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Evolution and Diverse Roles of the *CUP-SHAPED COTYLEDON* Genes in *Arabidopsis* Leaf Development [C][W]

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***CUP-SHAPED COTYLEDON2* (*CUC2*) and the interacting microRNA *miR164* regulate leaf margin dissection. Here, we further investigate the evolution and the specific roles of the *CUC1* to *CUC3* genes during *Arabidopsis thaliana* leaf serration. We show that *CUC2* is essential for dissecting the leaves of a wide range of lobed/serrated *Arabidopsis* lines. Inactivation of *CUC3* leads to a partial suppression of the serrations, indicating a role for this gene in leaf shaping. Morphometric analysis of leaf development and genetic analysis provide evidence for different temporal contributions of *CUC2* and *CUC3*. Chimeric constructs mixing *CUC* regulatory sequences with different coding sequences reveal both redundant and specific roles for the three *CUC* genes that could be traced back to changes in their expression pattern or protein activity. In particular, we show that CUC1 triggers the formation of leaflets when ectopically expressed instead of CUC2 in the developing leaves. These divergent fates of the *CUC1* and *CUC2* genes after their formation by the duplication of a common ancestor is consistent with the signature of positive selection detected on the ancestral branch to *CUC1*. Combining experimental observations with the retraced origin of the *CUC* genes in the Brassicales, we propose an evolutionary scenario for the *CUC* genes.**

INTRODUCTION

Development is based on the progressive restriction of the cell potential, which ultimately leads to the organization of differentiated cells into tissues and organs. Regulation of gene expression at the transcriptional level plays an essential role in this process, and the identity of a cell largely depends on regulatory networks entailing the combinatory action of transcription factors (TFs). Modification of the expression patterns of the TFs and/or changes in their activity contribute to the elaboration of regulatory networks, which in turn appears to underlie the evolution of developmental processes and the emergence of new morphologies. Such evolution in the function of TFs is facilitated by duplication events that, by providing additional gene copies, may reduce the evolutionary constraints and allow subfunctionalization or neofunctionalization of duplicates. Therefore, it is interesting to combine the functional analysis of regulatory networks that encompass related TFs with the investigation of the evolutionary history of these factors.

The *Arabidopsis thaliana* genome encodes 2315 TFs that fall into 64 families (Guo et al., 2008; Schmutz et al., 2010). The *NAC* (for *NAM*/*ATAF1*,*2*/*CUC2*) genes form one of the largest families of plant-specific TFs and contain more than 100 members in *Arabidopsis* (Ooka et al., 2003; Guo et al., 2008). *NAC* factors share a highly conserved N-terminal DNA binding domain, the NAC domain, and regulate different biological processes, such as shoot and root development or the response to biotic and abiotic stresses (Olsen et al., 2005).

Among the first identified *NAC* genes of *Arabidopsis* are the *CUP-SHAPED COTYLEDON1* to *3* genes (*CUC1*–*CUC3*). These genes were identified because double mutants show a defective shoot apical meristem (SAM) and cotyledon fusion (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Mutation of the *CUC* homologs in petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*), and tomato (*Solanum lycopersicum*), the *NO APICAL MERISTEM* (*NAM*), *CUPULIFORMIS*, and *GOBLET* genes, respectively, leads to similar developmental defects (Souer et al., 1996; Weir et al., 2004; Blein et al., 2008; Berger

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et al., 2009), revealing an evolutionarily conserved role for these genes in SAM function and organ separation. Several *NAC* genes, including *CUC1* and *CUC2*, are targeted by the microRNA (miRNA) *miR164* (Rhoades et al., 2002), and studies of *MIR164* gene loss-of-function mutants or lines expressing *miR164* cleavage-resistant *CUC1* or *CUC2* genes revealed the biological importance of miR164 regulation of the *CUC1/2* genes (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005; Nikovics et al., 2006; Peaucelle et al., 2007; Sieber et al., 2007; Raman et al., 2008; Larue et al., 2009).

Besides the role of the *CUC* genes in SAM function, a novel role for these factors has recently been identified during leaf development. Two main groups of leaves can be distinguished according to their degree of complexity: simple and compound leaves (Champagne and Sinha, 2004; Blein et al., 2010). Simple leaves are formed by a single unit that consists of a petiole that supports the blade, which can be entire (smooth) or dissected by lobes or serrations. Compound or dissected leaves are formed when the incisions of the margin reach the leaf main axis and generate several units called leaflets. Serration of the *Arabidopsis* leaf requires the activity of *CUC2* (Nikovics et al., 2006), and similarly, *CUC* genes are also required for the larger dissections that lead to compound leaf development of eudicots (Blein et al., 2008; Berger et al., 2009). This indicated that the "dissector" function of *CUC* genes is conserved from the SAM to the leaf and across species with contrasted leaf shapes. Work in *Arabidopsis* suggests that the specific expression of *CUC2* in the sinus of the serrations mainly results from transcriptional regulation, whereas regulation by *MIR164A* contributes to the regulation of *CUC2* expression level (Nikovics et al., 2006).

The *CUC* genes can be subdivided into two clades whose separation predates the monocot–dicot divergence (Zimmermann and Werr, 2005). Whereas *CUC3* is a single copy gene in all the species that were examined so far (Vroemen et al., 2003; Zimmermann and Werr, 2005; Blein et al., 2008), the number of genes in the *NAM/CUC1/CUC2* clade is more variable. Only one member has been identified in tomato and snapdragon, and the strong phenotype resulting from their inactivation suggests that there is no redundant gene (Weir et al., 2004; Blein et al., 2008; Berger et al., 2009). Two paralogs resulting probably from recent duplications are present in maize (*Zea mays*) and pea (*Pisum sativum*; Zimmermann and Werr, 2005; Blein et al., 2008). In contrast, *Arabidopsis* CUC1 and CUC2 are more divergent and only show limited conservation outside the NAC domain. Interestingly, *Arabidopsis* CUC1 together with *Cardamine hirsuta* (hairy bittercress) CUC1 forms a subclade separated from the other proteins of the NAM/CUC1/CUC2 clade (Blein et al., 2008). Genetic analysis revealed that the *CUC* genes share partially redundant functions; however, specificities emerge for some members. For instance, *CUC3* has a prominent role during axillary meristem development, whereas the contribution of *CUC2* to embryo development is greater than that of *CUC1* (Hibara et al., 2006; Raman et al., 2008). Although differences between the expression of individual *CUC* genes have been reported, the basis for their partially redundant functions is not yet understood.

To address the basis of the specific and redundant functions of the *CUC* genes, we performed here a detailed analysis of their roles during *Arabidopsis* leaf development. By combining mutant analysis with the expression of chimeric transgenes, in which coding and regulatory sequences were exchanged, we reveal specific functions for the *CUC* genes and assign these functions to changes in the protein sequence or to variation in the expression patterns. Reconstruction of the origin of the *CUC1/CUC2* genes allows us to propose a scenario for the evolution of *CUC* genes in Brassicales.

RESULTS

CUC2 and *CUC3* Are Expressed in Leaf Primordia and Are Required for Wild-Type Serration

CUC2 was previously shown to be essential for *Arabidopsis* wild-type leaf serration, as its inactivation leads to smooth margins, whereas leaf shape was not affected by *CUC1* inactivation (Nikovics et al., 2006). To investigate the role of the third *CUC Arabidopsis* gene, *CUC3*, we examined the leaf phenotype of *cuc3* loss-of-function mutants in the Columbia-0 and Wassilewskija backgrounds (Figures 1A–1D and 1A′–1D′). Both *cuc3-105* and *cuc3-2* showed reduced serrations, even if shallow serrations could still be observed in these mutants in contrast to the smooth *cuc2-3* mutant (Figures 1B, 1B′, 1D, and 1D′, Nikovics et al., 2006). As the *cuc3-105* and *cuc3-2* alleles are likely to be knockout alleles (see Supplemental Figure 1 online; Vroemen et al., 2003; Hibara et al., 2006), we concluded that *CUC3* contributes to leaf serration, but in a minor way compared with *CUC2*.

Next, we examined *CUC3* expression during leaf development. RT-PCR indicated that *CUC3* mRNAs, like *CUC2* mRNAs, were detected in developing leaves (see Supplemental Figure 2 online). In contrast, no *CUC1* mRNA could be detected in developing leaves, linking the absence of leaf phenotype of *cuc1* mutants with the absence of detectable expression of this gene in the leaf. To determine more precisely the expression pattern of *CUC3*, we used a Pro_{CUC3}:*GUS* (for β-glucuronidase) reporter that was shown to faithfully reproduce *CUC3* expression in the embryo (Kwon et al., 2006; Figure 1E). GUS activity was detected at the base of the detached leaves (asterisks in Figure 1E). This region marks the junction of the leaf with the apex, a region from which an axillary meristem will be initiated and that expresses *CUC3* (Hibara et al., 2006). In young, smooth leaf primordia, faint GUS expression can be detected in the margin region where the first pair of teeth will form (arrows in Figure 1Ea). Later, GUS activity marks the sinus of the developing serrations (Figures 1Eb–1Ed). GUS activity is absent from the sinus of older teeth (arrowheads in Figure 1Ee).

