Cytokinin Regulates Compound Leaf Development in Tomato [C][W]

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Leaf shape diversity relies on transient morphogenetic activity in leaf margins. However, how this morphogenetic capacity is maintained is still poorly understood. Here, we uncover a role for the hormone cytokinin (CK) in the regulation of morphogenetic activity of compound leaves in tomato (*Solanum lycopersicum*). Manipulation of CK levels led to alterations in leaf complexity and revealed a unique potential for prolonged growth and morphogenesis in tomato leaves. We further demonstrate that the effect of CK on leaf complexity depends on proper localization of auxin signaling. Genetic analysis showed that reduction of CK levels suppresses the effect of Knotted1 like homeobox (KNOXI) proteins on leaf shape and that CK can substitute for KNOXI activity at the leaf margin, suggesting that CK mediates the activity of KNOXI proteins in the regulation of leaf shape. These results imply that CK regulates flexible leaf patterning by dynamic interaction with additional hormones and transcription factors.

INTRODUCTION

Compound leaf development is a flexible process that responds sensitively to changes in genetic, hormonal, and environmental factors, making leaves an attractive system to study mechanisms of morphogenesis (Efroni et al., 2010). Leaf development can be roughly divided into three successive and overlapping stages. In the first stage, termed initiation (I), the leaf emerges from the flanks of the shoot apical meristem (SAM). During the second stage, primary morphogenesis (PM), leaves expand laterally, the basic leaf form is determined, and lateral structures such as leaflets, lobes, and serrations are elaborated from the leaf margin. In the final stage, secondary morphogenesis, the leaf grows substantially and differentiates by producing cell types typical of the mature leaf (Hagemann and Gleissberg, 1996; Poethig, 1997; Dengler and Tsukaya, 2001; Kaplan, 2001; Holtan and Hake, 2003; Efroni et al., 2008). Final leaf shape and size are affected by events that take place during all these stages. The developmental state of leaf primordia is followed by plastochrons, the interval between two successive leaves. Thus, the youngest leaf primordium is termed P1; it becomes P2 when the next primordium arises, and so on. Leaf growth is determinate, in that it lasts for a limited duration, but developing leaves retain transient organogenesis activity during PM in specific regions at their margins, termed marginal blastozones (Hagemann and Gleissberg, 1996). This results in the coexistence of tissues at different developmental stages and maturation states within the developing leaf.

Two basic leaf forms can be described with respect to the blade structure: simple leaves, in which there is a single undivided blade; and compound leaves, composed of multiple leaflets, each resembling a simple leaf. Classic and recent research has led to the hypothesis that compound-leaf development requires prolonged activity of the marginal blastozone during PM. Partially overlapping genetic mechanisms have been identified for leaf development from the SAM flanks and leaflet development from the leaf marginal blastozone (Hagemann and Gleissberg, 1996; Floyd and Bowman, 2010; Koenig and Sinha, 2010).

For example, *Knotted1 like homeobox* (*KNOX1*) genes are essential for the maintenance of indeterminate growth and morphogenetic activity of the SAM and also play essential roles in maintaining the transient indeterminacy and morphogenetic activity of the marginal blastozone of many species with compound leaves (Hareven et al., 1996; Janssen et al., 1998; Bharathan et al., 2002; Hake et al., 2004; Hay and Tsiantis, 2006; Kimura et al., 2008; Barth et al., 2009; Ramirez et al., 2009; Shani et al., 2009; Hay and Tsiantis, 2010). Conversely, TCP (teosinte branched-cycloidea-proliferating cell factor) domain proteins, such as LANCEOLATE (LA) in tomato (*Solanum lycopersicum*), negatively control SAM and marginal blastozone activity by promoting differentiation, and their downregulation during early leaf development is essential for compound-leaf development (Nath et al., 2003; Palatnik et al., 2003; Ori et al., 2007; Efroni et al., 2008). Thus, in the dominant *La* mutant, precocious LA activation leads to earlier differentiation and to a simplified leaf shape (Ori et al., 2007). In other species with compound leaves, such as pea (*Pisum sativum*) and *Medicago truncatula*, other factors play similar antagonistic roles in defining the window of morphogenetic activity (Hofer et al., 1997; Wang et al., 2008; Chen et al., 2010).

In species with either simple or compound leaves, CUC (cup-shaped cotyledon) transcription factors mark and define boundaries and maintain morphogenetic activities at both the SAM and the leaf's marginal blastozone (Nikovics et al., 2006; Blein et al., 2008, 2010; Berger et al., 2009). Auxin maxima were shown to define both leaf and leaflet initiation sites, likely by a yet undefined interaction with CUC proteins (Reinhardt et al., 2000, 2003; Benková et al., 2003; Heisler et al., 2005; Barkoulas et al., 2008; Koenig et al., 2009; Blein et al., 2010; Canales et al., 2010).

