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A role for BELLRINGER in cell wall development is supported by loss-of-function phenotypes

J Peter Etchells1,3*, Lucy Moore1†, Wen Zhi Jiang1,4, Helen Prescott1, Richard Capper1,5, Nigel J Saunders2,6, Anuj M Bhatt1 and Hugh G Dickinson1*

Abstract

Background: Homeodomain transcription factors play critical roles in metazoan development. BELLRINGER (BLR), one such transcription factor, is involved in diverse developmental processes in Arabidopsis, acting in vascular differentiation, phyllotaxy, flower and fruit development. BLR also has a redundant role in meristem maintenance. Cell wall remodelling underpins many of these processes, and BLR has recently been shown to regulate expression of PECTIN METHYL-ESTERASE 5 (PME5), a cell wall modifying enzyme in control of phyllotaxy. We have further explored the role of BLR in plant development by analysing phenotypes and gene expression in a series of plants over-expressing BLR, and generating combinatorial mutants with blr, brevipedicellus (bp), a member of the KNOX1 family of transcription factors that has previously been shown to interact with blr, and the homeodomain transcription factor revoluta (rev), required for radial patterning of the stem.

Results: Plants over-expressing BLR exhibited a wide range of phenotypes. Some were defective in cell size and demonstrated misregulation of genes predominantly affecting cell wall development. Other lines with more extreme phenotypes failed to generate lateral organs, consistent with BLR repressing transcription in the shoot apex. Cell wall dynamics are also affected in blr mutant plants, and BLR has previously been shown to regulate vascular development in conjunction with BP. We found that when bp and blr were combined with rev, a set of defects was observed that were distinct from those of bp blr lines. In these triple mutants xylem development was most strikingly affected, resulting in an almost complete lack of vessels and xylem parenchyma with secondary thickening.

Conclusions: Our data support a role for BLR in ordering the shoot apex and, in conjunction with BP and REV, playing a part in determining the composition and organisation of the vascular system. Microarray analysis strongly indicates that the striking vascular phenotypes of blr bp rev triple mutants and plants over-expressing BLR result from the misregulation of a suite of genes, targets of BLR in wild type plants, that determine cell size and structure in the developing vasculature.

Background

BELLRINGER (BLR), also known as VAAMANA, PENNYWISE, LARSON and REPLUMLESS, is a member of the BELL family of homeodomain transcription factors and functions in diverse processes in the development of Arabidopsis [1-5]. BLR was identified independently as a suppressor of the floral homeotic gene AGAMOUS (AG) [5], as a factor required in fruit development for specification of the replum [1] and as a gene necessary for normal phyllotactic patterning [2,4]. BELL transcription factors were shown to enhance DNA binding of maize KNOTTED1 [6], the founding member of the KNOX1 gene family [7], and BLR was subsequently shown to act synergistically with the KNOX1 homeodomain transcription factor BREVIPEDICELLUS (BP) [3]. BLR and KNOX1 proteins heterodimerize [3] and, in Nicotiana epidermal cell transient assays, BLR translocates to the nucleus on binding to KNOX1 proteins [2]. This event is

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likely to have evolved early in eukaryotic development as it is required for the haploid-diploid transition in the green algae *Chlamydomonas* [8].