CUC2 and *CUC3* Are Required for the Formation of Serrations in a Large Selection of Mutants/Transgenics

CUC2 (Nikovics et al., 2006) and now *CUC3* (Figure 1) are among the few documented genes that lead to leaves with smooth margins when inactivated. Therefore, we wondered whether these genes were obligatory actors of leaf dissection in *Arabidopsis*. To test this, we selected a collection of nine serrated or

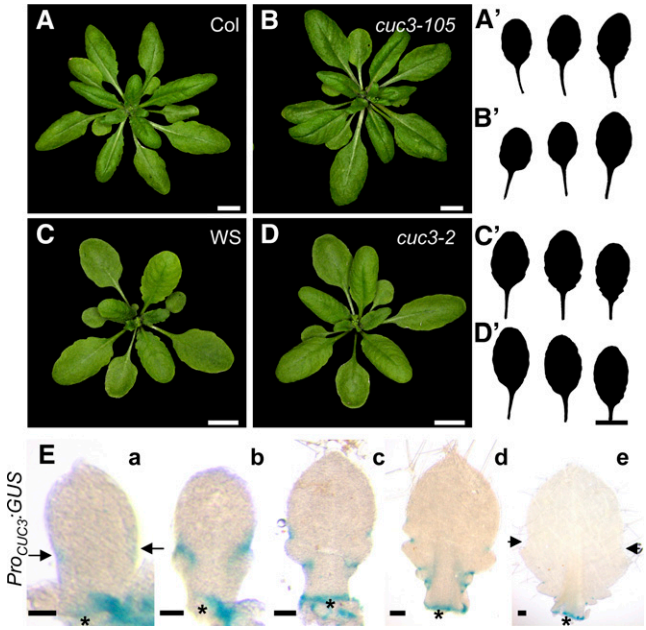

Figure 1. *CUC3* Is Required for Leaf Serration in *Arabidopsis*.

(A) to **(D)** Serrations are partially suppressed in *cuc3* mutants compared with the wild type. Rosette at bolting and leaves 5, 6, and 7 of wild-type Columbia-0 (**[A]** and **[A´]**), *cuc3-105* (**[B]** and **[B´]**), wild-type Wassilewskija (**[C]** and **[C´]**), and *cuc3-2* (**[D]** and **[D´]**) are shown. Bars = 1 cm.
(E) Expression of *CUC3* during leaf development. Weak activity of a Pro_{CUC3}:*GUS* reporter is observed along the margin of young smooth primordia at the position where the first pair of teeth is expected (arrows in **[a]**). In older primordia, Pro_{CUC3}:*GUS* activity marks the sinus of the outgrowing teeth (**[b]**–**[e]**) and disappears in larger teeth (arrowheads in **[e]**). Pro_{CUC3}:*GUS* activity is also detected at the base of the petiole at the junction point with the apex (asterisks). Bars = 100 μm.

lobed mutants or transgenic lines affected in diverse biological processes and analyzed the contribution of the *CUC2* or *CUC3* genes to their leaf phenotype. We selected the *serrate* (*se-1*; Grigg et al., 2005), *cap binding protein20* (*cbp20*; Papp et al., 2004), and *argonaute1* (*ago1-27*; Morel et al., 2002) mutants, the *sawtooth1 sawtooth2* (*saw1-1 saw2-1*; Kumar et al., 2007) double mutant, and transgenic lines overexpressing *UNUSUAL FLORAL ORGANS* (*UFOoexp*; Wang et al., 2003), *STYMPY/WOX9* (*stip*-D; Wu et al., 2005), *KNAT1/BP* (*KNAT1oexp*; Lincoln et al., 1994), *miRJAW/miR319* (*jaw*-D; Palatnik et al., 2003), or *NICOTIANA TOMENTOSIFORMIS KINASE INTERACTING SUBUNIT A* (*NtKIS1a-oexp*; Jasinski et al., 2002). These lines are affected in proteins with different biochemical functions, such as RNA binding, TFs, F-box proteins, or cyclin-dependent kinase inhibitors, that contribute to different biological processes, such as RNA, including miRNA, metabolism and function, organ identity, meristem function, or cell cycle regulation. When *CUC2* was inactivated in these backgrounds, the serrations were suppressed (*se-1*, *cbp20*, *ago1-27*, *stip*-D, *UFOoexp*, and *NtKIS1a-oexp* lines; Figures 2A–2F, 2I, 2J, 2M, 2N, 2S, and 2T) or strongly reduced (*jaw*-D and *saw1-1 saw2-1* lines; Figures 2G, 2H, 2L, and 2P). Suppression of the dissection of the *KNAT1oexp* line by the *cuc2-3* mutation was observed (Figures 2Q and 2R), although the mixed genetic background in the progeny affected the intensity of dissection (*KNAT1oexp* and *cuc2-3* are in the Nossen and Columbia-0 backgrounds, respectively; see Supplemental Figure 3 online). Examination of early stages of leaf development in *cbp20 cuc2-3*, *stip*-D *cuc2-3*, *se-1 cuc2-3*, and *jaw*-D *cuc2-3* lines indicated that serrations were not initiated in these backgrounds, whereas smaller teeth were observed in the *saw1-1 saw2-1 cuc2-3* line (see Supplemental Figure 4 online). Interestingly, in all combinations tested, only the leaf margin phenotype was modified by *CUC2* loss of function, leaving other parts of the leaf or plant unaffected. For instance, *jaw*-D *cuc2-3* had wavy leaves like *jaw*-D (Figures 2G and 2H) and *NtKIS1a-oexp cuc2-3* plants were small like *NtKIS1a-oexp* (Figures 2S and 2T). This indicates that *cuc2-3* is not a general suppressor of the phenotype of these lines but has a specific effect on the leaf margin. Together, these results suggest that *CUC2* is required for *Arabidopsis* leaf dissection.

miR164 targets the *CUC1* and *CUC2* genes, and this regulation is important for leaf development, as inactivation of *MIR164A*, one of the three *MIR164* genes, or expression of a *miR164* cleavage-resistant *CUC2* gene led to enhanced leaf serration (Nikovics et al., 2006; Larue et al., 2009). Inactivating *MIR164A* in the serrated mutant/transgenic lines suggested that *jaw*-D, *stip*-D, *saw1 saw2*, and *UFOoexp* contribute to leaf margin dissection independently of *MIR164A*, whereas *SE* and *CBP20* act via a pathway requiring *MIR164A* (see Supplemental Figure 5 online), in agreement with a role of SE and CBP20 in the processing of miRNA precursors into mature miRNAs (Chen, 2009; Voinnet, 2009). Furthermore, double mutants with *cbp20* or *se-1* and *cuc1-13* indicate that, as in the wild type, *CUC1* does not contribute to leaf serration in *cbp20* and *se-1* (see Supplemental Figure 5 online).

Next, we tested whether *CUC3* was also involved in the leaf phenotype of some of these mutants. *stip*-D *cuc3-105* and *UFOoexp cuc3-105* lines showed a partial suppression of the serration compared with the single *stip*-D and *UFOoexp* lines, respectively (Figures 2I, 2K, 2M, and 2O). This indicates that, like in the wild type, *CUC3* contributes to the serration of these transgenic lines, but to a lesser extent than does *CUC2*.

CUC2 Is Required Early in Serration Formation, Whereas *CUC3* Acts Later to Maintain Serration

To determine the developmental origin of the leaf serration defects of the *cuc2* and *cuc3* mutants, we performed a morphometric analysis of the first and second teeth of leaf 6, a leaf that shows clear serrations (Figure 3; see Supplemental Figure 6 online). In the wild type, and similarly in the *cuc1-13* mutant, the first and second teeth pair appeared in a basipetal order, symmetrically on both sides of primordia of ~200 and 400 μm long, respectively (Figure 3; see Supplemental Figures 6A–6D, 6G, and 6H online). In the *cuc2-3* mutant, no teeth appeared until the primordium reached ~400 μm. Small protrusions, which were not symmetrically distributed on both sides of the margin, occasionally appeared later and grew slowly to ~50 to 100 μm in height and kept a symmetrical shape, whereas teeth of the wild type grew and became asymmetrical (Figure 3; see

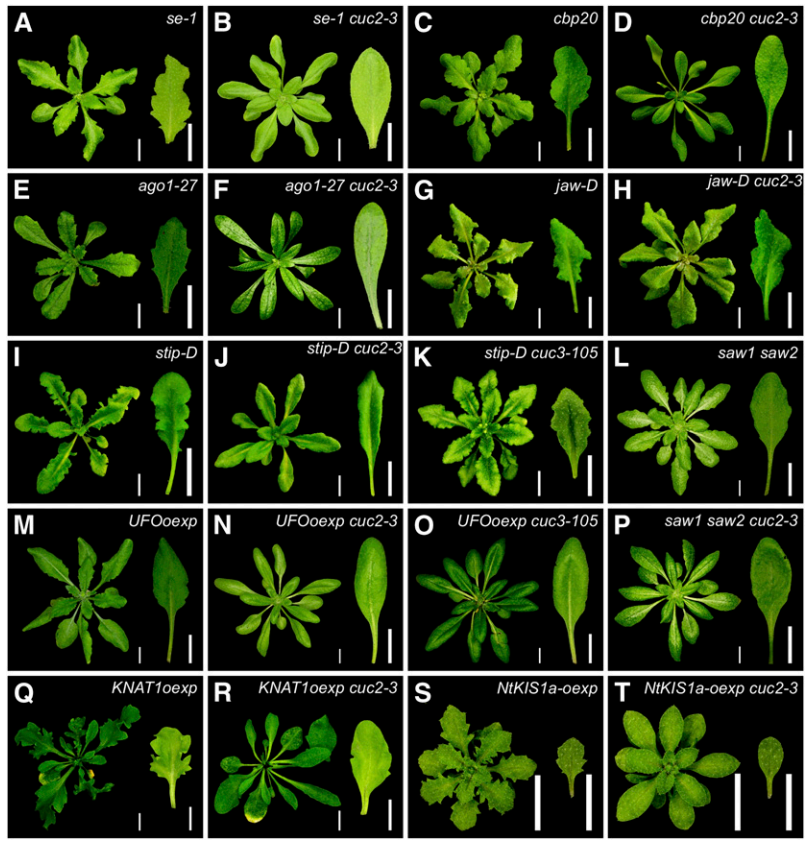

Figure 2. The *CUC2* and *CUC3* Genes Are Essential for *Arabidopsis* Leaf Serration.

