Evolution of polarity protein BASL and the capacity for stomatal lineage asymmetric divisions

Highlights

- BASL is a eudicot-specific regulator of stomatal lineage asymmetric cell divisions
- BASL features polarity domains added to an ancestral MAPK-binding chassis
- Cellular quiescence and BASL-guided polarity generate stomatal spacing in tomato
- Cell size and fate asymmetries are uncoupled in the tomato stomatal lineage

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In brief

Nir, Amador, et al. trace the evolution of Arabidopsis polarity protein AtBASL and show that eudicot proteins of similar domain organization but low sequence conservation polarize and function in Arabidopsis. They show SlBASL also mediates asymmetric divisions in tomato, but the repertoire of epidermal division types diverges between species.
Evolution of polarity protein BASL and the capacity for stomatal lineage asymmetric divisions

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SUMMARY

Asymmetric and oriented stem cell divisions enable the continued production of patterned tissues. The molecules that guide these divisions include several “polarity proteins” that are localized to discrete plasma membrane domains, are differentially inherited during asymmetric divisions, and whose scaffolding activities can guide division plane orientation and subsequent cell fates. In the stomatal lineages on the surfaces of plant leaves, asymmetric and oriented divisions create distinct cell types in physiologically optimized patterns. The polarity protein BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is a major regulator of stomatal lineage division and cell fate asymmetries in Arabidopsis, but its role in the stomatal lineages of other plants is unclear. Here, using phylogenetic and functional assays, we demonstrate that BASL is a eudicot-specific polarity protein. Dicot BASL orthologs can polarize in heterologous systems and rescue the Arabidopsis BASL mutant. The more widely distributed BASL-like proteins, although they share BASL’s conserved C-terminal domain, are neither polarized nor do they function in asymmetric divisions of the stomatal lineage. Comparison of BASL protein localization and loss of function BASL phenotypes in Arabidopsis and tomato revealed previously unappreciated differences in how asymmetric cell divisions are employed for pattern formation in different species. This multi-species analysis therefore provides insight into the evolution of a unique polarity regulator and into the developmental choices available to cells as they build and pattern tissues.

INTRODUCTION

Patterned surfaces adorn representatives of all major clades of multicellular life. These patterns serve functional roles in mature organisms and can bear imprints of the developmental trajectories underlying their production. Asymmetric and oriented stem cell divisions are often employed in the creation and patterning of tissues, particularly when development is flexible and environmentally responsive. In plants, stomatal lineages on the surfaces of leaves are prime models for investigating how cell polarity and asymmetric cell divisions (ACDs) link to tissue-wide patterns.1

In the stomatal lineage of the dicot angiosperm Arabidopsis thaliana, BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is the central regulator of cell size and fate asymmetries.2–4 Asymmetric “entry” divisions among protodermal cells initiate the stomatal lineage (Figure 1A, stages I and II). During these entry divisions, BASL becomes polarly localized in a cortical crescent (Figure 1A, stage II) that guides the division plane to create cells of different size and identity and ensures its own asymmetric inheritance (Figure 1A, stage III). Fate asymmetry between the smaller, meristemoid (M) daughter and larger, BASL-inheriting stomatal lineage ground cell (SLGC) daughter is critical for proper patterning of the leaf epidermis as the subsequent division types available to these cells differ (Figure 1A, stage IV). Through continued “amplifying divisions” in a spiral pattern, meristemoids can surround themselves with a buffer zone of non-meristemoid cells before differentiating into stomata.5 SLGCs can immediately differentiate or generate new meristemoid and SLGC pairs through “spacing divisions” precisely oriented to avoid placing meristemoids next to existing stomata.6 Polarity and cell-cell signaling regulate ACD propensity and orientation, resulting in stomata obeying a “one-cell spacing” rule.7 Loss of BASL has profound effects on division plane placement, self-renewing divisions, and cell fates in meristemoids and SLGCs, resulting ultimately in the accumulation of clustered stomatal precursors and clustered stomata (green and purple shading, respectively, in Figure 1B). A central role for BASL in polarity and ACDs is supported by genetic and protein interaction experiments showing that BASL is required for...
asymmetric localization and inheritance of signaling cascades linked to cell fates as well as other polarity proteins.

Despite a pivotal role in Arabidopsis stomatal lineage development and the fact that asymmetric divisions have been observed in the stomatal lineages of angiosperms, gymnosperms, and ferns, it is not known whether BASL acts in diverse stomatal lineages. This is due, in part, to debates as to whether BASL homologs are even present in other plants. Here, we use a combination of phylogenetic analyses, cross-species complementation, and functional analysis in native contexts to identify likely BASL orthologs. We find proteins that can polarize and function as BASL are restricted to the dicots but are related to more broadly distributed proteins, suggesting that BASL may be built from an ancestral MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) interacting domain, to which polarization capacity was added. Analysis of SlBASL dynamics and loss-of-function mutants in tomato reveal conservation in cell-autonomous BASL roles but unique implementation of those roles in the context of divergent patterning regimes, including novel cell fate transitions and spacing mechanisms. This multi-species analysis provides insight into the evolution of a unique polarity regulator and into the developmental choices available to cells as they build coordinated tissues and organs.

