Coordinating the morphogenesis-differentiation balance by tweaking the cytokinin-gibberellin equilibrium

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Abstract

Morphogenesis and differentiation are important stages in organ development and shape determination. However, how they are balanced and tuned during development is not fully understood. In the compound leaved tomato, an extended morphogenesis phase allows for the initiation of leaflets, resulting in the compound form. Maintaining a prolonged morphogenetic phase in early stages of compound-leaf development in tomato is dependent on delayed activity of several factors that promote differentiation, including the CIN-TCP transcription factor (TF) LA, the MYB TF CLAU and the plant hormone Gibberellin (GA), as well as on the morphogenesis-promoting activity of the plant hormone cytokinin (CK). Here, we investigated the genetic regulation of the morphogenesis-differentiation balance by studying the relationship between LA, CLAU, TKN2, CK and GA. Our genetic and molecular examination suggest that LA is expressed earlier and more broadly than CLAU and determines the developmental context of CLAU activity. Genetic interaction analysis indicates that LA and CLAU likely promote differentiation in parallel genetic pathways. These pathways converge downstream on tuning the balance between CK and GA. Comprehensive transcriptomic analyses support the genetic data and provide insights into the broader molecular basis of differentiation and morphogenesis processes in plants.
Author summary
Morphogenesis and differentiation are crucial steps in the formation and shaping of organs in both plants and animals. A wide array of transcription factors and hormones were shown to act together to support morphogenesis or promote differentiation. However, a comprehensive molecular and genetic understating of how morphogenesis and differentiation are coordinated during development is still missing. We addressed these questions in the context of the development of the tomato compound leaf, for which many regulators have been described. Investigating the coordination among these different actors, we show that several discrete genetic pathways promote differentiation. Downstream of these separate pathways, two important plant hormones, cytokinin and gibberellin, act antagonistically to tweak the morphogenesis-differentiation balance.

Introduction
Morphogenesis, originating from the Greek words \textit{morphe}/shape and \textit{genesis}/formation, is a fascinating biological process that has attracted human eyes since ancient times \cite{1,2}. Several model systems have been used to study morphogenesis, from the first examination of chicken embryos by Aristotle \cite{3–6}. Plants provide an excellent model system to investigate the shaping of an organism during the adult life cycle \cite{7,8}. Despite the ancient origin of morphogenesis studies in both the animal and plant kingdoms, our understanding of the molecular mechanisms governing morphogenesis, in particular the connection between gene regulatory networks, function, and shape formation—is still not complete.

Aristotle’s philosophy shaped our thinking of the term ‘form’ as fulfilling the full potential and destiny of oneself \cite{3}. Leaves are vital photosynthetic, lateral organs produced by the plant throughout its life cycle. The development of plant leaves follows a common basic program, adjusted flexibly according to species, developmental stage and environment \cite{9–12}. Morphogenesis and differentiation are important stages in leaf development, and the spatial and temporal balance between these processes influences leaf size and shape \cite{10,13,14}. In compound leaved plants such as tomato, the ratio between these two stages favors longer morphogenesis, allowing for initiation of leaflets, resulting in the compound form \cite{15}. The length of the morphogenetic window is thus a key determinant of final leaf shape. The flexibility of the morphogenetic window is regulated through a coordinated interplay between transcription factors and hormones \cite{16–25}. Tomato leaf development is therefore an attractive system to investigate the contribution of the morphogenesis-differentiation balance to organ shaping.

The hormone gibberellin (GA) promotes leaf differentiation, while cytokinin (CK) promotes morphogenesis. Therefore the balance between the activity of these two hormones is key to the modulation of the morphogenesis-differentiation balance \cite{17,26,27}.

CIN-TCP transcription factors affect leaf shape by promoting differentiation, and maintenance of the morphogenetic window is dependent on low CIN-TCP activity during the early stages of leaf development \cite{28–37}. A subset of CIN-TCPs, including LANCEOLATE (LA) from tomato, is negatively regulated by the microRNA miR319. In the tomato semi-dominant gain-of-function mutant \textit{La}, a mutation in the miR319 binding site leads to early ectopic LA expression, resulting in precocious differentiation and small, simplified leaves \cite{33,34,38,39}. Concurrently, premature expression of the miR319-insensitive TCP4 in Arabidopsis plants causes early onset of maturation, resulting in a range of leaf patterning defects \cite{37}. Downregulation of \textit{CIN-TCP} genes by overexpression of \textit{miR319} results in a substantial delay in leaf maturation and prolonged indeterminate growth in the leaf margin \cite{29,33,34,36,40}.
Differences in the timing of leaf growth and maturation among species and leaf positions are associated with altered LA expression dynamics [34]. Thus, the LA-miR319 balance defines the morphogenetic window at the tomato leaf margin that is required for leaf elaboration. LA activity is mediated in part by positive regulation of the hormone GA [21]. In Arabidopsis, the LA homolog TCP4 reduces CK response during leaf development [41]. Whether this effect of TCPs on CK is conserved in tomato is still unknown.

Maintenance of the morphogenetic window is also restricted by activity of the MYB transcription factor CLAUSA (CLAU) [42]. CLAU has evolved a unique role in compound-leaf species to promote an exit from the morphogenetic phase of leaf development [22]. clau mutants have highly compound, continuously morphogenetic leaves, in which meristematic tissues constantly generate leaflets on essentially mature leaves throughout the life of the plant [22,43]. clau mutants can be extremely variable in phenotype, showing that tight regulation of the morphogenetic window is also required for shape robustness [43]. CLAU regulates the morphogenetic window by attenuating cytokinin signaling and sensitivity [22]. GA application was shown to suppress the increased complexity of clau leaves [44], raising the possibility that CLAU also affects the GA pathway.