Rosettes at bolting and the sixth leaf are shown for the indicated genotypes. Inactivation of *CUC2* largely suppresses serrations of the different lines but does not affect other aspects of leaf shape (such as waviness of *jaw*-D and small size of *NtKIS1a-oexp*). Inactivation of *CUC3* only partially suppresses the serrations. Bars = 1 cm.
[See online article for color version of this figure.]

Supplemental Figures 6A, 6B, 6E, and 6I online). The early stages of teeth formation were unchanged in the *cuc3-105* mutant, as teeth were initiated when the leaf had a similar length as the wild type, and teeth initially showed a similar increase in width and height (Figure 3, insets; see Supplemental Figures 6A, 6B, 6F, and 6J online). However, when *cuc3-105* teeth were ~150 μm high, their increase in height slowed down, whereas their increase in width was unaffected. These observations indicate that *CUC2* is required for the initiation and early stages of teeth development, whereas *CUC3* acts later to maintain their growth.

CUC2 Contributes to Wild-Type Leaf Serration via Two Pathways, Dependent or Independent of *CUC3*

Next, we tested the genetic interaction between *CUC2* and *CUC3* during leaf serration. As the strong *cuc2-3* serration defect precluded a direct analysis of the contribution of *CUC3* to this phenotype, we turned to lines with higher *CUC2* activities. The *mir164a-4* mutant and the *CUC2g-m4* transgenic line have higher *CUC2* expression levels as a result of defective *miR164*-dependent regulation and show higher serration levels (Figures 4A and 4B; Nikovics et al., 2006). We compared the leaf phenotype of the double *CUC2g-m4 cuc3-105* and *mir164a-4 cuc3-105* mutants with that of the corresponding *CUC2g-m4*, *mir164a-4*, and *cuc3-105* parental lines (Figure 4). Serration in the *mir164a-4 cuc3-105* and *CUC2g-m4 cuc3-105* lines was weaker than in the *mir164a-4* and *CUC2g-m4* lines, respectively (Figures 4A, 4B, 4D, and 4E), indicating that part of *CUC2* function is *CUC3*-dependent. However, serration in the *mir164a-4 cuc3-105* and *CUC2g-m4 cuc3-105* lines was also stronger than in the *cuc3-105* line, revealing a *CUC3*-independent action of *CUC2* on serration (Figures 4C–4E). This indicates that *CUC2* leads to leaf serration via two distinct pathways, either dependent or independent of *CUC3*.

The CUC Proteins Have Partially Redundant Functions

The results described above point to both specific and redundant functions of *CUC2* and *CUC3* and to no role of *CUC1* during *Arabidopsis* leaf serration. To further investigate the basis of this, we functionally analyzed a series of chimeric gene constructs

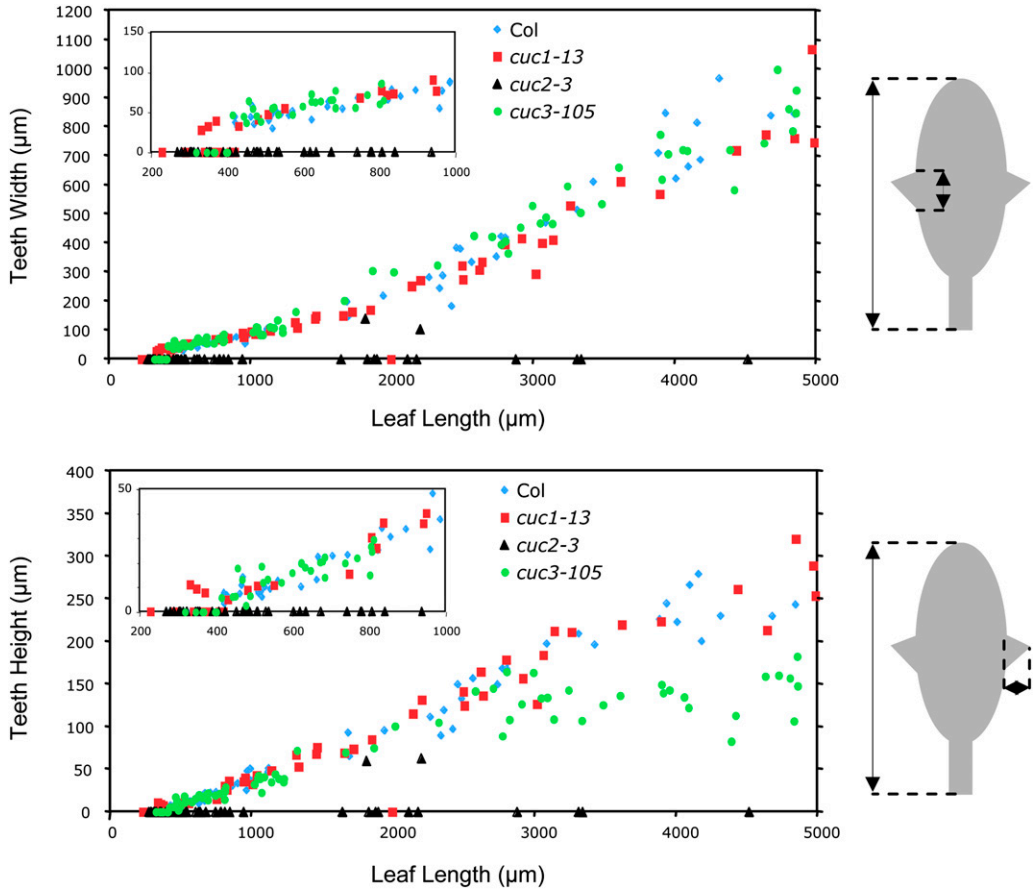

Figure 3. *CUC2* Is Required for the Early Stages of Teeth Formation, Whereas *CUC3* Acts Later to Maintain Teeth Growth.

Morphometric analysis of the second teeth in wild-type Columbia-0, *cuc1-13*, *cuc2-3*, and *cuc3-105* is shown. Teeth width or height is represented in relation to leaf length for the second teeth of the sixth leaf. Each point represents the data from one leaf. Insets are expanded details of the graphs showing the early phases of leaf formation. *cuc2-3* mutants show a defective initiation of the serration, whereas serration proceeds normally in the *cuc3-105* mutants until the teeth reach ~150 µm and the growth rate slows down.

expressing NAC open reading frames under the control of the *CUC1* or *CUC2* promoter in a *cuc2* loss-of-function background (Figures 5 and 6). To identify evolutionarily conserved functions of the CUC proteins, we enlarged this study to CUC open reading frames from pea and tomato that have a role in the dissection of compound leaves (Blein et al., 2008). The effects of each construct on leaf dissection in 11 to 38 independent transgenic lines were scored on a scale of increasing dissection ranging from 1 to 5 (see Methods; the parental *cuc2-1* smooth line has a score of 1 and the wild type has a score of 2, and scores above 2 indicate a stronger dissection; Figures 5A–5K). *cuc2-1* mutants not only show smooth margins (Figures 5L and 5M) but also severely reduced expression of the Pro_{CUC2}:*GUS*, Pro_{CUC3}:*GUS*, and $Pro_{MIR164A}$:*GUS* reporters: expression of all these markers is absent from the blade of the *cuc2-1* mutant and limited to the blade–petiole junction and leaf base for Pro_{CUC2}:*GUS*, to the leaf base for Pro_{CUC3}:*GUS*, and to the leaf tip for $Pro_{MIR164A}$:*GUS* (Figures 6A, 6H, and 6O; compare with Figures 6G, 6N, and 6U for wild-type patterns). Therefore, in addition to the morphological changes, we also characterized the effects of some chimeric constructs on *CUC2*, *CUC3*, and *MIR164A* promoter activities (Figure 6).

Expression of the control construct Pro_{CUC2}:*CUC2* in the *cuc2-1* background restored leaf serration (serration score = 2.1 ± 0.1; Figures 5A and 5N) and proper activities of the *CUC2*, *CUC3*, and *MIR164A* promoters at the blade margin (Figures 6B, 6I, and 6P). On the other hand, expression of the more distant NAC1 and ANAC019 proteins could not restore leaf serration (serration scores = 1.2 ± 0.2 and 1.0 ± 0.0, respectively; Figures 5B, 5C, 5O, and 5P). Expression of the Pro_{CUC1}:*CUC2* and Pro_{CUC1}:*CUC1* constructs could not restore leaf serration (serration scores = 1.2 ± 0.2 and 1.1 ± 0.1, respectively; Figures 5D, 5E, 5Q, and 5R), indicating that the *CUC1* promoter is not active in the developing leaves, in agreement with the absence of any detectable *CUC1* mRNA (see Supplemental Figure 2 online).

