The transcription factor BELLRINGER modulates phyllotaxis by regulating the expression of a pectin methylesterase in Arabidopsis

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SUMMARY
Plant leaves and flowers are positioned along the stem in a regular pattern. This pattern, which is referred to as phyllotaxis, is generated through the precise emergence of lateral organs and is controlled by gradients of the plant hormone auxin. This pattern is actively maintained during stem growth through controlled cell proliferation and elongation. The formation of new organs is known to depend on changes in cell wall chemistry, in particular the demethylesterification of homogalacturonans, one of the main pectic components. Here we report a dual function for the homeodomain transcription factor BELLRINGER (BLR) in the establishment and maintenance of the phyllotactic pattern in Arabidopsis. BLR is required for the establishment of normal phyllotaxis through the exclusion of pectin methylesterase PME5 expression from the meristem dome and for the maintenance of phyllotaxis through the activation of PME5 in the elongating stem. These results provide new insights into the role of pectin demethylsterification in organ initiation and cell elongation and identify an important component of the regulation mechanism involved.

KEY WORDS: Cell wall, Pectins, Pectin methylesterase (PME), Shoot apical meristem, Phyllotaxis, Arabidopsis

INTRODUCTION
The multitude of shapes adopted by multicellular organisms is controlled by complex networks of regulatory genes. How these regulatory networks are translated into the local changes in tissue growth that underlie morphogenesis remains a central question in developmental biology.

Plants are useful models with which to study organogenesis as new organs are formed throughout their lives, in contrast to animals in which organogenesis is restricted to embryogenesis. The shoot apical meristem (SAM), which gives rise to all aerial organs of the plant, has a dual function: maintaining a pool of undifferentiated cells, and initiating new lateral organs according to a specific temporal and spatial pattern, known as phyllotaxis (Carraro et al., 2006). Beyond the meristem, the phyllotactic pattern is actively maintained during stem growth. This process requires the restriction of the CUC2 transcription factor to the boundary domain, which involves miR164 (Peaucelle et al., 2007; Sieber et al., 2007).

The homeodomain transcription factors belonging to the KNOX and BEL groups play key roles in both meristem maintenance and organ patterning (Carraro et al., 2006; Smith et al., 2004). The KNOX and BEL transcription factors, which can form various heterodimers, regulate the expression of different sets of downstream effectors. BELLRINGER (BLR; also known as PENNYWISE, REPLUMLESS or VAAMANA) (Byrne et al., 2003; Kanrar et al., 2006; Roeder et al., 2003; Smith and Hake, 2003) is one of the members of the BEL group expressed in the meristem. The blr mutant was first described for its abnormal phyllotaxis, but also presents other developmental defects (Byrne et al., 2003; Kanrar et al., 2006; Kanrar et al., 2008; Roeder et al., 2003; Smith and Hake, 2003). BLR also shows complex genetic interactions with other BEL and KNOX genes (Smith and Hake, 2003; Smith et al., 2004; Ragni et al., 2008; Rutjens et al., 2009; Ung et al., 2011) and directly represses the flower organ identity gene AGAMOUS during flower development (Bao et al., 2004).

The initiation of new lateral organs depends on the local accumulation of the phytohormone auxin (Bayer et al., 2009; Reinhardt et al., 2000; Reinhardt et al., 2003). The auxin distribution within the meristem is highly dynamic and is the result of passive diffusion and active cell-to-cell transport involving the efflux carrier PIN1 and various influx carriers (Bainbridge et al., 2008). In several mathematical models, auxin patterning is autoregulated through feedback loops linking auxin flux or auxin accumulation (Jonsson et al., 2006; Smith et al., 2006; Stoma et al., 2008) to the polar intracellular localisation of PIN1. At the position of the auxin maximum a new organ primordium is initiated. The developing organ is thought to act as an auxin sink, which leads to the redistribution of the auxin and to the emergence of a new maximum at the position of the future primordium. In parallel to auxin patterning, the chemical modification of a cell wall component – the demethylsterification of the pectic polysaccharide homogalacturonan (HG) – also plays a key role in triggering primordia formation (Peaucelle et al., 2008).

