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Review

BTB/POZ ankyrin repeat genes identify leaf homologies in monocots and eudicots

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ABSTRACT

Monocot leaves possess fundamentally different morphologies compared to those of eudicots, making the identification of homologies extremely difficult. Although leaves from both groups originate from the flanks of the shoot apical meristem, each has novel structures that, from first appearance, seem unrelated. For example, many grasses have an epidermally derived fringe of tissue at the blade sheath boundary called the ligule that functions to repel water from the stem. In contrast, the leaves of several eudicots have paired foliar appendages called stipules that have a variety of functions ranging from photosynthesis to plant defense. Recent molecular evidence indicates that the stipule and ligule do in fact share common genetic mechanisms, both requiring expression of ankyrin repeat proteins belonging to the *BLADE-ON-PETIOLE* (*BOP*) clade at the leaf base. Knockouts of *BOP* orthologues in the monocot barley, as well as the eudicots *Medicago*, *Arabidopsis* and pea affect development and initiation of the ligules and stipules, respectively. These results indicate that ligules and stipules may in fact be homologous structures originating from the lower leaf zone, both requiring the formation of boundaries defined by *BOP* genes in order to initiate.

KEYWORDS: leaf, stipule, ligule, *BOP*, ankyrin repeat, BTB/POZ domain

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INTRODUCTION

The profound morphological differences between monocot and eudicot leaves have made establishing homologies difficult. Since monocots are a monophyletic group [1] that branched off from eudicots approximately 140-150 million years ago [2] the two groups must have a common ancestor and presumably share some developmental mechanisms. Indeed, we know that some molecular pathways controlling leaf development are well conserved between monocots and eudicots [3] despite their morphological divergence. Leaves of both groups of plants also share some histological and functional characteristics, as shown by the presence of differing zones of activity, such as those at the lamina and petiole portions of the leaf versus the leaf base [4, 5], also described as the upper and lower leaf zones. Some classical plant morphologists view eudicot and monocot leaves as having radically different developmental programs. For example, the phyllode theory of leaf development posits that monocot leaves are simply elaborated petioles with highly reduced leaf blades [6]. Recently, a group of genes has been described that may at least confirm or challenge some of these morphogenic models in both monocots and eudicots. Due to combined efforts of several labs working with a variety of plant systems, we now have molecular and genetic data supporting the concept of a basal leaf morphogenetic zone as well as a distal boundary that defines it. In addition, these genes can shed light on the putative homology of foliar appendages within these zones, including stipules and ligules.

Taken together, these findings provide a context for understanding the evolution of developmental novelty in plants.

Leaf development in monocots and eudicots

Very few similarities can be found between monocot and eudicot leaves early in development other than the fact that they all initiate as lateral primordia from the flanks of the shoot apical meristem. For example, many eudicot leaves display a variety of paired foliar appendages located at the base of the leaf collectively known as stipules (Figure 1A). A review of the classical literature by Ansel in 1897 [7] indicated that the only common features for stipules are a lateral position located at the base of a leaf, a presumed early function in protection of the associated axillary bud, and frequently shared vasculature with the associated leaf. The origin and homology of eudicot stipules are unclear. Some classical studies considered stipules as accessory leaflets, but others considered them underdeveloped lobes of the lamina blade or even stem scales [7].

Monocot leaves, in contrast, have no such clearly defined structures at their base. Considering only the members of the grass family, these leaves have unpaired structures called ligules that are located farther up the leaf base near the blade sheath boundary (Figure 1B). Ligules prevent the flow of water from the upper blade surface into the space between the sheath and stem, thereby protecting axillary buds in a way different from stipules. In addition, ligules appear to have a distinct mode of development, forming from periclinal cell divisions from the L1 epidermal layer well after leaf initiation [8]. In addition, maize ligules are not vascularized, in contrast to many eudicot stipules. Thus, ligules and stipules occupy different positions in the leaf, appear to initiate using different developmental mechanisms, and have completely different morphologies. Because of these differences, establishing homology between ligules and stipules has been challenging. Some researchers have treated the ligule as an evolutionary novelty with unique origin [9], while others have proposed them to be composite structures of fused stipules to the sheathing leaf base [10]. In 1887, however, Colomb very clearly indicated the potential homology between stipules and ligules when he stated, “Stipules and the ligule are then organs of the same nature, between which it is possible to find all forms of intergradation” [11]. This view was supported by Majumdar who considered ligules and stipules to be homologous structures that are common products of the lower leaf zone [12]. More recently, Mooney and Freeling proposed homology between pea stipules (Figure 1A) and maize ligules (Figure 1B) [13], a view not shared by others [14]. Unfortunately, molecular genetic support for these models has been lacking since genes that specifically function in the lower leaf zone in both monocots and eudicots had not yet been identified.

