Evolution and Diverse Roles of the CUP-SHAPED COTYLEDON Genes in Arabidopsis Leaf Development

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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 combinatorial 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 hybridra), 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...
duplications are present in maize (Zea mays) (Berger et al., 2009). Two paralogs resulting probably from recent strong phenotype resulting from their inactivation suggests that a member has been identified in tomato and snapdragon, and the miR164/CUC1 genes, including genes in SAM function and organ separation. Several 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 transgenics, 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 ProCUC3-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 1Ec). 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
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), miRuaW/miR319 (jaw-D; Palatnik et al., 2003), or NICOTIANA TOMENTOSIFORMIS KINASE INTERACTING SUB-UNIT A (NtkKIS1a-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 NtkKIS1a-oexp lines; Figures 2A–2F, 2l, 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 NtkKIS1a-oexp cuc2-3 plants were small like NtkKIS1a-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 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 ProCUC3 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, ProCUC3 GUS activity marks the sinus of the outgrowing teeth ([b]–[e]) and disappears in larger teeth (arrowheads in [e]). ProCUC3 GUS activity is also detected at the base of the petiole at the junction point with the apex (asterisks). Bars = 100 μm.
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.

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.

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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...
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 con-
struct 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 ProCUC2:GUS, ProCUC3:GUS, and
ProMIR164A: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 ProCUC2:GUS, to the
leaf base for ProCUC3:GUS, and to the leaf tip for ProMIR164A:GUS
(Figures 6A, 6H, and 6O; compare with Figures 6G, 6N, and 6U
for wild-type patterns). Therefore, in addition to the morpholog-
ical changes, we also characterized the effects of some chimeric
constructs on CUC2, CUC3, and MIR164A promoter activities
(Figure 6).
Expression of the control construct ProCUC2: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 ProCUC1:CUC2 and
ProCUC1:CUC1 constructs could not restore leaf serration (ser-
ration 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 SINAM 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 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.
was also restored in the blade margins of plants expressing PsNAM1 or SINAM (Figures 6E, 6F, 6L, 6M, 6S, and 6T). This suggests that SINAM, 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, ProCUC2:CUC1 lines showed a higher level of dissection compared with the wild type and the ProCUC2:CUC2 control construct (serration scores for the ProCUC2:CUC1 and ProCUC2:CUC2 lines = 2.8 ± 0.2 and 2.1 ± 0.1, respectively; t test P < 0.001; 9 out of the 31 ProCUC2:CUC1 lines have a serration score ≥ 3, which was not observed in any of the 27 ProCUC2: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 ± 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 ProCUC2: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 ± 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

SINAM, like CUC1 and CUC2, possesses a miR164 binding site (Blein et al., 2008). Therefore, to confirm the activation of MIR164A in the ProCUC2:CUC2, ProCUC2:CUC1, and ProCUC2:SINAM lines, we introduced the mir164a-4 loss of function in these backgrounds. Leaf dissection was increased following MIR164A inactivation in the ProCUC2:CUC2, ProCUC2:CUC1, and ProCUC2:SINAM lines, confirming that MIR164A was active in these lines (see Supplemental Figure 8 online). Interestingly, mir164a-4-ProCUC2: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 7F–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 ProCUC2:CUC1 (Figures 7O–7Q) and mir164a-4 ProCUC2:CUC2 (Figures 7O–7T) backgrounds. In the mir164a-4 ProCUC2: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 ProCUC2: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 ProCUC2:NAM2, which had a similar phenotype as the ProCUC2:NAM1 line). Bar = 1 cm.

[See online article for color version of this figure.]
precursors (Figure 8A). These duplications were followed by the ancestors, followed by duplication (a) only two Brassicaceae species, Arabidopsis divergent million years ago (Wikström et al., 2001; Zimmermann and Werr, diversification of these two groups occurred more than 150 CUC2 clade in both monocots and dicots, suggesting that 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 ω < 1 suggests purifying selection, ω = 1 indicates neutral evolution, and ω > 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 (ω0 = 0.052, ωCUC1 = 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 6.** Effects of CUC Chimeric Constructs on ProCUC2::GUS, ProCUC3::GUS, and ProMIR164A::GUS Reporter Activities.

