Phylogenetic Analyses of the BARREN STALK1/LAX PANICLE1 (BA1/LAX1) Genes and Evidence for Their Roles During Axillary Meristem Development

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Abstract

The diversity of plant architectural form is largely determined by the extent and duration of axillary meristem (AM) derived lateral growth. The orthologous basic helix-loop-helix (bHLH) proteins maize BARREN STALK1 (BA1) and rice LAX PANICLE1 (LAX1) are essential for the formation of AMs during vegetative development and all lateral structures during inflorescence development, but whether BA1/LAX1 co-orthologs exist outside of the grass family is unclear. Here, we present Bayesian phylogenetic evidence of a well-supported BA1/LAX1 clade comprised monocots and eudicots, estimating an origin for the lineage at least near the base of flowering plants. Genomic analyses in Arabidopsis, papaya, medicago, rice, sorghum, and maize indicate that BA1/LAX1 genes reside in syntenic regions, although there has also been a complex pattern of gene duplication and loss during the diversification of the angiosperm clade. BA1/LAX1 mRNA expression coincided with the initiation of leaves and associated AMs in the vegetative meristems of broccoli, medicago, and papaya implicating a role for the lineage in the formation of AMs in eudicots as well as monocots. Expression on the adaxial surface of lateral inflorescence structures was conserved in all sampled flowering plants, whereas mRNA expression in leaves of Arabidopsis, broccoli, and papaya also links BA1/LAX1 co-orthologs with roles in regulating leaf development, possibly as a downstream target of auxin regulating genes. Together these data point to roles for BA1/LAX1 genes during AM formation, leaf, and inflorescence development in diverse flowering plants and lend support to the hypothesis that the same genetic mechanisms regulate the development of different AM types.

Key words: basic helix-loop-helix (bHLH), evolutionary developmental genetics, genome evolution, axillary meristems, auxin.

Introduction

Plants exhibit a great diversity of architectural forms, ranging from single-stemmed herbs and trees to highly branched shrubs. Much of this architectural diversity is determined by the extent and duration of secondary branching during shoot development (McSteen and Leyser 2005; Schmitz and Theres 2005; Oikawa and Kyozuka 2009). Secondary axes of growth, such as vegetative branches, inflorescence branches, and flowers are derived from the growth of meristems in the axils of leaves or bracts (Okada et al. 1991; Reinhardt et al. 2003; McSteen and Leyser 2005; Schmitz and Theres 2005; Evert and Esau 2007; Oikawa and Kyozuka 2009). Two hypotheses have been proposed to describe the mechanisms regulating axillary meristem (AM) development. The first proposes that AMs develop from either 1) a set of cells that bud off from the shoot apical meristem (SAM) and maintain meristematic activity in the axils of initiating leaf primordia (detached meristems) or 2) a set of differentiated cells that regain meristematic activity (de novo meristems) (Snow M and Snow R 1942; Steeves and Sussex 1989; Long and Barton 2000; Evert and Esau 2007). This hypothesis proposes detached and de novo AMs form via different and distinct mechanisms. In the detached model, AM cells are different from their neighbors and retain meristematic activity, whereas in the de novo model, AM cells are equivalent to their neighbors and attain meristematic capacity only after they receive an external signal (Greb and Bleecker 2000; Long and Barton 2000). Most taxa are described as having either detached or de novo AMs (Steeves and Sussex 1989; Evert and Esau 2007), but the second hypothesis proposes that the two models are opposite extremes of the same underlying mechanism (Leyser 2003; Bennett and Leyser 2006). Support for this hypothesis comes from developmental analyses in Arabidopsis and tomato which produce a diversity of AMs within the same individual (Leyser 2003; Bennett and Leyser 2006) and from genetic analyses of LATERAL SUPPRESSOR co-orthologs in Arabidopsis, tomato, and rice that regulate the development of both detached and de novo AMs in the respective taxa (Schumacher et al. 1999; Greb et al. 2003; Li et al. 2003; Bennett and Leyser 2006). It is currently unknown whether species possessing...
detached, de novo, and intermediate AMs are widespread in vascular plants, but the identification of additional genes that regulate development of diverse AM types will lend further support to the hypothesis proposing that AM development is regulated by the same underlying mechanisms.