Genetic establishment of leaf zones in *Arabidopsis*

Support for the classical division of leaves into distinct upper and lower zones came from the identification of novel genes that help establish leaf boundaries. The *Arabidopsis thaliana* genome includes two closely related *BLADE-ON-PETIOLE* (*BOP*) genes named *BOP1* and *BOP2* (Figure 1C). *bop1/bop2* double mutants have misshapen leaves elongated along the proximal-distal axis, and blade outgrowths along the petiole (Figure 2). This phenotype indicates that *BOP1* and *BOP2* establish a boundary between proximal and distal regions of the leaf, and in their absence distal leaf fates are shifted proximally [15, 16, 17]. Accordingly, lower leaf zone appendages such as stipules (that are highly reduced in *Arabidopsis* and visible only via microscopy) are missing in *bop1/bop2* mutants [18] (Figure 2).

Cloning of *BOP1* in *Arabidopsis* showed that it belongs to the BTB/POZ (Broad-complex, Tramtrack, Bric-a-brac/Pox virus and zinc finger) domain protein family [15]. The BTB/POZ domain is a protein-protein interaction domain present at the N-terminus of several transcription factors [19, 20, 21]. Some BTB/POZ transcriptional co-activators have an additional domain ankyrin repeat domain located at the C-terminus. Proteins with ankyrin repeats are known for interacting with a diversity of different proteins with a broad range of functions [22, 23].

Consistent with their functions in the lower leaf zone, *BOP1* and *BOP2* show similar expression patterns near the leaf base. *BOP1* can first be detected in the embryo at the base of the cotyledons

