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Interplay of miR164, *CUP-SHAPED COTYLEDON* genes and *LATERAL SUPPRESSOR* controls axillary meristem formation in *Arabidopsis thaliana*

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Summary

Aerial architecture in higher plants is established post-embryonically by the inception of new meristems in the axils of leaves. These axillary meristems develop into side shoots or flowers. In Arabidopsis, the NAC domain transcription factors *CUP SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *CUC3* function redundantly in initiating the shoot apical meristem and establishing organ boundaries. Transcripts of *CUC1* and *CUC2* are targeted for degradation by miR164. In this study, we show that *cuc3-2* mutants are impaired in axillary meristem initiation. Overexpression of miR164 in the *cuc3-2* mutant caused an almost complete block of axillary meristem formation. Conversely, *mir164* mutants and plants harbouring miR164-resistant alleles of *CUC1* or *CUC2* developed accessory buds in leaf axils. Collectively, these experiments reveal that, in addition to *CUC3*, redundant functions of *CUC1* and *CUC2* as well as miR164 regulation are required for the establishment of axillary meristems. Studies on *LAS* transcript accumulation in *mir164* triple mutants and *cuc3-2* plants overexpressing miR164 suggest that regulation of axillary meristem formation by miR164 is mediated through *CUC1* and *CUC2*, which in turn regulate *LAS*.

Keywords: axillary meristem, *CUC*, miR164, Arabidopsis, *LAS*.

Introduction

Shoot branching in plants is initiated by the formation of new meristems in the axils of leaves, which develop into secondary axes of growth. Axillary meristems recapitulate the function of the shoot apical meristem (SAM) by initiating several leaf primordia, resulting in the formation of axillary buds, which either grow out or remain dormant depending on their position along the shoot axis, the developmental phase of the plant and environmental factors (Mcsteen and Leyser, 2005).

The *LATERAL SUPPRESSOR* genes in Arabidopsis (*LAS*; Greb *et al.*, 2003) and tomato (*Solanum lycopersicum*; Schumacher *et al.*, 1999) encode putative transcription factors belonging to the GRAS family, and specifically regulate the initiation of axillary meristems during the vegetative phase of development. Mutations in *MONOCULM1*, the rice orthologue of *LAS*, cause defective tiller formation, altered rachis branches, and modified spikelets (Li *et al.*, 2003). The tomato *blind* mutant displays a strong reduction in axillary meristem formation during vegetative and reproductive development (Schmitz *et al.*, 2002). The *Blind* gene encodes a transcription factor of the R2R3 MYB class. In Arabidopsis, the *Blind*-homologous *RAX* genes control the formation of axillary meristems in overlapping zones along the shoot axis (Keller *et al.*, 2006; Mueller *et al.*, 2006). Transcripts of *LAS*, *RAX1* and *RAX3* accumulate in similar domains in the developing leaf axil where axillary meristems will form (Greb *et al.*, 2003; Keller *et al.*, 2006; Mueller *et al.*, 2006), suggesting that the axillary region has a special identity which may be a prerequisite for the formation of new meristems.

CUC1, *CUC2* and *CUC3* encode NAC domain transcription factors (Ernst *et al.*, 2004; Olsen *et al.*, 2004). *CUC1* and *CUC2*

are redundantly involved in the initiation of the SAM through the regulation of *STM* expression and in the establishment of cotyledon and floral organ boundaries (Aida *et al.*, 1999; Long *et al.*, 1996). The discovery of *CUC3*, encoding a protein with high functional similarity to CUC1 and CUC2, uncovered an additional level of redundancy in the function of these genes (Vroemen *et al.*, 2003). When combined with *cuc1* or *cuc2*, mutations in *CUC3* lead to the formation of cup-shaped cotyledons, organ fusions and defects in shoot branching (Hibara *et al.*, 2006; Vroemen *et al.*, 2003).

A subset of NAC-domain transcription factors comprising *CUC1*, *CUC2*, *NAC1*, *At5g07680*, *At5g61430* and *At5g39610* is post-transcriptionally regulated by microRNA164 (miR164; Rhoades *et al.*, 2002; Kasschau *et al.*, 2003; Laufs *et al.*, 2004; Mallory *et al.*, 2004; Baker *et al.*, 2005; Schwab *et al.*, 2005). Plant microRNAs are endogenous, single-stranded, non-translated RNA molecules which are highly complementary to their target mRNAs and induce post-transcriptional gene silencing by catalysing cleavage of their targets (Bartel and Bartel, 2004). *CUC1* and *CUC2* mRNAs are cleaved within their miR164-binding site (Kasschau *et al.*, 2003), and plants containing miR164-resistant versions of *CUC1* or *CUC2* show severe alterations in embryonic, vegetative and floral development due to an enlargement of various boundary domains (Baker *et al.*, 2005; Laufs *et al.*, 2004; Mallory *et al.*, 2004; Nikovics *et al.*, 2006). Constitutive overexpression of mirR164 was shown to phenocopy the *cuc1 cuc2* double mutant by downregulating *CUC1* and *CUC2* transcript accumulation (Laufs *et al.*, 2004; Mallory *et al.*, 2004).

miR164 is encoded by three genes: *MIR164A*, *MIR164B* and *MIR164C* (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Reinhart *et al.*, 2002; Wang *et al.*, 2004). *mir164a* and *mir164b* mutants show increased lateral root formation (Guo *et al.*, 2005), and a loss of function mutation in *MIR164C* leads to the formation of extra petals in early arising flowers (Baker *et al.*, 2005). *miR164a* and *miR164b* mutations enhance the floral defects of *mir164c* plants substantially, showing that these miRNAs control flower development in a redundant manner and revealing a role of miR164 in the architecture of the inflorescence stem (Peaucelle *et al.*, 2007; Sieber *et al.*, 2007). *MIR164A* was also shown to regulate the development of leaf margins (Nikovics *et al.*, 2006).

In this study, we have analysed the role of the Arabidopsis *CUC* genes and miR164 in the process of shoot branching. Characterization of plants containing a *cuc3-2* knockout allele in combination with or without miR164 overexpression uncovered partially redundant functions of *CUC1*, *CUC2* and *CUC3* in axillary meristem formation. Analysis of *mir164* knockout mutants and of transgenic plants carrying miR164-resistant *CUC1* and *CUC2* alleles demonstrated that miR164 regulation of *CUC1*/*CUC2* transcript accumulation is required to achieve a wild-type shoot branching pattern. Expression studies suggest that *CUC1* and *CUC2* control axillary meristem development through regulation of *LAS*, whereas *CUC3* may function in an *LAS*-independent manner.

Results

cuc3-2 *is strongly compromised in axillary meristem formation during vegetative development*

CUC3 encodes a putative NAC-domain transcription factor and its role in the establishment of boundaries between the cotyledons as well as between the SAM and lateral organs has been described previously (Vroemen *et al.*, 2003). Because axillary meristems develop in the boundary zone between the SAM and leaf primordia, we have analysed the pattern of axillary bud formation in plants homozygous for the null allele *cuc3-2* (Vroemen *et al.*, 2003). In contrast to the Wassilewskija (Ws) wild type, *cuc3-2* plants grown under short-day (SD) conditions show a strong reduction in the number of axillary buds originating from the axils of rosette leaves (Figure 1h). Most of the rosette leaves formed in the early and middle phase of vegetative development did not support the formation of axillary buds. However, axillary buds developed from a high proportion of the rosette leaves formed late in vegetative development (Figure 1h). Closer inspection of the empty leaf axils using a stereomicroscope and scanning electron microscopy (SEM) uncovered barren leaf axils without any indication of morphologically distinguishable axillary structures (Figure 1c,e).