The tomato KNOTTED1-LIKE HOMEOBOX (KNOXI/TKN2) is a key regulator of compound leaf development. TKN2 delays leaf differentiation and preserves the meristematic identity of the leaf margin [30,45–51]. KNOXI proteins affect CK and GA levels [52–55] and both clau and La mutants show altered TKN2 expression. These findings suggest a complex interaction among these three TFs and two central plant hormones in the regulation of the morphogenesis-differentiation balance [42,56–58].

CLAU, LA, TKN2, CK and GA were shown to modulate the morphogenetic window during tomato leaf development. However, how their activities are coordinated is not clear. In this work, we investigate the relationship between the transcription factors LA, CLAU and TKN2, and the plant hormones GA and CK, in the regulation of the morphogenesis-differentiation balance. We show that LA and CLAU effect essentially similar outcomes in tomato leaf development via likely partially parallel genetic pathways. These genetic pathways converge on the modulation of the CK/GA balance. Global transcriptomic analysis of genotypes with altered activity of LA, CLAU and TKN2 provide a molecular context by which the activity of additional regulators of morphogenesis and differentiation can be investigated.

**Results**

**LA and CLAU operate in parallel genetic pathways**

To better understand the genetic regulation of the balance between morphogenesis and differentiation, we examined the genetic relationships between lines with different activity levels of CLAU and LA (Fig 1 and [22,33]). To overexpress CLAU specifically in leaves we used the FIL promoter, which is expressed from early stages of leaf development (Fig 1E and [48]). Increasing CLAU levels in a high LA expression background (FIL>>CLAU La-2/+ (Figs 1C, 1D and 1N and S1) exacerbates the highly differentiated La-2/+ phenotype (Fig 1B). LA acts in a dose-dependent manner, with two mutated copies of the gain-of-function allele La-2 promoting differentiation and reducing leaflet numbers more strongly than one mutated copy (Fig 1B). Interestingly, overexpressing CLAU from the FIL promoter in a heterozygous La-2/+ background had the same effect as two mutated copies of the La-2 allele, as demonstrated in FIL>>CLAU La-2/+ leaves that were similar in size and complexity to La-2 homozygous leaves (Compare Fig 1B and 1D), suggesting an additive, dose-dependent interaction (Fig 1B and [33,39]). Decreasing CLAU levels in a low LA expression background such as the LA loss-of-function allele la-6 or FIL>>miR319 results in a significant increase in leaf elaboration.
Fig 1. CLAU and LA function in parallel pathways. (A-D, F-M) Genetic interactions between genotypes with altered CLAU and LA expression levels. La-2/+: a semi-dominant LA allele with increased and precocious expression due to miR319 resistance. La-6, FIL>>MIR319: LA null or LA downregulation, respectively. clausa: CLAU null. FIL>>CLAU: CLAU upregulation. All leaves depicted are fully expanded fifth leaves. Bars = 1 cm. White and red arrowheads represent primary leaflets and missing primary and intercalary leaflets, respectively. (E) Cartoon depicting the expression domain of the FIL promoter. (N-O) Quantification of leaf complexity in genotypes with altered CLAU and LA expression levels. Graphs represent mean ± SE of six independent biological repeats. Different types of leaflets are indicated according to the color code. Statistical significance of differences in the total leaflet
Decreasing CLAU levels in a high LA expression background (Figs 1G and 1O and S1) partially rescues the highly differentiated La-2/+ phenotype, while increasing CLAU levels in a low LA expression background (Figs 1I, 1M and 1O and S1) results in a decrease in leaf elaboration when compared with the decreased LA expression genotypes. Interestingly, though the number of primary leaflets is a relatively stable trait, and deficiency in either LA or CLAU does not affect this trait, deficiency in both genes significantly increases primary leaflet number (S1C Fig). The effect of CLAU and LA deficiencies is additive when examining secondary and tertiary leaflet numbers (S1E and S1F Fig), while CLAU deficiency alone affects intercalary leaflet number (S1D Fig), and is not augmented by LA deficiency in this trait, suggesting that CLAU operates in the leaf rachis area while LA does not. These genetic analyses demonstrate that the effect of CLAU and LA on leaf development is partially additive, indicating that they promote differentiation in at least partially parallel pathways.

LA activity defines the developmental window in which CLAU is expressed

Our previous results demonstrated that LA has a wider expression window than CLAU, and is active earlier in development [22,33,34]. Examination of the dynamics of CLAU expression in the first and fifth leaves of the plant, which represent a relatively limited and a relatively extended morphogenetic window, respectively [34], confirmed that CLAU is expressed mostly during the extended morphogenetic window (S2 Fig). As the morphogenetic window is partially defined by LA [33,34], this raised the possibility that low LA activity enabled the recruitment of CLAU in the regulation of leaf differentiation. To explore this possibility and gain more insight into the molecular basis of the additive interaction of LA and CLAU in promoting differentiation, we examined how LA activity affects CLAU expression, by assaying the expression of CLAU and its promoter in successive stages of leaf development in genotypes with altered LA activity (Fig 2). Early maturation caused by increased LA expression in the gain-of-function mutant La-2, led to a decrease in CLAU expression (Fig 2E–2H and 2M). Conversely, delayed maturation resulting from decreased LA expression in FIL>>miR319 resulted in increased CLAU expression (Fig 2I–2L and 2M). Interestingly, expressing a miR319-resistant form of LA (op:La-2) from the CLAU expression domain mimicked the La-2/+ phenotype (S3 Fig), with a slightly weaker effect when compared to expressing the same La-2 version from its own expression domain [59]. We conclude that LA activity defines the (spatial and temporal) developmental window in which CLAU is active. This window is reduced in La-2/+ and is prolonged in FIL>>miR319. Therefore, these TFs act in partially distinct spatial and temporal domains to promote differentiation.