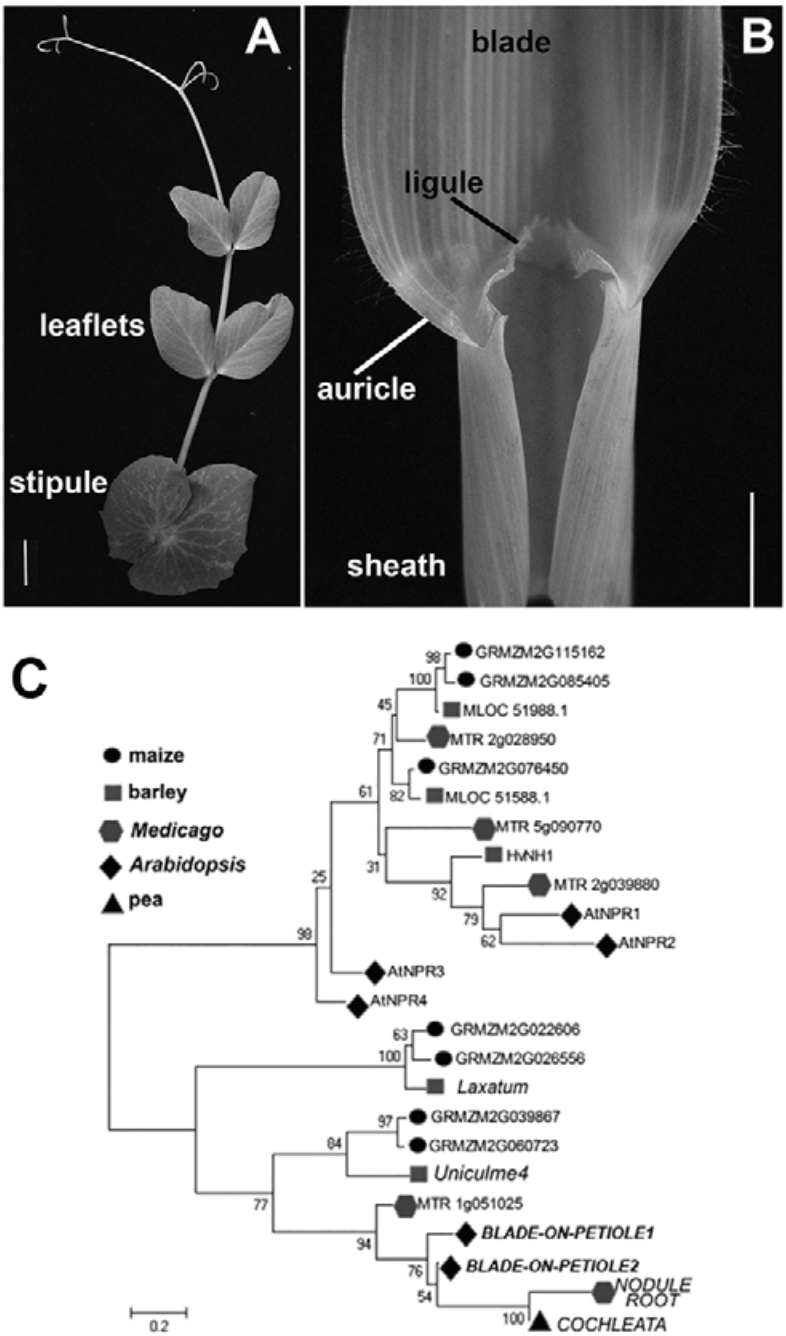

Figure 1. Comparison of eudicot vs monocot leaves and phylogenetic analysis of *BOP* genes. **A.** Pea leaf with proximal stipule and distal leaflets. **B.** Blade sheath boundary of maize leaf with ligule. Blade is distal and sheath is proximal. Scale bar = 1 inch. **C.** Neighbor-joining tree of monocot and eudicot *BOP*-like genes. Scale bar = 0.2 amino acid substitutions per site.

near the boundary with the shoot apical meristem [16]. At later stages of vegetative development, *BOP1* and *BOP2* continue to be expressed at the base of the leaf primordia and petiole. During the floral stage, *BOP1* and *BOP2* were found in early floral primordia, as well as at the base of sepals and petals [16, 24]. Within the cell, BOP1 and BOP2 localize to both the nucleus and cytoplasm

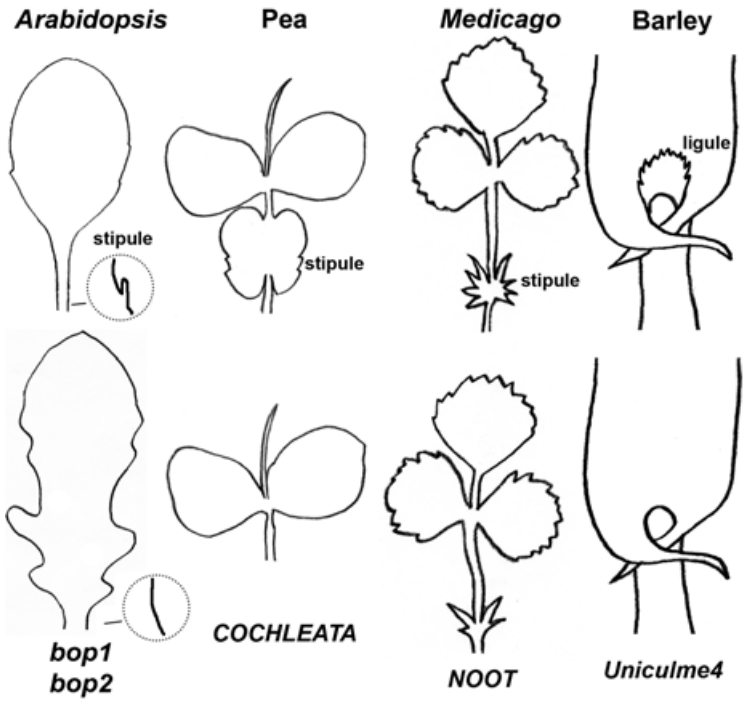

Figure 2. Comparison of *BOP-like* mutant phenotypes in monocots and eudicots. Wildtype on top and corresponding mutants on bottom.

[17, 25], and can form homo- and heterodimers [25].

The molecular mechanism of *BOP* function at the leaf/meristem boundary was revealed through chromatin immunoprecipitation studies. Shoot apical meristem identity is regulated by *knotted1-like homeobox* (*KNOX)* gene activity that maintains cells in an undifferentiated meristematic state [26]. Earlier studies indicated that *BOP1* and *BOP2* repress *KNOX* activity within leaf primordia [15, 24, 27], thus allowing leaf cells to differentiate. This negative regulation of *KNOX* occurs through direct activation of the *KNOX* repressor *ASYMMETRIC LEAVES2* (*AS2*) by the BOP1 protein [25].