The plant hormone cytokinin (CK) is involved in coordinating many developmental processes in the plant (Werner and Schmülling, 2009), including positive regulation of SAM size and activity (Werner et al., 2001, 2003; Giulini et al., 2004; Leibfried et al., 2005; Kurakawa et al., 2007; Sablowski, 2007; Galinha et al., 2009; Gordon et al., 2009; Veit, 2009; Perilli et al., 2010; Skylar et al., 2010; Zhao et al., 2010). CK biosynthesis was shown to be positively regulated by KNOXI proteins (Jasinski et al., 2005; Yanai et al., 2005; Sakamoto et al., 2006; Shani et al., 2006), and a mutual positive regulation was shown to exist between CK and WUSCHEL, an important regulator of SAM maintenance (Leibfried et al., 2005; Lindsay et al., 2006; Gordon et al., 2009). Conversely, SAM size was shown to be negatively regulated by type A response regulators (ARRs), which are induced by CK and negatively regulate CK
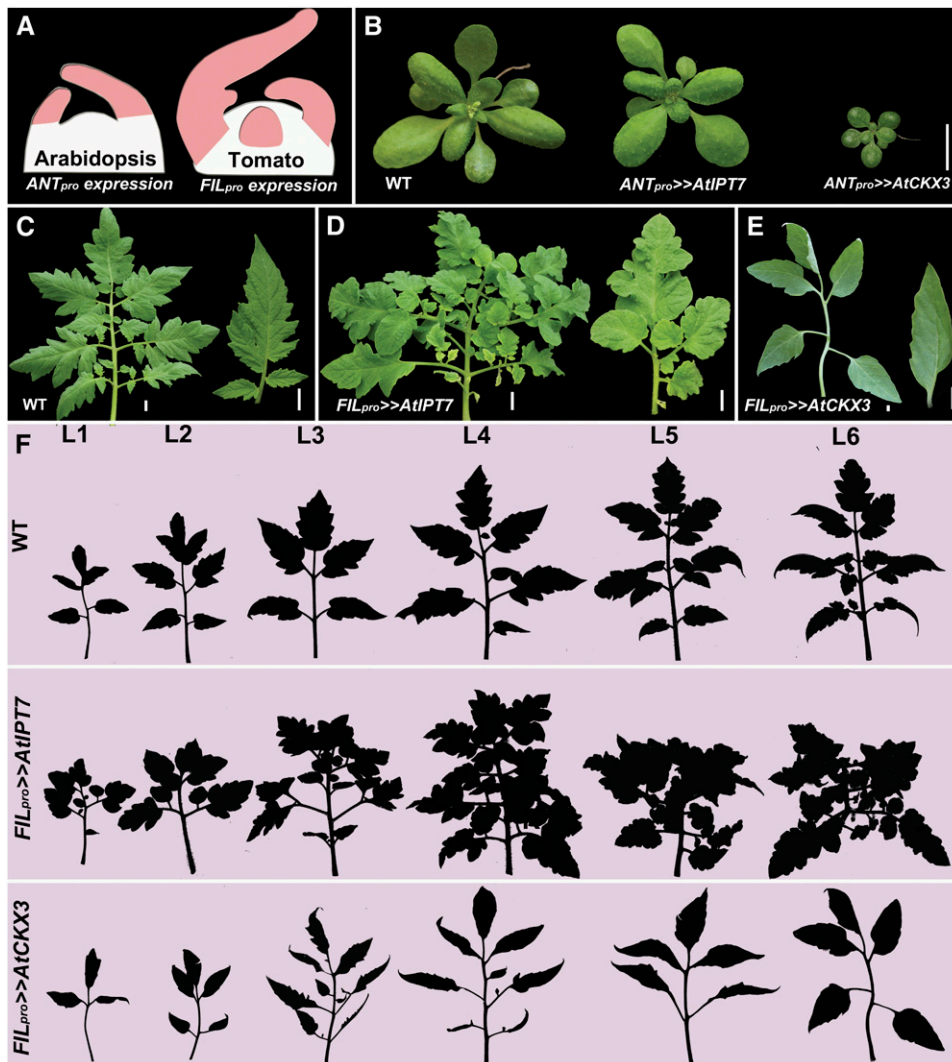

Figure 1. CK Regulates Compound Leaf Development.

(A) A schematic illustration of the expression domains (shading) directed by the indicated promoters.

(B) Three-week-old *Arabidopsis* seedlings of the indicated genotypes.

(C) to **(E)** The sixth leaf (left) and a magnification of the second primary leaflet (right) of the indicated tomato genotypes.

(F) Successive leaves were removed from the plants and are shown in an acropetal sequence from left to right. L1 to L6, leaf 1 to leaf 6.

X_{pro}>>Y, plants expressing the Y gene under the control of the X promoter, using the LhG4 transactivation system. WT, wild type. Bars = 1 cm.

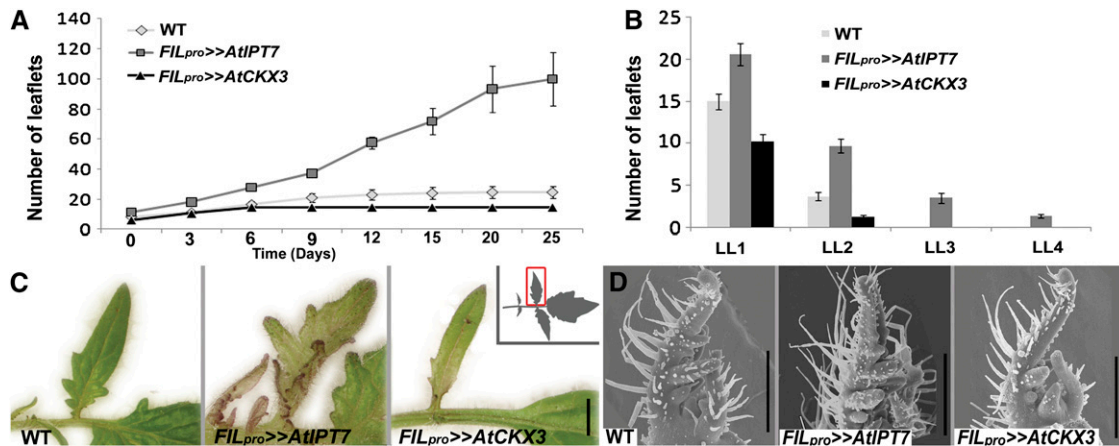[See online article for color version of this figure.]

Figure 2. CK Regulates a Prolonged Morphogenetic Activity of Tomato Leaf Margins.

(A) Total number of leaflets on the sixth leaf at successive time points during leaf growth. Time 0 refers to the day at which the leaf has emerged from the apex and expanded. The same leaves were analyzed every 3 to 5 d. Shown are averages ± SE (n = 10).

(B) Number of leaflets in each order of leaflet reiteration, at time point 20 d in **(A)**. LL1, number of primary and intercalary leaflets; LL2-LL4, number of secondary, tertiary, and quaternary leaflets, respectively, of the second primary leaflet. Shown are averages ± SE (n = 10).

(C) A leaflet from a leaf at the P8 stage. The inset shows schematic illustration of the context (rectangle) of the images.

(D) Scanning electron micrographs of young leaf primordia at the P4 stage.

WT, wild type. Bars = 1 mm in **(C)** and 500 μm **(D)**.

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signaling through a feedback loop (Giulini et al., 2004; Leibfried et al., 2005).

Here, we tested the possibility that CK is also involved in maintaining the activity of the leaf's marginal blastozone. We demonstrate a role for CK in the regulation of compound-leaf development in tomato. Manipulation of CK levels in developing leaves led to alteration of the level of leaf complexity in tomato. We further show that CK acts downstream of KNOXI proteins in maintaining prolonged morphogenetic activity at the leaf margin but that the activities of KNOXI and CK only partially overlap.

