INTRODUCTION

The plant shoot is derived from a group of undifferentiated stem cells within the shoot apical meristem (SAM). Lateral organs such as leaves arise on the flanks of the SAM by the coordinated repression of stem cell fate, determination of founder cells, and elaboration of the incipient primordium. Early events specify the spatial and temporal positioning of leaf and flower primordia and ultimately establish shoot phyllotaxy (Greek, ‘leaf order’). Later events define organ patterning along proximodistal, dorsoventral and mediolateral axes (reviewed by Kidner et al., 2002).

Initiation of successive lateral organs on the flanks of the SAM proceeds in predictable patterns generating a phyllotaxy. Spiral phyllotaxy observed in the vast majority of plants derives from the regular initiation of successive lateral organ primordia at a fixed angle of divergence but variable patterns of physical contact. Such patterns ultimately give rise to individual leaves and flowers at positions related to each other by consecutive terms in the mathematical series first described by Leonardo Fibonacci. We demonstrate that a BELL1 related homeodomain protein in Arabidopsis, BELLRINGER, maintains the spiral phyllotactic pattern. In the absence of BELLRINGER, the regular pattern of organ initiation is disturbed and lateral organs are initiated more frequently. BELLRINGER is also required for maintenance of stem cell fate in the absence of the regulatory genes SHOOT MERISTEMLESS and ASYMMETRIC LEAVES1. We propose a model whereby BELLRINGER coordinates the maintenance of stem cells with differentiation of daughter cells in stem cell lineages.

SUMMARY

Lateral organs in plants arise from the meristem in a stereotypical pattern known as phyllotaxy. Spiral patterns result from initiation of successive organs at a fixed angle of divergence but variable patterns of physical contact. Such patterns ultimately give rise to individual leaves and flowers at positions related to each other by consecutive terms in the mathematical series first described by Leonardo Fibonacci. We demonstrate that a BELL1 related homeodomain protein in Arabidopsis, BELLRINGER, maintains the spiral phyllotactic pattern. In the absence of BELLRINGER, the regular pattern of organ initiation is disturbed and lateral organs are initiated more frequently. BELLRINGER is also required for maintenance of stem cell fate in the absence of the regulatory genes SHOOT MERISTEMLESS and ASYMMETRIC LEAVES1. We propose a model whereby BELLRINGER coordinates the maintenance of stem cells with differentiation of daughter cells in stem cell lineages.

Key words: Phyllotaxy, Homeobox, Shoot apical meristem, KNOX, BELLRINGER, BREVIPEDICELLUS, SHOOT MERISTEMLESS, ASYMMETRIC LEAVES1

Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER

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Accepted 21 May 2003
class 1 KNOX homeobox transcription factors. Recessive mutations in the Arabidopsis KNOX gene SHOOT MERISTEMLESS (STM) and in the closely related maize gene knotted1 have defects in meristem function indicating a requirement for SAM initiation and/or maintenance (Barton and Poethig, 1993; Long et al., 1996; Vollbrecht et al., 2000; Vollbrecht et al., 1991). Consistent with a role in SAM function both STM and knl are expressed in vegetative and reproductive SAMs but are down-regulated in founder cells and lateral organ primordia (Jackson et al., 1994; Long et al., 1996). STM maintains stem cell fate by negative regulation of the myb domain transcription factor ASYMMETRIC LEAVES1 (AS1) and a member of the LOB-like transcription factor family ASYMMETRIC LEAVES2 (AS2) (Byrne et al., 2000; Byrne et al., 2002; Iwakawa et al., 2002; Shuai et al., 2002). AS1 is related to rough sheath2 in maize and PHANTASTICA in Antirrhinum. All three genes are expressed in lateral organ primordia where they function as negative regulators of KNOX genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Timmermans et al., 1999; Tsiantis et al., 1999).

Arabidopsis has three additional class I KNOX genes, BREVIPEDICELLUS (BP, formerly KNAT1), KNAT2 and KNAT6. Like STM, these genes are expressed in SAMs and downregulated in lateral organs, although the pattern and timing of expression differs from that of STM (Byrne et al., 2002; Dockx et al., 1995; Lincoln et al., 1994; Pautot et al., 2001; Semiarti et al., 2001). Mutations in BP alone do not cause meristem defects (Byrne et al., 2002; Douglas et al., 2002; Venglat et al., 2002). BP is, however, redundant with STM and has a role in SAM function in as1 stm and as2 stm mutant backgrounds (Byrne et al., 2002). Disruption of KNAT2 gene expression has no phenotypic effect, probably because of redundancy with the duplicate gene KNAT6 (Byrne et al., 2002).

