Control of stem-cell niche establishment in Arabidopsis flowers by REVOLUTA and the LEAFY-RAX1 module

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Abstract

Plants retain the ability to produce organs throughout their life by maintaining active stem cell niches called meristems. The shoot apical meristem (SAM) is responsible for the growth of aerial plant structures. In *Arabidopsis thaliana*, the SAM initially produces leaves during the vegetative phase and later flowers during reproductive development. In the early stages of floral initiation, a group of cells first emerges from the SAM to form a stereotypically organized meristematic structure on its flank. However, the molecular mechanisms underlying the acquisition of this specific meristematic organization remain elusive. We show here that the transcription factors LEAFY (LFY) and REVOLUTA (REV) control two partially redundant pathways controlling meristematic organization in early flower primordia. We found that LFY acts through the transcription factor REGULATOR OF AXILLARY MERISTEM1 (RAX1) and we provide mechanistic insights in how RAX1 allows meristem identity establishment in young flowers. Our work provides a molecular link between the processes of meristem formation and floral identity acquisition in the nascent flower.
Introduction

Plants retain the capacity to initiate new organs throughout their life. To this end, they maintain self-sustaining pools of stem cells in organized niches called meristems. The SAM gives rise to most post-embryonic aerial organs thanks to stem cells present in its central zone (CZ). These cells are maintained in an undifferentiated state by a genetic network including the two transcription factors (TF) WUSCHEL (WUS) and SHOOTMERISTEMLESS (STM). WUS is expressed in the organizing centre (OC) of the SAM, located a below the CZ where it migrates to repress cell differentiation programs (Mayer et al., 1998; Yadav et al., 2011, 2013, 2010). STM is widely expressed in the SAM and acts at least in part via the regulation of the CK levels (Endrizzi et al., 1996; Jasinski et al., 2005; Lenhard et al., 2002; Yanai et al., 2005). Maintenance of the stem cell pool in the SAM involves a regulatory feedback loop between WUSCHEL (WUS) and the CLAVATA (CLV) signalling module (Schoof et al., 2000; Gaillochet et al., 2015; Brand et al., 2000). WUS induces the expression of the stem cell marker CLAVATA3 (CLV3) in the CZ (Brand et al., 2002). CLV3 encodes a signalling peptide that binds its CLV1 receptor in the cells surrounding the OC (Ogawa et al., 2008), leading to negative regulation of WUS (Brand et al., 2000). This regulatory loop controls stem cell homeostasis by regulating the size of the stem cell niche. CK is also important for meristem maintenance by regulating both WUS and CLV3 expression and promoting stemness (Zhao et al., 2010; Gordon et al., 2009). In the peripheral zone of the SAM, primordia develop as leaves during the vegetative phase and as flowers after floral transition at sites primarily determined by auxin maxima. Both leaf and flower primordia initiation require an initial downregulation of the KNOX genes STM and KNAT1. However, during flower development, expression of KNOX and of genes controlling stem cell fate such as WUS and CLV3 are regained at a later stage. Indeed, at stage 2 (referred to here as flower meristem, FM), the flower primordia become patterned with distinct meristematic domains: they acquire WUS expression domain, marking the establishment of the OC, closely followed by that of CLV3 marking the CZ, and of UNUSUAL FLORAL ORGANS (UFO), another gene expressed both in the SAM and in stage 2 FM (Yoshida et al., 2011; Samach et al., 1999; Wilkinson and Haughn, 1995).
In parallel to its development as a meristem, the flower primordium acquires its floral fate. Several flower meristem identity genes (LFY, APETALA1, CAULIFLOWER and other MADS-box TF) confer to the nascent FM its flower specific features: determinate growth, whorled phyllotaxis, whorled pattern of floral organ identity genes expression (Chandler and Werr, 2014; Kaufmann et al., 2010; Denay et al., 2017). Among those, LFY is expressed first and throughout floral development. It acts as a key floral regulator, building on the meristematic pre-patterning defined by WUS or UFO to locally induce floral organ identity genes (Laux et al., 1996; Lohmann et al., 2001; Parcy et al., 1998).

How the floral primordium grows and acquires its meristematic organization still remains elusive (Denay et al., 2017). The proposed mechanisms involve a combination of growth and hormone signalling (Gruel et al., 2016), but the executive transcriptional signals remain largely uncharacterized. An efficient way to coordinate the establishment of the meristematic organization with floral fate acquisition is probably to couple both processes. LFY was proposed to participate in both (Moyroud et al., 2010) and genetic evidence have indeed accumulated, documenting its contribution to meristem emergence (Yamaguchi et al., 2013; Wu et al., 2015; Sawa et al., 1999; Chahtane et al., 2013). This function is particularly obvious in rice where LFY participates in tiller growth and panicle meristem development, and in legumes where LFY triggers compound leaf development; all processes requiring the acquisition of meristematic features (Moyroud et al., 2010). In Arabidopsis, such function for LFY remains cryptic as lfy mutants develop lateral structures such as meristems and leaves, suggesting LFY might act redundantly with other pathways (Moyroud et al., 2010). However, constitutive expression of LFY triggers ectopic flower production in the axils of rosette leaves or cotyledons (Chahtane et al., 2018; Sayou et al., 2016). This effect on floral meristem production can even be uncoupled from floral identity by impairing LFY dimerization. Expression of a LFY variant triggers the development of precocious or ectopic inflorescence meristems instead of flowers in the axil of rosette leaves, through the induction of the R2R3 TF REGULATOR OF AXILLARY MERISTEMS1 (RAX1) (Chahtane et al., 2013). Thus, in Arabidopsis too, LFY appears to be able to trigger meristem formation. Whether the LFY-RAX1 module that acts at the axil of rosette leaves is also active in flowers is unknown: RAX1 is expressed in flower meristems but rax1 mutants do not exhibit any floral
phenotype (Keller et al., 2006; Müller et al., 2006). Just as for LFY, we surmised that a role of RAX1 on floral meristem might be masked by redundancy with other pathways. Such pathways might involve the HD-ZIPIII family of TFs that is linked to de novo meristem formation in aerial tissues. Triple mutants of revoluta (rev) phabulosa phavoluta fail to form an embryonic SAM (Prigge et al., 2005) and single rev mutants show pleiotropic defects (Otsuga et al., 2001; Talbert et al., 1995). These defects include failure to form axillary stems and occasionally flowers, resulting in the formation of filaments or of flowers similar to those of weak wus mutants (Otsuga et al., 2001; Laux et al., 1996). Additionally, REV was shown recently to be an essential component of axillary shoot meristem formation by stimulating the expression of STM in the leaf axils and determining adaxial fate in young developing organs (Shi et al., 2016; Caggiano et al., 2017; Zhang et al., 2018).