When either the tomato SlNAM or pea PsNAM1 or PsNAM2 protein was expressed in *cuc2-1* under the control of the *CUC2* promoter, leaf serration was restored (serration scores = 2.0 ± 0.2, 2.3 ± 0.1, and 2.5 ± 0.3, respectively; Figures 5H–5J, 5U, and 5V). Expression of the *CUC2*, *CUC3*, and *MIR164A* reporters

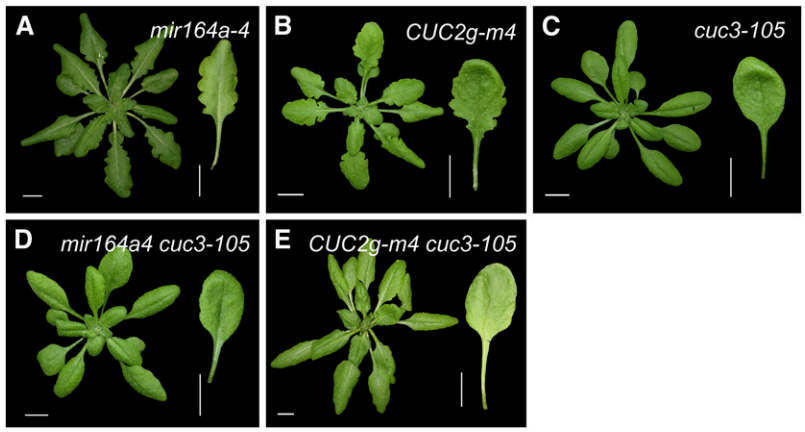

Figure 4. Genetic Interactions between the *CUC* Genes during *Arabidopsis* Leaf Serration.

Rosettes at bolting and sixth leaves are shown for the indicated genotypes. *mir164a-4* and *CUC2g-m4* leaves are strongly serrated (**[A]** and **[B]**), whereas *cuc3-105* leaves have shallow serrations **(C)**. *mir164a-4 cuc3-105* **(D)** and *CUC2g-m4 cuc3-105* **(E)** lines show a serration level intermediate between the parental *cuc3-105* and *mir164a-4* or *CUC2g-m4* lines, respectively, showing that *CUC2* contributes to leaf serration via two pathways, one dependent and one independent of *CUC3*. Bars = 1 cm.
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was also restored in the blade margins of plants expressing PsNAM1 or SlNAM (Figures 6E, 6F, 6L, 6M, 6S, and 6T). This suggests that SlNAM, PsNAM1, and PsNAM2 proteins, like CUC2, coordinate the activities of the CUC2, CUC3, and MIR164A promoters at the leaf margin and can induce the formation of serrations.

Expression of the CUC1 protein under the control of the *CUC2* promoter led to leaf dissection (Figures 5F and 5S) and activated *CUC2*, *CUC3*, and *MIR164A* promoter expression in the leaf blade margin (Figures 6C, 6J, and 6Q). Interestingly, Pro_{CUC2}:*CUC1* lines showed a higher level of dissection compared with the wild type and the Pro_{CUC2}:*CUC2* control construct (serration scores for the Pro_{CUC2}:*CUC1* and Pro_{CUC2}:*CUC2* lines = 2.8 $\pm$ 0.2 and 2.1 $\pm$ 0.1, respectively; *t* test $P < 0.001$; 9 out of the 31 Pro_{CUC2}:*CUC1* lines have a serration score ≥ 3, which was not observed in any of the 27 Pro_{CUC2}:*CUC2* lines; Figures 5A and 5F). This indicates that, although *CUC1* is normally not expressed in the developing leaf, the CUC1 protein can replace CUC2. However, the functions of CUC1 and CUC2 are not fully interchangeable, as the CUC1 protein seems to exhibit stronger and/or additional activities.

Most of the *cuc2-1* mutant lines expressing CUC3 under the control of the *CUC2* promoter did not show restoration of leaf serration (serration score = 1.6 $\pm$ 0.2; Figures 5G and 5T), whereas 15% of the lines showed deeply dissected and disorganized leaves. A higher expression level of *CUC3* was observed in dissected compared with smooth Pro_{CUC2}:*CUC3* lines (see Supplemental Figure 7 online), suggesting that differences in the activity levels of the transgene contributed to phenotypic variability. Expression of *CUC2* was weakly activated at the margin of these leaves, whereas *MIR164A* and *CUC3* expression remained faint (Figures 6D, 6K, and 6R). Expression of the pea PsCUC3 ortholog could not restore leaf serration (serration score = 1.0 $\pm$ 0.0; Figures 5K and 5W). This suggests that CUC3 function only slightly overlaps with that of CUC2.

Modulation of CUC Activity during Leaf Development Leads to Compound Leaf-Like Structures and Ectopic Meristems

SlNAM, like *CUC1* and *CUC2*, possesses a *miR164* binding site (Blein et al., 2008). Therefore, to confirm the activation of *MIR164A* in the Pro_{CUC2}:*CUC2*, Pro_{CUC2}:*CUC1*, and Pro_{CUC2}:*SlNAM* lines, we introduced the *mir164a-4* loss of function in these backgrounds. Leaf dissection was increased following *MIR164A* inactivation in the Pro_{CUC2}:*CUC2*, Pro_{CUC2}:*CUC1*, and Pro_{CUC2}:*SlNAM* lines, confirming that *MIR164A* was active in these lines (see Supplemental Figure 8 online). Interestingly, *mir164a-4* Pro_{CUC2}:*CUC1* plants showed an extreme dissection (Figures 7A and 7B), which did not depend on whether or not a functional endogenous *CUC2* gene was present (see Supplemental Figure 8 online). Leaflet-like structures, sometimes associated with stipules, developed in the proximal half of the blade (Figures 7D and 7E). Older leaves developed higher orders of leaflets (Figure 7B). Observation of early stages of leaf development indicated that leaflets formed as exaggerations of the teeth (Figures 7K–7N). In addition, foci of small undifferentiated and dividing cells could be observed on specific regions along the petiole and on the leaf blade (Figures 7F–7J). Ectopic meristems were formed from these islands and gave rise to ectopic inflorescences (Figure 7C). As formation of leaflets is often associated with *KNOX* expression, we investigated *KNAT1*/*BP*, *KNAT2*, and *SHOOT MERISTEMLESS* (*STM*) expression by introducing GUS reporters of these genes in the *mir164a-4* Pro_{CUC2}:*CUC1* (Figures 7O–7Q) and *mir164a-4* Pro_{CUC2}:*CUC2* (Figures 7O–7T) backgrounds. In the *mir164a-4* Pro_{CUC2}:*CUC1* line, *KNAT1*, *KNAT2*, and *STM* promoter activity was observed in foci within the blade that could correspond to the developing ectopic meristems and in the sinus region between outgrowing leaflets (Figures 7O–7T). By contrast, KNOX reporter activity was limited to the base of the petiole of both the wild type and *mir164a-4* mutants (see Supplemental Figure 9 online). In the

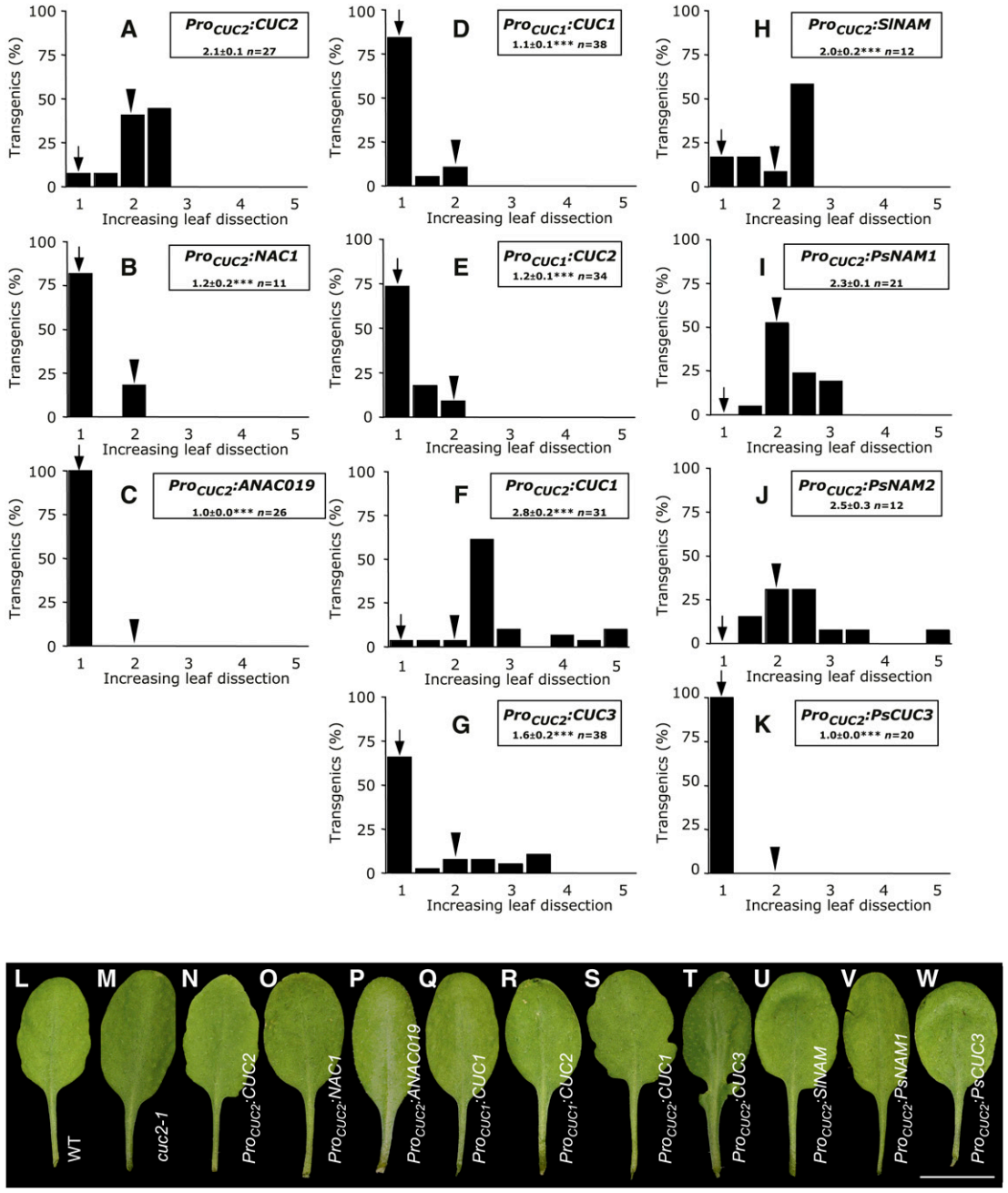

Figure 5. Effects of *CUC* Chimeric Constructs on Leaf Shape.

