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CHAPTER

15

CUC Transcription Factors: To the Meristem and Beyond

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OUTLINE

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15.1 INTRODUCTION

In 1996, Souer et al. reported the phenotypic characterization of a petunia mutant that fails to develop a shoot apical meristem (SAM) called "*nam*" (*no apical meristem*). The *NAM* gene is expressed in the boundaries of meristems and primordia, and the NAM protein shares a conserved N-terminal domain with other proteins, suggesting that it is part of a novel class of proteins. Indeed, the following year, Aida et al. (1997) identified an *Arabidopsis* mutant with no apical meristem but with cotyledons fused along their edge resulting in a cup-like structure, hence named "*cuc*" (*cup-shaped cotyledon*). This phenotype results from the combination of two mutations, one of which affects *CUC2*, a gene showing strong homology with the petunia *NAM* gene. These two papers began the story of the *NAM/CUC3* genes and founded the basis for the NAM, ATAF1, ATAF2, and CUC (NAC) family of plant-specific transcription factors. Here, we retrace the phylogenetic and evolutionary context of *NAM/CUC3* genes and review the important roles they play as boundary-defining actors during plant development. In particular, we discuss the mechanisms that regulate their expression patterns and how they affect plant development via their effects on cellular behavior.

15.2 EVOLUTION AND STRUCTURE OF NAM/CUC3 PROTEINS

15.2.1 The NAM/CUC3 Proteins are Part of the Large Plant-Specific Family of NAC Transcription Factors

15.2.1.1 New Insights into the Origin of the NAC Family

Together with ATAF1 and ATAF2, the petunia NAM and *Arabidopsis* CUC proteins are the founding members of the NAC family of plant-specific transcription factors (Aida et al., 1997). In an effort to trace the evolutionary origin of NAC proteins, Zhu et al. (2012) searched the full genome or expressed sequence tag (EST) data of 16 different species including eudicots, monocots, a lycophyte and a moss, chlorophytes, a red algae, and glaucophytes. Whereas a large number of NAC proteins have been identified in flowering plants (66–44, depending on the species) only 30 and 20 have been identified in *Physcomitrella patens* and *Selaginella moellendorffii*, respectively. This suggests that NAC proteins expanded as land plants evolved. Interestingly, no NAC proteins could be identified in the aquatic species analyzed, which suggests that NAC proteins may be specific to land plants. However, the analysis of Zhu and coworkers did not include any representatives of the charophytes which are thought to contain the sister group to land plants (Finet et al., 2010). Using Basic Local Alignment Search Tool (BLAST) searches we identified transcriptome shotgun assembly (TSA) sequences from the charophytes *Coleochaete* sp. (loci JO249122 and JO249294), *Penium margaritaceum* (locus JO233410), *Chaetosphaeridium globosum* (locus JO158096), and *Nitella mirabilis* (locus JV748667) whose putative translation yields proteins showing a conserved NAC domain (Figure 15.1). This observation strongly suggests that NAC proteins appeared before the transition from water to land, about 450 million years ago.

15.2.1.2 Origin and Early Evolution of the NAM/CUC3 Family

The phylogeny of NAC proteins has been analyzed by several groups who often determined the position of NACs from a particular species in relation to *Arabidopsis* and rice NACs (e.g., Fang et al., 2008; Hu et al., 2010; Ooka et al., 2003; Pinheiro et al., 2009; Shen et al., 2009; Zhu et al., 2012). Results from these phylogenetic analyses show some variability: for instance, the number of subfamilies varies from 5 (Fang et al., 2008) to 21 (Zhu et al., 2012). Despite these variations, NAM/CUC3 proteins are often associated with the same group of proteins, although with a variable topology, forming an entire or part of a subfamily (Figure 15.2A).

Proteins that belong to the NAM/CUC3 family can be clearly divided into two clades: the NAM clade that includes the petunia NAM and *Arabidopsis* CUC1 and CUC2 proteins, and the CUC3 clade (Blein et al., 2008, Zimmermann and Werr, 2005). These two clades are based on the sequence of the NAC domain (Section 15.2.2.1), but also overlap with the presence/absence of a microRNA-binding site. Indeed, all *NAM* genes possess a binding site for the *microRNA164* (*miR164*), whereas *CUC3* genes do not. Members of these two clades can be found in eudicots, monocots, and early-diverging angiosperms such as *Amborella trichopoda* (Adam et al., 2011; Blein et al., 2008; Zimmermann and Werr, 2005; Vialette-Guiraud et al., 2011). On the other hand, gymnosperm genes possessing a *miR164*-binding site are not grouped within angiosperm NAM or CUC3 clades, rather they occupy a sister position to the combined NAM + CUC3 clade. This suggests that a unique NAM + CUC3 lineage regulated by *miR164* was present in the last common ancestor of extant seed plants, and that a duplication event generated the NAM and CUC3 clades in the angiosperm lineage after its divergence from gymnosperms. In this scenario, loss of *miR164* regulation would have occurred later in the CUC3 lineage (Vialette-Guiraud et al., 2011; Figure 15.2B).

15.2.1.3 Recent Evolution Within the NAM/CUC3 Family

Additional duplication events further complicated the phylogeny of NAM/CUC3 proteins in angiosperms. Such duplication events can either be recent, resulting in two closely related paralogs (such as the pea proteins

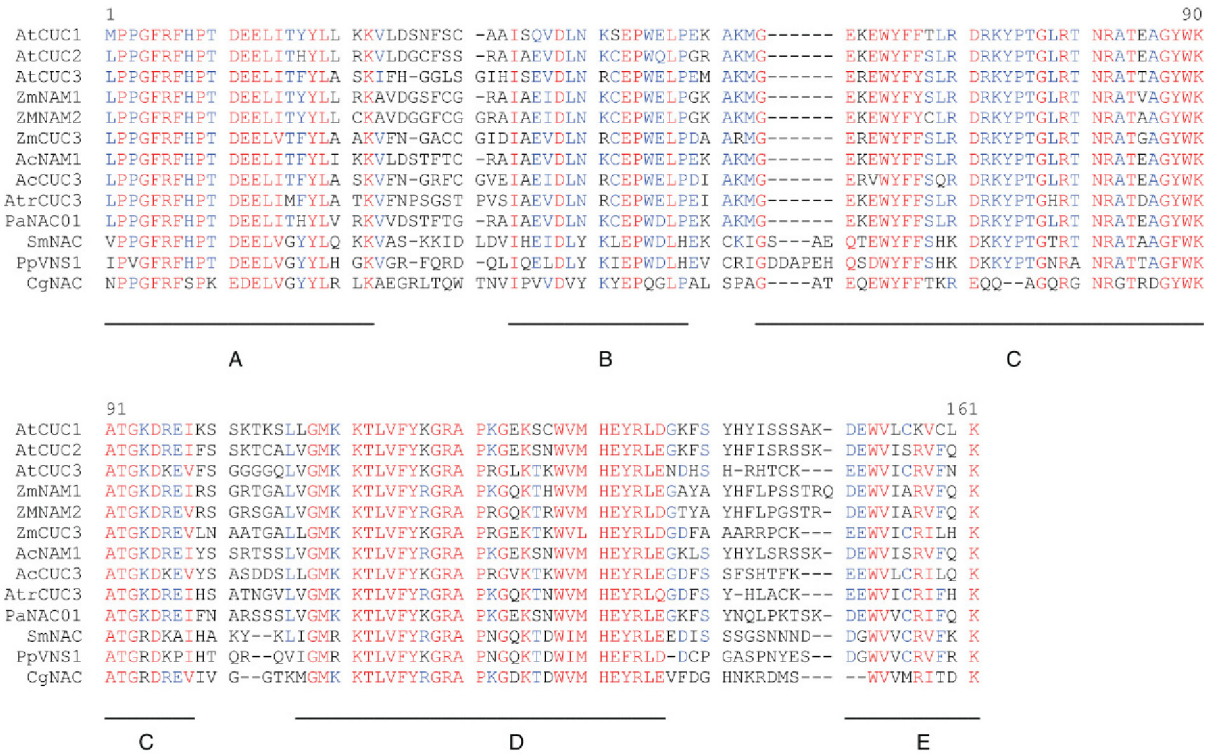

FIGURE 15.1 **Alignment of the NAC domain of representatives of different plant groups showing five conserved subdomains named "A to E."** At, *Arabidopsis thaliana*; Zm, *Zea mays*; Ac, *Aquilegia coerulea*; Atr, *Amborella trichopoda*; Pa, *Picea abies*; Sm, *Selaginella moellendorffii*; Pp, *Physcomitrella patens*; Cg, *Chaetosphaeridium globosum*. The alignment was produced using MultAlin (Corpet, 1988).

PsNAM1 and PsNAM2 that share 95% identity) or more ancient leading to more divergent genes like the CUC1 and CUC2 proteins found in the Brassicaceae lineage (the *Arabidopsis* CUC1 and CUC2 proteins share only 50% identity mostly concentrated in the NAC domain). Indeed, phylogenetic analysis and reconstruction of genome duplication events suggest that two rounds of gene duplication followed by gene loss led to the distinct CUC1 and CUC2 lineages in Brassicaceae, which have partially divergent functions (Hasson et al., 2011, Vialette-Guiraud et al., 2011). Because the CUC1 and CUC2 lineages are specific to Brassicaceae and possibly other closely related Brassicales, the names "CUC1" and "CUC2" should be exclusively used for genes identified in these groups, while for other species "NAM" should be used. Here, we use "*NAM/CUC3*" when referring to genes belonging to either of the two clades, and "*CUC*" when referring specifically to *Arabidopsis* genes.