Although the expression patterns of *BLR* and *KNOX1* overlap, they are not identical [3] and *blr* and *knox1* mutants show distinctive phenotypes, suggesting that *BLR* function is unlikely to be confined to acting with *KNOX1* proteins alone. Nevertheless, similarities are apparent, such as the short stature of both *blr* and *bp* plants [2-4] and the ability of *blr* and *bp* to enhance weak alleles of *shoot meristemless* (*stm*) [2,4], a *KNOX1* gene required for shoot apical meristem maintenance [9,10], which points to a redundant role for *BLR* and *BP* in stem cell fate establishment [2-4]. Reports of genetic interactions between *blr* and *bp* mutants have, however, been conflicting, with the short stature phenotype of the *bp blr* double mutation being variously interpreted as additive [4], synergistic [3], or novel - because inflorescence elongation is observed in the single mutants but not in the double [2]. Phenotypic differences between *blr* and *bp* mutants are unlikely to result from redundancy between family members because multiple knock-out lines within the *KNOX1* and *BELL* families exhibit diverse phenotypes. For example, vegetative to reproductive transition defects are observed when *blr* mutants are combined with mutations in *poundfoolish* (*pfn*), the gene with which *BLR* shares most similarity in *Arabidopsis* [11], which are not observed in multiple *knox1* mutants [12,13]. Similarly, multiple *knox1* mutants do not resemble *blr* plants as one role of *BP* is repression of *KNAT2* and *KNAT6* [12]. *BLR* is thus likely to have functions both dependent on and independent of *KNOX1*.

One *KNOX1* independent function of *BLR* may include control of phyllotaxy as similar defects are not present in *knox1* mutants. Normal phyllotaxy requires correct patterning of cell divisions [11,15] which depend in turn on strictly regulated cell-wall remodelling events [16] themselves driven by mechanical constraints [17]. Primordia outgrowth is accompanied by the de-methyl-esterification of pectic polysaccharides in the cell wall, and perturbation of the methyl-esterification status of pectins within the meristem by altering expression levels of *PECTIN METHYL-ESTERASE 5* has been shown to alter phyllotactic pattern [18]. *PME5* has been demonstrated to be negatively regulated by *BLR* in the meristem dome and this interaction is essential for maintenance of phyllotaxy, as incorrect positioning of organ initiation in *blr* mutants is suppressed in *blr pme5* double mutants. The relationship between *BLR* and *PME5* appears to be context specific as *BLR* promotes *PME5* expression in internodes [19].

The functions of *BP* and *BLR* do overlap in vascular development. Typically in *Arabidopsis* stems, vascular tissue is radially arranged in collateral bundles with xylem present towards the centre of the stem and phloem positioned towards the outside, such that the xylem, vascular meristem (procambium) and phloem are positioned along the mediolateral axis [20]. Xylem is characterised by the presence of large vessels for water transport and smaller xylary fibres, both of which have secondary cell walls with the adjacent procambium forming an arc of dividing cells. Within the stem, the vascular tissue represents a developmental series with most recently derived tissue at the top and the oldest at the base [20]. A continuous vascular ring has been described in *blr* lines, a phenotype enhanced in *hp blr* double mutants [3] by the appearance of undeveloped vascular bundles [3] containing cells previously only observed occurring in small, isolated strips overlaying some vascular bundles of *hp* plants [21,22].

Here we have exploited transgenic plants over-expressing *BLR* as a tool to identify putative downstream targets of this transcription factor. In addition to the previously described target of *BLR*, *PME5* [19], our microarray analysis has identified misregulation of many other genes encoding cell-wall-associated proteins following *BLR* over-expression. Defects in cell wall metabolism can affect cell size [23] and we therefore examined *blr* mutant vascular tissue, where cell size defects have previously been described [3]. We uncovered a genetic interaction between *blr*, *hp* and *revoluta* (*rev*), a homeo-domain transcription factor of the HD-ZipIII family that has previously been shown to specify adaxial identity, radial patterning in the stem [24] and xylem differentiation [25]. Our results add further support to the notion [19] that in addition to regulating genes at the shoot apex, *BLR* plays a wider role in development through its control of the extracellular matrix.

**Results and discussion**

**Over-expression of *BELLRINGER* reduces organ size**

Over-expression studies have been informative in functional analysis of *KNOX1* [26-28]. Using a similar strategy, *BLR* cDNA was used to generate 35S::*BLR* constructs from which *BLR* over-expression (*BLR-OX*) transgenic lines were generated. Plants were observed with short hypocotyls, small rosette leaves, narrow cauline leaves, and short siliques (Figure 1A, 1F, 1H). *BLR-OX* plants also had shorter, thinner stems than wild type with more axillary branches (Figure 1G, 1I). Northern blot analysis showed eight lines to exhibit increased *BLR* levels; *BLR* transcript levels in two of these lines, *BLR-OX1* and *BLR-OX2*, are shown in Figure 2A.

