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RESEARCH ARTICLE

Gene regulatory interactions at lateral organ boundaries in maize

Michael W. Lewis[1], Nathalie Bolduc[1,*], Kayley Hake[1,‡], Yadanar Htike[1], Angela Hay[1,§], Héctor Candela[1,¶] and Sarah Hake[1,2,]**

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 (Doebley and Stec, 1993; Doebley 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 grasses decreased losses due to lodging (Evans, 1993).

Leaf angle has also been important for crop improvement, especially for increased maize yields (Duvick, 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

[1]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA. [2]Plant Gene Expression Center, Agricultural Research Service, United States Department of Agriculture, Albany, CA 94710, USA. *Present address: Clontech Laboratories, Mountain View, CA 94043, USA. ‡Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA. §Present address: Max Planck Institute for Plant Breeding, 50829 Cologne, Germany. ¶Present address: Universidad Miguel Hernández de Elche, 03202 Alicante, Spain.

**Author for correspondence (hake@berkeley.edu)

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Fig. 1. Loss of *lg1* affects leaf and tassel development. (A) A normal maize leaf (+/+) contains proximal sheath (s) and distal blade (b) separated by ligule (arrow) and auricle (a). (B) *lg1-R* lacks ligule and auricle. (C-E) Close-up of leaves split at the midrib (C) normal, (D) *lg1-R*, (E) *lg1-n2375*. (F,G) Tassel branch profile of normal compared with *lg1-R*. (H-J) The pulvinus (arrow) is visible in normal but missing in *lg1* mutants.

(Fig. 1E). Tassels of both *lg1* alleles were upright with reduced or small pulvini (Fig. 1F-J). Tassel branch angle as well as tassel branch number were reduced in *lg1-R* (Table 1A). The tassel branch number was not different for *lg1-n2375* but the angle was significantly narrower (Table 1A).

We generated a LG1 antibody to follow its accumulation through time and in mutant backgrounds (Fig. 2). LG1 was detected in leaves and tassel branches of wild-type plants but absent in *lg1-R* and reduced in *lg1-n2375* (Fig. 2E,F). In developing leaves, LG1 accumulated by Plastochron 7 (P7) at a region coincident with the preligule band (Fig. 2A,B). In P8 and later leaves, LG1 accumulation marked the differentiating ligule band and, later, the morphologically distinct ligule (Fig. 2C,D). In *lg2* mutant leaves, the ligule and auricle are missing, except near the margin (supplementary material Fig. S1A). LG1 accumulation was only detected in the marginal domain in *lg2* mutant leaves (supplementary material Fig. S1B-D). These data suggest that accumulation of LG1 is required for correct ligule differentiation and outgrowth.

In wild-type tassels, LG1 was detected at the adaxial base of developing tassel branches near the junction with the rachis (Fig. 2G-J). Expression could be detected in young tassels (~1 mm, Fig. 2G) and persisted as the tassel grew (>3 mm, Fig. 2H). The accumulation did not spread far from the branch-rachis junction into the branch and was usually detected in one branch at a time through transverse serial sections (Fig. 2I,J). LG1 was also expressed at the junction of secondary tassel branches (Fig. 2J). Thus, LG1 accumulation marks two distinct boundaries in the maize plant, the junction between sheath and blade and the junction of tassel branches.

Table 1. Tassel measurements

	TBA	TBN	*n*
A			
wt sibs	26.67°	6.17	6
lg1-R	11.75°	3.77	8
	P<0.001	*P*<0.02	
wt sibs	17.5°	10.83	12
lg1n-2375	0.6°	8.8	10
	P<0.001	*P*=0.15	
B			
wt sibs		5.82	22
wab1-rev		1.3	10
		P<0.001	
C			
wt sibs	6°	5.714	7
Wab1-R/+	25.07°	4.5	14
	P<0.001	*P*<0.07	

TBA, tassel branch angle; TBN, tassel branch number.