Pectins, which represent ~35% of the dry weight in dicotyledonous species, are complex polysaccharides rich in galacturonic acid (Caffall and Mohnen, 2009; Mohnen, 2008). HG, one of the main pectic constituents, is a linear homopolymer of α-(1→4)-linked D-galacturonic acids, which can be methylesterified at

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the C-6 carboxyl residue. HG is synthesized from nucleotide sugars by a variety of glycosyl transferases (Lerouxel et al., 2006) in the Golgi apparatus and secreted in a highly methylesterified form into the cell wall (Sterling et al., 2001). Subsequently, its structure can be altered by the activity of cell wall-based enzymes. For example, the degree of methylesterification can be modified by pectin methylesterases (PMEs, EC 3.1.1.11), the activity of which is in turn regulated by proteaceous PME inhibitors (PMEIs) (Pelloux et al., 2007). Both PMEs and PMEIs are members of large gene families (66 and 69 members, respectively, in Arabidopsis).

The similarity of the defects in phyllotaxis observed in the Arabidopsis blr mutant and following ectopic pectin demethylesterification prompted us to investigate the link between these two factors. Here, we report that ectopic primordia formation in the floral meristem of blr is the result of meristem-specific changes in the PME-mediated methylesterification status of HG as a result of the ectopic expression of one member of the PME family, PME5. The ectopic primordia formation was reversed in the blr/pme5 double mutant, confirming unequivocally the role of PME5 in this process. Furthermore, we show that, in the blr mutant, the downregulation of PME5 expression in the internode leads to a defect in internode elongation that is associated with reduced cell expansion. In addition to identifying part of the regulatory network that controls the methylesterification status of HG in the stem, our results further confirm the crucial role of the demethylesterification of HG in the regulation of cell elongation.

MATERIALS AND METHODS
Plant material and growth conditions
The blr-6 mutant was identified, based on its phenotype, in the Versailles T-DNA insertion collection (WS ecotype). Tests for allelism were carried out by crossing plants homozygous for pny-42016 (Smith and Hake, 2003) with plants homozygous for blr-6. F1 plants displayed the blr mutant phenotype, showing that blr-6 and pny-42016 are allelic. The right and left flanking sequences of the T-DNA insertion site were amplified by PCR in the blr-pme5 mutant using the T-DNA-specific primer Tag5 (5'-CTACAAATGCCCCCTTTCTATGCAC-3') and the gene-specific primers pny-04 (Smith and Hake, 2003) and Tag3 (5'-CTGATACCAGACGTTGCCCGCATAA-3') and pny-3, respectively (Smith and Hake, 2003). Sequencing of the PCR products showed that the T-DNA was inserted in the first intron, at position 1262 to 1271 relative to the translation initiation site as described (Mouille et al., 2003). Statistical analyses were performed as described (Mouille et al., 2003).

PME activity measurements
PME activity was assayed using the alcohol oxidase-coupled assay on cell wall-enriched total protein extracts as described (Klavons and Bennet, 1986). Protein concentration in extracts was measured according to Bradford (Bradford, 1976) using a protein assay kit (Bio-Rad, Marne-la-Coquette, France). Data are the mean of four to six independent replicates. Data were statistically analysed by the Mann-Whitney test (Statisca, Softmas, Maisons-Alfort, France).

Real-time quantitative PCR (RT-qPCR)
Following RNA extraction from floral buds and cDNA synthesis, A15g09760, A13g19730, A15g47500, A14g33220 and A13g49220 transcripts were quantified by RT-qPCR using the following specific primers (5' to 3'): A15g09760, GGAGGGCCATGGAAGAGATTA and AGCACAGACACAGCCACCTC; A13g19730, AAAGCTAGGAGGAGCTGGA and ATTCAGAGCCGCTCGATAGAG; A15g47500, ATGGCCGCCCTCGATGTAAGAG; A13g49220, CATGGCTGTTGGTCTTCG and AAATCGGCCAAAGACAG; A13g49220, CCGGAAGAGAATGTACACCAAG and CACAGGGTCTGTCATG. Reactions were performed in a Roche LightCycler using the FastStart DNA MasterPLUS SYBR Green I Kit (Roche). Data are the mean of four to six replicates. These data were exported into ReQuant (Roche), which provides efficiency-corrected normalised quantification results. For each candidate gene, the expression in blr-6 is given relative to that in the wild type, which was set at 1.