15.2.2 NAM/CUC3 Protein Organization and Specific Domains

NAM/CUC3 proteins, like other NAC transcription factors, can be subdivided into two main functional domains: an amino-terminal domain including the conserved NAC domain, and a more divergent carboxy-terminal domain (CTD; Duval et al., 2002; Taoka et al., 2004). Domain-swapping experiments between the NAC or CTD domains of CUC1, CUC2 and the more distantly related ATAF1 protein showed that the ability of the CUC1/2 proteins to promote *in vitro* adventitious shoot formation lies in their NAC domain, suggesting that this part of the CUC1 and CUC2 proteins determines their specific functions (Taoka et al., 2004).

15.2.2.1 The Amino-Terminal NAC Domain

This domain can be subdivided into five highly conserved regions (Figure 15.1) and has been implicated in the DNA-binding properties of several NAC proteins (e.g., Duval et al., 2002; Jensen et al., 2010). Most mutations disrupting CUC1 function fall into its NAC domain, thus highlighting its importance (Figure 15.3).

The DNA-binding mechanisms of NAC proteins have begun to be elucidated. ANAC019 and ANAC092 bind to a CGT[A/G] consensus site (Olsen et al., 2005; Tran et al., 2004; Xu et al., 2013). Binding affinity to this motif varies between NAC proteins (Jensen et al., 2010). Lindemose et al. (2014) showed that 12 NACs can be divided into three groups with different binding specificities. Two groups recognize variants of the previously identified CGT[A/G] target sequence while the third recognizes an unrelated motif. These groups largely match the phylogenetic differences between NAC proteins.

NAC proteins can form both homo- and heterodimers via interaction of their NAC domains, for which both the

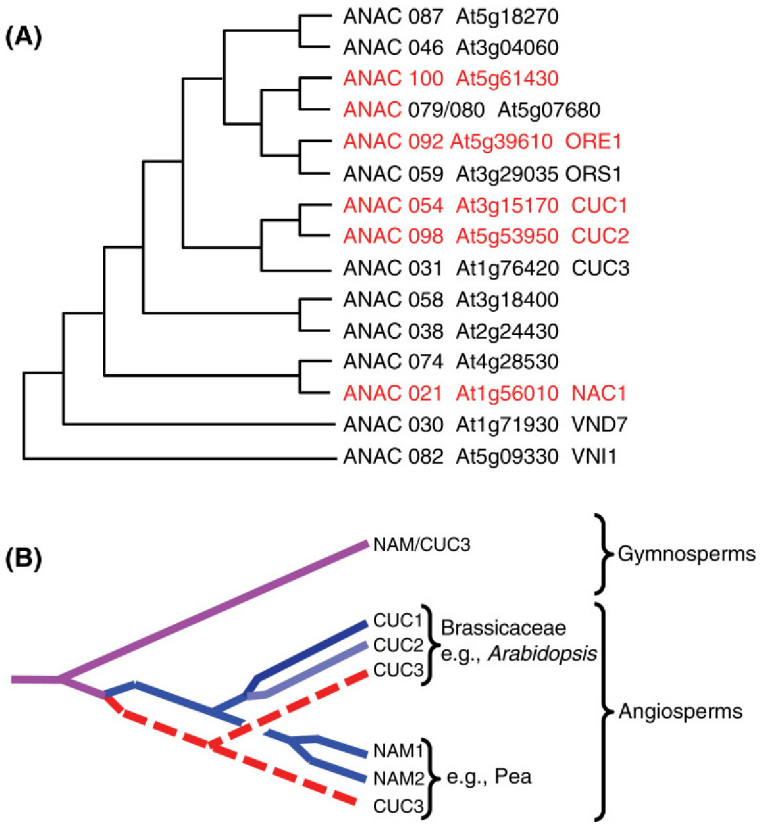

FIGURE 15.2 (A) Schematic phylogeny of the subgroup of *Arabidopsis thaliana* NAC proteins containing the CUC1, CUC2 and CUC3 proteins (adapted from Zhu et al., 2012). The genes targeted by the *miR164* are indicated in red. (B) Schematic evolution of the *NAM/CUC3* genes in seed plants. Members of the NAM + CUC3 clade are indicated in violet, members of the NAM clade are in blue, and members of the CUC3 clade are in red. Solid lines indicate lineages targeted by *miR164* while dotted lines are lineages not targeted by *miR164*. (Vialette-Guiraud et al., 2011).

interacting surface and two essential salt-bridge-forming residues have been identified (Ernst et al., 2004; Olsen et al., 2005). NACs stably bind DNA as dimers by recognizing two palindromic binding sites, but a single binding site is also sufficient for NAC binding, both *in vitro* (Olsen et al., 2005) and *in vivo* (Tran et al., 2004; Xu et al., 2013). ANAC019 dimers can exist either in an open or a closed conformation (Welner et al., 2012). While the open conformation is predominant in solution, dimers mostly adopt a closed conformation when bound to DNA. Variation in dimer conformation may account for the recognition of DNA stretches with either single binding sites or a variable number of base pairs separating two binding sites.

The NAC domain of ANAC019 contains a central twisted antiparallel ß-sheet, which is packed between two α-helices on both extremities (Ernst et al., 2004; Chapter 4). Part of this ß-sheet formed by conserved WKATGTD amino acids protrudes into the major groove of DNA and interacts with the sugar/base region of DNA providing specificity to the recognition, while other parts interact with the DNA backbone potentially increasing affinity (Welner et al., 2012; Chapter 13). This mode of interaction shows similarities with those of plant WRKY and mammalian glial cells missing (GCM) transcription factors.

To date, no structure data on any of the NAM/CUC3 proteins nor their DNA binding sites have been determined. A recent study suggests that, like ANAC019 and other phylogenetically related proteins, NAM/CUC3 may recognize a TT[A/G]CGT[A/G] motif (Lindemose et al., 2014). However, because the WKATGTD residues that contribute to ANAC019 specificity are replaced by WKATGKD in NAM/CUC3 proteins, it is not clear how conserved the core binding site can be. It is therefore essential to determine the binding specificity of NAM/CUC3 proteins experimentally.

15.2.2.2 The Carboxy-Terminal Domain

The CTD of NAM/CUC3 proteins is more variable than the NAC domain, but several small domains can be recognized. However, these domains are not all found in all NAM/CUC3 proteins and neither are they specific to these proteins, as they can be found in other NACs. Initially, Taoka et al. (2004) identified three domains called the V (TEHVSCFS), L (SLPPL), and W motifs (WNY) as well as a serine-rich domain, but further analyses identified additional domains (Adam et al., 2011; Larsson et al., 2012; Zimmermann and Werr, 2005; Figure 15.3). When fused to the GAL4 DNA-binding domain, the CTD of CUC1 and CUC2 proteins, like that of other NAC proteins, shows transcription activation in yeast cells and tobacco BY-2 cells (Taoka et al., 2004). Its serine-rich and W domains are necessary for transcriptional activity in yeast, while the V and L motifs are dispensable. The W motif is also important *in planta* as the strong *cuc1–3* and weak *cuc1–6* alleles affect this domain (Hibara et al., 2006; Takada et al., 2001; Figure 15.3). In contrast to the serine-rich and