RESULTS

CK Regulates Compound-Leaf Development

To test whether CK is involved in compound-leaf development, we manipulated CK levels in simple *Arabidopsis thaliana* leaves or compound tomato leaves by expressing either the CK biosynthesis gene *Arabidopsis* (At) *ISOPENTENYL TRANSFERASE7* (At *IPT7*) or the CK degradation gene *CYTOKININ OXIDASE3* (At *CKX3*) (Miyawaki et al., 2006; Werner and Schmülling, 2009). To distinguish between the roles of CK in
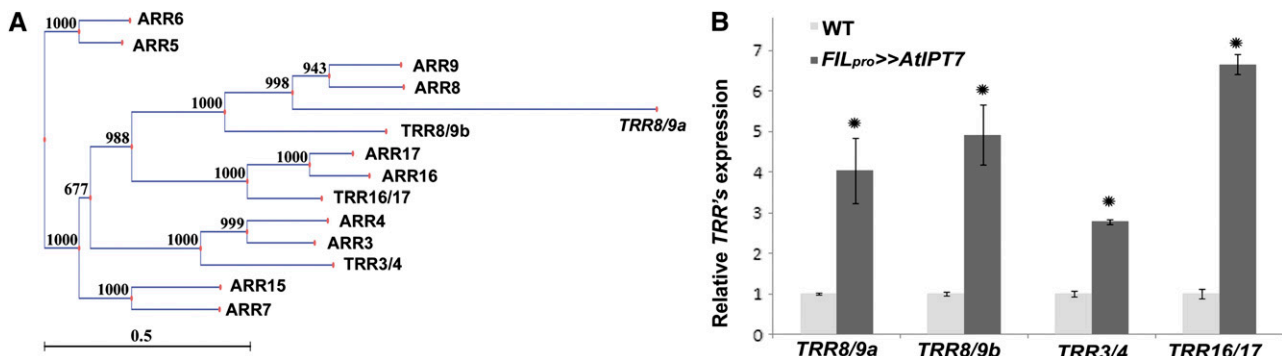
Figure 3. At *IPT7* Overexpression Leads to Increased Expression of CK Response Markers.

(A) Unrooted tree showing the phylogenetic relationship among type-A response regulator proteins from tomato (*TRR*) and *Arabidopsis* (*ARR*). The scale bar indicates the distances in substitutions per amino acid.

(B) Quantification of *TRR* mRNA level in third leaves at the P5 stage of 17-d-old plants by quantitative RT-PCR. Shown are averages ± SE (n = 3 biological repeats). *Significantly different relative to the respective wild type (WT) at P ≤ 0.05.

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the SAM and in developing leaves, we expressed these genes using the lateral organ–specific promoters ANT_{pro} and FIL_{pro} in *Arabidopsis* and tomato, respectively (Figure 1A). The *Arabidopsis* ANT_{pro} drives expression in the regions at the SAM flanks from which organs will initiate, and during early lateral organ development it is expressed throughout initiating organs. Later its expression becomes restricted to internal and basal regions (Elliott et al., 1996; Long and Barton, 1998; Schoof et al., 2000). In tomato, the FIL_{pro} from *Arabidopsis* drives expression throughout the primordia, including initiating leaflets and the intercalary region between them, until relatively late in leaf development (see Supplemental Figure 1 online; Lifschitz et al., 2006; Berger et al., 2009; Shani et al., 2009).

Leaves of ANT_{pro}>>*AtIPT7 Arabidopsis* plants, expressing At *IPT7* under the control of the ANT_{pro} using the LhG4 transactivation system (Moore et al., 1998; see Methods), were similar to wild-type leaves (Figure 1B). ANT_{pro}>>*AtCKX3 Arabidopsis* leaves were very small, round, and dark green, as described previously (Werner et al., 2003; Figure 1B). By contrast, At *IPT7* expression in tomato leaves under the control of the FIL_{pro} led to the development of super-compound leaves with up to four orders of leaflets, compared with two orders in the wild-type leaf (Figures 1C, 1D, 1F, 2A, and 2B). In addition, leaflets were rounder than in the wild type (Figures 1C and 1D), and ectopic meristems and flowers were formed on the adaxial side of the leaf rachis (see Supplemental Figure 2 online). Conversely,
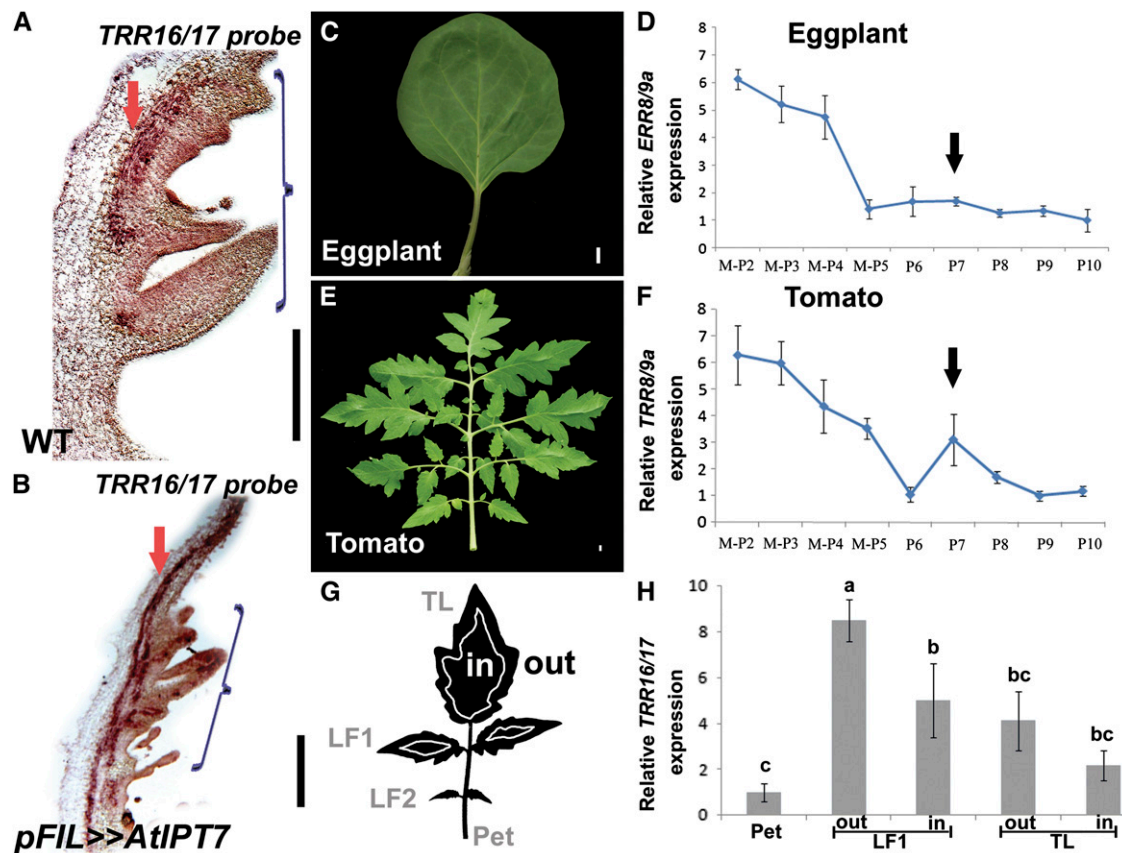

Figure 4. Enhanced CK Response in Leaf Margins of Compound Leaves.

(A) and **(B)** *TRR16/17* expression in P4 leaf primordia of the indicated genotypes, assayed by mRNA in situ hybridization. Arrows point to procambial expression; brackets indicate the expression at the leaf margin. **(A)** was exposed for 36 h and **(B)** for 12 h. WT, wild type.

(C) and **(E)** Mature fifth leaf of eggplant **(C)** and tomato **(E)**.