We have isolated several insertion alleles of BELLRINGER (BLR), a BELL1-like homeobox gene. Prominent defects in blr mutants include an increase in the number of leaves and disruption to the normal spiral pattern of primordia initiation. Genetic interactions also demonstrate that BLR is required for stem cell maintenance. Previously we reported that BP plays a role in meristem function in the absence of AS1 and STM (Byrne et al., 2002). BLR is also necessary for meristem function in the absence of AS1 and STM. BLR probably interacts directly with STM and BP in meristem function.

MATERIALS AND METHODS

Plant material and growth conditions

Mutant alleles, blr-1 (GT7797) and blr-2 (ET6411), were obtained from gene trap and enhancer trap lines generated as previously described (Springer et al., 1995; Sundaresan et al., 1995). The blr-3 allele was obtained from the The Salk Institute Genomic Analysis Laboratory (SIGnAL) collection. bell-1, was-1, clv1-1 and clv3-2 were obtained from the Arabidopsis Biological Resource Center (ABRC). Other mutants (asl-1, sm-1, sm-11, sm-2, and bp-2) were obtained as described previously (Byrne et al., 2002). All mutants other than blr-3 were in a Landsberg erecta background. Plants were grown either on soil or on MS medium supplemented with sucrose, with a minimum day length of 16 hours.

Plant genetics

Tests for allelism were carried out by crossing plants homozygous for blr-2 and blr-3 to plants homozygous for blr-1. F1 plants from both crosses displayed the blr mutant phenotype. All other genetic interactions and phenotypic studies were carried out with the blr-1 allele in a Landsberg erecta background. To generate double mutants, plants homozygous for bp, as1, bel1, clv1 or clv3 were crossed to plants homozygous for blr. All double mutants segregated in the F2 progeny in a 1:1 ratio. Double blr was, blr sm-11 and blr sm-2 mutants were generated by crossing plants homozygous for blr to plants heterozygous for wus, stm-11 or stm-2. Double blr was mutants segregated 1:3 in F3 seed from blr mutant plants. In lines carrying either sm-11 or sm-2 F3 seed from homozygous blr plants segregated 1:3 stm mutants.

Triple blr as1 sm-11 mutants were generated by crossing double homozygous blr as1 plants to as1/as1 sm-11/+ plants. F2 seed from blr/blr as1/as1 sm-11/+ plants segregated a meristemless phenotype in a 1:3 ratio. Triple bel1 as1 sm-11 mutants were generated by crossing as1/as1 sm-11/+ plants with bel1/bel1. F3 seed from plants homozygous for as1 and heterozygous for sm11 and bel1 segregated only wild type, as1 sm11 and as1 bel1 phenotypes in a 9:4:3 ratio.

Molecular biology

DNA extraction and manipulation were carried out using standard protocols (Sambrook et al., 1989). The Ds element copy number in lines carrying blr-1 and blr-2 was determined using Southern gel blot analysis as described previously (Vongs et al., 1993). The Ds-specific hybridization probe was obtained by PCR amplification of the Ds element using the primers ageccgatgaaatatactcgag and tggctgctgaagttgctgag. Identification of the tagged gene in blr-1 and blr-2 was carried out by thermal asymmetric interlaced-PCR (Liu et al., 1995). To confirm the Ds element insertion sites in blr-1 and blr-2, PCR products were generated using Ds and gene-specific primers. Ds primers were accgcgagctgtatcctag and actgctggaagaatcctac. blr-1 primers were ctcgctgctgaagattcag and tgcagctgatagacaag. blr-2 primers were atcgctgctgaagacag and cgcagagaatcctag. PCR products were sequenced using dye terminator cycle sequencing (Applied Biosystems).

Total RNA for northern and RT-PCR analysis was purified using Trizol reagent (Life Technologies). For northern hybridization, 20 μg of RNA was separated on a 1.4% glyoxal/DMSO denaturing gel. RNA was transferred to a membrane and hybridized using Ultrasynch buffer (Ambion). The BLR probe for northern analysis was a PCR product synthesized with the primers taagtgctgctggtattga and aggacgatgatcagaaaa. RT-PCR was carried out as previously described (Byrne et al., 2002). Following DNase treatment and synthesis of complementary DNA with M-MuLV reverse transcriptase (New England Biolab) PCR reactions were performed with gene-specific primers. BLR primers were as above. RBC primers were gacacgctttctctgctg and cagagggctcaggtctg. PCR products were subject to Southern hybridization using gene-specific probes.

