Unequal Redundancy in Maize knotted1 homeobox Genes¹[C][W][OPEN]

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The knotted1 (kn1) homeobox (knox) gene family was first identified through gain-of-function dominant mutants in maize (Zea mays). Class I knox members are expressed in meristems but excluded from leaves. In maize, a loss-of-function phenotype has only been characterized for kn1. To assess the function of another knox member, we characterized a loss-of-function mutation of rough sheath1 (rs1). rs1-mum1 has no phenotype alone but exacerbates several aspects of the kn1 phenotype. In permissive backgrounds in which kn1 mutants grow to maturity, loss of a single copy of rs1 enhances the tassel branch reduction phenotype, while loss of both copies results in limited shoots. In less introgressed lines, double mutants can grow to maturity but are shorter. Using a KNOX antibody, we demonstrate that RS1 binds in vivo to some of the KN1 target genes, which could partially explain why KN1 binds many genes but modulates few. Our results demonstrate an unequal redundancy between knox genes, with a role for rs1 only revealed in the complete absence of kn1.

Meristems are self-organizing groups of cells that produce plant organs. The shoot apical meristem (SAM) initiates leaf primordia from meristem flanks, the region known as the morphogenetic zone (Kaplan and Hagemann, 1991). Inside the morphogenetic zone lies the central zone, which divides to replenish cells lost to primordia initiation. Branch meristems initiate from the axes of leaf primordia. The pattern of leaf initiation and the extent to which the branch meristems grow determine the overall shoot architecture of a given plant species.

Genetic analysis of plant architecture has led to the discovery of key genes that function in meristems, including the maize (Zea mays) transcription factor knotted1 (kn1), the founding member of the kn1 homeobox (knox) gene family (Hake et al., 2004). Class I members of this family are expressed in meristems and excluded from leaves. In some species with dissected leaves, knox gene expression resumes in the leaf and contributes to leaf shape (Hay and Tsiantis, 2010). In maize, kn1 and other knox genes (rough sheath1 [rs1], gnarley1 [gn1], liguleless3 [lg3], and lg4) were discovered from their dominant gain-of-function phenotypes (Schneeburger et al., 1995; Foster et al., 1999; Muehlbauer et al., 1999; Bauer et al., 2004). In these mutants, knox genes are ectopically expressed in leaves, leading to proximal-distal patterning defects.

Loss-of-function mutants highlight the requirement for knox genes in shoot function. The first discovered knox loss-of-function mutant, shoot meristemless (stm) of Arabidopsis (Arabidopsis thaliana), fails to produce shoot organs after the cotyledons (Barton and Poethig, 1993; Long et al., 1996). The loss-of-function mutant of kn1 was discovered by reverting the gain-of-function phenotype (Kerstetter et al., 1997; Vollbrecht et al., 2000). Interestingly, the kn1 loss-of-function phenotype is dependent upon inbred background. In restrictive backgrounds that have small meristems, the phenotype of the kn1 mutant is a limited shoot, similar to stm. The scutellum and coleoptile form, which together are analogous to the cotyledon, and occasionally one or two leaves. In permissive backgrounds having larger shoot meristems, vegetative development continues without interruption, but defects are seen in male and female inflorescences. A similar phenotype has been described for the ortholog of kn1 in rice (Oryza sativa), OSH1 (Tsuda et al., 2011). osh1 single mutants make three leaves before shoot meristem termination, while double mutants with osh15 initiate only a coleoptile.

Chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (ChIP-seq) in combination with whole transcriptome shotgun sequencing identified a set of genes that are bound by KN1 and differentially expressed in either the gain- or loss-

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of-function mutants (Bolduc et al., 2012). Although over 5,000 genes were bound by KN1, only 643 were differentially expressed in one or multiple tissues. We hypothesized that KNOX redundancy partially explains the restricted overlap between the two data sets. In the absence of kn1, other KNOX proteins may also bind the same targets and modulate transcription.

In order to address the question of KNOX redundancy, we turned our attention to rs1, which has an expression pattern overlapping that of kn1 (Jackson et al., 1994). We show that RS1 shares many in vivo binding targets with KN1. Although the rs1 loss-of-function allele has no phenotype alone, it exacerbates several aspects of the kn1 phenotype. Our results indicate that KN1 and RS1 have unequal, redundant functions during development.