Because REV appears as a possible candidate to act in parallel with the LFY/RAX1 pathway in flowers, we combined mutations in both pathways to study their potential role in the acquisition of the meristematic structure of the flower primordium. We show that REV and the LFY-RAX1 module control the establishment of meristematic domains in flowers and that RAX1 may act in part by repressing CLV1 expression in the young flower bud, thereby enabling proper WUS expression and meristem patterning. This work reveals a molecular coupling between the establishment of the floral meristem structure and the acquisition of its floral identity through the action of LFY in both processes.

Results

LFY and REV act in parallel pathways during flower meristem development

To gain insight into the role of LFY in early floral meristem development, we analysed the effect of ify mutations in the rev mutant background. As LFY and REV are genetically linked, we used CRISPR/Cas9 to target the third and ninth exons of the REV gene (Supplemental Figure 1A) to simplify the isolation of ify rev double mutants. Several rev alleles were recovered in a pWUS:2xVENUS-NLS:tWUS reporter (pWUS:Venus; Supplemental Figure 2A), carrying insertions or deletions leading to premature stop codons in the third exon (Supplemental Figure 1A). These plants showed similar phenotypes to the previously described rev-6 mutant allele
(Otsuga et al., 2001). One representative line (thereafter named rev-c1) was further characterized: its leaves were slightly over-curved downward, the number of axillary stems was reduced, 20% of flowers lacked internal whors, and some rare flowers were replaced by filaments (Supplemental Figure 3 A,B).

We then crossed this line still containing the REV-targeting CRISPR construct into lfy-12/+ mutants. In the F2, we observed plants with typical lfy and rev mutant aspects as well as plants showing a dramatically enhanced phenotype with nearly all flowers replaced by small filamentous structures. These plants were clearly distinct from rev-like plants that bear distorted flowers (sometimes lacking the inner whorls) and from lfy mutants that lack flowers but display fully developed lateral structures (secondary shoots or shoot/flower intermediates) (Figure 1A-D). We selected one plant with a clear rev phenotype that was heterozygous for the lfy-12 allele, and which no longer carried the CRISPR construct. Sequencing of REV around the site of Cas9 nuclease activity in this plant revealed a homozygous one base deletion resulting in a premature stop codon in the third exon of REV. This mutation was called rev-c4 (Supplemental Figure 1A). Co-segregation analysis after one back-cross to wild-type showed that the newly observed filamentous phenotype is specific to lfy-12 rev-c4 double mutants (Supplemental Table 1).

The growth of short determinate filaments instead of flowers suggests that the lfy-12 rev-c4 mutant phenotype might arise from a failure to establish a functional floral meristem. To test this hypothesis, we analysed the activity of the pWUS:Venus reporter in inflorescences of the rev-c4 and lfy-12 rev-c4 mutants (Figure 1E-L). In wild-type plants, WUS expression is detectable from late stage 1 onwards, when the flower primordium forms a bulge; this expression is, however, very weak and restricted to only a few cells (Supplemental Figure 2D). At early stage 2, WUS expression is enhanced in the centre of the flower meristem (Supplemental Figure 2E) and absent from the peripheral zone and the L1 layer in almost all observed flowers (32/33). However, it is strongly expressed in the L2 of flower meristems (Supplemental Figure 2E) in contrast to the SAM, where WUS expression is restricted to the L3. These observations contrast with some previous reports of WUS promoter activity in the L2 of the SAM and the L1 of FMs (Yadav et al., 2011), but are in accordance with several independent data obtained by in situ hybridization (Yadav and Reddy, 2012; Mayer et al., 1998; Leibfried et al., 2005). Similar to wild-type
plants, the rev-c4 mutants showed WUS expression in flower primordia at stage 2. However, when combined with the Ify-12 mutation, the WUS-Venus signal was strongly altered in primordia (Figure 1E-N): more than 50% of the primordia at stage 2 or more advanced stages lacked detectable WUS expression (Supplemental Figure 4) and about 20% of the primordia showed WUS expression restricted to the axil of the developing filament (Figure 1I-L, Supplemental Figure 4C).

The presence of filaments that fail to establish a floral stem cell niche in the Ify rev double mutant suggests that REV and LFY act partially redundantly to build a functional floral meristem. Thus, the rev mutant represents a sensitized background suitable to investigate LFY’s molecular function in the formation of the floral stem cell niche.

*The LFY target RAX1 contributes to floral meristem development with REV*

Next we wondered whether the LFY-RAX1 module acting on axillary meristems (Chahtane et al., 2013) also participates in FM meristematic organisation. It was previously shown that RAX1 mRNA levels in inflorescences are not altered by mutations in Ify but that RAX1 expression is increased in plants expressing a constitutively active form of LFY, which is derived from a fusion between LFY and the VP16 trans-activation domain under the control of LFY promoter (LFY-VP16) (Chahtane et al., 2013; Parcy et al., 1998). To study the spatial effect of LFY-VP16, we compared RAX1 expression between wild-type plants and plants expressing LFY-VP16 using both *in situ* hybridisation and a RAX1:GUS transcriptional reporter (Supplemental Figure 5). We found that in the presence of LFY-VP16, RAX1 expression was stronger in early floral meristems and broader in inflorescences, confirming that LFY can promote RAX1 expression in these tissues.

Next, we probed the role of RAX1 during FM emergence. Since rax1 mutants have no floral defects (Keller et al., 2006; Müller et al., 2006), we introduced the rax1-3 mutation into the rev-6 background. In this case, the rev-6 Ler allele was backcrossed 3 times into Col-0. rev-6 [Col-0] displayed a higher proportion of flowers either lacking floral organs (63%) or replaced by filaments (27%) (Figure 2I) than reported for rev-6 in Ler (12% of flowers presenting defects) (Otsuga et al., 2001) or observed in the rev-c1 allele described above (20% of flowers lacking internal whorls, and 1% is replaced by filaments) (Supplemental Figure 3B). The presence of the
rax1-3 mutation considerably enhanced the rev-6 phenotypes (Figure 2). rev-6 rax1-3 plants produced only a few fertile flowers (1 to 2 per plant on average) and the proportion of filaments was much higher than in rev-6 (75% of flowers replaced by filaments and 22% lacking internal whorls). Also, the plants seldom formed axillary stems (Supplemental Figure 6A-D) and main axis growth was prolonged, resulting in the formation of an abnormally long main stem.

