Temporal control of leaf complexity by miRNA-regulated licensing of protein complexes

Ignacio Rubio-Somoza[1], Chuan-Miao Zhou[2], Ana Confraria[3], Claudia Martinho[3], Patrick von Born[1], Elena Baena-Gonzalez[3], Jia-Wei Wang[1,2*], and Detlef Weigel[1*]

[1]Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

[2]National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 200032 Shanghai, China

[3]Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal.

Author Information Correspondence and requests for materials should be addressed to J.-W.W. (email jwwang@sibs.ac.cn) or D.W. (e-mail: weigel@weigelworld.org).

Running title: SPL-dependent aging through licensing of protein complexes.

Highlights:

- miR319-targeted TCPs regulate CUC protein activities.

- SPL-dependent temporal cue is integrated by licensing protein complexes.

- Protein complex dynamism sustains age-dependent leaf shape.

Summary

The tremendous diversity of leaf shapes has caught the attention of naturalists for centuries. In addition to inter- and intra-specific differences, leaf morphologies may differ in single plants according to age, a phenomenon known as heteroblasty [1]. In *Arabidopsis thaliana*, the progression from the juvenile to the adult phase is characterized by increased leaf serration. A similar trend is seen in species with more complex leaves, such as the *A. thaliana* relative *Cardamine hirsuta*, where the number of leaflets per leaf increases with age. While the genetic changes that led to the overall simpler leaf architecture in *A. thaliana* are increasingly well understood [2-4], less is known about the events underlying age-dependent changes within single plants, in either *A. thaliana* or *C. hirsuta.* Here, we describe a conserved miRNA-transcription factor regulon responsible for an increase in leaf complexity. In early leaves, miR319-targeted TCP transcription factors interfere with the function of miR164-dependent and -independent CUC proteins, preventing the formation of serrations in *A. thaliana* and of leaflets in *C. hirsuta*. As plants age, accumulation of miR156-regulated SPLs act as a timing cue that destabilizes TCP-CUC interactions. The destabilization licenses activation of CUC protein complexes and thereby the gradual increase of leaf complexity in the newly formed organs. These findings point out to posttranslational interaction between unrelated miRNA-targeted transcription factors as a core feature of these regulatory circuits.

Results and Discussion

Similar to many other flowering plants, the progression from the juvenile to the adult phase in *Arabidopsis thaliana* is characterized by increased leaf serration and shifts in the distribution of specialized cell types, such as trichomes on the upper and lower leaf surface [5]. *Cardamine hirsuta* has more complex leaves than its relative *A. thaliana*, with older leaves having more leaflets instead of additional serrations (Figure 1A). Recent years have seen considerable advances in our understanding of the genetic causes that underlie the shift to a simpler leaf architecture in *A. thaliana* [2-4]. In contrast, less is known about the molecular events responsible for age-dependent changes within individuals in either *A. thaliana* or *C. hirsuta*. The sculpting of the leaf margin relies in species with both simple and compound leaves on CUP-SHAPED COTYLEDON (CUC) transcription factors, which function in a conserved framework for organ initiation and demarcation [6-8]. *CUC2* and *CUC3* are expressed at the boundaries of incipient serrations and leaflets [7, 9], with *CUC2* function partially depending on *CUC3* activity [9] (Figure S1). *CUC2*, but not *CUC3*, is a target of the microRNA (miRNA) miR164 [10]. Similar to the phenotype of *cuc3* mutants,

when *CUC2* activity was reduced because of inactivating mutation or because of miR164 overexpression, all leaves developed smoother margins in *A. thaliana* and fewer leaflets in *C. hirsute* [7, 9-11] (Figure 1A, Figure S1). Conversely, reduced miR164 activity increased the number of serrations or leaflets throughout development, without affecting the timing of the change in leaf complexity [10, 12] (Figure 1A).

Transcription factors of the NAC (NAM, ATAF1/2, CUC2) family often form DNA-binding dimers [13, 14]. Together with the overlap in *CUC2* and *CUC3* expression domains and similar genetic activities [6-9], this led us to hypothesize that CUC2 and CUC3 function in the same regulatory complex. Yeast two-hybrid (Y2H) assays indicated that CUC2 can homo- as well as heterodimerize with CUC3 (Figure 1B). These interactions were confirmed by bimolecular luminescence complementation (BiLC) in *Nicotiana benthamiana* leaf cells, using a miR164-insensitive form of the *CUC2* expression construct [15, 16] (Figure 1B, Figure S2). *CUC2* and *CUC3* expression are partially dependent on CUC2 activity [9], suggesting a feed-forward loop: After CUC2 homodimers have contributed to CUC3 activation, CUC2 and CUC3 heterodimerize, and the CUC2/CUC3 complexes promote leaf complexity.

Another group of miRNA-regulated transcription factors known to regulate leaf complexity belong to the TCP (TEOSINTE BRANCHED 1/CYCLOIDEA/PCF) family. MiR319-targeted TCPs sensitize cells at the leaf periphery to growth arrest signal in a range of species with different leaf morphologies [17-21]. Overexpression of miR319 induced the formation of additional serrations in *A. thaliana* and leaflets in *C. hirsuta* (Figure 1A, 2B), while *MIM319* plants deficient in miR319 activity had opposite phenotypes (Figure 1A, 2A).

To investigate how the miR164-CUC and miR319-TCP nodes might be connected, we examined the expression of miR164 and miR319. MiR164 amounts did not change substantially in consecutive *A. thaliana* leaves, but rose in parallel with increasing leaf complexity in *C. hirsuta* (Figure 2B), while miR319 expression appeared to be unaffected by age in both species. TCP3, one of the miR319 targets, has been suggested to down-regulate *CUC2* by directly activating *MIR164A* [22], but decreased miR319 activity did not obviously change miR164 expression in *A. thaliana* (Figure S3). To confirm this observation, we introduced the *mir164a*-4 mutation, which relieves miR164-dependent CUC repression, into *MIM319* plants. The increased leaf complexity caused by *mir164a*-4 was reversed by *MIM319* (Figure 1A, 2C). Taken together, these results indicated that TCPs can interfere with CUC2 activity in a manner that does not necessarily involve miR164. Moreover, overexpression of miR164 reduced the serrations and leaflets induced by miR319

overexpression in *A. thaliana* and *C. hirsuta* (Figure 2A, C), consistent with miR319 overexpression and a *cuc2* mutation having opposite effects [9].