The organs of *BLR-OX* lines were smaller than wild type, so differences in cell size and number were investigated in 7 day epidermal hypocotyl cells from *BLR-OX* and wild type plants (Figure 3A-D). *BLR-OX* epidermal cells were significantly shorter than controls (Figure 3B); similar reductions were observed in longitudinal sections...
of inflorescence stem epidermis (Figure 3G-I) and transverse sections of pith parenchyma (Figure 3E-F). This reduction in cell size is thus likely to contribute to the smaller organ size of BLR-OX plants. Other histological features, including those in vascular tissue were indistinguishable from wild type.

Expression changes in BLR-OX plants
To explore the changes in transcription underlying the BLR-OX phenotype, transcripts from hypocotyls of BLR-OX and control plants were subject to microarray analysis at stage 1.01 [29] of development. Using the 'Significance Analysis of Microarrays' strategy [30], 3109 genes demonstrated consistent expression changes and acceptable spot confidence of which 2957 had appropriate q-values (<0.1%). 927 genes demonstrated >1.5 fold expression change (Additional file 1: Table S1). Three lines of evidence point to the microarray data reflecting gene expression changes in planta; first, BLR was up-regulated 3.6 fold in BLR-OX compared to Col-0 (Table 1). Second, the expression of PME5, previously shown to be negatively regulated by BLR is reduced 2.3 fold (Table 1) and third, the promoters of 70 of the 100 genes showing the greatest fold change carried predicted BLR consensus DNA binding sites (Table 1). In contrast, the DNA binding sequence for ATHB2, which in common with BLR has a 9 base pair consensus sequence, occurs in only 9 of the 100 promoter regions. The array data was validated by testing expression of MERI-5 (At4g30270; 4.3 fold increase in BLR-OX), a xyloglucan endotransglucosylase which acts to hydrolyze glycosyl bonds, HPRG (At5g65660; 1.6 fold increase), a hydroxyproline-rich plasma membrane localised glycoprotein, and LPT6 (At3g08770; 1.9 fold decrease) and LTPV.2 (At3g53980; 6.3 fold decrease), both of which encode endomembrane localised lipid binding/transport proteins, by northern blot (Figure 2B). In all instances expression reflected the array data.

To understand better the significance of transcriptional differences between BLR-OX and control plants, a gene ontology (GO) analysis was carried out. Strikingly, the only significant change proved to be in cell wall associated gene expression, which was enriched from 1.3% in controls to 6.8% in BLR-OX lines (Table 1) pointing to a role for BLR in regulating cell wall metabolism or organisation. Changes in cell size are regularly accompanied by expression changes in cell wall associated genes and the GO analysis thus draws together the gain-of-function phenotypes (Figures 1, 3) and the array data (Table 1). Lipid transfer proteins (LTPs) [31] and peroxidases [32] loosen cell walls and induce cell expansion; thus a 6 fold decrease in expression of LTP and a 2.5 fold reduction in peroxidase expression in BLR-OX plants (Table 1) is consistent with the reduction in cell size observed in BLR-OX lines. PME5, which is negatively regulated by BLR (2.3 fold down-regulated in BLR-OX), alters the gelling properties of cell walls [33], while other genes with altered expression are implicated by homology to have roles in cell expansion. For example members of the thaumatin family (2.8 fold down-regulated) have potential endo-β-1,3 glucanase activity [34].