Additionally, we studied the expression of the shoot meristem marker *STM* in *cuc3-2* vegetative apices. Similar to its expression pattern in Ws wild-type apices (Figure 1f), *STM* transcript accumulation was also observed in the SAM and the interprimordial regions of *cuc3-2* apices (Figure 1g). In accordance with previously reported studies (e.g. Long and Barton, 2000), *STM* expression was excluded from incipient and existing leaf primordia. However, the focused *STM* expression found in the adaxial boundary of older leaf primordia in the Ws wild type (Figure 1f, arrowhead; Greb *et al.*, 2003) was conspicuously absent in *cuc3-2* plants. These focused *STM* expression domains in wild-type plants are indicative of the organization of a new meristem in the axillary region, and thus their absence suggests that *cuc3-2* plants are incapable of initiating these axillary meristems. From these histological and *in situ* hybridization experiments we concluded that the reduction in side-shoot formation in *cuc3-2* was due to a failure in axillary meristem initiation rather than due to a defect in axillary bud outgrowth. During reproductive development, axillary bud formation in *cuc3-2* did not deviate from the Ws wild type (Figure 1h). A strong reduction in the number of axillary buds in the axils of early rosette leaves was also observed in

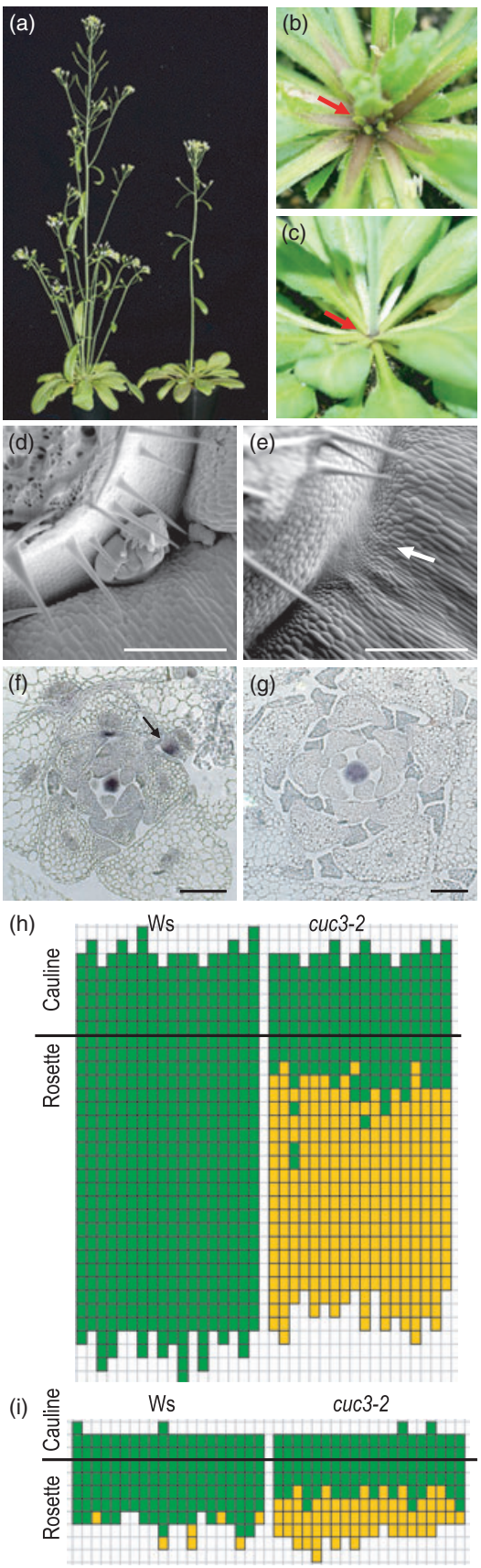

Figure 1. *CUC3* regulates axillary bud formation during vegetative development.
(a) Growth habit of a Wassilewskija (Ws) wild type (left) and a *cuc3-2* (right) plant grown for 35 days in short photoperiods and subsequently induced to flower in long days. (b, c) Close up of rosette leaf axils in Ws (b) and in *cuc3-2* (c) showing presence (b, arrow) and absence (c, arrow) of axillary bud, respectively. (d, e) Scanning electron microscopy (SEM) micrographs of a Ws rosette leaf axil showing an axillary bud (d) and a barren *cuc3-2* rosette leaf axil (e, arrow). (f, g) Comparison of *STM* mRNA accumulation in cross-sections through vegetative shoot apices of Ws (f) and *cuc3-2* (g) plants. In addition to expression in the meristem and the interprimordial regions, focused *STM* expression was seen in the adaxial centre of older leaf primordia in the wild type (f, arrow) which is absent in *cuc3-2*. Bar = 400 μm in (f) and (g). (h, i) Schematic representation of axillary bud formation in leaf axils of *cuc3-2* plants in comparison with Ws plants grown either under short-day conditions (h, $n = 18$) or in long photoperiods (i, $n = 18$). Leaf axils of plants were examined under a binocular microscope. Each column in (h) and (i) represents a single plant, with each square within a column representing an individual leaf axil. The horizontal line represents the border between the youngest rosette leaf and the oldest cauline leaf, with positions of progressively older rosette leaves below the line, and positions of progressively younger cauline leaves above it. The green colour denotes the presence of an axillary bud and yellow the absence of an axillary bud in any particular leaf axil.

cuc3-2 plants grown under long photoperiods, but meristem development remained unaffected in the axils of cauline leaves (Figure 1i). Our results extend observations by Hibara *et al.* (2006), who reported a reduction in tertiary shoot formation in *cuc3-101* and *cuc3-105* plants. Taken together, these results demonstrate that the *CUC3* gene plays a critical role in the genetic control of the formation of axillary meristems.

CUC1 *and* CUC2 *transcripts accumulate in the axils of leaf primordia*

CUC1 and *CUC2*, a pair of closely related genes, belong to a different clade of the NAC family of transcription factors than *CUC3* (Vroemen *et al.*, 2003; Zimmermann and Werr, 2005). However, they have been shown to function redundantly with *CUC3* in the formation of the SAM and specification of cotyledon boundaries (Vroemen *et al.*, 2003). Here, we monitored the distribution of *CUC1* and *CUC2* mRNA in vegetative shoot apices by RNA *in situ* hybridization experiments on tissue sections of Columbia (Col) wild-type plants that were grown under SD conditions and fixed 28 days after sowing. *CUC1* transcripts were detected in the axils of young leaf primordia from P0 to P6/P7 (Figure 2a). The *CUC1* expression domain was about three to five cell layers deep, including the L1–L3 layers of the SAM, and extended one or two cell layers into the adaxial–abaxial dimension. Transverse sections revealed that *CUC1* transcripts accumulated in a band-shaped domain along the adaxial boundary of the leaf primordium (Figure 2b). Expression of *CUC1* was not detectable in the axils of P7/P8 to P20/P21 primordia, but was again found from P21/P22 onwards, indicating the onset of axillary meristem activity in these leaf axils (data not shown).