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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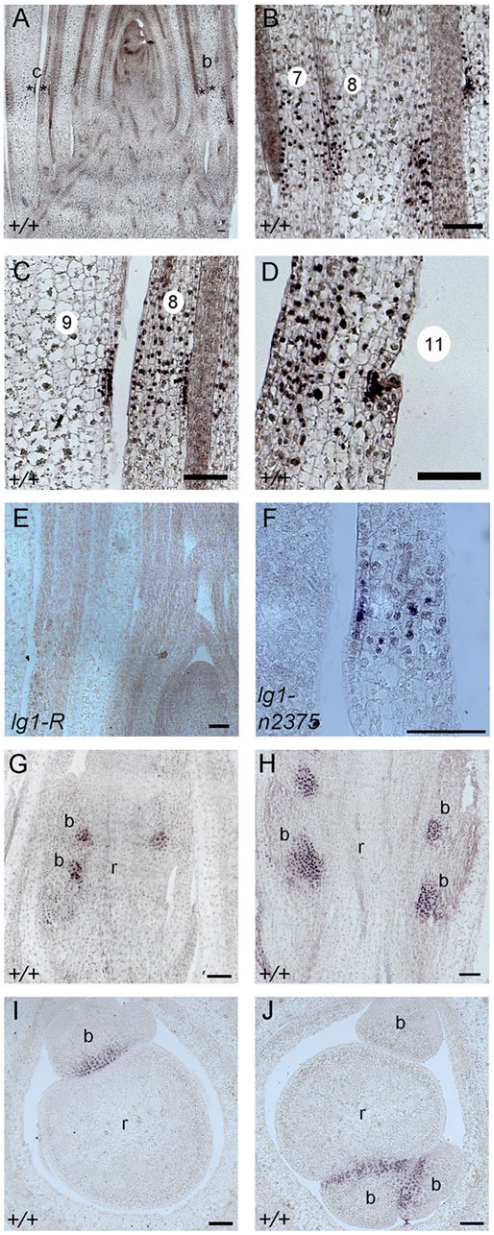

Fig. 2. LG1 immunolocalization in leaves and tassels. (A-D) Longitudinal sections through a wild-type shoot apex. (B,C) Higher magnification of ligule regions marked with asterisk in A, showing LG1 protein in the preligule band. By P9 (C), LG1 accumulation is more restricted to the incipient ligule. (D) Accumulation in the notch of a young ligule and throughout the blade-sheath boundary at P11. (E) No LG1 protein is detected in *lg1-R*. (F) Reduced LG1 protein is detected in *lg1-n2375*. (G,H) LG1 is detected on the adaxial side of developing tassel branches (G, ~1 mm tassel; H, ~3 mm tassel). (I,J) Transverse serial sections through a wild-type tassel. b, tassel branch; r, rachis. Scale bars: 50 µm.

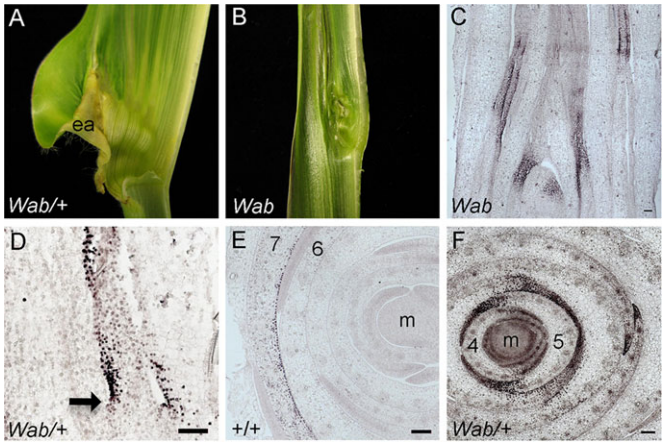

Fig. 3. LG1 is ectopically expressed in *Wab1-R* leaf blades. (A) In *Wab1-R* heterozygous plants (*Wab*/+), ectopic auricle (ea) spreads into the blade. (B) In *Wab1-R* homozygous plants (*Wab*), sheath tissue extends into the blade. The adaxial side of a split leaf is presented with midrib to right. (C-F) LG1 immunolocalizations. (C) Longitudinal sections of *Wab1-R* homozygotes (Fig. 2B-D or supplementary material Fig. S5A serves as wild-type comparison). (D) Accumulation of LG1 extends distally into the blade beginning at the ligule (arrow). (E) Transverse section, showing LG1 accumulating only in P7 leaf in wild-type vegetative apex. (F) Expanded and precocious accumulation of LG1 in *Wab1-R*/+ apex. Scale bars: 50 µm. m, shoot apical meristem.