RESULTS

BASL is a rapidly evolving eudicot-specific gene

Previous efforts to reconstruct the evolutionary history of BASL were hampered by low overall sequence conservation beyond close relatives of Arabidopsis, although select protein domains could be found in more distantly related species. Here, we use a combination of phylogenetic analyses, cross-species complementation, and functional analysis in native contexts to identify likely BASL orthologs. We find proteins that can polarize and function as BASL are restricted to the dicots but are related to more broadly distributed proteins, suggesting that BASL may be built from an ancestral MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) interacting domain, to which polarization capacity was added. Analysis of SlBASL dynamics and loss-of-function mutants in tomato reveal conservation in cell-autonomous BASL roles but unique implementation of those roles in the context of divergent patterning regimes, including novel cell fate transitions and spacing mechanisms. This multi-species analysis provides insight into the evolution of a unique polarity regulator and into the developmental choices available to cells as they build coordinated tissues and organs.
BSLLs, but not BSLLs, polarize in developing leaves

BSLL genes have not been characterized in any species. In Arabidopsis single-cell RNA sequencing (RNA-seq) profiles, BSLL1 is limited to young shoot tissue, whereas BSLL2 is more broadly distributed, but their peak expression differs from BASL and from each other (Figure S2A). Outside of Arabidopsis, however, BSLL proteins could be alternatives to BASL. In fact, BSLL gene expression is enriched in the stomatal precursor zone at the base of leaves of rice and maize, two species lacking BASL. Therefore, to characterize BASL and BSLL homologs, we began by assaying the feature that best defines BASL—its polarized subcellular localization, which can be replicated in non-native contexts. We expressed BASL and/or BSLL coding sequences from Arabidopsis thaliana, Solanum lycopersicum, Aquilegia coerulea, and the mono-cot outgroup Brachypodium distachyon in developing Nicotiana benthamiana leaves (Figures S2B and S2C). We found that proteins encoded by putative BASL orthologs from tomato and Aquilegia localized to a polar crescent at the edge of pavement cell lobes in N. benthamiana, much like AtBASL, although only AtBASL accumulated in the nucleus (Figure S2B). In contrast, all BSLL candidates exhibited diffuse cortical localization with no visible polarized domain (Figure S2C).

Based on these data, we selected three candidates for more extensive functional analysis: the tomato protein SlBASL (Solyc3g114770), which conserves all three major domains but displays modest overall sequence conservation (23% AA identity and 50% AA similarity); the Aquilegia protein AqBASL (Aqco7g067300), which conserves only D2 and D3 domains; and the Brachypodium protein BdBSLL1 (Bradi3g47127), which is the protein most similar to AtBASL in that genome but whose similarity is restricted to the D3 domain (see Method details for gene names and gene codes used in this paper).

We expressed these candidates in Arabidopsis as fluorescently tagged fusion proteins under control of the native AtBASL promoter. Both SlBASL (AtBASLpro:VENUS-SlBASL) and AqBASL (AtBASLpro:VENUS-AqBASL) reporters exhibited robust polarization, with the polar domain consistently located in the larger daughter cell post-division, similar to a native AtBASL reporter (Figures 2A–2C). In contrast, the BdBSLL1 reporter (AtBASLpro:VENUS-BdBSLL1) was cortically enriched but failed to polarize and was inherited symmetrically by both daughters following division (Figure 2D).

Figure 2. BASLs, but not BSLLs, polarize and rescue the atbasl mutant

(A–D) Confocal images of BASL and BSLL translational reporters (yellow) in abaxial epidermis of 4 dpg atbasl cotyledons. Cell outlines (magenta) visualized by ML1pro:mCherry-RCI2A are shown. White arrowheads mark cell enlarged in inset to highlight reporter distribution during ACD. Scale bars, 30 μm. Cells were hand outlined in inset (C) for clarity.

Data show quantification of stomatal clustering phenotypes in atbasl as rescued by reporters noted on x axis. For SlBASL, AqBASL, and BdBSLL constructs, results from two independent lines are shown. n = 120 stomata in each of 8–20 leaves. For letters above each sample, see STAR Methods.

(F) Quantification of the origin of stomatal pairs in atbasl and the atbasl; SlBASL rescue line, n, all trackable pairs in each of 4 leaves. Numerical data in (E) and (F) are represented as mean ± 95% confidence interval. Bonferroni-corrected p values from Mann-Whitney U test are shown. See also Figure S2.
The terminal phenotype of Arabidopsis BASL (atbasl) mutants is clustered stomata, which can originate from two distinct errors: misoriented spacing divisions or failures to differentiate cell fates after ACD.\(^{18}\) We measured the ability of BASL and BSSL reporter constructs to rescue the stomatal clustering phenotype of atbasl mutants and observed a graded response: whereas an AtBASL construct nearly perfectly rescued the mutant phenotype (99% rescue capacity; details in STAR Methods), rescue was partial with SiBASL (87%) and modest with AqBASL (50%) constructs (Figure 2E). As expected from its lack of polarization, BdBSLL1 had no significant capacity to correct stomatal clustering (Figure 2E). Finer dissection of SiBASL rescue function through lineage tracing showed that SiBASL nearly completely rescued stomatal pairs that arose from fate errors but was only moderately effective at correcting spacing errors (Figure 2F).