An *Antirrhinum majus* TCP protein that does not belong to the miR319-targeted TCP subgroup can interact with a CUC2 homolog in vitro [23], but the biological relevance of this interaction has not yet been studied. We found that TCP4 interacted with both CUC2 and CUC3 in yeast and in *N. benthamiana* (Figure 2D, Figure S2). This result, together with the opposite phenotypic effects, suggested that TCPs modulate CUC function either by altering their ability to dimerize, by affecting their interactions with other partners, or by blocking their access to general transcription factors. Any of these scenarios could explain the negative effect of increased TCP activity on CUC-dependent formation of serrations and leaflets. To test the first possibility, we performed yeast-three-hybrid (Y3H) assays with CUC2, CUC3 and TCP4. We observed that TCP4 interfered with dimerization of the two CUC proteins (Figure 2E). The formation of CUC2-CUC2 and CUC2-CUC3 dimers could also be titrated by TCP4 in BiLC assays (Figure 2E). In addition, TCP4 compromised CUC3 transactivation ability to a similar extent as other proteins that form a bridge between DNA binding factors and transcriptional repressors such as TOPLESS [24-26]. When fused to the GAL4-DNA binding domain and tested in protoplasts with a UAS-Firefly reporter, CUC3 had substantial transactivation potential, which was reduced to about half by adding TCP4 (Figure 2F).

The inhibitory effect of TCP4 on CUC dimerization and transactivation potential provides a potential means of CUC2 and CUC3 post-transcriptional control that is independent of miR164. In *A. thaliana*, there was no obvious age-dependent change in expression of miR319, which modulates TCP4 activity (Figure 2B). However, there is a suite of interconnected regulatory molecules that is known to change in expression during the vegetative life cycle: miR172 and miR156 along with their transcription factor targets from the APETALA2 **(**AP2) and SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) families [13, 14, 27, 28]. As in *A. thaliana*, miR156 levels declined in successive leaves of *C. hirsuta* (Figure 3A), and by manipulation of miR156 activity, we could either slow or accelerate the acquisition of leaf complexity (Figure 3B). miR156-targeted SPLs fall into two size classes that share some downstream targets [27-29]. Both the three short SPLs (SPL3, SPL4 and SPL5) and long SPLs such as SPL9 promote both flowering and the acquisition of certain adult leaf traits [27], but only long SPLs contribute to changes in leaf shape [27]. As long SPLs have several additional domains in addition to their DNA binding domain, an obvious hypothesis is that their specialized activities rely on protein interactions mediated by these additional sequences.

We performed epistasis tests to determine whether the miR164-CUC node was required for the precocious serrations or leaflets in plants with reduced miR156 activity. We found that miR164 and miR319 expression did not change in response to altered miR156 levels (Figure S4, S5, S6), and that, different from TCP4, there was no evidence for CUC heterodimers being affected by SPL9 protein (Figure S5). Because SPL9 can regulate other processes through licensing of active transcription factor complexes [15], we hypothesized that the link between CUCs and SPLs is indirect and mediated by TCPs. Dimerization of SPL9 with TCP4, via the SPL9 carboxy-terminal domain, (Figure 3C, 3D Figure. S2) and interference of SPL9 with TCP4-CUC dimerization (Figure 4A) indicated that SPLs promote CUC-controlled acquisition of leaf complexity by competing with CUCs for TCP interaction, thereby preventing disruption of functional CUC dimers by TCPs. In agreement, the precocious formation of serrations or leaflets caused by increased SPL activity was partially suppressed by a simultaneous increase in TCP activity (Figure 4B, 4C).

In summary, our findings describe a conserved miRNA-transcription factor regulatory circuit that is responsible for age-related increase in leaf complexity. We have provided evidence for a posttranslational interaction between the miR164-CUC and miR319-TCP nodes that is separate from the previously suggested link via TCP-dependent activation of the *MIR164A* promoter [22]. The observation that TCPs regulate CUC activity by titrating CUC dimers and altering CUC activation potential underscores the importance of protein-protein interactions between targets of unrelated miRNAs as a recurring feature in miRNA network architecture [30]. MiR319-targeted TCP transcription factors prevent the formation of functional CUC complexes in early-arising leaves, which therefore lack serrations or leaflets. As the plant ages, miR156 levels decline, alleviating repression of its SPL targets. MiR156-targeted SPLs compete with CUC proteins for access to TCPs, and thereby enable the formation of active CUC complexes in later-arising leaves (Figure 4D). Hence, in addition to their role as direct transcriptional regulators [27, 28], SPLs license protein complexes that instruct morphological modifications typical for the adult phase of development.

Supplemental Information

Supplemental Information includes 6 figures, 2 tables, and Supplemental Experimental Procedures and can be found with this article online at XXXX.

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Author Contributions

I.R.-S., J.-W.W. and D.W. conceived the study; I.R.-S., C.-M.Z., A.C., C.M., P.B., E.B.-G. and J.-W.W. performed the experiments;. I.R.-S., J.-W.W. and D.W. wrote the paper with contributions from all authors.

Competing Financial Interests

The authors declare that no competing interests exist.

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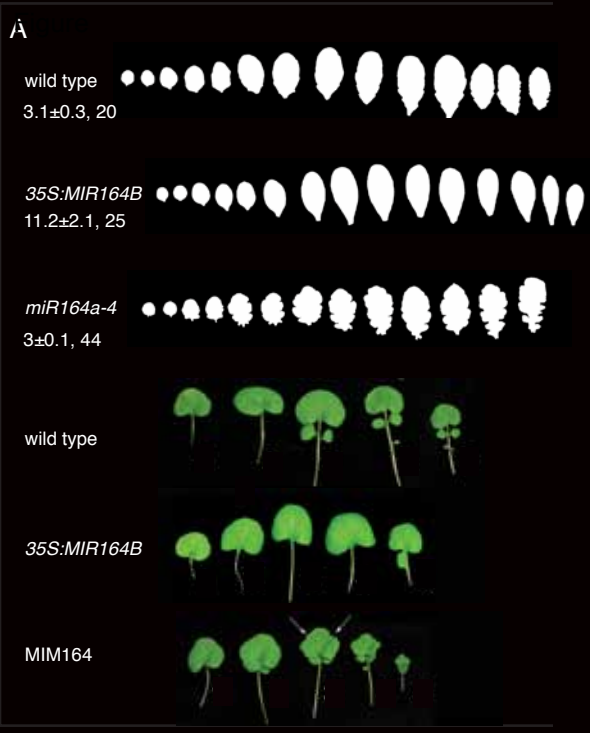
Figures

Figure 1. Promotion of leaf complexity by CUC proteins. (A) The later leaves are initiated in *A. thaliana* (outlines on top), the more serrations they have. In *C. hirsuta* (photographs), the number of leaflets increases. In both species, the effects of overexpressing miR164 in *35S:MIR164A* plants are the opposite of those caused by inactivation of miR164 through a mutation or the *MIM164* target mimic. (B) CUC2 interacts with itself and with CUC3 in Y2H (top) and *N. benthamiana* BiLC (bottom) assays. Color scale on the right indicates luciferase activity. A miR164-non-targetable version of *CUC2* was used here and in all other BiLC assays. Numbers below the plant background in Arabidopsis leaves (A) mean: first leave with serrations, SD and number of plants assayed. See also Figures S1, S2.