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

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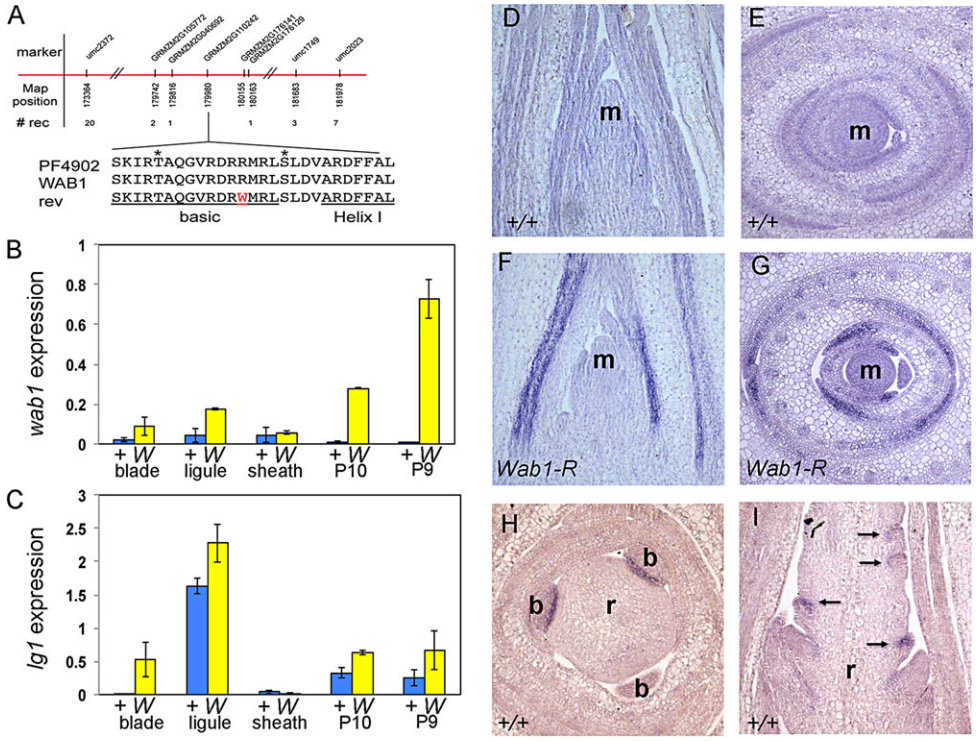

Fig. 4. Reverting the dominant mutant phenotype reveals that *wab1* encodes a TCP. (A) The *wab1* revertant (*rev*) contains a single nucleotide substitution (marked in red) in GRMZM2G110242 resulting in a R130W mutation within a highly conserved domain. *wab1-bad1* (T122M) and *wab1-bad2* (S134F) are denoted with an asterisk (Bai et al., 2012). The number of recombinants (# rec) is shown for markers in the interval. (B,C) qRT-PCR of *wab1* (B) or *lg1* (C) relative expression in non-mutant (+) or *Wab1-R* homozygous (*W*) leaves. For P11 leaves, primordia were separated into blade, ligule and sheath. For P10 and P9 leaves, the ligule and sheath were collected together. Note that the relative expression scales differ. (D-I) *In situ* hybridizations with a *wab1* probe. *wab1* is not detected in longitudinal (D) or transverse (E) sections of wild-type seedlings. Strong expression of *wab1* is apparent in leaves of *Wab1-R* seedlings in longitudinal (F) and transverse sections (G). (H) *wab1* is detected in the axils of tassel branches in a wild-type transverse section. (I) *wab1* is expressed in the axil of spikelet pair meristems and branch meristems in a longitudinal section of a wild-type tassel (arrows). m, shoot apical meristem; r, rachis; b, tassel branch. Standard deviations are shown in graphs.

