Gene regulatory interactions at lateral organ boundaries in maize

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ABSTRACT

Maize leaves have distinct tissues that serve specific purposes. The blade tilts back to photosynthesize and the sheath wraps around the stem to provide structural support and protect young leaves. At the junction between blade and sheath are the ligule and auricles, both of which are absent in the recessive liguleless1 (lg1) mutant. Using an antibody against LG1, we reveal LG1 accumulation at the site of ligule formation and in the axil of developing tassel branches. The dominant mutant Wavy auricle in blade1 (Wab1-R) produces ectopic auricle tissue in the blade and increases the domain of LG1 accumulation. We determined that wab1 encodes a TCP transcription factor by positional cloning and revertant analysis. Tassel branches are few and upright in the wab1 revertant tassel and have an increased branch angle in the dominant mutant. wab1 mRNA is expressed at the base of branches in the inflorescence and is necessary for LG1 expression. wab1 is not expressed in leaves, except in the dominant mutant. The domain of wab1 expression in the Wab1-R leaf closely mirrors the accumulation of LG1. Although wab1 is not needed to induce lg1 expression in the leaf, LG1 is needed to counteract the severe phenotype of the dominant Wab1-R mutant. The regulatory interaction of LG1 and WAB1 reveals a link between leaf shape and tassel architecture, and suggests the ligule is a boundary similar to that at the base of lateral organs.

KEY WORDS: Maize, Leaf, Inflorescence

INTRODUCTION

Alteration of plant architecture has been a critical driver of crop domestication. Structural modifications that occurred often resulted from changes in branching architecture by altering the number, length or angle of branches. During maize domestication, axillary branch number and length decreased, leading to the maize ear (Doebly and Stec, 1993; Doebly et al., 1997). In rice, selection led to more upright panicle branches to reduce grain loss before harvest (Sweeney and McCouch, 2007). Reduction of height in many cereal grains decreased losses due to lodging (Evans, 1993).

Leaf angle has also been important for crop improvement, especially for increased maize yields (Duivick, 2005). Modern maize has been bred for upright leaves that allow increased numbers of plants per acre. The angle of a leaf is determined by the presence of auricles found at the junction of the sheath and blade (Fig. 1A). The auricles act as hinges to allow the blade to tilt back for optimal photosynthesis while the sheath grasps the stem and protects younger leaves. The ligule, a flap of epidermal outgrowth, is found on the inner surface of the leaf. The ligule is thought to keep water and pests from entering the stem (Chaffey, 2000).

Two genes, liguleless1 and 2 (lg1 and lg2, respectively) are important for the leaf angle in maize and rice (Lee et al., 2007; Moreno et al., 1997; Walsh et al., 1998). lg1 encodes a squamosa promoter-binding protein and lg2 encodes a bZIP transcription factor (Moreno et al., 1997; Walsh et al., 1998). In the absence of lg1, both ligules and auricles are missing and the leaf is upright. lg2 mutants are missing the ligule across the midrib but auricle and accompanying ligule are found at the margins. Real-time (RT)-PCR showed that lg1 mRNA accumulates at the ligule region (Moreno et al., 1997) and this finding was confirmed by whole-mount in situ hybridization (Moon et al., 2013). lg2 is expressed more widely (Walsh et al., 1998). A large quantitative trait locus (QTL) study using 2000 recombinant inbred lines identified both lg1 and lg2 among the 30 QTL that contribute to leaf angle in maize (Tian et al., 2011). A role for lg1 in the inflorescence has also been identified. A QTL for rice domestication mapped to a region 10 kb upstream of lg1. Expression of lg1 was increased in undomesticated varieties that had a larger branch angle than domesticated varieties (Ishii et al., 2013; Zhu et al., 2013). In maize, lg2 mutants have few, upright tassel branches (Walsh and Freeling, 1999). Thus, a connection between leaf and tassel-branch angle exists in both maize and rice.

Given a role for LG1 in both tassel and leaf, we sought to further understand its regulation. Earlier reports showed that lg1 RNA levels increase in the dominant mutant Wab1-R (Foster et al., 2004). We cloned Wab1-R and found that it encodes a TCP transcription factor implicated in tassel branch angle, previously described as branch angle defective1 (bad1) (Bai et al., 2012). Using an antibody against LG1, we show that LG1 requires WAB1 in the tassel and is misexpressed in a pattern that is identical to that of WAB1 in the dominant mutant. Our results demonstrate that WAB1 regulates LG1 directly or indirectly and suggest that the ligule forms at a boundary similar to that found between lateral organs initiating from the meristem.