Analysis of rev-6 rax1-3 inflorescences by scanning electron microscopy revealed either filaments or ‘empty flowers’ only made of a whorl of sepals lacking a meristematic dome (Figure 2 E-H,J-M). Both phenotypes suggested a failure to establish a flower meristem of adequate size to allow development of all four whorls of organs. The strong genetic interaction between rax1 and rev mutations was further illustrated by the following observations: 1) the rax1-3 mutation behaved semi-dominant in the rev-6 background (Supplemental Figure 7): rev-6 rax1-3/RAX1 plants displayed for instance less axillary stems than rev-6 mutants. 2) In short-day conditions, where the LFY pathway is less active (Blázquez et al., 1997), the rev-6 phenotype was enhanced, with inflorescences essentially made of filaments subtended by bract primordia. In these growth conditions, the rax1-3 rev-6 phenotype was drastic: filaments were extremely reduced and stipule-like organs became visible on the flanks of rudimentary bracts (Supplemental Figure 8).

In conclusion, although the single rax1 mutation does not display any flower phenotype, our results using the rev-6 background show that RAX1 and REV both act to regulate development of early flower primordia.

RAX1 and REV are required for FM meristematic structure

The development of flowers lacking internal whorls or of filaments suggested that the flower meristematic structure is not properly established or maintained in rax1 rev mutants. To characterize possible meristematic defects of these double mutants, we monitored the expression of the CLV3 and WUS meristem patterning markers using in situ hybridization. Whereas the expression of CLV3 and WUS was not altered in the SAM, they were strongly reduced or even absent in some rax1-3 rev-6 “flower” primordia (Supplemental Figure 9). Other primordia displayed a detectable and normally localized WUS and CLV3 expression consistent with the fact that rax1 rev
mutants showed a mixture of severely affected structures (empty flowers or filaments) as well as some more normal flowers (Figure 2).

In order to more finely track the establishment of the floral OC over developmental time, we introduced CRISPR/Cas9 constructs targeting both RAX1 and REV in the pWUS:Venus reporter line. We validated the isolation of rax1 and rev single mutants and rax1 rev double mutants by phenotypic characterization and genotyping (Supplemental Figures 1 and 3). We recovered several types of mutations that all resulted in frameshifts at the same sequence site, which led to stop codons at different downstream positions (rev-c2, -c3 and rax1-c1, -c2 and -c3; Supplemental Figure 1). For the rax1 rev double mutant, we studied the progeny of a double heteroallelic plant (rax1-c2/3 rev-c2/3). In this rax1-c2/3 rev-c2/3 double mutant, the pWUS:Venus signal was weaker in the first few primordia than in either rax1-c1 or rev-c1 single mutants, but still detectable (Figure 3A-C). However, the main effect of this mutation combination was an expansion of the WUS expression domain in young flowers. Such ectopic WUS expression was observed in only 12% (N=40) of rax1-c1 flowers, while single rev-c1 mutant showed ectopic WUS expression in 75% (N=35) of observed flowers, either restricted to the apical domain of the L1 (44%) or throughout the L1 (31%). In contrast, double mutants exhibited ectopic WUS expression in almost all observed flowers (95%, N=23), in the apical domain of the L1 and throughout the L1 in 30% and 65% of the flowers, respectively (Figure 3J-L’, O). Additionally, the WUS expression domain appeared shifted apically in the rev-c1 mutant and to a higher extent in the double mutant where WUS expression was restricted to the topmost 3-5 cell layers, while it reached much deeper layers in rax1-c1 and WT (Figure 3D-L’). The SAM was also considerably enlarged in double mutants, however we could not detect any defect in WUS expression there (Figure 3A-C and Supplemental Figure 10).

Taken together, these data indicated that REV and RAX1 are important regulators controlling both the level and spatial expression of WUS in the centre of emerging flower primordia. While the contribution of RAX1 may be hidden due to redundancy in a single mutant situation, its role became clear when REV function was also compromised: double mutants showed a reduction in WUS expression levels combined with a dramatic expansion of the WUS expression domain in the L1 and the peripheral domains of floral primordia. Since the proportion of flowers showing
expression of WUS in the peripheral zone was strongly increased in double mutants, we hypothesised that RAX1 plays a more specific role in preventing OC expansion in the peripheral zone while REV acts mostly in positioning the OC below the L1. The defects in OC positioning, and thus FM organization, is likely the cause for the floral defects observed in these mutants.

RAX1 induces WUS expression

To understand how RAX1 regulates meristem formation, we generated plant lines expressing a mCherry-RAX1-GR (iRAX1) fusion protein under the control of the moderate constitutive UBQ10 promoter (Geldner et al., 2009). The hormone binding domain of the glucocorticoid receptor (GR) allows the retention of the fusion protein in the cytoplasm and its conditional translocation to the nucleus upon dexamethasone (DEX) treatment (Padidam, 2003). RAX1 protein expression was validated by western blot (Supplemental Figure 11I). When RAX1 activity was induced over a long period of time by periodic DEX treatments, plants appeared stunted, retarded in growth and produced very small siliques with only a few seeds. Mock treated iRAX1 plants were slightly smaller than a control transgenic line expressing mCherry-GR fusion proteins (iMock), indicating a residual activity of the construct in the absence of DEX (Supplemental Figure 11A-H).

In the iRAX1 line, we could observe ectopic WUS expression in the L1 of young flowers as early as 6h after DEX treatment in 7 out of 12 plants (Figure 4A), while control pWUS:Venus plants showed normal WUS expression up to 24h after DEX treatment (10 out of 11 plants) (Figure 4B). This indicated that ectopic activation of RAX1 transcriptional activity in young flowers could trigger ectopic WUS expression throughout the FM.

RAX1 acts on multiple signalling pathways

In order to gain insight into RAX1 TF molecular function, we examined gene expression in response to RAX1 post-transcriptional induction in the iRAX1 line in comparison to iMock. Paired-end RNA-sequencing analysis was performed on 14-day-old seedlings expressing the above-mentioned iRAX1 line or iMock mock line, 4h after DEX or mock treatment, with three biological replicates for each condition, yielding libraries of 26 to 37 million reads. Principal component analysis of the top 500 variable genes across all datasets showed a clear segregation of the DEX-
treated RAX1 inducible line along the first component axis (Figure 5A), indicating a specific transcriptome response in these samples. We identified 822 differentially expressed genes (DEG; FDR ≤ 0.01, 482 down- and 340 up-regulated) in response to both the treatment and the presence of RAX1 (Figure 5B, Supplementary Dataset 1). Gene ontology (GO) analysis showed an enrichment of genes involved in cell modifications and phenylpropanoid synthesis amongst the genes up-regulated. The down-regulated genes were enriched in genes involved in various immune and hormone responses (Figure 5C; Supplementary Datasets 2-3).