TKN2 plays an essential role in extended morphogenesis

The class I KNOX homeobox transcription factor TKN2 is a key factor promoting morphogenesis in compound leaves [48,49,60–65]. Therefore, we set to examine the role of TKN2 in mediating the extended morphogenesis in plants with reduced CLAU or LA activity, by combining them with expression of TKN2-SRDX, in which TKN2 is fused to a repressive domain. Expressing TKN2-SRDX from the leaf-specific promoter BLS, which is expressed from the P4 stage of leaf development (Fig 3H), lacks any observable phenotype in the WT background (Figs 3A, 3D and 3G and S4 and [48]), likely due to the lack of TKN2 activity at this stage in
wild type leaves. Interestingly, BLS >> TKN2-SRDX suppresses the increased complexity of CLAU or LA deficient backgrounds (Figs 3E–3G and S4). In addition, expression of TKN2-SRDX from the LA expression domain (LA >> TKN2-SRDX) resulted in similar phenotypes to those of La-2/+ mutants (S3B Fig). In contrast, expressing TKN2 in either the LA or CLAU expression domains (pLA >> TKN2 and pCLAU >> TKN2, respectively) produced highly compound leaves (S3B–S3D Fig). Together, these results indicate that TKN2 takes part in the increased complexity resulting from compromised CLAU and LA activities, and that LA and CLAU may both act via TKN2. Alternatively, TKN2 is essential for the morphogenetic stage in both the wild-type context and when this window is extended by reduced CLAU and LA activities. In agreement with previous findings [42,56], the TKN2 promoter is more strongly activated at the leaf margin of CLAU or LA deficient backgrounds than in the wild type, while remaining mostly restricted to meristems in WT. Its expression is further elevated in the clau la-6 double mutant background (S5 Fig). This effect can again be an effect of the extended morphogenetic window and of TKN2 expression being one of the molecular characteristics of this window.

To further investigate the functional interaction among these factors, we examined the effect of combining altered CLAU and LA expression with TKN2 overexpression (Fig 4). Overexpressing TKN2 (Fig 4G) in a CLAU (Fig 4B) or LA (Fig 4D and 4E) deficient background (Fig 4H, 4J, 4K and 4N), leads to highly compound leaf forms. Overexpressing TKN2 in a CLAU (Fig 4C) or LA (Fig 4F) overexpression background (Fig 4I, 4L and 4N) leads to increased relative leaf elaboration and a rescue of the simplified leaf forms generated by overexpression of CLAU or LA. This rescue is substantial in the case of CLAU, and more moderate in the case of La-2/+ (Fig 4). Terminal leaflets are exemplified in shading in Fig 4M. These results indicate that the phenotypes observed upon loss of function of CLAU or LA are not solely due to TKN2 and that there are other morphogenesis-differentiation processes mediated
Overall, these results suggest that TKN2 acts antagonistically to CLAU and LA in tuning the morphogenesis-differentiation balance, and that reduced CLAU and LA activities enable the extension of morphogenesis, in which TKN2 plays a central role.

**CLAU and LA converge on the CK-GA balance**

We hypothesized that LA, CLAU and TKN2 may converge on common downstream pathways that are involved in leaf development. Several evidences suggest that both these TFs regulate the balance between the two plant hormones GA and CK. We previously demonstrated that CLAU functions through attenuation of CK signaling [22]. We and others have also previously shown that LA functions in part through GA signaling [21,66]. Previous work has also demonstrated that the Arabidopsis LA homolog, TCP4, reduces leaf CK response through binding and promoting expression of the CK response inhibitor ARR16 [41]. TKN2 was shown to affect both GA and CK [52,53,64]. Since both CLAU and LA promote differentiation in different pathways and spatiotemporal windows, and since CK and GA are partially antagonistic in leaf development [22,27,33,67], we examined the relationship between CLAU and GA, and LA and CK. In agreement with the antagonistic relationship between CK and GA in leaf morphogenesis, reducing CK content by overexpression of the CK inactivation gene CKX, or application of GA, led to simplification of leaf form (Figs 5B, 5D and 5N), and combining reduced CK with increased GA further reduced leaf complexity (Figs 5G and 5N and S6). Interestingly, the leaves of simultaneously reduced CK and increased GA levels resulted in
phenotypes that were very similar to that of La-2 and CLAU overexpressing plants (Fig 1). Conversely, inhibition of GA response via overexpression of a GA-resistant form of the GA response inhibitor DELLA/PROCERA (PRO) (PROΔ17) results in increased leaf complexity (Fig 5E). Interestingly, FIL >> PROΔ17 had an increased number of intercalary and secondary leaflets, similar to clausa mutants (Figs 1F and S1B and S1D), suggesting that CLAU could act
Fig 5. *clausa* has an altered GA profile and reduced sensitivity to GA treatment. (A-D) Phenotypes of leaves with reduced CK (*FIL*>>*CLAU*), or that received exogenous GA, (E-G) Phenotypes of leaves with reduced GA response (*FIL*>>*ΔPRO*), or increased CLAU and reduced GA response (*FIL* >> *CLAU+ΔPRO*), or reduced CK content (*FIL* >> *CKX*) treated with GA. (H-J) Effect of GA treatment on WT and *clausa*. All leaves depicted are fully expanded fifth leaves. Bars = 1 cm. White and red arrowheads represent primary leaflets and missing primary and intercalary leaflets, respectively. (K) Quantification of GAs in WT and *clausa*. Asterisks indicate significant differences between WT and *clausa* for each GA in an unpaired two-tailed t-test, *p*≤0.05, **p**≤0.01, ***p***≤0.001. (L) Expression of *SIGA20ox-1*, the enzyme that converts GA19 to GA20, in WT and *clausa*, was determined in young shoots (m+6) of 2-week-old plants using RT-qPCR. Graphs represent mean ± SE of five independent biological repeats. Asterisks indicate significant differences in an unpaired two-tailed t-test, *p* = 0.0078. (M) Depiction of GA biosynthesis pathways arrested in *clausa*. The compounds GA24, GA53 and GA19 accumulate (red underline) while the active GA4 and the GA1 precursor GA20 (green underline) are reduced, suggesting reduced GA20ox function. (N) Quantification of leaf complexity. Graphs represent mean ± SE of at least three independent biological repeats. Different types of leaflets are indicated according to the color code. Statistical significance of differences in the total leaflet number was examined in a one-way ANOVA, *p*<0.0001. Different letters indicate significant differences between samples in an unpaired two-tailed t-test with Welch’s correction (*p*<0.015). Statistical significance of differences in
via GA. In agreement, the simplified leaf phenotype caused by CLAU overexpression (Fig 5C) is rescued by co-expression of PROΔ17 (Figs 5F and 5N and S6), suggesting that GA may mediate the effect of CLAU on leaf differentiation.