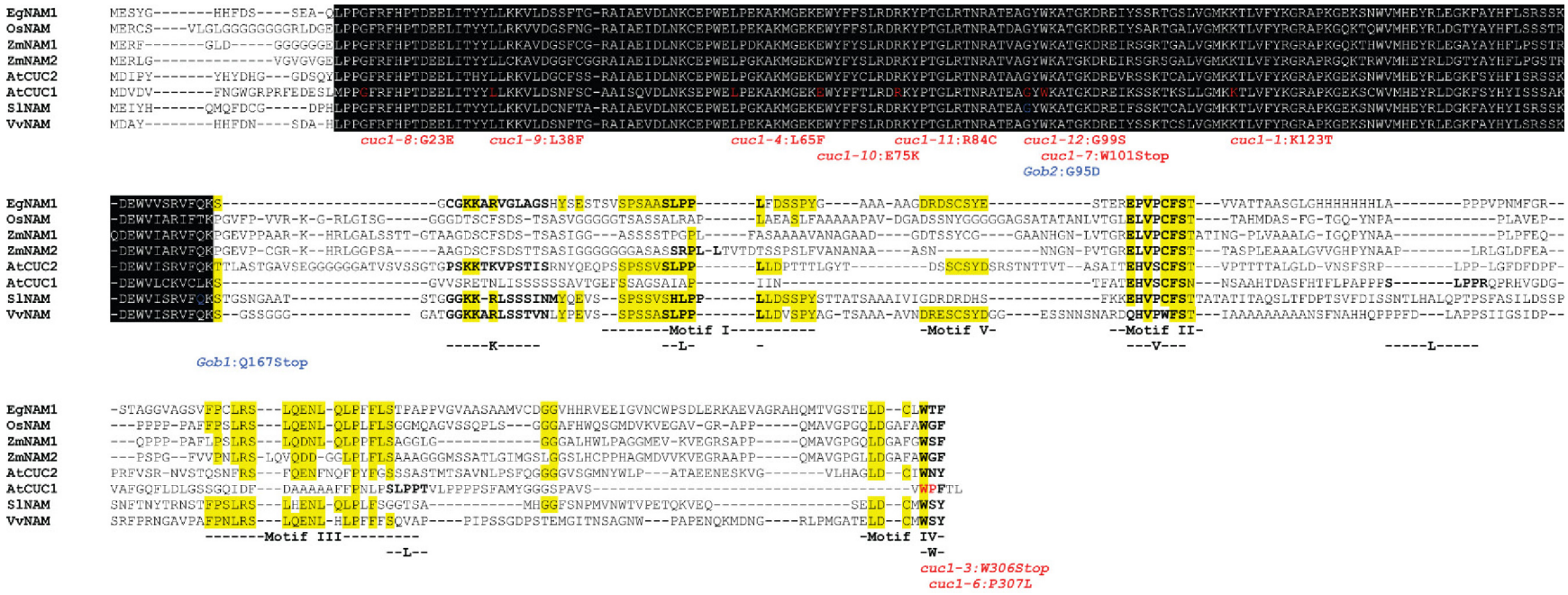

FIGURE 15.3 **Alignment of the NAM proteins (adapted from Adam et al., 2011).** The NAC domain is on a black background. In the CTD, conserved motifs, as defined in Adam et al. (2011; from I to IV), are on a gray background while those defined by Taoka et al. (2004) and Larsson et al. (2012); (domains K, L, V, and W) are in boldface. Mutations affecting either AtCUC1 or SlNAM (also known as GOB) are indicated in red and blue, respectively. Eg, *Elaeis guineensis*; Os, *Oriza sativa*; Zm, *Zea mays*; At, *Arabidopsis thaliana*; Sl, *Solanum lycopersicum*; Vv, *Vitis vinifera*.

W domains that positively contribute to the transcriptional activity of CUC1/2 proteins, a hydrophobic region that contains the K domain (described by Larsson et al., 2012) represses CUC2 activity: deletion of this domain leads to a 14-fold increase in transcriptional activity in yeast (Taoka et al., 2004). Interestingly, the K domain is not only absent from monocot NAM proteins but also from CUC1, which correlates with a higher activity of CUC1 compared to CUC2 (Hasson et al., 2011).

15.3 NAM/CUC3 GENES DEFINE BOUNDARIES IN MERISTEMS AND BEYOND

As mentioned in the introduction, *NAM/CUC3* genes were identified in genetic screens in petunia and *Arabidopsis* as arrested-development mutants showing seedlings with fused cotyledons. Here we present the mutant phenotypes, genetic studies, and expression pattern analysis that led to the characterization of the *NAM/CUC3* functions during plant development.

15.3.1 Identification of the NAM/CUC3 Genes: Role in Boundary and Meristem Formation

15.3.1.1 Identification of NAM/CUC3 in Petunia and Arabidopsis

Petunia *nam* and *Arabidopsis cuc1–cuc2* seedlings share similar phenotypes characterized by fused cotyledons and no SAM (Souer et al., 1996, Aida et al., 1997). This phenotype appears early on during embryonic development with an ectopic bulging at the central apical part of heart-shaped embryos. Simultaneous bulging within this region and at cotyledon primordia effectively leads to fusion of the two cotyledons (Figure 15.4A, B). Therefore, the role of *CUC1/2* and *NAM* genes in cotyledon separation has been ascribed to inhibition of growth in the boundary region. Cotyledon fusion in these mutants is accompanied by a lack of embryonic SAM development. Indeed, presumptive SAM cells in *cuc1–cuc2* double-mutants do not express the meristem marker *SHOOT MERISTEMLESS* (*STM*; Aida et al., 1999). Together, these observations suggest that, in addition to their role in cotyledon separation, *CUC1*, *CUC2*, and *NAM* genes are also implicated in SAM initiation. Accordingly, these genes are expressed during embryogenesis in a region encompassing the presumptive SAM (Aida et al., 1999; Takada et al., 2001). In later stages, this expression disappears from the initiating SAM and becomes restricted to the boundaries between the developing cotyledons and the SAM (Figure 15.4G). This observation suggests that *CUC1*, *CUC2*, and *NAM* have an early role in separating cotyledons and specifying SAM initiation, and a later one separating the undifferentiated SAM from the differentiating cotyledons.

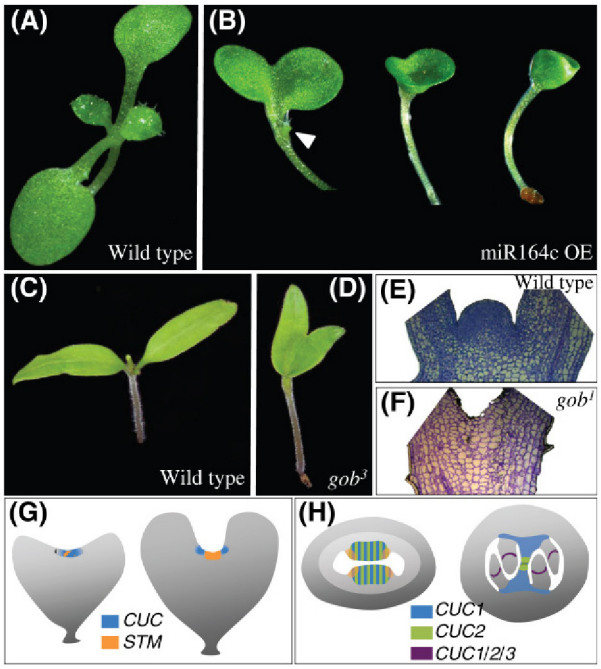

FIGURE 15.4 Mutant phenotypes and expression patterns of *NAM/CUC3* genes in *Arabidopsis* and tomato. (A) Wild-type *Arabidopsis* seedlings and (B) transgenic seedlings showing various degrees of cotyledon fusion phenotypes resulting from reduced expression of *CUC1/2* due to overexpression of its regulator *miR164*. Weak phenotypes show partial cotyledon fusion and reduced meristematic activity (arrowhead points to young leaves) and strongly silenced lines show complete cotyledon fusion and no meristematic activity. (C) Tomato wild-type seedlings and (D) *gob* mutant seedlings showing partial cotyledon fusion. (E, F) The cotyledon fusion phenotype of *gob* mutants is accompanied by an absence of meristem that can be identified in the wild type as a bulge between cotyledon primordia. (G) The expression domain of *CUC* genes at the central apical region of heart-stage embryos (left) overlaps that of shoot apical meristem marker *STM*. At later stages (right) *CUC* expression is restricted to the boundary between cotyledon primordia and the meristematic zone. (H) *CUC* genes are expressed during gynecium development at the adaxial side (lighter gray at the left) of the medial region in the presumptive septum in a region encompassing the future placenta (in orange at the left). This expression is sustained at the medial ridge tips during septum development and eventual fusion. *CUC* genes are also expressed in the boundaries and at the base of ovule primordia and in a ring at the boundary between the nucellus and chalaza.

Much like in the embryo, *CUC* genes are expressed at a variety of frontier regions in the mature plant, such as the boundary between the apical meristem and leaf primordia, between the inflorescence and floral meristems, or even between different floral organs. Accordingly, regenerated shoots of *cuc* double-mutants show organ fusions at all these levels (Aida et al., 1997). Therefore, these genes have been classified as general regulators of organ separation, or, simply put, boundary genes.

While *cuc1–cuc2* double-mutants show strong fusion phenotypes with no SAM initiation, single *cuc1* or *cuc2* mutants are phenotypically normal for the most part, with few showing incomplete cotyledon fusions that produce heart-shaped seedlings. The incomplete penetrance of

single mutations and the overlap of *CUC1* and *CUC2* expression domains suggest that a certain degree of functional redundancy exists between these genes (Takada et al., 2001). The characterization of the *CUC3* gene increased the degree of functional redundancy between *CUC* genes. Indeed, this paralog has overall similar expression patterns to *CUC1* and *CUC2* as well as additive phenotypic effects (Vroemen et al., 2003).

15.3.1.2 Role in Other Dicots and Monocots

Since the characterization of *nam* and *cuc* mutants in petunia and *Arabidopsis*, similar roles in boundary specification and organ separation have been revealed for *NAM/CUC3* genes in other species. For example, *nam* mutants in *Medicago truncatula* have fused cotyledons and lack primary apical meristems (Cheng et al., 2012). In *Antirrhinum majus,* the *CUPULIFORMIS* (*CUP)* gene has been identified through its mutant phenotype, which presents strong organ fusion both at the embryonic and vegetative level (Weir et al., 2004). Despite strong defects in meristem initiation, *cup* mutants can produce secondary meristems at the hypocotyl. These develop severe fusions of leaves and floral organs as well as phyllotaxis perturbations. Overall, organ fusion defects in *cup* mutants are more severe than observed in *Arabidopsis cuc1–cuc2*, suggesting that the redundancy level between *NAM/CUC3* paralogs can vary. Tomato (*Solanum lycopersicum*) *goblet* (*gob*) mutants show similar phenotypes of cotyledon fusion and SAM absence (Figure 15.4C–F; Berger et al., 2009; Blein et al., 2008; Brand et al., 2007). The role of *NAM/CUC3* genes in monocots has not yet been functionally tested, but characterization of the maize *ZmNAM1*, *ZmNAM2*, and *ZmCUC3* genes as well as the oil palm *EgNAM1* and *EgCUC3* genes showed that they have similar expression patterns to *Arabidopsis* homologs, with transcripts being found in meristematic tissues and in cells separating adjacent organs. Interestingly, small differences may exist between monocots and dicots: for instance, *ZmCUC3* is expressed later than *ZmNAM1/2* during maize embryo development whereas *CUC3* is activated earlier than *CUC1/2* in *Arabidopsis* (Zimmermann and Werr, 2005). Nevertheless, these proteins appear to have a conserved function, as oil palm homologs are capable of increasing leaf serration when ectopically expressed in *Arabidopsis* (for the role *NAM/CUC3* plays in leaf development see Section 15.3.3) and restore organ fusion defects in *cuc* mutants (Adam et al., 2011).