Figure 2. **MiR164-independent effects of TCPs on CUC activity.** (A) The effects of overexpressing the *MIR319B* precursor are the opposite of those caused by inactivation of miR319 through a target mimic. (B) MiR164 and miR319 expression in leaf series from *A. thaliana* (left) and *C. hirsuta* plants (right). "1" is the first (oldest) leaf. (C) Reducing miR319 activity with *MIM319* suppresses increased leaf complexity caused by a *mir164a*-1 mutation in *A. thaliana* (top). Similarly, overexpression of miR164 reduces the increased leaf complexity caused by miR319 overexpression in *A. thaliana* (middle) and *C. hirsuta* (bottom). (D) Interaction of TCP4 with CUC2 and CUC 3 in Y2H (top) and BiLC assays. A miR319-non-targetable version of *TCP4* was used here and in all other BiLC assays. (E) Disruption of CUC dimers in Y3H (top) and BiLC assays. (F) CUC transactivation activity in *A. thaliana* protoplasts. Values are normalized to the controls expressing *UAS:Luc* with *35S:BD* alone or in combination with *35S:rTCP4*, as represented by the dotted line. Bars represent standard error of the mean (s.e.m.; $n = 6$). p, Student's t-test (two-tailed, paired). See also Figures S2, S3. Numbers below the plant background in Arabidopsis leaves (A,C) mean: first leave with serrations, SD and number of plants assayed.

Figure 3. The miR156-SPL node provides an aging cue through protein-protein interactions with miR319-regulated TCPs. (A) MiR156 expression in leaf series from *A. thaliana* (left) and *C. hirsuta* plants (right). (B) MiR156 contributes to age-dependent increase in leaf complexity. (C) Interaction of SPL9 with TCP4 in Y2H (top) and BiLC (bottom) assays. (B) SPL9 interacts with TCP4 in Y2H assays via the carboxy-terminal domain; such sequences are missing in the short SPLs SPL3, SPL4 and SPL5. N-SPL9 and C-SPL9 refer to the amino- and carboxy-terminal portions of the protein. Numbers below the plant background in Arabidopsis leaves (B) mean: first leave with serrations, SD and number of plants assayed. See also Figure S2.