β-glucuronidase (GUS) staining and imaging
A 1 kb region of the A15g47500 promoter was amplified using Phusion Hot Start F-Taq Polymerase (Finnzyme, Saint Quentin en Yvelines, France). The PCR product was cloned into the pGEM-T Easy vector (Promega, Charbonnières-les-Bains, France), sequenced and subcloned into the binary vector pBI1103.1 upstream of the GUS coding sequence (Clontech, Saint-Germain-en-Laye, France). Plant transformation, using Agrobacterium tumefaciens strain LBA4404, was performed by the floral dip method (Clough and Bent, 1998). Transfomers were selected on 80 μg/ml kanamycin. GUS staining was carried out as described (Sessions et al., 1999), with 10 mM K3Fe(CN)6 and 10 mM K4Fe(CN)6, to limit stain diffusion. Plant samples were destained in 75% ethanol and digital images were taken with a Coolpix 995 camera (Nikon, Champigny sur Marne, France).

A 4.9 kb stretch of KNAT1 (BP) promoter sequence upstream of the translation initiation site was amplified from wild-type WS Arabidopsis using primers 5'-CGGCGCGGCTGCGGATAGTTAGTATG-3' and 5'-ACTGATACCCAGATGAGTAAAGG. Reactions were performed in a Roche LightCycler using the FastStart DNA MasterPLUS SYBR Green I Kit (Roche). Data are the mean of four to six replicates. Data were statistically analysed by the Mann-Whitney test (Statisca, Softmas, Maisons-Alfort, France).

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Electron microscopy and phyllotactic pattern measurement
Scanning electron microscopy, confocal microscopy, epidermal cell length measurement and phyllotactic pattern measurement have been described previously (Deveau et al., 2003). Plant growth in controlled chambers under short-day or long-day conditions was as described previously (Peaucelle et al., 2008). For each experiment, phyllotaxis measurements were performed on a minimum of five plants and meristic phyllotaxis measurements on a minimum of ten plants. Cell measurements were performed on a minimum of five plants and phyllotactic pattern measurement have been described (Peaucelle et al., 2008).

Fourier transform infrared (FTIR) microscopy
Ten 12 μm slices from ten meristems of ten different wild-type and blr-6 plants were obtained by Vibratome sectioning of dissected dried inflorescences embedded in 5% low-melting-point agarose. For each meristem slice, ten FTIR spectra were collected from the central zone of the meristem. Spectra were baseline and area-normalised as described (Mouille et al., 2003). Statistical analyses were performed as described (Mouille et al., 2003).
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ARABIDOPSIS blr-6

RESULTS

Altered phyllotaxis in the Arabidopsis blr-6 mutant

We identified blr-6, a new allele of the BLR gene in the wild-type

WS background (see Materials and methods). The blr-6 mutant is

characterised by the production of ectopic flowers at the shoot apex

and by a high proportion of short internodes (Fig. 1A,B). To

characterise the phyllotactic defect, we studied the pattern of organ

initiation in wild-type and blr-6 SAMs by measuring the angles

between successive primordia (Fig. 1B). As shown in Fig. 1C,D,

the divergence angle showed a bimodal distribution with peaks at

120-149° and 240-279°. The distribution of the angles around 137°

was similar to that of the wild type but with a higher variability

(s.d.=46 versus s.d.=16 in wild type). The primordia with angles of

240-279° can be considered ectopic as they do not follow the normal phyllotactic pattern. In conclusion, the pattern of organ

initiation in the blr-6 meristem presents two defects: higher variability in the positioning of successive primordia within a normal phyllotactic pattern and ectopic primordia formation.

Increased pectin demethylesterification in the SAM of blr-6

To investigate whether the ectopic primordia of the blr-6 mutant could be related to changes in cell wall composition within the SAM, Fourier transform infrared (FTIR) microspectroscopy was carried out on a 50 \( \mu \text{m} \times 50 \mu \text{m} \) region delimiting the central meristem dome on a 12 \( \mu \text{m} \) transverse section through the shoot apex (Fig. 2A,B). We compared wild-type and blr-6 FTIR spectra using a \( t \)-test for each wave number. Only two wave numbers (1720 cm\(^{-1}\) and 1780 cm\(^{-1}\)), which correspond to ester bonds, showed significant differences in absorbance (higher in wild type than in blr-6). Esters are mainly found in cell wall pectin. These results suggest a lower degree of HG esterification in the blr-6 meristem in the absence of other changes in cell wall composition detectable with this sensitive technique.