Figure 1 Phenotype of BLR-OX lines. 7 day-old (top panel) and 21 day-old (lower panel) wild type (A), transformed control (B), BLR-OX1 (C) and BLR-OX2 (D). (E) Dissected rosette leaves of 4 week-old WT, BLR-OX1 and BLR-OX2 plants. (F) WT and BLR-OX1 hypocotyls at 2 weeks-old. (G-I) Comparison of 5 week-old BLR-OX1, BLR-OX2 and control plant inflorescence stems (G), mature siliques (H) and stature (I).
Extreme BLR gain-of-function plants lack organ initiation
Organised phyllotaxy, lacking in blr mutants [2-4] requires accurate cell expansion and division during organogenesis [15], processes reliant on close regulation of cell-wall formation and expansion [16,18]. We therefore anticipated that phenotypes observed in BLR-OX lines could be strengthened to include disruption of organ initiation due to altered cell wall metabolism, particularly as BLR-OX lines analysed above exhibited only a relatively small expression increase (3.6 fold). Furthermore, in some contexts, BLR is localised in the cytoplasm, and its nuclear localisation is dependent on an interacting partner [2,35]. Plants were therefore generated expressing a steroid-inducible version of BLR in the form of a BLR-GR fusion regulated by the 35S CaMV promoter which would result in nuclear localisation of BLR in a steroid inducible manner, showing independence of an interacting partner. To confirm that expression changes of cell wall modifying enzymes following BLR-GR induction were consistent with those observed in BLR-OX lines, plants were subjected to dexamethasone (dex) induction for 72 h, as maximum activity of direct transcriptional targets in this system has been demonstrated as occurring between 64 and 128 hours [36]. Following dex induction, expression of both MERI-5 and EXP17 was elevated (Figure 2C), as in BLR-OX lines (Table 1) providing further evidence for BLR regulation of the extracellular matrix.

When BLR-GR seeds were germinated on 10 µm dex, only cotyledons were visible after 14 days (Figure 4A), suggesting that high levels of BLR in the nucleus lead to extremely limited growth. This observation may explain the relatively low increases in BLR expression in BLR-OX as plants with higher expression levels may not have been recovered in the T1 generation. Seedlings germinated for 3 days in the absence of steroid and subsequently transferred to 10 µm dex (BLR-GR), produced leaves with severely reduced expansion (Figure 4B-E). Visible leaves no longer emerged after 21 days and the apex became enlarged (Figure 4F), suggesting that growth continued in the absence of organ initiation. Sections cut through these enlarging BLR-GR apices revealed an absence of lateral organ primordia or any other clearly differentiated cell types (Figure 4G-H), suggesting that high levels of nuclear BLR results in anisotropic growth.

Normal organ initiation and phyllotaxy in Arabidopsis requires interplay between BLR repression of PME5 in the shoot apical meristem [19], and loss of this repression during formation of organ primordia [4]. Loss of PME5 repression has been proposed to allow other cell wall modifying components to access the wall. Our results support this hypothesis for, in our BLR-GR plants, BLR would continue to negatively regulate PME5 with the consequent inhibition of organ initiation we observed.

Organ initiation requires both the correct patterning of cell divisions [14,15] and strictly regulated cell wall remodelling [16,18]. The organisation of cell divisions in the apex has been shown to depend on mechanical pressures [17], which are likely to be influenced by the cell wall [37]. The current model for cell-wall remodelling during organ initiation at the apex holds that the breakage and annealing of cell-wall components is controlled by connections with microtubules, possibly through regulation of cellulose-microfibril length [38]. Treatment of Arabidopsis apices with oryzalin, which depolymerises microtubules and prevents ordered division, results in a bulbous apex and loss of lateral-organ initiation when combined with disrupted auxin signalling [39]. The cell wall plays a central part in the transmission of chemical and mechanical signals between cells, and the positioning of developmental cues at the apex of sunflowers is reported to be sensitive to mechanical interventions [17]. Our data suggest that BLR modulates the expression of genes encoding cell wall associated proteins, and our observation that plants strongly over-
expressing BLR-GR fusions generate bulbous apices incapable of forming lateral organs is consistent with the view that closely-regulated cell wall development is essential for ordered initiation of lateral organs at the shoot apex.