15.3.2 Role of CUC Genes in Other Meristematic Territories

As full cup-shaped mutants usually lack a SAM, the study of their effect at the postembryonic level is dependent on the formation of escape or regenerated shoots. Studies of such regenerated shoots have allowed for additional roles of *NAM/CUC3* genes during later vegetative and flowering stages to be characterized.

15.3.2.1 Axillary Meristems

Axillary meristems form near the shoot apex during vegetative and reproductive development at the axils of developing rosette and cauline leaves (Grbic and Bleecker, 2000). *CUC1/2/3* transcripts have been detected in axillary meristems at the boundary between leaf primordia and the shoot apex, and *cuc3* mutants fail to initiate axillary meristems in rosette leaf axils (Aida et al., 1999; Hibara et al., 2006; Ishida et al., 2000; Raman et al., 2008; Takada et al., 2001). This phenotype is greatly enhanced by the *cuc2* mutation but is not observed in other single-mutants or combination of mutants, suggesting that, although both *CUC2* and *CUC3* are required for axillary meristem specification, *CUC3* contribution is greater (Hibara et al., 2006; Raman et al., 2008). Alternatively, plants expressing *miR164*-resistant variants of *CUC1/2* genes form accessory axillary meristems (Raman et al., 2008). Collectively, these results show that *CUC* genes redundantly promote shoot meristem formation both during embryonic and postembryonic development.

15.3.2.2 Floral Organ Boundaries

Flowers of *Arabidopsis cuc1–cuc2* double-mutants show strong organ fusions between sepals and stamens and also have fewer petals and stamens (Aida et al., 1997). Floral phenotypes in single-mutants are much less severe, suggesting once again a certain degree of functional redundancy between *CUC* genes (Hibara et al., 2006). Accordingly, *CUC1/2/3* have mostly overlapping expression patterns in the boundaries between floral organs, both between organs of the same whorl and between different whorls (Hibara et al., 2006; Ishida et al., 2000; Takada et al., 2001; Vroemen et al., 2003). Similar to the way they function in the SAM, CUCs act at the boundaries of organ primordia suppressing cell proliferation and bulging, which allows for clean organ separation. The roles *CUC1/2* play in floral organ number and separation are also dependent upon their regulation by *miR164*. Indeed, *eep1,* a mutant allele of *MIR164C*, leads to the production of supernumerary petals in regions adjacent to normal organs, which is associated with an increase in *CUC1* and *CUC2* expression (Baker et al., 2005).

The role of *NAM/CUC3* genes in floral organ patterning and separation also appears to be conserved across angiosperms. Floral organ fusions are observed in *Medicago truncatula nam*, tomato *gob*, and *Antirrhinum cup* mutants, with the corresponding genes being expressed at floral organ boundaries (Berger et al., 2009; Cheng et al., 2012; Weir et al., 2004). Interestingly, the expression of a *miR164*-resistant variant of *SlGOB* leads to the production of accessory organs mostly in the petal and carpel whorls,

suggesting conservation of the *miR164/CUC* module during tomato flower development (Berger et al., 2009).

15.3.2.3 Gynecium

Arabidopsis CUCs are also expressed within carpel tissues and around developing ovules, suggesting they play a role in gynecium and ovule development (Galbiati et al., 2013; Ishida et al., 2000; Kamiuchi et al., 2014; Nahar et al., 2012; Takada et al., 2001; Vroemen et al., 2003). In *Arabidopsis*, gynecia are composed of two carpels that fuse along two opposing longitudinal medial ridges. The two medial ridge meristems form the placenta, a tissue with meristematic properties, which develops ovules and central outgrowths that fuse to form the septum. Gynecia that lack *CUC* activity fail to initiate medial ridge meristems resulting in severe septum fusion defects and fewer ovules (Ishida et al., 2000; Kamiuchi et al., 2014; Nahar et al., 2012). The early expression of *CUC1/2* at presumptive medial ridges, the absence of meristem marker *STM* expression in the double mutant, and the enlargement of carpel margins in plants expressing *miR164*-resistant forms of *CUC1* or *CUC2* indicate that these genes act both to initiate medial ridge meristems and to maintain their meristematic state (Figure 15.4H; Kamiuchi et al., 2014). In some mutants, incipient medial ridge meristems are formed in an asymmetric fashion suggesting that *CUC1/2* are also required for proper positioning of meristems. In a more extreme case, *miR164*-dependent *CUC2* misregulation leads to incomplete carpel fusion, as medial ridges are incompletely formed (Larue et al., 2009; Nikovics et al., 2006; Sieber et al., 2007). Several lines of evidence suggest *CUC* genes play a role in ovule development, notably the reduced number of ovules in *cuc* double-mutants and the expression of *CUC1/3* between ovule primordia and *CUC1/2/3* between nucellus and chalaza (Aida et al., 1999; Ishida et al., 2000; Vroemen et al., 2003). Although, the exact mechanisms through which *CUC* genes regulate ovule development are still unknown, a recent model involving the integration of auxin signaling has been proposed (Section 15.4.1.1; Galbiati et al., 2013).

Other results are also suggestive of conservation of *NAM/CUC3* roles in gynecium and ovule development across angiosperms. In *Medicago truncatula, nam* mutant carpel margins are incompletely fused and fewer ovules with altered embryo sac development are formed, leading to female sterility (Cheng et al., 2012). *Antirrhinum cup* mutants not only produce fewer ovules and/or fused ovules, they are also female sterile (Weir et al., 2004).

15.3.2.4 Organ Abscission

Abscission – the detachment of aged, mature, or diseased organs such as leaves and seeds – occurs in specific regions that display a set of characteristics reminiscent of meristematic tissues such as small cells with dense cytoplasms (Nakano et al., 2013). These abscission zones situated at key hinge regions share characteristics with boundaries. Indeed, in tomato, the *GOB* gene and other genes known to promote meristematic identity in axillary meristems are expressed in the abscission zone.

15.3.3 Role of CUC Genes in Leaf Development

Arabidopsis leaves are simple with small serrations on their margins. While *cuc2* mutants produce leaves with smooth margins, plants with increased *CUC2* expression as a result of defective *miR164* regulation show deeper and larger serrations than the wild type (Nikovics et al., 2006). *cuc3* mutants also show reduced serrations, while *CUC1,* which is not expressed in leaves, plays no role in *Arabidopsis* leaf development. Whereas *CUC2* acts early on with the onset of teeth, *CUC3* is thought to act only at later stages to sustain teeth outgrowth (Hasson et al., 2011). Interestingly, chimeric constructs, where the *CUC2* promoter drives the expression of CUC1 rescue normal leaf serration in *cuc2* mutants, also induce leaflet formation in genetic backgrounds lacking *miR164*. These results show that, even though *CUC1* is not expressed in developing leaves, the CUC1 protein is partially functionally interchangeable with CUC2.

In species with compound leaves the role of *NAM/CUC3* genes is extended to specify the boundaries between leaflets. Indeed, these genes are expressed at the boundaries of leaflet primordia, and their inactivation results in fused and fewer leaflets (Berger et al., 2009; Blein et al., 2008; Cheng et al., 2012; Wang et al., 2013). Alternatively, tomato plants expressing the gain-of-function *miR164*-resistant allele *Gob4-d* produce deeply lobed leaflets (Berger et al., 2009). Altogether, these observations are suggestive of a conservation of the mechanisms controlling boundary specification between the apex and leaf primordia with different architectures.

15.4 MULTIPLE REGULATORY PATHWAYS CONTRIBUTE TO THE FINE REGULATION OF NAM/CUC3 GENES

Section 15.3 focused on *nam/cuc3* mutant phenotypes and highlighted the precise expression patterns of these genes during development. *NAM/CUC3* genes are expressed in narrow and discontinuous domains, often restricted to a few cells at the boundary between two outgrowing structures. Regulation of this expression pattern is essential for proper organ development as *CUC* overexpression leads to severe phenotypes (Hibara et al., 2006; Laufs et al., 2004). When *CUC2* is uniformly expressed across the leaf margin instead of its discrete expression pattern at the teeth sinuses, a smooth leaf margin is formed in place of the typical serrated form (Bilsborough et al., 2011).