**In silico prediction of putative direct RAX1 targets**

We aimed at identifying putative direct targets of RAX1 amongst the differentially expressed genes. For this, we determined RAX1 DNA-binding properties using protein-binding micro-array (PBM) (Franco-Zorrilla et al., 2014). We used the RAX1 protein fused to Maltose Binding Protein (MBP) and a 6-Histidine tag (RAX1\textsubscript{full}-MBP-6H) produced recombinantly in *E. coli*. The best motif obtained by PBM matches well previously described Myb R2R3 motifs (Franco-Zorrilla et al., 2014) (Figure 5D). Using this DNA-binding model, we determined both the best RAX1 predicted binding sites (RAX1bs) and the probability of occupancy (pOcc) (Granek and Clarke, 2005) by RAX1 in the genomic region of each differentially expressed gene. The scores obtained for RAX1 DNA-binding model ranged from -47.87 (worst possible RAX1bs) to 14.37 (best RAX1bs). In order to focus on the genes most likely to be direct targets, we arbitrarily set a score threshold of 12 and a pOcc threshold of 0.2 corresponding to the top 51 and 33 % respectively. Based on these predictions, we identified 272 genes (out of 822) as best direct targets candidates of RAX1 (Figure 5E, Supplementary Dataset 4). These included a set of genes with experimental evidence for SAM expression (Yadav et al., 2014, 2009) (Supplemental Table 2).

Among the predicted RAX1 direct targets, we identified ABF2, a protein linked to abscisic acid signalling, confirming previous evidence of ABF2 regulation by RAX1 (Kim et al., 2004; Yu et al., 2016). We also identified the transcription factors ETHYLENE RESPONSE FACTOR (ERF) 1 and 2, involved in ethylene and jasmonate signalling, and in pathogen response (Cheng et al., 2013). Numerous cell-wall remodelling enzymes as well as the two pectin receptors WALL ASSOCIATED KINASE (WAK) 1 and 2 were also identified, all of which have roles in cell growth regulation (Goh et al., 2012; Hewezi et al., 2008; Liang et al., 2013; Wu et al., 2010;
Wagner and Kohorn, 2001; Kohorn et al., 2006). Finally, high-score RAX1 binding sites were detected in the UPBEAT1 (UPB1) and CLV1 genomic regions. UPB1 is involved in the control of ROS balance and was shown to control root and shoot stemness (Tsukagoshi et al., 2010; Zeng et al., 2017). The inhibition of UPB1 and CLV1 observed in response to RAX1 activity suggests that RAX1 may contribute in stem cell niche maintenance by repressing these genes (Supplemental Table 2).

RAX1 directly regulates CLV1 expression

CLV1, a known negative regulator of WUS, was identified as a putative direct RAX1 target and was repressed over two-fold in seedlings in response to RAX1 induction. CLV1 carries two high-score (> 12) RAX1bs in its promoter and coding sequence (Figure 6A). In order to determine if CLV1 is a genuine target of RAX1 in the shoot apex, we analysed CLV1 expression by qRT-PCR in mock- or DEX-treated iRAX1 inflorescences. CLV1 mRNA levels were mildly reduced in response to DEX treatment (Figure 6B) and this reduction was still observed in the presence of the protein synthesis inhibitor cycloheximide (CHX) indicating that this regulation is likely direct. Consistent with this result, we found that recombinantly produced RAX1 full-MBP-6H as well as a tagged truncated version of RAX1 carrying only the Myb domain (RAX1_myb-6H) were able to specifically bind oligonucleotides carrying either the best predicted RAX1bs or the one present in CLV1 cis-regulatory region (Figure 6C). Taken together, these results indicated that RAX1 most likely binds the CLV1 promoter via its Myb domain, and is able to reduce CLV1 expression.

Discussion

REV and the LFY-RAX1 module control flower meristem development

Since it was first proposed (Moyroud et al., 2010), several studies suggested that LFY could also be involved in the establishment of meristematic structures of FMs, in addition to its well-studied role in flower identity determination (Sawa et al., 1999; Yamaguchi et al., 2013; Wu et al., 2015; Chahtane et al., 2013; Moyroud et al., 2010). However, lfy mutants do initiate lateral structures on shoots, indicating that either LFY does not play any role in this process or that other pathways can compensate for the loss of LFY function. Because rev mutants are affected in flower initiation and meristem formation (Talbert et al., 1995; Otsuga et al., 2001; Prigge et al., 2005), we used rev as a sensitized background to study the role of LFY in FM
formation. We found that combinations of *lfy* and *rev* mutations almost completely abolished the formation of flowers, which were replaced by small filamentous organs. These structures often lacked proper *WUS* expression, a likely explanation for their failure to establish floral meristems. These results unambiguously showed that *LFY* acts in parallel with *REV* in the acquisition of meristematic features in FM.

It was proposed earlier that *LFY* ectopically induces meristem formation through the regulation of *RAX1* (Chahtane et al., 2013). Although *RAX1* is expressed in flower primordia, *rax1* single mutants do not display any floral phenotype (Keller et al., 2006; Müller et al., 2006) and it was unclear whether *RAX1* participates in floral meristem initiation as it does for axillary branches. We show here that *RAX1* is a likely target of *LFY* in early flowers. This regulation was not detected in *lfy* mutants (Chahtane et al., 2013) likely because in this background flowers are replaced by shoot/flower intermediates that express *RAX1* through independent mechanisms, probably similar to the situation in leaf axils (Guo et al., 2015). When combined with mutations in *rev*, the loss-of-*rax1* mutations drastically enhanced flower developmental defects and many flowers were either replaced by filamentous structures or lacked internal organ whorls. The defects observed in *rax1 rev* mutants were milder than that of *lfy rev* mutants either because the *rax1* alleles used here contain mutations in the C-terminal part of the proteins, which can retain some activity, or because *LFY* regulates additional genes involved in meristem formation such as A-type ARRs (Chahtane et al., 2013).

*Filaments likely result from failed flower meristem establishment*

Filaments replacing flowers in *lfy rev* or *rax1 rev* double mutants are structures that do not differentiate further and seem to have a determinate growth. To characterize those structures, we monitored the expression of *WUS* and *CLV3* as meristematic markers for the OC and stem cell zone, respectively. We found that some flowers of the *rax1-3 rev-6* mutant lacked the expression of both *WUS* and *CLV3* in the first flower development stages where they normally appear. In weaker mutant contexts (such as *rax1-c2/3 rev-c2/3*), we observed lower *WUS* expression but shifted upwards in the L1 and underlying layers, indicating that both *RAX1* and *REV* are involved in the regulation of *WUS* expression and its exclusion from the L1. However, *RAX1* specifically prevents *WUS* expression in the flower PZ, including in the L1. When *RAX1* activity was ectopically induced, *WUS* expression became present
throughout the flower primordia. Since REV is not expressed in the PZ of flower primordia (Otsuga et al., 2001), we conclude that RAX1 likely induces WUS expression independently of REV. RAX1 and REV are thus required for WUS activation and the proper definition of the FM domains, and filaments appeared to result either from loss of primordia meristematic organization leading to the development of a determinate structure, or from the consumption of the stem-cell pool, resulting in flowers lacking internal whorls.