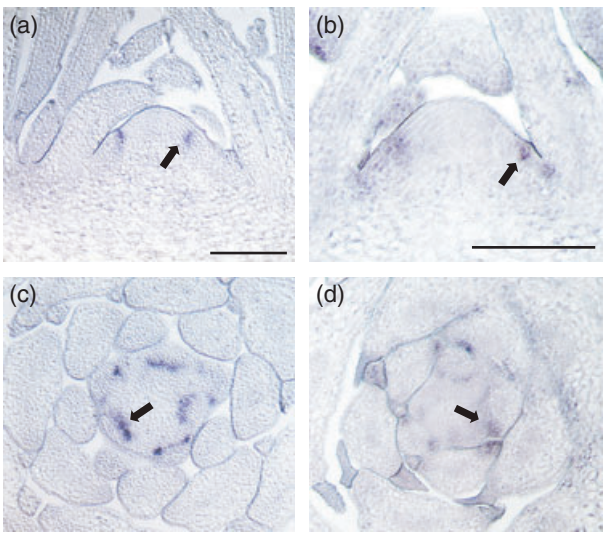

Figure 2. Patterns of *CUC1* and *CUC2* transcript accumulation in the vegetative shoot apex.
Longitudinal (a, c) and transverse (b, d) sections through shoot apices of 28-day-old Columbia (Col) plants grown under short-day conditions were hybridized with a *CUC1* (a, b) or a *CUC2* (c, d) antisense probe. The arrows point to the *CUC1* and *CUC2* expression domains.
Bars = 200 μm in (a) for (a) and (b), and in (c) for (c) and (d).

CUC2 mRNA was found to accumulate in a domain similar to the *CUC1* domain (Figure 2c). Different from *CUC1*, *CUC2* transcripts were detected in the axils of leaf primordia from P0 to P17. Transverse sections revealed accumulation of *CUC2* transcript along the adaxial border of leaf primordia (Figure 2d). Vroemen *et al.* (2003) reported that during vegetative development *CUC3* transcripts also accumulate in a narrow domain in the axils of leaf primordia. Taken together, these data suggest that during vegetative growth *CUC1*, *CUC2* and *CUC3* mRNAs accumulate in the leaf axil zone from which axillary meristems develop.

Since *cuc3-2* mutants displayed a clear defect in axillary shoot development, we also analysed axillary bud formation in plants homozygous for null alleles of *CUC1* and *CUC2*. Under long-day as well as SD growing conditions the shoot branching patterns of *cuc1-1* and *cuc2-1* mutants showed no aberration compared with their corresponding wild-type Landsberg *erecta* (L*er*). The early growth arrest in *cuc1 cuc2* double mutants (Aida *et al.*, 1997) rendered it impossible to examine the pattern of shoot branching in these double mutants.

Overexpression of MIR164A *or* MIR164B *in a* cuc3-2 *background enhances the* cuc3-2 *branching defect*

CUC1 and *CUC2* were predicted to be post-transcriptionally regulated by miR164 (Schwab *et al.*, 2005). Overexpression of this miRNA was shown to reduce *CUC1* and *CUC2* RNA levels and phenocopy the *cuc1 cuc2* double mutant phenotype (Laufs *et al.*, 2004; Mallory *et al.*, 2004). To uncover any function of *CUC1* and/or *CUC2* in the process of axillary meristem formation that might be masked by *CUC3* activity, we characterized the shoot branching pattern of transgenic lines harbouring a *2x35S::MIR164A* or *2x35S::MIR164B* construct (Laufs *et al.*, 2004), thus overexpressing miR164, in a Ws or a *cuc3-2* background. One *2x35S::MIR164A* and two *2x35S::MIR164B* transgenic lines in the Ws background were analysed. All three transgenic lines showed fusions of cotyledons, sepals and stamens, as described previously (Laufs *et al.*, 2004; Mallory *et al.*, 2004). However, in both short and long photoperiods, overexpression of *MIR164A* or *MIR164B* in Ws plants did not lead to any deviation from the wild-type branching pattern, neither in the vegetative phase nor in the reproductive phase of development (Figure 3a).

The branching patterns of two populations of plants homozygous for *cuc3-2* and segregating for *2x35S::MIR164A* (population A) and *2x35S::MIR164B* (population B), respectively, were analysed. These plants were grown in short photoperiods for 30 days and then shifted to long days to induce flowering. From these populations we selected plants that formed cup-shaped cotyledons indicating a strong downregulation of *CUC1* and *CUC2* activity (Figure 3d). The PCR analysis demonstrated that these cup-shaped seedlings contained the *2x35S::MIR164* T-DNA. The majority of these plants initiated a new SAM in the hypocotyl region (Figure 3e), which produced rosette leaves and developed into a flowering shoot (Figure 3b). During vegetative (Figure 3f) as well as reproductive (Figure 3g) development, these plants failed to develop axillary buds in most of their leaf axils. Very rarely, in some plants a single axillary shoot was observed in a cauline leaf axil (Figure 3c). In addition, these plants were characterized by a reduction in the number of leaves, fusion of leaf petioles to the main axis, distorted phyllotaxis, curled cauline leaves, immature siliques and a dark green colour (Figure 3b,c,f,g). The enhancement of the *cuc3-2* branching defect by overexpression of *MIR164A* or *MIR164B* suggests a redundant role for *CUC1* and/or *CUC2* in the control of axillary meristem formation.

Plants expressing miR164-resistant transcripts of CUC1 *and* CUC2 *develop accessory side shoots*

To further analyse the function of *CUC1* and *CUC2* in axillary bud formation, we investigated miR164-resistant *CUC1* and *CUC2* lines, *5mCUC1* and *CUC2g-m4,* respectively, for their pattern of axillary shoot development. *5mCUC1* and *CUC2g-m4* are transgenic lines containing the miR164-resistant *CUC1* and *CUC2* genes driven by their endogenous promoters in a wild-type background (Mallory *et al.*, 2004; Nikovics *et al.*, 2006). These miR164-resistant alleles have silent mutations in their miR164-binding sites, which insulate them from miR164 regulation, leading to the accumulation of high levels of *CUC1* and *CUC2* mRNA.

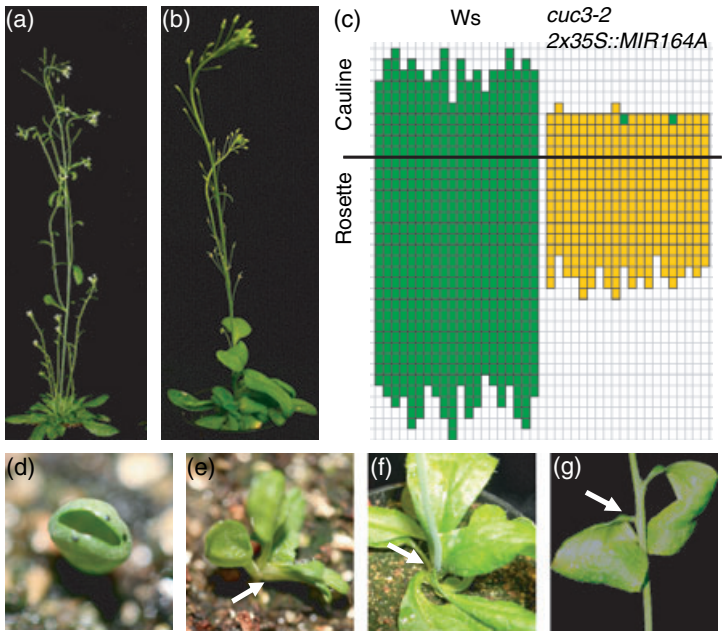

Figure 3. Axillary bud formation in *2x35s::MIR164B* plants.
Plants were grown for 35 days in short photoperiods and subsequently induced to flower in long photoperiods.
(a, b) Growth habits of a transgenic plant harbouring the *2x35s::MIR164A* construct in a Wassilewskija (Ws) wild-type background (a) and in a *cuc3-2* mutant background (b).
(c) Schematic representation of axillary bud formation in leaf axils of *cuc3-2 2x35s::MIR164A* in comparison with Ws wild-type plants. As explained in the legend to Figure 1, green and yellow boxes denote the presence or absence of an axillary bud, respectively, in leaf axils along the main shoot.
(d) Ten-day-old *cuc3-2 2x35s::MIR164A* plant grown showing cup-shaped cotyledons.
(e) Twenty-day-old *cuc3-2 2x35s::MIR164A* plant showing formation of a new shoot apex (arrow) at the hypocotyl.
(f) Rosette leaves fused to the main shoot and displaying empty leaf axils (arrow) and distorted phyllotaxis in a mature *cuc3-2 2x35s::MIR164A* plant.
(g) Empty leaf axils (arrow) and curled cauline leaves in a mature *cuc3-2; 2x35s::MIR164A* plant.