Figure 4: Age dependent modulation of TCP4-CUC interactions by miR156-regulated SPLs. (A) Disruption of CUC-TCP4 heterodimerization by SPL9 in Y3H (left) and BiLC assays (right). A miR156-non-targetable version of *SPL9* was used for the plant experiments. (B) *MIM156* partially suppresses the *MIM319* effects in short-day grown *A. thaliana* (left) and *C. hirsuta*.(right). (C) Onset of leaf serration in *A. thaliana* in plants grown in short-day conditions. (D) Diagram for age-dependent control of leaf complexity through licensing of active CUC complexes by miR156-regulated SPLs. See also Figures S2, S4, S5 and S6.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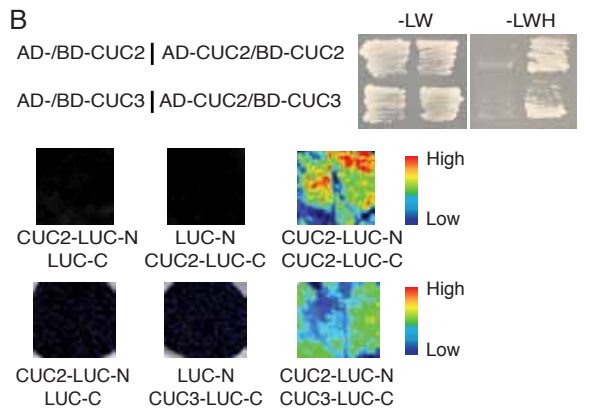
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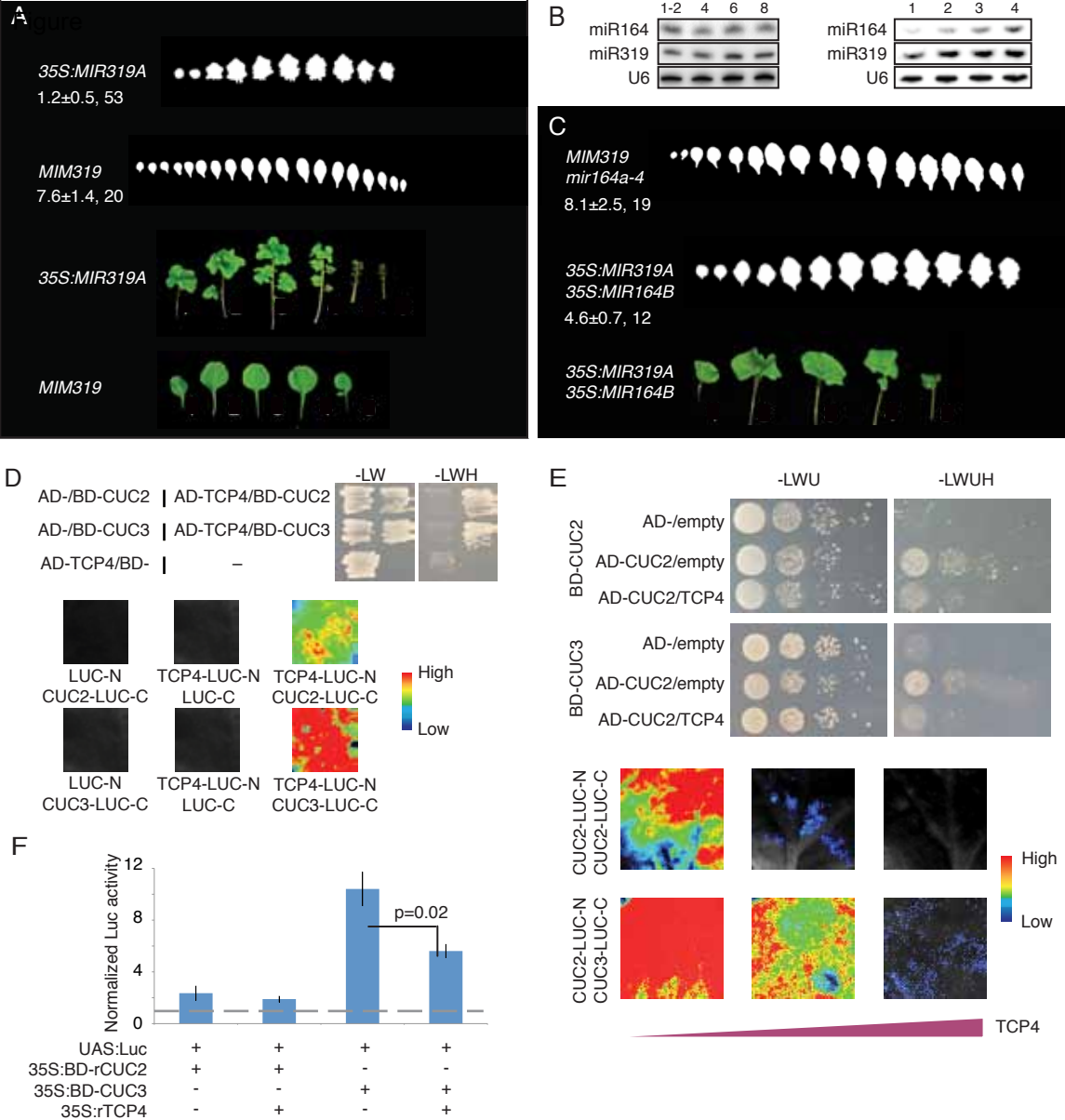
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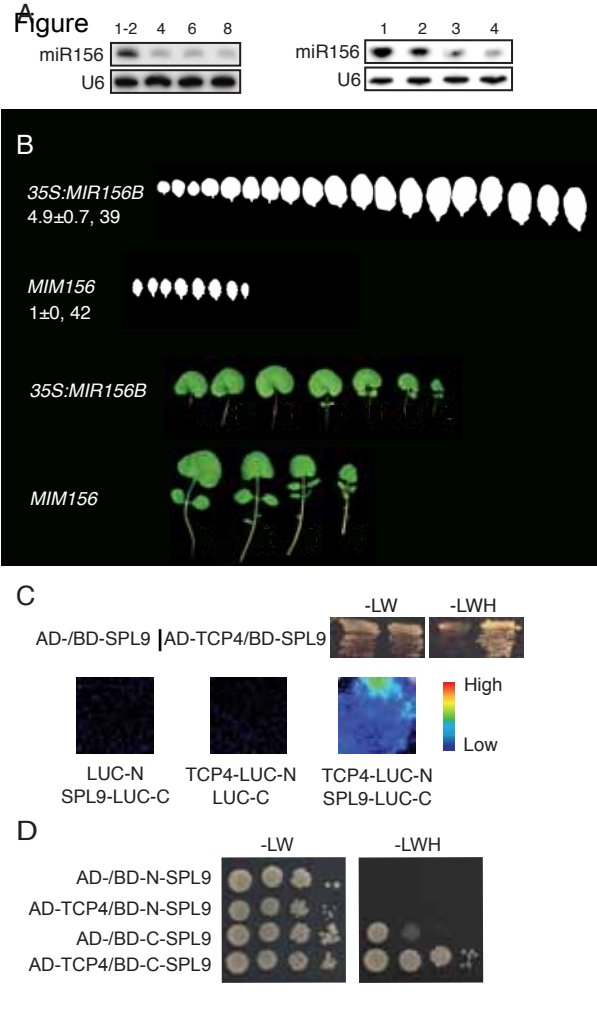
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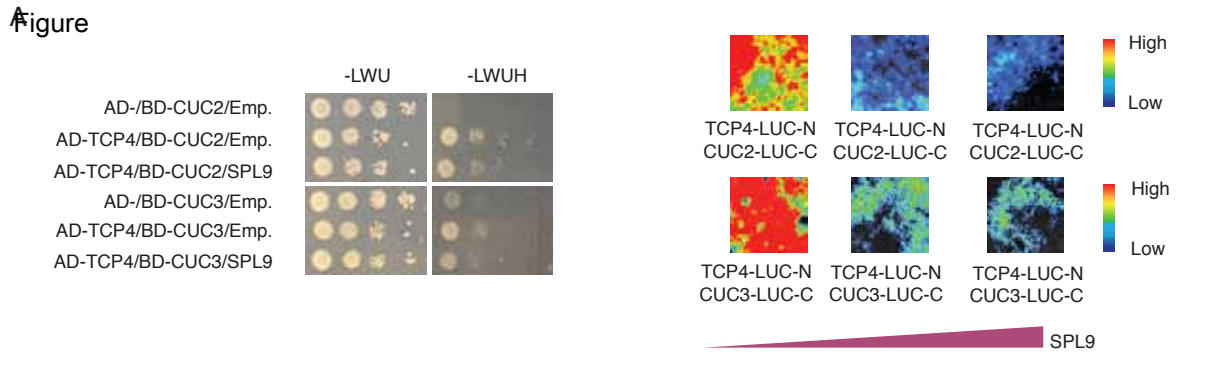
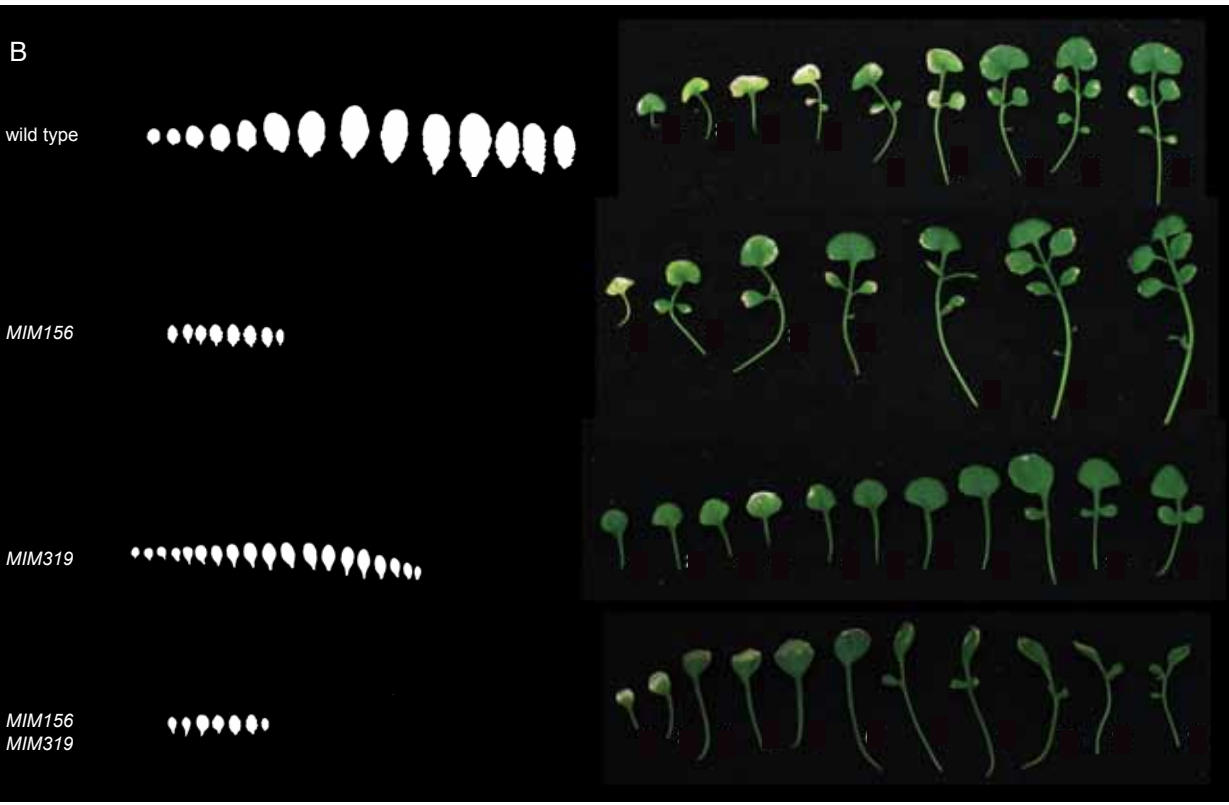
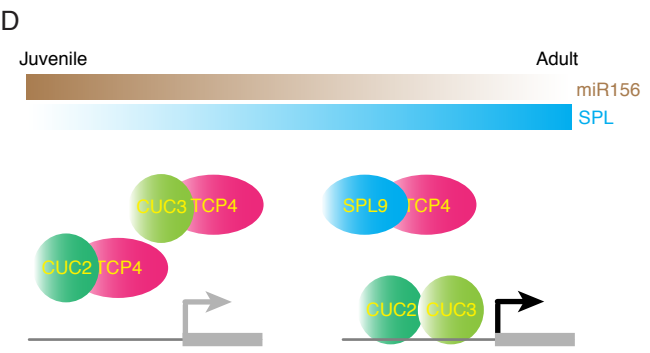
Figure