These results suggested that CLAU may act via increased GA activity in addition to its inhibition of CK response, and that LA may act on the CK/GA balance also by reducing CK, as has been shown in Arabidopsis [41]. To test this hypothesis, we examined the effect clausa on GA content. Interestingly, GA4 and GA20 amounts were substantially reduced in 14-day-old clausa shoot apices, while the content of the more upstream GAs GA53 and GA19 increased (Fig 5K). This demonstrates that the GA pathway is blocked in clausa mutants at the step of the conversion of GA19 to GA20, a step catalyzed by GA20ox. In agreement with the accumulation of GA19 and decrease in GA20, and with previous findings [44], the expression of the tomato GA biosynthesis gene GA20oxidase-1 (SIGA20ox-1) was reduced in clausa mutants (Fig 5L). These results suggest that CLAU promotes differentiation by regulating GA biosynthesis, and that in clausa mutants, reduced levels of the GAs GA20 and GA4 and/or GA response facilitate prolonged morphogenesis and compound leaf shape.

To examine whether CLAU also influences the leaf sensitivity to GA, we treated WT and clausa plants with increasing GA concentrations (Figs 5H, 5I, 5J and 5O). The clausa mutant displayed a strong and significant reduction in GA sensitivity at the leaf margin, remaining highly compound despite GA treatments at WT-responsive concentrations (0.01–1 uM GA), and responding only to a whopping 10 uM of GA (Figs 5O and S7). Therefore, CLAU exerts its role in regulating differentiation through regulation of both GA levels and response.

In kind, The La-2/+ simple-leaf phenotype is exacerbated by overexpression of the CK inactivation gene CKX (La-2/+ FIL>>CKX) (Fig 6B). The reduced CK levels resulting from CKX overexpression were phenotypically equivalent to an extra mutated La-2 copy, similar to the effect of CLAU overexpression in a La-2/+ background (compare Figs 1B and 1D and 6B) [22]. In agreement, La-2/+ is partially rescued by overexpression of CK biosynthesis gene IPT (La-2/+ FIL>>IPT) (Fig 6D). Reducing CK in a LA deficient background shortens the morphogenetic window, partially rescuing the super compound phenotype of FIL>>miR319 (Fig 6F). In addition, similar to the arabidopsis TCP4, we found that LA reduced leaf sensitivity to CK, as evident from both the reduced signal of VENUS driven by the synthetic CK responsive promoter TCSv2 [68] in La-2/+ primordia (Figs 6G–6I and S8), and the increase in CK-dependent anthocyanin accumulation in LA deficient plants. As expected, in WT leaf primordia, the mean TCSv2 signal decreases during development (S8G Fig), as the CK signal decreases, while the corrected total fluorescence increases (S8H Fig), because the area being measured is larger as the primordia grows. We observed no differences in the fluorescence level in the meristem of the different genotypes (S8G and S8H Fig), and a reduction in TCSv2 driven expression in La-2/+ leaf primordia compared with WT, in both mean fluorescence (from the P2 stage) and corrected total fluorescence (from the P3 stage). CK promotes anthocyanin accumulation [27,41,69]. We therefore measured anthocyanin levels in the different LA genotypes as an additional measure of the effect of LA activity on CK sensitivity. LA deficiency caused increases in CK dependent anthocyanin accumulation (S9 Fig). LA was found to bind in vitro to the promoters of tomato response regulators (A-type TRRs) involved in CK...
signaling (S10 Fig). We found that LA, CLAU and the CK/GA balance also affect inflorescence complexity in a similar manner to their effect in leaves (S11 Fig). Thus, we conclude that both CLAU and LA enhance differentiation by reducing the plant’s sensitivity to CK and by elevating GA levels and/or response. Together, LA and CLAU affect the GA/CK balance, in turn tuning the morphogenesis-differentiation balance.
Global transcriptomic approach to identify common molecular pathways of morphogenesis and differentiation

To gain insights on leaf morphogenesis at the molecular level, we compared global transcriptomic data among the genotypes included in this study. Our findings suggest that the key regulators: LA, CLAU and TKN2 act in partially parallel pathways but also converge on the same downstream processes in the regulation of the balance between morphogenesis and differentiation. We thus compared several transcriptomic data sets from various genetic backgrounds with different activity of CLAU (the meristem and the four youngest leaf primordia of WT vs clausa) [22], LA (the meristem and the two youngest leaf primordia)-of La-2/+ gain-of-function), WT, la-6 loss-of-function, and FIL>>miR319 that down regulates LA and three additional CIN-TCPs TCP3, TCP10 and TCP24) [59] and TKN2 (the meristem and the five youngest leaf primordia of BLS>>TKN2 vs WT and BLS>>TKN2-SRDX) [48] (Fig 7). Microarray data sets for the LA genotypes and TKN2, and RNAseq data for the clausa mutant, were analyzed for Fold change. All data sets were generated by the Ori group, using the M82 background, with plants grown under essentially similar conditions in a controlled growth chamber. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was conducted to identify significantly differential pathways. Each genotype was compared to the M82, wild type background for the analysis. DEGs confirm dependencies between the LA genotypes, with between about a third to half of the genes significantly upregulated in La-2/+ being significantly downregulated in la-6 and upon miR319 overexpression (S1 Data). Likewise, about a third of the genes significantly downregulated in La-2/+ are significantly upregulated in la-6 or upon miR319 overexpression (S1 and S2 Datatas).