**rev enhances the bp blr phenotype**

The array experiments identified genes involved in cell expansion to be misregulated in BLR-OX plants. Expression changes in genes involved in cell expansion are also a feature of ectopic knox1 (STM) expression in poplar stem vascular tissue [40], and several putative BLR targets identified in BLR-OX arrays are reciprocally regulated in bp mutants [41] (Table 1). BP is required for patterning the inflorescence stem as sectors of differentiation-defective epidermal, cortical and vascular cells are present in bp mutant stems [22,42,43]. Furthermore, BP negatively regulates biosynthesis of lignin [41], a cell wall polymer, that is present in cells that have secondary walls including those of the vascular tissue. Despite exhaustive analysis, blr and wild type hypocotyls proved indistinguishable (not shown); however we saw clear differences in the vascular tissue of the inflorescence stem where BLR is expressed [4]. Loss of function blr phenotypes have been described in this region [3] in the relatively newly formed vascular tissue at the top of the stem (between the third and fourth siliques). Here blr mutants were found to form a continuous ring of vascular tissue, while bp blr double mutants demonstrated an enhanced blr phenotype as cells in the continuous vascular ring failed to differentiate and contained small, densely stained cells where xylem cells with secondary cell walls formed in wild type plants [3].

We analysed vascular tissue at a later stage in development, adjacent to the first cauline leaf on the primary inflorescence stem. blr vascular bundles were patterned
differently from those of wild type, as the arc of procambial cells present in wild type appeared shallower in \textit{blr} (Figure 5A, C). \textit{blr} mutants with the strongest phenotypes also had vascular bundles lacking large xylem vessels and containing apparently only xylary fibres, suggesting that there was either a failure of vessel specification, or a defect in vessel cell size (Figure 6A, C). 'Stripes' of differentiation-defective cells overlying some vascular bundles in \textit{bp} mutants gave rise to two distinct vascular morphologies (Figures 5B, 6B); Within the stripes xylem differentiation was altered because as with \textit{blr} mutants, xylem lacked large vessels, however, in contrast to \textit{blr} mutants, many of the smaller cells had not generated secondary cell walls \cite{22,42} (Figure 6B). In areas outside the differentiation defective

<table>
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<th>AGI number</th>
<th>Common name</th>
<th>Expression</th>
<th>BLR-OX</th>
<th>WT</th>
<th>Fold Change</th>
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<td>At1g10070 1,4</td>
<td>Branched-chain amino acid aminotransferase 2 (BCAT-2)</td>
<td>469 ± 320</td>
<td>47 ± 12</td>
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<td>At4g30270 1,2,4</td>
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<td>522 ± 38</td>
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<td>Glutamine dependent asparagine synthetase 1</td>
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<td>281 ± 41</td>
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<td>At1g75750 1,4</td>
<td>GA-responsive GAST1 protein homolog (GASA1)</td>
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<td>700 ± 357</td>
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<td>At5g56870 1,4</td>
<td>Beta-galactosidase 4 (BGAL4)</td>
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<td>265 ± 57</td>
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<td>1110 ± 353</td>
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<td>3685 ± 133</td>
<td>2007 ± 193</td>
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<td>At4g36850 2,4</td>
<td>PQ-loop repeat family/transmembrane family protein</td>
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<td>At1g53910 1,4</td>
<td>AP2 domain-containing protein RAP2.12</td>
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<td>Lipid transfer protein 6 (LT6P)</td>
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<td>At1g21890 1,4</td>
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<td>775 ± 194</td>
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<td>At3g08770 1,4</td>
<td>Lipid transfer protein 6 (LT6P)</td>
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<td>359 ± 61</td>
<td>598 ± 66</td>
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</table>

Genes with putative roles in extracellular matrix regulation (GO analysis), altered expression in \textit{bp} mutants \cite{36} or having a BLR consensus binding site in the promoter (http://arabidopsis.med.ohio-state.edu/AtcisDB/) are shown.

1Role, or putative role in modification of the extracellular matrix.
2Reduced expression in \textit{bp} compared to wild-type plants (Mele et al., 2003).
3Increased expression in \textit{bp} compared to wild-type plants (Mele et al., 2003).
4BLR DNA binding consensus sequence present in promoter.

