

CLAUSA Is a MYB Transcription Factor That Promotes Leaf Differentiation by Attenuating Cytokinin Signaling

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Leaf morphogenesis and differentiation are highly flexible processes, resulting in a large diversity of leaf forms. The development of compound leaves involves an extended morphogenesis stage compared with that of simple leaves, and the tomato (*Solanum lycopersicum*) mutant *clausa* (*clau*) exposes a potential for extended morphogenesis in tomato leaves. Here, we report that the *CLAU* gene encodes a MYB transcription factor that has evolved a unique role in compound-leaf species to promote an exit from the morphogenetic phase of tomato leaf development. We show that *CLAU* attenuates cytokinin signaling, and that *clau* plants have increased cytokinin sensitivity. The results suggest that flexible leaf patterning involves a coordinated interplay between transcription factors and hormones.

INTRODUCTION

Leaves are determinate organs, which differentiate to become major providers for the plant following a limited growth period. However, a transient period of indeterminate growth, termed primary morphogenesis, occurs during early stages of leaf development. During this stage, the leaf attains its basic shape and generates lateral appendages such as leaflets (Dengler and Tsukaya, 2001; Hagemann and Gleissberg, 1996; Kaplan, 2001). The spatial and temporal extent of this morphogenetic phase responds flexibly to the genetic, developmental, and environmental context. This flexibility gives rise to a wide diversity of leaf sizes and shapes that vary within and between species. Compound leaves, such as those of tomato (*Solanum lycopersicum*), are composed of multiple leaflets and are characterized by a prolonged morphogenetic phase. This extended morphogenesis is enabled by the activity of a meristematic region at the leaf margin, termed marginal blastozone (Hagemann and Gleissberg, 1996). The morphogenetic stage is particularly long in tomato, resulting in a wide diversity of leaf forms and unique developmental flexibility (Bar and Ori, 2014; Bar et al., 2015; Burko and Ori, 2013). Recent studies have uncovered an even higher potential for prolonged morphogenesis in tomato leaf development,

which is normally restricted by differentiation-promoting factors (Bar et al., 2015; Shani et al., 2010; Yanai et al., 2011). Characterization of mutants with extended or restricted morphogenesis has pointed to this potential and led to the identification of factors that balance morphogenesis and differentiation during leaf development (Sluis and Hake, 2015; Canales et al., 2010; Efroni et al., 2010; Bar and Ori, 2014; Blein et al., 2010).

Transcription factors from the class I knotted1-like homeobox (KNOXI) family were implicated in promoting the extended morphogenesis in leaves of many plant species, including tomato (Hareven et al., 1996; Janssen et al., 1998; Kimura et al., 2008; Bharathan et al., 2002; Hay and Tsiantis, 2006; Shani et al., 2009; Barth et al., 2009; Hake et al., 2004; Rast-Somssich et al., 2015). The plant hormone cytokinin acts downstream of KNOXI proteins to promote morphogenesis, mainly during relatively late stages of leaf development (Shani et al., 2010). By contrast, transcription factors from the CIN-TCP (CINNCINATA-TEOSINTE BRANCHED1-CYCLOIDEA-PCF) family, belonging to class II TCPs, restrict the morphogenetic window and promote differentiation (Burko et al., 2013; Efroni et al., 2008; Shleizer-Burko et al., 2011; Ori et al., 2007; Palatnik et al., 2003). The hormone gibberellin also promotes differentiation and partially mediates CIN-TCP function in this process (Yanai et al., 2011; Fleishon et al., 2011; Van Tuinen et al., 1999; Jasinski et al., 2008). The ratio of SINGLE FLOWER TRUSS and SELF PRUNING has also been shown to affect the balance between growth and differentiation in many developmental processes including leaf development, in addition to their role in regulating flowering time (Lifschitz et al., 2014; Shalit et al., 2009).

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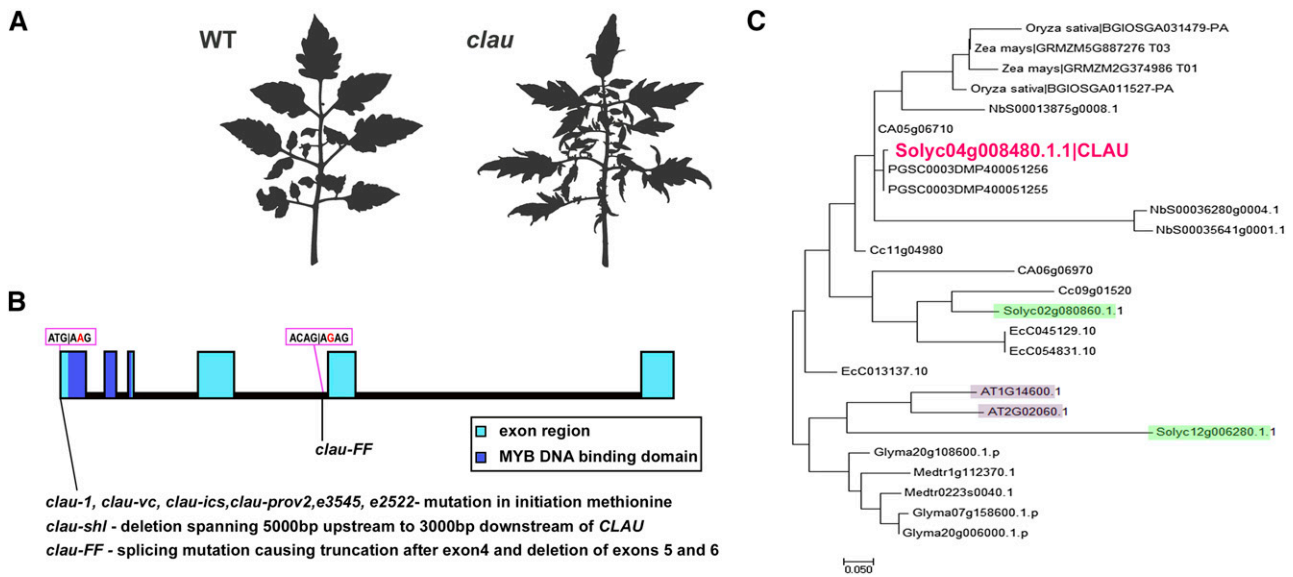


Figure 1. CLAUA Is a MYB Transcription Factor.

(A) Leaf 5 of wild-type and *clau:e3545* M82 tomato plants.

(B) Graphic representation of the *CLAU* ORF and the mutations found in the different *clau* alleles.

(C) Phylogenetic tree of *CLAU* homologs in different species. *CLAU* is in red. The two closest tomato homologs are marked in green, and the two closest Arabidopsis homologs are marked in purple. Rice, *Oryza sativa*; maize, *Zea mays*; *Nicotiana benthamiana*, Niben (Nb); pepper, *Capsicum annuum* (CA); potato, *Solanum tuberosum* (Stub, PGSC); coffee, *Coffea canefora* (Cc); eucalyptus, *Eucalyptus camaldulensis* (Ec); soybean, *Glycine max* (Glyma); Medicago, *Medicago truncatula* (Medtr).

clausa (*clau*), first described in the 1960s (Khush and Rick, 1967), is a classical tomato mutant characterized by increased morphogenetic potential that leads to abnormal, excessively divided leaves, often carrying shoot-like structures on the rachis. *CLAU* is a potential negative regulator of KNOX1 genes and of the boundary gene *GOBLET* (*GOB*) (Bar et al., 2015; Avivi et al., 2000; Jasinski et al., 2007, 2008; Naz et al., 2013).

CLAU therefore emerges as a factor that puts a hold on the morphogenetic potential of tomato leaves, enabling controlled maturation and morphogenesis. Here, we identify *CLAU* as a unique MYB transcription factor that promotes differentiation and attenuates CK responses.