Interestingly, commonly up/downregulated genes are overrepresented between LA data-sets and TKN2 datasets, with 2–3 times more DEGs than expected being commonly upregulated in La-2/+ and downregulated upon TKN2 overexpression or upregulated upon TKN2-SRDX overexpression (S1 and S2 Datatas). In agreement with our genetic and molecular analyses, genes upregulated upon low LA expression (la-6, miR319 overexpression), or genes downregulated upon high LA expression (La-2/+), correlate best with those upregulated upon TKN2 overexpression or downregulated upon TKN2-SRDX overexpression (Fig 7A and 7B). This demonstrates that, to a degree that is significantly higher than expected from random sampling, the extended morphogenesis upon absence of LA activity correlates with increased TKN2 activity, and with downregulation of processes which are affected by inhibition of TKN targets.

KEGG pathway analysis of DEGs in all samples revealed that, in agreement with the results of this study (Figs 5 and 6) and with published data [21,22], plant hormone signal transduction pathways are affected in TKN2, CLAU and LA genotypes (Fig 7C and S1 and S2 Datatas). For example, GA signaling is altered in these genotypes, with DELLA/PROCERA upregulated in clausa and the GA-receptor GID1 upregulated in miR319 and TKN2 and downregulated in La-2/+ and TKN2-SRDX, providing molecular context for the altered sensitivity of these genotypes to GA. Interestingly, jasmonate pathways are upregulated in all the "morphogenetic" genotypes, most strongly in clausa, and ethylene signaling is uniquely upregulated in clausa, in addition to upregulation of plant pathogen interaction (p = 0.00058) and MAPK signaling (p = 0.0096) pathways (S1 and S2 Datatas). We have previously demonstrated that clausa is immuno-active and pathogen resistant [70]. Direct comparisons of the CLAU data generated by RNaseq, to the LA and TKN2 data sets, generated by microarray hybridization, was not undertaken in depth due to the methodological differences.

Analysis of increased morphogenesis genotypes (la-6, miR319 overexpression, clausa and TKN2 overexpression) revealed a significant increase in metabolic processes, carbon fixation,
biosynthesis of amino acids and glycolysis—perhaps required for the increase in activities required for leaflet generation (S1 and S2 Datas). Furthermore, it emerges that LA and TKN2 co-regulate protein processing and protein modification, with pathways of ER protein processing being upregulated in la-6 and TKN2 and downregulated in La-2/+, and Glycosylphosphati-dylinositol (GPI)-anchor biosynthesis being upregulated in la-6 and miR319 overexpression, and down regulated in La-2/+ and TKN2-SRDX (S1 and S2 Datas).
termed 'morphogenesis genes', and on genes that were successively upregulated throughout the developmental stages in the M82 background, to whom we referred to as 'differentiation genes'. Many classical morphogenesis genes were identified in the dataset based on this rule, validating our approach (see Fig 7F and 7G and S3 Data). When comparing these sets of 'morphogenesis' and 'differentiation' genes with the DEGs in our genotypes (Fig 7D and 7F) we found that, in nearly all cases, morphogenesis genes were significantly enriched in the morphogenetic genotypes clausa, la-6, and overexpression of miR319 or TKN2, while differentiation genes were significantly depleted in these genotypes (Fig 7 and S3 and S4 Datas). In agreement, differentiation genes were significantly enriched in La-2/+ and depleted in the morphogenetic genotypes (S3 and S4 Datas). Interestingly, the morphogenetic genes upregulated in clausa and miR319 overexpression showed no overlap, while the differentiation genes depleted in clausa and miR319 overexpression showed 10–15% overlap, supporting the hypothesis that emerges from our results, that CLAU and LA may regulate different genetic pathways in the leaf developmental program (Fig 7). While the absence of CLAU or LA activity can result in highly compound leaf forms, which may be similar in leaflet number (Figs 1, 3 and 4), their patterning results in visibly different leaf forms. This can be a result of spatial differences in their expression (S2 and S3 Figs) as well as their participation in different downstream pathways, as evidenced by their genetic interaction (Fig 1) and their influence on different "morphogenetic" genes (Fig 7D, 7F and 7H). While there are several patterns in which a leaf can be compound, the most simplified leaf forms generally resemble each other (Fig 7H).