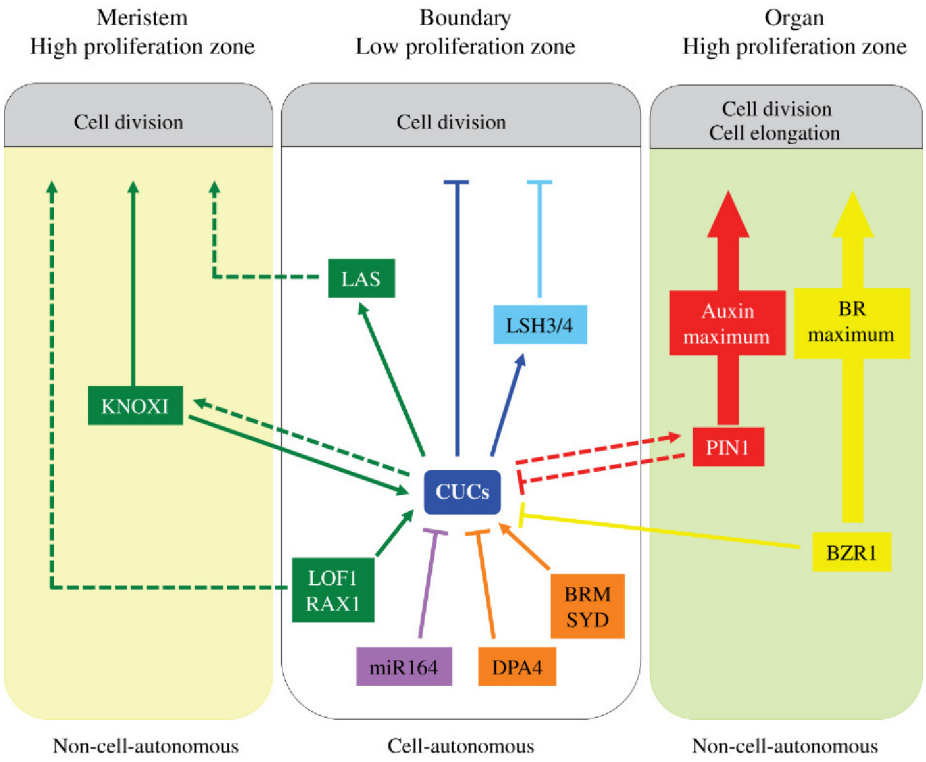

FIGURE 15.5 **CUC regulatory network.** CUC transcription factors are regulated by chromatin regulators such as BRM, SYD, and DPA4. CUC1 and CUC2 are negatively regulated by *miR164*. LOF1 and RAX1 induce CUCs during axillary meristem formation. BRs and auxin, which promote lateral organ proliferation, participate in the CUC discrete boundary expression pattern. BR modify BZR1 activity, which in turn negatively regulates CUCs. Auxin maxima, formed via a PIN1-dependent mechanism in growing primordia, restrict CUC expression to boundaries. In turn, CUCs act via a nonidentified mechanism dependent on PIN1 to modify auxin levels. KNOX genes, which are essential for meristem maintenance, induce CUC expression and activate KNOX expression in a feedforward regulatory loop as well. KNOX, LOF, RAX1, and LAS all contribute to maintaining the high division rate in meristematic zones. CUCs directly activate LSH3 and LSH4 in boundary cells. This complex regulatory network allows both the definition of the boundary by locally repressing growth and organ outgrowth, and meristem initiation and maintenance by promoting growth in a non-cell-autonomous manner. Solid arrows represent direct interaction; dashed arrows represent nonlocal genetic interactions.

This section discusses the factors that contribute to establishing the precise expression patterns of *NAM/CUC3* genes throughout plant development (Figure 15.5). First, we discuss how hormonal regulation shapes *NAM/CUC3* expression. Then, we consider the role of *miR164* in the posttranscriptional regulation of *NAM* genes. Finally, we describe *NAM/CUC3* transcriptional regulation.

15.4.1 Hormonal Regulation of NAM/CUC3 Gene Expression

15.4.1.1 The Interplay between NAM/CUC3 *Genes and Auxin*

Numerous works suggest that *CUC2* expression is repressed by PIN1-generated auxin maxima. The *PIN-FORMED1* (*PIN1*) gene encodes an auxin efflux carrier that has a polar distribution within the cell thus contributing to differential auxin accumulation in *Arabidopsis*. In developing embryos, PIN1-dependent auxin maxima induce cotyledon formation (Friml et al., 2003). In *pin1* mutants, the *CUC1* expression domain is extended to the entire apical region whereas *CUC2* is expressed in patches restricted to the center and sides of the embryo (Aida et al., 2002). The *PINOID* (*PID*) gene encodes a serine/threonine kinase that acts as a positive regulator of PIN1-mediated polar auxin transport. *pin1–pid* double-mutant embryos completely lack cotyledons and show broad expression of *CUC1* and slight enlargement of the *CUC2* expression domain. Additionally, *pin1–pid–cuc1* triple-mutants form small cotyledons which suggests that ectopic expression of *CUC1* in *pin1–pid* embryos is responsible for the absence of cotyledons (Furutani et al., 2004). *pasticcino1* (*pas1*) mutants show defective cotyledon development and associate altered membrane localization of PIN1 with an enlargement of the domain expressing *CUC2* (Roudier et al., 2010). Overall, these results indicate that PIN1-mediated auxin transport is necessary to regulate *CUC1/2* expression in the embryo.

During postembryonic development, *pin1* mutants produce a naked inflorescence (Okada et al., 1991). In similarly to what as happens in the embryo, primordia positioning in the SAM is determined by PIN1-driven auxin maxima (Reinhardt et al., 2003). In *pin1* mutants, *CUC2* expression is enlarged forming a circle around

the inflorescence SAM (Vernoux et al., 2000). Moreover, live imaging experiments suggest that *CUC2* expression is downregulated in tissues where convergent PIN1 polarities are expected to accumulate high auxin levels (Heisler et al., 2005). Together, these results suggest that *CUC2* expression in the SAM is inhibited by PIN1-generated auxin activity maxima. As explained in Section 15.3.2.1, *CUC2* genes redundantly promote axillary meristem formation (Raman et al., 2008). Two articles suggest that an auxin minimum is required for axillary meristem formation in *Arabidopsis* and tomato (Wang et al., 2014a; Wang et al., 2014b). Although this has not been tested, this auxin minimum could allow *CUC* expression thus inducing axillary meristem formation.

pin1 Arabidopsis mutants form leaves that lack serrations. PIN1-mediated auxin response foci at the leaf margin are interspaced with regions showing high *CUC2* and *CUC3* expression (Hasson et al., 2011; Hay et al., 2006; Nikovics et al., 2006). Auxin treatments are able to abolish expression of a *CUC2* reporter in leaf primordia, suggesting that auxin negatively regulates *CUC2* expression during simple leaf development (Bilsborough et al., 2011).

As mentioned in Section 15.3.2.3, *CUC1* and *CUC2* are involved in carpel margin meristem initiation required for ovule initiation. In this context, *MONOPTEROS* (*MP*) is expressed in a similar pattern to *CUC1* and *CUC2*. Moreover, in *mp* mutants, *CUC1* and *CUC2* expression is reduced in inflorescences and leaves. Chromatin immunoprecipitation (ChIP) experiments have established that MP directly binds *CUC1* and *CUC2* genomic regions (Galbiati et al., 2013). These results strongly suggest that MP positively regulates *CUC1* and *CUC2*, providing a molecular link between auxin signaling and *CUC* genes. However, these results are difficult to reconcile with data obtained in the embryo where *CUC1* expression domain is enlarged in *mp* mutant embryos (Aida et al., 2002), indicating that *MP* negatively regulates *CUC1* expression in the embryo. Moreover, it is surprising that an auxin response factor would positively regulate *CUC* expression when in most organs auxin maxima negatively regulate *CUC* expression.

To date, it is not clear to what extent the relationship between auxin and *CUC* genes identified in *Arabidopsis* is conserved. In *Cardamine hirsuta*, a close relative of *Arabidopsis* with compound leaves, it is not known whether *ChCUC* expression is controlled by polar auxin transport as in *Arabidopsis*. Interestingly, the expression of *GOB* in tomato is not modified upon auxin treatment. Moreover, *ENTIRE*, an auxin-response repressor, acts on leaf dissection in a parallel pathway independent of *GOB* (Ben-Gera et al., 2012; Berger et al., 2009). Alternatively, auxin-induced downregulation of *NAM* genes seems to be a general feature during embryonic and postembryonic development, and some evidence points to conservation of this role. Indeed, in the gymnosperm *Picea abies* a *NAM/CUC3* ortholog is also regulated by polar auxin transport (Larsson et al., 2012).

A recent work reported a link between cytokinins and *CUCs* (Li et al., 2010). A line overproducing cytokinins produces more flowers, a phenotype that is dependent on *CUC2* and *CUC3* overexpression. Moreover, in the cytokinin receptor *ahk2–ahk3* double-mutant, *CUC1* and *CUC2* expression is strongly reduced, suggesting that cytokinin signaling promotes *CUC* expression. Interestingly, there is increasing evidence that cytokinin signaling controls polar auxin transport (Marhavy et al., 2014). Therefore, further investigations are required to determine whether regulation of *CUC* genes by cytokinins is mediated by auxin.

15.4.1.2 *Brassinosteroids, New Regulators of* CUC *Expression*

Brassinosteroids (BRs) are plant steroid hormones that regulate cell proliferation and other developmental processes (Kim and Wang, 2010). They act through a complex signaling pathway that leads to activation of two transcription factors, BZR1 and BES1, which in turn modify the expression of over 1000 genes.