Identification of *BOP* homologues in eudicots

All plants with sequenced genomes have genes encoding both BTB/POZ and ankyrin repeat domains similar to BOP1 [28]. While the ultimate functions of these genes varies depending on what proteins they interact with, a subset of them appear to be involved in plant defense. For example, in *Arabidopsis* the BTB/POZ domain has been shown to interact with *TGA* transcription factors, many of which are involved in pathogen response [17, 29]. A phylogenetic tree containing *BTB/POZ ANKYRIN* domain genes from maize, barley, *Medicago*, *Arabidopsis* and pea shows that they fall into two main groups, one involved in plant defense being similar to *Arabidopsis NPR1* (*non-expressor of pathogenesis-related genes1*), and one involved in plant development, being similar to *BOP1* and *BOP2* (Figure 1C). From this analysis, it is clear that closely related *BOP* homologues are present in *Medicago*, pea, and barley. Recent isolation of loss-of-function mutations in these genes sheds light on the conflicting models of ligule and stipule development discussed earlier.

The pea *COCHLEATA* (*coch*) loss-of-function mutant of the *BOP2* homologue was initially described as affecting stipule development [30]. In the first few nodes, *coch* leaves are missing stipules (Figure 2) while upper nodes have modified stipules that can be small and strap-like, knifeblade-like, thread-like, elliptical or spatulate. At higher nodes, the stipules might be replaced by leaflets, a distal to proximal identity shift reminiscent of

bop1/bop2 mutants [30, 31, 32]. Knockouts of *NODULE ROOT* (*NOOT*), the *BOP1/BOP2* ortholog in *Medicago*, also affect stipule formation: mutant stipules are reduced and have fewer serrations than wild type [32] (Figure 2). *In situ* expression analysis showed that *COCH* is localized at the base of the developing pea leaf specifically within stipule primordia [32].

It should be noted that, besides stipule development, mutations in *coch and noot* also affect flower symmetry and floral organ abscission similar to *bop1/bop2* [17, 24, 32]. This finding indicates that genetic modules required for boundary formation in one tissue may be redeployed in different parts of the plant to serve a similar function [33].

BOP mutants in monocots

In order to understand the molecular program underlying ligule development in maize, RNA sequencing was performed on ligule specific libraries [33]. A close maize paralogue of *BOP1* identified in this study, (GRMZM2G060723) (Figure 1C), was enriched in the ligule as well as the sheath, but excluded from the blade. *In situ* hybridization experiments showed this gene to be specifically expressed in the ligule and sheath, thus defining the lower leaf zone boundary. In keeping with the idea that ligule-specific genes have wide functions, GRMZM2G060723 was also expressed in young axillary meristems. While functional analysis of this gene in maize is still pending, the barley orthologue of this gene is mutated in the *Uniculme4* mutant (*cul4*) [34] (Figure 2). *cul4* mutants have little or no tillers, demonstrating that axillary meristem development is defective, perhaps due to loss of the meristem/ leaf boundary. In addition, mutant leaves have no ligule (Figure 2), and show displacement of auricle tissue proximally down the blade. This phenotype shows that *CUL4* is important for establishment of the lower leaf zone boundary much like *BOP1* and *BOP2* in *Arabidopsis*, and in its absence, proximal leaf fates are shifted downwards. Thus, in monocots and eudicots, *BOP* genes are expressed in ligules as well as stipules, and loss of *BOP* gene function affects initiation of both structures (Figure 2), supporting the hypothesis that they are in fact homologues. In light of these results, it is interesting that ligules and stipules occupy different positions within the leaf despite their homology. It is possible that the lower leaf zone in grasses (consisting of sheath and ligule) is greatly expanded, resulting in the ligule being shifted away from the stem up to the blade sheath boundary. While position is a common criteria used to establish homology, in this case it seems clear that it should not be used by itself, but interpreted in combination with other supporting data.

CONCLUSION

Expression of *BOP*-like genes in monocots and eudicots marks the lower leaf zone boundary where they function to prevent the expression of upper leaf zone identities. In addition, these genes are expressed at high levels in various foliar appendages including ligules and stipules. When these genes are mutated, cell fates of upper leaf zones are shifted downwards into the petiole in eudicots, or into the sheath in monocots. This is manifested by the presence of ectopic blade on the petioles of *Arabidopsis*, the appearance of ectopic leaflets at the base of the pea leaf, or the presence of ectopic auricle tissue along the margins of the sheath in barley. The absence of the lower leaf zone in the mutants prevents the formation of foliar appendages normally made in this zone, including stipules in eudicots and ligules in monocots. Taken together, these data imply that stipules and ligules are in fact homologous structures, a finding that was unclear for many years. Thus, molecular genetic analysis can be used with morphological analysis within a wide range of plants to identify presumptive homologies, and ultimately give insights on the evolution of complex structures.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

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