To confirm the changes in pectin structure in the SAM of the blr-6 mutant, pectic epitopes were immunolocalised on successive transverse sections of the meristem with monoclonal antibody 2F4, which specifically labels demethylesterified HG (Liners et al.,

Fig. 1. Altered phyllotaxis in the meristem of the Arabidopsis blr-6 mutant. (A) Overall architecture of wild type (ecotype WS) (left) and blr-6 mutant (right) presenting abnormal phyllotaxis. (B) Ectopic primordia formation in blr-6 compared with wild type as revealed by scanning electron microscopy. Meristem (m), incipient primordia (i) and growing primordia (p1-10) are indicated. Note the ectopic primordium p1’ in the blr-6 mutant. Scale bars: 200 \( \mu \text{m} \). (C,D) Merismatic phyllotaxis in wild type [total number of measurements (n)=298, average (av)=137.5, s.d.=16.1] and blr-6 (n=333, av=142.0, s.d.=45.9). Divergence angles between two successive flowers in the meristem were allocated into twelve 30° classes and the percentage of total measurements (n) falling into each class is displayed.

Fig. 2. Reduced pectin methylesterification in the meristem in blr-6. (A) A 12 \( \mu \text{m} \) meristem slice, indicating the area (square) in which the FTIR spectrum in B was acquired. (B) Student’s \( t \)-test on the comparison between FTIR spectra collected from wild-type and blr-6 meristems. Arrows indicate wave numbers for which differences are significant (\( P<0.01 \)). (C,D) Immunolabelling of demethylesterified pectins using 2F4 antibody on four successive 6 \( \mu \text{m} \) transverse sections (starting from the meristem surface, shown on the right) of WS and blr-6 meristems. Arrows indicate the central zone of the meristem. Scale bars: 100 \( \mu \text{m} \).
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Fig. 3. PME5 transcript levels are upregulated in blr-6. (A) Transcript levels of five putative meristem-expressed pectin methylesterase genes in floral buds of blr-6. Levels in wild type are set at 1. Error bars indicate s.d. (n=4-6). (B,C) In situ hybridisation of PME5 transcripts on transverse sections of WS and blr-6 meristem. (D,E) Binocular observations of the meristem of WS and blr-6 Arabidopsis plants containing a pPME5::GUS construct, following GUS staining and tissue-clearing in ethanol. Arrows indicate the central zone of the meristem. Scale bars: 100 μm.

1989). As previously described, in the wild type the centre of the meristem dome remained unlabelled, while 2F4 labelling was observed in the walls of young primordia (Fig. 2C) (Peaucelle et al., 2008). By contrast, in the blr-6 mutant the 2F4 signal was more widespread and stronger throughout the entire section (Fig. 2D). These results, together with the FTIR data, show that the degree of methylesterification of HG was greatly reduced in the mutant meristem. Such a decrease in the degree of methylesterification of blr-6 most likely reflects higher activity of the pectin-modifying enzyme pectin methylesterase (PME).

Ectopic expression of PME5 in blr-6
To investigate whether higher pectin demethylesterification in the floral buds of the blr-6 mutant was the result of increased expression of PME genes, we first exploited microarray databases (http://bar.utoronto.ca/epf/cgi-bin/epfWeb.cgi) to identify PMEs expressed in the SAM. The expression of five meristem-expressed PMEs was further analysed by RT-qPCR (Fig. 3A). PMEs At15g47500 and At4g33220 were upregulated (1.6- and 1.2-fold, respectively) in the blr-6 mutant compared with wild type. At15g47500 (referred to as PME5) is the most highly expressed PME family member in wild-type inflorescence meristems (data not shown) and its overexpression in the SAM induces ectopic flower primordia formation (Peaucelle et al., 2008). PME5 was therefore chosen for more detailed analysis.

In situ hybridisation on wild-type floral buds revealed the presence of PME5 transcripts exclusively in young primordia in a salt and pepper pattern typical for cell cycle-regulated transcripts (Fig. 3B). In the blr-6 mutant, however, PME5 transcripts were observed at ectopic positions in the meristem dome in addition to the young primordia (Fig. 3C).