The phytohormone auxin is essential for the initiation of AMs and the regulation of secondary branching (Benjamins and Scheres 2008; Mockaitis and Estelle 2008; McSteen 2009). Auxin maxima form where AM primordia will initiate and are depleted once growth occurs causing a new auxin sink to form at the site of the next primordium (Reinhardt et al. 2003; Heisler et al. 2005; Gallavotti et al. 2008). Several genes have been identified that regulate auxin biosynthesis or transport, with loss of function mutants characteristically having a pin-shaped inflorescence phenotype with few or no flowers (McSteen 2009). Two such mutants in grasses are rice lax panicle1 (lax1) and maize barren stalk1 (ba1). In strong rice lax1 mutants, the number of inflorescence and vegetative branches (tillers) is reduced and the initiation of lateral spikelets is completely suppressed (Komatsu et al. 2003; Oikawa and Kyozuka 2009). Maize ba1 mutants lack tillers and female inflorescence branches (ears), and the male inflorescence (tassel) is unbranched, shorter than wild type, and almost completely devoid of spikelets (Ritter et al. 2002; Gallavotti et al. 2004). LAX1 and BA1 are orthologous basic helix-loop-helix (bHLH) transcription factors (Malcomber et al. 2006). BA1 is expressed in a cup-shaped pattern above initiating AMs during maize inflorescence development (Gallavotti et al. 2004) and is hypothesized to integrate a signal from the suppressed bract with upstream components of the polar auxin transport pathway to create the auxin maximum required for the formation of a new AM (Gallavotti et al. 2008). During vegetative development, BA1 is expressed on the adaxial surface of developing tiller buds linking mRNA expression patterns with the tiller-less ba1 mutant phenotype (Gallavotti et al. 2004). Rice LAX1 RNA is also expressed in a cup-shaped pattern on the adaxial surface of initiating primordia during vegetative and reproductive development (Komatsu et al. 2003) with the onset of LAX1 mRNA expression coinciding with the formation of AMs in the axes of P4 (Oikawa and Kyozuka 2009). It is noteworthy that based on the rice lax1/LAX1 and maize ba1/BA1 mutant phenotypes/mRNA expression patterns that meristem fate is determined by transcripts that are not expressed within the meristem itself (Kellogg 2007). Oikawa and Kyozuka (2009) resolved this apparent conundrum by demonstrating that outgrowth of the AM only occurs once LAX1 protein has been trafficked into the site of primordium initiation from the arc of surrounding LAX1 mRNA. Whether this two-step regulation of AM development by BA1/LAX1 is conserved in other taxa remains to be tested.

Thus far, attempts to identify BA1/LAX1 co-orthologs in eudicots using phylogenetic methods have produced mixed results. Li et al. (2006) identified 167 bHLH genes in rice and 162 genes in the Arabidopsis genome and used a neighbor-joining phylogenetic analysis to place OsLAX1 as an early diverging member of clade “A” and only distantly related to similar Arabidopsis genes. In contrast, neighbor-joining phylogenetic analysis of 118 Arabidopsis and 131 rice bHLH by Buck and Atchley (2003) and the recent maximum likelihood analysis of 638 bHLH genes from Arabidopsis, poplar, rice, moss, four green algae, and one red alga by Carretero-Paulet et al. (2010) both estimate that the BA1/LAX1 clade comprises eudicots and monocots. However, support for this relationship was either weak (Buck and Atchley 2003) or not reported (Carretero-Paulet et al. 2010). Identification of BA1/LAX1 co-orthologs in other taxa and their putative functions during plant development has yet to be explored.

In this paper, we use a combination of phylogenetics and genomic approaches to investigate the evolution of the BA1/LAX1 gene lineage and identify BA1/LAX1 co-orthologs in diverse eudicots and monocots. We then use comparative expression analyses in grasses and eudicots to investigate the roles of BA1/LAX1 genes in AM, leaf, and inflorescence development.

Materials and Methods

Gene Isolation

Plants were grown in the California State University—Long Beach (CSULB) greenhouses under standard conditions. Total RNA was extracted from inflorescences, leaves, and vegetative apices using a hot phenol protocol (Janssen et al. 1998) and then DNAsed (DNAFree kit; Ambion Inc., Austin, TX). Total DNA was extracted using a cetyltrimethylammonium bromide-polyvinylpyrrolidone (Lodhi et al. 2004) or GenCatch Plant Genomic DNA purification Kit (Epoch Life Science, Sugar Land, TX). cDNA was generated from total RNA using Superscript III reverse transcription kits (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. BA1/LAX1 and SHOOT MERISTEMLESS (STM)-like genes were isolated using either a semi-nested rapid amplification of cDNA ends–polymerase chain reaction (RT-PCR) approach anchored using a polyT + adaptor primer (5’- CCG CAT CCT CTA GAG CGG CCG CTT TTT TTT TTT TTT V-3’) or using species-specific primers with the reverse primer located in the 3’ untranslated region (UTR) (supplementary file 1, Supplementary Material online). All primers were designed using Primaclade (Gadberry et al. 2005). PCR products were cleaned using silica spin columns (Epoch Biolabs, TX) and subcloned using a pGEM-T vector kit (Promega, Madison, WI). Both DNA strands were then sequenced using standard dideoxy sequencing protocols. Sequence data from this article have been deposited with the GenBank Data Libraries under the accession numbers: HM855961–HM855969.