RESULTS

Isolation of the rs1-mum1 Mutant

Many inbreds, such as B73, Mo17, A619, and A188, are permissive of the absence of kn1 during vegetative growth. Others, including W22 and W23, are restrictive, exhibiting limited shoots (Vollbrecht et al., 2000). We reasoned that other knox genes must compensate for the absence of kn1 in the SAM in permissive backgrounds. We took advantage of whole transcriptome shotgun sequencing data from different maize tissues to assess the expression of kn1 and other knox genes (Supplemental Fig. S1). The expression level varies depending on tissue, suggesting specific functions of each knox gene throughout development. kn1 is abundantly expressed in organs that show phenotypes in its absence: the SAM, ear, tassel, and embryo.

Considering the high expression level of rs1 and its overlapping expression pattern with kn1 in ears and tassels (Jackson et al., 1994), we pursued analysis of rs1. We obtained an insertion allele, rs1-mum1, which carries a Mutator (Mu) element in the second exon (Fig. 1A). Reverse transcription-PCR and sequencing confirmed that the insertion leads to an abnormal transcript with an early stop codon (Fig. 1B), resulting in a predicted chimeric protein consisting of the first 146 amino acids from RS1 followed by 25 amino acids encoded by the Mu element. Expression analysis by quantitative reverse transcription (qRT)-PCR indicates that rs1 mRNA accumulates to a very low level in the rs1-mum1 SAM compared with the wild type. As controls, we also verified the accumulation of lg3 and kn1 in the same samples and found that they are not differentially expressed (Fig. 1C). Thus, rs1-mum1 represents a true loss-of-function allele. However, after six backcrosses to inbreds B73, Mo17, and W23, rs1-mum1 plants exhibit normal vegetative and reproductive development (Fig. 1D; Supplemental Fig. S2). Quantitative comparison of rs1-mum1 tassels and their wild-type siblings after six backcrosses to the B73 inbred detected no significant differences (Supplemental Table S1). Thus, RS1 is not essential, and its function may be hidden due to KNOX redundancy.

Genes Bound and Modulated by KN1 in Leaves Are Also Modulated by RS1 and LG3

To test redundancy between RS1 and KN1, we examined the expression levels of select KN1 targets in Rs1 and Lg3 dominant mutants introgressed into B73. In a previous study, we had reported that the KN1 target ga2ox1 was up-regulated in leaves of the dominant knox mutants, Gna-R and Lg3-O (Bolduc and Hake, 2009). Because Rs1 and Lg3 mainly affect the sheath-blade boundary (Fig. 2, A–F; Freeling, 1992; Fowler and Freeling, 1996), we dissected this region in developing leaves and used qRT-PCR to evaluate the expression of known KN1 targets and nontargets. As expected, lg3 and rs1 were up-regulated in their respective dominant mutants and lg3 was up-regulated in the leaves of Rs1-1025 (Fig. 2G), as described before (Bauer et al., 2004). Interestingly, lg3 was also detected...
suggest that at least some of the genes directly bound and modulated by KN1 are similarly modulated by LG3 and Rs1 and may explain the lack of phenotype observed for rs1-mum1 as well as for lg3 loss-of-function alleles (Bauer et al., 2004).

Genes Bound in Vivo by KN1 Are Also Bound by Other KNOX Proteins

To investigate if Rs1 in vivo binding targets overlap with those of KN1, we took advantage of antibodies reacting against the full-length KN1 protein that also cross-react with other KNOX proteins in maize and other species (Bharathan et al., 2002; Nowak et al., 2011). When used for western blots with chromatin derived from B73, kn1-e1, and rs1-mum1 shoot apices, these “KNOX” antibodies recognized a major band corresponding to KN1 as well as another band of slightly smaller Mr, present in B73 and kn1-e1 but not in rs1-mum1 (Fig. 3A). The absence of this band in the rs1 mutant, as well as its smaller size compared with KN1, indicates that it likely corresponds to the RS1 protein, which has a predicted size of 38.8 kD, compared with 39.9 kD for KN1. Furthermore, analysis of the efficiency of immunoprecipitation showed that the intensity of both bands decreased when the KNOX antibodies were used for pull down, while the smaller band remained unchanged when the KN1-specific antibodies were used for pull down (Supplemental Fig. S4). Thus, the KNOX antibodies can detect and pull down both KN1 and RS1.