RESULTS

LG1 protein accumulation defines boundary regions in leaf and tassel

Maize plants that carry two different lg1 mutations were grown to assess their phenotypes in the leaf and tassel. The ligule and auricles were missing in the lg1 reference allele (lg1-R) (Fig. 1A-D), which carries a deletion (Moreno et al., 1997). In lg1-n2375, which contains a mutation in a highly conserved residue within the predicted DNA-binding domain (V206M) (personal communication, David Braun, University of Missouri), occasional patches of ligule were visible...
was not different for \( lg1-n2375 \) and tassel branches of wild-type plants but absent in time and in mutant backgrounds (Fig. 2). LG1 was detected in leaves narrower (Table 1A).

...domain in are missing, except near the margin (supplementary material distinct ligule (Fig. 2C,D). In developing leaves, LG1 marked the differentiating ligule band and, later, the morphologically preligule band (Fig. 2A,B). In P8 and later leaves, LG1 accumulation was also expressed at the junction of secondary tassel branches (Fig. 2H). The accumulation did not spread far from the branch-junction (Fig. 2G-J). Expression could be detected in young tassels developing tassel branches near the junction with the rachis (Fig. 2I,J). LG1 accumulation marks two distinct boundaries in the maize plant, the junction between sheath and blade and the junction of tassel branches.

**LG1 accumulation in Wab1-R leaves explains the dominant phenotype**

Prior work showed that \( lg1 \) is upregulated in \( Wab1-R \) mutants, which have a normal sheath but an abnormal ligular region and blade (Foster et al., 2004; Hay and Hake, 2004). In heterozygotes, ectopic auricle can be found in the blade, either as isolated patches or continuous with the auricle (Fig. 3A). In homozygotes, leaves are very narrow and ectopic auricle is replaced by sheath tissue that continues up into the blade (Fig. 3B).

In both \( Wab1-R \) homozygous and heterozygous leaves, the LG1 domain expanded into the blade but was still excluded from the sheath (Fig. 3C,D). LG1 accumulation was detected in younger leaves in \( Wab1-R \) compared with wild type (Fig. 3E,F). When examining transverse sections of wild-type plants, LG1 was detected in the adaxial epidermis in only one leaf in a section (Fig. 3E). We detected LG1 throughout the width of the leaf in \( Wab1-R \) (Fig. 3F). The timing and location of LG1 accumulation in \( Wab1-R \) is consistent with the phenotypic defects found in the mutant leaves (Foster et al., 2004; Hay and Hake, 2004) and suggests that WAB1 is a positive regulator of LG1.

**Cloning of Wab1**

To identify the gene responsible for \( Wab1-R \), we combined positional cloning with isolation of an intragenic suppressor. \( Wab1-R \) was mapped to an interval containing two genes, one encoding a bHLH-containing TCP transcription factor that had previously been identified in a forward genetic screen for upright tassel branches (Bai et al., 2012), and the other encoding a different bHLH transcription factor (Fig. 4A). Neither gene revealed any sequence differences when compared with the parent PF4902 and no polymorphisms were detected by Southern blot hybridization (supplementary material Fig. S2). To identify which gene was responsible for \( Wab1-R \), we used EMS mutagenesis. A single revertant, \( wab1-rev \), that had lost the \( Wab1-R \) phenotype and carried the PF4902 polymorphism was identified. Sequencing revealed a C389T mutation that resulted in a R130W substitution (Fig. 4A) in the highly conserved DNA-binding domain of the TCP gene (Martín-Trillo and Cubas, 2010). No sequence differences were detected in the other bHLH gene. The identification of an intragenic mutation that suppresses the dominant \( Wab1-R \) phenotype confirmed that \( wab1 \) encodes this TCP gene.

\( wab1-rev \) was made homozygous and grown to maturity to assess the inflorescence phenotype. Homozygotes had fewer, upright tassel branches (Fig. 5A,B). Following three back-crosses to \( A619 \), revertants had an average of 1.3 tassel branches compared with 5.8 in the normal siblings (Table 1B). The upright angle was similar to that identified in the branch angle.