The BLR::GUS construct was derived as follows. A 3.9 kb genomic fragment containing the BLR promoter was amplified from Landsberg erecta using the primers tgccacagctagttgctag and ctgcggcggttgagga. The product was cloned into pRITA, which contains the GUS reporter gene and NOS terminator sequences (a gift from John Bowman). The resulting plasmid, pRIP3, contains an in frame fusion of the start codon of BLR with GUS. This gene fusion fragment was cloned into a binary vector and introduced into plants using Agrobacterium transformation.

Histology and microscopy

Inflorescences were prepared for sectioning by fixation in glutaraldehyde (2.5% in 0.1 M sodium phosphate buffer pH 7.0), dehydration through an ethanol series and infiltration with Histoclear prior to embedding in paraffin wax. All sections were 8 μm thick. Sections were cleared of paraffin wax using Histoclear, rehydrated to 50% ethanol, stained for 20 minutes in 0.1% safranin in 50% ethanol, rinsed in 70% ethanol, then stained for 3 minutes in 0.1% Fast Green.
in 95% ethanol. The sections were then dehydrated in 100% ethanol, and moved to Histoclear. For meristem size comparisons measurement were taken from longitudinal sections of 8 wild-type and 9 blr plants that were 23 days old.

GUS staining was carried out as previously described (Gu et al., 1998) using a substrate solution containing 100 mM sodium phosphate pH 7.10 mM EDTA, 0.1% Triton X-100, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D glucuronic acid (X-Gluc), 100 µg/ml chloramphenicol, and either 2 mM or 5 mM each of potassium ferricyanide and potassium ferrocyanide. After 24-48 hours at 37°C samples were cleared in 70% ethanol at room temperature. Samples were viewed with a Leica MZ8 microscope and images captured with a Spot RT digital camera (Diagnostic instruments).

For scanning electron microscopy (SEM) inflorescences from wild-type and blr plants were first fixed overnight in 2.5% glutaraldehyde, then rinsed in 0.1 M sodium phosphate buffer and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 95%, 100%) prior to critical point drying using a Tousimis Autosamdr-815. Samples were subsequently mounted on silver tape and sputter coated with gold (Emitech K550) before viewing with an Hitachi S-3500N SEM under high vacuum and with a beam accelerating voltage of 3-5 kV. Measurements of meristem radius and organ divergence angle were derived from SEM images of 16 wild-type and 16 blr mutant that were 18 days old. Plastochron ratios were measured from 7 wild-type and 7 blr plants.

**In situ hybridization**

In situ hybridizations were performed using digoxigenin-labeled probes (Long and Barton, 1998; Long et al., 1996). Antisense and control sense BLR transcripts were synthesized from the plasmid pBlue028, which carries a 531 bp fragment, encompassing the 3’ end of BLR, in pBluescript. Antisense STM transcripts were synthesized from the plasmid Meri HB1 (a gift from Kathy Barton). All sections were 8 µm thick.

**Yeast 2-hybrid assay**

Full-length BLR cDNA was amplified with the primers ggtcgacgggctgatgcatacgagcct and agcggccgcatttcaattcccccatatc. The PCR product was digested with SauI and NotI cloned into the GAL4 transcriptional activation domain (TA) vector pBI-771 (Kohalmi et al., 1997) forming the plasmid TA-BLR. STM cDNA was amplified with the primers acgcgtcgacgtatggagagtggttccaac and ggtcgacgggctgatgcatacgagcct and cloned into the GAL4 DNA-binding domain (DB) vector pBI-770. Other constructs, DB-BP, DB-KNAT4 and TABEL1 were kindly provided by George Haughn and are as previously described (Bellauoi et al., 2001). All plasmids were transformed into the yeast strain p69A using a lithium acetate/polyethylene glycol protocol (Schiestl et al., 1993).