Expression values are presented as the mean ± standard error for three biological replicates.

Table 1 Genes prominently mis-regulated in BLR-OX

Genes with putative roles in extracellular matrix regulation (GO analysis), altered expression in \textit{bp} mutants \cite{36} or having a BLR consensus binding site in the promoter (http://arabidopsis.med.ohio-state.edu/AtcisDB/) are shown.

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1Role, or putative role in modification of the extracellular matrix.
2Reduced expression in \textit{bp} compared to wild-type plants (Mele et al., 2003).
3Increased expression in \textit{bp} compared to wild-type plants (Mele et al., 2003).
4BLR DNA binding consensus sequence present in promoter.

Expression values are presented as the mean ± standard error for three biological replicates.
stripe, vascular patterning was indistinguishable from wild type (Figure 5B).

bp blr vascular tissue has been described as reminiscent of that present in differentiation defective areas of bp mutants [3]. In bp blr bundles outside the differentiation-defective stripe, small darkly staining cells as described by Smith et al. [3], were observed towards the centre of the stem (Figure 5F) suggesting that these cells were derived early in vascular development. However, xylem cells generated later in development (closest to the procambium) underwent significant differentiation in bp blr double mutants because secondary cell walls were present, but as with extreme blr mutants, all xylem cells were reminiscent of xylary fibres (Figure 6D). In contrast, we observed very few xylem cells with secondary walls in bp blr vascular bundles within the differentiation-defective tissue associated with the bp mutation, even at this late stage of development (Figures 5F, 6D). Consequently, and consistent with other reports [3], blr enhances bp in the context of the vascular tissue and this interaction is particularly evident in mature bp blr vascular tissue within differentiation-defective regions of the stem (Figures 5A-C, 5F, 6A-D). We reasoned that analysis of bp and blr mutant combinations in a background of altered xylem identity may reveal redundant functions of bp and blr. REV has been associated with radial patterning in the stem [24] and has previously been shown to be required for development of interfascicular fibres [25,44], for xylem differentiation and maintenance of xylem cell number [25]. The role of REV in the xylem is likely to be partially redundant as phenotypes appear enhanced in rev phb/+ or rev phv mutants [45]. Furthermore in the shoot apex, as with bp [13] and blr [2], rev enhances the phenotype of weak alleles of stm [46] suggesting that REV may have overlapping functions with BLR. We therefore examined inflorescence stem vascular tissue in bp, blr and rev combinatorial mutants. Within rev vascular bundles xylem tissue resembled wild type in that both vessels and parenchyma were present, however the arc of procambium was shallower as in blr plants (Figure 5D), a phenotype also occurring in bp rev mutant vascular tissue, outside of differentiation defective tissue (Figure 5G). Within differentiation defective tissue, the xylem cells were generally similar to those of bp single mutants except that the relatively few
cells that developed secondary walls were extremely small compared with those of bp (Figure 6F). blr rev and blr vascular bundles were similar but, strikingly, the xylem in vascular bundles of blr bp rev triple mutants comprised small and darkly stained xylem cells, whether or not the bundles lay within the stripe of differentiation-defective cells (Figures 5H, 6G). In extreme cases, no cells with secondary walls were seen in vascular bundles (Figure 5H).

Genetic interactions between BLR, REV and BP in the stem vary depending on tissue type. Vascular cell division between vascular bundles is one of the hallmarks of secondary growth (radial expansion). BP and BLR repress this process as bp blr mutants have a continuous ring of vascular tissue [3]. However, REV appears to be required for the large increase in vascular tissue present in bp blr because bp blr rev lines demonstrate a reduction in vascular tissue compared to bp blr (Figure 5F, H). This observation supports a role for REV in contrast to BP and BLR in promoting secondary growth, a function that has previously been suggested for its poplar orthologue [47]. BLR, REV and BP do not have contrasting roles in regulating xylem differentiation. The presence of xylem cells with secondary walls was reduced in double and triple mutant combinations (Figure 6) suggesting that BLR, REV and BP act together in this process. Consequently, BLR, REV and BP interactions are context dependent.