Altogether these data indicate a role of RAX1 downstream of LFY in parallel to REV to regulate the acquisition or the maintenance of the flower meristem structure.

SAM size is increased when filaments replace flowers

In addition to their role in flower primordia, LFY/RAX1 and REV showed a synergistic effect on restricting the size of the OC and the SAM. The size of the OC in the double rax1 rev mutants remained proportional to the overall SAM size, in accordance to the existence of a scaling mechanism linking meristem shape and size to WUS expression (Gruel et al., 2016). A similar role in meristem size regulation was already described for FILAMENTOUS FLOWERS (FIL) and YABBY3 (YAB3) (Goldshmidt et al., 2008). It has been suggested that the enlarged SAM in the fil yab3 mutants results from decreased auxin synthesis in filamentous structures replacing flowers, which causes auxin depletion in the SAM and thereby increases meristem activity (Shi et al., 2018). The same mechanism could also explain the enlarged SAM observed here, that would be connected only indirectly to LFY/RAX1 and REV pathways.

RAX1 regulates a variety of pathways

To gain insights into the molecular mechanisms controlled by RAX1, we studied RAX1 regulated genes in seedlings, unravelling links between RAX1 and proteins involved in cell wall modifications, a step know to be important for FM emergence (Armezzani et al., 2018; Tucker et al., 2018). Amongst these, we predicted that the genes encoding the cell wall modifying enzymes FUCOSYL TRANSFERASE4 (FUT4), PECTIN METHYLESTERASE3 (PME3) and EXPANSIN10 (EXPA10) and the pectin receptors WAK 1 and 2 to be likely direct targets of RAX1. All were linked to cell growth and expansion (Liang et al., 2013; Wu et al., 2010; Hewezi et al., 2008; Goh et al., 2012; Kohorn et al., 2006; Wagner and Kohorn, 2001). Additionally,
members of the WAK family have been linked to the regulation of cell differentiation (Lally et al., 2001).

This analysis also revealed a role of RAX1 in inhibiting plant defence response. This seems to be a common feature of floral regulators as it is also observed for the genes LFY and ANT/AIL6 (Winter et al., 2011; Krizek et al., 2016). Consistently, we predicted the ethylene receptors ERF1 and 2 as being direct RAX1 targets. The ERFs can induce both abiotic and biotic defence pathways in response to a variety of stresses (Cheng et al., 2013). Interestingly, we also detected a role of RAX1 in regulating ABA and ROS responses which can also be linked to cell differentiation in the SAM (Wilson et al., 2016). In particular, the ABA response regulator ABF2 (previously proposed to be regulated by RAX1 (Yu et al., 2016)) and the ROS homeostasis regulator UPB1 are predicted direct RAX1 targets. UPB1 was shown to regulate the balance of cell proliferation and differentiation in the growing root via control of ROS homeostasis (Tsukagoshi et al., 2010). More recently, UPB1 and ROS levels were shown to regulate WUS expression in the SAM (Zeng et al., 2017).

Surprisingly, we did not detect any enrichment in CUC2 transcripts upon RAX1 induction. CUC2 was shown to be a direct target of RAX1 (Tian et al., 2014). However, CUC2 is a target for microRNA degradation and therefore its ectopic accumulation in the tissues used for this analysis may be prevented (Laufs et al., 2004).

The transcriptome analysis yielded a low overlap of targets between RAX1 and REV (Reinhart et al., 2013), with none clearly related to meristem homeostasis (Supplemental Table 3). Despite differences in the age and growth conditions of the samples between these two datasets, it suggests that RAX1 and REV act in different pathways that can compensate for each other.

**RAX1 regulates CLV1 expression**

Arguably one of the most interesting targets of RAX1 in the FM homeostasis context is the plasma membrane receptor CLV1, which acts as a negative regulator of WUS, restricting the size of the OC (Lenhard and Laux, 2003). We confirmed that RAX1 was able to bind *in vitro* to an element from CLV1 cis-regulatory region. Additionally, induction of RAX1 activity led to a decrease in CLV1 transcripts in inflorescences, even in the presence of the protein synthesis inhibitor cycloheximide. Altogether our
data indicate that RAX1 most likely directly regulates CLV1 in inflorescences, providing a direct mechanistic link between RAX1 and the regulation of meristem activity. It was shown that WUS-mediated repression of CLV1 fine-tunes its expression, promoting the adaptation of the CLV3/WUS equilibrium (Busch et al., 2010). A similar mechanism might be leveraged by RAX1, prior to the establishment of the meristem. Fine-tuning of CLV1 expression during FM emergence would participate to establish the new stem cell niche and the balance of CLV3 and WUS, and thus define the different meristematic domains. Failure to do so would not allow a self-maintaining meristem to emerge and lead to developmental arrest, as observed in the rax1 rev double mutants.

This work yielded two apparently contradictory observations: both weak rax1 rev mutants and RAX1 over-expressers are characterized by ectopic WUS expression in the L1. However, we think they can be reconciled by the fact that RAX1 represses CLV1 expression. When ectopically inducing RAX1 activity in pre-existing flower meristems, CLV1 inhibition allows the invasive expression of WUS throughout the flower. In the rax1 rev double mutants, lack of RAX1 at the very early stages of primordium formation (stage 1) would lead to a perturbation of the CLV pathway, a likely cause for a slightly decreased WUS expression, a shift upwards of the OC and an overall loss of meristem organization.

Recent publications showed that REV and other members of the HD-ZIPIII family directly regulate stem cell niche formation by (1) inducing STM expression, which was shown to potentiate stemness partly through activation of CK signalling (Zhang et al., 2018; Jasinski et al., 2005; Yanai et al., 2005), and (2) binding to the WUS promoter in a complex with B-type ARRs (Zhang et al., 2017a, 2017b). Therefore, we propose that REV and the LFY/RAX1 module control two synergistic pathways controlling the establishment of a self-sustaining floral stem-cell niche.

Our work shows that the LFY and REV pathways are essential to establish WUS and CLV3 expression in floral meristems. How these regulators can be integrated into the recently proposed model where the floral stem cell niche arise from L1 signals controlling WUS and CLV3 expression is not straightforward (Gruel et al., 2016). Since there is no evidence that LFY/RAX1 or REV act downstream of the L1 signals, we can imagine that they are required to regulate WUS level in parallel of the L1 signals. Still, the extension of WUS signal in L1 layer in some mutant combinations
we generated also suggests a more direct involvement in the action of L1 signal inhibiting WUS.