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. 5F-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. 5J,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

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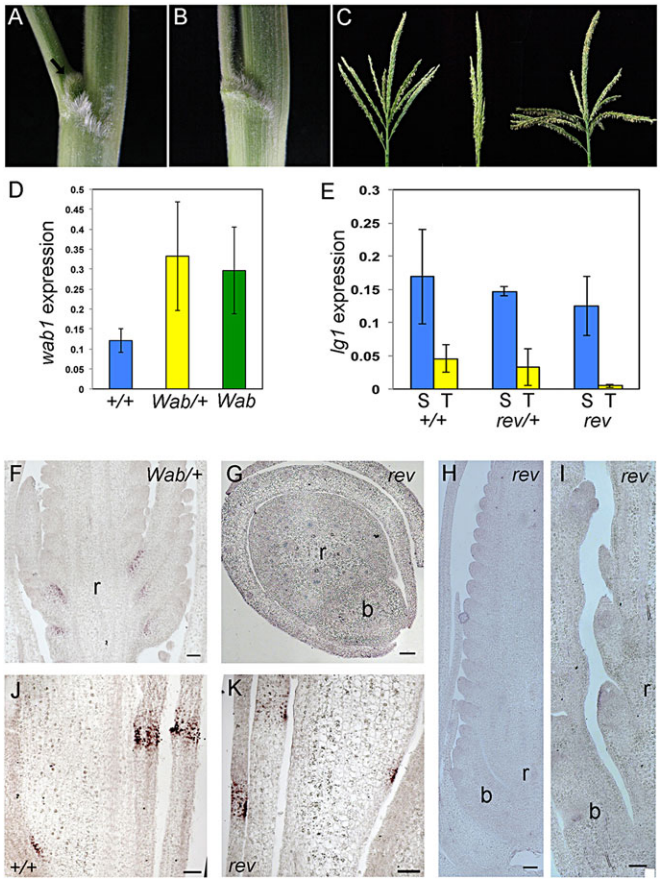

Fig. 5. *wab1* regulates LG1 during tassel branch development. (A) The pulvinus (arrow) of normal compared with *wab1-rev* tassel (B). (C) From left to right: A619/*wab1-bad1*, *wab1-bad1*/*wab1-bad1*, *Wab1-R*/A619 or *Wab1-R*/*wab1-bad1* tassels. (D) qRT-PCR of *wab1* in tassels. (E) qRT-PCR analysis in 1-3 mm tassels (T) compared with seedlings (S). (F) LG1 domain in *Wab1-R* is similar to wild type (see Fig. 2G,H for comparison). (G) No LG1 is detected in transverse sections through emerging tassel branches in *wab1-rev*. (H,I) LG1 is absent in *wab1-rev* tassels (~1 mm tassel; E, ~3 mm tassel; F). (J,K) LG1 accumulation pattern in *wab-rev* leaves is similar to normal siblings. (See supplementary material Fig. S5 for a large zoom out of the images shown in J and K.) b, tassel branch; r, rachis. Scale bars: 50 µm. Standard deviations are shown in graphs.