**Discussion**

Investigating the underlying molecular mechanism of shape formation is crucial for our understanding of organ function. In this work, we used partially elucidated pathways to probe the convergence of two important transcription factor developmental regulators, CLAU and LA, on the balance between CK and GA, from different angles. We examined how the key regulators LA, CLAU and TKN2 interact in promoting and tuning differentiation during leaf development. Analysis of the interaction between CLAU and LA indicates that they operate in mostly parallel pathways (Figs 1 and S1), as LA exacerbates the effect of CLAU deficiency, leading to super-elaboration, while CLAU overexpression rescues LA deficiency, and LA overexpression rescues CLAU deficiency. This is also evident from the bioinformatics analyses (Fig 7 and S3 and S4 Datas). Our work suggests that LA might determine the developmental window during which CLAU acts (S2 Fig) [34]. Thus, despite the mostly parallel genetic pathways, decreased or delayed LA expression during early wild-type leaf development enables the existence of an extended morphogenesis window during which CLAU is expressed. CLAU in turn restricts further expansion of this window. Precocious LA activity thus renders CLAU activity irrelevant, since the spatio-temporal domain in which CLAU is active is essentially abolished upon an early increase in LA activity. This is in agreement with the unique role of CLAU in compound leaf species [22]. It will be interesting to identify additional compound-leaf specific regulators that were recruited in the context of extended morphogenesis. Similarly, the class I KNOX homeobox transcription factor TKN2 was also investigated in the context of extended morphogenesis of compound leaves [48,49,63–65], and was shown here to partially mediate the effect of both LA and CLAU in the regulation of the morphogenesis-differentiation balance. Here, we show that the CK-GA balance is a common mechanism that mediates both LA and CLAU activity. Leaf development has been reported to depend on the balance between CK, which promotes morphogenesis, and GA, which promotes differentiation [27,52,53,64,72,73]. The genetic interaction shown here between LA and CK (Fig 6) suggests that LA acts in part by reducing CK sensitivity. This is also supported by a previous report
showing an effect of TCP on the sensitivity to CK [41], suggesting that the role of TCP4 and LA is conserved between Arabidopsis and tomato. LA differentiation-promoting activity was shown to also depend on GA response [21,66,74]. CLAU promotes differentiation by elevating GA levels, and, in its absence, the plant becomes less sensitive to GA treatment at the leaf margin (Fig 5). We previously demonstrated that CLAU reduces CK sensitivity [22]. It thus emerges that CLAU and LA converge on the CK-GA balance: both promote differentiation by increasing the plants’ response to GA and reducing its response to CK. The length of the morphogenetic window within leaf differentiation can thus be viewed as an almost binary “lever” of sorts: pulling the lever towards CK will lengthen the window, while pulling it towards GA will shorten the window. It seems that the differentiation-morphogenesis and CK-GA balances are regulated and interpreted in a dose dependent manner (Fig 8). The mutation in the miR319 recognition site in La-2 is dominant, with the homozygote being more severely affected than the heterozygote (Fig 1B) [33,74]. Our results demonstrate a "dose" effect of transcription factor activity and hormone levels that is translated to leaflet number. Overexpression of both CLAU and LA, or either one of these transcription factors overexpressed with CKX (Figs 1 and 6; [22]), or the homozygous version of the dominant La-2 mutant, all exhibit simple leaves without any leaflets, indicating that the capacity for morphogenesis is embodied in the activity of LA, CLAU, CK and GA, acting in concert. It may suggest that additional regulators that were co-opted into the developmental program of compound leaves are regulating this balance. For example, KNOX proteins such as TKN2 regulate the CK-GA balance, by negatively regulating the expression of the GA biosynthesis gene GA20oxidase (GA20ox) and positively regulating the GA deactivation gene GA2oxidase (GA2ox) [52,54,55,64]. KNOXI proteins also activate CK biosynthesis genes and promote CK accumulation [52,53,55]. Here we show that GA20ox-1 is positively regulated by CLAU (Fig 5). It is therefore possible that the regulation of the CK-GA balance by CLAU and LA may be mediated in part through pathways common with TKN2. The GA-CK balance also plays a key role in meristem maintenance, which highlights the similarities between the shoot apical meristem and the transient meristematic phase in the leaf primordia that is required to preserve and enable organogenesis [75].

Fig 8 details a model depicting the roles of CLAU, LA and TKN2 in the CK/GA balance during leaf development. Both LA and CLAU may promote differentiation via inhibition of TKN2, though they also appear to have TKN2 independent activity. The activity of different transcription factors may affect the location of the lever between CK and GA and can do so within different spatial-temporal domains of the developmental program.

We conclude that CLAU and LA operate in mostly parallel pathways, having different spatial-temporal expression patterns and likely different downstream targets, based on our transcriptomic analyses. Their activity “funnels” from different pathways into the CK-GA balance, which is a common downstream mechanism to regulate morphogenesis and differentiation in plants. Overall, the genetic, molecular, and transcriptomic analyses we present here, provide insights into the molecular basis of differentiation and morphogenesis processes in plants. Our transcriptomic approach and generated data could be a potential starting point to identify and examine additional potential morphogenesis and differentiation factors in additional species and developmental processes.

Materials and methods

Plant material

Tomato seeds (Solanum lycopersicum cv M82 or as indicated) were sown in a commercial nursery and grown in the field or in a glasshouse under natural daylight with 25:18 °C (day: night) temperatures and a maximum light intensity of 450 μmol m⁻² s⁻¹. For developmental
and expression analyses, plants were grown in a controlled growth chamber, 300 μmol m⁻² s⁻¹ 18 h/6-h light/dark regime. All complexity analyses were conducted on leaf No. 5 of 7–8 week old plants.

Genotypes used in the present study were previously described: *clausa* [22,43,76]. *pFIL>>CLAU* [22]. *La-2/+* and *pFIL>>miR319* [33,34]. *pFIL>>IPT* and *pFIL>>CKX* [20]. *pBLS>>TKN2* and *pBLS>>TKN2-SRDX* [48]. *pFIL>>PRO17* [72]. *pTKN2::nYFP* was generated by amplifying ~5500 bp of genomic DNA upstream to the tomato *TKN2* atg using the primers detailed in S1 Table, fusing them to YFP with a nuclear localization signal, and transforming tomato plants—essentially as previously described for *pCLAU::nYFP* [22]. Additional genotypes were generated by crossing these genotypes, where indicated. *pTKN2::nYFP*,
pCLAU::nYFP [22], and pTCSv2:3XVENUS [22,78,79], were backcrossed into the relevant backgrounds.