(D) and **(F)** Relative *ERR8/9A* **(D)** and *TRR8/9A* **(F)** mRNA expression at successive stages of leaf maturation of wild-type eggplant or tomato, respectively, as measured by quantitative RT-PCR. Averages ± SE (*n* = 3 to 6 biological repeats) are indicated. M-P2, SAM and the two youngest leaf primordia (P1 and P2). Arrow points to the second peak of elevated expression at the P7 stage.

(G) A schematic illustration of the dissected domains of P8, used for the *TRR16/17* quantification shown in **(H)**.

(H) Relative TRR16/17 mRNA levels in different domains of a fifth wild-type tomato leaf at the P8 stage. The domains are illustrated in **(G)**. mRNA levels were assayed by quantitative RT-PCR. Shown are averages ± SE (*n* = 3). Bars with different letters are significantly different at P < 0.05. TL, terminal leaflet; LF1 and LF2, lateral leaflet 1 and 2, respectively; Pet, leaf petiole.

Bars = 200 μm in **(A)** and **(B)** and 1 cm in **(C)** and **(E)**.

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expression of At *CKX3* in tomato leaves led to the production of simplified leaves that made only primary leaflets (Figures 1E, 1F, 2A, and 2B). Furthermore, leaf margins of *FIL*$_{pro}$*>>AtCKX3* plants were smooth, compared with the lobed margins of the wild-type leaf (Figure 2C). *FIL*$_{pro}$*>>AtIPT7* leaves showed an opposite effect of enhanced morphogenetic activity at their margins, as manifested by an appearance of initiating primordia and the development of additional marginal structures (Figure 2C). In agreement, *FIL*$_{pro}$*>>AtCKX3* leaves ceased leaflet initiation at a relatively early developmental stage relative to the wild type, while *FIL*$_{pro}$*>>AtIPT7* continued to initiate leaflets much longer than the wild type (Figure 2A). Thus, tomato leaves have a potential for extended morphogenetic activity that lasts for weeks, and CK positively regulates this potential.

Examination of young primordia showed only mild effects during early leaf development of *FIL*$_{pro}$*>>AtIPT7* and *FIL*$_{pro}$*>>AtCKX*3 leaves: Leaflet initiation was slightly accelerated in *FIL*$_{pro}$*>>AtIPT7* primordia and slightly delayed in *FIL*$_{pro}$*>>AtCKX3* primordia (Figure 2D; see Supplemental Figure 3 online). Constitutive expression of At *CKX3* in tomato plants using the *35S* promoter led to a strong phenotype of small plants with inhibited growth and small, simplified leaves (see Supplemental Figure 4 online), similar to *Arabidopsis 35S*$_{pro}$*:CKX3* (Werner et al., 2003).

In *Arabidopsis*, *ARRs* have been shown to be rapidly induced by CK application (Imamura et al., 1998; D'Agostino et al., 2000; To and Kieber, 2008). A search in the tomato genomic and EST databases (http://solgenomics.net/) has identified seven *ARR* homologs. For further analysis, we used four of these genes that showed a relatively high degree of similarity. Interestingly, two pairs of closely related *ARR*s had a single close tomato homolog, and an additional pair had two close tomato homologs. The

identified tomato genes were termed *Tomato Response Regulator 3/4* (*TRR3/4*), *TRR8/9a*, *TRR8/9b*, and *TRR16/17*, according to the respective most similar *Arabidopsis* genes (Figure 3A; see Supplemental Figure 5 online).

Quantitative RT-PCR analysis showed that mRNA levels of four of the identified *TRRs* were upregulated 2.5- to 6.5-fold in leaf primordia at the P5 stage of *FIL*$_{pro}$*>>AtIPT7* plants (Figure 3B), suggesting that CK response was affected as expected in these plants. CK is a positive regulator of cell division and shoot regeneration (Miller et al., 1955). In *Arabidopsis*, the D-type cyclin (*CYCD3*) genes were shown to be induced by CK and to mediate its activity. CYCD3s were also shown to affect stage transition during lateral organ development in *Arabidopsis* (Riou-Khamlichi et al., 1999; Dewitte et al., 2007). We thus tested the effect of manipulating CK levels in developing leaves on the expression of tomato (SI) *CYCD3.3* (Kvarnheden et al., 2000). SI *CYCD3.3* was upregulated 2-fold in P5 and P7 primordia of *FIL*$_{pro}$*>>AtIPT7* plants relative to the wild type but was unaffected in *FIL*$_{pro}$*>>AtCKX3* (see Supplemental Figure 6 online).

Cumulatively, these observations suggest that CK regulates the level of leaf complexity by enabling extended morphogenetic activity at the leaf margin and regulating its duration but cannot cause the conversion of the *Arabidopsis* simple-leaf structure into a compound one.

Compound-Leaf Development Is Correlated with Extended CK Response at the Leaf Margin

To further understand the role of CK in the development of the compound tomato leaf, we examined the spatial distribution of
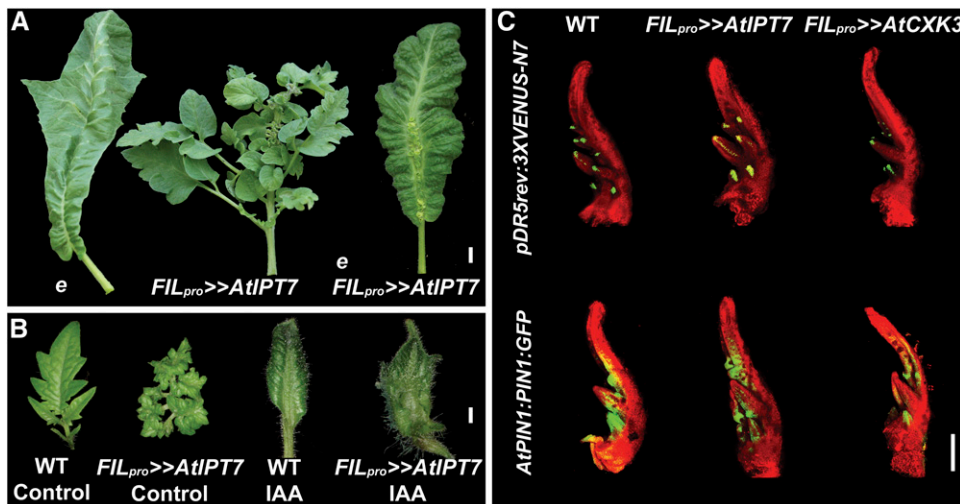

Figure 5. CK Requires Proper Localization of Auxin Response to Regulate Leaf Shape.

