showed cotyledons positions at 12, 36, 60 and 84 hours upon exposure to constant light conditions. Circadian period of the cotyledons from Solanum lycopersicum (cv. M82) and Solanum pimpinellifolium. Data presented are
mean ± SE (n = 8), and an asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between cotyledon 1 and cotyledon 2.

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Figure 2. Developmental and physiological traits over development from domesticated and wild species of tomatoes. Leaflet traits were assessed in older leaflets of third (L3, dark-green) and fourth (L4, pale-green) leaves from domesticated tomato (Solanum lycopersicum cv. M82), S. pimpinellifolium, S. habrochaites, S. neorickii, and S. pennellii. A, Leaflet area (cm²); B, SLA: specific leaf area (cm² g⁻¹); C, Chlorophyll (mg g⁻¹ fresh weight); D, ratio chlorophyll a/b. Data presented are mean ± SE (n = 7), and an asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between L3 and L4 leaflets in the specific point. DAS: days after sowing.

Figure 3. Net photosynthesis (AN) curves in response to sub-stomatal (Ci) CO₂ concentration and metabolite levels in segments of domesticated and wild tomatoes. A, AN/Ci curves of Solanum lycopersicum (Cv. M82), S. pimpinellifolium, and S. pennellii determined in leaflets of (dark-green) and fourth (pale-green) leaves. Data are means ± SE (n = 7), and an asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between the third and fourth leaflets. B, Metabolite levels at the middle day were assessed in seven segments. Samples were taken from the third and fourth leaves from the apex of 4-week-old plants. Data are means ± SE (n = 5).
Figure 4. The diel regulation of sucrose metabolism on tomatoes. Older leaflets of (L3, dark-green) and fourth (L4, pale-green) leaves from domesticated tomatoes (Cv. M82), introgression lines (IL) and wild tomato *Solanum pennellii* were analyzed, as represented at left, while the right shows sucrose levels and the ratio between the fourth and third leaflets. Sucrose levels were assessed in leaflets harvested every 6 hours over a diurnal cycle. Data are means ± SE (n = 5), and an asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between L3 and L4 leaflets in the specific point. Yellow bars indicate the light period while grey bars indicate the dark period. DW: Dry weight.

Figure 5. Expression profile of genes associated with tomato domestication in *Solanum lycopersicum* cv. M82 and *S. pennellii*. Diurnal oscillations of transcript levels were determined in leaflets of the third (dark-green) and fourth (pale-green) leaves. An asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between the third and fourth leaflets. Data represent the average expression of three biological replicates ± SE. TIMING OF CAB EXPRESSION 1 (TOC1); LATE ELONGATED HYPOCOTYL (LHY); GIGANTEA (GI); NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 2 (LNK2); SELF PRUNING 5G (SP5G); chlorophyll ab binding protein 13 (CAB13).

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image6.emf available at https://authorea.com/users/591967/articles/644305-inter-leaf-
Figure 6. The establishment of leaf development hierarchy with domestication de novo. Leaflet traits were assessed in older leaflets of (L3, dark-green) and fourth (L4, pale-green) leaves from *Solanum pimpinellifolium* genotypes, wild-type (WT) and multiplex lines harbouring gene editions in key genes associated with domestication. Data presented are mean ± SE (n = 7), and an asterisk (*) indicates different values that were determined by the two-sided Student’s t-test to be different (P < 0.05) between L3 and L4 leaflets in the specific point. Leaflet length (cm); Leaflet area (cm$^2$); Leaflet mass (g); SLA: specific leaf area (cm$^2$ g$^{-1}$); DAS: days after sowing.