**RESULTS**

**bellringer affects shoot architecture**

In a screen for mutants affecting shoot architecture, we recovered gene and enhancer trap insertions in a gene we named BELLRINGER. A third allele carrying a T-DNA insertion was also identified. All three alleles produced comparable phenotypes. Compared to wild type, blr mutants are reduced in stature and have a bushy appearance due to precocious growth of axillary branch meristems (Fig. 1A,B). There is an increase in the number of leaves and flowers (Table 1) although time to flowering is not significantly delayed (average of 16 days in wild type compared with 18 days in blr-1). In addition phyllotaxy is disrupted in these mutants such that the regular spiral arrangement of flowers on the inflorescence shoot is not strictly maintained (Fig. 1C,D). This is reflected in the relative displacement of flowers along both the radial and longitudinal axis of the stem. In the radial dimension, flowers can occur both closer together and further apart than in wild type. Inflorescence internodes are variable so that flowers occur at irregular intervals along the stem. blr mutants also have shorter siliques and ovary septum fusion defects (data not shown). The severity of these phenotypes is dependent on the genetic background and growth conditions. Principally the reduction in stature and loss of apical dominance are less severe under low light conditions and in a Columbia background.

Since phyllotaxy is often associated with changes in meristem size or shape, we compared meristems in wild type and blr mutants. In longitudinal section the size and shape of blr inflorescence meristems is comparable to that of wild type (Fig. 2A,B). Furthermore, the radius of blr mutant inflorescence meristems, estimated from SEM images of 18-day-old plants, was not significantly different from that of wild type. The average radius of wild-type meristems was 24.8 µm.
(range 22.6-28.1 \textmu m) while that of \textit{blr} mutants was 23.6 \textmu m (range 20.5-27.0 \textmu m).

Spiral phyllotaxy can be described by two parameters, the plastochron ratio and the divergence angle (Richards, 1951). The plastochron ratio can be used to predict the number of contact parastiches and reflects alterations in the packing of lateral organs. This parameter is derived by calculating the ratio of the distance of two successive primordia from the meristem, where distances are measured from the center of the meristem to the center of each primordium. To compare initiation patterns in wild type and mutant we measured the plastochron ratio for the youngest 4-6 primordia on inflorescence apices from 18-day-old plants. Average plastochron ratios between initiating floral primordia on \textit{blr} mutant inflorescence apices were not significantly different from wild type (1.1481 versus 1.1465). In plants with spiral phyllotaxy the divergence angle between successive primordia, measured from the center of the meristem to the middle of two successive organs, is approximately 137.5°. The average divergence angle between successive primordia in wild-type inflorescence apices was 136.72° (range 121.49°-152.31°), which was comparable with most \textit{blr} mutant inflorescence apices at 137.52° (range 127.54°-154.91°). However, 2 out of 16 \textit{blr} mutants had significantly reduced divergence angles between successive primordia (range 79.35°-112.13°) indicating abnormal sites of floral meristem initiation (Fig. 2C,D). This was consistent with the irregular phyllotaxy observed in mature plants (Fig. 2E-H). In agreement with these quantitative parameters, the number of clockwise and counterclockwise contact parastichies was unchanged. However, the angle between spiral parastichies was increased, accommodating the extra organs in a tighter spiral than normal (Fig. 2G,H).

\textit{BELLRINGER} is related to the homeodomain transcription factor \textit{BELL1}

The recessive \textit{blr-1} mutation was found to cosegregate with a single Ds element inserted into a homeobox gene, At5g02030, located on chromosome 5 (http://cshl.genetrap.org) (The Arabidopsis Genome Initiative, 2000). This gene encodes a protein most closely related to members of the \textit{BELL1} subclass of homeodomain transcription factors, of which there

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**Table 1. Average number of leaves and flowers in wild type and \textit{blr} mutants**

<table>
<thead>
<tr>
<th></th>
<th>Rosette leaves</th>
<th>Cauline leaves</th>
<th>Total leaves</th>
<th>Flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type L. \textit{erecta} (n=20)</td>
<td>6.3 (6-7)</td>
<td>2.5 (2-3)</td>
<td>8.8</td>
<td>29 (26-32)</td>
</tr>
<tr>
<td>\textit{blr-1} (n=20)</td>
<td>9.3 (8-11)</td>
<td>2.9 (2-3)</td>
<td>12.2</td>
<td>41.8 (39-47)</td>
</tr>
<tr>
<td>\textit{blr-2} (n=20)</td>
<td>8.5 (7-11)</td>
<td>3.0 (2-4)</td>
<td>11.4</td>
<td>39.7 (35-44)</td>
</tr>
<tr>
<td>Wild type Columbia (n=15)</td>
<td>6.9 (6-8)</td>
<td>2.7 (3-5)</td>
<td>9.4</td>
<td>29.3 (25-36)</td>
</tr>
<tr>
<td>\textit{blr-3} (n=15)</td>
<td>9.9 (9-12)</td>
<td>2.9 (2-4)</td>
<td>12.8</td>
<td>36.2 (34-40)</td>
</tr>
</tbody>
</table>