When we used the KNOX antibodies to perform ChIP starting with rs1-mum1 shoot apices, we detected enrichment at the ga2ox1 locus similar to that detected in B73. This enrichment was reduced when using kn1-e1 shoot apices. By contrast, no enrichment was detected in the kn1-e1 apices when using KN1-specific antibodies (Fig. 3B; Bolduc and Hake, 2009; Bolduc et al., 2012). These results clearly show that KNOX transcription factors other than KN1 bind in vivo to the ga2ox1 locus. To evaluate if we could reproduce these results with other KN1-bound genes, we selected four loci previously identified by KN1 ChIP-seq that exhibited weaker or stronger binding than that to ga2ox1 (Bolduc et al., 2012). As observed for ga2ox1, ChIP using KN1-specific and KNOX antibodies led to comparable enrichment between B73 and rs1-mum1, but the overall enrichment detected with the KNOX antibodies was 50% lower in kn1-e1. Thus, at least for the five loci tested, KNOX transcription factors other than KN1 can bind in vivo to the chromatin regions occupied by KN1. Given that the two proteins identified in B73 shoot apices disappear in either kn1 or rs1 mutants (Fig. 3A), we propose that RS1 is the other major protein binding the KNOX targets in our ChIP assay.

RS1 and LG3 Bind in Planta to the cis-Regulatory Element Previously Identified for KN1

Although we are confident that the KNOX antibodies recognize RS1 in addition to KN1, the ChIP
assay highlights binding to a particular chromatin region but does not allow the assessment of binding to individual cis-regulatory elements. In a previous study, we showed in vitro binding of GN1 (which is 98.4% identical to RS1 across its homeodomain [HD]) and LG3 to the KN1 binding motif identified at the ga2ox1 locus (Bolduc and Hake, 2009). To determine if RS1 and LG3 could bind in vivo to the same cis-regulatory element, we took advantage of the in planta luciferase reporter assay previously developed for KN1 (Bolduc and Hake, 2009). Effector and reporter constructs (Fig. 4A) were introduced into Nicotiana benthamiana leaves, along with a transformation control, using Agrobacterium tumefaciens. RS1 behaved similarly to KN1 and induced a 5-fold increase of firefly luciferase (FiLuc) activity when fused to the activation domain of VP16 and 2-fold when used alone (Fig. 4B). LG3 also promoted an increase of FiLuc activity, although more modest than KN1 and RS1, with only a 2-fold increase when fused to VP16 and 1.5-fold when used alone. The weaker activation observed for LG3 suggests more divergent binding properties, also suggested by differences in the HD (Supplemental Fig. S5). Removal of the HD abolished any increase in reporter activity, and none of the constructs induced any changes when the mutated binding site was used, confirming that the changes in FiLuc reporter activity depend on the DNA-binding properties of the transcription factors. Thus,

![Figure 4](https://academic.oup.com/plphys/article/164/1/229/6112751)

Figure 4. RS1 and LG3 bind in planta to the cis-regulatory element previously identified for KN1. A and B, RS1, LG3, and KN1 (KNOX) with or without the activation domain of VP16 or lacking the HD (KNOX-HD) were produced in vivo in N. benthamiana leaves along with a reporter plasmid containing a minimal 35S promoter (min 35S) driving a FiLuc gene. Two copies of the wild-type or mutated KN1 binding site (BS) were inserted upstream of the minimal 35S promoter. Data were normalized with the Renilla luciferase (RiLuc) control. A, Drawing of the constructs used for the experiment. In the binding site sequences, the core TGAC motifs are boxed, with introduced mutations underlined. B, Fold activation of FiLuc activity is expressed relative to the basal value obtained with the minimal 35S promoter only for each protein. Error bars show the so from two biological replicates.

![Figure 3](https://academic.oup.com/plphys/article/164/1/229/6112751)

Figure 3. Other KNOX proteins are capable of binding in vivo to KN1-bound loci. A, Immunodetection of KNOX proteins in chromatin samples using KN1-specific antibodies or KN1 antibodies that cross react with other KNOX proteins. The same membrane was used for both antibodies with stripping and reblotting. KN1 (dots) is absent in the kn1-e1 mutant. A lower molecular weight protein (arrow) is detected with the KNOX antibodies in B73 and kn1-e1 but not in rs1-mum1, indicating that the antibodies cross react with the RS1 protein. A nonspecific band of about 60 kD detected with the KN1-specific antibodies indicates similar loading of the gel and uniform transfer to the membrane. B, ChIP, performed with either KN1-specific (top) or KNOX (bottom) antibodies on chromatin extracted from shoot apices, was followed by quantitative PCR on KN1 targets previously identified by ChIP-seq (Bolduc et al., 2012). Error bars show the so from two biological replicates.
both RS1 and LG3 can bind in planta to the KN1 cis-regulatory element, suggesting that this is a common KNOX binding site. These results support the hypothesis that RS1 also binds KN1 targets and is capable of modulating transcription in the absence of KN1.