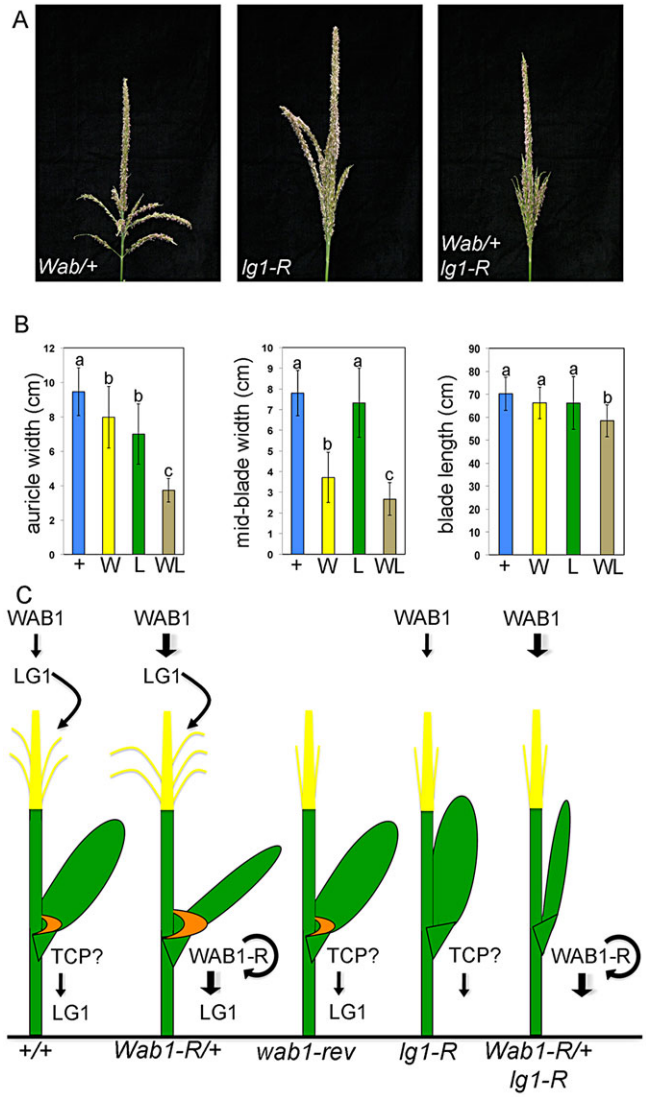

Fig. 6. Genetic interactions of *wab1* and *lg1*. (A) *Wab1-R*/+ *lg1-R* double-mutant tassels have upright tassel branch architecture similar to *lg1-R*. (B) Leaf measurements from families segregating *lg1-R* (L), *Wab1-R*/+ (W), normal (+), and the double mutant (WL). Each letter (a, b, c) is significantly different from another letter at $P<0.003$. (C) Model of WAB1 and LG1 function in tassel and leaf. *wab1* turns on *lg1* in the tassel and a different TCP or other transcription factor turns on *lg1* in the leaf. In *Wab1-R*, misexpression of *wab1* leads to an increase in tassel branch angle, and in the leaf, it leads to misexpression of *lg1*. Because *wab1* expression returns to background levels in *wab1-rev* leaves (but not in *wab1-rev* tassels), we assume that *Wab1-R* promotes its own expression in the leaf. In the *wab1* loss-of-function, there are fewer tassel branches and no *lg1* in the tassel; however, *lg1* is still expressed in the leaf. Standard deviations are shown in graphs.

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 anther 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

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fringe. The first sign of a preligule 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 axils of tassel branches early in their development. This pattern overlaps that of *wab1* and is dependent on *wab1*. In the *wab1* loss-of-function mutant, fewer branches form and LG1 protein fails to accumulate. Branches that do form are upright. In the gain-of-function mutant, an increase of LG1 and *wab1* mRNA occurs with a concomitant increase in tassel branch angle. Unlike the leaf, where *wab1* is normally excluded, the increase in *wab1* and LG1 in the tassel is confined to their normal regions of expression, affecting only tassel branch angle.

Given that LG1 still accumulates in leaves of *wab1-rev* plants, WAB1 is sufficient to turn on *lg1* in the leaf but is not necessary. We hypothesize that in wild type an unknown transcription factor, perhaps a different TCP, turns on *lg1* in the leaf.

TCP expression in boundaries

The narrow leaf phenotype of *Wab1-R* is consistent with a role in restricting growth suggested in studies of other TCP genes. *teosinte branched1* (*tb1*) is expressed in maize lateral branches and suppresses elongation of the branch (Doebley et al., 1997; Hubbard et al., 2002). *Cycloidea* is expressed in the dorsal part of *Antirrhinum* floral meristems, which leads to the zygomorphic floral form (Luo et al., 1996). *cincinnata* mutants show extra growth at the margins, revealing a lack of growth restraint (Nath et al., 2003). *BRC1*, the closest *Arabidopsis thaliana* gene to *wab1*, is expressed in axillary meristems to promote growth arrest (Aguilar-Martínez et al., 2007).

In contrast to the expression domain of *BRC1* and *tb1* within the lateral meristem, *wab1* is expressed in the boundary between lateral organs and the meristem. Rather than repressing growth of the lateral organ, *wab1* expression may repress growth in the axil. The reduction in branch number seen in *wab1* loss-of-function mutants suggests that this boundary expression is required for branch initiation.