(A) to **(K)** Distribution of the leaf phenotype of independent *cuc2-1* mutants transformed with the indicated constructs. The serration level was expressed as an arbitrary score ranging from 1 (smooth margin) to 5 (strongly dissected), with the starting *cuc2-1* mutant having a score of 1 (arrows) and the wild type a score of 2 (arrowheads). Mean serration score, SE, and number of lines (*n*) are indicated for each construct. Phenotypes statistically different from those obtained with the Pro_{CUC2}:*CUC2* construct are indicated (*** $P < 0.001$, Student's *t* test).

(L) to **(W)** A representative sixth leaf is shown for the wild type and each construct (except for Pro_{CUC2}:*NAM2*, which had a similar phenotype as the Pro_{CUC2}:*NAM1* line). Bar = 1 cm.

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

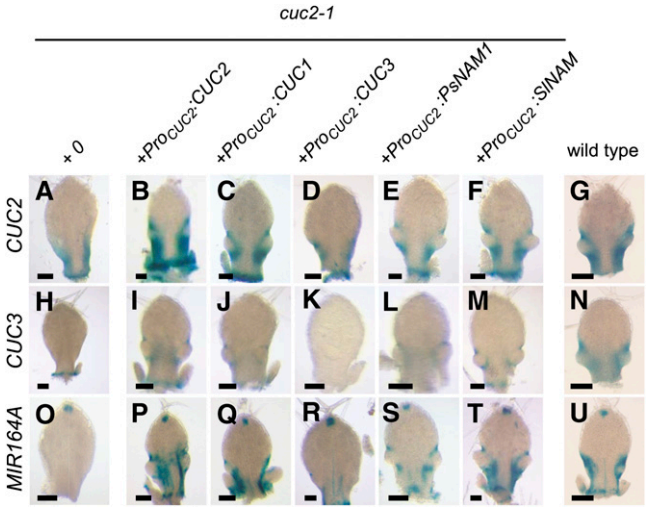

Figure 6. Effects of *CUC* Chimeric Constructs on Pro_{CUC2}:*GUS*, Pro_{CUC3}:*GUS*, and $Pro_{MIR164A}$:*GUS* Reporter Activities.

Expression of the Pro_{CUC2}:*GUS*, Pro_{CUC3}:*GUS*, and $Pro_{MIR164A}$:*GUS* reporters is restored in a *cuc2-1* background upon expression of CUC2, CUC1, PsNAM1, and SlNAM proteins under the control of the *CUC2* promoter. In contrast, expression of the CUC3 protein under the control of the *CUC2* promoter does not restore, or only partially restores, *CUC2*, *CUC3*, and *MIR164A* activities. Bar = 100 μm.

mir164a-4 Pro_{CUC2}:*CUC1* line, *CUC2*, *CUC3*, and *MIR164A* reporters exhibited stronger and/or ectopic expression in regions where ectopic *KNOX* expression occurred (see Supplemental Figure 10 online). Therefore, expression of *CUC1* in the margins of the developing leaf is sufficient to change its architecture from simple to compound. This change is associated with modified expression patterns of *KNOX*, *CUC2*, *CUC3*, and *MIR164A* promoters.

CUC1 and *CUC2* Resulted from Duplications of a Unique Ancestral Gene and Show Different Patterns of Evolution

To investigate the evolutionary origin of the functional differences between the *Arabidopsis* CUC proteins, we reconstructed the CUC phylogeny. CUC3 forms a clade distinct from the CUC1/CUC2 clade in both monocots and dicots, suggesting that diversification of these two groups occurred more than 150 million years ago (Wikström et al., 2001; Zimmermann and Werr, 2005). In contrast, within the NAM/CUC1/CUC2 clade, two divergent *CUC1* and *CUC2* genes have so far been identified in only two Brassicaceae species, *Arabidopsis* and *C. hirsuta* (Blein et al., 2008). To investigate the possible origin of *CUC1* and *CUC2*, we combined genome-wide chromosomal duplication data in *Arabidopsis* (Bowers et al., 2003), data mining, and cloning of *CUC* putative orthologs in a sample of other Brassicaceae species. Data from Bowers et al. (2003) showed that the *CUC1* and *CUC2* genomic regions underwent two rounds of duplications, the first (β21) generating the *CUC1* and *CUC2* ancestors, followed by duplication (α8 and α22) of each of these precursors (Figure 8A). These duplications were followed by the loss of one member of each duplicated gene (Figure 8A). Loss of the *CUC1* gene on chromosome I left three discontinuous stretches that showed similarities with the promoter, exon 1, or exon 2 of *CUC1*, suggesting that several independent deletions had occurred (Figure 8B). The duplicated *CUC2* region on chromosome IV was replaced by three genes, *At4g27530*, *At4g27540*, and *At4g27550* (Figure 8A). The α duplications postdate separation of the Cleomaceae and the Brassicaceae within Brassicales (Baker et al., 2005; Schranz and Mitchell-Olds, 2006) and predate the divergence of *Arabidopsis* from *Brassica* (Bowers et al., 2003; Figure 8C). The time of the β duplication is less clear; although it was initially suggested that it may predate the *Arabidopsis* separation from other dicots (Bowers et al., 2003), it is now more likely that it occurred later, possibly after the divergence of *Arabidopsis* from papaya (*Carica papaya*; Caricaceae, Brassicales; Ming et al., 2008; Tang et al., 2008; Soltis et al., 2009). Consistently, we found a single *CUC1/2* gene in the papaya genome, while distinct *CUC1* and *CUC2* genes could be identified in several Brassicaceae species (*Arabidopsis lyrata*, *C. hirsuta*, *Raphanus sativus*, and *Brassica oleracea*; Figures 8C and 8D). Together, this indicates that the *Arabidopsis CUC1* and *CUC2* genes were generated by two duplications occurring after papaya diverged from other Brassicales species 68 to 72 million years ago (Wikström et al., 2001) and before the divergence of *Brassica* from *Arabidopsis* 16 to 21 million years ago (Koch et al., 2001), followed by the loss of one of the most recently duplicated copies (Figure 8C).

Alignments showed that the papaya CUC2 protein has 137 and 69 amino acids conserved with the *Arabidopsis*, *A. lyrata*, and *C. hirsuta* CUC2 proteins, respectively within and outside the NAC domain, whereas only 122 and 33 amino acids were similarly conserved between papaya CUC1 and the CUC1 proteins of the three same species (see Supplemental Figure 11 online). This suggested that the CUC1 and CUC2 proteins evolved differentially. To test this, we investigated the ratio of the rate of synonymous to nonsynonymous substitutions (ω) in the *CUC* phylogeny. An $\omega < 1$ suggests purifying selection, $\omega = 1$ indicates neutral evolution, and $\omega > 1$ is interpreted as evidence of positive selection. Using the branch model, which enables ω to vary among branches (Yang, 2007), we detected a significant increase of ω in the *CUC1* branch (red branch in Figure 8D) but not in the *CUC2* branch (blue branch in Figure 8D; see Supplemental Data Set 1 online), compared with all other branches ($\omega_0 = 0.052$, $\omega_{CUC1} = 0.15$; $P < 0.01$), confirming that the two genes were subjected to different selective pressures. Next, we focused on the *CUC1* branch using the branch-site model to detect selective events at precise amino acid residues. It appeared that both constraint relaxation and positive selection took place on the *CUC1* branch (see Supplemental Figure 12 online for details). Within the NAC domain, more than 13% of sites had been subjected to accelerated evolution in the *CUC1* branch while being constrained or neutral in the other branches of the tree, and 12 sites were identified as potentially being under positive selection (posterior probability [PP] > 0.95), with three of them having a PP of higher than 0.99 using the Bayes Empirical Bayes procedure (see Supplemental Figure 12 online). Together, this analysis provides evidence for different patterns of evolution of the Brassicaceae *CUC1* and *CUC2* genes and for