Recent findings suggest a link between BRs and *CUC* genes. Plants with increased BR content or signaling show axillary shoot, stamen, and cotyledon fusions, reflecting abnormal boundary establishment (Gendron et al., 2012). Alternatively, mutants with reduced biosynthesis or sensitivity to BRs have deeper axillary separations and form ectopic boundaries. This suggests that low BR signaling is sufficient and necessary for proper boundary formation. Genetic and pharmacological experiments show that low BR signaling induces *CUC* expression in the SAM, whereas high BR signaling inhibits it. Additionally, ChIP experiments indicate that BZR1 strongly binds the *CUC3* promoter suggesting direct regulation. Overall, these results indicate that BR signaling negatively regulates *CUC* gene expression.

15.4.2 *miR164* FineTunes NAM Gene Expression

miR164 was among the first identified plant miRNAs. In *Arabidopsis*, it is encoded by three loci, *MIR164A*, *B*, and *C*. It regulates the expression of six transcription factors of the NAC family: *CUC1* and *CUC2*, *NAC1* which is known to regulate lateral root induction, *ORESARA1* (*ORE1*) which controls leaf senescence, and two uncharacterized *NACs* (At5g61430 and At5g07680; Schwab et al., 2005).

15.4.2.1 miR164 *Regulation is Essential for Shoot Development*

miR164 controls inflorescence and floral development. Plants expressing *CUC1* or *CUC2 miR164*-resistant variants

show extra petals and enlarged sepal boundaries (Laufs et al., 2004; Mallory et al., 2004). Another *CUC2 miR164*-resistant allele has shorter and wider siliques with tissue projections along the valve margins (Larue et al., 2009). Accordingly, *early extra petals1* (*eep1*), a *mir164c* mutant, also presents extra petals and defects in carpel fusion (Baker et al., 2005). This indicates *MIR164C* plays a role in regulating *CUC1* and *CUC2* during flower development. A similar role has been proposed for *SlmiR164* in tomato, in which expression of a *SlmiR164*-resistant *GOB* variant results in extra petals and ectopic carpels (Berger et al., 2009). Expression of *miR164*-resistant *CUC2* leads to modified phyllotaxy compared to wild type (Peaucelle et al., 2007), as also observed in the *mir164abc* triple-mutant (Sieber et al., 2007). Strikingly, in both genotypes, the formation of primordia in the meristem appears to be normal. Taken together, this reveals that phyllotaxy is postmeristematically maintained via *miR164*-dependent negative regulation of *CUC2*.

miR164 also regulates leaf development. Both *mir164a Arabidopsis* mutants and *CUC2 miR164*-resistant lines present leaves with deeper serrations than the wild type. Moreover, *MIR164A* is expressed in leaf margin sinuses in a pattern overlapping *CUC2* expression (Nikovics et al., 2006). Thus, in *Arabidopsis*, *MIR164A* regulates the level of *CUC2* expression, which in turn governs the level of leaf serration. Interestingly, quantitative trait locus (QTL) mapping has revealed a single nucleotide polymorphism in *MIR164A* miRNA* which modifies *MIR164A* biogenesis and drastically reduces its accumulation (Todesco et al., 2012). This indicates that natural variation in *MIR164A* maturation can contribute to leaf serration polymorphism. In the compound leaves of tomato, the *GOB* miRNA-resistant allele, *Gob-4d*, harbors leaflets with deeper and wider lobes than the wild type, whereas *gob* mutants show smooth fused leaflets (Berger et al., 2009). In contrast to *Arabidopsis*, *GOB* and *SlMIR164* show complementary expression profiles and the *GOB* expression pattern becomes wider in the *Gob-4d* allele. This suggests, that *SlmiR164* defines the sharp domain of the *GOB* expression pattern rather than controling its expression level as in *Arabidopsis*.

miR164 controls axillary meristem development. Expression of *CUC1* or *CUC2 miR164*-resistant variants leads to the formation of accessory buds in leaf axils, a phenotype also observed in *mir164abc* mutants. Concurrently, *MIR164A* and *MIR164C* are expressed in the boundary between the leaf primordium and the SAM, from where the axillary meristem subsequently emerges (Raman et al., 2008). Overall, *CUC1* and *CUC2* mRNA cleavage by *miR164* is required to negatively regulate the formation of accessory buds in leaf axils.

These results establish *CUC1/2–miR164* as a conserved genetic module that is recruited multiple times during the evolution of aerial organs (Figure 15.6). Moreover, *miR164* plays a crucial role in regulating *NAC1* during lateral root induction (Guo et al., 2005) and inhibiting *ORE1* expression during leaf senescence (Kim et al., 2009). *miR164* is therefore an important regulator of plant development (Pulido and Laufs, 2010). Interestingly, *miR164* does not seem to regulate its targets always in the same manner: while it regulates the timing of *ORE1* expression, it regulates the *GOB* expression pattern spatially during tomato leaf development and controls the level of *CUC1* and *CUC2* expression during flower and leaf development in *Arabidopsis*.

15.4.2.2 *Evolution and Specialization of the* MIR164 *Genes*

miR164 is found in dicots, monocots, and gymnosperms, indicating that, much like its target *NAC* genes, it was likely present in the last common ancestor of

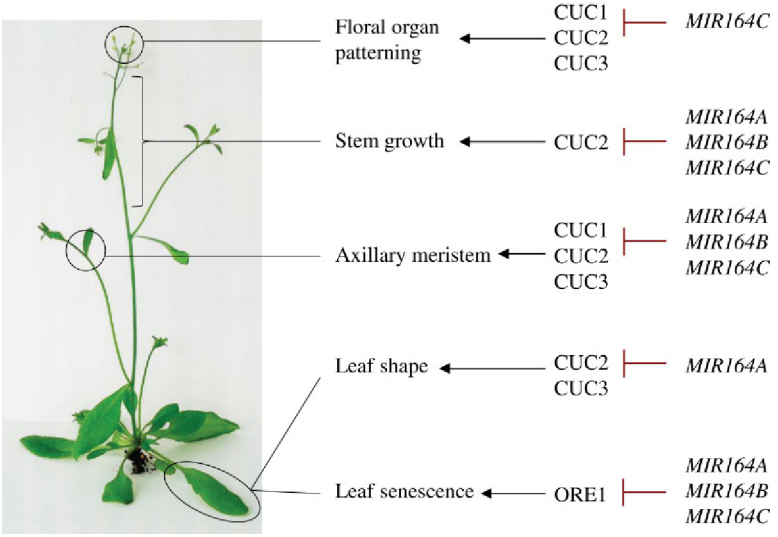

FIGURE 15.6 ***CUC/miR164*, a genetic module essential for plant shoot development.** The roles played by each *CUC* (and the related NAC gene, *ORE1*) and *MIR164* genes during *Arabidopsis* development are indicated.

gymnosperms and angiosperms (Section 15.2.1.2; Axtell and Bartel, 2005). Multiple genes code for *miR164*: 3 in *Arabidopsis* but up to 12 in soybean. Mature *miR164* genes encoded by different members can be identical, such as in soybean, or show small sequence variations as in *Arabidopsis* in which mature *miR164c* differs by one nucleotide from mature *mir164a* or *mir164b*. An evo-devo study of the *MIR164* family suggests that two lineages were present in the last common ancestor of extant angiosperms: a B clade containing the *Arabidopsis MIR164B* gene whose members tend to be highly expressed in roots; and another clade whose members, such as the *Arabidopsis MIR164A* and *C* genes, tend to be less expressed in roots than in other tissues (Jasinski et al., 2010).

In some developmental processes, the three *Arabidopsis MIR164* genes seem to be functionally redundant. For instance, *miR164a*, *miR164b* , and *miR164c* act redundantly to downregulate *CUC2* expression during the postmeristematic maintenance of phyllotaxy (Sieber et al., 2007). In contrast, *MIR164C* plays a more important role during floral development than the two others (Sieber et al., 2007), while *MIR164A* is the negative regulator of *CUC2* that controls leaf shape (Nikovics et al., 2006). The extent to which each *MIR164* gene regulates different developmental processes varies and is likely to result from differences in their expression patterns.

15.4.2.3 Transcriptional Control of miR164 Expression

Transcription factors of the plant-specific TCP family (TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTOR) are well known for the role they play in regulating developmental processes. They are divided into two classes (Martin-Trillo and Cubas, 2009; Chapter 16). In particular, class II *CINCINNATA-like* (*CIN-like*) genes redundantly regulate cell proliferation and promote differentiation during leaf development. Plants expressing TCP3-EAR, a fusion with the EAR repression domain, have rosette leaves with exaggerated serrations and lobed cotyledons with ectopic shoot meristems (Koyama et al., 2007). CUC genes are overexpressed in these plants and *miR164* accumulation is decreased. Moreover, *cuc1* and *cuc2* mutations suppress the *TCP3–EAR* phenotype, indicating that it mainly results from increased *CUC* expression. *TCP3* has been found to directly activate *MIR164A* transcription (Koyama et al., 2010). Interestingly, some class I TCP members also regulate *CUC* expression (Uberti-Manassero et al., 2012). Thus, TCPs appear to be positive regulators of *miR164* and indirect inhibitors of *CUC* expression.