**Evolutionary perspective**

Whereas LFY was initially described for its role during flower development, it is becoming clearer that LFY ancestral role was to control cell division and apical growth (Moyroud et al., 2010; Tanahashi et al., 2005). This role is essential in the moss *Physcomitrella patens* sporophyte first divisions (Tanahashi et al., 2005) and the fern *Ceratopteris* gametophyte and sporophyte apical cells (Plackett et al., 2018). As evolution proceeded, LFY could have been co-opted as a flower regulator with the meristematic function becoming more redundant and cryptic in species such as Arabidopsis but still obvious in SAM (Zhao et al., 2018; Ahearn et al., 2001), leaves (Hofer et al., 1997; Wang et al., 2008) and axillary shoot (Rao et al., 2008) of some species. Because of its trajectory, it is likely that LEAFY has been interacting with meristematic regulator very early in evolution. Its double role is probably an efficient way to synchronize growth and identity of floral meristems. It will be interesting in the future to establish whether the LFY-RAX1 module at work in Arabidopsis flowers also plays a role in other angiosperms LEAFY related process and to determine the time of origin of the LFY-RAX1 module.

**Experimental Procedures**

**Plant material and treatments**

The *rax1-3, rev-6, and lfy-12* alleles have been previously described (Müller et al., 2006; Weigel et al., 1992; Otsuga et al., 2001). *rev-6* mutants (Ler) and *rax1-3* mutants (Col) were crossed and the double mutants were backcrossed 3 times to Col. Further work was performed on the progeny of the backcrossed plants. Plants were cultivated in long-day conditions (16h light) unless specified otherwise. The *pLFY:LFY-VP16* line was previously described (Parcy et al., 1998). For confocal and scanning electron microscopy, plants were grown in short-day conditions (8 h of light) for 6 weeks and transferred to long-day conditions for 2 weeks. Mutants phenotyping was performed three weeks after bolting. For DEX treatment the plants were sprayed
with either 10 µM DEX in 1/10 000 DMSO or ethanol or 1/10 000 DMSO or ethanol (mock) every other day from two weeks on.

**Reporter constructs**

The *pRAX1:GUS* construct contains 2.1 kb of *RAX1* promoter driving GUS expression. The *pWUS:Venus* construct was generated by combining pGGA003, pGGB002, 2xVenus, pGGD007, pGGE002 and pGGF003 in PGGZ001 in a single step GreenGate reaction (Lampropoulos et al., 2013). All constructs were transformed by the floral dip method (Logemann et al., 2006), several independent lines were analysed and a representative one was selected for further work.

**CRISPR constructs**

CRISPR spacers were designed using CHOPCHOP (Montague et al., 2014). Spacers with no predicted off-targets were selected (Supplemental Table 4). Spacers were cloned in pAtU6-26-v4, pAtU6-26:gRNA and pUBQ10:Cas9:tNOS expression cassettes were then combined in pCAMBIA1300 (Yan et al., 2016).

**RAX1 inducible constructs**

*RAX1* cDNA in pDONR221 (DQ446976) was acquired from ABRC. The internal *Bsa*I site was removed by mutagenesis using oGD122 and oGD123 (Supplemental Table 5). The sequence was subsequently amplified with oGD115 and oGD116, which added the compatible GreenGate overhangs and flanking *Bsa*I sites, and was cloned in pGGC000, producing pGD41. The GR coding sequence was amplified from plants carrying an APETALA1-GR construct (Wellmer et al., 2006) with oGD109 and oGD110 to be cloned in pGGC000 to produce pGD38. For the cloning of GR in pGGD000, a linker sequence was amplified from pGGD001 with oGD118 and oGD119 and the GR sequence was amplified with oGD110 and oGD111. Both fragments were cloned with compatible ends in pGGD000 in a single step ligation to produce pGD39. The Alligator selection cassette (*At2S3:GFP*) was amplified from pALLIGATOR1 (Bensmihen et al., 2004) with oGD120 and oGD121 and cloned in pGGF000 to produce pGD43. The construct *pUBQ10:mCHERRY-RAX1-GR:tUBQ10:Alligator* (iRAX1) was produced in a single step GreenGate reaction with the plasmids pGGZ001, pGGA006, pGGE009, pGD43, pGGB001, pGD41 and pGD39. The construct *pUBQ10:mCHERRY-GR-NLS:tUBQ10:Alligator* (iMock) was
produced with the plasmids pGGZ001, pGGA006, pGGE009, pGGB001, pGD38 and pGD002. Constructs were transformed in Arabidopsis by the floral dip method (Logemann et al., 2006) and T1 plants were selected based on seed fluorescence. Several independent lines were analysed in the T2 generation for mCherry translocation in the nuclei and phenotypical effects upon DEX treatment. A single line was selected for further analysis.

**RAX1 constructs for in vitro expression**

The internal Ncol site in RAX1 cDNA was removed by mutagenesis with oGD03 and oGD04. The resulting sequence was amplified with oGD01 and oGD02 for the cloning of the full-length sequence and oGD01 and oGD05 for the cloning of the Myb domain. The latter primers added Ncol and NotI restriction sites which were used to transfer the amplicon to the destination plasmid. The full length cDNA was transferred to pETM41 (Dümmler et al., 2005), which contains the sequence for an 6xHis tag and an MBP tag, producing pGD19. The sequence corresponding to the Myb domain was transferred to pETM11 (Dümmler et al., 2005), which contains the sequence for a 6xHis tag, producing pGD14.

**Identification and isolation of CRISPR mutants**

CRISPR constructs were transformed in pWUS:VENUS background by the floral dip method (Logemann et al., 2006). Several T1 plants were selected based on Hygromycin resistance. CRISPR-induced mutations were identified in the T2 using poly-acrylamide gel electrophoresis (PAGE) separation of DNA heteroduplexes (Zhu et al., 2014). The regions surrounding RAX1 spacers 1 and 2 were amplified with oGD124 and oGD126, and oGD125 and oGD134 respectively. The regions surrounding REV spacers 1 and 2 were amplified with oGD127 and oGD129, and oGD128 and oGD135 respectively. These regions were subcloned in pCR-Blunt (ThermoFisher) for sequencing. Selected lines for the single mutants in either RAX1 or REV carried a homozygous mutation (namely rax1-c1 and rev-c1), however the double mutant line carried heteroallelic mutations at each locus (rax1-c2/c3 rev-c2/c3; see Figure S1). Progeny of these plants was used for further characterization. CRISPR lines targeting REV were crossed to lfy-12 and T2 was screened for double mutant genotype. A line carrying homozygous mutation at the REV loci (rev-c4) and heterozygous lfy-12 mutation was selected.
**Western blotting**

mCHERRY expression was detected in seedlings whole extract using the [6G6] anti-RFP antibody (ChromoTek) at a dilution of 1/1000 and revealed with HRP-coupled anti-mouse antibody. Western blotting was performed as described previously (Sayou et al., 2016).