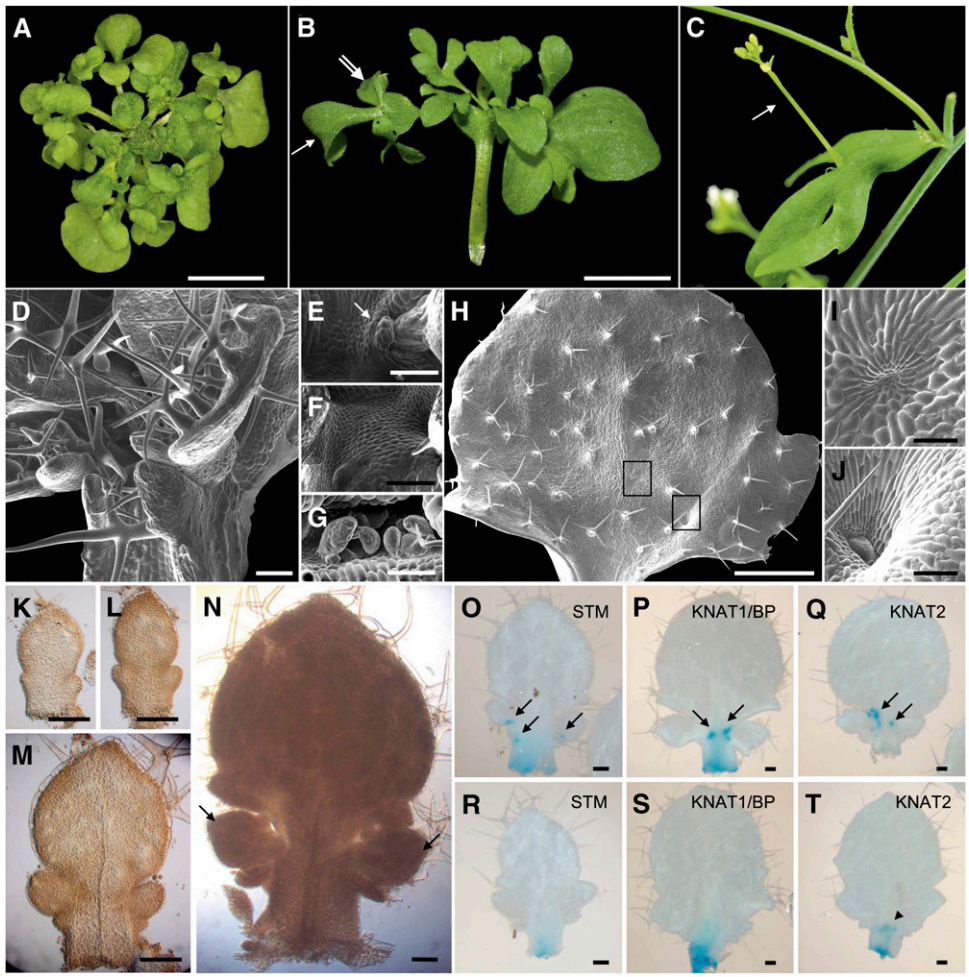

Figure 7. Modulation of CUC Activity Is Sufficient to Promote Leaflet Formation.

(A) to **(C)** Phenotype of *cuc2-1 mir164a-4 Pro$_{CUC2}$:CUC1* plants. Rosette at bolting **(A)**, detail of a rosette leaf showing leaflet-like structures of increasing order (single- and double-line arrows in **[B]**), and ectopic inflorescence developing on a cauline leaf (arrow in **[C]**) are shown.
(D) to **(J)** Scanning electron microscopy views of the *cuc2-1 mir164a-4 Pro$_{CUC2}$:CUC1* line, showing leaflet-like structures initiated from the edges of the petiole **(D)** and sometimes associated with stipules (arrow in **[E]**). Islands of undifferentiated, proliferating cells are found on the adaxial side of the petiole **(F)** and leaf blade (**[H]** and **[I]**) and initiate ectopic meristems (**[G]** and **[J]**). **(I)** and **(J)** are details of the boxed regions in **(H)**.
(K) to **(N)** Developmental series of *cuc2-1 mir164a-4 Pro$_{CUC2}$:CUC1* leaves. Teeth are properly initiated **(K)** but show an exaggerated development (**[L]** and **[M]**) and turn into leaflet-like structures (arrows in **[N]**).
(O) to **(U)** *KNOX* expression in *mir164a-4 Pro$_{CUC2}$:CUC1* (**[O]**–**[Q]**) and *mir164a-4 Pro$_{CUC2}$:CUC2* (**[R]**–**[T]**) leaf 5 or 6. In leaves of *miR164a-4 Pro$_{CUC2}$:CUC1* plants, expression of the *STM*, *KNAT1/BP*, and *KNAT2 GUS* reporters is observed in the sinus and in small spots within the lamina that possibly correspond to the ectopic meristems (arrows in **[O]**–**[Q]**). In contrast, no expression of these reporters is observed in leaves of *mir164a-4 Pro$_{CUC2}$:CUC2* plants, except for the *KNAT2* reporter, which shows diffuse GUS staining at the blade–petiole junction (arrowhead in **[T]**).
Bars = 1 cm in **(A)**, **(B)**, and **(H)** and 100 μm in **(D)** to **(G)** and **(I)** to **(T)**.

neofunctionalization of *CUC1*, which corroborates our functional analysis that showed that the *Arabidopsis* CUC1 and CUC2 proteins had different functions.

DISCUSSION

Here, we show that *CUC2* and, to a lesser extent, *CUC3* are essential for leaf serration in *Arabidopsis*. Furthermore, we demonstrate that *CUC3* acts at a later stage than *CUC2* to maintain growth of the developing teeth. Using leaf serration as a functional test, we reveal both redundant and specific roles for the three *Arabidopsis CUC* genes and propose an evolutionary scenario for the origin and the specific fates of the *CUC1* and *CUC2* genes.

CUC2 and *CUC3* Contribute Differentially to *Arabidopsis* Leaf Serration

We show here that, in addition to *CUC2* (Nikovics et al., 2006), *CUC3* is also involved in *Arabidopsis* leaf serration. Interestingly, whereas inactivation of either *CUC2* or *CUC3* leads to leaf margin

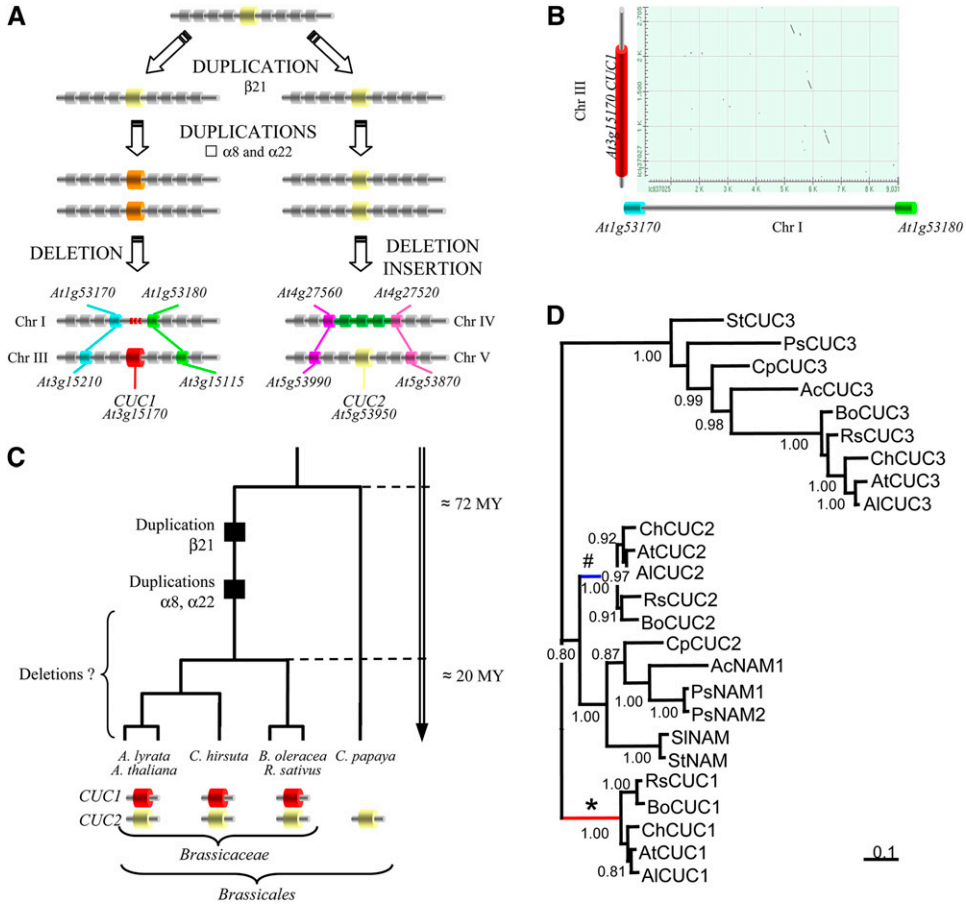

Figure 8. Evolution of the *CUC* Genes in Brassicales.

(A) Reconstruction of the history of the *CUC* genes in the *Arabidopsis* lineage. The duplications are named according to Bowers et al. (2003).
(B) Alignment between the *CUC1* region on chromosome III and the corresponding region on chromosome I, showing three stretches of conserved regions.
(C) Scheme illustrating the history of the *CUC* genes in Brassicales. The likely timing of the duplications is indicated. Timing of the deletions relative to the phylogeny of the species is uncertain. MY, Million years.
(D) Phylogeny of *CUC* genes inferred by Bayesian analysis (MrBayes version 3.1.2). Model GTR + Γ + I 2,000,000 generations, two runs, three chains each. Matrix 492 nucleotide positions. The alignment was partitioned according to codon position for Bayesian analysis. Posterior probabilities of nodes are indicated when above 0.8. The branch to the Brassicaceae *CUC1* genes is shown in red, and the branch to the Brassicaceae *CUC2* genes is shown in blue. Sequences were named according to species names: At, *Arabidopsis*; Al, *A. lyrata*; Rs, *R. sativus*; Ch, *C. hirsuta*; Bo, *B. oleracea*; Ps, *P. sativum*; Sl, *S. lycopersicum*; St, *Solanum tuberosum*; Ac, *Aquilegia coerulea*; Cp, *C. papaya*. The scale bar shows the rate of expected number of substitutions per site.
[See online article for color version of this figure.]

smoothening, our morphometric characterization reveals that their contribution to serration is different: *CUC2* acts early, promoting teeth emergence and outgrowth, possibly through growth limitation in the sinus and/or growth promotion in the teeth (Nikovics et al., 2006; Kawamura et al., 2010), whereas *CUC3* appears to act later to sustain teeth growth. A differential contribution of the *CUC2* and *CUC3* genes to leaf serration is also supported by our genetic analysis, which shows that *CUC2* promotes leaf serration via two different pathways, one requiring *CUC3* and one independent of *CUC3*. Together, these observations suggest that leaf serration occurs in two different phases: an early step, requiring *CUC2*, during which leaf serration is initiated, and a later step, requiring both *CUC2* and *CUC3*, which sustains teeth formation.