Phylogenetic Analysis

BA1/LAX1-like genes were identified by Blast searches at CoGe (http://synteny.cnr.berkeley.edu/CoGe), NCBI (http://www.ncbi.nlm.nih.gov), Phytozome (http://www.phytozome.org), and PlantGDB (http://www.plantgdb.org) (all URLs last accessed February 19, 2011). The sequence alignment was assembled and translated into an amino acid sequence using MacClade 4.0 (Maddison DR and Maddison WP 2003) and then aligned using MUSCLE (Edgar 2004)
before being manually adjusted using MacClade 4.0. Bayesian phylogenetic analyses used MrBayes 3.2 (Ronquist and Huelsenbeck 2003) on the Grethor parallel processing cluster at the University of Missouri—St. Louis and consisted of two separate searches of 20 million generations using flat priors and the General Time Reversible (GTR) model of sequence evolution with invariant sites and gamma-distributed rates (GTR + I + G) partitioned according to coding position with trees being sampled every 1,000 generations. Convergence between the two runs was determined by examining the average standard deviation of the split frequencies. After convergence had been assured, the first 25% of trees were removed as burn-in and clade credibility values estimated using MrBayes.

Comparative Genomics
Evidence of conserved gene order in syntenic chromosomal regions of Arabidopsis thaliana, Carica papaya, Medicago truncatula, Oryza sativa, Sorghum bicolor, and Zea mays were investigated using the GEvo software package within CoGe (Lyons and Freeling 2008).

Expression Analysis
RNA in situ hybridization was conducted on developing vegetative apices, leaves, and inflorescences using 3′ UTR probes derived from reverse transcription polymerase chain reaction (RT-PCR) gene fragments as described by Malcomber and Kellogg (2004). Slides were imaged using an Olympus BX51 compound microscope with a DX4 digital camera. Hybridizations were repeated at least three times to check for consistency. Images were adjusted for white balance using Adobe Photoshop CS3.

Scanning Electron Microscopy
Inflorescences from a wide range of developmental stages were harvested and fixed in FAA (3.7% formalin/5% glacial acetic acid/42.5% ethanol v/v) before being dehydrated through a graded ethanol series and critically point dried using a Tousimis Samdri-PVT-3D. Samples were sputter coated with gold/palladium and imaged on an FEI Quanta 200 environmental scanning electron microscope (ESEM).

Results
Bayesian Phylogenetic Analyses Estimate a Well-Supported Eudicot and Monocot BA1/LAX1 Clade
Relationships among the 62 BA1/LAX1-like bHLH genes isolated from nonmodel species and identified from Blast searches were estimated using Bayesian phylogenetic methods from an alignment comprising the 198 bp bHLH region plus an additional 132 bp of aligned upstream sequence. This analysis estimated a BA1/LAX1 clade comprised eudicots and monocots (fig. 1), consistent with the recent analysis by Carretero-Paulet et al. (2010). Within the BA1/LAX1 clade, the eudicot, Brassicaceae, Fabaceae, and Salicaceae subclades are all well supported (≥0.95 posterior probability (PP); 1.00, 0.99, 0.96, and 1.00 PP, respectively). In contrast, the monocot clade was not strongly supported (0.90 PP) which we attribute to limited phylogenetic signal.

Unresolved relative to the BA1/LAX1 clade (unresolved grade, fig. 1) are five grass bHLH sequences from rice (Os09g045300 and Os08g36740), maize (GRMZM2G045341, GRMZM2G471635), and Brachypodium distachyon (Bd3g38250). Sister to the BA1/LAX1 and unresolved sequence clade is another clade containing three separate lineages: 1) a well-supported (1.00 PP) clade of grass and eudicot sequences including Arabidopsis At4g00120, At5g09750, and Oryza Os08g01700 (Clade A, fig. 1), 2) a well-supported (1.00 PP) clade of grass sequences including Oryza Os01g38610 (Clade B, fig. 1), and 3) a clade of grass and eudicot sequences including Arabidopsis At3g21330 and Oryza Os01g51140 (Clade C, fig. 1). The sister relationship between clades B and C is well supported (1.00 PP), but clade C itself is poorly supported (0.81 PP). The clade of BA1/LAX1, unresolved sequences, plus A, B, and C clades is sister to a clade of eudicot sequences including Arabidopsis At3g50330 and At5g67060 (Clade D, fig. 1), although this sister relationship is not well supported (0.92 PP). These phylogenetic analyses indicate that BA1/LAX1 co-orthologs are distributed across the asterid, rosid, and monocot clades of flowering plants and suggest an origin for the gene lineage at least near the base of flowering plants and potentially deeper within the land plant clade.

Genomic Analyses Estimate Partial Micro-synteny in Monocot and Eudicot BA1/LAX1-Containing Chromosomal Regions
To infer whether the BA1/LAX1 co-orthologs identified in the phylogenetic analyses reside in micro-syntenic chromosomal regions, we used CoGe (Lyons and Freeling 2008) to explore the A. thaliana, C. papaya (papaya), M. truncatula (medicago), rice, S. bicolor (sorghum), and maize genomes (fig. 2). These comparative genomic analyses reveal evidence of partial gene conservation, duplication, and loss in syntenic regions of eudicots (Arabidopsis, medicago, and papaya) and monocots (maize, rice, and sorghum) but support the general hypothesis that the BA1/LAX1 co-orthologs identified from the phylogenetic analysis are also syntenogs (Lyons and Freeling 2008).