(A) Tomato sixth leaves of the indicated genotypes. The *e* mutant phenotype is epistatic to that of *pFIL>>AtIPT7*.
(B) Tomato leaves of the indicated genotypes, with or without microapplication of 1 mM IAA. Microapplication of IAA led to the development of simple leaves and suppressed the super-compound leaf phenotype of *pFIL>>AtIPT7*. WT, wild type.
(C) Distribution of the *pDR5rev:3XVENUS-N7* and *AtPIN1:PIN1-GFP* markers. Shown are confocal microscope images of P4-staged leaves. The background leaf represents chlorophyll autofluorescence. Genotypes are as described in Figure 1.
Bars = 1 cm in **(A)**, 1 mm in **(B)**, and 200 μm in **(C)**.
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the CK response marker *TRR16/17* in developing wild-type and *FIL_{pro}>>AtIPT7* tomato leaves using in situ hybridization. In wild-type plants, *TRR16/17* expression was observed at the margins of the leaf procambium (Figure 4A), as expected from the recently described role of CK in *Arabidopsis* root procambium development (Mähönen et al., 2006). Expression was also observed in leaf margins and leaflets, consistent with the involvement of CK in coordinating the morphogenetic activity of the tomato leaf margin (Figure 4A). In *FIL_{pro}>>AtIPT7* leaves, *TRR16/17* distribution was similar to that in wild-type leaves (Figure 4B), although expression was elevated, as revealed by a shorter exposure required to detect a signal.

To examine whether CK response is prolonged during compound-leaf development, we compared the expression dynamics of representative CK response genes at successive developmental stages between tomato and eggplant (*Solanum melongena*), a *Solanum* species with simple leaves (Figure 4C). *TRR8/9a* and *TRR16/17*, as well as *ERR8/9a* (*EGGPLANT RR8/9a*, the closest homolog of *TRR8/9a* in eggplant), showed relatively high mRNA expression in SAM and young leaf primordia of tomato and eggplant, respectively, and a gradual reduction in expression was observed as leaves matured. However, *ERR8/9a* downregulation in eggplant leaves was earlier and steeper than in tomato (Figures 2D and 2F; see Supplemental Figure 7 online). Moreover, while eggplant primordia retained low *ERR8/9a* expression at later stages (Figure 4D), in tomato primordia, *TRR8/9a* and *TRR16/17* expression was re-elevated at stage P7, around the developmental stage at which secondary leaflets develop (Figure 4F; see Supplemental Figure 7 online). This is consistent with the elimination of secondary leaflets and marginal lobing in *FIL_{pro}>>AtCKX3* leaves and with the expression of *TRR16/17* in leaf margins (Figures 1E, 4A, and 4B).

Different parts of the young tomato leaf primordium are at different developmental stages, and vascular CK response could account for much of the expression at later stages. Therefore, to test the relevance of the marginal morphogenetic activity to the extended *TRR* expression, we quantified *TRR16/17* expression at different domains of dissected tomato fifth leaf primordia at the P8 stage (Figure 4G). *TRR16/17* expression was relatively high in basal and marginal domains of the leaf (Figure 4H), regions that are considered younger and still meristematic at this developmental stage (Sun, 1957). Expression was relatively low in the petiole, implying that it does not reflect expression in mature vasculature (Figure 4H).

Together, these results imply that an extended CK activity in leaf margins is correlated with leaf complexity, confirming its role in controlling leaf-marginal morphogenetic activity.

The Control of Leaf Complexity by CK Requires Proper Localization of Auxin Response

The plant hormone auxin has been recently implied in the regulation of leaflet initiation and growth (Barkoulas et al., 2008; Berger et al., 2009; Koenig et al., 2009). *entire* (*e*) tomato mutants have a simpler leaf form relative to the wild type (Figure 5A). The *E* gene was recently shown to encode Sl IAA9, a protein from the AUX/IAA (Auxin/indole-3-acetic acid) family of auxin response repressors, suggesting that auxin response is upregu-

lated between leaflets in *e* mutant leaves (Wang et al., 2005; Berger et al., 2009). To examine the interaction between CK and auxin in the context of compound-leaf development, we expressed *FIL_{pro}>>AtIPT7* in the background of the *e* mutant. The *e* phenotype was epistatic to that of At *IPT7* overexpression with respect to the level of leaf complexity, preventing the formation of super-compound leaves, although *e FIL_{pro}>>AtIPT7* leaves still produced ectopic meristems from their rachises (Figure 5A). In agreement, microapplication of auxin (IAA; 1 mM) directly to both wild-type and *FIL_{pro}>>AtIPT7* P2 leaf primordia resulted in simple mature leaves and a suppression of the super-compound phenotype caused by At *IPT7* (Figure 5B). Auxin (IAA; 5 μM or 1 mM) suppressed the *FIL_{pro}>>AtIPT7* phenotype also when applied to older primordia (see Supplemental Figure 8 online).

These findings imply that CK-regulated morphogenesis at the leaf margin depends on proper localization of the auxin signal. To
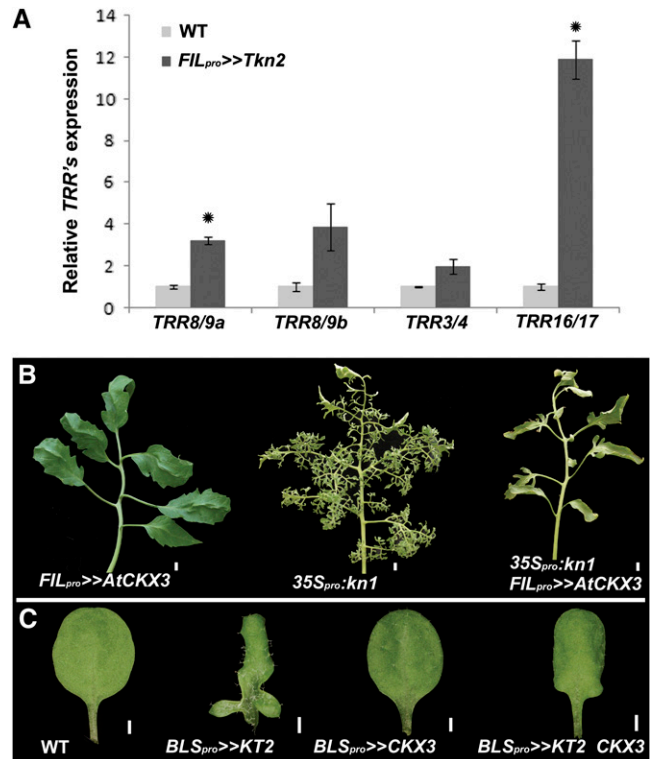
Figure 6. CK Acts Downstream of KNOXI in the Regulation of Leaf Complexity.

(A) Relative mRNA expression of the indicated *TRR* genes in 17-d-old tomato apices as measured by quantitative RT-PCR. Averages ± SE (*n* = 3) are indicated for each genotype. *Significantly different relative to the respective wild type (WT) at P ≤ 0.05.

(B) Tomato fifth leaves. *p35:kn1* plants express the maize *KNOXI* gene *knotted1* by direct fusion to the *35S* promoter, and all other transgenes are expressed using the transactivation system and are as in Figure 1.