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**Table 1. Tassel measurements**

<table>
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<tr>
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<th>TBA (°)</th>
<th>TBN</th>
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<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
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<tr>
<td>wt sibs</td>
<td>5.77</td>
<td>11.37</td>
<td>10</td>
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<tr>
<td>( lg1-R )</td>
<td>0.6°</td>
<td>10.83</td>
<td>12</td>
</tr>
<tr>
<td>( lg1-n2375 )</td>
<td>17.5°</td>
<td>3.77</td>
<td>8</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>wt sibs</td>
<td>6°</td>
<td>5.714</td>
<td>7</td>
</tr>
<tr>
<td>( wab1-rev )</td>
<td>25.07°</td>
<td>5.714</td>
<td>7</td>
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TBA, tassel branch angle; TBN, tassel branch number.
defective1 (bad1) mutant (Bai et al., 2012), although they did not describe a change in tassel branch number. Following a cross of wab1-rev to bad1, all F1 progeny had upright and fewer tassel branches, demonstrating that the two mutations are allelic. No visible changes were detected in the leaf. We refer to bad1 as wab1-bad because Wab1-R was identified and named first (Hake et al., 1999; Hay and Hake, 2004). The wab1-bad1 and wab1-bad2 lesions affect the same highly conserved bHLH domain as Wab1-R (Fig. 4A).

LG1 and WAB1 have overlapping expression patterns
We quantified wab1 and lg1 transcript accumulation in Wab1-R leaves by RT-PCR. Expression of wab1 and lg1 increased in P9 and P10 Wab1-R primordia compared with wild type (Fig. 4B,C). At the P11 stage, where a leaf could be dissected into blade, ligule and sheath, wab1 misexpression was detected in the ligule and blade but not the sheath of Wab1-R leaves (Fig. 4B). In these same tissue samples, lg1 was increased in the Wab1-R blade and absent in the sheath (Fig. 4C), suggesting that wab1 spatially regulates LG1 accumulation.

We carried out in situ hybridizations with wab1 in order to determine its expression domain (Fig. 4D-I). We were unable to detect wab1 in normal leaves (Fig. 4D,E) but did find expression at the base of both spikelet pair and branch meristems (Fig. 4H,I). From serial transverse sections of 1-2 mm tassels, a sharp zone of expression was detected on the adaxial side of the branch meristem, adjacent to the main rachis (Fig. 4H). This expression pattern was transient and not detected at the base of more mature tassel branches.

As predicted from the quantitative (q) RT-PCR results, we found strong expression in Wab1-R leaves (Fig. 4F,G). Expression was visible in Wab1-R starting with a P4 leaf and continued into the next few leaves. Expression was stronger towards the margins than the midrib (Fig. 4G). Both the timing and position of wab1 expression were consistent with the dominant Wab1-R mutant phenotype (Foster et al., 2004; Hay and Hake, 2004) and similar to the misexpression of LG1 in Wab1-R.

LG1 requires WAB1 in the tassel
Given the tassel branch angle phenotype in wab1-rev (Fig. 5A,B), we measured the branch angle of Wab1-R-Dominant mutants, segregating in the B73 background. Branch angle was 6° in normal siblings compared with 25° in Wab1-R/+ heterozygotes (Table 1C). Plant phenotypes were examined for a family that segregated both gain- and loss-of-function mutants in the A619 background (Fig. 5C; supplementary material Fig. S3). The tassel branch angle was greater in Wab1-R plants compared with both
normal siblings and loss-of-function mutants (Fig. 5C). qRT-PCR showed that wab1 expression levels were higher in tassels of Wab1-R mutants (Fig. 5D). Thus, the dominant Wab1 mutation causes a larger branch angle, whereas loss of wab1 function results in a small angle and fewer branches.

We examined wab1 mRNA levels in seedlings and tassels of wab1-rev plants. In the seedling, where wab1 was normally excluded, wab1 misexpression disappeared. In the tassel, where wab1 was normally expressed, wab1 levels were still high (supplementary material Fig. S4). Thus, the mutation in the DNA-binding domain did not interfere with the increased expression conferred by the dominant mutation in the tassel, but it did in the leaf. These results suggest that a functional WAB1 protein is needed to maintain high levels of wab1 expression in Wab1-R leaves, a zone where wab1 is normally absent.