Although the primary defect observed in BLR-OX was one of reduction in cell size, similar to that observed in vascular tissue of blr mutant combinations, the relationship between cell size and expression of particular cell wall modifying genes is not always straightforward [48–53]. For example, over-expression of an E2ff transcription factor causes a reduction in cell size, accompanied by a decrease in expression of expansins (EXP3, 7 and 9), a UDP-glucose-glucosyl transferase (UGT) and MERI-5 [52] (up-regulated 4.6 fold in BLR-OX). However, over-expression of MERI-5 is reported elsewhere as promoting precocious elongation [54]. BLR control of the extracellular matrix is thus likely to be similarly complex. BLR-OX plants have smaller cells than those of wild-type, but blr mutants, when combined with bp and rev also resulted in vascular tissue with smaller cells.
Conclusions
Control of cell expansion is essential for normal plant development. Our results demonstrate that BLR controls cell expansion and differentiation in vascular development in a process also involving BP and REV transcription factors. Microarray analysis of plants over-expressing BLR indicates that BLR targets many genes involved in cell wall regulation. As dynamic control of the cell wall is essential for cell expansion, we suggest that the striking phenotypes of plants over- and under-expressing BLR may result from the disruption of this process.

Closely regulated cell wall dynamics are also required for a normal pattern of organ initiation at the shoot apex and this process has previously been shown to be regulated by BLR repression of PME5 [19]. Our experiments with BLR-GR suggest that further transcriptional targets of BLR may also play important parts in this process.

Methods
BLR overexpression
Full-length BLR cDNA was cloned into pGEM-T Easy (Promega), subcloned as an EcoRI fragment into pART7 [55] and sequenced to identify plasmids with BLR in the sense orientation. The resultant CaMV35S::BLR::OCS-terminator cassette was subsequently excised from pART7 using NotI and subcloned into pMLBART. The resultant BLR-OX binary vector was introduced into Arabidopsis (Col-0) using standard methods [56]. For steroid-
inducible BLR lines, two PCR products were amplified, one containing BLR cDNA and a second containing the glucocorticoid receptor (GR). The GR forward primer (AAAAAGCAGGCTTAATGACCACTCAATCGT) contained a partial attB1 site, while the reverse primer (GCTCCACCTCCATCTCATGCT) contained an Ala–Gly linker and part of the 5′ BLR sequence. Similarly, the forward BLR primer (GGAGGTGGGCTGCAATGG) contained the Ala–Gly linker while the reverse primer (AGAAAGCTGGGTCAAACC TACAAAATCATG) contained an attB2 site. The two PCR products were mixed in equimolar concentrations and used as templates for overlap PCR with attB1 and attB2 primers resulting in a BLR-GR in-frame fusion, which was cloned into pDONR Zeo, sequenced, and subcloned into pEarleyGate100 using Gateway technology. BASTA-resistant Col-0 transgenic lines were generated as described above, and Arabidopsis transformants that had a wild-type phenotype when uninduced but responded to 10-μM dexamethasone were used for subsequent analysis.

Gene expression analysis
For Microarray experiments total RNA was extracted from Col-0 and BLR-OX plants using an RNeasy Plant Mini Kit (Qiagen). RNA integrity was checked on a 2100 BioAnalyzer (Agilent, CA, USA) prior to cDNA synthesis from 2 μg total RNA using a 3DNA Array 900™ kit (Genisphere Inc., Hatfield, PA, USA) with RT-primers that contained a dendrimer [57] capture sequence. Labelled cDNAs were hybridised for 16 hours to the Arabidopsis Atv3.3.2.z. Array printed with the Operon Arabidopsis thaliana version 3 Array-Ready Oligo Set (Galbraith, USA) using a Slidebooster (Advalytix, Brunthald, Germany) with a power setting of 27 and a pulse setting of 7/3. Array slides were washed in 2× SSC, 0.2% SDS (10 min, 55°C), 2× SSC (10 min, Room temp.) and 0.2× SSC (10 min, room temp.). Fluorescent-labelled Genisphere 3DNA dendrimers containing approximately 850 Cy3 or Cy5 dyes were hybridized for 4 hours to the arrays. Following a set of washes as above, arrays were scanned using a ScanArray Express HT Microarray Scanner (Perkin Elmer).