During the reproductive phase, *CUC2g-m4* plants frequently developed clusters of cauline leaves with highly reduced internodes (Figure 4a,d, arrows; Peaucelle *et al.*, 2007). Notably, *CUC2g-m4* plants developed accessory side shoots in the axils of cauline leaves (Figure 4a,b, arrows) and rosette leaves (Figure 4c, arrow). These additional side shoots developed in the zone between the primary side shoot and the leaf and appeared later than the primary side shoot. During vegetative development in short photoperiods, accessory buds were formed predominantly in the axils of late and middle rosette leaves (Figure 4f). In the reproductive phase, the formation of accessory buds was found to be more enhanced in early cauline leaf axils than in late cauline leaf axils (Figure 4f). An increase in accessory bud formation was observed irrespective of the length of the photoperiods that these plants experienced (data not shown). Accessory side shoots were also found at a very low frequency in cauline leaf axils of the Ws wild-type control (Figure 4f).

Formation of accessory buds was also observed in four independent *5mCUC1* lines. Similar to *CUC2g-m4* plants, accessory bud formation was found mostly in early cauline leaf axils and decreased during late reproductive development (Figure 4g). However, unlike in *CUC2g-m4* plants, accessory bud development was observed in *5mCUC1* plants, particularly during the middle phase of vegetative development; very few early and late rosette leaf axils harboured accessory buds (Figure 4g). Furthermore, the overall increase in accessory bud formation during vegetative as well as reproductive development was lower in *CUC1-5m* plants when compared with *CUC2g-m4* plants. For example, almost 90% of *CUC2g-m4* plants displayed accessory buds in early cauline leaf axils, whereas in *5mCUC1* plants fewer than 70% of early cauline leaf axils supported accessory bud formation (Figure 4f,g).

MIR164A *and* MIR164C *are expressed in the boundary region between leaf primordia and the SAM*

In Arabidopsis the *MIR164* gene family comprises three members: *MIR164A, MIR164B* and *MIR164C* (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Reinhart *et al.*, 2002; Wang *et al.*, 2004). Previous studies using northern hybridization, RT-PCR, GFP-based transcriptional reporters and mRNA *in situ* hybridization have shown that these three miRNAs accumulate in leaves, floral organs and roots, and that miR164b is the most abundant of the three (Baker *et al.*, 2005; Guo *et al.*, 2005; Mallory *et al.*, 2004; Nikovics *et al.*, 2006; Sieber *et al.*, 2007). In this study, we examined the expression patterns of the three *MIR164* genes in vegetative shoot apices of Col wild-type plants grown for 30 days in short photoperiods, using β-glucuronidase (GUS)-based reporters. Expression from the *MIR164A* promoter was detected in the axillary regions of the shoot apex and within leaf primordia (Figure 5a,b). In the shoot apex, GUS staining was restricted to a few cells in the L1 layer of the boundary region between leaf primordia and the shoot meristem (Figure 5a,b). In leaf primordia, the *MIR164A* promoter directed GUS expression to the epidermal layer of both, the abaxial and the adaxial side, and to vascular bundles (Figure 5a,b). On the other hand, GUS staining was not observed in the SAM of plants transformed with the *MIR164B* promoter construct (Figure 5c,d). However, GUS staining was detected in the epidermal layer and the

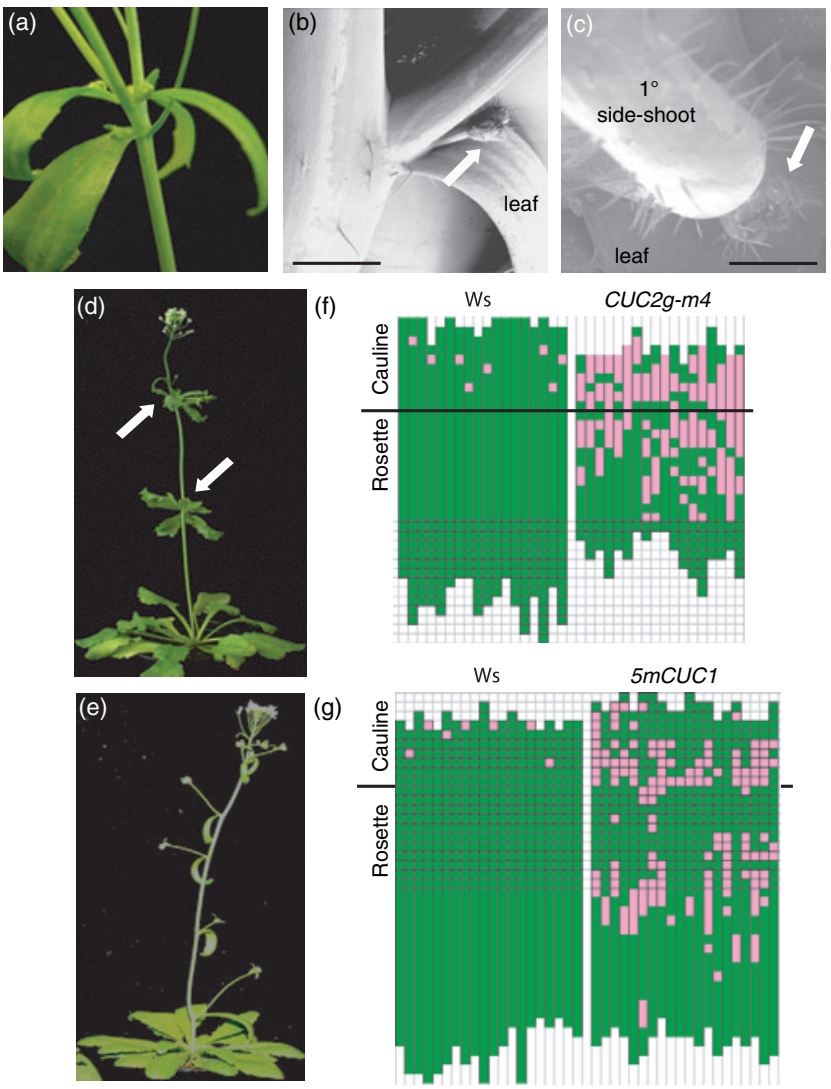

Figure 4. Analysis of plants expressing miR164-resistant alleles of *CUC1* and *CUC2*.
(a) Close-up of cauline leaf axils in a *CUC2g-m4* plant showing accessory bud formation and distorted phyllotaxis.
(b) A SEM micrograph of a *CUC2g-m4* cauline leaf axil displaying an accessory bud (arrow). Bar = 125 μm.
(c) A SEM micrograph of a *CUC2g-m4* rosette leaf axil showing an accessory bud (arrow). Bar = 250 μm.
(d, e) Habitus of *CUC2g-m4* (d) and *5mCUC1* (e) plants grown for 30 days in short photoperiods and subsequently in long days.
(f, g) Schematic representation of accessory bud formation in leaf axils of a population of *CUC2g-m4* (f) and *5mCUC1* (g) plants in comparison to Wassilewskija (Ws) plants. The pink colour denotes the presence of an accessory bud in a particular leaf axil, and green denotes the absence of accessory bud. Plants were grown for 30 days in short photoperiods and subsequently for 35 days under long-day conditions.