Together, these data suggest that, among dicots, there are true BASL orthologs that retain polarization capacity and the ability to regulate division plane orientation and fate asymmetry during ACDs. BSSLs, as exemplified by BdBSLL1, do not. The apparent lack of functional BASL orthologs in the grasses may be related to the differences in ontogeny of grass stomata, where stomatal guard cell formation is preceded by a single ACD that is invariantly oriented relative to the overall leaf axis.\(^{19}\) Interestingly, we found that an AtBASL reporter expressed in leaves of the pooid grass Brachypodium distachyon polarly localized in crescents oriented toward the base of the leaf and was preferentially expressed in the larger daughter cells resulting from ACDs (Figure S2D). In newly formed stomatal complexes, AtBASL also localized to the junction between guard cells and subsidiary cells (Figure S2D). These behaviors indicate that BASL can recognize tissue-wide polarity information created through other, more ancient polarity networks, even if it is not a functional component of those networks itself.

**SiBASL is polarized and involved in stomatal patterning in tomato**

If proteins sharing AtBASL’s polar localization exist outside of Brassicaceae and can substitute for AtBASL in ACDs of the Arabidopsis stomatal lineage, what is their role in their native species? To address this, we generated reporters and CRISPR-Cas9-induced mutation lines to test localization and function of BASL in another dicot. We chose Solanum lycopersicum (tomato) for these analyses because of its phylogenetic position among dicots, efficient transformation protocols, and the rich history of developmental and physiological studies on tomato leaves and stomata.\(^{20-22}\)

Stomata on the M82 tomato cotyledon and true leaf are spaced to avoid direct contact, and mature organs feature stomata distributed among lobed pavement cells in a pattern resembling that found in mature Arabidopsis leaves (Figures 3A and S3A). Earlier, during cotyledon development (3 days post-germination [dpg] on MS-agar plates), when small epidermal cells with the morphological characteristics of meristemoids are abundant, we begin to see expression of our SiBASL translational reporter (SiBASLpro:VENUS-SiBASL; Figure 3B). In still images, SiBASL appeared in a polarized cortical crescent consistently associated with the larger daughter cell after ACD but lacked the symmetrically inherited nuclear localization typical of AtBASL (Figures 2B and 3B). To monitor SiBASLpro:VENUS-SiBASL dynamics during a single cell division cycle, we used time-lapse imaging, collecting images at 30-min intervals from 3 dpg abaxial cotyledons. As shown for two representative cells in Figure 3C (n = 24 cells tracked), SiBASL first appeared several hours before division. In some cells, SiBASL was polarized before division, but in all cells, the polar signal intensified post-division (Figure 3C, white arrowheads). Given SiBASL’s polar localization and its ability to complement atbasl, we hypothesized that it would be required for stomatal patterning in tomato. We targeted the SiBASL locus for CRISPR-Cas9 mutagenesis using multiplexed guides at the 5’ UTR and exons 2, 3, and 5 and obtained three independent lines bearing mutations at the SiBASL locus (slbasl cr#2, cr#4, cr#6; Figures 3D and S3B). In the T2 generation, plants bearing these slbasl mutations were not noticeably different from control M82 plants in overall size, growth habit, or fertility, but each line displayed stomatal clusters on the epidermis, suggesting that SiBASL function was disrupted (Figures 3A and S3B). For detailed phenotypic and quantitative characterization, we selected the slbasl-cr#4 allele, where a 1-bp deletion in exon 2 is predicted to cause a frameshift and premature stop codon after 63 amino acids (shortly after domain D1; Figures 3D, S3C, and S3D).

We imaged wild-type (M82) and slbasl-cr#4 soil-grown cotyledons representing early (0 days post-emergence [dpe]), young (2 dpe), and mature (7 dpe) stages of epidermal development. At early stages, many small, box-like cells and physically asymmetric divisions (Figure 3A, orange shading) could be observed in both M82 and slbasl-cr#4. At 2 dpe, lobed pavement cells and all classes of stomatal lineage cells—meristemoids, SLGCs, GMCs, and mature guard cells—could be identified in both genotypes, but slbasl-cr#4 also exhibited occasional stomatal pairs (Figure 3A, purple shading). At maturity (7 dpe), the abaxial cotyledon and true leaf epidermis of slbasl-cr#4 plants exhibited many clustered stomata relative to wild-type plants (Figures 3A and S3A).