Approximately 20 kb upstream of OsBA1/LAX1 in rice is a hypothetical protein, Os01g0830700, containing the domain of unknown function (DUF) 213 that also occurs in syntenic regions in sorghum, maize, and Arabidopsis but is lacking from syntenic regions of papaya and medicago (A, fig. 2). Further upstream of OsBA1/LAX1 are a 2OG-Fe(II) oxygenase protein, Os01g0830500 (B, fig. 2) and Os01g0830300 (C, fig. 2) that are found in syntenic regions of the other sampled grass genomes but are lacking from sampled eudicots. Downstream of OsBA1/LAX1 is an Ubiquitin-domain–containing protein, Os01g0831200 (D, fig. 2) that is also found in syntenic regions of sorghum, papaya, and medicago but is lacking from syntenic regions of maize and Arabidopsis. Further downstream of Os01g0831200 are the ammonium transporters Os01g0831300 (E, fig. 2) and Os01g0831900 (F, fig. 2) that appear to be two serial paralogs in rice and sorghum of single genes in medicago and papaya.
(fig. 2). No ammonium transporters similar to E and F were identified in syntenic regions of Arabidopsis and maize.

The region surrounding AtBA1/LAX1 on Arabidopsis chromosome 5 shows greatest synteny with papaya contig 120, although several genes are also shared with medicago. Ten kilobase pairs upstream of AtBA1/LAX1 are two estimated pyruvate carboxylase proteins that are similar to a single protein in papaya (120.23) and two duplicated proteins in medicago (CT967302_23 and CT967302_19), suggesting an independent loss of one of the duplicate genes in the papaya lineage. No pyruvate carboxylases were identified in syntenic regions of the sampled grass genomes. Immediately downstream of the AtBA1/LAX1 protein is an estimated phosphatidylethanolamine-binding family protein that is also present in papaya but is absent from syntenic regions of the other sampled flowering plants.

Using the genome analysis as a guide, we then asked whether gene structure was conserved among the putative BA1/LAX1 co-orthologs. Although all other BA1/LAX1 genes are composed of a single exon of approximately 450–650 bp with the bHLH domain near the C-terminus of the gene, the Arabidopsis BA1/LAX1 co-ortholog (At5g01310) is annotated as comprising 2.736 kb of nucleotide sequence arranged in six exons and with the bHLH domain near the N-terminus. In papaya, the single exon BA1/LAX1 gene and a second aprataxin-like gene comprising five exons are syntenic with Arabidopsis At5g01310. These two genes are separated by ~20 kb of intergenic sequence in papaya.
but are annotated as a single gene in Arabidopsis. These analyses suggest At5g01310 as currently annotated comprises two genes: 1) the BA1/LAX1 co-ortholog comprising a single exon of 502 bp and 2) an aprataxin-like gene comprising five exons and a total coding sequence of 2234 bp. This interpretation is supported by RT–PCR analyses (supplementary file 2, Supplementary Material online).

Broad Level mRNA Expression Patterns of BA1/LAX1 Genes in Grasses and Eudicots

The identification of BA1/LAX1 co-orthologs from other monocots and eudicots allows us to investigate the extent of expression conservation and divergence in taxa spanning the flowering plant clade. We first used RT–PCR with species-specific BA1/LAX1 primers on inflorescence and leaf cDNA to investigate broad level patterns of gene expression in: S. bicolor, a relative of maize in the grass subfamily Panicoideae, and three eudicots, medicago (Fabaceae, Fabales), and two members of the Brassicales, papaya (Caricaceae), and broccoli (Brassicaceae) (fig. 3). BA1/LAX1 was expressed in inflorescence tissue in the four sampled taxa and not in the leaves of sorghum and medicago, consistent with published analyses in rice and maize (Komatsu et al. 2003; Gallavotti et al. 2004). In contrast, RT–PCR analyses detected BA1/LAX1 expression in leaf material of papaya and broccoli suggesting a potential role for BA1/LAX1 genes during leaf development of these related taxa with dissected leaf margins.

BA1/LAX1 mRNA Is Expressed on the Adaxial Surface of Developing Lateral Structures in Grass Inflorescences

BA1/LAX1 mRNA is expressed as an arc above lateral meristems throughout vegetative and inflorescence
development in rice and maize (Komatsu et al. 2003; Gallavotti et al. 2004; Oikawa and Kyozuka 2009), but the pattern of \textit{BA1/LAX1} expression during the development of other grasses is unknown. To investigate \textit{BA1/LAX1} expression during inflorescence development in other grasses, we conducted mRNA in situ hybridization expression analyses on \textit{Chasmanthium latifolium}, \textit{Hordeum vulgare}, and \textit{S. bicolor}. These grasses span the major diversification of the family and also display a diverse range of inflorescence branching patterns.

\textit{Sorghum bicolor} (subfamily Panicoideae, PACCMAD clade) has up to six orders of branching with branches initiating and branching repeatedly early in development and continuing to produce spikelet pairs, spikelets, and florets only later in development (fig. 4A, B). We detected an arc of \textit{S. bicolor} \textit{BA1/LAX1} (\textit{SbBA1/LAX1}) mRNA expression on the adaxial surface of all initiating branches and also between the upper and lower florets of the two flowered spikelets (fig. 4C–E), similar to published expression patterns in maize (Gallavotti et al. 2004).