\textit{n}, number of plants used for determining leaf and flower number. Numbers in brackets are range of organs. Organ numbers were determined from wild-type and mutant plants grown for the same length of time under identical growth conditions. \textit{blr-1} and \textit{blr-2} are in \textit{L. erecta} background. \textit{blr-3} is in a Columbia background.

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Fig. 2. Phyllotaxy defect in \textit{blr} mutants. (A,C,E,G) Wild-type inflorescence apex; (B,D,F,H) \textit{blr} mutant inflorescence apex. (A,B) Longitudinal sections of 23-day-old plants showing comparable size and shape of wild-type (A) and \textit{blr} (B) inflorescence meristems. The width of the stem is also similar in wild type and \textit{blr}. (C-F) 18-day-old plants. In wild type (C,E) organs are initiated on average 137.5° apart forming a continuous spiral. In wild type (C) the divergence angle between primordia 2 and 3 is 130.6°. In \textit{blr} mutants (D,F) flowers can initiate in aberrant positions as in primordia 3 in D and 5 in F. In D the divergence angle between primordia 2 and 3 is 79.3°. (G,H) Inflorescence apex from 23-day-old plants. Clockwise (red) and counterclockwise (yellow) contact parastichies forming a tighter curve than in wild type. Numbering of each inflorescence starts at the youngest visible primordium. Scale bars: 0.5 mm (A,B), 50 \textmu m (C,D), 200 \textmu m (E,F) and 500 \textmu m (G,H).
Phyllotaxy and stem cell function in *Arabidopsis* are 12 members in *Arabidopsis* (Becker et al., 2002). BLR comprises 4 exons and 3 introns (Fig. 3A). The Ds insertion in *blr-1* is located in the second intron whereas *blr-2* has a Ds insertion 2 bp upstream of the BLR ATG initiation codon. In both alleles the insertion does not activate the GUS reporter on the Ds element. The third allele, *blr-3*, carries a T-DNA insertion in the first intron of the gene. For all three alleles full-length BLR transcripts were not detected in homozygous mutant plants (Fig. 3B).

**BELLRINGER expression pattern**

The expression pattern of BLR in wild-type plants was examined by northern hybridization. Low levels of BLR transcript were detected in 8-day-old seedlings and in leaf tissue (Fig. 3C). Higher transcript levels were detected in inflorescence shoots, including the inflorescence meristem and flower buds.

BLR expression was further examined by driving GUS reporter gene expression from the BLR promoter. In the embryo, BLR expression was confined to the SAM (Fig. 4A) but weak expression could also be detected in the root tip (data not shown). In young seedlings, BLR was highly expressed in the SAM and could be detected in cotyledon and leaf vasculature (Fig. 4B). In the inflorescence, BLR was expressed in the inflorescence meristem, stem, flower pedicel and in developing flowers (data not shown).

In situ hybridization was carried out to confirm the authenticity of the *BLR:GUS* expression pattern. BLR expression in late heart stage embryos was confined to the SAM region between the two developing cotyledons (Fig. 4C). The expression pattern is similar to that of STM (Fig. 4D) (Long et al., 1996). In addition, BLR is expressed in the inflorescence SAM in a region similar to that of STM (Fig. 4E,F). BLR expression is initially downregulated in incipient floral primordia (Fig. 4G-I). Strong expression is subsequently downregulated in incipient floral primordia (Fig. 4G-I).
Genetic interactions between bellringer and KNOX genes

BELR-like proteins belong to a class of homeodomain transcription factors that can interact directly with KNOX class homeodomain transcription factors (Bellaoui et al., 2001; Müller et al., 2001; Smith et al., 2002). Since protein-protein interactions between heterologous homeodomain transcription factors are required in animals (Mann and Chan, 1996) it is likely that such interactions are functionally significant in plants. We therefore investigated genetic interactions between BLR and the class 1 KNOX genes STM, KNAT1 and KNAT2.