RESULTS

CLAU Is a Unique MYB Transcription Factor

The classical tomato mutant *clau* possesses highly compound leaves (Figure 1A). We previously quantified the *clau* phenotype, demonstrating that *clau* leaves are on average 11 times more compound than wild-type leaves (Bar et al., 2015). The *clau* mutant phenotypes suggest that *CLAU* plays a central role in modulating the flexibility of leaf morphogenesis. We therefore wished to identify the *CLAU* gene to further understand this flexibility. We obtained eight *clau* alleles (Figure 1B) and, for the new alleles, confirmed allelism by complementation tests. Of these, *clau:e3545* and *clau:e2522* were identified in an EMS population (Menda et al., 2004), generated in the *S. lycopersicum* M82 background. Leaves of all eight alleles were similar, allowing for

slight differences resulting from the different background cultivars (Supplemental Figure 1). *CLAU* was previously mapped to the short arm of chromosome 4 using radiation ablation mutagenesis (Khush and Rick, 1967). We created an F2 mapping population from a cross between *clau:e3545* in the *S. lycopersicum* M82 background and *Solanum pimpinellifolium* and used this mapping population to pinpoint the physical location of *CLAU* between bases 2094866 and 2122247 on the short arm of chromosome 4, an area which contains two genes. RNA sequencing of shoot apices of the EMS allele *clau-e3545*, in comparison to wild-type M82 apices, revealed a mutation in the first methionine codon of a gene encoding a MYB transcription factor (*Solyc04g008480*, named *CLAU* hereafter), which is physically located within this interval. The mutation is predicted to prevent the formation of any protein from the *CLAU* open reading frame, which encodes a 297-amino acid SHAQKYF-class MYB transcription factor, possessing six exons (Figure 1B). *clau* and five additional alleles contain the exact same mutation, an ATG-to-AAG substitution in the codon for the initiation methionine. *clau-shl* is a deletion allele, where no other annotated genes are deleted, and *clau-ff* results from a splicing mutation leading to a truncated protein of 150 amino acids, lacking exons 5 and 6 (Figure 1B). We found *CLAU* homologs in several plant species (Figure 1C; Supplemental Data Set 1), including *Arabidopsis thaliana* (*AT1G14600* and *AT2G02060*). Figure 1C depicts a phylogenetic analysis of *CLAU* homologs in a variety of different plant species that have published annotated genomes. *CLAU* is not highly conserved in Arabidopsis, as the Arabidopsis homologs, *AT1G14600* and *AT2G02060*, possess only 35 and 32% global identity, respectively, to *CLAU*, with over

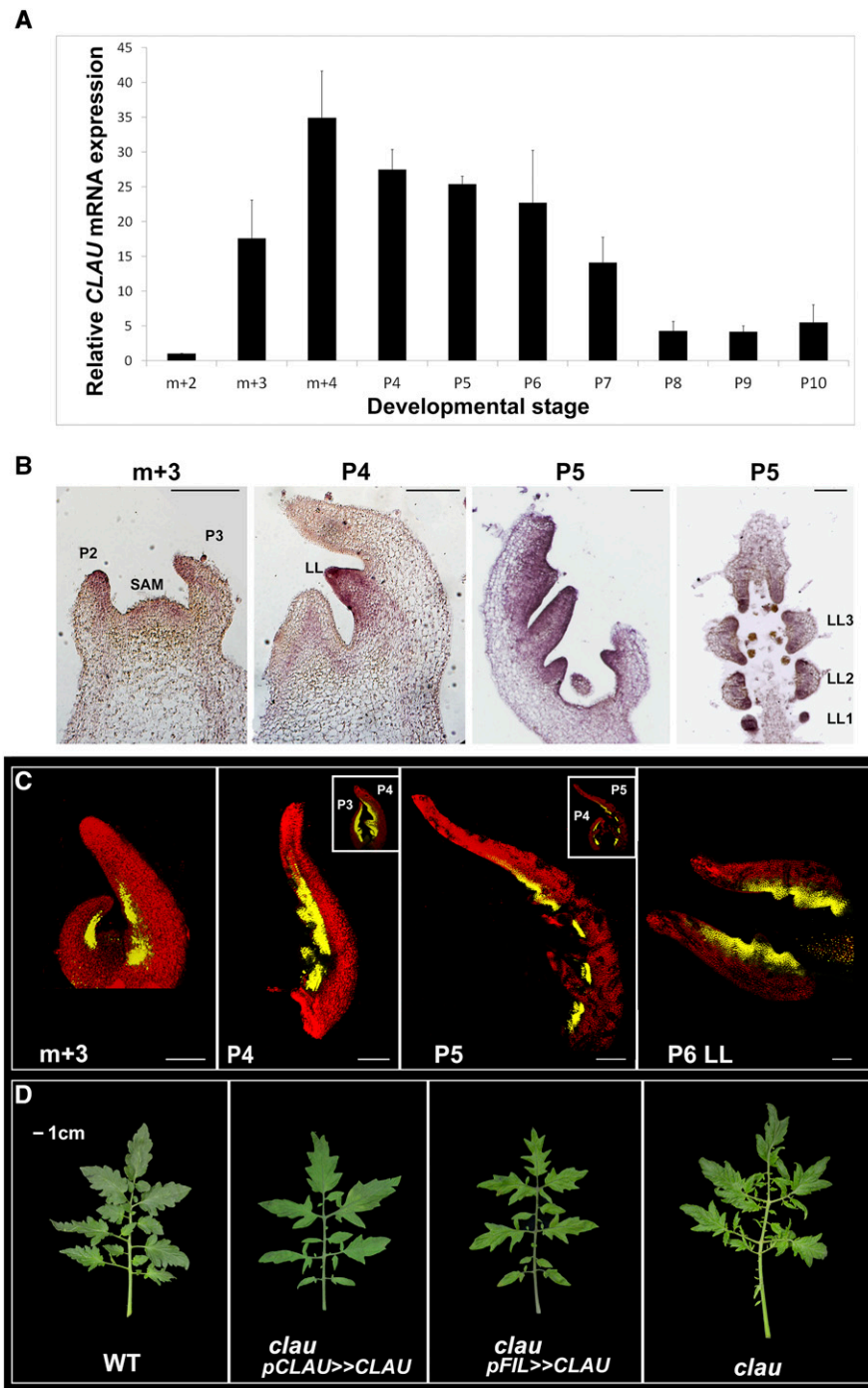


Figure 2. *CLAU* Is Expressed during Leaflet Morphogenesis at the Leaf Margin.

Analysis of *CLAU* expression during development. Leaf development follows plastochrons, such that P1 is the most recently initiated leaf primordium, and it becomes P2, P3, etc., as new primordia emerge from the SAM, with leaflets initiating basipetally from the P3 stage on.

(A) qRT-PCR analyzing *CLAU* mRNA expression levels at successive stages of leaf development. Bars are the SE of at least four biological replicates.

(B) In situ hybridization of *CLAU* mRNA in tissue sections of wild-type apices and leaf primordia, as indicated.

(C) Confocal micrographs of wild-type apices and leaf primordia, expressing YFP driven by the *CLAU* promoter. Insets show the plant shoot containing the depicted stage. Bars = 200 μ m.

(D) Fifth leaf of the indicated genotypes. Expression of *CLAU* from the *CLAU* promoter rescues the *clau* mutant phenotype. m+2, m+3, m+4, SAM and 2, 3, or 4 youngest leaf primordia; LL, lateral leaflet.

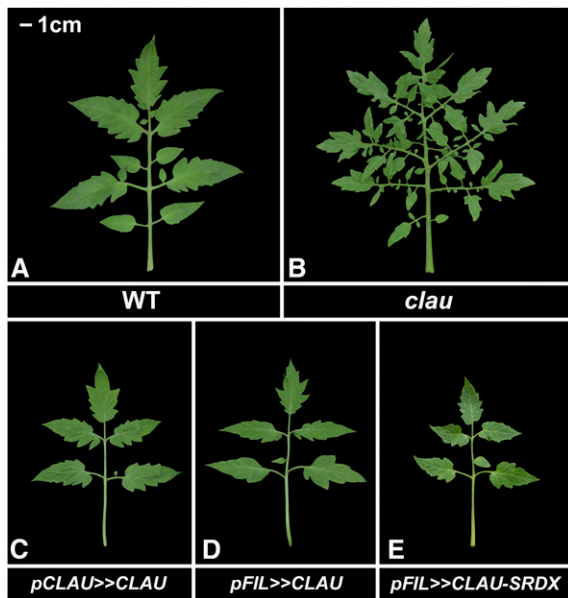


Figure 3. Overexpression of *CLAU* Promotes Differentiation.

Leaves of the wild type (**A**), *clau* (**B**), plants overexpressing *CLAU* under the control of the indicated promoters (**C**) and (**D**), and plants overexpressing *CLAU* fused to the *SRDX* repressor domain under the control of the *FIL* promoter (**E**). All leaves depicted are leaf 5. Bar = 1 cm.