C

Background	First leaf with serrations (±SD)	n
Empty Vector	3.1±0.3	20
MIM156	1.0±.0.0	42
MIM319	7.6±1.4	20
MIM319xMIM156	3.5±0.7	76



Supplemental Information

Temporal control of leaf complexity by miRNA-regulated licensing of protein complexes

Ignacio Rubio-Somoza, Chuan-Miao Zhou, Ana Confraria, Claudia Martinho, Patrick von Born, Elena Baena-Gonzalez, Jia-Wei Wang, and Detlef Weigel

Supplemental Tables

Table S1. DNA constructs.

Table S2. Oligonucleotide sequences.

Supplemental Figures

Figure S1. Epistatic interactions between *CUC2* and *CUC3*. Loss of miR164a in *mir164a*-4 mutants increases leaf serrations, while inactivation of *CUC3* has the opposite effect. The cuc3-3 mutation largely suppresses the *mir164a*-4 defects.

Figure S2. BiLC assays.

Figure S3. MiR164 expression in leaves of short-day grown *A. thaliana* plants with altered miR319 activity. "1" is the first leaf.

Figure S4. Epistatic interactions between miR156 and CUCs in *A. thaliana* and *C. hirsuta*. (A) Precocious serrations of *MIM156* plants, which are deficient in miR156 activity, are lost when CUC2 activity is compromised by overexpressing the *MIR164A* precursor or by mutating the gene encoding the CUC2 partner CUC3. (B) CUC2 function is required for the formation of leaflets in *MIM156* plants.

Figure S5. Absence of miR156-SPL effects on miR164 expression or CUC dimerization. (A) MiR164 expression in leaves of short-day grown *A. thaliana* plants. (B) Effects of SPL9 and TCP4 on CUC2-CUC3 dimerization in BiLC assays.

Figure S6. MiR319 expression in leaves of short-day grown *A. thaliana MIM156* plants. "1" is the first leaf.

Supplemental Experimental procedures

Plants. Plants were grown on soil in long days (16 hr light/8 hr dark) or in short days (8 hr light/16 hr dark) under a mixture of warm and cold white fluorescent light at 23°C and 65% humidity. *Arabidopsis thaliana* plants constitutively expressing *MIM156, MIM164, MIM319, MIR319A, MIR156B, MIR164B* [1-5] and the *miR164a*-4 [6] mutant have been described. The *cuc3-3* (GABI_218D03) mutant line was from the European Arabidopsis Stock Centre (http://arabidopsis.info). *MIM* and *MIR* overexpression constructs were introduced into *C. hirsuta* by *Agrobacterium tumefaciens*-mediated transformation. Detailed information about the constructs can be found in Table S1.

Leaf imaging. Leaves from *A. thaliana* plants grown in short days where collected in a mixture of ethanol:glycerol:lactic acid (3:1:1) and kept at 4°C. Cleared leaves were fully flattened, placed between two transparency films and imaged on a flat-bed scanner. Image segmentation was made using ImageJ and Adobe Photoshop.

RNA analysis. Total RNA was extracted using TRIzol Reagent (Invitrogen). For small RNA blots, about 30 leaves were pooled for each sample. Sequences for oligonucleotide probes can be found in Table S2.

Protein interaction assays. Interactions in yeast were assayed in selective medium (Leu^-, Trp^-, His^-) supplemented with 15 to 45 mM 3-amino 1,2,4-triazole (3-AT). Yeast three-hybrids assays were performed as described [7] in selective medium (Leu^-, Trp^-, His^-, Ura^-) containing from 60 to 80 mM 3-AT. Assays were repeated at least 3 times.

Bimolecular luciferase complementation (BiLC) assays were performed in *N. benthamiana* leaves as described [8] with *Agrobacterium tumefaciens* cultures for the LUC-fusions at OD_{600} = 0.2, mixed in equal ratios with a P19 silencing suppressor [9] culture at OD_{600} = 0.1 and *35S:GUS* culture at OD_{600} = 0.1. For the

experiments were protein complex composition was assayed, the *35S:GUS* culture was replaced with increasing amounts of *35S:rTCP4* or *35S:rSPL9* (OD_{600} = 0.05 and OD_{600} = 0.1). Assays were repeated at least 3 times and 3 leaves in 3 independent plants were infiltrated in each repetition

Transactivation assays in protoplasts. Transient expression assays were performed as described [10], using the *UBQ10:GUS* reporter as internal control for transfection efficiency. Protoplasts were prepared from four week-old *A. thaliana* leaves and co-transfected with a firefly luciferase reporter driven by a promoter containing five GAL4-binding sites (*UAS:LUC*) [11], the *UBQ10:GUS* control reporter, and the indicated effector constructs. After transfection, protoplasts were incubated for 6 hr, harvested and lysed for measurements of LUC and GUS activities as described [10]. Samples transfected with *35S:rTCP4* were normalized to the control sample expressing *35S:GAL4-BD* and *35S:rTCP4*; all other samples were normalized to the control samples expressing *35S:GAL4-BD* alone. The *35S:rTCP4* construct has been described [3]. GAL4-DBD fusions were created by MultiSite Gateway cloning employing pEN-L4-2-R1 (35S promoter), pEN-R2-GAL4DBD-L3, an rCUC2 or CUC3 entry clone and pm43GW7 as destination vector [12].