Embryos homozygous for strong stm alleles, including stm-1 and stm-11 (Fig. 5A), lack a SAM and develop cotyledons that are fused at their base (Barton and Poethig, 1993; Clark et al., 1996; Long et al., 1996). Double blr stm-11 mutants are similar to stm-11 mutants (Fig. 5B). However, blr enhances the phenotype of the weak allele stm-2. Single stm-2 mutants germinate with slight fusion at the base of the cotyledons (Clark et al., 1996; Endrizzi et al., 1996). After a brief delay, vegetative shoot development is initiated (Fig. 5C). In contrast, blr stm-2 double mutants do not form any vegetative shoot and resemble mutants of strong alleles of stm (Fig. 5D). This genetic interaction indicates BLR is required for SAM function when there is reduced STM activity.

The blr mutant phenotype shares some characteristics with bp mutants including reduced stature and precocious outgrowth of axillary meristems (Fig. 6A). In addition, in bp mutants differentiation of subepidermal chlorenchyma extending from the abaxial side of the pedicel into the adjacent stem is disrupted (Douglas et al., 2002; Venglat et al., 2002). We analyzed blr bp double mutants to determine if there is any interaction between these two genes. Plants homozygous for both blr and bp were much reduced in stature compared with either single mutant (Fig. 6B). In other respects the double mutant has features of both blr and bp single mutants. The number of rosette and cauline leaves in blr bp mutants is comparable to that of blr (data not shown) while flower pedicels and siliques are reduced in length and generally point downward as in bp (Fig. 6A,B), and subepidermal chlorenchyma below the flower is pale relative to surrounding tissue. The phenotype of the blr bp double mutant therefore appears additive. blr knat2 double mutants are indistinguishable from blr mutants indicating that the blr phenotype is not influenced by loss of KNAT2 expression (data not shown).

To further explore the possibility of genetic interactions between BLR and BP we took advantage of the conditional role of BP in SAM function. BP maintains the SAM in asl stm double mutants (Byrne et al., 2002). Vegetative development in these double mutants resembles that of asl, whereas hp asl stm mutants lack a SAM, fail to form a vegetative shoot, and are indistinguishable from stm single mutants (Byrne et al., 2000; Byrne et al., 2002). The phenotype of blr asl double mutants is additive in all respects (Fig. 6C). However, blr asl stm triple mutants have severely reduced SAM function. Following germination only one or two leaves are produced (Fig. 6D). Occasionally development is resumed to form a disorganized vegetative shoot (Fig. 6E,F). BLR is therefore required for SAM function in the absence of STM and ASL.

In contrast to blr mutants where phenotypic effects are evident in the shoot, mutations in the related gene bel1 only affect ovule development (Modrusan et al., 1994; Reiser et al., 1995; Robinson-Beers et al., 1992). blr bel1 double mutants display a blr shoot phenotype and are sterile as is bel1 (Fig. 6G,H). This demonstrates that BEL1 is not redundant with BLR in shoot development. Since BEL1 protein directly interacts with BP we also investigated whether BEL1 is required for SAM function in an asl stm background. In contrast to blr asl stm mutants, the triple bel1 asl stm mutants form a vegetative shoot similar to asl stm double mutants (data not shown). Furthermore no novel phenotype is detected in progeny of blr asl plants also segregating bel1 and stm. Therefore BEL1 is not required for SAM function in these contexts.

SAM function is also regulated by WUSCHEL (WUS) and the CLAVATA (CLV) genes, acting independently of KNOX genes. WUS is a homeodomain protein expressed in inner central zone stem cells of the SAM. Mutations in WUS result in loss of meristem function. CLV1 and CLV2 are transmembrane receptors and CLV3 encodes a secreted peptide. Mutations in all three CLV genes result in a much enlarged meristem. CLV genes maintain meristem homeostasis by limiting WUS function (reviewed by Brand et al., 2001; Clark, 2001; Fletcher, 2002). We found blr wus, blr clv1 and blr clv3 double mutants are additive (not shown). Therefore, BLR appears to affect meristem function via a KNOX gene-specific pathway.

![Fig. 5. blr enhances a weak allele of stm. Twelve-day-old seedlings of stm-11 (A), blr stm-11 (B), stm-2 (C) and blr stm-2 (D). blr stm-11 double mutants are similar to stm-11 single mutants. No vegetative shoot is produced and the base of the cotyledons are fused. Plants homozygous for the weak stm-2 allele show limited fusion at the base of the cotyledons and initiate a vegetative shoot. The meristem defect is enhanced in blr stm-2 double mutants which do not develop vegetative shoot structures and there is significant fusion of the cotyledons.](image-url)
Genetic analysis demonstrated that blr enhances a weak allele of 
stm and is required for SAM function in the as1 stm background.