(C) *Arabidopsis* fifth leaves of the indicated genotypes. At *CKX3* coexpression suppresses the *KT2* lobbed-leaf phenotype.

Bars = 1 cm in **(B)** and 1 mm in **(C)**.

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further understand the interaction between auxin and CK, we examined the effect of manipulating CK levels on the distribution of auxin response and transport markers. A slight reduction in the distribution of the auxin response marker *pDR5rev:3XVENUS-N7* and the auxin transporter *AtPIN1:PIN1-GFP* (Heisler et al., 2005; Bayer et al., 2009) was observed in *FIL$_{pro}$>>AtCKX3* leaves (Figure 5C). However, these alterations were likely secondary to the developmental effects of the transgene, as the principal distribution of these markers remained similar to that in wild-type leaves. The distribution of these markers was not altered in *FIL$_{pro}$>>AtIPT7* leaves (Figure 5C).

CK Acts Downstream of KNOXI Proteins in Compound-Leaf Development

CK and KNOXI transcription factors both regulate compound-leaf development (Hareven et al., 1996; Janssen et al., 1998; Hay and Tsiantis, 2006; Shani et al., 2009; this study). In the SAM, KNOXI proteins were shown to act upstream of CK by positively regulating CK biosynthesis, and CK could partially compensate for loss of KNOXI function (Jasinski et al., 2005; Yanai et al., 2005; Sakamoto et al., 2006). This prompted us to ask whether CK mediates the activity of KNOXI proteins in the context of compound-leaf development. We thus tested the effect of *KNOXI* overexpression on the expression of the *TRR* genes. *TRR* expression was upregulated 2- to 12-fold in apices of tomato plants overexpressing the *KNOXI* gene *Tkn2* specifically in lateral organs, relative to the wild type (Figure 6A).

To test the biological relevance of this activation, we examined whether reducing CK levels will suppress the *KNOXI* overexpression phenotype. Leaf-specific expression of At *CKX3* suppressed the super-compound leaf phenotype of *35S$_{pro}$:Kn1* tomato plants, which ubiquitously overexpress the maize (*Zea mays*) *KNOXI* gene *kn1* (Figure 6B). Similarly, coexpression of At *CKX3* suppressed the phenotype of *BLS$_{pro}$>>Tkn2*, expressing

Tkn2 at later stages of leaf development (see Supplemental Figure 9 online). At *CKX3* coexpression also suppressed the leaf lobing phenotype of *Arabidopsis BLS$_{pro}$>>KT2* plants, expressing the *KNOXI* gene *KT2* in leaves (Figure 6C).

If CK acts downstream of KNOXI proteins in compound leaves, elevating CK levels is expected to compensate for downregulation of KNOXI activity. To test this prediction, we coexpressed At *IPT7* and *Tkn2-SRDX*, a fusion of *Tkn2* with a repression domain (Shani et al., 2009), in tomato leaves. At *IPT7* overexpression was epistatic to the aberrant and simple leaf phenotype of *Tkn2-SRDX*, when coexpressed in leaves (Figure 7A). Examination of the mRNA expression levels of *Tkn2*, representing the combined expression of the endogenous *Tkn2* gene and the *Tkn2-SRDX* transgene, verified that the *Tkn2-SRDX* transgene was not suppressed by the coexpression of At *IPT7* (see Supplemental Figure 10 online). Examination of early stages of leaf development by scanning electron microscopy showed that At *IPT7* coexpression specifically rescued the meristematic activity of the marginal blastozone, as manifested by the lack of trichome development and the initiation of leaflets (Figure 7B). In summary, these results imply that CK acts downstream of *KNOXI* genes in compound-leaf elaboration.

DISCUSSION

During leaf development, the leaf margin transiently retains morphogenetic potential that is responsible for the extremely flexible process of leaf morphogenesis. As a result, leaves show enormous size and shape diversity both within and among species. However, how this morphogenetic capacity is maintained is still poorly understood. Here, we show that the tomato leaf keeps initiating leaflets for weeks after its emergence and that CK regulates the extent of this prolonged morphogenetic activity. In agreement, CK response markers showed extended expression
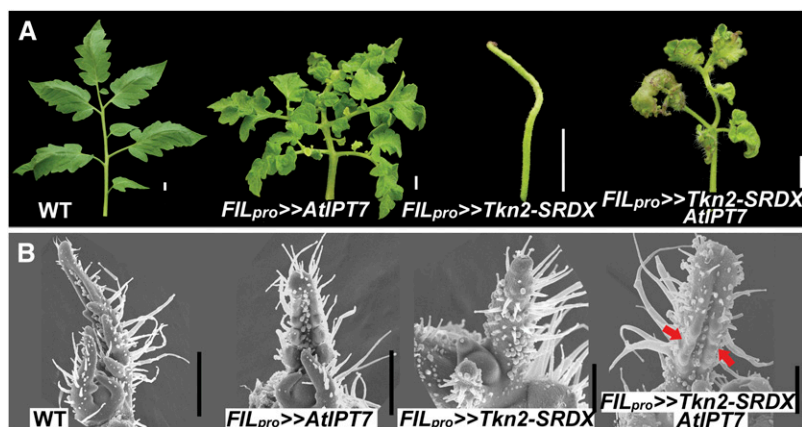

Figure 7. Cytokinin Can Compensate for KNOXI Activity in Tomato Compound Leaves.

(A) Tomato fourth leaves of the indicated genotypes. Coexpression of At *IPT7* suppresses the *pFIL>>TKN2-SRDX* simple-leaf phenotype.
(B) Scanning electron micrographs showing early leaf development. Arrows mark the sites of leaflets initiation in *pFIL>>TKN2-SRDX AtIPT7* leaf margins.
WT, wild type. Bars = 1 cm in **(A)** and 500 μm in **(B)**.
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during the development of compound tomato leaves in comparison to simple-leaved eggplant. The activity of CK in promoting SAM activity was thus recruited for the transient indeterminate growth of developing leaves during their evolution from shoots.

We show that CK acts downstream to KNOXI proteins in the context of the leaf margin. The suppression of the simple leaf phenotype of *FIL_pro>>Tkn2-SRDX* by coexpression of At *IPT7* is striking, as in most cases a simplified leaf phenotype is epistatic to a more compound leaf phenotype, in both tomato and other species (Hareven et al., 1996; Ori et al., 2007; Chen et al., 2010). Furthermore, both the *e* and the *La* mutant backgrounds, which have simplified leaves due to a defect in leaflet separation and precocious leaf maturation, respectively, were epistatic to the *FIL_pro>>IPT7* phenotype. This further supports the conclusion that CK mediates KNOXI activity in compound-leaf development. It should be noted, however, that tomato leaves react differently to manipulations of CK levels and KNOXI activity in similar developmental contexts. For example, *KNOXI* genes were shown to dramatically extend both the I and PM stages of leaf development (Shani et al., 2009). By contrast, CK elevation throughout early leaf development using *FIL_pro* did not affect the transition to PM but only affected the morphogenetic activity during PM. Expression of *KNOXI* genes by *BLS_pro* extended PM, while overexpressing At *IPT7* by *BLS_pro* caused uncontrolled growth between veins but did not affect marginal growth and did not delay the transition to secondary morphogenesis (see Supplemental Figure 11 online).