We examined LG1 accumulation in the tassel to see whether it was affected by loss of WAB1 function. lg1 levels disappeared in the wab1-rev mutant tassels compared with seedlings (Fig. 5E). LG1 immunolocalization confirmed an absence of protein accumulation in axils of tassel branches in wab1-rev mutants compared with Wab1-R/+ plants (Fig. S5-I), consistent with the hypothesis that WAB1 positively regulates lg1 in the tassel. We detected normal LG1 accumulation in the blade-sheath boundary of wab1-rev mutants (Fig. S5-K; supplementary material Fig. S5), suggesting that WAB1 is not needed for lg1 expression in the leaf. The combined increase of lg1 in Wab1-R and loss of lg1 expression in wab1-rev supports the hypothesis that WAB1 regulates lg1.

**Genetic interactions of Wab1-R and lg1-R**

To determine whether lg1 and wab1 interact genetically, we examined the phenotype of double mutants in a segregating family. Tassel branch angles were similarly narrow between lg1-R and the lg1-R; Wab1-R/+ double mutant, suggesting that lg1 acts epistatically to Wab1-R (Fig. 6A). These results are consistent with WAB1 being upstream of LG1.

We quantified blade width and length in the lg1-R; Wab1-R double-mutant families. lg1-R mutants were narrower than wild type at the auricle but not at the mid-blade point. The double-mutant leaves were narrower than either single mutant at both positions and significantly shorter (P<0.01) (Fig. 6B), suggesting a synergistic interaction. These results highlight regulatory interactions between wab1 and lg1 (Fig. 6C). In the context of the tassel, where wab1 is normally expressed, lg1 is dependent on wab1, and lg1-R is epistatic to Wab1-R. In the leaf, where wab1 is normally excluded, the double-mutant phenotype is synergistic, suggesting that these gene products have opposing effects on leaf shape and that the ectopic accumulation of LG1 counteracts some of the effects of wab1 in the leaf.

**DISCUSSION**

We show that LG1 protein accumulation is normally confined to a narrow band of cells at the blade-sheath boundary of the leaf and in the axil of tassel branches. In Wab1-R leaves, LG1 accumulates earlier and expands up into the blade when compared with wild type. We cloned Wab1-R and discovered it encodes a protein with a TCP bHLH domain that had recently been identified as a tassel
branch angle mutant (Bai et al., 2012). wab1 is expressed in tassel branch axils, overlapping with LG1 accumulation. In the absence of wab1, LG1 accumulation disappears. wab1 is normally excluded from wild-type leaves but is ectopically expressed in Wab1-R mutant leaves, mirroring LG1 accumulation. Our data reveal a gene regulatory interaction that normally functions in the tassel but was recruited to the leaf in the Wab1-R mutant, leading to the dominant phenotype. Our data also suggest that the ligule functions as a boundary that separates tissue types and that creation of this boundary is correlated with correct medial-lateral growth.

The nature of the dominant mutant phenotype
The nature of the Wab1-R mutant is unknown. We did not detect any changes in the coding region compared with the progenitor nor did we identify any large polymorphisms, such as an insertion or deletion by Southern blot hybridization. Sequencing outside of the coding region was confounded by differences in the Wab1-R progenitor PF4902, compared with the reference genome. Given that Wab1-R arose following another tissue culture (Hake et al., 1999), it may have been caused by an epigenetic mutation (Manning et al., 2006) or a distal transposon, such as the transposon 60 kb upstream of tb1 (Studer et al., 2011). wab1 is normally silenced in leaves, but is expressed in Wab1-R. Another mutation, wab2, has a similar ectopic auricle phenotype when homozygous but no tassel phenotype. Future research into wab2, which may be a negative regulator of wab1, should lead to an understanding of what normally keeps wab1 silent in leaves.