RS1 Acts Redundantly with KN1 to Promote Shoot Initiation

Given the finding that other KNOX proteins bind and activate at least a subset of the genes targeted by KN1, we investigated whether the combination of kn1 and rs1 loss-of-function mutations would expose additional phenotypes not observed in single mutants. We carried out these crosses in B73 and Mo17, two permissive inbreds for the loss of kn1. kn1-e1 and rs1-mum1 were backcrossed six times into the B73 and Mo17 inbreds before combining them. In families segregating kn1 and rs1 loss-of-function mutations, we observed segregation of a limited shoot phenotype (Fig. 5, A and B) at a ratio of about 1:10. Eighty-two percent to 88% of the limited shoot individuals were homozygous for both mutations, while 12% to 18% were homozygous for kn1 and heterozygous for rs1 (Table 1). To determine if the limited shoot phenotype was similar to that described for kn1-e1 in the restrictive background W23, we looked at the histology of mature embryos (Fig. 5, C–J). In both kn1-e1 in W23 and kn1-e1; rs1-mum1 in B73, the meristem is flat with large vacuolated cells. Only the surrounding coleoptile is formed. Thus, loss of rs1 eliminates the background dependency of the kn1 limited shoot phenotype.

To quantify the penetrance of the limited shoot phenotype, we classified all the plants from two families segregating rs1-mum1 and kn1-e1 by genotype and evaluated shoot growth. All greenhouse-grown plants with limited shoots were homozygous for kn1-e1 and either rs1-mum1/rs1-mum1 or rs1-mum1/+. No normal seedlings were double mutants (Supplemental Tables S2 and S3). Field conditions precluded the observation of limited shoots, so we assessed only mature phenotypes. No double mutants were among the 125 individuals analyzed, although 17 were expected (Supplemental Table S3; Supplemental Fig. S6). These results show that both KN1 and RS1 are necessary for shoot development in B73 and Mo17.

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**Figure 5.** Loss of meristem maintenance in the rs1;kn1 double mutant. A and B, The rs1;kn1 double mutant does not grow past the coleoptile (Co) stage. C, Closeup of the double mutant shown in A (left). C to J, Mature embryos were allowed to rehydrate for 24 h before fixation. Sections were stained with toluidine blue to reveal histology. C to F, In the W23 inbred (C and E), kn1-e1 single mutants display loss of meristem maintenance, while in the B73 inbred (D and F), the absence of kn1 (D) does not affect vegetative development. Similarly, no defect is observed in the B73 inbred carrying the rs1-mum1 single mutation (F). G to J, In the rs1;kn1 double mutant embryos, vegetative development is compromised beyond the coleoptile stage. In B73 (wild-type) embryos (G and I), the coleoptile encircles the first five leaves initiated during embryonic development, while in the double mutant (H and J), no leaf structure other than the coleoptile is visible. I and J show closeups of the dotted regions in G and H. L1 or L5, Leaf 1 or leaf 5. Bars = 100 μm.
A possible explanation for the difference between the permissive and restrictive backgrounds for kn1 loss of function could be that rs1 is not expressed in W23 but is expressed in B73. To address this question, we assayed the expression of rs1 and lg3 in the W23 background using qRT-PCR. We used a line carrying kn1-e1 introgressed four times into W23 for which limited shoots were 100% penetrant. We isolated RNA from embryos dissected 13 d after pollination. While kn1 was expressed at very low levels in mutants compared with normal siblings, rs1 and lg3 were expressed at normal levels (Supplemental Fig. S7). Sequencing of rs1 mRNA did not identify deleterious mutations. Thus, the background modifier that regulates the limited shoot phenotype in W23 does not significantly affect rs1 or lg3 mRNA levels.