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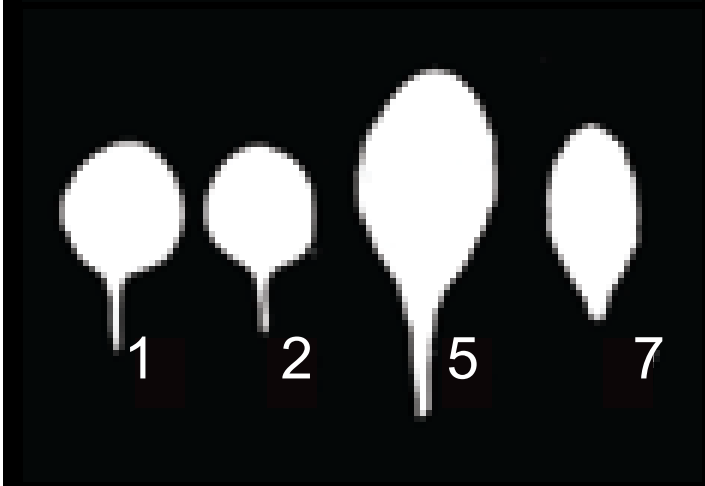
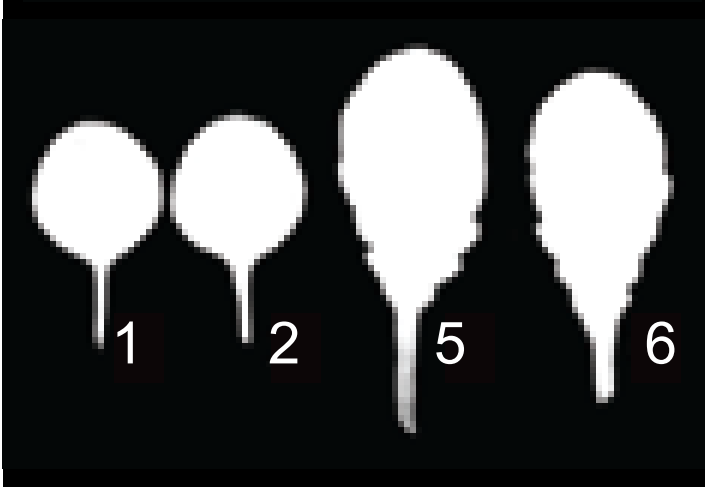
Supplemental Data

mir164a-4

cuc3-3



mir164a-4
cuc3-3



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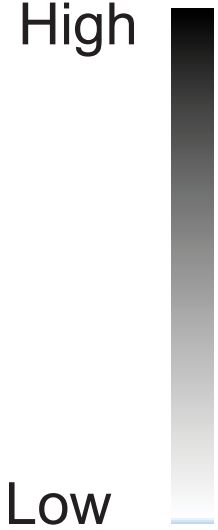
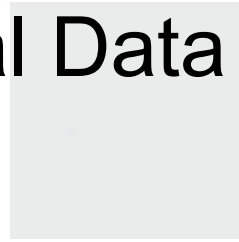
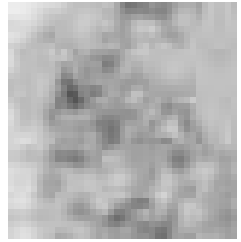
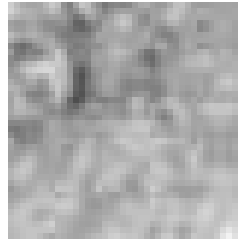




LUC-N
LUC-C

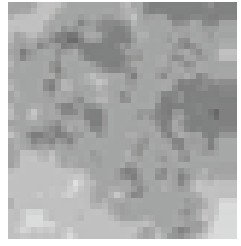


CUC2-LUC-N
CUC2-LUC-C



CUC2-LUC-N
CUC3-LUC-C

TCP4-LUC-N
CUC2-LUC-C



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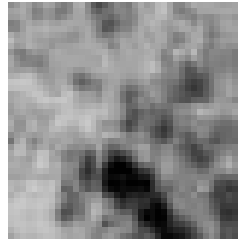
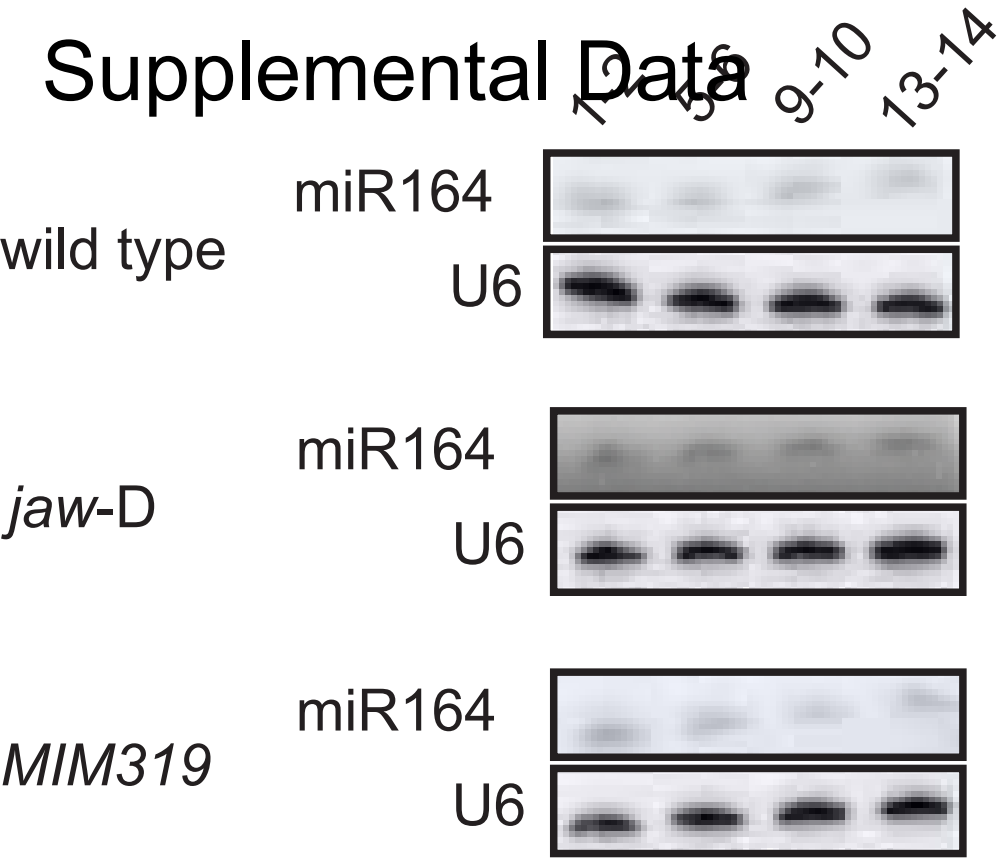