**In situ hybridization**

Plant samples were harvested shortly after bolting. Older flowers were swiftly removed, apices were collected in fixative, and *in situ* hybridization was performed as previously described (Carles et al., 2010). The *RAX1*, *WUS*, and *CLV3* probes were described in previous studies (Fletcher et al., 1999; Brand et al., 2000; Keller et al., 2006).

**Scanning electron microscopy**

Older flowers were removed from the inflorescences. Apices were collected and swiftly fixed on a stub by carbon tape. A drop of water was added at the base of the inflorescence and the samples were placed in the FEI Quanta 250 chamber. Imaging was performed in ESEM mode with a pressure between 700 and 550 Pa and a temperature of 1°C to 2°C with a tension of 14 kV.

**Confocal microscopy and image treatment**

Apices were dissected and placed on 2% agarose and cell walls were counterstained with FM4-64 or propidium iodide. Cell wall and VENUS signals were recorded in two separate channels. Imaging was performed on a Zeiss 780 (for the *lfy rev* mutants) or a Leica SP2 (for the *rax1 rev* mutants) with a 40X Water immersion long-distance objective. Image treatment was performed with FIJI (Schindelin et al., 2012). Minimal and maximal values were set to improve signal-to-noise ratio, and are indicated next to the images. For dexamethasone (DEX) induction, the apices were placed on apex culture medium (Hamant et al., 2014) containing 1/10 000 DMSO or ethanol with or without 10 µM DEX. Samples were imaged after 6-24 h of incubation.

**RNA sequencing and analysis**

14-day-old seedlings grown on MS plates were shortly immersed with a solution containing 0.03% L-77 Silwett and 1/10 000 DMSO (mock), or 10 µM DEX (DEX).
Whole seedlings were harvested 4 h after treatment and immediately flash-frozen in liquid nitrogen. RNA was extracted with the RNAeasy kit (Qiagen) and DNA was removed with the TURBO DNA-free kit (Ambion) according to manufacturer’s instructions. Libraries were synthesized with the TruSeq Stranded kit (Illumina) and paired-end sequencing was performed on an HiSeq2000 (Illumina) at the POPS platform (IPS2, Paris-Saclay). Adapter sequences were trimmed and duplicated and low-quality reads were discarded. Mapping was performed on TAIR10 assembly with HISAT2 (Kim et al., 2015) and mapped reads with a mapping quality score below 30 or mapped at several locations were discarded, resulting in an average of 97% of uniquely mapped read pairs. Reads mapped to exons or untranslated regions were counted with HTSeq (Anders et al., 2015). DEG discovery was performed with EdgeR (Robinson et al., 2010) using a multiparametric GLM model for the interaction genotype:treatment after TMM normalization (McCarthy et al., 2012; Zhou et al., 2014; Robinson and Smyth, 2007). Genes were considered differentially expressed if the likelihood-ratio test FDR was equal or below 0.01. GO-term enrichment analysis was performed in Araport (Krishnakumar et al., 2015).

**RAX1 binding-site prediction**

PBM was performed as previously described using an MBP tagged full-length RAX1 fusion protein (Franco-Zorrilla and Solano, 2014). Binding sites were predicted using the Biopython package for python 2.7 (Cock et al., 2009). pOcc was calculated as described for the GOMER program (Granek and Clarke, 2005). All analyses were performed on the extended genomic sequence spanning 3 kb upstream to 3 kb downstream of transcribed regions.

**qRT-PCR**

Shortly after bolting, inflorescences were treated with a drop of solution containing 0.03% L-77 Silwett, 1/10 000 DMSO, and 1/1 000 ethanol (mock), and alternatively 10 µM DEX (DEX), 50 µM cycloheximide (CHX) or both (DEX+CHX). Older flowers were dissected and 6 inflorescences per replicate were harvested. RNA was extracted with the RNAeasy kit (Qiagen). Gene expression was quantified using AT2G28390 and AT4G34270 as internal reference as they were shown to be stable across a wide range of conditions (Czechowski et al., 2005). Statistical analysis was
performed on the ΔCq values and fold-change was calculated solely for graphical representation purpose.

In vitro DNA-binding assay

Protein production was performed as previously described (Sayou et al., 2016). Proteins were purified on nickel-sepharose column in purification buffer (Tris-HCl 20 mM; dithiothreitol 1 mM; pH 7.5) and eluted with 150 mM imidazole before dialysis in purification buffer. Electrophoretic mobility-shift assay was performed as previously described (Sayou et al., 2016) in binding buffer (Tris-HCl 10 mM; NaCl 50 mM; MgCl₂ 1 mM; 1% glycerol; EDTA 0.5 mM; DTT 1 mM; pH 7.5). Probe sequences are indicated in Supplemental Table 6.

Accessions

RNA sequencing raw and processed files are available from ArrayExpress (E-MTAB-7050).

Supplementary Data

Supplemental Figure 1. CRISPR/Cas9-induced mutations in REV and RAX1.
Supplemental Figure 2. Characterization of the WUS transcriptional reporter expression in flower primordia.
Supplemental Figure 3. Phenotypic characterization of the rax1, rev and rax1 rev CRISPR lines.
Supplemental Figure 4. Expression of WUS in the rev-c4 and lfy-12 rev-c4 mutants.
Supplemental Figure 5. LFY induces RAX1 expression in inflorescences.
Supplemental Figure 6. Growth habit of rax1-3, rev-6 and double mutant in long-day inductive conditions.
Supplemental Figure 7. Axillary organ formation in rax1-3 rev-6 F2 population.
Supplemental Figure 8. rax1-3 and rev-6 mutant phenotype in non-inductive short-day conditions.
Supplemental Figure 9. Abnormal flower primordia in rax1 rev lack detectable WUS and CLV3 transcripts.
Supplemental Figure 10. Enlarged shoot apical meristems in the rax1 rev mutants.
Supplemental Figure 11. Effects of RAX1 activity induction on growth and development.
Supplemental Table 1. Segregation analysis of lfy-12 and rev-c4 mutations.
Supplemental Table 2. Non exhaustive list of predicted putative direct RAX1 targets.
Supplemental Table 3. Genes co-regulated by RAX1 and REV.
Supplemental Table 4. CRISPR spacers sequences.
Supplemental Table 5. Primers used in this study.
Supplemental Table 6. EMSA probes used in this study.
Supplementary Dataset 1: List of DEG in response to RAX1 induction
Supplementary Dataset 2: GO term enrichment in DEG
Supplementary Dataset 3: Gene-sorted GO term enrichment
Supplementary Dataset 4: RAX1 best binding score and predicted occupancy in DEG promoter regions.