To investigate whether the higher PME5 mRNA levels could be related to modified transcriptional regulation, we analysed PME5 promoter activity in wild-type and blr-6 mutant backgrounds using a PME5 promoter::GUS reporter. Increased GUS staining was observed in the blr-6 mutant, in particular in the meristem dome, compared with the wild type (Fig. 3D,E). However, in contrast to the in situ hybridisation results, the GUS staining was homogenous, suggesting that either the promoter fragment was lacking the information needed for cell cycle regulation, or that this regulation occurred at a post-transcriptional level. Nevertheless, the higher promoter activity of PME5 in the mutant background suggests that the BLR protein negatively regulates PME5 transcription in the wild-type meristem dome.

Reduced PME activity in floral buds and reduced variability in primordia position in pme5 mutants
We previously showed that ectopic PME activity leads to ectopic primordia formation, whereas inhibition of PME activity blocks primordia outgrowth (Peaucelle et al., 2008). Here, we investigated whether a reduction in PME5 expression also could affect primordia positioning. We first isolated two mutants, pme5-1 (175B12) and pme5-2 (232E12), which carry T-DNA insertions in the first and third exon, respectively (see Fig. S1A in the supplementary material). Both mutants can be considered null as no PME5 transcripts are detected in floral buds by RT-qPCR (see Fig. S1B in the supplementary material). The absence of PME5 transcripts in the pme5-1 meristem was further confirmed by in situ hybridisation (see Fig. S1C in the supplementary material). PME5 assays (using standard conditions, see Materials and methods) on cell wall-enriched protein extracts from flower buds showed that total PME activity was ~60% of that of the control in both pme5-1 and pme5-2 lines (Fig. 4A and see Fig. S1D in the supplementary material).

We next compared the phyllotaxis of wild-type and pme5-1 meristems. Interestingly, pme5-1 differed significantly from the wild type in the divergence angle distribution in the meristem (P=0.011, P=0.009 and P=0.0089 by K-S test in three independent experiments) and with a smaller standard deviation (pme5, s.d.=10.1, 7.4, 9.0; wild type, s.d.=16, 15, 19).

As a control, the statistical test failed to distinguish between the distributions of the three biological repeats for the wild-type samples (P=0.70, P=0.70 and P=0.71 for experiment 1 versus 2, 1 versus 3, and 2 versus 3, respectively) (Fig. 4B,C). The reduced PME activity in pme5-1 therefore correlates with a reduced variability in primordia positioning in the meristem.

pme5-1 complements the ectopic primordia phenotype of blr-6
We next investigated the relationship between the altered phyllotaxis, the increased pectin demethylesterification and the ectopic expression of PME5 in the blr-6 meristem. We first assayed total PME activity in floral buds. The activity was 3-fold higher in the blr-6 mutant than in the wild type (Fig. 4A), which could suggest post-transcriptional or post-translational control of PME activity.

Next, we generated and analysed a blr-6/pme5-1 double mutant. In the double mutant, PME activity was greatly reduced compared with blr-6, indicating that the ectopic expression of PME5 explains most of the increased PME activity in blr-6 (Fig. 4A). Interestingly, the divergence angle distribution in the meristems of the double mutant was indistinguishable from that of pme5-1 (P=0.3, P=0.78, P=0.45 by K-S test in three independent experiments) and significantly different from that of blr-6 (P<1×10^{-4}, P<1×10^{-4}, P=0.001 by K-S test in three independent experiments) (Fig. 4B-C-E).

To confirm that the phyllotactic pattern observed in blr-6/pme5-1 is due to the absence of PME5 activity, we ectopically expressed PME5 under the control of the constitutive 35S promoter in the double mutant. Double mutants expressing 35S::PME5 indeed
showed a higher PME activity than that of blr-6/pme5-1 (Fig. 4A) and a phyllotaxis in the meristem that was indistinguishable from that of the blr-6 mutant (Fig. 4D,F).

Together, these results indicate that the increased PME activity and the abnormal phyllotactic pattern in the meristem in blr-6 are solely the result of the ectopic expression of PME5. Strikingly, as discussed below, the disordered post-meristematic phyllotaxis phenotype observed in blr-6 was not rescued in blr-6/pme5-1 (see Fig. S2 in the supplementary material). We show that this can be explained by an independent role for PME5 in the regulation of internode length.