Auxin regulates *CUC* expression both directly (Section 15.4.1.1) and indirectly via modification of *miR164* levels. Indeed, auxin treatments can induce *miR164* expression in roots (Guo et al., 2005), and some mutants with disrupted auxin signaling show enhanced leaf serration due to reduced *MIR164A* expression (Bilsborough et al., 2011). Thus, auxin regulates *miR164* levels during root and leaf development.

miR164 expression is regulated by ethylene during leaf aging (Kim et al., 2009). In the *ethylene insensitive 2* (*ein2*) mutant, no reduction of *miR164* levels is observed, which suggests that *EIN2* is required for *MIR164* downregulation. EIN3 acts downstream of EIN2 and binds to *MIR164A*, *MIR164B*, and *MIR164C* promoters to repress their activity (Li et al., 2013). Overall, ethylene negatively regulates *miR164* levels by activating EIN2 which in turn promotes EIN3 transcriptional repression activity.

Interestingly, all three *MIR164* genes are marked with H3K27me3 repressive histone modification during leaf development (Lafos et al., 2011), suggesting that modification of chromatin dynamics also contributes to their regulation.

During floral development, a C_2H_2 zinc finger transcriptional repressor named RABBIT EARS (RBE) is specifically required for proper formation of second-whorl boundaries (Krizek et al., 2006). *rbe* mutants show fused sepals and aberrant petals, which recapitulate the floral phenotype of *cuc1–cuc2* double-mutants. RBE directly binds to the *MIR164C* promoter, negatively regulating its activity in floral boundaries. Moreover, genetic analyses reveal that RBE negatively regulates *MIR164B* expression while activating *MIR164A* expression in floral buds (Huang et al., 2012). Thus, RBE would differentially regulate the expression of *MIR164* genes during floral development, promoting their functional differentiation.

15.4.3 Transcriptional Regulation of NAM/CUC3 Expression

15.4.3.1 Transcription Factors Regulating CUC Expression During Embryogenesis

Class I KNOTTED-like homeobox genes (KNOXI) that code for homeodomain transcription factors are essential for SAM initiation and maintenance (for reviews see Hamant and Pautot, 2010; Hay and Tsiantis, 2009; Chapter 14). Plants mutated in the *KNOXI* gene *STM* lack a SAM and show reduced *CUC1* and *CUC3* expression, which is restricted to a stripe in the center of the boundary between two cotyledons (Takada et al., 2001; Vroemen et al., 2003). *CUC2* expression is even more modified in *stm* embryos, being limited to small spots at variable positions between developing cotyledons (Aida et al., 1999). Thus, *STM* regulates *CUC* expression contributing to its localization at the center of the embryo. Alternatively, the absence of *CUC* downregulation in the center of the embryo could also be attributed to the lack of meristematic cells in the *stm* mutant. Spinelli et al. (2011) demonstrated that inducing *STM* expression activates *CUC1* transcription in a direct manner since induction is maintained in the

presence of a translational inhibitor. A binding site for STM in the *CUC1* promoter has been identified and validated *in vitro*, in yeast, and *in planta.* Overall, this indicates that *STM* directly induces *CUC1* expression.

Besides *STM*, there are other *KNOXI* genes that contribute to the establishment and maintenance of the SAM. Notably, their inactivation aggravates the phenotype of a weak *stm* allele, *stm-2*. For example, *knat6-1–stm-2* double-mutants show no SAM and strong cotyledon fusion compared with *stm-2*. Although *CUC3* expression is not altered in *knat6* mutants, it is completely lost in *knat6-1–stm-2* double-mutants indicating that *KNAT6* and *STM* redundantly contribute to *CUC3* activation (Belles-Boix et al., 2006).

In addition to KNOXI proteins the homeobox transcription factor WUSCHEL (WUS) also contributes to stem cell maintenance (Chapter 14). Although there is no evidence to suggest that WUS regulates *CUC* genes, other members of the same *WUSCHEL-RELATED HOMEOBOX* (*WOX*) clade control *CUC* expression in the cotyledon boundary. *wox2 stimpy-like* (*stpl/wox8*) double-mutants show partial cotyledon fusion, which correlates with asymmetrical *CUC2* and *CUC3* expression at one side of the embryo (Lie et al., 2012). Conversely, *CUC1* expression in some embryos is expanded in the protodermal layer. Overall, *WOX2* and *STPL* differentially regulate *CUC* genes, restricting *CUC1* expression and allowing symmetrical expression of *CUC2* and *CUC3*.

15.4.3.2 *Transcription Factors Regulating CUC Expression During Axillary Meristem Formation*

Two independent groups identified three genes coding for MYB domain transcription factors –*REGULATOR OF AXILLARY MERISTEM 1* (*RAX1*), *RAX2*, and *RAX3* – which are redundantly required for early induction of axillary meristems in *Arabidopsis* (Keller et al., 2006; Muller et al., 2006). Like the CUC genes, *RAX1* and *RAX3* are expressed in the axils of leaf primordia. Interestingly, *in situ* hybridizations show that *CUC2* expression in *rax1* is missing at the exact position of a future axillary meristem, indicating that *RAX1* induces local *CUC2* expression to promote axillary meristem formation (Keller et al., 2006).

Like *RAX1–3* genes, *LATERAL ORGAN FUSION1* (*LOF1*) also encodes a MYB domain transcription factor involved in axillary meristem formation and expressed in leaf axils. *lof1* mutants show reduced expression levels of *CUC1/2/3* and *RAX1* that could be indirectly mediated by changes in *RAX1* activity (Lee et al., 2009). Overall, LOF1 and RAX1 are transcription factors that act upstream of *CUC* genes during axillary meristem formation.

15.4.3.3 *Transcription Factors Regulating* NAM/CUC3 *Expression During Leaf Development*

Besides their central role in meristem formation, *KNOXI* genes are also involved in the development of most compound leaves (Blein et al., 2010; Chapter 14). *KNOXI* expression is initially downregulated both in simple and compound incipient leaf primordia. Such downregulation is permanent in species with simple leaves, whereas it is transient in primordia of compound leaves, being reactivated later during primordia development (Bharathan et al., 2002). In *Cardamine hirsuta*, the expression of *KNOXI* genes is required for leaflet formation and their overexpression leads to more leaflets and deeper serrations (Hay and Tsiantis, 2006). *KNOXI* overexpression increases *CUC* expression, while silencing *CUC* genes in plants overexpressing *KNOXI* suppresses their phenotype, indicating that *KNOXI* genes promote leaflet formation by activating *CUC* expression (Blein et al., 2008).

Although observed in many species, the reactivation of *KNOXI* genes during compound leaf development is not a general mechanism. Instead, some *Fabacea* show activation of *UNIFOLIATA*, an ortholog of the *Arabidopsis LEAFY* (*LFY*) gene, which controls leaflet formation in these species (Hofer et al., 1997). In *Pisum sativum*, the *uni* mutant forms simple smooth leaves where neither *NAM* nor *CUC3* expression could be detected (Blein et al., 2008). Interestingly, *CUC2* has been shown to be a possible target of LFY in the *Arabidopsis* inflorescence (Winter et al., 2011). All in all, *UNI/LFY* could be a positive regulator of NAM/CUC3 expression.

15.4.3.4 *Transcription Factors Regulating* GOB *Expression During Abscission*

Two MADS box domain transcription factors, JOINTLESS and MACROCALYX (MC), promote abscission zone formation during tomato fruit development (Nakano et al., 2012). Transcriptional studies on plants misexpressing *JOINTLESS* or *MC* show that *GOB* expression is probably positively regulated by the *JOINTLESS/MC* heterodimer (Nakano et al., 2012). Another gene induced by the JOINTLESS/MC heterodimer is the AP2/ERF transcription factor *ETHYLENE RESPONSE FACTOR 52* (*SlERF52*). Plants with reduced *SlERF52* levels are impaired when pedicel abscission is activated and present reduced *GOB* expression, indicating that SlERF52 is also a positive regulator of *GOB* expression (Nakano et al., 2014). Overall, this designates JOINTLESS/MC as early activators of *GOB* expression and SlERF52 as a late *GOB* activator during fruit abscission.

15.4.3.5 *Regulation of CUC Expression by Chromatin Modifications*

Gene expression regulation depends not only on the presence of transcription factors that bind to specific promoter domains but also on chromatin availability to transcription factors. The chromatin dynamic is regulated by nucleosome-modifying enzymes that catalyze histone and DNA-covalent modifications as well as chromatin-remodeling complexes that remodel histone

octamers/DNA interactions. Switch/sucrose nonfermentable (SWI/SNF) complexes are chromatin-remodeling factors conserved between yeast, mammals, and plants. Remodeling SWI/SNF complexes are recruited to promoters and regulate the accessibility of binding sites to transcription factors (Jerzmanowski, 2007).

In an enhancer screen of the *cuc2* cotyledon fusion phenotype, three mutations in the *BRAHMA* (*BRM*) gene were identified. BRM is an adenosine triphosphatase (ATPase) of the SWI2/SNF2 family (Kwon et al., 2006). A mutation in another SWI2/SNF2 member, *splayed* (*syd*), also enhances the cotyledon fusion phenotype of *cuc1* and *cuc3* mutants. Real time polymerase chain reaction (RT-PCR) and β-glucuronidase gene (GUS) reporter analyses established that *BRM* positively regulates the expression of the three *CUC* genes, and that *SYD* induces *CUC2* expression. This result indicates that general regulators of gene expression are also required for proper *CUC* expression.