vascular bundles of leaf primordia (Figure 5c,d). In the epidermis, staining was more prominent on the abaxial side than on the adaxial side, whereas in vascular tissues GUS activity seems to be restricted to the phloem (Figure 5c,d). In contrast to the *MIR164A* and *MIR164B* expression patterns, *MIR164C*-directed GUS staining was specifically confined to the boundary region between the meristem and leaf primordia as well as to older leaf axils (Figure 5e,f). In young leaf axils, the expression was restricted to the L1 layer, whereas it comprised two or three cell layers in older leaf axils (Figure 5e,f). Notably, GUS staining in the *MIR164C* transgenic lines was not detected within leaf primordia, neither in the epidermis nor in the vasculature (Figure 5e,f).

miR164 regulates the number of axillary buds in leaf axils

Since interference in the miR164 regulation of *CUC1* and *CUC2* led to the formation of accessory side shoots, we further investigated the role of miR164 in axillary meristem development by characterizing the shoot branching patterns of plants homozygous for the loss of function alleles *mir164a-4* (Nikovics *et al.*, 2006), *mir164b-1* (Mallory *et al.*, 2004), *mir164c-1* (*eep1*, Baker *et al.*, 2005; Sieber *et al.*, 2007), the three double mutants and the triple mutant.

In short photoperiods, *mir164a-4*, *mir164b-1* and *mir164c-1* single mutants developed accessory buds in their leaf axils (Figure 6c). In *mir164a-4* and *mir164b-1* mutants, accessory bud formation was observed only in the cauline leaf axils, with *mir164a-4* displaying a much stronger phenotype than *mir164b-1* (Figure 6d). This phenotype was accentuated in *mir164a-4 mir164b-1* double mutants (Figure 6d). On the other hand, accessory bud formation was restricted to vegetative development in *mir164c-1* (Figure 6d). Axils of rosette leaves formed during the middle phase of vegetative development displayed a greater tendency to harbour an accessory bud than early or late rosette leaf axils. *mir164c-1*

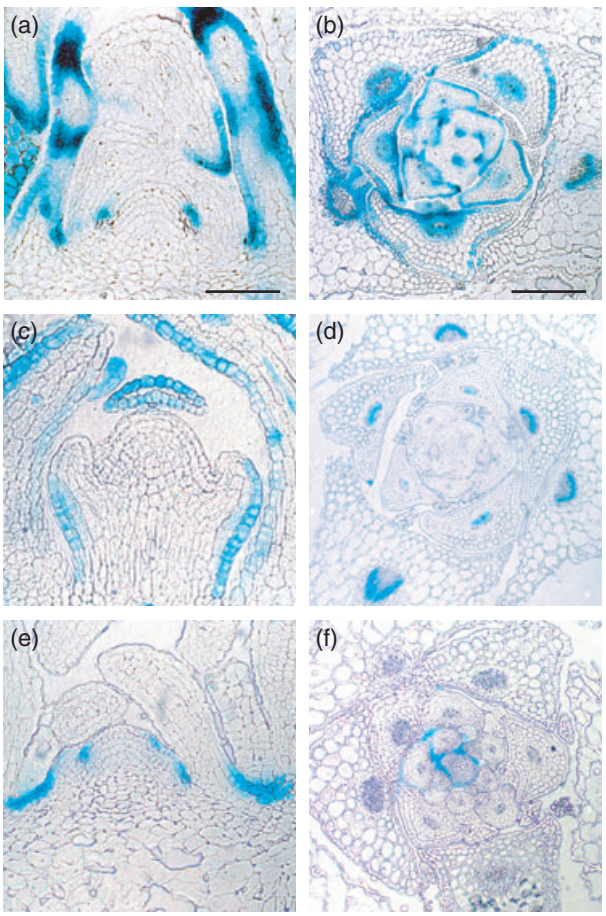

Figure 5. Patterns of *MIR164A*-, *MIR164B*- and *MIR164C*- promoter driven GUS expression in the vegetative shoot apex.
Longitudinal (a, c, e) and transverse (b, d, f,) sections through shoot apices of *MIR164A::GUS* (a, b), *MIR164B::GUS* (c, d) and *MIR164C::GUS* (e, f) plants. Apices were harvested from plants grown for 30 days under short-day conditions and were incubated in X-Gluc staining buffer. Bars = 100 μm in (a) for (a), (c) and (e), and 400 μm in (b) for (b), (d) and (f).

enhanced the mutant phenotype of *mir164b-1*, with the *mir164b-1 mir164c-1* double mutant displaying a low number of accessory buds in late rosette leaf axils and an increase in the tendency to form accessory buds during reproductive development (Figure 6d). Surprisingly, *mir164a-4 mir164c-1* plants developed accessory buds exclusively in cauline leaf axils (Figure 6d). However, these double mutants formed a small rosette within each rosette leaf axil, making it difficult to analyse accessory bud formation during the vegetative phase of development. Accessory bud formation was most prominent in *mir164a-4 mir164b-1 mir164c-1* (*mir164abc*) triple mutants, both during the vegetative and the reproductive phase of development (Figure 6c,d). At a very low frequency, two accessory buds per cauline leaf axil were observed in the *mir164abc* mutant as well as in the *mir164ab* mutant. *mir164abc* frequently displayed extremely short internodes during reproductive development (Figure 6c) and all cauline leaf axils displayed an increased tendency to harbour an accessory bud (Figure 6d). During vegetative development, this tendency was stronger in the axils of late than of early rosette leaves (Figure 6d). Similar, though less pronounced, patterns of accessory bud formation were also seen in all *mir164* mutant combinations grown in long photoperiods (data not shown). These results show that *MIR164A*, *MIR164B* and *MIR164C* redundantly regulate the number of side shoots, with a greater contribution of *MIR164A* during reproductive development and *MIR164C* during vegetative development.

miR164 fine tunes axillary meristem initiation by regulating LAS *through* CUC1 *and* CUC2

Our studies on GUS expression from the promoters of the *MIR164* genes revealed that the pattern of miR164 accumulation in the shoot apex partially overlapped with the expression domains of its targets, *CUC1* and *CUC2*. Therefore we also examined the expression patterns of *CUC1* and *CUC2* in vegetative shoot apices of the *mir164abc* mutant grown for 30 days in short photoperiods. In *mir164abc* plants, *CUC1* transcripts accumulated in a similar pattern as in the corresponding wild type, but the expression levels seemed to be upregulated in the axillary regions of the triple mutant (Figure 7a,b). On the other hand, we observed not only strongly elevated levels of *CUC2* mRNA in *mir164abc* apices, when compared to wild-type apices, but also a broadening of its expression domain encompassing cells of the L1, L2 and L3 layers throughout the shoot apex (Figure 7c,d).