TCP4-LUC-N
SPL9-LUC-C

Supplemental Data



A

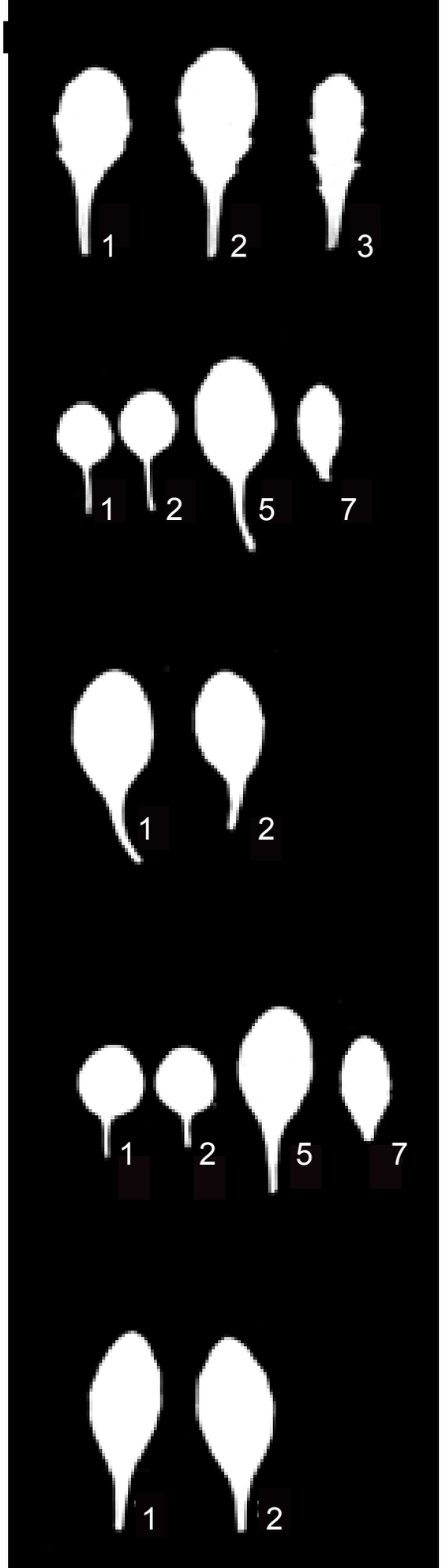
MIM156

35S:MIR164B

MIM156
35S:MIR164B

cuc3-3

MIM156
cuc3-3

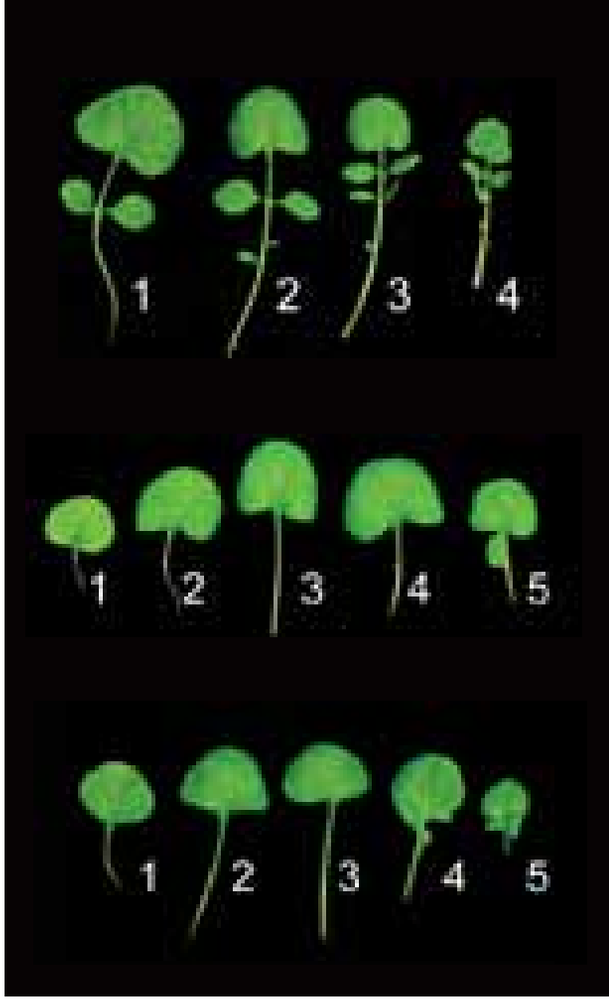


B

MIM156

35S:MIR164B

MIM156
35S:MIR164B



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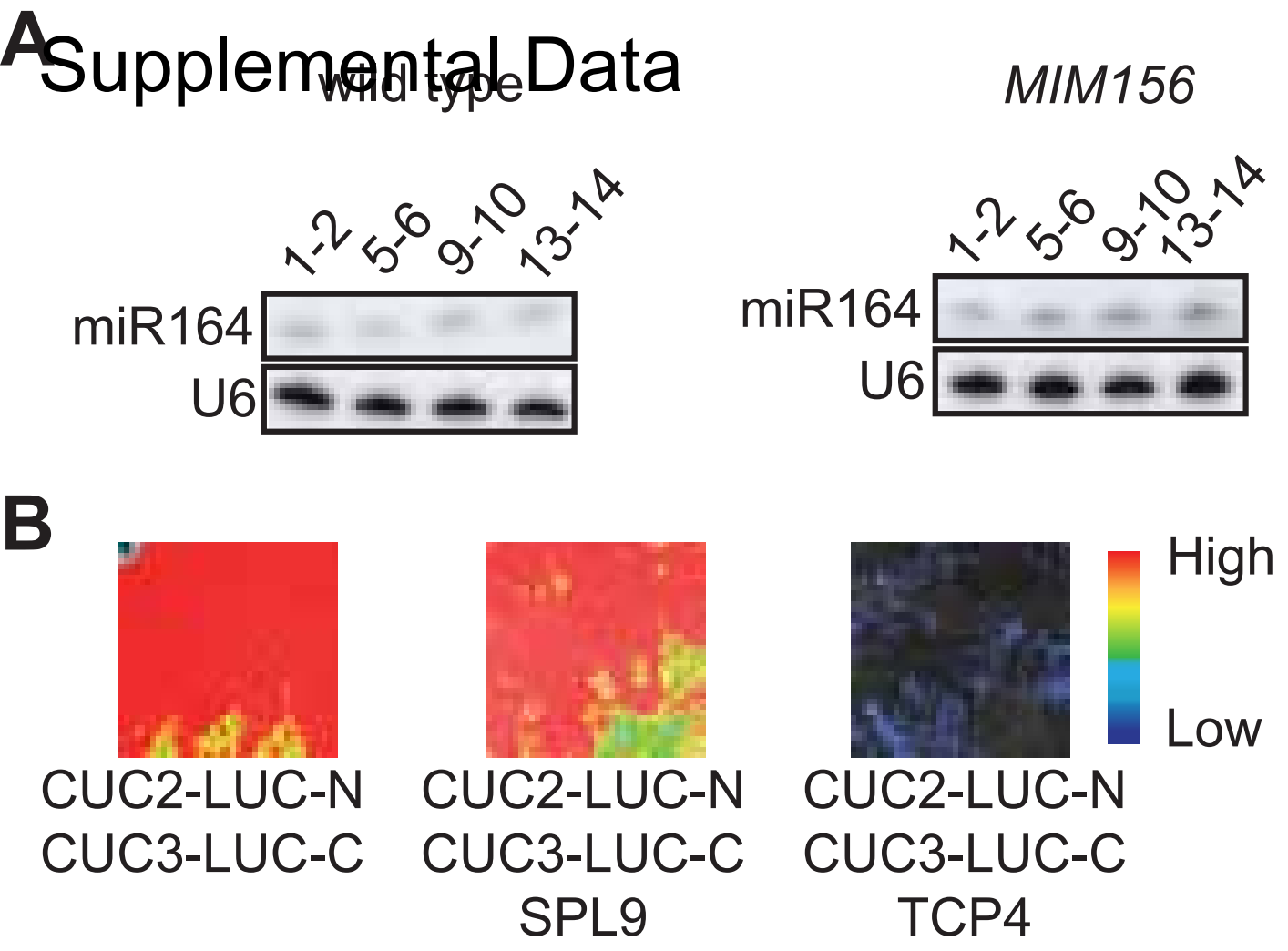