Although loss of SiBASL resulted in stomatal clusters in all scored cotyledons (Figure 3A), the phenotype in tomato was much milder than that generated by loss of AtBASL in Arabidopsis (Figure 3E). Because we had identified multiple mutant alleles of SiBASL (Figure S3B) and because we found no evidence for sequences that could encode redundant SiBASL genes (Method details), we hypothesized that differences in severity of basl phenotypes were not due to incomplete loss of BASL activity but rather might reflect different strategies for stomatal spacing between these two species.

To track stomatal development over time, and to identify the origin of the stomatal pairs in slbasl mutants, we used a long-term lineage-tracing strategy similar to Gong et al.,\(^{18}\) where daughters of each ACD were following through subsequent divisions and differentiation to terminal cell fates. We adapted this time course and lineage-tracing method for tomato by creating a live-cell marker for epidermal cell outlines (ML1pro:RC12A-mNeonGreen) and following epidermal development in soil-grown plants starting at 1 dpe to capture the widest variety of division competent cell types (e.g., Figures 4A and 4B).

When cells were tracked between 1 dpe and 3 dpe time points, we readily observed symmetric GMC divisions and asymmetric divisions of meristemoids (amplifying divisions; Figure 4A). However, asymmetric divisions of SLGCs (spacing divisions) were...
nearly undetectable (Figures 4A and 4D). As spacing divisions are common in Arabidopsis leaves (Figure 4D and S3E) and misoriented spacing divisions are major contributors to pattern defects in atbasl mutants,18 this suggested a reason for the mild slbasl phenotype in tomato.

Indeed, seven out of eight lineage-tracked stomatal pairs in slbasl-cr#4 arose from a fate defect (Figures S3F and S3G).

If spacing divisions are not major contributors to epidermal pattern in tomato, then other patterning mechanisms may enforce 1-cell spacing. We therefore returned to the time course images, focusing on outcomes of asymmetric divisions and on the behaviors of cells surrounding young stomata. We found that spacing (and all other) divisions were suppressed in the first ring of cells around a stoma (Figures 4B and 4C), as was SlBASL expression (Figure 4C). In contrast, AtBASL expression in Arabidopsis, where spacing divisions are common, was not excluded from ring 1 (Figure 4C). Because the SlBASL promoter can drive expression during ACDs in transgenic Arabidopsis plants (Figure S2E) and conserves several SPCH binding motifs that may drive expression during ACDs (Figure S2F), the exclusion of SlBASL from this first ring of cells likely results from changes to the developmental system upstream of SlBASL. Interestingly, although asymmetric spacing divisions were reduced, there were still many physically asymmetric divisions in the tomato epidermis (Figure 4A, orange). Unexpectedly, many resolved symmetrically as pairs of pavement cells (Figures 4A and 4E), a phenomenon not observed in wild-type Arabidopsis. We termed this novel fate transition “meristemoid drop-out” (Figure 4F) due to the loss of stomatal precursors from the lineage. Considered together, the relationships between morphologically asymmetric divisions and the resultant daughter cell fates and behaviors point to remarkably divergent developmental trajectories converging on similar final epidermal patterns in Arabidopsis and tomato (Figure 4F).

DISCUSSION

Our data suggest that BASL is a recently evolved eudicot-specific plant polarity protein that has been co-opted into more ancient programs of asymmetric divisions in stomatal development. Comparison of BASL expression and function in Arabidopsis and tomato, however, revealed that, even among eudicots,
Figure 4. Rewired fate transitions in tomato stomatal development

(A) Confocal images of developmental time course of abaxial cotyledon epidermis. Cell outlines visualized by ML1pro:RCI2A-NeonGreen are shown. White arrowheads mark division and differentiation events (a–c) shown below. Cell types are false colored as indicated in key. Scale bars, 50 μm.

(B) Image to illustrate typical spatial organization of cells surrounding a stoma (purple) at 4 dpe, including cells immediately adjacent (ring 1, green) and one cell removed (ring 2, blue and orange). Scale bar, 30 μm.

(C) Percentage of cells expressing BASL in successive rings surrounding a stoma. n = ~250 cells in each of 6 leaves.

(D) Percentage of ACDs that underwent additional amplifying or spacing divisions. ACDs displaying both amplifying and spacing divisions in our imaging window (2 days) are counted once for each class of division. n = ~45 ACDs in each of 6 imaging fields.

(E) Percentage of ACDs that resolve into asymmetric (one stoma and one pavement cell) or symmetric (two pavement cells) cell fates. n = ~45 ACDs in each of 6 imaging fields (~0.125 mm² at 1 dpe; ~0.3 mm² at 3 dpe).

(F) Model of symmetric and asymmetric division types that contribute to stomatal fate and pattern featured in Arabidopsis and tomato stomatal lineages, with shifts in predominant types between the species summarized to the right.