The remarkable similarities between the expression patterns of miR164, the *CUC* genes and *LAS*, a well-known regulator of axillary meristem initiation in plants (Greb *et al.*, 2003), together with the corresponding mutant phenotypes, suggested a hierarchical interaction between these genes in controlling axillary meristem development. To test this hypothesis, we examined *LAS* expression in vegetative apices of the *cuc* mutants. *LAS* transcript accumulation was found to be unaltered in apices of *cuc1-1* and *cuc2-1* mutants (Figure S1). Furthermore, we detected no change in *LAS* mRNA distribution in apices of the *cuc3-2* mutant, which displays a defect in axillary meristem initiation, when compared with the corresponding wild type (Figure 7e,f). Apices of *cuc3-2 35S::MIR164A* plants, on the other hand, displayed a reduction in *LAS* mRNA accumulation (Figure 7g). Conversely, a substantial upregulation of the *LAS* transcript was observed in *mir164abc* mutant apices (Figure 7h). From these lines of evidence we conclude that *LAS* expression is positively regulated by concerted *CUC* activities. Taken together, these data suggest that the initiation of axillary meristems is confined by miRNA164 through the restriction of *CUC1* and *CUC2* mRNA accumulation, which in turn regulate *LAS* expression.

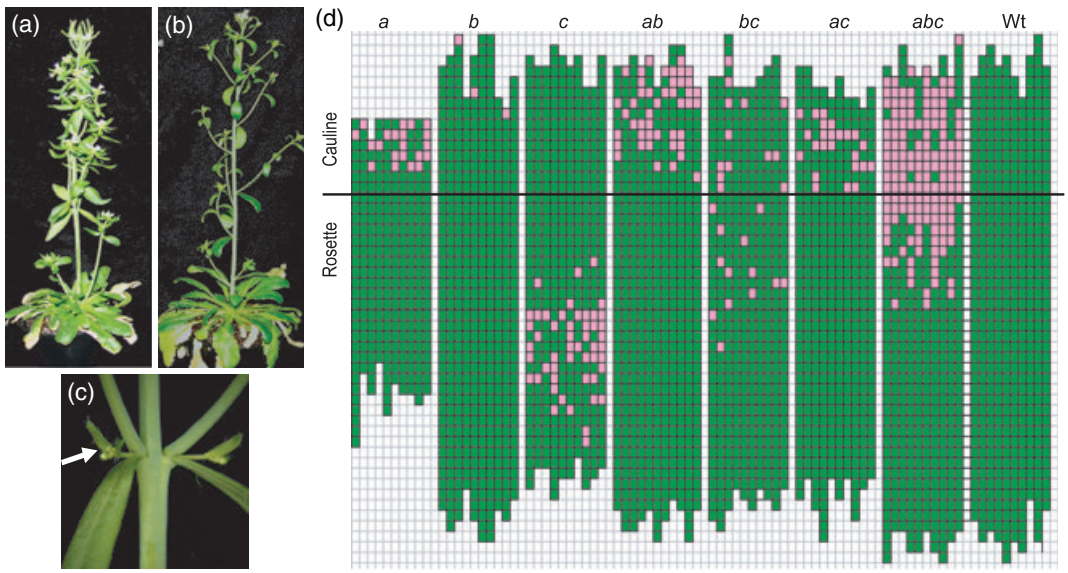

Figure 6. *mir164* mutants develop accessory buds.
(a, b) Habitus of a *mir164c* (a) and a *mir164b mir164c* plant (b) grown for 30 days in short photoperiods and subsequently in long days.
(c) Close up of cauline leaf axils of a *mir164abc* plant showing accessory bud formation (arrow) and distorted phyllotaxis.
(d) Schematic representation of accessory bud formation in leaf axils of *mir164a* (*a*), *mir164b* (*b*), *mir164c* (*c*), *mir164a mir164b* (*ab*), *mir164b mir164c* (*bc*), *mir164a mir164c* (*ac*) and *mir164a mir164b mir164c* (*abc*) mutants in comparison to wild-type plants. The pink colour denotes the presence of an accessory bud in a particular leaf axil, and green denotes the absence of an accessory bud. *mir164a* and *mir164b* are in the Columbia (Col) background, and *mir164c* in Landsberg *erecta* (L*er*). The wild type (Wt) depicted here is from the F_2 of a cross between Col and L*er*, and did not differ from Col or L*er* with respect to accessory bud formation. A representative population of 10 plants out of 18 plants of each genotype, grown for 30 days in short photoperiods and subsequently for 30 days under long-day conditions, is shown here.

Discussion

CUC *genes are redundantly required to initiate axillary meristem formation*

The *CUC* genes are expressed in a variety of boundary regions between organs as well as between the SAM and developing organs (Keller *et al.*, 2006; Takada *et al.*, 2001; Vroemen *et al.*, 2003). Because axillary meristems develop from the boundary region between a leaf primordium and the inner part of the shoot apex, we tested for a possible role of these genes in axillary meristem formation. Phenotypic analysis demonstrated that *cuc3-2* plants do not develop axillary buds in rosette leaf axils in the early and middle phase of vegetative development. Axillary bud formation was much less compromised towards the top of the rosette and in the reproductive phase. The SEM analysis demonstrated that empty leaf axils did not contain any morphological structures that could be traced back to the activity of an axillary meristem. The absence of axillary meristem initiation in *cuc3-2* was confirmed by the lack of a focused *STM* expression in the axils of older leaf primordia in these plants. Hibara *et al.* (2006) observed the lack of axillary shoots at a low frequency in *cuc3-105* mutants. Our detailed analysis further establishes and refines the role of *CUC3* as a key regulator of axillary meristem formation during the early and middle phases of vegetative development.

The block in axillary bud formation in *cuc3-2* was not absolute, because axillary buds developed during the late vegetative phase and the reproductive phase. On the other hand, *cuc1-1* and *cuc2-1* mutants as well as wild-type plants overexpressing miR164 did not show a defect in axillary bud formation. To test for a possible redundant involvement of *CUC1* and *CUC2* in the process of axillary meristem initiation, we studied the consequences of misexpression of *MIR164A* and *MIR164B* under the control of the *CaMV 35S* promoter in the *cuc3-2* background. Those plants that were characterized by a strong reduction in *CUC1* and/or *CUC2* activity, as indicated by the formation of cup-shaped cotyledons, showed an almost complete block in axillary bud formation in both rosette and cauline leaf axils. Taken together, these results suggest that, in addition to *CUC3*, *CUC1* and/or *CUC2* also play a role in the regulation of axillary meristem initiation.