30% gaps in the alignment, and cluster in a different clade than *CLAU*, closer to its distant tomato homolog Solyc12g006280 (Figure 1C). To further examine possible *CLAU* homologs between different plant species, we examined the synteny of the genes within the tomato chromosomal region containing *CLAU* to Arabidopsis and several Solanaceae species. Although AT1G14600 is slightly more homologous to *CLAU* than AT2G02060, the genes within the respective region in Arabidopsis chromosome 2 that contains *AT2G02060* show greater synteny with the *CLAU* region in tomato chromosome 4 (Supplemental Figure 2). Comparison of these syntenic regions suggests several rearrangements. The additional Solanaceae species presented have significant gene synteny in this region to tomato, in particular potato (*Solanum tuberosum*; chromosome 4) and pepper (*Cap-sicum annuum*; chromosome 5). The other species have only partially annotated published genomes, so the analysis may not be complete. Comparison between *CLAU* and other tomato MYB transcription factors shows that *CLAU* is relatively unique in tomato (Supplemental Figure 3 and Supplemental Data Set 2; see below), as the two closest tomato homologs, Solyc02g080860 and Solyc12g006280 (Supplemental Figures 3A and 3C; Figure 1C), share only 28 and 22% global identity, respectively, with *CLAU*. Local identities of 78 and 49%, respectively, are limited to the DBD region, where the SHAQKYF motif is highly conserved (amino acids 10 to 100; Supplemental Figures 3A and 3B). Overall, the 20 tomato proteins closest to *CLAU* all contain SHAQKYF domains (interpro IPR006447) and cluster on separate but close branches to *CLAU*, when compared with several tomato MYBs that are involved in developmental processes and contain R2R3 MYB domains (interpro IPR17930) (Supplemental Figure 3A).

***CLAU* Is Expressed at the Leaf Margin to Restrict the Morphogenetic Window**

Pursuant to the identification of the *CLAU* gene, we proceeded to examine the expression patterns of the *CLAU* mRNA during leaf development using qRT-PCR (Figure 2). *CLAU* is expressed primarily during the P3-P7 stages of leaf development (Figure 2A), with expression peaking between P4 and P6. In situ mRNA hybridization localized *CLAU* mRNA expression to developing leaflets at the leaf margin (Figure 2B), correlating with the developmental stages during which the *CLAU* mRNA expression peaks. Expression of YFP under the control of the *CLAU* promoter, consisting of 2700 bp upstream of the *CLAU* translational start site, showed promoter activity at the leaf margin and in developing leaflets (Figure 2C), in a pattern similar to that observed for the *CLAU* mRNA in the in situ mRNA hybridization (compare Figures 2B and 2C). The 2700-bp *CLAU* promoter was able to rescue the *clau* mutant when driving *CLAU* cDNA expression (Figure 2D).

Loss of *CLAU* function results in extended morphogenesis and extremely elaborate leaves. Identification of the *CLAU* gene allowed us to examine the effect of *CLAU* gain of function. Overexpression of *CLAU* under the control of its own promoter or the *FIL* promoter (Lifschitz et al., 2006; Shani et al., 2009), using a *trans*-activation system (Moore et al., 1998), led to a simplified leaf possessing primary leaflets and very few secondary leaflets (Figure 3). This confirms that *CLAU* is involved in curbing leaf morphogenesis, in line with the more elaborate leaf observed in the *clau* mutant. The simplified-leaf phenotype caused by expressing *CLAU* under the control of its own promoter suggests that *CLAU* affects morphogenesis in a dose-dependent manner. This is also supported by the observation that *clau/+* heterozygotes occasionally showed a very mild *clau* phenotype. To further investigate the role of *CLAU* in leaf development, we overexpressed a fusion of *CLAU* with the *SRDX* repression motif (Hiratsu et al., 2003) in wild-type leaves, under the control of the *FIL* promoter, using the *trans*-activation system. The *CLAU-SRDX* fusion was expected to downregulate *CLAU* targets, affecting the downstream developmental pathways and providing insight into the usual mode of action of *CLAU*. Overexpression of *CLAU-SRDX* resulted in leaves very similar to those resulting from overexpression of *CLAU* alone (Figures 3E and 3D), suggesting that *CLAU* may act primarily as a repressor of its downstream targets.

***CLAU* Has a Unique Role in Compound-Leaf Development**

As detailed above, *CLAU* is relatively unique in tomato and its closest Arabidopsis homolog has low identity (32%) to *CLAU*. *CLAU* has close orthologs in additional Solanaceae species. To compare the nature of the *CLAU* genes in simple- and compound-leaf species, we analyzed the closest *CLAU* homologs in several additional Solanaceae species (Figure 4): potato, which has compound leaves, as well as eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*), and pepper, with simple leaves. A phylogenetic tree of the two closest *CLAU* homologs in several Solanaceae species is presented in Figure 4A (sequence alignment in Supplemental Data Set 3). Potato *CLAU* (St-*CLAU*, PGSCDMP400051255) is the closest ortholog to *CLAU*; pepper (CA0506710) and eggplant (Sme2.501601) sequences are

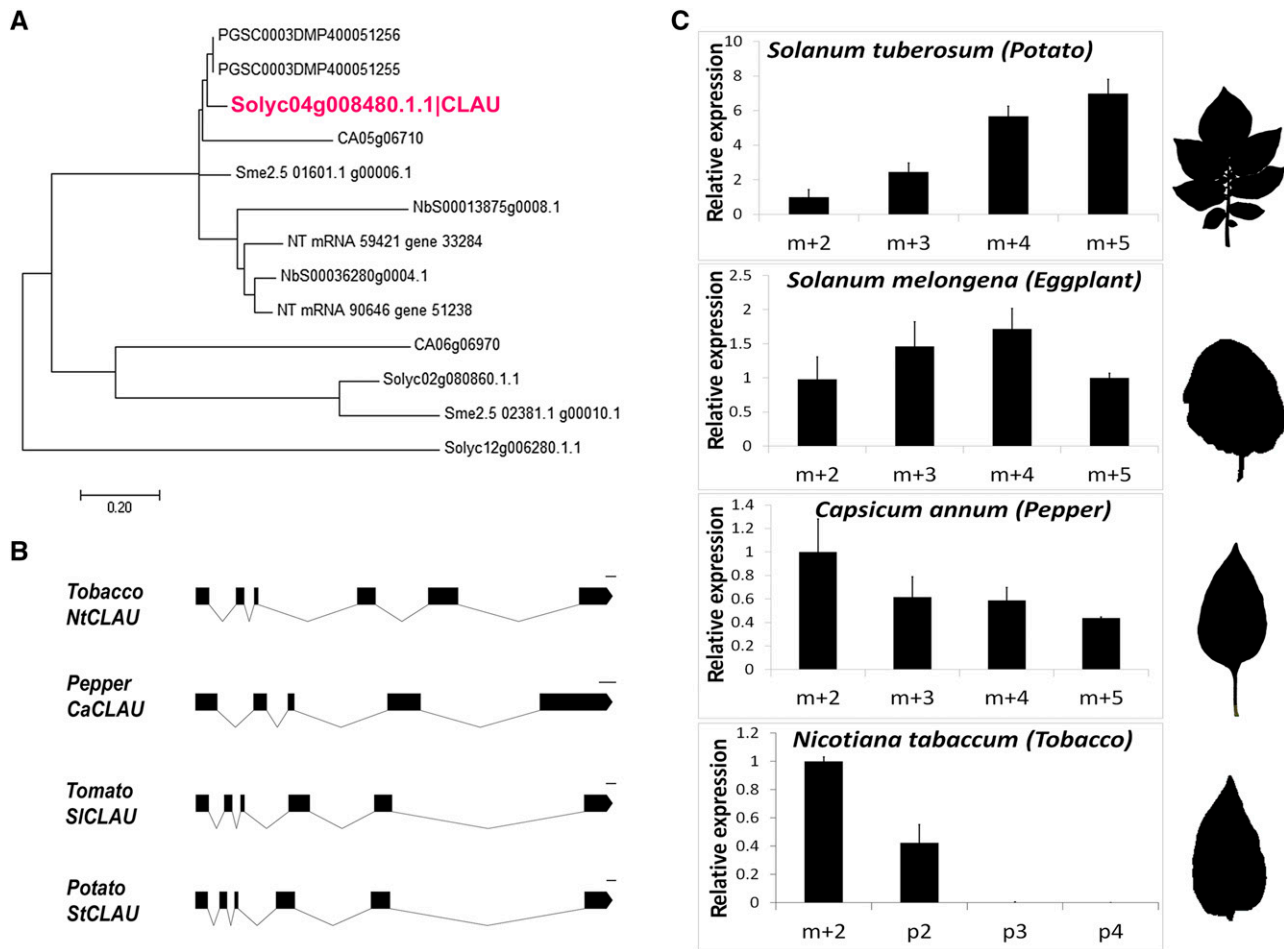


Figure 4. CLAU Has Unique Expression Dynamics during Compound Leaf Development.