Numerical data in (C)–(E) are represented as mean ± 95% confidence interval. Bonferroni-corrected p values from Mann-Whitney U test are shown.
the way polarity and ACDs are employed during stomatal development can be quite different (Figure 4F). In tomato cotyledons, there is a near-complete lack of spacing divisions and divisions surrounding young and mature stomata, but in Arabidopsis, ACDs adjacent to stomata are common, and their occurrence and orientation must be carefully regulated to avoid stomatal clustering. This regulation is disrupted in the atbasl mutant, where ~30% of stomatal clusters arise through defects in division orientation.\(^6\) In contrast, stomatal development in tomato employs quiescence of both sister and non-sister cells surrounding stomata to create a pavement cell “buffer zone” (Figure 4B) that ensures a degree of stomatal spacing, even in the sibas\(i\) mutant. Additionally, in tomato, approximately 30% of physically asymmetric divisions undergo “meristemoid drop-out” and resolve as pairs of pavement cells (Figure 4E). While such divisions are absent in wild-type Arabidopsis, similar phenotypes have been described in myoxi-i mutants, where nuclear migration and division plane defects accompany differentiation of both daughters into pavement cells.\(^23\) Similarly, late depletion of the transcription factor SPEECHLESS can “divert” meristemoids or GMCs toward pavement cell fate.\(^15\) In tomato, such decoupling of physical and fate asymmetry can be used to populate the developing epidermis with additional pavement cells and promote proper spacing of stomata.

Different strategies for spacing stomata apart may incur different costs. As spacing divisions allow Arabidopsis to generate additional meristemoids under favorable environmental conditions,\(^24,25\) their absence in tomato may represent a constraint on the plasticity of stomatal development. Plasticity could be restored, however, by regulating flux along other paths in the lineage, such as in the number of entry divisions, or by converting “drop-outs” into meristemoids (Figure 4F). Alternatively, a latent spacing division capacity may be activated under appropriate environmental conditions not tested in our assays, as the SIBASL promoter appears to contain the necessary cis-regulatory information to operate in spacing divisions when expressed in Arabidopsis.

The paucity of spacing divisions in tomato may have relaxed selection on SIBASL protein activity, an idea supported by its effective rescue of atbasl stomatal pairs originating from fate defects but only modest rescue of pairs originating from spacing divisions (Figure 2F). When considering sources of specificity, it is notable that, although highly diverged, BASL orthologs from eudicots conserve three distinct protein domains, including a previously described MAPK docking site at the C terminus\(^1\) and two newly identified domains at D1 and D2 positions. Consistent with previous dissections of AtBASL domain structure,\(^2\) BASL orthologs conserving only D2 and D3 domains (as exemplified by AqBASL) appear to be sufficient for substantial rescue of the atbasl phenotype, despite broad divergence elsewhere in the peptide sequence. Arabidopsis D1 contains sequences directing nuclear import and export,\(^7\) but no positive role for AtBASL in the nucleus has been identified. Instead, current models suggest that nuclear localization may sequester AtBASL away from its site of action at the cell cortex because variants missing D1 are hyperactive.\(^2,21\) SIBASL’s capacity to function in cell polarity, without detectable nuclear accumulation, further suggests that positive BASL functions are at the plasma membrane. Whether sequestration or other ways of downregulating BASL function (such as posttranslational modifications) feature in plants outside of Arabidopsis remains to be shown, though interestingly, the MAPK target sites are not conserved between AtBASL and SIBASL.

Among these domains, D3 is most highly conserved and present in BSLLs, an ancient clade of proteins that do not polarize. This domain overlaps several residues where MAPKs phosphorylate AtBASL\(^3\) and a conserved specialized MAPK docking motif (FxFP) that, although phylogenetically widespread, occurs in only a modest number of proteins in each species.\(^26\) We speculate that D3-containing BSLL genes in early diverging angiosperms may have provided a MAPK-interacting “chassis” to which a polarity module was linked in eudicots, and the resultant protein became useful to enforce cell fate segregation during stomatal lineage ACDs.

BASL’s ability to polarize also requires information encoded in the D2 domain. This domain overlaps a region of AtBASL shown to interact with BREVIS RADIX (BRX) family proteins,\(^9\) which exhibit mutual dependency with BASL for polarization and function in the Arabidopsis stomatal lineage. In turn, BRX interacts with BASL through a pair of conserved BRX domains,\(^9\) which are also found in other proteins, such as PRAFs (plextrin, regulator of chromatin condensation and FYVE domain).\(^27\) Interestingly, BASL may interact with the BRX domain through a pair of well-conserved \(\beta\) strands in the D2 domain, which bear similarity in structure, but not sequence, to the BRX-interacting domain of LAZY3, a known PRAF binding partner.\(^28\) These data suggest possible convergence of a BRX interaction fold across two plant-specific polar protein families: BASLs and LAZYs. Convergent evolution of protein-protein interaction folds among polar proteins has also been described for DIX (Dishevelled and Axin) domains, found in the animal proteins for which they were named, and in plant SOSEKI proteins.\(^29\)