FIG. 3. Inflorescence, leaf morphologies, and \textit{BARREN STALK1/LAX1 PANICLE1} (\textit{BA1/LAX1}) RNA RT-PCR expression patterns in inflorescences and leaf tissues of broccoli, papaya, medicago, and sorghum. (A–B) Broccoli (\textit{Brassica oleracea} subsp. \textit{italica}) inflorescence (A) and leaf (B) morphology. (C–D) Papaya (\textit{Carica papaya}) inflorescence (C) and leaf (D) morphology. (E–F) Medicago (\textit{Medicago truncatula}) inflorescence (E) and leaf (F) morphology. (G–H) Sorghum (\textit{Sorghum bicolor}) inflorescence (G) and leaf (H) morphology. (I) \textit{BA1/LAX1} RT-PCR expression patterns in inflorescence (infl) and leaf tissues of broccoli (Bo), papaya (Cp), medicago (Mt), and sorghum (Sb). cDNA and genomic DNA amplifications of \textit{TRIOSE PHOSPHATE ISOMERASE} (\textit{TPI}) (Sb, Cp, and Bo) or \textit{GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE} (\textit{GAPDH}) (Mt) were included as positive controls. \textit{TPI} and \textit{GAPDH} amplicons both span an intron allowing a test for genomic DNA contamination in cDNA samples.
Chasmanthium latifolium (subfamily Centotheceae, PACCMAD clade) has up to two orders of branching with florets maturing acropetally within the 4- to 24-flowered spikelets (fig. 4G, Malcomber and Kellogg 2004). *Chasmanthium latifolium* BA1/LAX1 (CIBA1/LAX1) expression was detected above initiating meristems similar to that seen in sorghum where it demarcates inflorescence branching events (fig. 4i) and floret initiation within the spikelet (fig. 4j,k). CIBA1/LAX1 expression persists within the spikelet until differentiated stamens are clearly visible (fig. 4k).

The two-rowed variety of *H. vulgare* (barley, BEP clade) used in these analyses is characterized by an indeterminate inflorescence with one-flowered spikelets clustered together in triads on short secondary inflorescence branches (fig. 4M,N). Only the central spikelet is bisexual, whereas the two lateral spikelets are sterile and reduced to awns. *Hordeum vulgare* BA1/LAX1 (HvBA1/LAX1) is expressed within the triad (fig. 4), and floret initiation within the spikelet (fig. 4i). BA1/LAX1 expression persists within the spikelet until differentiated stamens are clearly visible (fig. 4K).

In our sample of phylogenetically disparate grasses, BA1/LAX1 expression is localized to the adaxial surface of all branching events during inflorescence development. These data, coupled with published analyses of inflorescence and vegetative development in rice and maize (Komatsu et al. 2003; Gallavotti et al. 2004; Oikawa and Kyozuka 2009), suggest that mRNA expression and potential roles of grass BA1/LAX1 co-orthologs in regulating the development of lateral structures during both vegetative and inflorescence development are likely conserved in the bulk of grasses.

**BA1/LAX1 mRNA Is Expressed Differently in Eudicots than in Monocots**

To investigate the potential role of BA1/LAX1 genes during eudicot development, we used mRNA in situ hybridization to examine gene expression in developing tissues of broccoli, medicago, and papaya.

*Medicago truncatula* is a scrambling herb with distichous phyllotaxy and peltate trifoliate leaves with a serrate margin. MtBA1/LAX1 mRNA was first detected as a longitudinal stripe through the vegetative apical meristem with expression persisting on the adaxial surface above developing leaves (fig. 5A). Consistent with the RT–PCR analyses, no expression was detected in developing leaves at any stage of development. During inflorescence development, MtBA1/LAX1 was detected on the adaxial surface of developing branches, bracts, and flowers (fig. 5B).

Broccoli is a monocaulous herb with spiral phyllotaxy, dissected simple leaves, and a highly branched inflorescence. BoBA1/LAX1 expression was first detected as a longitudinal stripe through the center of the vegetative apical meristem (fig. 5D) and persisted until P1 was clearly visible (fig. 5E). Later in shoot development, BoBA1/LAX1 expression persisted as an arc in the furrow between the adaxial surface of the developing leaf and the SAM (fig. 5F). Unlike the other taxa described so far and consistent with the RT–PCR analyses, expression was also detected later in leaf development on the adaxial surface as the leaves expanded (fig. 5G). Within inflorescences, BoBA1/LAX1 expression was detected on the adaxial surface of all developing lateral structures, including branches, bracts and flowers, and floral organs (fig. 5H).

*Carica papaya* is a monopodial tree with spiral phyllotaxy, palmate leaves, and short axillary inflorescences. As in broccoli, CpBA1/LAX1 mRNA was detected as an arc in the furrow within the SAM. Furthermore, like broccoli, expression was also detected on the adaxial surface of developing leaves and in the lobes of developing leaflet clusters (fig. 5J). Within developing inflorescences, CpBA1/LAX1 was detected as an arc on the adaxial surface of young floral meristems in the axils of bracts (fig. 5K).