(A) Phylogenetic analysis of CLAU in the Solanaceae. The Si-CLAU protein is closest to the potato proteins (PGSC), followed by pepper and eggplant. The two closest tomato homologs (Solyc02g080860 and Solyc12g006280) cluster further away than the other Solanaceae homologs and have homologs of their own in the other Solanaceae species.

(B) Gene structure (bar = 100 bp) of CLAU in several Solanaceae species. The gene is composed of six exons and five introns, except in pepper, which possesses five exons and four introns. The genomic sequence of the eggplant CLAU is not available.

(C) qRT-PCR analyzing CLAU mRNA expression levels at early stages of leaf development in the indicated plastochrons in potato, eggplant, pepper, and tobacco. Final leaf shape is depicted to the right. Bars are the SE of at least four biological replicates. m+2, m+3, m+4, SAM and 2, 3, or 4 youngest leaf primordia, respectively.

slightly more removed, and those of *Nicotiana benthamiana* (Nbs00013875) and tobacco (NT 33,284) are less similar (Figure 4A). St-CLAU bears 87% global identity to Si-CLAU, spanning the entire protein. The St-CLAU mRNA had similar expression dynamics to Si-CLAU during early leaf development (Figure 4C; compare with Figure 2A). The eggplant ortholog, Sm-CLAU, is 72% identical globally to CLAU. Its expression decreased at the P5 stage, when Si-CLAU and St-CLAU were still highly expressed (Figure 4C). The pepper ortholog Ca-CLAU contains only five exons (Figure 4B) and encodes a shorter protein than Si-CLAU. Ca-CLAU shares 54% global identity and 71% local identity with the N-terminal two-thirds of the Si-CLAU protein. Ca-CLAU mRNA was expressed at earlier developmental stages and its expression decreased at the P3 stage, when Si-CLAU and

St-CLAU started to increase in expression, toward the formation of leaflets at the leaf margin (Figure 4C). The *N. benthamiana* Nb-CLAU and *N. tabacum* Nt-CLAU proteins share ~55% global identity with Si-CLAU. Similar to the observed expression pattern in pepper, the expression of Nt-CLAU decreased at the P3 stage (Figure 4C; developmental stages collected in tobacco were slightly different than in the other species due to technical constraints). Thus, the degree of similarity to Si-CLAU and the expression dynamics and leaf shape were correlated in the tested species. This suggests that CLAU exerts part of its effect on leaf form during the formation of leaflets, in a developmental window that exists only in compound leaves. Simple leaves have a shorter morphogenetic window and thus lack the developmental window in which CLAU is active in compound leaves. In both leaf types,

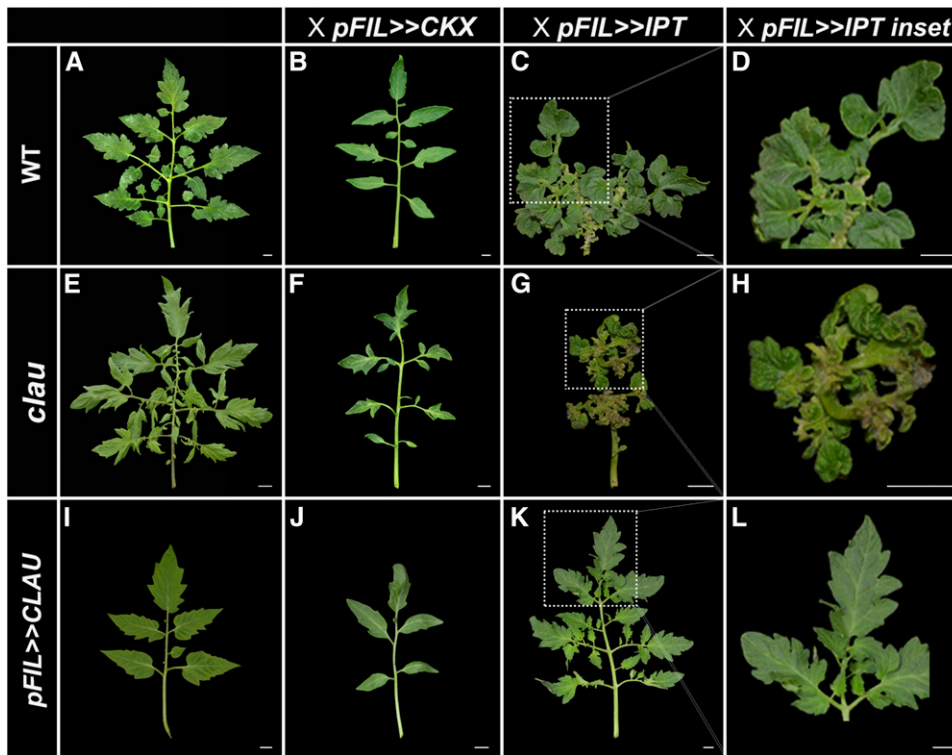


Figure 5. *CLAU* Attenuates CK Response.

Genetic interactions between *clau*, *pFIL>>CLAU*, and *pFIL>>CKX* or *pFIL>>IPT*. Genotypes are indicated above each panel. All leaves depicted are fully expanded leaf 5. Bars = 1 cm.

CLAU likely functions during early leaf development to attenuate meristematic activity and promote leaf fate.

CLAU Attenuates Cytokinin Signaling

Overexpression of the Arabidopsis cytokinin (CK) biosynthesis gene *ISOPENTENYLTRANSFERASE7 (IPT7)* in tomato leaves led to the formation of highly compound leaves, whereas decreasing the CK levels via the expression of the Arabidopsis CK degradation gene *CYTOKININ OXIDASE (CKX3)* resulted in reduced leaf complexity (Shani et al., 2010) (Figure 5). Some aspects of the leaf phenotype caused by *IPT7* overexpression are reminiscent of the *clau* phenotype, including a highly compound leaf, many ectopic meristems on the leaf, and an extension of the developmental window of leaflet morphogenesis (Bar et al., 2015). The simplified leaf phenotypes resulting from overexpression of *CLAU* and *CKX* were also similar. This prompted us to examine whether an altered CK response could be involved in the *clau* phenotype. Overexpressing *pFIL>>IPT7* in a *clau* background (Figures 5G and 5H) enhanced the phenotype caused by elevated CK levels alone (Figures 5C and 5D). Conversely, reducing CK biosynthesis in the *clau* background was substantially epistatic to *CKX* overexpression (Figure 5F), as the leaf was only slightly more complex than the leaf overexpressing *CKX* alone (Figure 5B), suggesting that the *clau* phenotype depends at least in part on CK. Interestingly, overexpressing *IPT7* and *CLAU* simultaneously under the control of the *FIL* promoter resulted in a significant rescue of the *FIL>>IPT7*

phenotype (Figures 5K and 5L), indicating that *CLAU* can affect the CK response. Taken together, these results suggest that CK dynamics may mediate *CLAU* function.

Expression of the CK-Response Reporter TCS Is Upregulated in *clau*

To further examine whether CK dynamics could be altered by *CLAU*, we compared the expression of a CK activity reporter TCS fused to VENUS (Zürcher et al., 2013; Steiner et al., 2016) between wild-type tomato plants and the *clau* mutant (Figure 6). We first confirmed that TCS is CK responsive in tomato as was reported in Arabidopsis (Supplemental Figure 4). To avoid positional effects, we compared a wild-type TCS-expressing line with the same line that was introgressed into *clau* by a cross. In wild-type shoot apices, TCS drove expression in the shoot apical meristem (SAM) and at the leaf margin of developing leaves (Figures 6A to 6E). TCS-driven expression was strongly upregulated in the *clau* background (Figures 6F to 6J). Quantification of TCS-driven fluorescence showed that TCS was significantly upregulated in *clau* at all developmental stages (Figure 6K), except in the SAM where its expression was already high in the wild-type background, and where *CLAU* expression was low (Figures 2A and 2B). In the wild type, TCS-driven expression was high in the SAM, decreased in P1, peaked again between P2-P5, and subsequently decreased (Figure 6K). In *clau*, however, all developmental stages showed similar, relatively high TCS-driven expression.