Expression of the ProCUC2::GUS, ProCUC3::GUS, and ProMIR164A::GUS reporters is restored in a cuc2-1 background upon expression of CUC2, CUC1, PsNAM1, and SINAM 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.

**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 ω < 1 suggests purifying selection, ω = 1 indicates neutral evolution, and ω > 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 (ω0 = 0.052, ωCUC1 = 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
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 7. Modulation of CUC Activity Is Sufficient to Promote Leaflet Formation.
(A) to (C) Phenotype of cuc2-1 mir164a-4 ProCUC2: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 ProCUC2: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 ProCUC2: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 ProCUC2:CUC1 ([O]–[Q]) and mir164a-4 ProCUC2:CUC2 ([R]–[T]) leaf 5 or 6. In leaves of mir164a-4 ProCUC2: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 ProCUC2: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).
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/tobed 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 ify mutant, ify7, 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 (Nikovic 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 Brassicales.

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 neo-functionalization.

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 Brassicaceae 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 (Hiibara 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 K3Fe(CN)6 and K4Fe(CN)6 for the ProCUC2GUS, ProCUC1GUS, ProKNAT1GUS, and ProMIR164AGUS reporters and with 10 mM K3Fe(CN)6 and K4Fe(CN)6 for the ProCUC2GUS and ProMIR164AGUS 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 NotI site was removed from pGreen0129 by NotI digestion, Kleenow-mediated blunt-ending, and self-ligation. A BamHI-XbaI cassette from the pL4 plasmid harboring the 35S terminator containing a GUS reporter was then inserted into the modified pGreen0129 to generate the pGreen0129-35S construct. The 1.5-kb CUC1 promoter was amplified from the CUC1 control plasmid (Mallory et al., 2004) to include an EcoRV site at the 5′ end and a NotI site at the 3′ end and inserted using these sites into the pGreen0129-35S plasmid to generate the pGreen0129-35S-ProCUC1 vector. The 3.7-kb CUC2 promoter was transferred as an EcoRV-BglII fragment from CUC2g-wt (Nikovics et al., 2006) into pGreen0129-35S to generate pGreen0129-35S-ProCUC2. The pGreen0129-35S-ProCUC1 and pGreen0129-35S-ProCUC2 vectors had a unique NotI 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 NotI fragment into the appropriate pGreen0129-35S-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: mrt164a-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% aceticone, 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 Brassicaceae 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 DY0272115.1, and Raphanus sativus RsCUC2 corresponds to accession EY940413.1. Brassicaceae 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 AINAC1 [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 + I + Γ 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 (ω0) 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 other two 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 (MA0). When the test was significant, the Bayes Empirical Bayes procedure (Yang et al., 2005) was implemented in codeml to estimate the ω value by a postdoctoral fellowship from the Region Ile-de-France.

**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), KNAT1 (At5g09790), KNAT2 (At2g17230), AcNAM (FJ435160.1), AcCUC3 (FJ435156.1), AICUC1 (XM_002882870.1), AICUC2 (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), PnAM1 (FJ435164.1), PnAM2 (FJ435165.1), PscCUC3 (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.** cuc2-3Suppresses 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 ProCUC2/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.

**ACKNOWLEDGMENTS**

We thank J.D. Faure, S. Jouannic, A. Maizel, F. Parcy, J.P. Palaquui, and V. Pautot for useful discussions and suggestions to improve the manuscript. We thank M. Aida, N. Glab, G. Haughn, V. Irish, P. Papp, C. Rameau, H. Vaucheret, D. Wagner, and D. Weigel for providing material used in this study and H. Ferry and B. Letarnec for plant care. A.H. and T.B. were supported by Ph.D. fellowships from the Ministere de l’Enseignement Superieur et de la Recherche, and A.P. was supported by a postdoctoral fellowship from the Region Ile-de-France.

Received November 17, 2010; revised December 2, 2010; accepted January 4, 2011; published January 21, 2011.

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Evolution and Diverse Roles of the CUP-SHAPED COTYLEDON Genes in Arabidopsis Leaf Development
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Plant Cell 2011;23;54-68; originally published online January 21, 2011;
DOI 10.1105/tpc.110.081448

This information is current as of November 6, 2017

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