Expression of PME5 is downregulated in the internodes of blr-6
To investigate the potential role of PME5 in regulating internode length and the post-meristematic maintenance of phyllotaxis, we first analysed the activity of the PME5 promoter in wild-type and blr-6 mutant backgrounds using PME5 promoter::GUS transformants. The PME5 promoter was active in wild-type but not in blr-6 internodes (Fig. 5). This suggests that, in the internode, BLR acts as an activator of PME5 transcription, in contrast to the meristem, where BLR appears to act as a repressor. In the context of the blr-6 mutant, the post-meristematic phyllotaxis phenotype (i.e. short internodes) might thus be related to the lack of PME5 expression in the internode.

pme5-1 does not complement the post-meristematic phyllotaxis phenotype of blr-6
To analyse the post-meristematic modifications of phyllotaxis, we first compared the internode length of blr-6, pme5-1 and blr-6/pme5-1 mutants with that of the wild type. No significant differences were detected between wild-type and pme5-1 plants for this parameter (Fig. 6A; \(P=0.139, P=0.131, P=0.611\) by K-S test in three independent experiments). By contrast, blr-6 and blr-6/pme5-1 plants presented a phenotype of large variability in internode length and, notably, a high frequency of shorter internodes (Fig. 6B,C). Differences were detected between wild-type and pme5-1 mutants (\(n=298, av=137.5, s.d.=16.1\), pme5-1 (\(n=227, av=137.8, s.d.=10.1\)), blr-6 (\(n=333, av=142.0, s.d.=45.9\)), blr-6/pme5-1 (\(n=215, av=136.4, s.d.=8.9\)) and p35S::PME5 (WS background, \(n=107, av=12.8, s.d.=32.8\)).

Divergence angles between two successive flowers in the meristem were allocated into twelve 30° classes and the percentage of total measurements \((n)\) falling into each is displayed. Reduction in PME activity in the pme5-1 mutant leads to reduced variability in the phyllotactic pattern observed in the meristem compared with the wild type. In the blr-6/pme5-1 double mutant, the variability in phyllotaxis observed in blr-6 is rescued. Ectopic expression of PME5 using a p35S::PME5 construct induced increased variability in the blr-6/pme5-1 double mutant.
This confirms that, in \textit{blr-6}, the post-meristematic defect in phyllotaxis is due to reduced \textit{PME5} expression in the internodes.

To investigate whether the ectopic PME activity in the meristem could be indirectly responsible for the reduced internode elongation and altered post-meristematic phyllotaxis, we expressed \textit{PME5} under the control of the meristem-specific \textit{UFO}-derived promoter. Similar internode and cell lengths as well as divergence angles at the post-meristematic levels were observed in \textit{UFO::PME5} lines and wild-type plants (see Fig. S4 in the supplementary material), confirming that internode elongation is not affected by ectopic \textit{PME5} expression in the meristem. These results show that the altered post-meristematic phyllotaxis in \textit{blr-6} is a result of the downregulation of \textit{PME5} in the internode rather than of ectopic \textit{PME5} expression in the meristem.

We conclude that, in wild-type plants, \textit{PME5} plays a dual role in controlling phyllotaxis at the meristematic and post-meristematic level. This involves organ-specific regulation of the expression of \textit{PME5} by BLR.

**DISCUSSION**

BLR was previously shown to be required for the maintenance of meristem identity in the SAM. BLR transcripts are present in the meristem and are downregulated in incipient primordia (Byrne et al., 2003). In loss-of-function \textit{blr} mutants, such as \textit{blr-6} used in this study, phyllotaxis is perturbed, with an increased variability in the divergence angles and ectopic primordia (Byrne et al., 2003; Smith et al., 2003). BLR forms heterodimers with the KNOX TALE homeodomain protein STM and acts in combination with two other BEL1-like homeodomain proteins, PNF and ATH1 (Belles-Boix et al., 2003).
al., 2006; Mele et al., 2003; Rutjens et al., 2009). blr/pnf/ath1 triple mutants phenocopy the extreme stm-1 phenotype, in which all cells in the meristem are recruited for the formation of a single primordium. By comparison, blr-6 represents a mild phenotype, in which the ability to maintain meristem identity is only partially affected.