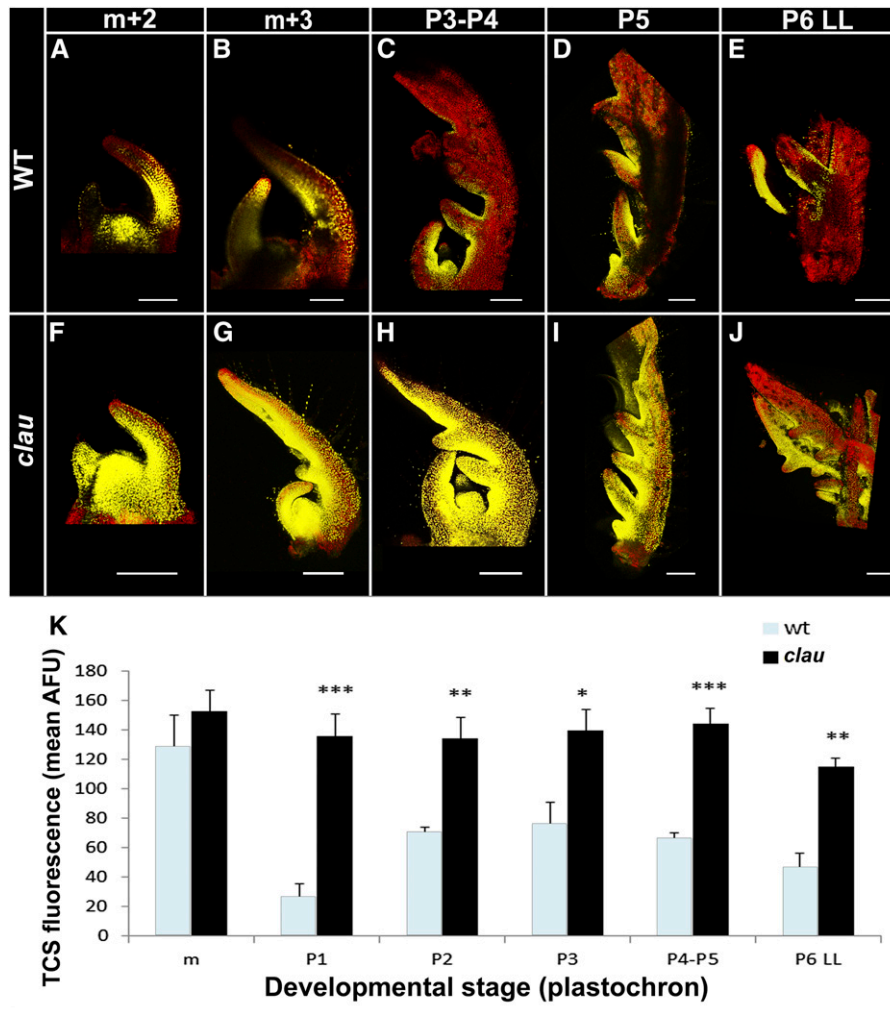


Figure 6. *clau* Has an Upregulated CK Response.

The CK response reporter TCS is upregulated in *clau*. Confocal micrographs of wild-type (**A**) to (**E**) and *clau* (**F**) to (**J**) apices and/or leaves at the indicated developmental stages, expressing VENUS driven by the CK reporter TCS. VENUS quantification is presented in (**K**); three to seven meristems or primordia were analyzed for each developmental stage. Error bars represent SE, and asterisks indicate statistical significance (two-tailed Student's *t* test) of up-regulation in *clau* over the wild type. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. LL, lateral leaflet. Bars = 200 μ m.

clau Has Increased CK Sensitivity

The genetic results suggesting an involvement of CK in the *clau* phenotype and the increase in TCS reporter-driven expression prompted us to examine whether *clau* could have increased CK content (Figure 7). Surprisingly, young *clau* primordia have a lower amount of CKs (Figure 7A), including *trans*-zeatin, which is the most abundant CK in these tissues. An increase in CK signaling coupled with a decrease in CK content led us to investigate whether *clau* affects CK sensitivity. In three independent biological assays (Coenen and Lomax, 1998; Fleishon et al., 2011), *clau* plants responded to CK concentrations a tenth (anthocyanin production; Supplemental Figure 5B) to a hundredth (root growth, Figure 7B; hypocotyl height, Supplemental Figure 5A) lower than the concentrations required to elicit a response in wild-type plants. Another hallmark of CK

response is an increase in the transcripts of type A response regulator (RR) genes (Keshishian and Rashotte, 2015; Shi et al., 2013; D'Agostino and Kieber, 1999; Brandstatter and Kieber, 1998). The Arabidopsis type A RRs *ARR3* and *ARR5* respond strongly to CK and inhibit CK response (Shi et al., 2013; To et al., 2004). Interestingly, the baseline transcript level of the tomato type A RRs *TRR3* and *TRR5* was significantly lower in *clau* than in the wild type (Figure 7C), suggesting that TRRs may mediate the effect of CLAU on cytokinin sensitivity. Examining the type A TRR transcript response to CK treatment demonstrated that the response of *TRR3* and *TRR5* to CK was stronger in *clau* than in the wild type (Figures 7D and 7E). We also examined the response of *TRR8/9A*, *8/9B*, *8/9C*, *16/17*, and *16B* to CK, and with the exception of *TRR8/9C*, all demonstrated a statistically significant higher transcriptional response following CK treatment in *clau* than in the wild type (Supplemental Figures 5C to

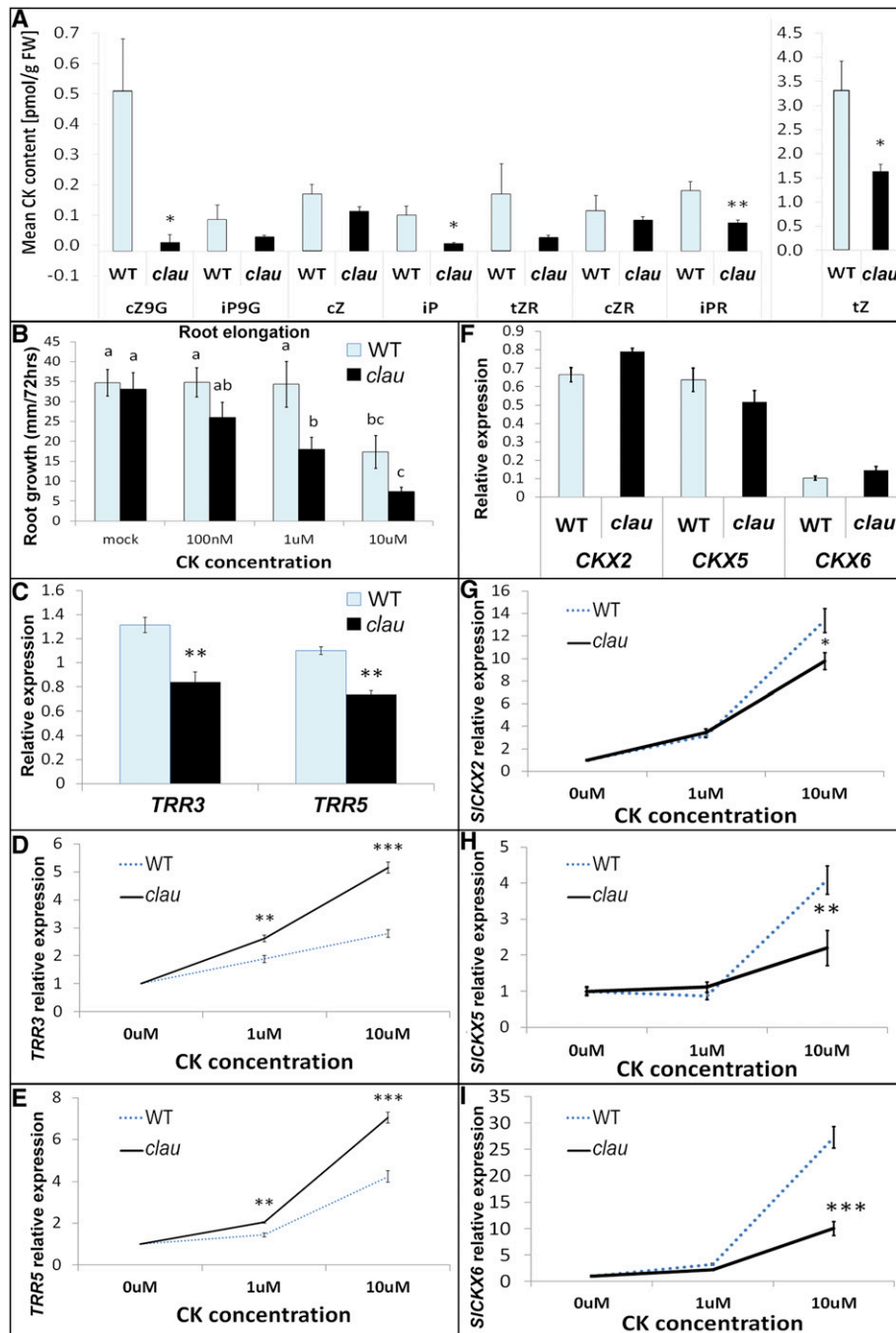


Figure 7. *clau* Has Decreased CK Content and Increased CK Sensitivity.