LG1 is downstream of WAB1
Grasses are characterized by a distinct leaf morphology that includes an enclosing sheath, a photosynthetic blade, two auricles and a ligule...
fringe. The first sign of a preiligule band is a zone of division that occurs in a P6-P7 leaf primordium. These divisions are parallel and perpendicular to the long axis of the leaf. At about P7-P8, divisions occur that are periclinal to the surface, causing growth out of the plane of the leaf (Sharman, 1941; Sylvester et al., 1990). The periclinal divisions initiate the actual ligule and do not occur in the lg1-R leaf, leading to a failure of ligule and auricle formation (Sylvester et al., 1990). Previous work, in which RT-PCR (Foster et al., 2004; Moreno et al., 1997) and whole-mount in situ hybridization (Moon et al., 2013) was used, has shown that lg1 is expressed at the ligule. We used an antibody to follow the timing of LG1 accumulation at a cellular resolution. LG1 protein is visible in a band of ~20 cells coincident with the first divisions. The band of LG1 accumulation narrows such that not all dividing cells accumulate LG1 protein. Accumulation is strongest on the adaxial epidermis, consistent with the formation of the ligule on the adaxial side of the leaf. Accumulation outside the adaxial domain may be required for development of the auricle.

In Wab1-R, the pattern of LG1 accumulation correlates well with the dominant mutant phenotype (Foster et al., 2004; Hay and Hake, 2004). LG1 is detected in Wab1-R starting at the P4 stage, the same stage at which ectopic wab1 is detected and when the phenotype first manifests as a narrow leaf primordium. Both LG1 accumulation and wab1 expression extend throughout the leaf width and distally into the blade. The increase in width at the auricle compared with mid-blade in Wab1-R mutants is consistent with the zone of increased LG1 expression.

In the inflorescence, LG1 protein accumulates in the axes of tassel branches early in their development. This pattern overlaps that of increased LG1 expression. LG1 is detected and when the phenotype first manifests as a narrow leaf primordium. Both LG1 accumulation and wab1 expression extend throughout the leaf width and distally into the blade. The increase in width at the auricle compared with mid-blade in Wab1-R mutants is consistent with the zone of increased LG1 expression.

The role of LG1 and WAB1 in plant architecture

Identifying the tissue-specific regulation of LG1 accumulation provides insight into its role in plant architecture. Quantitative trait mapping identified a region upstream of lg1 as a QTL for domestication in rice (Ishii et al., 2013; Zha et al., 2013). In maize, comparisons of ear and tassel expression showed that lg1 is normally excluded from ear primordia but is expressed in the highly branched ear of a ramosa1 mutant (Eveland et al., 2013). We identified a LG1 regulator that normally functions in the tassel. In the absence of wab1, tassel branches are upright and few, and lg1 is not expressed. In Wab1-R tassels, the increase in wab1 leads to an increase in LG1 and a larger branch angle. wab1 itself might also play a role in natural variation. Both wab1 and lg1 map to QTL for tassel branch number (Brown et al., 2011; Mickelson et al., 2002).

In Wab1-R leaves, the gene module is redeployed, with ectopic wab1 and expanded LG1 accumulation that affects leaf width and proximal-distal patterning. We speculate that this gene module is utilized in other examples of morphological variation – similar to the story of KNOX and CUC genes, and their roles in leaf diversity (Bharathan et al., 2002; Blein et al., 2008; Floyd and Bowman, 2010).

MATERIALS AND METHODS

Plant materials

Wab1-R arose from anther tissue culture following a cross of PF4902 and HF1. Sequencing revealed the parent was PF4902. Potential alleles Wab-DCL and Wab1-RM (Hay and Hake, 2004) carry the same polymorphisms as Wab1-R and PF4902 and are, thus, assumed to be pollen contaminants and not independent alleles. wab1-had1 was introgressed into A619 four times (Bai et al., 2012). The lgb1-R allele was obtained from the Maize Genetics Stock Center and introgressed at least seven generations into W23. The lgb1-n2375 line was obtained from the Maize Genetics Stock Center and introgressed twice into B73.

For expression and phenotypic analysis of young plants, seedlings were grown in the greenhouse. Dissected parts were fixed in FAA overnight or frozen in liquid nitrogen. For screens of mature plant phenotypes and/or large populations, plants were grown at the Gill Tract summer field in Albany, CA. For mature plants, leaf phenotypes were scored at the leaf above the ear. Tassels were measured at or just prior to dehiscence.
Recombinant mapping, revertants and genotyping

Families segregating 1:1 for Wab1-R/+;+/+ were screened for recombination between umc2372 and bmc1329. Recombinants were further tested with primers within this interval. A total of 1300 individuals were examined.