The function of *CUC1* and *CUC2* in lateral meristem formation was further studied by analysing the shoot branching patterns of transgenic lines containing miR164-resistant variants of *CUC1* and *CUC2*, namely *5mCUC1* and *CUC2g-m4*. These plants developed accessory buds in rosette and cauline leaf axils, demonstrating that increased *CUC1* or *CUC2* activities lead to additional axillary

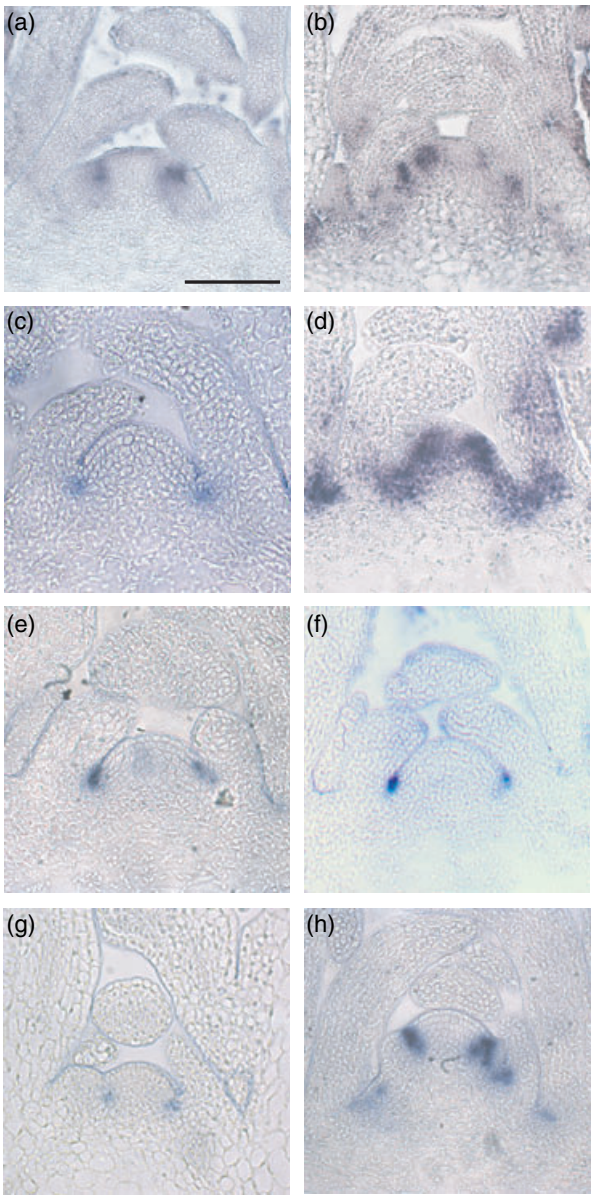

Figure 7. Interactions between miR164, the *CUC* genes and *LAS* in the control of axillary meristem formation.
Longitudinal sections through shoot apices of wild-type (a, c, e), *mir164abc* (b, d, h), *cuc3-2* (f) and *cuc3-2 2x35S::MIR164A* (g) plants grown for 30 days under short-day conditions, hybridized with *CUC1* (a, b), *CUC2* (c, d) and *LAS* (e–h) anti-sense probes. *mir164abc* and wild-type plants had a mixed Columbia (Col)l/Landsberg *erecta* (L*er*) background, whereas *cuc3-2* and *cuc3-2 2x35S::MIR164A* were in the Wassilewskija (Ws) background. *LAS* expression in Col/L*er* wild-type apices was comparable to that in Ws wild-type apices. Bar = 200 μm in (a) for (a) to (h).

meristems. However, the increase in accessory bud formation was considerably stronger in *CUC2g-m4* than in *5mCUC1*, indicating that *CUC2* has a more prominent role in axillary meristem initiation than *CUC1*. Altogether, these data suggest that *CUC* activity is not only required for axillary meristem formation but is also rate limiting during normal development and that additional axillary meristems form in response to an increase in *CUC1* or *CUC2* activity. Along the same lines, the phenotype of the *cupuliformis* mutant, which develops malformed leaf axils without lateral buds, put forward a role for the *Antirrhinum CUC1*/*CUC2* homologue in axillary meristem formation (Weir *et al.*, 2004).

miR164 controls the number of meristems per leaf axil

Schwab *et al.* (2005) have shown that six NAC-domain transcription factors contain binding sites for miR164, which is encoded by the genes *MIR164A*, *MIR164B* and *MIR164C*. From this subgroup of NAC-domain genes only *CUC1* and *CUC2* are expressed in the shoot apex (http://www.genevestigator.ethz.ch/). *CUC3*, the third redundant *CUC* gene that is expressed in the shoot tip, does not carry a recognition site for miR164. Phenotypic analysis revealed that *mir164a-4* and *mir164b-1* loss of function mutants developed accessory buds in the axils of cauline leaves, whereas in *mir164c-1* accessory buds were formed in the axils of rosette leaves. This phenotype was enhanced in *mir164a-4 mir164b-1* double mutants. On the other hand, accessory bud formation during the vegetative phase was reduced in *mir164b-1 mir164c-1* and absent in *mir164a-4 mir164c-1* mutants. This may be explained by the fact that the *mir164c-1* mutant has a different genetic background (L*er*) than the *mir164a-4* and *mir164b-1* mutants (Col) indicating the influence of accession-specific modifiers on this phenotype. However, the triple mutant developed accessory buds in most of its leaf axils. These results suggest that the three genes encoding miR164 differentially regulate *CUC1* and *CUC2* transcripts in overlapping zones along the shoot axis.

In situ hybridization analysis of miR164 accumulation in *Nicotiana benthamiana* revealed specific temporal and spatial expression patterns in developing flowers, ovules, ovaries, pollen sacs and anthers, and high levels of miR164 in meristems, procambial strands and vascular bundles (Valoczi *et al.*, 2006). In Arabidopsis, *mir164a-1* and *mir164b-4* were shown to enhance the floral defects of *mir164c-1*, and the three genes, *MIR164A*, *MIR164B* and *MIR164C*, were reported to have partially overlapping and distinct expression patterns in floral apices, indicating partially redundant yet specific roles for these genes in regulating floral development (Sieber *et al.*, 2007). Our results further support this concept of partial redundancy and specialization in the functions of the three *MIR164* genes. Through GUS reporter studies, we showed that *MIR164A* and *MIR164C* are active in domains overlapping with the boundary between the SAM and leaf primordia, whereas *MIR164B* is not transcribed in these axillary regions. These observations correlate well with the phenotypes of the *mir164* single mutants. Additionally, the *mir164abc* triple mutant displayed changes in *CUC1* and *CUC2* mRNA accumulation. Loss of miR164

activity leads to a considerably broader domain of *CUC2* mRNA accumulation and an upregulation of *CUC1* transcription, resulting in the formation of more than one axillary meristem. In addition to spatial regulation, miR164 may be needed to restrict the developmental time window of *CUC1* and *CUC2* mRNA accumulation.

Interplay between miR164, CUP SHAPED COTYLEDON *genes and* LATERAL SUPPRESSOR *in the control of axillary meristem initiation*

The *cuc3-2* branching pattern is similar to the patterns of shoot branching observed in the Arabidopsis *las-4* and *rax1-3* mutants. In *cuc3-2* and *rax1-3* mutants, axillary meristem formation is impaired in the early phase of vegetative development and restored when the plant matures (Mueller *et al.*, 2006). *las-4* mutants do not usually develop axillary buds in the vegetative phase, but depending on growth conditions axillary shoots are formed in the axils of the topmost rosette leaves at a low frequency (Greb *et al.*, 2003). Transcripts of the *CUC1*, *CUC2*, *CUC3*, *RAX1* and *LAS* genes accumulate in overlapping domains in the axils of developing leaf primordia (Greb *et al.*, 2003; Keller *et al.*, 2006; Mueller *et al.*, 2006; Vroemen *et al.*, 2003). Taken together, the similarities in the mutant phenotypes and in the patterns of transcript accumulation raise the possibility that *CUC1*, *CUC2*, *CUC3*, *RAX1* and *LAS* interact to establish and maintain the competence for axillary meristem formation during the phase of vegetative development. The RNA *in situ* hybridization analysis of *CUC2* transcript accumulation in *rax1-2* mutants suggested that *RAX1* regulation of axillary meristem initiation is mediated through *CUC2* (Keller *et al.*, 2006; Figure 8). Hibara *et al.* (2006) showed that *LAS* and *CUC3* have overlapping roles in various aspects of boundary formation, including the establishment of axillary meristems.