**BA1/LAX1 and SHOOT MERISTEMLESS Expression in Vegetative Apices of Broccoli, Medicago, and Papaya**

Functional analyses reveal that BA1/LAX1 genes are essential for axillary bud initiation and development in model grasses. The onset of BA1/LAX1 mRNA expression in rice coincides with the formation of AMs, as indicated by expression of the meristem marker gene *O. sativa HOMEO-BOX1* (*OsH1*, Oikawa and Kyozuka 2009). To investigate whether expression of BA1/LAX1 co-orthologs are similarly correlated with axillary bud formation in eudicots, we compared expression of broccoli, medicago, and papaya BA1/LAX1 with expression of their respective STM co-orthologs. STM expression is restricted to meristems and is downregulated in leaves in disparate eudicots including *Arabidopsis* (Long et al. 1996), *Lotus* (Fabaceae, Alvarez et al. 2006), *Nicotiana* (Solanaeae, Sakamoto et al. 2001), and *Pisum* (Fabaceae, Hofer et al. 2001).

BA1/LAX1 and STM expression in the longitudinal sections through vegetative apices of broccoli, medicago, and papaya revealed an overlap in BA1/LAX1 expression with STM on the periphery of the STM expression domain (fig. 6), similar to the pattern reported in rice (Oikawa and Kyozuka 2009). However, these analyses also revealed differences between grasses and eudicots. First, rice and maize BA1/LAX1 expression is restricted to developing AMs, but all eudicot BA1/LAX1 co-orthologs were expressed in the SAM and AMs. In the SAM, the onset of BA1/LAX1 expression was correlated with the boundary of the next developing leaf and presumably its associated AM. Second, BA1/LAX1 expression in grasses persists after the initiation and initial outgrowth of the AM, whereas BA1/LAX1 expression in the sampled eudicots is short lived (particularly in medicago) and is only barely detectable in regions containing the presumed AM beyond P2. Lastly, grass BA1/LAX1 genes are expressed as an arc on the adaxial surface of the lateral meristem and, in rice at least, it is...
hypothesized that the LAX1 protein is trafficked into the primordium to initiate the AM and stimulate outgrowth. BoBA1/LAX1 and CpBA1/LAX1 mRNA were expressed more broadly than in grasses with BA1/LAX1 and STM expression overlapping the site of the presumed AM (fig. 6A–D). In contrast, MtBA1/LAX1 expression was only

**Fig. 5.** In situ hybridization of BA1/LAX1 RNA expression in developing tissues of medicago (A–C), broccoli (D–I) and papaya (J–L) using antisense (A–B, D–H, and J–K) and sense (C, I, and L) digoxygenin-labeled RNA probes. (A–C) Developing *Medicago truncatula* shoot material showing expression on the adaxial surface of P1 (A) and within the floral meristem (B). (D–I) Developing *Brassica oleracea* subsp. *italica* vegetative (D–G, I) and inflorescence (H) material showing expression within the vegetative meristem (D–F), in developing leaves (G) and on the adaxial surface of developing stamens (H). (J–L) Developing *Carica papaya* vegetative (J) and inflorescence (K) material showing expression on the adaxial surface of the leaf base and in developing leaflets (J) and on the adaxial surface of an emerging flower (K). Control sections showing lack of hybridization signal using sense probes (C, I, and L). Bars = 100 μm.

**Fig. 4.** Scanning electron microscopy (SEM) analysis (A–B, G–H, and M–N) of inflorescence development and in situ hybridization of *BARREN STALK1/LAX PANICLE1* (BA1/LAX1) RNA expression (C–F, I–L, and O–R) in developing inflorescences of *Sorghum bicolor* (A–F), *Chasmanthium latifolium* (G–L), and *Hordeum vulgare* (M–R) using antisense (C–E, I–K, and O–Q) and sense (F, L, and R) digoxygenin-labeled RNA probes. (C–E) Developing *Sorghum bicolor* inflorescence material showing expression on the adaxial surface of inflorescence branches (C–D), spikelets and florets (E). (I–K) Developing *C. latifolium* inflorescence material showing expression on the adaxial surface of inflorescence branches, spikelets (I), and florets (J–K). (O–Q) Developing *H. vulgare* inflorescence material showing expression on the adaxial surface of inflorescence branches, spikelets, and florets (O–Q). Control sections showing lack of hybridization signal using sense probes (F, I, and R). Bars, 100 μm.
visible in the apical meristem and no evidence of expression surrounding AMs was detected later in development (fig. 6E–F), suggesting a difference in BA1/LAX1 expression within eudicots between the Fabales and Brassicales clades.