The wab1-1 rev allele was identified following EMS mutagenesis of Wab1-R. Pollen from 20 homozygotes introgressed into B73 was treated with ethylmethane sulfonate (EMS) (Neuffer, 1982). A 1% solution of EMS in paraffin oil was stirred overnight. A further dilution was made with 1 ml of pollen, 1 ml of 1% EMS and 14 ml of paraffin oil. After 40 min of shaking, pollen was applied to silks of inbred A619 with a paintbrush. Five thousand plants were screened for normal-appearing plants in the Gill Tract field (2011). All normal plants were screened for a CAPS marker in the TCP gene with primer set P1 and P2 (supplementary material Table S1), followed by Dral digestion. One of the normal plants carried the P1/P12 allele and the other the P1/P11 allele of the normal plants carried the P1/P12 allele and it was again crossed to A619. Southern blotting was performed as previously described (Kerstetter et al., 1994). 15 μg of genomic DNA from Wab1-R homozygotes and their progenitor (PF4902) was digested with seven enzymes (supplementary material Fig. S2) and blotted with a probe created using primers P1 and P2 (supplementary material Table S1).

The identification of wab1-bad1 plants was confirmed by sequencing analysis with primer set P3 and P4. Both alleles of lg1 were identified by phenotype. Sequencing of the BHLH gene within the mapping interval used P5/P6 and P7/P8 primer combinations (supplementary material Table S1).

In situ hybridization

In situ hybridization was performed as described (Jackson, 1991) with modifications (Bortini et al., 2006), except that tissue was fixed for 16-18 h instead of 1 h. Two different wab1 probes were generated, corresponding to the 5’-end of wab1 cDNA but excluding the conserved TCP domain (P9/P10 and P11/P12, supplementary material Table S1). The two RNA probes were synthesized by using a DIG RNA labeling mix (Roche) and mixed prior to hybridization after verifying that individual probes generated the same pattern.

Expression analysis

Quantitative real-time PCR (qRT-PCR) was performed as described (Bolduc and Hake, 2009). The primer sets used for wab1 were P13/P14, for lg1 P15/P16 and for the gap run control P17/P18 (supplementary material Table S1). For each experiment, at least three biological replicates and two technical replicates were assayed on a BioRad CFX instrument, and averaged – except for those shown in Fig. 4B, C, which come from two biological replicates pooling four individuals each. Normalization against a gapdh gene and relative fold enrichment were calculated by using the Normalized Expression mode (ΔΔCq) (Hellemons et al., 2007) integrated in the instrument software.

Antibody creation and immunolocalization

The antibody against full-length LG1 was created as described (Chuck et al., 2006). Sequence encoding full-length LG1 protein was cloned into pENTR D-TOPO (Invitrogen) to generate the N-terminal fusion as a GST-tagged N-terminal fusion protein.

The antibody against full-length LG1 was created as described (Chuck et al., 2006). Sequence encoding full-length LG1 protein was cloned into pENTR D-TOPO (Invitrogen) to generate the N-terminal fusion to pDEST15 or pDEST17 (Invitrogen) to generate the N-terminal fusion protein as a GST-tagged N-terminal fusion protein.

Maize plants were grown and sampled as described above and fixed in FAA under vacuum infiltration, dehydrated through an ethanol series into Histoclear and embedded in Paraplast plus. Tissue was sectioned to 10 μm using a Leica microtome. Localization was performed as previously described (Jackson, 1991). Primary anti-LG1 (guinea pig) was used at a 1:5000 dilution, and guinea pig anti-LG1 (guinea pig) was used at a 1:500 dilution (Abcam). The alkaline phosphatase color reaction was stopped in water and the slides were mounted in aqueous mounting medium for imaging.

Statistical analysis

Unpaired Student’s t-test using mean, standard deviation and number was used to determine significance between populations. Standard deviations are shown in graphs throughout the paper.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Y.H. carried out experiments. K.H. designed and carried out experiments. S.H., M.W.L., N.B., A.H. and H.C. designed and carried out experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.111955/-/DC1

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