Tissue collection and RNA analysis
Tissue collection, RNA preparation, and qRT-PCR analysis were performed as previously described [34]. Expression of all assayed genes was normalized relative to tomato EXPRESSED (EXP). Primer sequences used in qRT-PCR analyses are detailed in S1 Table.

Imaging
Leaves were photographed using a Nikon D5200 camera. For analysis of pTKN2::nYFP, pCLAU::nYFP, and pTCSv2:3XVENUS expression, dissected whole-leaf primordia were placed into drops of water on glass microscope slides and covered with cover slips. The pattern of YFP or VENUS expression was observed using a confocal laser scanning microscope (CLSM model SP8; Leica), with a solid-state laser set at 514 nm for excitation/530 nm for emission. Chlorophyll expression was detected at 488 nm excitation/700 nm for emission.

GA content analysis
Giberellins were isolated and purified according to the method described by [80].

Anthocyanin measurement
For anthocyanin measurement, plants were sprayed with the indicated CK concentrations three times a week for 3 weeks prior to analysis, starting upon emergence of the first leaf. Anthocyanins were extracted from the terminal leaflet of the third leaf by incubation overnight in methanol supplemented with a final concentration of 1% HCl. OD was measured in a plate spectrophotometer and anthocyanin content was calculated according to the following formula: \( \text{OD}_{530} \cdot \left(0.25 \cdot \text{OD}_{660}\right) \), normalized to the starting tissue weight. Three technical replicates of 3–8 biological repeats were performed for each sample.

Electrophoresis mobility shift assay (EMSA)
DNA probes were generated by end labeling of a 60-base single-stranded oligonucleotide using the DNA 3’ End Biotinylation Kit (Pierce 89818) and hybridization to complementary synthetic oligonucleotides (S1 Table) spanning binding sites for LA (GGNCC) which were identified using Sequencer 4.9, and generated with mutations disrupting the binding sites in the case of the mutant probe. Probes were generated by hybridizing the two complementary oligos by boil/cool. EMSAs were performed using the Light-Shift chemiluminescent EMSA kit (Pierce 20148). Briefly, 10 µL of purified recombinant MBP-LA fusion protein was incubated at room temperature in 1× binding buffer, 50 ng/µL poly(dI/dC), 2.5% glycerol, 0.05% Nonidet P-40, 50 fmol biotin-labeled probe, and 3.75 µg BSA for 30 to 40 min. The samples were resolved on 6% non-denaturing polyacrylamide gels, electrotransferred onto 0.45 µm Biodyne B nylon membrane (Pierce 7701), and cross-linked to the membrane. The migration of the biotin-labeled probe was detected on x-ray film (5-h exposure) using streptavidin–horseradish peroxidase conjugates and chemiluminescent substrate according to the manufacturer’s protocol.

Supporting information
S1 Fig. (Supplement to Figure 1): CLAU and LA function in parallel pathways–leaflet quantification. (A–F) Quantification of leaf complexity in genotypes with altered CLAU and LA expression levels. Graphs represent mean ± SE of six independent biological repeats. (A–B)
Stacked bars of total leaflet number split into leaflet types, bars = SE. Statistical significance of differences in the total leaflet number was examined in a one-way ANOVA, \( p < 0.0001 \). Different letters indicate significant differences between samples in an unpaired two-tailed t-test with Welch’s correction. (C-F) Graphs representing each leaflet type separately. Statistical significance of differences was examined in a one-way ANOVA, \( p < 0.0001 \). Different letters indicate significant differences between samples in a Tukey post hoc test (C) or a two-tailed t-test (D-F), (C) \( p < 0.0443 \), (D) \( p < 0.0039 \), (E) \( p < 0.045 \), (F) \( p < 0.022 \).

S2 Fig. CLAU expression in successive developmental stages of different leaves. Expression level of CLAU was determined using RT-qPCR. (A) CLAU expression in the fifth plastochron in leaves 1 through 4. Leaves 1 and 2, which have reduced complexity, also have reduced CLAU expression. Graph represents mean ± SE of three independent biological repeats. Letters indicate significant differences in a one-way ANOVA with a Tukey post-hoc test, \( p \leq 0.037 \). (B) CLAU expression in successive leaf developmental stages, comparing the first and fifth leaves. Dashed line indicates expression trend. Graph represents mean ± SE of three independent biological repeats. Asterisks indicate significant differences in an unpaired, two-tailed t-test, \( p \leq 0.0019 \).

S3 Fig. LA and CLAU expression domains and activity and TKN2. (A-D) Phenotypes of leaves of the indicated genotypes. All leaves depicted are fully expanded fifth leaves. Bars = 2 cm.

S4 Fig. (Supplement to Figure 3). TKN2 mediates the increased morphogenesis in CLAU- and LA-deficient backgrounds—leaflet quantification. (A–E) Quantification of leaf complexity upon overexpression of TKN2-SRDX in the background of CLAU and LA deficiency. Graphs represent mean ± SE of at least three independent biological repeats. (A) Stacked bars of total leaflet number split into leaflet types, bars = SE. Asterisks indicate significant differences of the total leaflet number from the background genotype (without TKN2-SRDX overexpression) in an unpaired two-tailed t-test with Welch’s correction, \( p \leq 0.0355 \). (B–E) Graphs representing each leaflet type separately. Statistical significance of differences was examined in a one-way ANOVA, \( p < 0.0001 \) (C–E). Different letters indicate significant differences between samples in a two-tailed t-test (B,C,E) or a Tukey post hoc test (D), (B) \( p = \text{ns} \) (no significant differences), (C) \( p < 0.0249 \), (D) \( p < 0.0052 \), (E) \( p < 0.049 \).