Lateral organ boundaries and the ligule

Support for the idea of a boundary between lateral organs and the shoot apical meristem is well-supported by analysis of boundary genes (Aida and Tasaka, 2006). Whether boundary genes function at the ligule has not been determined. However, *LATERAL ORGAN BOUNDARY* (*LOB*) regulates brassinosteroid (BR) function, and manipulation of BR ameliorates the *lob* defect (Bell et al., 2012; Gendron et al., 2012). BR has a well-documented role in the ligule in rice. Mutants that fail to perceive BR have upright leaves with small auricles, whereas addition of BR to leaves causes the blade to lean out because of expanded auricle cells (Wada et al., 1981; Yamamuro et al., 2000).

We propose that the ligule functions as a boundary. The mature sheath and blade contain different epidermal cell types, distinct venation and unique abaxial-adaxial patterning (Candela et al., 2008; Russell and Evert, 1985; Sylvester et al., 1990). At this boundary, reorientation of growth occurs with the ligule growing out of the plane of the leaf. LG1 or a gene downstream may act as an organizer of tissue polarity (Green et al., 2010). In mutants without the ligule, such as *Liguleless narrow* (Moon et al., 2013) and *Wab1-R* homozygotes, a lack of integrity is documented with proximal identity (sheath) spreading into distal regions (blade). Boundaries are also needed for lateral growth; mutants that lack ligules are narrower. Interestingly, leaves of dominant *Kn1* mutants with ectopic ligule and auricle in the blade are wider than those of their normal siblings (Freeling and Hake, 1985; Ramirez et al., 2009), suggesting that the ectopic boundary formation supports additional medial-lateral growth.

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; Zhu 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-bad1* was introgressed into A619 four times (Bai et al., 2012). The *lg1-R* allele was obtained from the Maize Genetics Stock Center and introgressed at least seven generations into W23. The *lg1-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.

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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-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 *Dra*I digestion. One of the normal plants carried the PF4902 polymorphism 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 (Bortiri 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) (Hellemans 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., 2010). Sequence encoding full-length LG1 protein was cloned into pENTR (using P19/P20, supplementary material Table S1) and, subsequently, into pDEST15 or pDEST17 (Invitrogen) to generate the N-terminal fusion proteins. The LG1 protein used for immunization of guinea pigs (Cocalico Biologicals) was produced in *E. coli* and purified by using a N-terminal HIS-tag. The serum was affinity purified against full-length LG1 expressed 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 1:500 dilution, and guinea pig α-alkaline-phosphatase-conjugated secondary antibody was used at a 1:5000 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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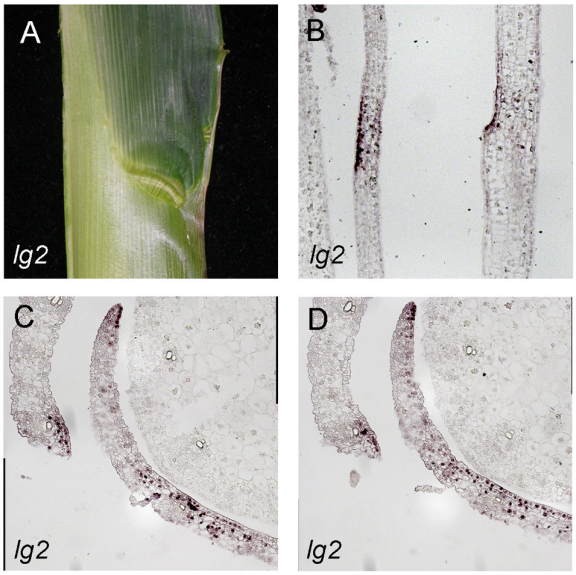

Figure S1. Phenotype and LG1 accumulation in *lg2-R* leaves.

A) Leaves of *lg2-R* plants develop ligule and auricle only at the margin (right). B) Accumulation of LG1 is only detected in the narrow marginal domains of *lg2* leaves. C, D) In transverse sections, LG1 accumulation typically appears in one side of a leaf at a time. This accumulation pattern reflects the phenotype of *lg2* mutant plants in which the marginal ligule has displaced symmetry across the midrib, with ligule higher on one side than the other.

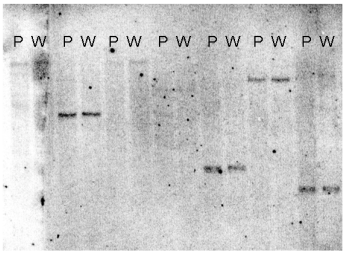

Figure S2. Southern blot analysis of *wab1* mapping interval.