Therefore, while KNOXI proteins act through CK to promote the marginal morphogenetic activity, the context-specific response of the developing leaf to these factors only partially overlaps. The low sensitivity of the *Arabidopsis* leaves to At *IPT7* expression may result from a combination of the relatively low sensitivity of the *Arabidopsis* leaf to KNOXI activity during the I stage (Hay et al., 2003; Shani et al., 2009) and the relatively short PM in these leaves; while KNOXI proteins can extend PM also in *Arabidopsis*, CK acts more locally within an extended PM. In agreement, while overexpression of At *IPT7* at different stages of *Arabidopsis* leaf development did not affect leaf shape, coexpression of At *IPT7* enhanced the *BLS_pro>>KT2* phenotype (see Supplemental Figure 12 online). We propose that KNOXI proteins act in two pathways: (1) activating factors that delay stage transition, partially by inhibiting gibberellins (Hay et al., 2002; Bolduc and Hake, 2009) and (2) activating CK to maintain the meristematic potential of the compound-leaf margins.

Proper localization of auxin maxima and response is shown here to be required for the regulation of leaf development by CK. The ratio between auxin and CK rather than their absolute amounts is known to affect many developmental processes (Skoog and Miller, 1957; Moubayidin et al., 2009). Recently, several mechanisms by which the plant assesses this ratio have been elucidated in embryos, roots, and shoots. These include repression of auxin response and transport by CK (Dello Ioio et al., 2008; Galinha et al., 2009; Lee et al., 2009; Ruzicka et al., 2009; Wolters and Jürgens, 2009; Moubayidin et al., 2010), as well as repression or activation of CK response by auxin (Müller and Sheen, 2008; Zhao et al., 2010). Here, the distribution of an auxin response marker and the auxin trans-

porter PIN1 appeared to be essentially similar between the wild type and the genotypes with altered CK levels during early stages of leaf development, although the prolonged morphogenesis is likely accompanied by prolonged auxin signaling at the leaf margin. Thus, the reason that disruption of the auxin response gradients suppresses the effect of CK on leaf shape is still unclear.

We propose that CK is required for morphogenetic activity of the marginal blastozone but that differential auxin distribution is required in the context of this morphogenetic activity for leaflet initiation and separation. Alternatively, the suppression of the CK effect by uniformly high auxin levels or response could be a result of an earlier activity of auxin, leading to the elimination of the leaf domain that is responsive to CK. In conclusion, the genetic and environmental flexibility of compound leaves shape is regulated by the balance between the activities of CK, other hormones, and transcription factors.

METHODS

Plant Material

Tomato (*Solanum lycopersicum* cv M82, *sp*) and eggplant (*Solanum melongena*) plants were grown initially in a growth room at 16-h-day and 8-h-night conditions at 24 to 25°C. Four-week-old seedlings were transferred to greenhouse conditions at natural daylength (~14 and 10 h light in the summer and winter, respectively) 2°C at night and 25°C during the day. *Arabidopsis thaliana* plants were grown under long-day fluorescent light (16 h light/8 h dark, 21°C). The described *Arabidopsis* plants are all in the Landsberg *erecta* background. Most of the described transgenic plants were produced using the LhG4 transactivation system (Moore et al., 1998). In this system, a driver line (PRO:LhG4) that expresses the synthetic transcription factor LhG4 under a specific promoter is crossed to a responder line (OP:GENE), expressing a gene of interest downstream to several copies of the *Escherichia coli* Operator, recognized by LhG4. The resulting plants that harbor both constructs (PRO>>GENE) express the gene of interest in the expression domain of the specific promoter. The following *Arabidopsis* and tomato transgenic plants and mutants were described before: *Arabidopsis BLS_pro:LhG4* and *ANT_pro:LhG4* (Efroni et al., 2008), *OP:KNAT2* (*KT2*), *OP:Tkn2*, *OP:Tkn2-SRDX*, tomato *BLS_pro:LhG4*, and *FIL_pro:LhG4* (Shani et al., 2009), *35S_pro:LhG4* (Alvarez et al., 2006), *35S_pro:Kn1* (Hareven et al., 1996), *AtPIN1:PIN1-GFP* (*Moneymaker* background) (Bayer et al., 2009), *e* (*e2-n0741*) (Berger et al., 2009), *OP:AtIPT7*, *OP:AtCKX3*, and *pDR5rev:3XVENUS-N7* lines were generated during this research as described below.

Cloning and Plant Transformation

The *Arabidopsis* (Columbia) At *IPT7* gene was amplified from genomic DNA, with annealing temperature of 58°C and 30 cycles, cloned downstream to an array of *E. coli* Operator (OP) sequences and transferred into the binary vector pART27 (Gleave, 1992). The At *CKX3* construct, kindly provided by Thomas Schmülling and Tomáš Werner (Freie Universität Berlin), was cloned downstream to an OP array and subcloned into the binary pART27 vector. The *pDR5rev:3XVENUS-N7* construct, kindly provided by Elliot Meyerowitz and Marcus Heisler (California Institute of Technology), was subcloned into pART27 vector. Kanamycin-resistant transformants for both species were selected as described (Shani et al., 2009). Primers used for At *IPT7* cloning are described in Supplemental Table 1 online.

Plant Genetics

Phenotypic analyses were performed with selected operator:GENE (OP: GENE) responder lines that were crossed to promoter:LhG4 driver lines. Ten to twelve independent responder lines were crossed to the *FIL_pro* (tomato) or *ANT_pro* (*Arabidopsis*) driver lines, and a representative line was selected for further analysis. *FIL_pro>>AtIPT7* plants were sterile. Therefore, to generate *FIL_pro>>AtIPT7 e* plants, homozygous *OP:AtIPT7* and *FIL_pro:LhG4* plants were each crossed separately to plants homozygous for the *e* mutation, which causes leaves to have a simpler form. F2 individuals displaying the *e* phenotype and carrying the respective transgene (selected by their resistance to kanamycin) were crossed to each other (*e OP:IPT7 × e FIL_pro:LhG4*). Three-quarters of the F1 plants showed the *e* phenotype, while the remaining one-quarter possessed both the *FIL_pro:LhG4* and *op:AtIPT7* transgenes. A similar strategy was used to introduce *FIL_pro>>AtIPT7* and *FIL_pro>AtCKX* into the *pDR5rev:3XVENUS-N7*, *AtPIN1:PIN1-GFP*, and op:*Tkn2-SRDX* backgrounds.