BA1/LAX1 and PINFORMED1 (PIN1) mRNA Are Coexpressed in Arabidopsis Leaves

Hay et al. (2006) have shown that PIN1-directed auxin maxima are essential for the formation of leaf serrations in Arabidopsis, and Koenig et al. (2009) demonstrated PIN1 and auxin maxima are essential for the formation of leaflets in tomato leaves. In maize, BA1 and ZmPIN1 proteins interact (Skirpan et al. 2008), with BA1 inferred to be a downstream target of ZmPIN1 (Wu and McSteen 2007), but it is unknown whether BA1/LAX1 and PIN1 also interact in eudicots. Using in situ expression analyses, we estimated colocalization of AtBA1/LAX1 and AtPIN1 mRNA in developing serrations along the margin of developing Arabidopsis leaves (fig. 7). Although these data are unable to confirm whether the polar auxin transport pathway described in maize is conserved in eudicots, overlapping expression of AtBA1/LAX1 and AtPIN1 mRNA demonstrates at least a potential for AtBA1/LAX1 and AtPIN1 proteins to interact and regulate the formation of auxin maxima during Arabidopsis leaf development.

Discussion

Although BA1/LAX1 genes play a key role in regulating AM development during vegetative and inflorescence development in rice and maize, their roles in other flowering plants has yet to be determined. Our phylogenomic and comparative expression analyses provide evidence of a well-supported monocot and eudicot BA1/LAX1 clade, conservation of BA1/LAX1 mRNA expression in a phylogenetically disparate sample of grass species during inflorescence development, and similar but not identical patterns of expression for BA1/LAX1 orthologs in eudicot shoot development.

BA1/LAX1 and the Initiation of Axillary Meristems

It is unclear based on STM expression patterns whether broccoli, medicago, and papaya have detached or de novo AMs. STM expression within the SAM of all taxa is consistent with the detached mechanism, but these data could also be interpreted as consistent with the hypothesis that de novo AMs form immediately after leaving the meristic zone (fig. 6). BA1/LAX1 expression in broccoli, medicago, and papaya was detected at the edge of the STM expression domain as leaves and the associated axillary buds were initiating within the vegetative SAM (fig. 6). This is different from analyses in rice and maize which are traditionally considered to have de novo AMs where BA1/LAX1 expression is not detected in the SAM (Hubbard

Fig. 6. Comparison of BA1/LAX1 and STM expression in vegetative apices of broccoli (A, B), papaya (C, D) and medicago (E, F). (A, B) Developing Brassica oleracea subsp. italica vegetative shoots showing BoBA1/LAX1 expression on the adaxial surface of developing leaves and associated AMs (A) and BoSTM expression in the SAM and AMs (B) on adjacent sections. (C, D) Developing Carica papaya vegetative shoots showing CpBA1/LAX1 expression on the adaxial surface of emerging leaf primordia and associated AMs (C) and CpSTM expression in the SAM and AMs (D) on adjacent sections. (E, F) Developing Medicago truncatula vegetative shoots showing MtBA1/LAX1 expression bisecting the SAM (E) and MtSTM expression (F) in the SAM but downregulated in the developing leaf primordia. Bars = 200 μm.

Fig. 7. Arabidopsis AtPIN1 (A, B) and AtBA1/LAX1 (C, D) mRNA are co-localized in developing leaf serrations. (A, B) Arabidopsis AtPIN1 antisense probe (A) and sense probes (B). (C, D) Arabidopsis AtBA1/LAX1 antisense (C) and sense (D) probes. Bars = 50 μm.
et al. 2002; Oikawa and Kyozuka 2009). BA1/LAX1 expression was visible in broccoli and papaya until the formation of the AMs in P2, based on STM expression patterns in adjacent sections (fig. 6A–D). In medicago, expression was only visible in the SAM and was not detected in more developed AMs (fig. 6E,F). Although our hypothesis will need to be tested by downregulating the BA1/LAX1 transcript in amenable eudicot taxa using either interference RNA or virus induced gene silencing (VIGS) and testing for the presence of AMs, these expression data coupled with published analyses in rice and maize suggest that BA1/LAX1 genes play a role in AM development of both monocots and eudicots and supports the hypothesis that the same genetic mechanisms regulate different types of AM development.

Is the Polar Auxin Biosynthesis and Transport Genetic Pathway Conserved in Flowering Plants?

By regulating the initiation and subsequent development of lateral structures in plants including leaves, branches, and floral organs, polar auxin biosynthesis and transport play major roles in shaping plant architectural diversity. BA1 is a downstream target of BIF2/PID and ZmPIN1 in maize (Wu and McSteen 2007), but whether this auxin transport genetic pathway is conserved in eudicots has yet to be determined.

Colocalization of AtPIN1 and AtBA1/LAX mRNA in developing serratations of Arabidopsis leaves indicates there is a potential for the two proteins to interact and regulate the formation of auxin maxima necessary for blade outgrowth. Similarly, BA1/LAX1 mRNA expression patterns on the adaxial surface of lateral structures in broccoli, medicago, and papaya and in the leaves of broccoli and papaya are consistent with AtPID and AtPIN1 expression patterns (Galweiler et al. 1998; Benjamins et al. 2001; Furutani et al. 2004; Benjamins and Scheres 2008), suggesting that BA1/LAX1 could also interact with PID and PIN1 in these taxa. However, definitive evidence of an interaction between BIF2/PID and BA1/LAX1 in nongrass taxa and whether BA1/LAX1 proteins also regulate the formation of auxin maxima as in grasses remains to be tested.