DNA from *Wab1-R* homozygotes and their progenitor, PF4902, was digested with 7 enzymes (from left to right: *Bste II, EcoRI, EcoRV, HindIII, NcoI, SstI* and *Xba*) electrophoresed, blotted and hybridized with a 5' fragment of *wab1*. No differences were detected.

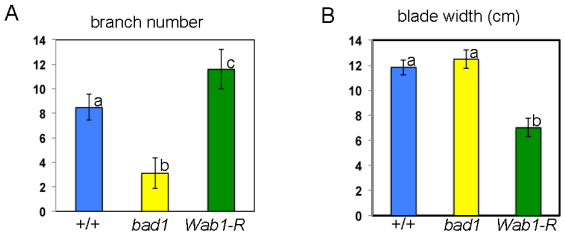

Figure S3. Quantification of tassel branch number and blade width.

Maize plants from family 1013, segregating A619/*wab1-bad1* (+), *wab1-bad1/wab1-bad1 (bad), Wab1-R*/A619 or *Wab1-R/wab1-bad1 (Wab1-R*), were scored for tassel branch number and mid-blade width. Tassel branch number is significantly different between each genotype (A) while significant differences in leaf width at mid-blade can be seen when comparing A619*/wab1-bad1* or *wab1-bad1* to *Wab1-R/wab1-bad1* (B). Standard deviation is shown. Significance is indicated by letter (A, B or C): each letter is significantly different from another letter (i.e. B is similar to B but significantly different from A or C at $p<0.001$).

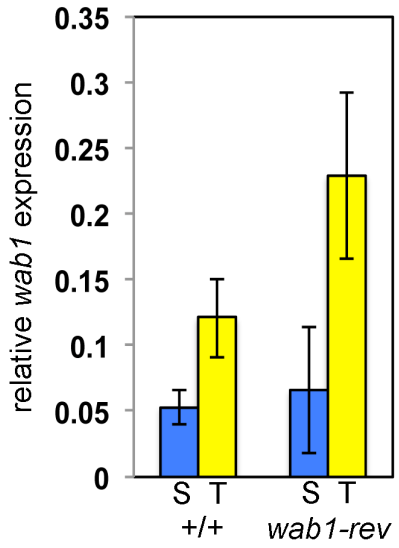

Figure S4. *wab1* expression in *wab1-rev* shoots and tassels.

Quantitative real time PCR analysis of *wab1* in *wab1-rev* seedlings (S) or tassels (T) compared to normal siblings shows that ectopic expression caused by the *Wab1-R* mutation remains in the tassel but not seedlings. This suggests that in the leaf, an active DNA binding domain is required to keep *wab1* levels high. Standard deviations are shown in graphs.