BELLRINGER interacts directly with KNOX proteins

Blr directly interacts with and affects the activity of STM and BP. To test this possibility a yeast two-hybrid assay was carried out (Fig. 7). Yeast strains carrying the plasmids TA-BLR and DB-BP or DB-STM were viable in the absence of histidine. In contrast, yeast carrying TA-BRL and DB-KNAT4 failed to grow in the absence of histidine. Therefore in this system BLR interacts with the class1 KNOX proteins STM and BP but not with the class 2 KNOX protein KNAT4. The negative control yeast strain carrying the plasmid TA-BLR in the presence of the DB vector showed no growth in the absence of histidine.

DISCUSSION

BLR is a BELL class gene which, like KNOX genes, are members of the TALE homeobox transcription factor family. TALE homeodomain proteins have an atypical homeodomain, characterized by three additional amino acids between the first and second helix. Several features distinguish BELL and KNOX proteins (Becker et al., 2002; Bharathan et al., 1999; Bürglin, 1997; Reiser et al., 2000). The 12 members of the BELL class share 54% amino acid identity in the homeodomain. Likewise, the 8 KNOX proteins share 54% amino acid identity in the homeodomain. However, between these two classes amino acid conservation in the homeodomain is 32%. In addition, BELL proteins have conserved SKY and BELL domains upstream of the homeodomain, whereas KNOX proteins are defined by conserved MEINOX, ELK and GSE domains upstream of the homeodomain (Bellaoui et al., 2001; Bürglin, 1997; Kerstetter et al., 1994).

BLR is strongly expressed in the embryonic SAM, and has a conditional role in SAM function as revealed by genetic interactions. Whereas as1 stm mutants form a vegetative shoot indistinguishable from that of as1 single mutants, shoot development is greatly reduced in the triple blr as1 stm mutant. This triple mutant strongly resembles pinhead/zwille mutants where a solitary vegetative organ appears to consume the meristem (Lynn et al., 1999; Moussian et al., 1998). BLR is therefore required to maintain the SAM in the as1 stm background. Meristem function in as1 stm mutants is dependent on BP (Byrne et al., 2002), so that it is probable that BP activity is compromised in blr. We tested this possibility by making bp blr double mutants, which were additive in all respects. Thus BP is still functional in a blr mutant, meaning
that loss of \( bp \) function cannot completely explain the \( blr \) phenotype.

Strong alleles of \( stm \) are formally epistatic to \( blr \), although the \( blr \) phenotype is difficult to observe in strong \( stm \). However, a weak allele of \( stm \) is greatly enhanced by \( blr \). This effect is far stronger than that of \( bp \), which also enhances weak \( stm-2 \) (Byrne et al., 2002). Thus it is possible that \( blr \) is required for \( STM \) function, consistent with its expression pattern in the embryo. However, \( blr \) must also have an \( STM \)-independent role, revealed by the strong phenotype of \( blr \ asl \) \( stm \) triple mutants. A likely explanation is that \( blr \) is required for both \( BP \) and \( STM \) function. Consistent with this possibility, yeast two-hybrid assay demonstrates the \( BLR \) protein interacts directly with both \( STM \) and \( BP \). The requirement for \( BLR \) must be only partial, as \( blr \) has a much milder phenotype than either \( bp \) or \( stm \), and, as noted above, \( BP \) is still functional in a \( blr \) mutant. One explanation is that \( BLR \) is itself partially redundant. A strong candidate would be the BELL class gene (\( At2g27990 \) most closely related to \( BLR \)). Conservation between these two genes is 90% within the homeodomain and 48% overall.