(A) Quantification various CK derivatives in *clau* and wild-type young leaves (P5-6 developmental stage). cZ9G, *cis*-zeatin-9-glucoside; iP9G, iso-pentenyl-9-*N*-glucoside; cZ, *cis*-zeatin; iP, iso-pentenyl-adenine; tZR, *trans*-zeatin riboside; cZR, *cis*-zeatin riboside; iPR, iso-pentenyl riboside; tZ, *trans*-zeatin.

(B) Root growth response to CK in *clau* and wild-type seedlings.

(C) qRT-PCR analyzing *TRR* mRNA expression levels in untreated *clau* and wild-type shoot apices.

(D) and (E) qRT-PCR analyzing *TRR* mRNA expression levels in response to CK in *clau* and wild-type shoot apices.

(F) qRT-PCR analyzing *CKX* mRNA expression levels in untreated *clau* and wild-type shoot apices.

(G) to (I) qRT-PCR analyzing *CKX* mRNA expression levels in response to CK in *clau* and wild-type shoot apices.

In (D), (E), and (G) to (I), the expression in untreated apices is set to 1. In all cases, the SAM and five youngest leaf primordia were collected, and bars represent the mean \pm SE of at least five biological repeats. Asterisks indicate statistical significance (two-tailed Student's *t* test) of upregulation or downregulation in *clau*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005.

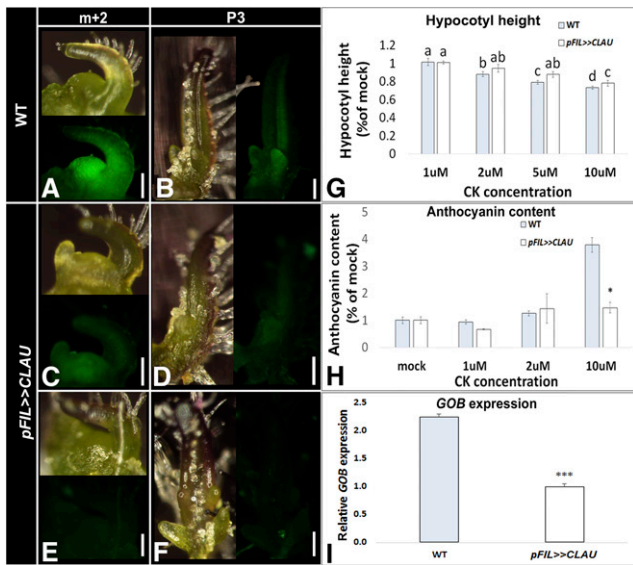


Figure 8. *pFIL>>CLAU* Has Decreased CK Sensitivity and Decreased *GOB* Expression.

(A) to (F) The CK response reporter TCS is downregulated in *pFIL>>CLAU*. Stereomicroscope images of wild-type (A) and (B) and two lines of *pFIL>>CLAU* (C) to (F) apices and/or leaves at the indicated developmental stages, expressing VENUS driven by the CK reporter TCS. Bars = 100 μ m. (G) Hypocotyl height response to CK in *pFIL>>CLAU* and wild-type seedlings.

(H) Anthocyanin production response to CK in *pFIL>>CLAU* and wild-type leaves.

(I) qRT-PCR analyzing *GOB* mRNA expression levels in *pFIL>>CLAU* and wild-type shoot apices (m-P5).

Bars represent the mean \pm SE of 5 to 12 biological repeats, and asterisks indicate statistical significance (two-tailed Student's *t* test). **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001.

5H). We conclude that an absence of an active *CLAU* protein leads to increased CK sensitivity, suggesting that *CLAU* functions in attenuating the response to CK during development, possibly via positive regulation of TRR expression. A possible mechanism to counterbalance the increased CK sensitivity found in *clau* could be through CKX regulation. *CKX2*, 5, and 6 were all induced in tomato leaves following CK treatment (Shi et al., 2013). Interestingly, *clau* had similar levels of *CKX2*, 5, and 6 prior to CK treatment (Figure 7F). However, while *CKX2* (Figure 7G), *CKX5* (Figure 7H), and *CKX6* (Figure 7I) were induced by 1 μ M CK to a similar degree as in the wild type, they were induced to a lower degree in *clau* than in the wild type by 10 μ M CK. This suggests that CKXs may feed back to the already low levels of CKs in *clau* rather than respond to the increased CK sensitivity.

Overexpressing *CLAU* Decreases CK Sensitivity

To complement the analysis presented in Figure 7, we examined whether overexpression of *CLAU* could decrease CK sensitivity. We first compared the expression of the *TCSv2:3XVENUS* reporter detailed above between wild-type tomato plants and

several transgenic lines overexpressing *pFIL>>CLAU* (Figure 8). Once again, we compared a wild-type TCS-expressing line with the same line that was introgressed into *pFIL>>CLAU*. TCS-driven expression was downregulated in a *pFIL>>CLAU* background (Figures 8C to 8F). *pFIL>>CLAU* plants also showed a decrease in CK sensitivity, requiring higher CK concentrations to elicit a decrease in hypocotyl height (Figure 8G) or an increase in anthocyanin content (Figure 8H).

Overexpressing *CLAU* Leads to Reduced *GOB* Expression

In previous work (Bar et al., 2015), we showed that part of the *CLAU* mechanism of action in tomato involves negative regulation of the *NAM/CUC* gene *GOB*. We previously demonstrated that *GOB* is upregulated in a *clau* background and that downregulation of *GOB* via overexpression of its regulatory microRNA, miRNA164, results in a dramatic rescue of the *clau* phenotype (Bar et al., 2015). Upon identification of the *CLAU* gene, we wanted to examine whether upregulation of *CLAU* has an effect on *GOB* expression. Figure 8I shows that overexpression of *CLAU* caused downregulation of *GOB*, strengthening the assertion that *CLAU* negatively regulates *GOB* in leaf development.

CK Application Affects *CLAU* Expression

To further understand the interaction between *CLAU* and cytokinin, we examined whether *CLAU* expression is affected by CK (Figure 9). CK application led to a decrease in *CLAU* expression (Figure 9A), suggesting that *CLAU* promotes differentiation by inhibiting CK responses, and CK promotes morphogenesis by inhibiting *CLAU* expression (Figure 9B).

DISCUSSION

Tomato leaves undergo extended morphogenesis, resulting in elaborate and variable leaf forms (Burko and Ori, 2013). Among tomato leaf phenotypes, the *clau* mutant is extreme in its leaf elaboration, exemplifying the potential of further extending the

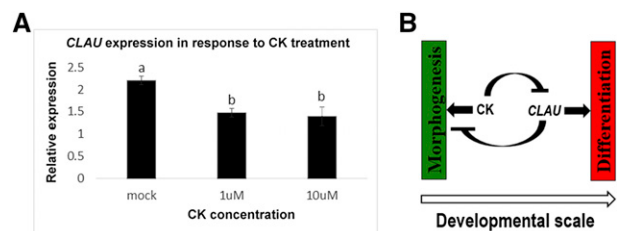


Figure 9. CK Regulates *CLAU* Expression.

(A) qRT-PCR analyzing *CLAU* mRNA expression levels in mock and CK-treated wild-type shoot apices. Bars represent the mean \pm SE of 3 to 5 biological repeats, and letters indicate statistical significance (two-tailed Student's *t* test).

(B) Diagram indicating relationships between CK and *CLAU* during development. CK promotes morphogenesis and *CLAU* promotes differentiation. *CLAU* inhibits morphogenesis by inhibiting CK response, while CK in turn can inhibit differentiation by inhibiting *CLAU* expression.

tomato leaf morphogenetic window and its possible results. Overexpressing *CLAU* curbs leaf morphogenesis, demonstrating that *CLAU* regulates the length of the leaf morphogenetic window.