After calibration to obtain scans within the optimum dynamic range, image analysis and spot feature quantification were performed using BlueFuse version 3.1 (BlueGnome, Cambridge, UK). Data from each slide were confirmed to be essentially linear and globally median-normalized before further analysis. Spots were excluded when the combined confidence values of biological replicates was <0.005. Microarray data were median-normalized in Cy5 and Cy3 channels. Spots that showed expression changes in the same direction in all three replicates were analysed further.

Normalized Cy5/Cy3 log₂ ratios (three values for each gene for BLR-OX lines and three for Col-0) were used to perform a significance analysis of microarray (SAM) [30] using the TIGR Multi Experiment Viewer (TMEV V3.0.3). The imputation engine was set as 10-nearest neighbour imputer and the number of permutations was 100. The delta value in the Significance Analysis was adjusted so that estimated false discovery rate (FDR) was 0% for significant genes. The expression-fold change was set as 1.5 as a threshold for changes of potential biological importance. The microarray data described here have been submitted to MIAMExpress (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-852). GO annotation analysis was performed using http://www.arabidopsis.org/tools/bulk/go/index.jsp.

For northern blot analysis of primary transformants, two micrograms of total RNA extracted from leaf tissue was subjected to electrophoresis on a 1.2% formaldehyde agarose gel, which was subsequently blotted onto Hybond-NX membrane and hybridised with a full-length 32PdCTP-labelled cDNA probe. RNA was also visualised by ethidium bromide staining and the filter hybridised with GAPDH as loading controls. Validation of microarray targets was carried out on RNA from 6–7-day-old seedlings. Primers were used to amplify the 3′ UTR and adjoining exons for specific probes, which were 32PdCTP-labelled.

qRT-PCR analysis was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma) and an ABI Prism 7000 machine (Applied Biosystems) with the standard sybr green detection programme. A melting curve was produced at the end of every experiment to ensure that only single products were formed. Gene expression was determined using a version of the comparative threshold cycle (Ct) method. The average amplification efficiency of each target was determined using LinReg [58] and samples were normalised to 18S rRNA (not shown) and ACT2 (shown). Results were similar independent of control used. Samples were measured in technical triplicates on biological triplicates.

Plant materials, growth conditions and imaging
Plants were grown in long-day conditions at 20°C. Mutant lines, blr (van-1) [2], rev-6 [46], and bp-1 [22,42], have been previously described. Digital images of the plants were analysed on Scion Image (Scion Corporation, Maryland, USA) for hypocotyl measurements. For histological analysis of BLR-OX and BLR-GR, plants were fixed, wax-embedded and sectioned using standard methods. Sections between 6 and 10 micrometers were stained with toluidine blue for light microscopy. For visualisation of hypocotyl epidermal cells, seedlings were stained with 10 μg ml⁻¹ propidium iodide (Molecular Probes Europe BV, Netherlands) for 10 minutes in the dark before transfer to water. Seedlings were viewed by confocal microscopy on a Zeiss (Welwyn, UK) CLSM 510.

Toluidine blue stained resin sections for visualisation of transverse vascular bundle sections in inflorescence
stems were taken from 5 week old plants. Sections were taken above the first cauline leaf and prepared as described in [59].

Additional file

Additional file 1: Genes changed in expression in BLR-OX in the same direction in all 3 replicates with an average of >1.5-fold change, arranged in order of spot confidence.

Authors’ contributions

AMB and HGD designed the experiments. JPE, LM, W2J, HP, NC, NS and AMB carried out the experiments. JPE, AMB and HGD wrote the manuscript. All authors read and approved the final manuscript.

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