Isolation of *TRR* Genes

The BLASTN and TBLASTX search programs and the SOL genomics network (SGN; www.sgn.cornell.edu) were used to identify sequences of *ARR* homologs from tomato. Eggplant *ERR8/9a* was amplified from eggplant cDNA using tomato *TRR8/9a* primers.

Phylogenetic Analysis

Multiple sequence alignment (see Supplemental Data Set 1) and phylogenetic tree calculation were conducted using CLC Main Workbench 5.6.1 program, using the neighbor-joining algorithm (www.clcbio.com). Bootstrap values are from 1000 trials.

Tissue Collection, RNA Analysis, and Statistical Analysis

Each leaf is characterized by its position on the plant (for example, L1 is the first leaf produced by the plant and L5 the fifth) and by its developmental stage. Thus, L5 P1 is the fifth leaf when it has just initiated from the SAM, and it becomes L5 P2 after the next primordium initiates. For analysis of gene expression during leaf development, tissue was collected at successive stages of plant development, such that the fifth leaf of the plant (L5) was at the corresponding developmental stage in all samples. For very young leaf primordia at the P1-P3 stages, the leaf was at the respective developmental stage and was collected with younger leaf primordia and the SAM. At least three biological repeats, each consisting of pooled material from three different plants, were collected for each developmental stage. Quantitative real-time RT-PCR analysis was performed using the TaKaRa SYBR Premix Ex Taq II (RR081Q) kit and a Corbett Research Rotor-Gene 6000 cycler. For each gene, a standard curve was obtained using dilutions of a cDNA sample, and only reactions in which the standard curve was linear and the sample values fell within the standard curve (efficiency of 0.96 to 1.02 and $R^2 > 0.99$) were used. Quantification of each gene was performed using the Corbett Research Rotor-Gene software. The levels of each gene and of the *TUBULIN* (*TUB*) reference gene were separately calculated at the logarithmic phase relative to the respective standard curve. At least three independent technical repeats were performed for each cDNA sample, and at least three biological repeats were used for each genotype. Relative expression of each sample was calculated by dividing the expression level by that of TUB (in arbitrary units). Gene/TUB ratios were then averaged and presented as a ratio of a control treatment, the value of which was set to 1. Primer sequences are detailed in Supplemental Table 1 online.

Statistical analysis was performed using JMP software (SAS Institute). Student's *t* test was used for comparison of means, which were deemed significantly different at P < 0.05.

In Situ Hybridization

Antisense and sense probes were produced by in vitro transcription with digoxigenin-11-UTP (Roche) using T7 or SP6 RNA polymerase (Promega). Tissue samples were fixed overnight in 4% paraformaldehyde, gradually transferred to ethanol and then to Histoclear (Gadot), and embedded in Paraplast Plus (McCormick Scientific). Eight-micrometer-thick tissue sections were produced and mounted on ProbeOnPlus slides (Fisher Biotech). Slides were treated successively with Histoclear, an ethanol series, water, Proteinase K (1 μg/mL), PBS, 4% paraformaldehyde, PBS, triethanolamine (0.1 M, with stirring), PBS, and increasing ethanol series up to 100% ethanol. Hybridization was performed overnight at 55°C. After hybridization, slides were washed successively several times with 0.2× SSC at 55°C, several times with NTE (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) at 37°C, and blocked with 1% fresh Boehringer block and then with 1% BSA solution (1% BSA, 100 mM Tris, pH 7.5, 150 mM NaCl, and 0.3% Triton X-100). Slides were incubated in pairs with antidigoxigenin antibodies (Roche) for 2 h at room temperature and then washed three times with 1% BSA solution and two times with 100 mM Tris, pH 9.5, and 100 mM NaCl. Slides were incubated with NBP/BCIP substrate solution (Roche) for 1 to 3 d and then washed with water, mounted, and analyzed. The expression pattern detected by the *TRR16/17* antisense probe was compared with a control *TRR16/17* sense probe, which showed only background signal. Primer sequences are detailed in Supplemental Table 1 online.

Imaging, Microscopy, and Microapplication of IAA

The pattern of GFP and VENUS expression was detected by a confocal laser scanning microscope (model LSM510; Zeiss) with the argon laser set at 488 nm for excitation, a long-pass 560-nm filter for chlorophyll emission, and 505- to 530-nm filter for GFP and VENUS emission, as described (Shani et al., 2009). Scanning electron microscopy was performed using a JEOL 5410 LV microscope as described (Shani et al., 2009). Microapplication of 1 mM IAA (Duchefa Brand I0901) was performed as described (Reinhardt et al., 2000).

Accession Numbers

Sequence data for *Arabidopsis* (At) or tomato (Sl) genes used in this study can be found in the Arabidopsis Genome Initiative or Sol Genomics Network under the following accession numbers: At *IPT7* (AT3G23630), At *CKX3* (AT3G23630), At *KT2* (AT1G70510), Sl *TRR3,4* (SGN-U577676), Sl *TRR8/9a* (SGN-U572841), Sl *TRR8/9b* (SGN-U572839), Sl *TRR16,17* (SGN-U601012), Sl *Tkn2* (SGN-U321206), Sl *E* (AF022020), and Sl *CYCD3.3* (SGN-U586344).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *FIL_pro* Drives Expression throughout the Leaf until Relatively Late Stages of Leaf Development.

Supplemental Figure 2. *FIL_pro>>AtIPT7* Plants Grow Indeterminately.

Supplemental Figure 3. Early Leaf Development in Genotypes with Altered CK Levels.

Supplemental Figure 4. Constitutive Overexpression of At *CKX3* in Tomato Results in Stunted Plants with Simple Leaves.

Supplemental Figure 5. Sequence Comparison of Tomato and *Arabidopsis* Type A *RR* Genes.

Supplemental Figure 6. *CYCD3.3* Is Upregulated in *FIL_{pro}>>AtIPT7* Plants.

Supplemental Figure 7. A Second Peak of a CK Response Marker in P7 Tomato Primordia.

Supplemental Figure 8. Exogenous Auxin Application Suppresses the *FIL_{pro}>>AtIPT7* Super-Compound Leaf Phenotype.

Supplemental Figure 9. Coexpression of At *CKX3* Suppresses the Super-Compound Leaf Phenotype Caused by *Tkn2* Expression in Leaf Margins.

Supplemental Figure 10. *Tkn2-SRDX* Expression Remains High When Coexpressed with At *IPT7*.

Supplemental Figure 11. Late Expression of At *IPT7* Does Not Affect Leaf Complexity but Causes Ectopic Growth between Veins.

Supplemental Figure 12. Coexpression of At *IPT7* Enhances the Leaf Phenotype Caused by *KT2* Overexpression at Late Stages of *Arabidopsis* Leaf Development by *BLS_{pro}*.

Supplemental Table 1. Primers Used in This Study.

Supplemental Data Set 1. Text File of the Sequences and Alignment Used for the Phylogenetic Analysis Shown in Figure 3A.

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