Regardless of how BASL homologs are refined for participation in different types of ACDs, we are still left with the conundrum of how non-eudicots coordinate ACDs without BASL. Stomatal development has been extensively studied in grasses, such as maize, rice, and Brachypodium dis-tachyon, where meristemoids typically undergo a single, uniformly oriented asymmetric division before terminal differentiation.\(^19\) This mode contrasts with the flexible program exhibited by many eudicots, including Arabidopsis and tomato, where the number and orientation of asymmetric divisions is variable and responsive to a variety of internal and external inputs. It is tempting to speculate that BASL may have been co-opted in eudicots to coordinate some aspect of this flexible developmental program that is dispensable in grasses. Alternatively, BASL functions in non-eudicots may be performed by an unrelated protein family, such as BRX family proteins, which polarize alongside BASL in the Arabidopsis stomatal lineage and appear to be deeply conserved across land plants.\(^9,27,30\) Recent work\(^26\) to reconstruct the long-term evolutionary history of Arabidopsis proteins, combined with studies of protein function in their native contexts, may shed light on the genetic toolkit and behaviors available to ancient and modern stem cells as they progress through development.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.11.013.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.N., G.A., and D.C.B.; methodology, I.N., G.A., and Y.G.; investigation, I.N., G.A., Y.G., N.K.S., L.C., and H.S.; writing – original draft, I.N., G.A., and D.C.B.; writing – review & editing, I.N., G.A., and D.C.B.; funding acquisition, I.N., G.A., and D.C.B.; resources, D.C.B.; supervision, D.C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR+METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| TOP10 Competent cells | N/A | N/A |
| Agrobacterium tumefaciens, GV3101 cells | Koncz and Schell\(^{11}\) | N/A |
| Agrobacterium tumefaciens, AGL1 cells | Bragg et al.\(^{32}\) | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Propidium iodide | Life Technologies/Thermo Fisher Scientific | P3566 |
| FM 4-64 | Life Technologies/Thermo Fisher Scientific | T3166 |
| Nitsch media for Tomato | Caisson labs | NNP03-50LT |
| MS media for Arabidopsis | Caisson labs | MSP01-50LT |
| Micropropagation Agar-Type 1 | Caisson labs | A038 |
| **Experimental models: Organisms/strains** | | |
| Arabidopsis thaliana ecotype Col-0 | Arabidopsis Biological Resources Center | CS60000 |
| Tomato (*Solanum lycopersicum* cv. M82) | Nir et al.\(^{22}\) | N/A |
| Nicotiana benthamiana | N/A | N/A |
| Brachypodium distachyon Bd21-3 | Raisig et al.\(^{33}\) | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A | Gong et al.\(^{18}\) | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A, *AtBASL*\(_{pro}\)-Venus-mDBox-AtBASL | Gong et al.\(^{18}\) | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A, *AtBASL*\(_{pro}\)-Venus-SlBASL | This paper | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A, *SiBASL*\(_{pro}\)-Venus-SlBASL | This paper | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A, *AtBASL*\(_{pro}\)-Venus-mDBox-AqBASL | This paper | N/A |
| Arabidopsis: basl-2 *AtML1*\(_{pro}\)-mCherry-RCI2A, *AtBASL*\(_{pro}\)-Venus-mDBox-BdBSLL1 | This paper | N/A |
| Brachypodium: *Ub*\(_{pro}\)-YFP-AtBASL | This paper | N/A |
| Tomato: *SiBASL*\(_{pro}\)-Venus-SiBASL | This paper | N/A |
| Tomato: *AtML1*\(_{pro}\)-RCI2A-mNeonGreen | This paper | N/A |
| Tomato: *sibasl-cr*\(_{4}\) | This paper | N/A |
| Tomato: *sibasl-cr*\(_{2}\) | This paper | N/A |
| Tomato: *sibasl-cr*\(_{6}\) | This paper | N/A |
| **Oligonucleotides** | | |
| Promoter cloning, *SiBASL*\(_{pro}\) F1: GGCA TCGTTTGTTAAAAAGTGTAG | This paper | N/A |
| Promoter cloning, *SiBASL*\(_{pro}\)-mid F: TTA CAcTCTTTCTTGCACATACCATTACAC | This paper | N/A |
| Promoter cloning, *SiBASL*\(_{pro}\)-mid R: GTAAA GCTACTTTAGTAACTATTATTCACAC | This paper | N/A |
| Promoter cloning, *SiBASL*\(_{pro}\)-R: TCTTATT CAATTACAACAGAATTACAAGGGGAA | This paper | N/A |
| Genotyping PCR, *SiBASL*\(_{476}\) up F: TTGA AAGAAGTGAAACTCAACACC | This paper | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dominique Bergmann (dbergmann@stanford.edu).
Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and code availability
- Multiple sequence alignments and Newick tree files are at https://doi.org/10.6084/m9.figshare.15109575
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis growth conditions
*Arabidopsis thaliana* Col-0 seeds were surface-sterilized with 75% ethanol, rinsed twice with distilled water and stratified for 2 days. After stratification, seedlings were grown on ½ strength Murashige and Skoog (MS) media (Casson Labs) with 1% agar for 3 – 14 days under long-day conditions (16 hr light/8 hr dark at 110 μmol m² s⁻¹ and 22°C) in a Percival growth chamber, model CU36L5.