Here, we provided insight into the mechanism underlying the allocation of meristem cells to the developing primordium. We provide evidence that BLR represses, directly or indirectly, the expression of PME5 in the meristem. In the blr-6 mutant, the altered phyllotaxis in the meristem can be explained entirely by the ectopic PME5 expression in the meristem dome, as shown by the complete restoration of meristem phyllotaxis in a blr-6/pme5-1 double mutant. These observations support a scenario in which the demethylsterification of HG in the cell wall is a critical step in the loss of meristem identity and in the allocation of the cell to a primordium. The maintenance of meristem identity during cell division requires the inhibition of PME activity, at least partly through inhibition of the accumulation of the PME5 transcript by BLR. This is consistent with the observation that PME13 overexpression completely prevents primordia formation (Peaucelle et al., 2008). At the meristem periphery, the observed downregulation of BLR expression (Byrne et al., 2003) is likely to cause PME5 transcript accumulation. Demethylsterification of HG in the newly deposited cell wall is sufficient for the recruitment of the cell into the primordium, as shown by the formation of ectopic primordia upon ectopic PME5 expression. In this scenario, it is interesting to note that the primordium fate is determined negatively by the loss of meristem identity, as a result of derepression of PME5. Primordia patterning is based on the positive determination of cells in the initiation by local accumulation of the phytohormone auxin (Bayer et al., 2009; Reinhardt et al., 2000; Reinhardt et al., 2003; Yu et al., 2009). This might impinge on PME5 expression directly or indirectly, possibly via BLR.

It should also be noted that pme5-1 rescues the altered phyllotactic pattern in the blr-6 meristem but not the altered distribution of flowers and siliques in the mature inflorescence. This defect reflects changes in the post-meristematic elongation of the internodes, as revealed by the decreased length of internode epidermal cells in blr-6. Since the promoter activity of PME5 was repressed in the internode of blr-6 mutants, this suggests that, depending on the tissue, BLR can either act as an inhibitor or activator of PME5 expression (Fig. 8). This hypothesis is in accordance with the reported expression of BLR in stems (Byrne et al., 2003). The constitutive, or inducible, expression of PME5 in the internodes complemented the blr-6 phenotype, which confirms the role of the pectin methylesterification status in the control of internode cell elongation. Such a role of PME-mediated changes in cell elongation is in accordance with previous data obtained on hypocotyls (Pelletier et al., 2010). However, the absence of a post-meristematic phenotype in the pme5-1 single mutant could be related to potential redundancy within the large PME gene family.

How does demethylsterification of HG lead to primordium formation? This chemical change is expected to cause dramatic changes in the physicochemical properties of the polymer, but also removes the protection against hydrolysis by the pectin-hydrolysing enzymes pectate lyase and polygalacturonase. Cleavage of HG reduces its viscosity and presumably leads to an increase in the porosity of the cell wall, which in turn may facilitate the access of other cell wall-loosening agents, such as expansins, to their substrates (Fleming et al., 1997; Reinhardt et al., 1998). We recently investigated the role of PME5-mediated HG demethylsterification
Fig. 8. The opposing effects of BLR on the expression of PMES in the wild-type meristem and internode and the impact on the establishment and maintenance of the phyllotactic pattern. BLR acts, directly or indirectly, to regulate PMES transcription in a tissue-specific manner. BLR represses PMES expression in the meristem, leading to de-methylsterification of pectins that is restricted to primordia, thus influencing the establishment of the phyllotactic pattern. By contrast, BLR activates PMES expression in the internode, promoting cell elongation and the post-meristematic maintenance of phyllotaxis. The tissue-specific regulation of PMES expression by BLR thus directs regular phyllotaxy patterning.

in the mechanical properties of the meristem. Using micro-indentation we demonstrated that HG demethylsterification does not lead to the predicted stiffening of the pectic matrix but rather to a local reduction of cell wall rigidity (Peaucelle et al., 2011). This is likely to be related to the mode of action of PME isoforms (blockwise versus non-blockwise) that could generate substrates for pectin-degrading enzymes. Our data, together with data presented in previous work (Peaucelle et al., 2008), suggest that BLR-controlled HG demethylsterification could be one of the key elements controlling the mechanical changes that underlie organ formation. In this respect, it is likely that changes in pectin structure could act synergistically with the regulation of the microtubule cytoskeleton and changes in PIN polarity, affecting auxin transport through mechanical stresses and thus triggering morphogenesis at the shoot apex (Hamant et al., 2008; Heisler et al., 2010). Our results could pave the way to adding novel components to the current model for phyllotaxis through integrating PIN1, the regulation of cell wall structure and cell mechanics.

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References