How does *CLAU* regulate morphogenetic competence in the leaf? We recently reported that *CLAU* is involved in delimiting the expression of *GOB*, a central regulator of leaf morphogenesis (Bar et al., 2015; Ben-Gera et al., 2012; Berger et al., 2009; Brand et al., 2007; Busch et al., 2011; Rossmann et al., 2015). Here, *GOB* is shown to be downregulated by *CLAU* overexpression. In addition, *clau* has abnormally high expression of the KNOXI gene *LeT6/Tkn2*, which plays important role in promoting leaf morphogenesis (Avivi et al., 2000; Jasinski et al., 2007). Here, we show that a central mechanism via which *CLAU* regulates the leaf morphogenetic window is attenuation of CK response. Interestingly, KNOXI proteins positively regulate CK levels in the contexts of SAM maintenance and compound-leaf morphogenesis (Sakamoto et al., 2006; Scofield et al., 2013; Shani et al., 2010). It therefore appears that to promote differentiation and attenuate morphogenesis, *CLAU* interferes with the activity of several interconnected pathways that promote morphogenesis. It will be interesting to see which of these pathways are directly affected by *CLAU* and which are affected secondarily by the molecular and developmental context of differentiation.

How does *CLAU* affect CK response? *clau* mutants have decreased CK levels and increased CK sensitivity, and *IPT* overexpression phenotypes are enhanced by *clau*. These observations suggest that *CLAU* may negatively affect CK response, which in turn feeds back to CK levels. Notably, while TCS-driven reporter accumulation is increased and type A RRs respond to CK with higher sensitivity in *clau*, the steady state levels of these type A RRs are reduced in *clau*. These results suggest that *CLAU* is a positive regulator of type A RRs, which negatively regulate CK response (Kiba et al., 2004; To et al., 2004; Lee et al., 2008). In *clau*, their expression is reduced, leading to increased CK sensitivity. The effect of *CLAU* on type A RRs may be direct, or possibly, additional regulators are feeding back to downregulate type A RRs. In contrast to the increased CK sensitivity of TRR response in *clau*, *CKX* has reduced response to CK treatment in *clau* when compared with the wild type. These results suggest that the response of *TRR* and *CKX* expression to CK may be regulated by distinct pathways and that CK affects *CKX* expression in a pathway not affected by *CLAU*. In other words, some downstream components of the CK pathway respond to the elevated CK sensitivity mediated by *CLAU*, while other downstream components of the CK pathway respond to the decrease in CK content present in *clau*.

Our results show that *CLAU* is primarily expressed at the leaf margin and during the developmental stages where leaflets are formed, suggesting that *CLAU* functions to restrain morphogenesis. This is also evident from the fact that in wild-type leaves, *CLAU* expression decreases after leaflets have been determined (P8 onwards). Essentially, *CLAU* can be viewed as the gatekeeper for exiting morphogenesis, which can explain why its loss of function increases leaf complexity so drastically; *CLAU* functions as a repressor during the morphogenetic stage, and removing *CLAU* function causes an already elaborate leaf to continue elaboration indefinitely, subsequently being regulated only by the plant senescing (Bar et al., 2015).

TCP transcription factors, which also promote leaf differentiation and attenuate morphogenesis have been shown in Arabidopsis to decrease CK sensitivity by an interaction with the chromatin remodeling ATPase BRHAMA and direct binding to the *ARR16* gene, which is rapidly induced by CK and inhibits CK response (Efroni et al., 2013). Therefore, transcription factors that antagonistically affect the balance between morphogenesis and differentiation appear to converge on modulating the CK response. As the tomato TCP factor LA was shown to also affect GA metabolism, the window of morphogenesis may be regulated through the balance between CK and GA (Shani et al., 2010; Bolduc and Hake, 2009; Steiner et al., 2012; Efroni et al., 2013; Yanai et al., 2011; Hay et al., 2002).

CLAU encodes a MYB transcription factor that possesses a DNA binding SHAQYF domain. Several MYB transcription factors have been previously reported to be involved in leaf development (Barkoulas et al., 2007; Luo et al., 2012; Busch et al., 2011; Naz et al., 2013), but most of them encode proteins containing R2R3- or R3-type DNA binding domains, which cluster distantly from *CLAU* (Supplemental Figure 3). *CLAU* homologs exist in simple-leaf species, where they are possibly involved in *GOB* regulation and restriction of the meristematic morphogenetic potential, but *CLAU* appears to have an expanded function in compound-leaf development. In tomato, and possibly additional compound-leaf species, *CLAU* is expressed and functions primarily at the stage when leaflets are already being formed, suggesting that it may have a more restricted role in the development of leaves that lack leaflets. In the Solanaceae, *CLAU* increases in expression at developmental stages where leaflets are being formed in the compound-leaf tomato and potato, decreasing after leaflets have been formed, while its expression decreases much earlier in the simple-leaf pepper and tobacco, possibly supporting a role in restricting lateral organ morphogenesis from the meristem. Interestingly, the pepper *CLAU* homolog is truncated, possessing five exons rather than the six in tomato and potato. The portion of the protein missing in Ca-*CLAU* is somewhat comparable to the *clau-FF* mutation (Ca-*CLAU* is ~70% identical to amino acids 1 to 207 of *CLAU*, while the predicted protein in the *clau-FF* allele is composed of amino acids 1 to 150 of *CLAU*), which results in a loss-of-function *clau* phenotype. This suggests that the *CLAU* protein may not be functional or may have decreased or different function in pepper, demonstrating that the activity of *CLAU* in curbing leaf morphogenesis may not be required in the simple-leaf pepper. The function of *CLAU* in simple-leaf species remains to be investigated.

To summarize, we have shown here that *CLAU* is a MYB transcription factor that modulates leaf morphogenesis by curbing the morphogenetic potential, in part through attenuation of CK signaling. It appears that *CLAU* is of particular spatio-temporal relevance in compound leaves.

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 $\sim 450 \mu\text{mol m}^{-2} \text{s}^{-1}$. For in situ hybridization and expression analyses, plants were grown in a controlled growth chamber under fluorescent bulbs providing $\sim 300 \mu\text{mol m}^{-2} \text{s}^{-1}$ at an 18 h/6-h light/dark regimen. The *clau* allele used throughout all experiments, *clau:e3545*, is from a tomato EMS population (Menda et al., 2004) and was previously characterized (Bar et al., 2015). Tomato (M82, sp) plants expressing *pFIL>>IPT and pFIL>>CKX* were generated by the LhG4 transactivation system (Moore et al., 1998) and described previously (Shani et al., 2010). Tomato plants overexpressing TCSv2:3XVENUS were obtained from the Muller, Eshed, and Weiss labs (Steiner et al., 2016; Zürcher et al., 2013), confirmed to be CK responsive in tomato, and backcrossed into a *clau* background.

Cloning and Plant Transformation

The *CLAU* gene was amplified from tomato M82 cDNA and cloned into the pENTR/d vector using a TOPO isomerase cloning system (Invitrogen). To generate *op:CLAU* and *op:CLAU-SRDX*, *CLAU* from pENTR was subcloned downstream to an Operator array (Moore et al., 1998). The SRDX fusion was generated by assembly PCR. At least five independent kanamycin-resistant transgenic lines from each transgene were crossed to the *pFIL* and *pCLAU* (see below) driver lines and examined, and a representative line was selected for further analysis. The *CLAU* promoter was generated by amplifying 2700 bp upstream of the *CLAU* ATG from genomic DNA (M82 background) and subcloned upstream to NLS-YFP generating *pCLAU:nYFP* or subcloned upstream to LhG4, generating a vector for driving expression in the *trans*-activation system (Moore et al., 1998).

CLAU Mapping and RNA-Seq

We created an F2 mapping population from a cross between *clau:e3545* in the M82 background and *Solanum pimpinellifolium*. Markers used in the mapping of this population are presented in Supplemental Table 1. Using this population and these markers, we mapped *CLAU* between bases 2094866 and 2122247 on the short arm of chromosome 4. RNA was prepared from the shoot apices of 10-d-old *clau:e3545* and wild-type M82 plants (m-P4) and subjected to RNA-seq. Two libraries per genotype were prepared according to the Illumina TruSeq RNA protocol and sequenced on an Illumina HiSeq2000 platform at the Genome Center of the Max Planck Institute for Plant Breeding Research. We obtained between 17.9 and 29.9 million 96-bp single-end reads per library (average of 25.8 million).