Brachypodium growth conditions
*Brachypodium distachyon* Bd21-3 seeds were stratified for 2 – 8 days. After stratification, seedlings were grown on agar-solidified ½ MS media at 22-26°C, at 80 μmol m² s⁻¹ and 16 hr light/8 hr dark cycles in a Percival growth chamber, model CU36L5. For propagation, plants were transferred to soil in a greenhouse (20 hr light/4 hr dark, 250-300 μmol m² s⁻¹; day temperature: 28°C; night temperature: 18°C).

Tomato growth conditions
Tomato plants (*Solanum lycopersicum* cv. M82) were grown in a Percival growth chamber, model CU22L, or Percival growth room, model AR-1015L3, set to a photoperiod of 16 hr light/8 hr dark, light intensity of approximately 250 μmol m² s⁻¹ and 26°C. For propagation, plants were grown in a greenhouse under natural day length conditions, at 700–1200 μmol m² s⁻¹ and 18 – 29°C.

Nicotiana benthamiana growth conditions
*Nicotiana benthamiana* plants were grown in Percival AR66 growth chambers set a photoperiod of 16 hrlight/8 hr dark, light intensity of approximately 110 μmol m² s⁻¹, and 23°C day/22°C night. Plants were infiltrated with Agrobacterium at least six weeks after germination.

METHOD DETAILS

Identification of BASL and BSLL sequences
BASL sequences from 9 Brassicaceae species were aligned with MUSCLE and used to query the Viridiplantae section of the UniProtKB protein sequence database with jackhmmer. Candidate homologs from 33 land plant species with e-values below 0.01 were selected for alignment and manual inspection. After removing exact and near duplicates (> 95% identity), sequences were aligned using MAFFT and sites with > 80% coverage were used to estimate the phylogeny with the neighbor-joining algorithm as implemented on Geneious Prime 2.3. The tree shown in Figure 1C is trimmed to remove several clades of highly diverged, likely spurious orthologs.

In several cases, BASL orthologs appeared to be missing in eudicot proteomes as annotated on UniProtKB but could always be found in other proteome or genome annotations (Table S1). Similarly, parts of conserved domains at the extreme N- and C-termini that appeared to be missing in protein models could often be found in-frame in the corresponding DNA sequence; protein sequences were manually corrected to include such domains in Figure S1C. For that figure, intrinsic disorder predictions were derived from DISOPRED3. Secondary structure predictions were derived from PSIPRED 4.0 and the confidence score of the prediction is used to color the alignment. The location of splice junctions was derived from the primary transcript annotated on Phytozome v12 and exons were then shaded in alternating colors. Measurements are displayed for the following proteins: *Arabidopsis thaliana* AT5G60880, *Medicago truncatula* Medtr2g461550.1, *Populus trichocarpa* Potri.015G048600.1, *Solanum lycopersicum* Solyc03g114770.2.1, *Erythranthe guttata* (previously *Mimulus guttatus*) XP_012853883.1, *Daucus carota* DCAR_023155 and *Aquilegia coerulea* Aqcoe7G067300.1.

Identification of BASL pseudogenes in tomato
A single BASL ortholog and three clearly distinguishable BSLL proteins were found in the *Solanum lycopersicum* iTAG2.4 proteome. In the iTAG2.4 genome, one additional short ORF located within an intron of Solyc01g009450.2.1 could be recognized. Sequence inspection showed remnants of conserved D1 and D2 domains, suggesting orthology to SiBASL; however, several early stop codons and a frameshifting indel are predicted to produce a severely truncated 20 amino acid polypeptide from this locus.
Gene names used in this paper

Named genes in this paper correspond to the following proteins and genes (listed as gene name, UniProt ID, locus ID): AtBASL Q5BPF3 AT5G60880, AtBSLL1 Q9LMY2 AT1G13650, AtBSLL2 F4ITB8 AT2G03810, SibASL A0A3Q7GGD1 Solyc03 g114770.2.1, SibBSLL1 A0A3Q7FSN7 Solyc03 g114750.2.1, AqBASL Aqcoe7G067300.1, AqBSLL1 A0A2G5F5L9 Aqcoe9G377700.1 and BdBSLL1 I1A25 Bradi3g47127. Note that AqBASL has not been assigned a UniProt ID.

Arabidopsis and Nicotiana transformations

BSL candidates from tomato, Aquilegia coerulea and Brachypodium distachyon and BASL candidates from Aquilegia coerulea and Brachypodium distachyon were synthesized in vitro following the primary annotated CDS from Phytozome v12. For transient expression in Nicotiana benthamiana, 35Spro:AtBASL-YFP, 35Spro:AtBSLL1-YFP, 35Spro:SibBSLL1-YFP, and 35Spro:AqBSLL1-YFP, were cloned using the binary vector pH35YG.34 and 35Sx2pro:Venus-SiBASL, 35Sx2pro:Venus-AqBASL and 35Sx2pro:Venus-BdBSLL1 cloned using the Golden Gate system35 into the binary vector plCH47742 with NOS terminator. The binary vectors were introduced into Agrobacterium tumefaciens strain GV3101 and infiltrated into mature tobacco leaves as described in Zhang et al. For stable expression in Arabidopsis, AtBASL3pro:Venus-SiBASL, AtBSLL3pro:Venus-AqBASL and AtBSLL3pro:Venus-BdBSLL1 were cloned using the Golden Gate system into the binary vector pAGM4723 with NOS terminator. The constructs were introduced into A. tumefaciens as above and transferred to Arabidopsis by floral dipping.49 Transgenic founder plants were identified by kanamycin resistance. All analyses were performed on homozygous T3 plants.