The *CUC* Genes Define an Obligatory Pathway for Leaf Dissection

Inactivation of *CUC2* and, to a lesser extent, of *CUC3*, suppresses leaf dissection in a wide range of *Arabidopsis* mutants and transgenic lines, indicating that *CUC2* and *CUC3* are obligatory for *Arabidopsis* leaf dissection. Furthermore, activities of the *CUC2*, *CUC3*, and *MIR164A* promoters are severely affected in *cuc2* mutants, indicating that *CUC2* is required to coordinate gene expression at the leaf margin for proper teeth formation.

In the leaf, transcriptional control determines the pattern of *CUC2* expression, whereas miR164 regulates the level of its expression (Nikovics et al., 2006). Our genetic analyses indicate

that the increased level of serration following *SE* and *CBP20* inactivation is due to reduced *miR164* regulation of *CUC2*, in agreement with a role for these genes in miRNA function (Chen, 2009; Voinnet, 2009). By contrast, increased leaf dissection following modification of *UFO*, *JAW*, *STIP*, and *SAW1–SAW2* activities does not appear to rely on reduced *miR164* function. These genes may either act upstream of *CUC2* and/or *CUC3*, and for example modify the activities of their promoters, or may be active in parallel pathways. For instance, the *TCP* genes targeted by *miRJAW* promote the transition from proliferation to differentiation (Palatnik et al., 2003; Efroni et al., 2008; Pulido and Laufs, 2010), and their inactivation in the *jaw*-D line may lead to prolonged growth, exaggerating the dissection generated by the *CUC* genes. Nevertheless, determining precisely how the *CUC*/*miR164* regulatory unit contributes to variation in *Arabidopsis* leaf shape awaits a quantitative analysis of the activity of the *CUC*/*MIR164A* genes during the course of leaf development in serrated/lobed lines.

We show that ectopic expression of the *KNOX* gene *KNAT1* leads to higher *Arabidopsis* leaf dissection through the *CUC* genes, as shown before for the formation of leaflets in *C. hirsuta* (Blein et al., 2008). Inactivation of *CUC2* also suppresses the serrations of *UFO*-overexpressing lines. The serrated phenotype resulting from *UFO* overexpression depends on the function of the floral identity gene *LEAFY* (*LFY*; Lee et al., 1997; Chae et al., 2008). Furthermore, *LFY* orthologs are required for the formation of leaflets in some compound leaves (Hofer et al., 1997; Molinero-Rosales et al., 1999; Champagne et al., 2007; Wang et al., 2008), a process related to serration in simple leaves (Blein et al., 2010; Floyd and Bowman, 2010). However, a strong *lfy* mutant, *lfy7*, does not show any change in leaf serration, indicating that *LFY* is not involved in *Arabidopsis* leaf serration (see Supplemental Figure 13 online).

Not only are the *CUC* genes required for leaf dissection, but they are also involved in the elaboration of more complex structures. Increasing *CUC2* expression following the impaired regulation by *miR164* leads to enhanced serration and occasionally second order serrations (Nikovics et al., 2006; Larue et al., 2009; Kawamura et al., 2010). Now, our observations indicate that modulation of *CUC* activity (i.e., expressing *CUC1* in place of *CUC2* in the absence of repression by *MIR164A*) is sufficient to promote leaflet formation. Leaflet formation is also observed in lines overexpressing *KNOX* genes (Hay and Tsiantis, 2006; Barth et al., 2009; Shani et al., 2009), and indeed, development of leaflets upon *CUC1* expression is accompanied by ectopic *KNOX* expression and a modification of the expression patterns of the *CUC2*, *CUC3*, and *MIR164A* promoters. Taken together, these observations suggest that, upon ectopic *CUC1* expression in the leaf, a positive feedback loop between *KNOX* genes and *CUC1* is established in the simple *Arabidopsis* leaf, as it is in the compound *C. hirsuta* leaf (Blein et al., 2008).

Evolution of the *CUC* Genes in the Brassicales

The *CUC* genes form two separate clades, the NAM/CUC1/CUC2 clade and the CUC3 clade, which diverged before the dicot–monocot split 143 to 161 million years ago (Wikström et al., 2001). Within the NAM/CUC1/CUC2 clade, two strongly divergent genes have been identified in *Arabidopsis* (Aida et al., 1997; Takada et al., 2001) and *C. hirsuta* (Blein et al., 2008), and now also in other Brassicaceae species. In contrast, a single gene has been found within the NAM/CUC1/CUC2 clade in snapdragon and tomato, and inactivation of this gene leads to a strong phenotype, suggesting that it may indeed be unique in these species (Weir et al., 2004; Blein et al., 2008; Berger et al., 2009). Two genes that are possible paralogs resulting from recent duplications are found in maize and pea (Zimmermann and Werr, 2005; Blein et al., 2008). Therefore, the presence of two divergent *CUC1* and *CUC2* genes appears to be unique to Brassicaceae species and possibly to related taxons within Brassicales. The data of Bowers et al. (2003), the recently sequenced genome of papaya (Ming et al., 2008), and the cloning of Brassicaceae *CUC* genes allowed us to propose a possible evolutionary scenario for this, involving two successive duplications of an ancestral gene followed by two gene-loss events, leaving only two copies, that took place after the divergence between papaya and other Brassicales species and before the divergence of Brassicaceae.

Our data suggest that *CUC1* and *CUC2* evolved differentially since the initial duplication. Several observations indicate that *CUC2* did not diverge importantly from the ancestral gene. First, strong sequence conservation between CUC2 and NAM of other eudicots extends outside the NAC domain, and *CUC2* sequences appear close to *NAM* sequences in the phylogenetic tree. Second, CUC2 can be functionally replaced during *Arabidopsis* leaf development by NAM proteins of pea and tomato, two species that shared a common ancestor with *Arabidopsis* ~105 and 120 million years ago, respectively (Wikström et al., 2001). Third, *CUC2*, like the *NAM* genes of other species (Blein et al., 2008), is expressed in the leaf and regulates its development.

By contrast, *CUC1* appears to have diverged more profoundly from its ancestor. *Arabidopsis CUC1* is not expressed in the leaves and does not regulate their morphogenesis. Similarly, *CUC1* has a less important role than *CUC2* during embryonic development and axillary meristem formation (Hibara et al., 2006; Raman et al., 2008). The *CUC1* genes form a clade distinct from the *CUC2* genes, and analysis of their molecular evolution indicates that positive selection took place on the branch ancestral to the *CUC1* clade, pointing to neofunctionalization.

Conservation of the CUC1 and CUC2 proteins outside the NAC domain is limited to small motifs, including the so-called S, L, and V, that are also found in other members of the *NAC* family, suggesting that these small motifs are essential for their function (Taoka et al., 2004). One of these motifs, the V motif, corresponds to translation of the mRNA region that binds *miR164*. The conservation of the miRNA binding site underlines the importance of the regulation by *miR164* for proper *CUC1*/*CUC2* functioning, which is also illustrated by the strong developmental defects resulting from *CUC1* or *CUC2* escaping from *miR164* regulation (Laufs et al., 2004; Mallory et al., 2004). In addition, although CUC1 can functionally replace CUC2 during *Arabidopsis* leaf development, it significantly enhances leaf dissection compared with CUC2. This stronger effect of CUC1 may be due to CUC1 regulating more strongly the same targets as CUC2 or to CUC1 acting on a partially different range of target genes. The latter hypothesis is supported by the observation that *KNOX*

genes are expressed in the leaves following ectopic CUC1 expression. Such an evolution of the target genes following changes in a TF has been shown, for instance, for *LFY* (Maizel et al., 2005). As it is not clear whether the ancestor of Brassicales had simple or compound leaves (Bharathan et al., 2002), the different ability of the CUC1 and CUC2 proteins to activate *KNOX* gene expression may represent a function gained by CUC1 or a function lost by CUC2.

Together, our observations allow us to propose an evolutionary scenario for the origin and different fates of *CUC1* and *CUC2*. Following the duplication of an ancestral gene, the resulting *CUC2* gene may have conserved most of the ancestral role while *CUC1* diverged, with changes affecting both the regulatory and the coding regions of the gene. The two genes may have maintained overlapping roles, as they do during organ separation, while subfunctionalization may have occurred for other functions, such as axillary meristem formation (Hibara et al., 2006; Raman et al., 2008) and leaf development, which is regulated only by *CUC2* (Nikovics et al., 2006; this work). Neofunctionalization of *CUC1* may have contributed to developmental and morphological changes. In this respect, it may be significant that *CUC1* is expressed in the compound leaf of *C. hirsuta* (Blein et al., 2008), opening the possibility that changes in *CUC1* activity may be associated with variation in leaf shape within the Brassicaceae.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* lines used in this work are described in Supplemental Table 1 online. Plants were grown in growth chambers under long-day conditions (16 h of light at 23°C and 8 h of darkness at 15°C). Double mutants were identified in the F2 segregating population of a cross between the two single mutants based on their phenotype and, if necessary, were genotyped. Phenotypic analyses were performed in F3 or F4 double homozygous mutant populations.

GUS Staining and RT-PCR

GUS staining was performed as described (Sessions et al., 1999) in the presence of 0.5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ for the Pro_{CUC3}:*GUS*, Pro_{STM}:*GUS*, Pro_{KNAT1}:*GUS*, and Pro_{KNAT2}:*GUS* reporters and with 10 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ for the Pro_{CUC2}:*GUS* and $Pro_{MIR164A}$:*GUS* reporters. The reaction was stopped with 95% ethanol, which was also used to remove the chlorophyll from the tissues. Leaves were mounted in water, and photographs were taken with a ProgRes C10 plus Jenoptik digital camera on a Nikon Microphot-FXA microscope.