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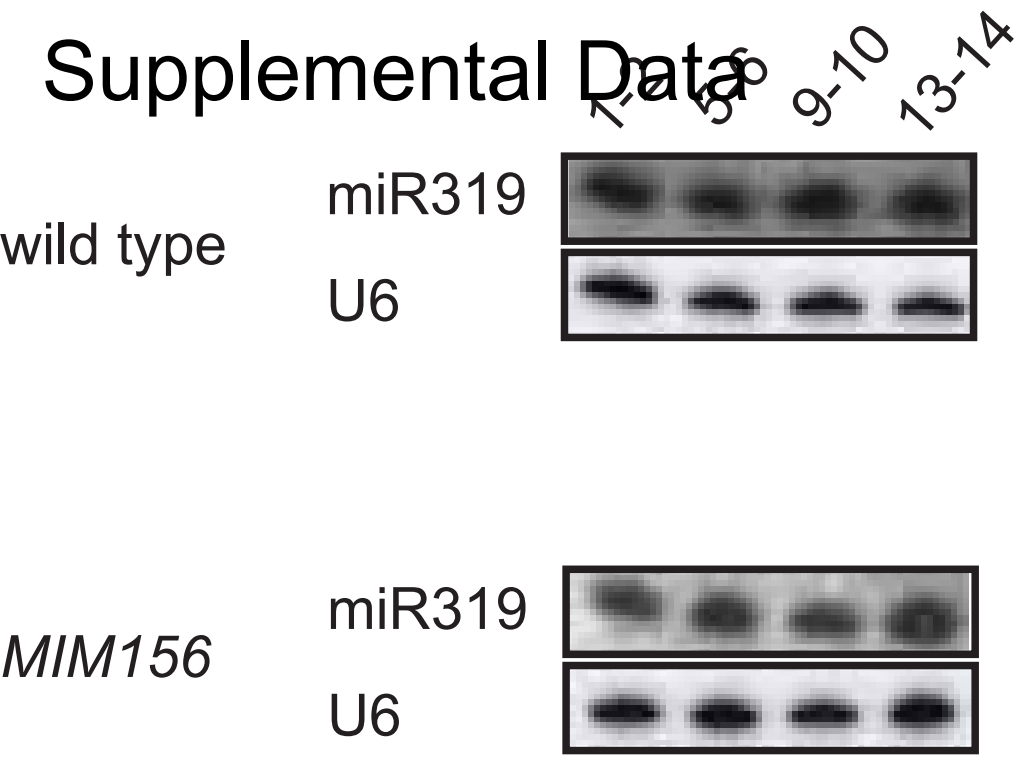

Table S1: Key to DNA constructs.

Description	Construct ID	Purpose
35S::miR156b	pRS105	miR156 OX plants
35S::MIM156	MT49	MIM156 OX plants
35S::miR164b	pRS111	miR164 OX in *A. thaliana*
35S::MIM164	MT249	MIM164 OX plants
35S::miR319a	pIR198	miR319 OX plants
35S::MIM319	MT54	MIM319 OX plants
CUC2 in pGADT7	JW616	Yeast assays
CUC3 in pGADT7	JW626	Yeast assays
TCP4 in pGADT7	JW630	Yeast assays
CUC2 in pGBKT7	JW624	Yeast assays
CUC3 in pGBKT7	JW617	Yeast assays
TCP4 in pYES52	pIR195	Yeast assays
SPL9 in pYES52	pIR196	Yeast assays
TCP4 in pDEST22	pIR190	Yeast assays
N-SPL9 in pDEST32	pIR220	Yeast assays
C-SPL9 in pDEST32	pIR221	Yeast assays
35S:rTCP4	JP74	Agroinfiltration and protoplast assays
35S:rSPL9	JW826	Agroinfiltration assays
35S:LUC-N	JW771	Agroinfiltration assays
35S::LUC-C	JW772	Agroinfiltration assays
rTCP4 in JW771	JW790	Agroinfiltration assays
rCUC2 in JW771	JW776	Agroinfiltration assays
CUC3 in JW772	JW777	Agroinfiltration assays
rCUC2 in JW772	pIR197	Agroinfiltration assays
rTCP4 in JW772	JW778	Agroinfiltration assays
35S::GUS	pPM180	Agroinfiltration assays
35S::rCUC2-GAL4BD	pIR199	Protoplast assays
35S::CUC3-GAL4BD	pIR200	Protoplast assays

Table S2: Oligonucleotide sequences.

Primer ID	Primer sequence	Purpose
G-28301	GTGCTCACTCTCTTCTGTCA	miR156 probe for RNA blot
G-16355	TGCACGTGCCCTGCTTCTCCA	miR164 probe for RNA blot
G-26748	GGGAGCTCCCTTCAGTCCAA	miR319 probe for RNA blot
G-20557	GCTAATCTTCTCTGTATCGTTCC	U6 probe for RNA blot