Among factors regulating the chromatin dynamic are the modifying enzymes of histone octamers. These enzymes catalyze posttranslational modifications of histones, thus changing their interaction with DNA. One of the best-characterized histone modifications is the trimethylation of histone 3 on lysine 27 (H3K27me3), which leads to chromatin compaction and transcriptional repression. This mark is deposited by Polycomb group (PcG) proteins assembled in the Polycomb repressive complex 2 (PRC2). H3K27me3 is subsequently recognized by PRC1, which mediates locus repression (Schatlowski et al., 2008). Interestingly, *CUC2* and *CUC3* carry the H3K27me3 repressive mark. *CUC2* shows this mark in the meristem and leaves, whereas *CUC3* specifically carries the H3K27me3 mark in the leaves (Lafos et al., 2011). Thus, developmentally regulated deposition of repressive histone marks is likely to contribute to proper *CUC2* and *CUC3* expression.

Engelhorn et al. (2012) screened for genes expressed in the plant apex which were regulated by PRC1. They characterized the *DEVELOPMENT-RELATED PCG TARGET IN THE APEX 4* (*DPA4*) gene, which encodes a transcriptional repressor containing a B3 DNA-binding domain. *DPA4*, like *CUC* genes, is expressed in the boundary domains of the meristem and leaf primordia. *DPA4* negatively regulates *CUC2* expression and, accordingly, *dpa4* mutants show increased leaf serration, whereas a *DPA4* overexpressor presents smooth leaves. Thus, DPA4 appears to be an upstream negative regulator of *CUC2* expression.

15.5 NAM/CUC3 CONTROL PLANT DEVELOPMENT VIA MODIFICATIONS OF THE CELLULAR BEHAVIOR

Organ boundaries act both as frontiers and growth organizer centers (Aida and Tasaka, 2006). Boundaries cells display typically reduced growth activity, delimiting the frontier between different cell types. Besides this role in organ/tissue separation, boundaries participate in organ initiation and meristematic activity maintenance. Therefore, CUCs are likely to play different roles in controlling multiple aspects of plant growth and morphogenesis. Here, we focus on the effects downstream of CUCs, exploring the cellular effects dependent on CUCs, how they are achieved, and what molecular actors are involved (Figure 15.5).

15.5.1 CUC-Dependent Cellular Effects

Genetic analysis of *cuc* mutant combinations, coupled with morphologic analysis, suggest that *CUC1/2/3* repress growth in boundaries thus allowing organ separation (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003). Growth integrates cell division and cell expansion parameters; therefore, reduced growth activity from cells localized at boundaries can be due to decreased cell division rate, reduced cell expansion, or both. Several pieces of work investigating various species report that cells located at boundaries display reduced cell division (Breuil-Broyer et al., 2004; Gaudin et al., 2000). However, experimental work linking cell proliferation and CUC transcription factors is scarce. In the wild-type *Arabidopsis* inflorescence meristem, floral primordia are formed 5–6 cells apart from each other (Heisler et al., 2005; Reddy et al., 2004). In the *mir164abc* triple-mutant, mature flowers are separated by roughly the same number of cells indicating that plant cell division in *mir164abc* is repressed between flowers during stem development. This correlates with local increase of *CUC1* expression suggesting a function for CUC1 in controlling cell division (Sieber et al., 2007). To test this hypothesis, Sieber and coworkers ectopically expressed CUC1 and examined sepal cells. Sepal length was dramatically reduced in plants overexpressing *CUC1*, but the cell number per area unit was not different from the wild type suggesting that CUC1 plays a role in cell division regulation. Taken together these results indicate that CUCs act as growth antagonists through local repression of cell division.

Leaf development constitutes an excellent model to study cellular parameters controlled by *CUC* genes. By analogy with cellular mechanisms occurring at lateral organ primordia boundaries, *CUC2* has been suggested to restrict growth of sinuses at the leaf margin (Nikovics et al., 2006). In contrast, *CUC2* promotes tooth outgrowth via a non-cell-autonomous pathway involving auxin (Bilsborough et al., 2011; Kawamura et al., 2010). These opposing results highlight the fact that CUCs control cell proliferation in different ways to allow differential growth.

15.5.2 How Does CUC Impact Cell Proliferation?

In plants, cell proliferation depends on the action of phytohormones. BRs, for example, constitute a major class

of polyhydroxysteroid hormones, structurally similar to steroid hormones in animals, promoting growth in various developmental processes (Mussig, 2005). BRs promote growth by controlling both cell elongation and cell division. BR-insensitive mutants display dwarf phenotypes, partially as a result of impaired mitotic activity (Gonzalez-Garcia et al., 2011; Zhiponova et al., 2013). The *LATERAL ORGAN BOUNDARIES* (*LOB*) gene negatively controls BR accumulation in boundaries, while BRs repress other boundary identity genes, such as *CUC* genes, in a feedback loop to control boundary formation (Section 15.3; Bell et al., 2012; Gendron et al., 2012). These studies reveal the fundamental role BRs play in boundary delimitation and link BR signaling to boundary identity genes.

Another hormone playing a key role in boundary formation is auxin. Spatiotemporal auxin accumulation relies on controlled expression and subcellular localization of auxin efflux transporters PIN1 (Friml et al., 2004; Okada et al., 1991). JAGGED LATERAL ORGAN (JLO), a boundary identity gene and member of the LATERAL ORGAN BOUNDARY DOMAIN (LBD) transcription factor family – to which LOB belongs – controls *PIN* expression (Bureau et al., 2010; Rast and Simon, 2012). CUC2 promotes auxin accumulation via an unknown PIN1-dependent mechanism in leaves and, in turn, auxin represses *CUC2* expression forming a regulatory feedback loop. *In silico* models accounting for such a regulatory loop recapitulate wild-type leaf margin development and teeth formation patterns (Bilsborough et al., 2011).

Interestingly, BRs and auxins act synergistically to regulate photomorphogenesis by modulating AUXIN RESPONSE FACTOR2 (ARF2) activity of BR signaling components (Vert et al., 2008). Therefore, it is probable that the integrated action of these two hormones regulates boundary domain formation as well.

Although it is clear from the work described above that the *CUC* genes, auxins, and BRs play important roles in boundary delimitation, the underlying molecular mechanisms still need to be elucidated.

15.5.3 CUC Direct Targets

cuc mutant boundary phenotypes can be enhanced by mutations in several other genes, including transcription factors (Gomez-Mena and Sablowski, 2008; Lee et al., 2009; Lie et al., 2012), chromatin-remodeling factors (Kwon et al., 2006), and auxin flux regulators (Furutani et al., 2004). Taken together these studies show that CUC transcription factors cooperate with various biological processes to regulate boundary formation. Despite efforts to identify the molecular factors responsible for boundary delimitation, little is known about the regulatory network involved in this developmental process.

So far, only two CUC direct targets have been identified. Using rat glucocorticoid-receptor-inducible cell lines overexpressing *CUC1*, Takeda et al. (2011) showed that *LIGHT-DEPENDENT SHORT HYPOCOTYL 4* (*LSH4*) and its homolog *LSH3* are directly activated by CUC1 in boundary cells. These genes encode proteins belonging to the ALOG family (*Arabidopsis* LSH1 and *Oryza* G1) which are predicted to bind DNA and modulate transcriptional activity (Iyer and Aravind, 2012). LSH3 (also known as OBO1; Cho and Zambryski, 2011) and LSH4 are located in the nuclei of boundary cells and, therefore, may play a role in boundary formation (Takeda et al., 2011). Constitutive *LSH4* expression results in developmental defects such as inhibition of leaf growth and formation of ectopic meristems highlighting its potential role during plant development. Conversely, constitutive LSH4 expression cannot rescue the developmental defects of *cuc1–cuc2* mutants, suggesting that other regulators act downstream of *CUC1* to delimit boundaries.

15.5.4 Other Regulators: KNOX, LFY, LAS

Other regulators are known to act downstream of CUC transcription factors, but their molecular links are still missing. This is the case for *KNOXI* genes. Hibara et al. (2003) have shown that *KNOXI* genes such as *STM* and *BREVIPEDICELLUS* (*BP*) are ectopically expressed in *Arabidopsis* plants overexpressing *CUC1*. More generally, the accumulation of *KNOXI/LFY*-like transcripts is reduced in leaves when *NAM/CUC3* genes are silenced (Blein et al., 2008). These results, together with the data presented in Section 15.4.3.1, reveal the existence of a feed-forward regulatory loop between *KNOXI/LFY*-like genes and *NAM/CUC3* genes during leaf development, which is likely to be conserved widely across eudicots. The *LATERAL SUPPRESSOR* (*LAS*) gene encodes a member of the GAI, RGA, SCR (GRAS) family of putative transcription factors, which is expressed at the SAM boundary (Greb et al., 2003). *LAS* expression decreases when *CUC* activity is reduced suggesting that LAS acts downstream of CUC (Raman et al., 2008). Accordingly, the higher level of *CUC* mRNA accumulation in *mir164abc* mutants correlates with an increase in *LAS* expression.

15.6 CONCLUSION

Since their first identification almost 20 years ago, a wealth of data have been accumulated on *NAM/CUC3* genes, establishing their central role in plant boundary formation. Fine analyses have shown a strong conservation of their function from species to species and in different organs of aerial parts, but have also underlined variations within this general trend. However, these conclusions are mostly based on genetic analyses. The challenge for the next years will be to reveal the molecular links, in particular, the genetic regulatory network between NAM/CUC3 transcription factors and boundary biology.

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