Tomato transformations

For tomato BASL marker lines, the SIBASL promoter and CDS were cloned into a level 0 MoClo part using the Golden Gate cloning system35 and then fused to Venus and a NOS terminator to form a level 1 construct. The level 1 was transferred to a level 2, together with a kanamycin resistance cassette. For primers used in cloning see Key resources table. The constructs were sub-cloned into the pAGM4723 binary vector and were introduced into A. tumefaciens strain GV3101 by electroporation. The constructs were transferred to M82 cotyledons, using transformation and regeneration methods from McCormick.50 Kanamycin-resistant T0 plants were grown and at least four independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines.

Brachypodium transformations

For expression of AtBASL in Brachypodium distachyon strain Bd21-3, a Gateway pENTR plasmid encoding a fusion of YFP with the coding region of BASL3 was recombined into the monocot transformation plPKb002 that drives expression of inserts with the maize Ubiquitin promoter31 following standard Gateway protocols.32 Brachypodium calli were transformed with A. tumefaciens strain AGL1, selected and regenerated according to standard protocols.35 Expression of the transgene was monitored in the development zone of the base of leaves from 22 dpg T1 plants as described in Abrash et al.51

Tomato CRISPR mutagenesis

Four single-guide RNAs (sgRNAs) were designed using the CRISPR-P tool.53 The gRNAs and promoter were assembled using the Golden Gate cloning system as described in Weber et al.35 The final binary vector including zCas9, the gRNAs and NPTII, assembled in pAGM4723, was introduced into Agrobacterium tumefaciens strain GV3101 by electroporation. The construct was transferred into M82 cotyledons using transformation and regeneration methods described by McCormick.50 T0 transgenic plants resistant to Kanamycin were grown and independent lines were selected and self-pollinated to generate homozygous lines. For genotyping of the transgenic lines, genomic DNA was extracted, and each plant was genotyped by PCR for the presence of the zCas9. The positive lines for the zCas9 were further genotyped for mutations in SIBASL (Solyc03 g114770) using a forward primer 400bp upstream to the ATG and a reverse primer 200 bp downstream to the stop codon, these pair primers cover the 4 gRNAs.

Microscopy, image analysis and processing

All fluorescence imaging experiments on Arabidopsis plants were performed on a Leica SP5 confocal microscope with HyD detectors using 40x NA1.1 water objective with image size 1024*1024 and digital zoom from 1x to 2x. To quantify rescue of the atbasl stomatal clustering phenotype, 14 dpg cotyledons were imaged and the fraction of stomata in clusters of size two or larger was computed from regions containing 120 stomata (approximately 0.5 – 1.5 mm² in size). Normalized rescue capacity was calculated as 1 – (% pairs in rescue - % pairs in WT) / (% pairs in atbasl - % pairs in WT). Still or time-course images of SIBASL (pSIBASLpro:VENUS-SiBASL, ML1pro:RCI2A-NeonGreen, propidium iodide (PI), and FM4-64 fluorescence in tomato were obtained from a Leica SP8 confocal microscope with HyD detectors using 20x oil objective with image size 1024*1024 and digital zoom from 1x to 2x. For time-course experiments on tomato, cotyledons of 1 dpe soil-grown seedlings were mounted with abaxial side toward coverslip, and 0.15% agarose was added to prevent root drying. Seedlings were carefully unmounted after imaging and returned to the soil until the next image acquisition. Time-lapse experiments of SIBASL in tomato were performed on a Leica SP5 confocal microscope with HyD detectors using 25x NA0.95 and 40x NA1.1 water objectives. Seedlings were mounted on a customized flow-through chamber54 and images were acquired at 30 min. Imaging data was analyzed on Fiji.45 All raw fluorescence image Z stacks were projected with SUM (Arabidopsis stills) or STD (tomato stills and time-lapse) slices. STD was found empirically to generate clearer images from tomato cells than other image processing tools, likely due to the high levels of autofluorescence in tomato cells. For all time-lapse images, drift was corrected after projection using the Correct 3D Drift plugin46 prior to any further analysis.
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses in this manuscript were performed in RStudio. Statistical parameters for each analysis are indicated in the figure legends. For significance testing, unpaired Mann-Whitney U tests were conducted with the wilcox_test function from the rstatix package. Bonferroni corrections were performed when more than 2 pairwise comparisons were conducted, and Bonferroni corrected p values are indicated in all figures where applicable. For Figure 2E, each letter indicates a group of samples with no statistically significant difference between their means (Bonferroni corrected p value > 0.05).