S5 Fig. TKN2 is expressed at the leaf margin in CLAU and LA deficient backgrounds (A–D) Expression pattern of the TKN2 promoter fused to YFP in WT M82 plants. (A) Vegetative meristem (m) and two youngest leaf primordia (P1, P2). (B) Floral meristem (FM), sympodial inflorescence meristem (SIM). (C–D) Weak expression is observed in P3/P4 leaf primordia (area of the leaf primordium depicted in C is indicated in the inset). (E) Confocal micrographs of pTKN::nYFP in the fourth plastochron (P4) in indicated genotypes. TKN2 promoter activation increases in the leaf margin in CLAU and LA deficient backgrounds. The pattern of YFP expression was detected by a confocal laser scanning microscope (CLSM model SP8; Leica), with the solid-state laser set at 514 nm for excitation and 530 nm for emission. Chlorophyll expression was detected at 488nm excitation/ 700nm emission. Bars = 200 um.

S6 Fig. (Supplement to Figure 5)/ clausa has reduced sensitivity to GA treatment—leaflet quantification. (A–E) Quantification of leaf complexity following GA treatment in WT and
clausa. Graphs represent mean ± SE of at least three independent biological repeats. (A) stacked bars of total leaflet number split into leaflet types, bars = SE. Statistical significance of differences in the total leaflet number was examined in a one-way ANOVA, p<0.0001. Different letters indicate significant differences between samples in an unpaired two-tailed t-test with Welch's correction (p<0.015). (B-E) Graphs representing each leaflet type separately. Statistical significance of differences was examined in a one-way ANOVA, p<0.0001. Different letters indicate significant differences between samples in a two-tailed t-test (C,D), (B) p<0.0043, (C) p<0.0111, (D) p<0.02, (E) p<0.03. (PDF)

S7 Fig. (Supplement to Figure 5). clausa has reduced sensitivity to GA treatment – leaflet quantification. (A-E) Quantification of leaf complexity following GA treatment in WT and clausa. Graphs represent mean ± SE of at least three independent biological repeats. (A) stacked bars of total leaflet number split into leaflet types, bars = SE. (B-E) Graphs representing each leaflet type separately. Statistical significance of differences was examined in a one-way ANOVA, p<0.0001 (C-E). Different letters indicate significant differences between samples in a two-tailed t-test, (B) p = ns (no significant differences), (C) p<0.0255, (D) p<0.0398, (E) p<0.0212. (PDF)

S8 Fig. CK response is reduced at the leaf margin upon LA overexpression. (A-F) Confocal micrographs of TCSv2::3XVENUS in successive developmental stages in indicated genotypes. TCSv2 driven signals are reduced in La-2/+ in the fifth plastochron. The pattern of VENUS expression was detected by a confocal laser scanning microscope (CLSM model SP8, Leica), with the solid-state laser set at 514 nm for excitation and 530 nm for emission. Chlorophyll emission was detected at 488nm excitation/700nm emission. Bars = 100 um. (G-H) Quantification of TCSv2 driven Venus fluorescence in indicated developmental stages of indicated genotypes. (J) Mean fluorescence % of WT meristem signal; (K) Corrected total fluorescence (CTF) was calculated as [integrated density–(area background mean fluorescence)]- presented as % of WT meristem CTF. Quantifications were done on at least 5 plants from each genotype in 3 experiments. Asterisks indicate significance from WT of the same developmental stage in a two-tailed t-test, *p<0.05, **p<0.01, ***p<0.001, ns = not significant. (PDF)

S9 Fig. LA deficiency enhances CK sensitivity to anthocyanin accumulation. Anthocyanin content in WT and altered LA genotypes with or without CK treatment was determined by measuring optical density following methanolic extraction. Graph represents mean ± SE of five independent biological repeats. Letters indicate significant differences in an unpaired, two-tailed t-test with Welch's correction, p<0.038. (PDF)

S10 Fig. LA binds in vitro to the promoters of TRRs. (A) LA binds in vitro to the promoters of TRRs. Recombinant MBP-LA caused a shift in the PAGE migration of biotinylated promoter derived probes (Probe-B) of the indicated TRRs. (B) Specific binding of LA to the TRR3/4 promoter. Recombinant MBP-LA caused a shift (s) in the PAGE migration of the biotinylated promoter derived probe (p) of TRR3/4. A non-biotinylated probe was able to compete with the biotinylated probe in a quantity depended manner (c1-c3). MBP (MBP empty) was used as a control protein and does not cause a probe shift in PAGE. (C) A biotinylated probe with a mutation in the binding site (m) does not shift in the presence of MBP-LA, and cannot compete with the WT probe (s+m1-m3). (PDF)
S11 Fig. **CLAU and LA function in parallel pathways in additional developmental processes.** (A–H) Genetic interactions between genotypes with altered **CLAU** and **LA** expression levels and **GA** levels or response. *La-2/+*: a semi-dominant **LA** allele with increased and precocious expression due to miR319 resistance. *FIL>>CLAU*: **CLAU** upregulation. Increased **GA** levels (**GA** application) or response: in **procera** (pro) loss-of-function. Bars = 2 cm. Red and white arrowheads represent flowers and leaf-like structures, respectively. (I) Quantification of inflorescence complexity in the indicated genotypes and treatments. Graphs represent mean ± SE of at least 4 independent biological repeats. Letters indicate significant differences between samples in a one-way ANOVA with a Tukey post-hoc test, p<0.0009.

(PDF)

S1 Table. Primers used in this work.

(PDF)

S1 Data. Lists of upregulated DEGs in all genotypes- excel file.

(XLSX)

S2 Data. Differential KEGG pathways in all genotypes- excel file.

(XLSX)

S3 Data. Morphogenesis and differentiation genes from Ichihashi et al., 2014- excel file. Classical morphogenesis genes identified in the data are highlighted.

(XLSX)

S4 Data. Representation factor and analysis of the overlap of "morphogenesis" and "differentiation" genes from Ichihashi et al., 2014, with **CLAU** and **LA** genotypes.

(PDF)

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