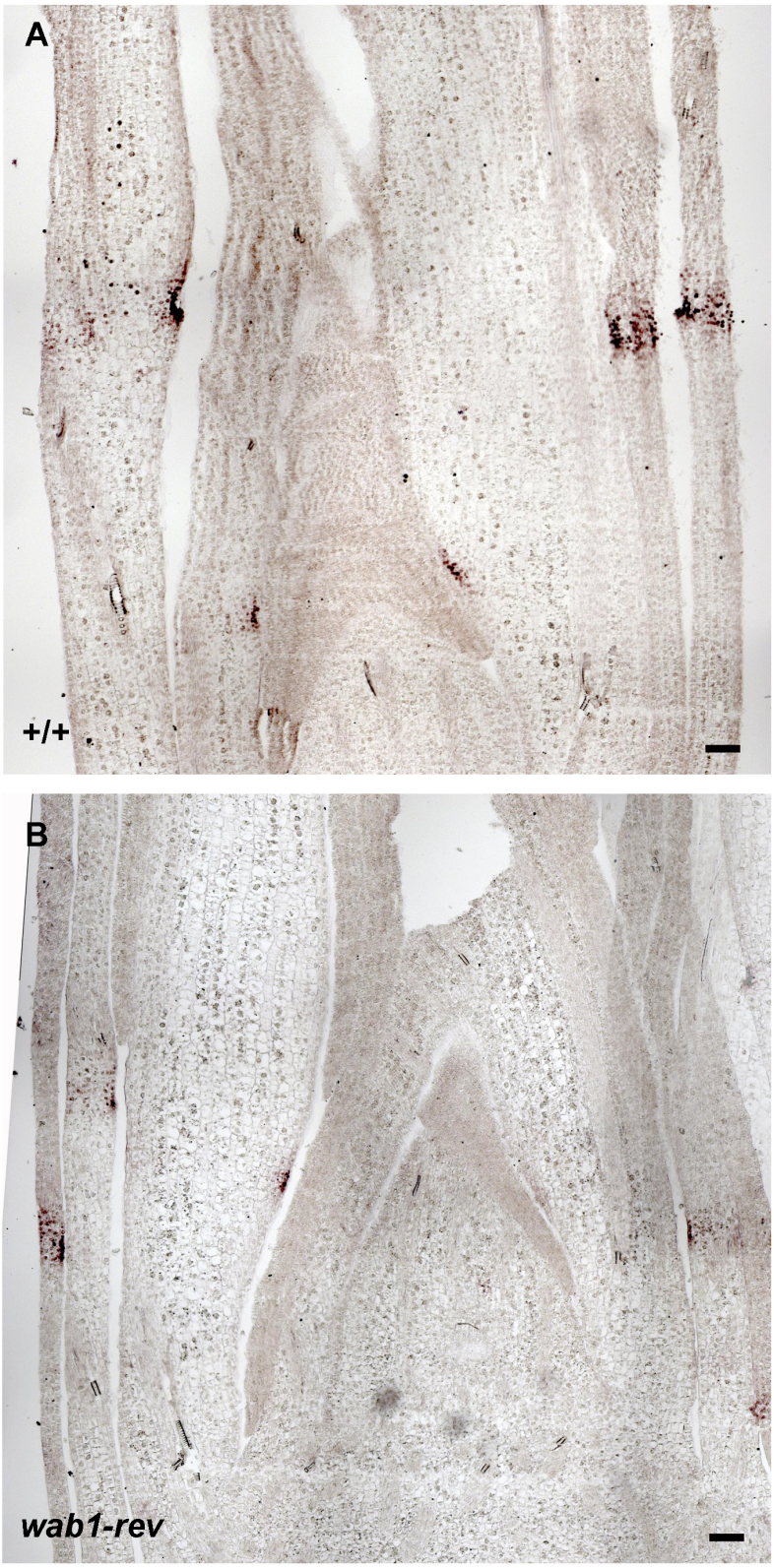
A
+/+
B
wab1-rev

Figure S5. LG1 accumulates in *wab1-rev* leaves.

Wild-type (A, +/+) and *wab1-rev* (B) leaves both accumulate LG1 at the blade sheath boundary. Longitudinal sections off-centered from the SAM reveal the earliest signs of LG1 accumulation. Figure 5 J,K are a zoom in taken from these images.

Table S1. Oligonucleotides used in this study.

Primer	Sequence
P1	GTGTGGGAGTTCTTGGGAGA
P2	TGCACGACCCAGTGTATTGT
P3	CACCATGTTGCCGTACCCTAACAACCC
P4	CCAACTTCTCGGCAACTCCTC
P5	ACATAAAACGCTCCCCACTG
P6	TGGCAGTACGACGACACCTA
P7	GGGGAGGCAAAAGTAGATCC
P8	GCTCGATAAAAAGGCACCAC
P9	CTCTCACATCTTTCTGCTTCCTG
P10	CGGCAGGCAGACTACCGTT
P11	GAGCGACGACGCAGCTTT
P12	AGTAACGTCAAAGCACAAGATCAG
P13	TCCAGGAAATCACCGTCTTC
P14	AGCGACGACGCAGCTTTCTCA
P15	GCTAAAGACAAAGCAGAGAG
P16	CTAGTGATCGAAGTCGAGATC
P17	CCTGCTTCTCATGGATGGTT
P18	TGGTAGCAGGAAGGGAAACA
P19	CACCATGATGAACCTATCGGCTGCCG
P20	CTAGTGATCGAAGTCGAGATCAAAC