Reads were aligned to the *S. lycopersicum* reference sequence v2.40 (ftp://ftp.solgenomics.net/tomato_genome/wgs/assembly/build_2.40/) using TopHat v2.0.6 (Kim et al., 2013) with the following parameters: `-max-insertion-length 12-max-deletion-length 12 -g 1-read-gap-length 12-read-edit-dist 20-read-mismatches 12-no-coverage-search-read-realign-edit-dist 0-segment-mismatches 3-splice-mismatches 1`. An average of 93.2% of all reads were uniquely aligned to the reference genome.

To detect polymorphisms between *clau:e3545* and wild-type M82, we first removed duplicated reads using default settings in Picard (<http://broadinstitute.github.io/picard/>), realigned indels, and called variants using default parameters in GATK v2.2-8 (DePristo et al., 2011). For all analyses, we retained only homozygous, biallelic variants covered in both genotypes with a phred-scaled variant quality higher than 30. This analysis revealed 26,995 variants between the mutant and its M82 background. We estimated the effect of each variant in annotated transcripts (ITAG 2.3) using ANNOVAR (Wang et al., 2010). Deleterious variants in the candidate region in chromosome 4 were evaluated manually.

Phylogenetic Analysis

Protein sequence alignment was performed using Muscle (Edgar, 2004). The evolutionary history was inferred using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992). In each

case, the tree with the highest log likelihood is shown. Initial trees for the heuristic searches were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Syteny

CLAU-like genes were chosen based on BLASTP to SI-*CLAU*. Chromosomal regions were checked for syteny by seeking neighbor gene annotations and verified by global pairwise sequence alignment (Needle, EMBOSS). Genomic syteny was analyzed for chromosome 2 in *Arabidopsis thaliana* (TAIR10) and chromosome 4 in potato (*Solanum tuberosum*; ITAG1). Gene models for the syteny were drawn using published gff3 files. The following genomes were used for syteny analyses: Tomato ITAG2.4; Arabidopsis TAIR10; Potato ITAG1, PGSC3.4, and 4.03; Pepper CAv1.55; Eggplant SME2.5.1; *Nicotiana benthamiana* Niben101.

Tissue Collection and RNA Analysis

Tissue collection, RNA preparation, and qRT-PCR analysis were performed as previously described (Shleizer-Burko et al., 2011). Values are means of at least three biological repeats, each containing the shoot apices, including the SAM and P1-P5 leaf primordia, of 8 to 12 seedlings. Expression of all assayed genes was normalized relative to tomato *EXPRESSED (EXP)*, except in the results presented in Figure 4, where each *CLAU* ortholog was normalized relative to the expression of *TUBULIN* within the same species. Primer sequences used for the qRT-PCR analyses are detailed in Supplemental Table 2. Student's *t* test (two-tailed) was used for comparison of means, which were deemed significantly different at $P \leq 0.05$. RNA in situ hybridization was performed as previously described (Shani et al., 2010). The *CLAU* mRNA antisense probe was generated as described previously (Berger et al., 2009) from a pENTR-*CLAU* construct (see above) containing the full-length *CLAU* cDNA, digested with *HindIII*.

Imaging

Intact leaves were photographed using a Nikon D5200 camera. For analysis of TCSv2:3XVENUS expression, dissected whole-leaf primordia were placed into drops of water on glass microscope slides and covered with cover slips. The pattern of VENUS expression was detected by a confocal laser scanning microscope (CLSMmodel SP8; Leica), with the solid-state laser set at 514 nm for excitation and 530 nm for emission. Chlorophyll expression was detected at 488 nm for excitation and 700 nm for emission. The pattern of VENUS expression was also observed with a Nikon SMZ1270 stereomicroscope equipped with a Nikon DS-RI2 camera and NIS elements software.

In situ hybridization slides were photographed with an Olympus 1X81 microscope using CellR software as described (Berger et al., 2009). LAS AF and ImageJ software were used for analysis, quantification, and measurements of captured images. Student's *t* test (two-tailed) was used for comparison of means, which were deemed significantly different at $P \leq 0.05$. Images were adjusted uniformly using Adobe Photoshop CS6.

CK Content Analysis

Cytokinins were isolated and purified as outlined by Novák et al. (2003) with some modifications. Frozen tomato tissue (30 mg) was homogenized using vibration mill MM 301 at a frequency of 30 Hz for 3 min (3-mm zirconium oxide beads; Retsch) with 1 mL ice-cold Bielecki solution

(methanol-chloroform-formic acid-water; 12:5:1:2) as extraction solution. The cytokinins were then extracted overnight at 4°C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific) after addition of heavy labeled internal standards (OChemIm). The samples were further purified by SPE (SCX), and the eluate was concentrated in vacuo at 37°C. For analysis, samples were redissolved in mobile phase and analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS; Acquity UPLC System, Xevo TQ) according to Novák et al. (2008). Cytokinins were ionized by electrospray in positive mode and detected using multiple-reaction monitoring. Masslynx 4.1 software (Waters) was used to analyze the data and processed by the standard isotope dilution method (Rittenberg and Foster, 1940)

CK Treatment and Root Growth, Hypocotyl Height, and Anthocyanin Measurements

For anthocyanin measurement and hypocotyl elongation, 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: (OD530-(0.25*OD660)), normalized to the starting tissue weight. Three technical replicates of 10 to 15 biological repeats were performed for each sample.

For root growth, seeds were germinated on sterile wetted paper filters and transferred to plates containing the indicated CK concentrations after 5 d, then grown upright on the CK concentrations for an additional 3 to 5 d in a growth chamber.

For qRT-PCR, 10-d-old seedlings were dipped in the indicated CK concentrations for 5 min and then incubated at room temperature for 3 h. Shoot apices containing the SAM and P1-P5 leaf primordia were then collected for RNA extraction. 6-Benzyl-aminopurine (Sigma-Aldrich) was used for all CK treatments.

Accession Numbers

Sequence data used in this study can be found in the Sol Genomic Network, The Arabidopsis Information Resource (TAIR), and the National Center for Biotechnology Information databases under the following accession numbers: CLAU, Solyc04g008480; CLAU homologs: tomato, Solyc02g080860.1.1 and Solyc12g006280.1.1; Arabidopsis, AT1G14600.1 and AT2G02060.1; coffee, Cc09g01520 and Cc11g04980; eucalyptus, EcC013137.10, EcC045129.10, and EcC054831.10; soybean, Glyma20g108600.1.p, Glyma07g158600.1.p, and Glyma20g006000.1.p; rice, *Oryza sativa*|BGIOSGA011527-PA and *Oryza sativa*|BGIOSGA031479-PA; *Nicotiana benthamiana*, NbS00036280g0004.1, NbS00013875g0008.1, and NbS00035641g0001.1; *Nicotiana tabacum*, NT_mRNA_59421_gene_33284 and NT_mRNA_90646_gene_51238; pepper, CA05g06710 and CA06g06970; potato, PGSC0003DMP400051256 and PGSC0003DMP400051255; eggplant, Sme2.5_01601.1_g00006.1 and Sme2.5_02381.1_g00010.1; maize, *Zea mays*|GRMZM2G374986_T01 and *Zea mays*|GRMZM5G887276_T03; and Medicago, Medtr0223s0040.1 and Medtr1g112370.1.

Supplemental Data

Supplemental Figure 1. Allelic representation of *CLAU*.

Supplemental Figure 2. Synteny of tomato *CLAU* chromosomal region with additional species.

Supplemental Figure 3. Analysis of various *CLAU* homologs and tomato MYB genes.

Supplemental Figure 4. TCSv2:3XVENUS is upregulated in response to CK treatment in tomato.

Supplemental Figure 5. Additional CK sensitivity experiments conducted on *clau* versus the wild type.

Supplemental Table 1. Markers used in *CLAU* mapping.

Supplemental Table 2. Primer pairs used in this work.

Supplemental Data Set 1. Text file of the *CLAU* alignment used for the phylogenetic analysis shown in Figure 1C.

Supplemental Data Set 2. Text file of the tomato MYB alignment used for the phylogenetic analysis shown in Supplemental Figure 3A.

Supplemental Data Set 3. Text file of the tomato MYB alignment used for the phylogenetic analysis shown in Figure 4A.

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AUTHOR CONTRIBUTIONS

M.B. and N.O. designed the research. M.B., A.I., H.B.G., M.L., J.M.J.-G., S.K., and P.T. performed research. M.B., A.I., M.L., H.B.G., N.O., J.M.J.-G., S.K., and P.T. analyzed data. M.B. and N.O. wrote the article.

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