RT-PCR was performed as described by Blein et al. (2008) using primers listed in Supplemental Table 2 online, and the gels were visualized by ethidium bromide.

Plasmids and Plant Transformation

All the chimeric *CUC* constructs were generated in the pGreen0129 backbone (Hellens et al., 2000). The endogenous *Not*I site was removed from pGreen0129 by *Not*I digestion, Kleenow-mediated blunt-ending, and self-ligation. A *Bam*HI–*Xba*I cassette from the pL4 plasmid harboring the 35S terminator containing a *Not*I site was then inserted into the modified pGreen0129 to generate the pGreen0129-t35S construct. The 1.5-kb *CUC1* promoter was amplified from the CUC1 control plasmid (Mallory et al., 2004) to include an *Eco*RV site at the 5′ end and a *Not*I site at the 3′ end and inserted using these sites into the pGreen0129-t35S plasmid to generate the pGreen0129-t35S-Pro_{CUC1} vector. The 3.7-kb *CUC2* promoter was transferred as an *Eco*RV–*Bgl*II fragment from CUC2g-wt (Nikovics et al., 2006) into pGreen0129-t35S to generate pGreen0129-t35S-Pro_{CUC2}. The pGreen0129-t35S-Pro_{CUC1} and pGreen0129-t35S-Pro_{CUC2} vectors had a unique *Not*I restriction site located between the specific promoter and the 35S terminator. NAC open reading frames were amplified from the first ATG codon to the last stop codon, cloned into pGEM-T, and transferred as a *Not*I fragment into the appropriate pGreen0129-t35S-promoter vector. Transfer of the final vectors into *Agrobacterium tumefaciens*, plant transformation, and transformant selection on hygromycin plates were performed as described before (Deveaux et al., 2003).

Phenotypic Analysis

For the scoring of the leaf phenotype of plants expressing *CUC* chimeric constructs, 20 T2 plants of each line were grown alongside four standard lines showing increasing dissection levels. At bolting, a serration score ranging from 1 to 5 was given to each line by comparing it with a smooth line: *cuc2-1*, serration score = 1; normal: wild type, serration score = 2; moderate increase of dissection: *mir164a-4*, serration score = 3; intermediate increase of dissection: *CUC2g-m4*, serration score = 4; and stronger increase of dissection: serration score = 5.

The morphometric analysis was performed on the sixth leaves of 11- to 23-d-old, long-day-grown plants that were collected daily, fixed for 20 min in 90% acetone, and cleared in 90% ethanol. Images of dissected leaves were obtained with a ProgRes C10 plus Jenoptik digital camera on a Nikon Microphot-FXA microscope. Leaf parameters were measured using ImageJ 1.42q, and a homemade plugin allowed us to extract lengths from user-defined characteristic points. For each leaf, we calculated the average parameter value of the pair of teeth located on each side of the leaf, unless only one tooth was visible, in which case we kept the parameters of the single tooth. Forty-nine to 86 leaves were observed per genotype.

Scanning Electronic Microscopy

Freshly sampled tissues were cooled to −33°C by a peltier cooling stage (Deben) and observed with a Hirox SH-1500 benchtop scanning electronic microscope.

Identification of Brassicales CUC Genes

Genomes of papaya (*Carica papaya*; http://www.plantgdb.org/) and *Arabidopsis lyrata* (http://www.phytozome.net/) were searched for *CUC* genes, and the putative coding sequences were reconstructed based on the predicted splicing sites (http://www.cbs.dtu.dk/services/NetPGene/) and on conservation with splicing sites in *Arabidopsis CUC* genes. *Brassica oleracea BoCUC1* corresponds to accession DY028115.1, and *Raphanus sativus RsCUC2* corresponds to accession EY940413.1. Brassicales ESTs were retrieved from databases, aligned, and used to design primers in conserved regions. These primers were used to amplify other *CUC* genes from genomic DNA of *B. oleracea* (cv Tete noire 3; Vilmorin) and *R. sativus* (cv Gaudray 2; Vilmorin), which were cloned into pGEM-T and sequenced.

Phylogenetic Analyses

Twenty-five CUC sequences and *Arabidopsis* NAC1 were aligned using ClustalW as implemented in BioEdit. The phylogenetic analyses were conducted on a portion of the alignment that included the CUC domain, where primary homology could be assessed without ambiguity (495 nucleotide positions for the analysis including AtNAC1 [including gaps

introduced to optimize alignments] and 492 positions without AtNAC1). Phylogenetic trees were reconstructed by Bayesian inference using MrBayes version 3.1, with a GTR + Γ + I model, and the alignment was partitioned according to codon position for substitution rates. The tree was rooted with AtNAC1, and the analysis was run twice with four chains, three heated, for 4,000,000 generations. Another analysis without AtNAC1 was conducted to obtain an unrooted tree that was used for molecular evolution analyses. In this case, two runs were done with three chains each (two heated) and 2,000,000 generations. In both analyses, convergence was checked with the average SD of split frequencies (below 0.01) and potential scale reduction factor (close to 1.0) for evolutionary model parameters.

Molecular Evolution Analyses

To detect particular selective pressure among *CUC* genes, we investigated the nonsynonymous–synonymous substitution rate ratio (dN:dS or ω) using the codeml package implemented in PAML version 4.3 (Yang, 2007). The codon substitution models were compared using a likelihood ratio test, and the F3x4 model was retained for subsequent analyses. First, it was tested whether the *CUC1* or *CUC2* branch had undergone a selective pressure different from other branches in the *CUC* phylogeny using the branch model, with a likelihood ratio test comparing a model with the same ω value for all branches (ω_0) versus a different value on the branch of interest. This test is expected to reveal major events concerning many amino acids in the branch under scrutiny. The branch-site model, which is able to detect selective events at precise amino acid residues on a given branch termed the foreground, was also used. This model assumes four site classes: class 1 sites and class 2 undergo the same selective pressure over the phylogeny, respectively purifying selection and neutral evolution. The two other classes, 2a and 2b, correspond to a proportion of sites from class 1 and 2, respectively, that come under positive selection in the foreground lineage. This model (MA) was tested against a null model, where sites in 2a and 2b classes evolved under neutrality (MA_0). When the test was significant, the Bayes Empirical Bayes procedure (Yang et al., 2005) was implemented in codeml to estimate the PP that a site evolved under positive selection. Model MA was also tested against model M1a, which considers two classes of sites, one being under purifying selection and the other one being neutral (Zhang et al., 2005).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: MIR164A (AT2G47585), CUC1 (AT3G15170), CUC2 (AT5G53950), CUC3 (AT1G76420), AcNAM (FJ435160.1), AcCUC3 (FJ435156.1), AlCUC1 (XM_002882870.1), AlCUC2 (XM_002865963), AlCUC3 (XM_002889035), BoCUC1 (DY028115.1), BoCUC2 (HQ703968), BoCUC3 (HQ703970), CpCUC2 (BK007973), CpCUC3 (BK007974), ChCUC1 (FJ435161.1), ChCUC2 (FJ435162.1), ChCUC3 (FJ435157.1), PsNAM1 (FJ435164.1), PsNAM2 (FJ435165.1), PsCUC3 (FJ435158.1), RsCUC1 (HQ703967), RsCUC2 (EY940413.1), RsCUC3 (HQ703969), SlNAM (FJ435163.1), StNAM (FJ435166.1), and StCUC3 (FJ435159.1).

Supplemental Data

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

Supplemental Figure 1. Molecular Analysis of the *cuc3-105* and *cuc3-2* Mutants.

Supplemental Figure 2. RT-PCR Expression Analysis of the *CUC* Genes.

Supplemental Figure 3. *cuc2-3* Partially Suppresses Lobing of *KNAT1oexp*.

Supplemental Figure 4. *cuc2-3* Suppresses Early Steps of Teeth Formation in Serrated Mutants and Transgenic Lines.

Supplemental Figure 5. Serration in Some Mutants Is Due to Defective *miR164* Function.

Supplemental Figure 6. *CUC2* Is Required for the Early Stages of Teeth Formation, Whereas *CUC3* Acts Later to Maintain Teeth Growth.

Supplemental Figure 7. RT-PCR Expression Analysis of the *CUC3* Gene in *cuc2-1* and *cuc2-1* Pro_{CUC2}:*CUC3* Lines.

Supplemental Figure 8. Morphological Consequences of *MIR164A* Inactivation in Lines Expressing *CUC* Chimeric Constructs.

Supplemental Figure 9. Expression Patterns of the *STM*, *KNAT1/BP*, and *KNAT2* Reporters in the Wild Type and the *mir164a-4* Mutant.

Supplemental Figure 10. Expression Patterns of the *CUC2*, *CUC3*, and *MIR164A* Reporters in *mir164a-4* Lines Expressing *CUC2*, *CUC1*, or *SlNAM* under the Control of the *CUC2* Promoter.

Supplemental Figure 11. Comparison of Brassicaceae CUC1 and CUC2 Proteins with the Papaya CUC2 Protein.

Supplemental Figure 12. Molecular Evolution of the CUC Proteins.

Supplemental Figure 13. *LFY* Does Not Contribute to *Arabidopsis* Leaf Serration.

Supplemental Table 1. Lines Used in This Study.

Supplemental Table 2. Primers Used in This Study.

Supplemental Data Set 1. Sequences Used to Generate the Phylogeny in Figure 8D.

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