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\text{BA1/LAX1 mRNA Is Detected in Some, but Not All, Sampled Eudicots with Dissected Leaf Margins}
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Our RT–PCR and in situ hybridization expression analyses detected BA1/LAX1 mRNA leaves of papaya and broccoli but not in leaves of medicago. If the PIN1-BIF2/PID-BA1/LAX1 pathway regulating polar auxin transport is partially conserved in eudicots, then expression of BA1/LAX1 in expanding leaf blades of broccoli and papaya is consistent with auxin-mediated leaf margin expansion in these members of the Brassicales. However, our inability to detect BA1/LAX1 mRNA in the trifoliate compound leaves with serrate leaf margins of medicago suggests that either the pathway is not completely conserved in rosids or the serrate leaves in medicago are generated via a different mechanism.

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\text{KNOTTED1-LIKE HOMEobox (KNOX) regulation of compound leaf development occurs in most studied flowering plants (Bharathan et al. 2002) with the exception of the Fabaceae Inverted Repeat Lacking Clade (IRLC), containing pea, medicago, Vicia, and Wisteria (Champagne et al. 2007). Within the IRLC clade, analysis of the pea (Pisum sativum) unifoliata (uni) and M. truncatula single leaflet1 (sgl1) mutants demonstrate that FLORICAULA/LEAFY (FLO/LFY) orthologs take on the role of KNOX genes in generating compound leaf morphologies, but not the formation of the serrate margin (Hofer et al. 1997; Wang et al. 2008).

Redeployment of the same auxin pathway required for primordium initiation in the SAM is hypothesized to help produce serrated leaves in Arabidopsis (Hay et al. 2006), but several other genes have also been has been linked with marginal outgrowth including the NO APICAL MERISTEM/CUP-SHAPED COTYLEDON (NAM/CUC) lineage (Blein et al. 2008). Downregulation of the pea NAM/CUC genes using VIGS produced stipules with smoother margins indicating a role for these genes during serrate margin development (Blein et al. 2008). Assuming that BA1/LAX1 orthologs are downstream of BIF2/PID in eudicots as they are in monocots, the lack of MtBA1/LAX1 expression in leaves suggests that BA1/LAX1-regulated auxin transport is not necessary for the formation of serrate leaf margins in medicago. These data are consistent with the findings of Champagne et al. (2007) that compound leaf development within IRLC legume clade is different from compound leaf development in other taxa.

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\text{BA1/LAX1 mRNA Expression During Inflorescence Development}
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BA1/LAX1 regulates the initiation of branches, spikelets, and florets during inflorescence development in rice and maize (Komatsu et al. 2003; Gallavotti et al. 2004; Oikawa and Kyozuka 2009), and the results presented here suggest that BA1/LAX1 orthologs also regulate the formation of lateral structures during inflorescence development in barley, chasmanthium, and sorghum (fig. 4), suggesting that BA1/LAX1 gene function is likely conserved in the vast majority of grasses. It is notable that BA1/LAX1 expression in sampled grasses extends beyond the initiation and initial outgrowth of the meristems. This extended period of expression is not required for the establishment of lateral meristems and suggests that continued mRNA expression (and possibly BA1/LAX1 protein trafficking) might be required for continued auxin-mediated outgrowth.

BA1/LAX1 expression on the adaxial surface of lateral structures in broccoli, medicago, and papaya inflorescences suggests the role demonstrated in grasses is partially conserved in rosids. There are, however, differences in the temporal distribution of BA1/LAX1 mRNA in sampled eudicots compared with the pattern of expression in grasses. In contrast to the extended duration of BA1/LAX1 expression in grasses, expression in sampled eudicots is much more transient and soon undetectable once the lateral structure has started to elongate. These data are consistent with the
hypothesis that BA1/LAX1 expression in eudicots is required for the initiation and early maintenance of AMs but not during later stages of outgrowth.

In this paper, we have used phylogenomic and comparative expression analyses to investigate the evolution of the BA1/LAX1 gene lineage in flowering plants and their roles in regulating AM, leaf, and inflorescence development. Our analyses estimate a well-supported BA1/LAX1 clade comprised both eudicots and monocots with an estimated origin at least near the base of the flowering plants, approximately 125 Ma. Comparative genomic analyses are consistent with results of the phylogenetic analysis and estimate partially conserved gene order among syntenic BA1/LAX1-containing chromosomal regions in Arabidopsis, papaya, medicago, rice, sorghum, and maize. Expression analyses suggest a role for BA1/LAX1 genes in the formation of AMs in all sampled taxa, plus several other periods of shoot development including inflorescence branching and leaf expansion in two of the three sampled eudicot species with divided leaves. The failure to detect BA1/LAX1 expression in the compound serrate leaves of medicago suggests that marginal outgrowth might be regulated by another genetic mechanism. The next step will be to expand the comparative analyses to other flowering plants, particularly non-grass monocots and asterids, to estimate where and when changes in BA1/LAX1 expression and function occurred during the diversification of the flowering plant clade and include biochemical analyses to infer whether BA1/LAX1 genes are downstream targets of polar auxin transport in eudicots as described in grasses.

Supplementary Material
Supplementary files 1 and 2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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