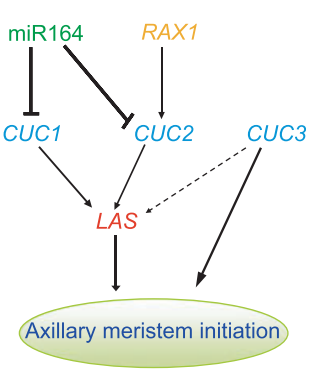

Figure 8. Regulation of axillary meristem initiation.
miR164 restricts expression of *CUC1* and *CUC2*, but not *CUC3*, in the axillary regions. *CUC1* and *CUC2* promote axillary meristem formation through the regulation of *LAS*. *CUC3* promotes the formation of axillary meristems, either through regulation of *LAS* or via an *LAS*-independent pathway. *RAX1* has been previously shown to regulate the development of axillary meristems through *CUC2* (Keller *et al.*, 2006).

We investigated a possible genetic interaction between *LAS* and *CUC3* in axillary meristem development by studying the accumulation of *LAS* mRNA in the *cuc3-2* shoot apex. Although failing to initiate axillary meristems, the *cuc3-2* mutant accumulates *LAS* mRNA in a wild-type-like pattern, suggesting that *CUC3* regulates axillary meristem development in a *LAS*-independent manner. However, because of the limitations of the RNA *in situ* hybridization technique, a slight downregulation in the level of *LAS* transcript would not have been detectable. Furthermore, *LAS* expression in *cuc1-1* and *cuc2-1* single mutants does not deviate from the wild-type pattern. On the other hand, overexpression of *MIR164* in a *cuc3-2* background results in a downregulation of *LAS* transcript accumulation. Along the same line, the *LAS* mRNA level is strongly upregulated in shoot apices of the *mir164* triple mutant. These two lines of evidence suggest that miR164 indirectly regulates *LAS* function, through *CUC1* and *CUC2* (Figure 8). *LAS* expression was also shown to be downregulated in embryos of the *cuc1 cuc2* double mutant (Hibara *et al.*, 2006), supporting the conclusion that *CUC1* and *CUC2* are regulators of *LAS* activity. Future experiments will have to show whether *CUC3* is redundantly involved in the same pathway or acts independently.

Experimental procedures

Plant material

Arabidopsis thaliana ecotypes Ws, Col and L*er* were used as wild types in this study. *cuc1-1*, *cuc2-1* and *cuc3-2* were obtained from the Nottingham Arabidopsis Stock Center (NASC). *mir164b-1* and *mir164a-4* were obtained from the Salk Institute Genomic Analysis Laboratory collection (SALK N636105) and the GABI insertion collection (GABI 867E03), respectively. *mir164c-1, mir164ac, mir164bc* and *mir164abc* were kindly provided by P. Sieber (University of Zurich, Switzerland). *2x35S::MIR164A* and *2x35S::MIR164B* constructs and the *5mCUC1* and *CUC2g-m4* transgenic line have been described elsewhere (Laufs *et al.*, 2004; Mallory *et al.*, 2004; Nikovics *et al.*, 2006). The *2x35S::MIR164A* and *2x35S::MIR164B* transgenic lines were crossed to *cuc3-2* homozygotes. Seventeen F_2 plants showing the sepal fusion phenotype of plants overexpressing miR164 (Laufs *et al.*, 2004) and additionally displaying fusions of rosette leaves and fusions between leaves and the inflorescence stem, were selected from these two crosses. The progeny of these F_2 plants segregated plants that were indistinguishable from the *cuc3-2* homozygous parent and plants with leaf fusions, indicating that the F_2 was homozygous for *cuc3-2* and hemizygous for the *2x35S::MIR164A* or *B* construct. Plants homozygous for both *cuc3-2* and the *2x35S::MIR164A* or *2x35S::MIR164B* construct could not be identified, suggesting that they may be embryo lethal or arrested very early in development. The *MIR164A::*GUS line has been described before ($Pro_{MIR164A2.1}$:GUS; Nikovics *et al.*, 2006).

Growth conditions

Plants were grown to maturity either in long days in greenhouses or in short days (8 h light 23°C and 16 h darkness 18°C) in a controlled environment. Wherever specifically mentioned in the text, SD plants

were induced to flower after 30–35 days by shifting to long photoperiods.

Analysis of side shoot formation

Axillary bud formation in the axils of rosette and cauline leaves was examined using a stereomicroscope. The analyses were done by sequentially checking the oldest to the youngest leaf axils for initiation of a bud. The older leaves were successively removed to make the younger leaf axils available for inspection. At least 18 plants of each genotype were analysed in every experiment. Each experiment was repeated at least once.

DNA isolation and PCR

Plant DNA preparation was carried out using Qiagen DNeasy®96 Plant Kit (http://www.qiagen.com/) as per the manufacturer's instructions. For the *MIR164B::*GUS reporter, 2043 bp of promoter sequence (from positions −2042 to −3 relative to the first nucleotide of mature miR164) were amplified from Col using miR164B-6 (TTG CTC ATC ACA CAC CTT CAT) and mir164B-18 attb1 (AAA AAA GCA GGC TTA ACT TGA CAT GAT ATA CAC CAC T) primers and used to drive the GUS reporter in the pBIB101.3 vector. A 2.3-kb promoter was shown by Guo *et al.* (2005) to complement the root phenotype of the *mir164b-1* mutant. For the *MIR164C::*GUS reporter, 804 bp of promoter sequence (from positions –808 to –5 relative to the first nucleotide of mature miR164) was amplified from Ws using mir164c-13-attb2 (AAG AAA GCT GGG TTC AAG TGT TAC TCA CCC ATT ACT) and mir164c-14-attb1 (AAA AAA GCA GGC TGG ACC CAA ACT CAT CAC CTA TCT) primers and used to drive the expression of a GUS reporter in the Gateway pBI101-R1R2-GUS plasmid (Divol *et al.*, 2007). The expression pattern driven by this promoter in the shoot apex is similar to that by a 1.8-kb promoter (A. Peaucelle and and P. Laufs, unpublished results) which contains a region sufficient for the function of *MIR164C* (Baker *et al.*, 2005). The PCR detection of *2x35S::MIR164* T-DNA in segregating *cuc3-2, 2x35S::MIR164/+* populations was performed using the primers MIR164A532F (TGG AGA AGC AGG GCA CGT GCA) and t35sR (CCT TAT CGG GAA ACT ACT CAC ACA T).

RNA in situ *hybridization*

Sample preparations and *in situ* hybridizations including probe hydrolysis were performed as described previously (Greb *et al.*, 2003). The *CUC1*, *CUC2* and *LAS* probes contained the nucleotide sequence 501–961, 496–1128 and 2–1348, respectively, relative to the ATG.

GUS assay

The GUS staining of apices harvested from plants grown for 30 days in short photoperiods was carried out as described in Sessions *et al.* (1999). Stained apices were embedded in Paraplast (Paraplast Plus, Kendall; http://www.kendallhq.com) and sectioned. Eight-micrometre sections were viewed by differential interference contrast microscopy after deparaffinization.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Accumulation of *LAS* mRNA in *cuc1* and *cuc2*.

This material is available as part of the online article from http://www.blackwell-synergy.com

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