Author Contributions

DG, TG and PF conceived and design the work. DG, TG, LMM, CH, HS, and LVI collected and analysed data. WC, FZJM, SR and LJ provided material and/or expertise essential for this work.

Acknowledgments

The authors wish to acknowledge the support of the Electron Microscopy facility of the ICMG Nanobio – Chemistry Platform and C. Lancelon-Pin in particular. The POPS transcriptomic platform and L. Soubignou-Taconnat. S. Figuet and D. Grunwald for shared facilities. K. Kaufmann and W. Yan for sharing plasmids ahead of publication, E. Delannoy, M-L. Martin-Magniette, P. Das, L. Comai and A. Larrieu for their inputs on the project. pRAX1:GUS seeds were a generous gift from P. Doerner. This work was supported by the French National Agency for Research programs Charmful (ANRBlanc–SVSE2– 2011) and Gral (ANR-10-LABX-49-01). The platform POPS benefits from the support of the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS).

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Figure 1. LFY and REV control floral meristem establishment.

(A-D) Inflorescences of WT (A; pWUS:Venus), Ify-12 (B), rev-c4 (C) and rev-c4 Ify-12 (D) mutants. Flowers of the double mutant are almost entirely replaced by filaments. Scale bar: 1 mm. (E-H) Maximum intensity projection (E, I) and orthogonal cross-sections (F-H, J-L) of confocal z-stacks of wild-type (E-H) and rev-c4 Ify-12 (I-L) inflorescence meristems expressing the pWUS:Venus reporter. The color frames around the cross-sections correspond to the position of the identically colored lines on the projected stacks. The stars indicate the position of the organ axil. Grey: cell-wall staining (propidium iodide), fire heatmap: Venus signal. Scale bars: 50 µm. (M) Intensity heatmap scale for (E,I). (N) Intensity heatmap scale for (F-H, J-L).
Figure 2. RAX1 and REV act together to control flower initiation.

(A-H) Inflorescences of WT (A,E), rax1-3 (B,F), rev-6 (C,G) and rax1-3 rev-6 (D,H) plants, observed by light (A-D; scale bar: 2 mm) or scanning electron microscopy (E-H; scale bar: 100 µm). (I) Plant architecture diagram of WT (Col-0), rax1-3 and rev-6 single and double mutants three weeks after bolting. Each column represents an individual plant and each square represents a single internode. Squares below the thick black line represent internodes on the rosette and squares above the thick black line, internodes on the main stem. Structures initiated are color-coded. Dark green: leaf and axillary stem; light green: leaf lacking the axillary stem; red: wild-type flower; blue: flower lacking one or more internal whorls; purple: filamentous structure. (J) Proportion of floral structures initiated in rev-6 and rax1-3 rev-6 mutants (N ≥ 9). Close up of the scored structures is shown on the right. Red: normal flower (K), blue: incomplete flower lacking a meristematic dome (star) between developing sepals (arrowheads) (L), violet: filament (M). Scale bar: 100 µm.
Figure 3. RAX1 and REV establish flower meristem organization.

(A-C) Maximum intensity projection of confocal z-stacks of rax1-c1 (A), rev-c1 (B) and rax1-c2/c3 rev-c2/c3 (C) inflorescences expressing the pWUS:Venus reporter. Grey: cell-wall staining (FM4-64), fire heatmap: Venus signal. Scale bars: 50 µm. Color arrowheads indicate the position of the primordia cross-sections (D-L’) with identically colored color-frames. (D-L’)

Orthogonal sections through flower primordia across the abaxial-adaxial (left) and lateral (right) axes of the rax1-c1 (D-F’), rev-c1 (G-I’) and rax1-c2/3 rev-c2/3 (J-L’) plants. White arrowheads mark the limits of WUS expression in the L1. Grey: cell-wall staining (FM4-64), fire heatmap: Venus signal. Scale bars: 20 µm. (M) Intensity heatmap scale for (A-C). (N) Intensity heatmap scale for (D-L’). (O) Frequency of flower primordia expressing WUS in the central domain only (white), the apical domain of the L1 (light grey), or throughout the L1 (dark grey). N ≥ 23.
Figure 4. RAX1 induction leads to activation of WUS expression.

Transverse sections through young flower primordia of iRAX1i (A) or wild-type (B) plants carrying the pWUS:Venus transgene after DEX treatment. Arrowhead indicates ectopic WUS expression in the L1. Stars indicate the position of the SAM. Grey: cell-wall staining (FM4-64), fire heatmap: Venus signal. Scale bars: 20 µm.
Figure 5. Identification of RAX1 target genes.

(A) Principal component analysis of the 500 differentially expressed genes with the highest variance across samples, separated along the two principal components (PC1 and PC2). The non-induced samples (Mock-treated \textit{iRAX1} (blue) and \textit{iMock} (Red) or DEX-treated \textit{iMock} (Green)) segregate together (blue cloud), while the induced RAX1 samples (DEX-treated \textit{iRAX1} (Violet)) are clearly separated. (B) Identification of differentially expressed genes by RNA-seq. The average Log of count per million read (logCPM) is plotted against the Log fold-change (logFC) value across samples for the interaction of genotype:treatment. Differentially expressed genes are indicated in red (FDR ≤ 0.01). (C) Enriched gene-ontology terms amongst up-regulated (blue) and down-regulated (red) genes. FDR ≤ 0.05. (D) DNA-binding model of RAX1 as determined by protein-binding microarray. Letter size indicates the information content at each position of the motif. (E) Identification of potential direct targets of RAX1. The probability of occupancy (pOcc) of RAX1 on each targets genomic sequence is plotted against the best binding score for RAX1 within this sequence. Distribution histograms of the pOcc (top) and best binding score (right) are represented. Targets were selected using a pOcc threshold of 0.2 and a binding score threshold of 12 (bold lines). The other sequences (greyed area) were not considered as potential direct targets.
Figure 6. CLV1 is a direct target of RAX1.

(A) Scheme of CLV1 genomic locus with the two best RAX1 predicted binding site (CLV1-bs1 and CLV1-bs2). Untranscribed regions and introns are indicated by a black line, exons and 5’ and 3’ UTR are indicated as dark and light orange boxes respectively. (B) Quantification of CLV1 transcripts in iRAX1 inflorescences treated with mock, dexamethasone (DEX), cycloheximide (CHX) and CHX + DEX. * p < 0.05, ** p < 0.01, determined by Student’s t-test (N = 4). Each point represents a single biological replicate. (C) In vitro binding assay of RAX1full and RAX1myb on sequences from CLV1 genomic region (CLV1-bs1), the best possible RAX1 binding site according to the DNA-binding model (RAX1bs) and a mutated version of this binding site (mRAX1bs).