\( BLR \) is more distantly related to the BELL class gene \( BEL1 \). The \( blr \ bel1 \) double mutant phenotype indicates a lack of functional overlap between these two genes. The phenotype of \( bel1 \) mutants is restricted to the ovule where the morphology of the outer integument is abnormal while the inner integument is completely absent (Modrusan et al., 1994; Robinson-Beers et al., 1992). Consistent with this phenotype, \( BEL1 \) expression in the ovule is restricted to the region where integuments initiate (Reiser et al., 1995). However, \( BEL1 \) is also expressed in vegetative tissues and roots. The lack of other plant phenotypes suggests \( BEL1 \) shares genetic redundancy. Similarly, \( BEL1 \) interacts physically with \( BP \), yet the \( bel1 \) phenotype indicates that it is not required for \( BP \) function in the inflorescence. We have demonstrated that \( BEL1 \) is not required for \( SAM \) function in \( asl \) \( stm \), indicating it has no effect on \( BP \) activity in the embryonic or vegetative \( SAM \). Again the apparent lack of \( BEL1 \) genetic interactions with \( BP \) is possibly the result of redundancy. Candidates for such redundancy are two genes, \( BLH2 \) and \( BLH4 \), most closely related to \( BEL1 \) (Becker et al., 2002).

Mutations in \( BLR \) result in phyllotaxy defects including both an increase in the number of lateral organs and displacement of organs along the stem. Although mechanisms governing phyllotactic patterning are still to be elucidated, early surgical experiments have shown that leaf primordia are positioned in response to preexisting primordia (Snow and Snow, 1931). This influence may be mediated by production of a diffusible inhibitor or may be biophysical in nature. A model where biophysical forces regulate phyllotaxy is supported by studies demonstrating induction of leaf formation by local concentration of the cell wall protein expansin (Fleming et al., 1990). Interestingly, phyllotaxy defects are also observed in plants expressing a constitutively active form of a RHO GTPase that may be involved in mediating plant responses to hormones such as auxin (Li et al., 2001). The inflorescence phenotype in this case resembles that of \( bp \) mutants.

The increase in organ number and organ displacement indicates \( blr \) mutants are no longer fully responsive to inhibitory signals from preexisting organs such that distances between organs are not maintained. Aberrant initiation along the apical-basal axis of the SAM potentially contributes to variable internode lengths. Alternatively, regular partitioning of cells between organs and internodes is affected. In this respect \( blr \) resembles \( tel \) in maize, which has also been interpreted as a phyllotaxy mutant (Veit et al., 1998).

Despite the phyllotactic defects in \( blr \) there is no appreciable difference in the size of the SAM compared with wild type. This may be coincident with more peripheral zone cells being specified as organ founder cells. Alternatively, in \( blr \) mutants an increase in recruitment of cells into lateral organs is offset by an increase in the number of stem cells, together maintaining SAM size. In this case \( BLR \) normally delays

![Fig. 7. BLR interacts directly with class 1 KNOX proteins. Yeast two-hybrid assay demonstrating interaction between BLR and KNOX proteins inferred through selective growth on medium lacking leucine, tryptophan and histidine (–leu –trp –his) compared with medium lacking leucine and tryptophan (–leu, –trp). All yeast strains grow on –leu –trp medium. Growth on –leu, –trp, –his medium is detected for strains carrying TA-BLR and DB-BP or DB-STM but not for strains carrying TA-BLR and DB-KNAT4 or the DB vector. Growth of a strain carrying TA-BEL1 and DB-BP is shown as a positive control.](image)
differentiation of stem cells in the SAM and slows their propagation. In each case, the function of BLR in delaying specification of lateral organs is consistent with BLR expression in peripheral cells of the inflorescence meristem, but not in initiating primordia.

Stem cell lineages expand according to the Fibonacci series when daughter cells are delayed from acquiring stem cell fate, raising the possibility that stem cells are responsible for phyllotactic patterns (Klar, 2002). In this respect, BLR fulfills a postulated stem cell function required for Fibonacci progression (Klar, 2002), in that BLR dictates how long daughter cells require to differentiate in the stem cell lineage. However, this model remains controversial and is yet to be substantiated (Fleming, 2002). For example, stem cell lineages are multicellular in higher plants, such that extensive coordination in the meristem would be required for stem cell lineages to regulate organ initiation in this way. The effects of the blr mutation on phyllotactic pattern are intriguing in this context and will be examined further.

We thank George Haughn for providing yeast two-hybrid constructs, John Bowman for the GUS expression vector, Julie Thomas for advice on yeast transformation, Marja Timmermans and members of Rob Martienssen’s lab for helpful discussion. We also thank Tim Mulligan for plant care, and Rulan Shen, Uma Umamaheswari, Amy Tang-Qu and the CSHL gene trap team for helping with plants and generating the blr line. This work was supported by USDA-NRI grant 2003-00967 to M.E.B. and R.A.M., and by NIH Postdoctoral Fellowship GM19974 and USDA-NRI 35301-10878 to A.T.G.

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