Given that 12% to 18% of seeds with limited shoot were homozygous for kn1 and heterozygous for rs1, we investigated the dosage requirement for RS1 in kn1 mutant tassels. In B73, kn1 tassels have fewer branches and reduced spikelet density (Kerstetter et al., 1997). We genotyped and measured all the plants from a family segregating kn1-r1 and rs1-mum1. Similar to the kn1-e1 allele, we observed limited shoots in families segregating rs1-mum1 and the kn1-r1 allele. We did not detect any quantifiable differences between the tassels of the various genotype combinations, with the exception of the kn1-r1 homozygous plants (Supplemental Fig. S6). Notably, rs1-mum1 homozygotes did not suffer from the loss of one copy of kn1. However, in plants homozygous for kn1, loss of one copy of rs1 reduced the tassel branches further (Fig. 6A). To better quantify this phenotype, we extended our analysis to a larger family carrying the kn1-e1 allele, focusing only on kn1 homozygotes, and noticed a statistically significant decrease in spikelet density and number of branches associated with the loss of one copy of rs1 (Fig. 6B).

We also evaluated material that was not as well introgressed to uncover additional phenotypes for loss of rs1. After four backcrosses of rs1-mum1 to B73, we crossed it to the kn1-e1/B73 stock. Although the limited shoot phenotype segregated in F2 families, a number of double mutants made it to maturity. These were significantly shorter than their siblings, although the leaf number was not changed (Supplemental Table S4), indicating that the height difference is due to shorter internode length. This result suggests that RS1 acts redundantly with KN1 to promote stem elongation, similar to its rice ortholog, OSH15 (Sato et al., 1999).

In summary, rs1 plays a critical, dosage-dependent role in SAM establishment and maintenance, stem elongation, and inflorescence development that is uncovered in a kn1 mutant background. When mutant alleles are not fully introgressed, KN1 and RS1 act together during internode elongation. When kn1 and rs1 mutant alleles are fully introgressed into the permissive backgrounds B73 and Mo17, the limited shoot phenotype detected only in restrictive backgrounds for the kn1 single mutant becomes fully penetrant. The reduction in tassel branch numbers is also enhanced with loss of one copy of rs1.

**DISCUSSION**

Class I knox transcription factors represent a small family of genes with as few as four members in Arabidopsis and eight in maize. In Arabidopsis, single, double, or higher order mutant combinations have helped pinpoint specific developmental functions of each gene. In maize, knox genes have been characterized through dominant leaf mutants, but their role during normal development has not been elucidated, with the exception of kn1. In this study, we identified a role for rs1 during multiple stages of development.

| Genotype            | B73    | Mo17   |
|---------------------|--------|--------|
| rs1/+;kn1/+         | 16 (87.5%) | 9 (81.8%) |
| rs1/+;kn1/kn1       | 2      | 2      |
| Total               | 18     | 11     |

**Table 1. Genotypes associated with the limited shoot phenotype**
Hierarchical Redundancy between kn1 and rs1

ChIP-seq experiments demonstrated that KN1 binds several thousand genomic loci, contributing to the regulation of 643 genes (Bolduc et al., 2012). We used anti-KNOX antibodies to show that KN1 targets are still bound in the absence of KN1, suggesting that additional KNOX proteins function in that capacity. Using an rs1 loss-of-function mutant, we demonstrate that RS1 is likely to be the other major KNOX protein recognized by this antibody. We confirmed that RS1 and other KNOX proteins, such as LG3, also bind in planta to the cis-regulatory element previously identified as KN1 bound using an in vivo luciferase reporter system, further reinforcing the idea that a subset of the KN1-targeted genes may be shared with other KNOX proteins. Although the rs1-mum1 mutant does not exhibit a phenotype on its own, it does show a phenotype when combined with kn1. When kn1 and rs1 are both absent, the limited shoot phenotype observed for kn1 single mutants in inbreds with small meristems, such as W23, becomes completely penetrant, regardless of genetic background. Occasionally, this phenotype was observed when only one copy of rs1 was mutated, illustrating a dosage effect. These results highlight a knox redundancy that is hierarchical. Only when kn1 is missing does the dose of rs1 have an impact, and rs1 cannot substitute for kn1.

Similar knox relationships have been uncovered in Arabidopsis and rice. stm has a limited shoot phenotype (Long et al., 1996), while brevipedicellus (BP) affects pedicel angle and plant height (Douglas et al., 2002; Venglat et al., 2002). The KNOX suppressor, asymmetric leaves1 (asl), suppresses the stm phenotype, but in the absence of BP, suppression is lost and the triple mutant (as1 bp stm) looks like stm. Thus, BP is redundant with STM in an as1 background (Byrne et al., 2002). In addition, bp enhances a weak stm allele (Byrne et al., 2002). Similarly, knat6 mutants have no phenotype but enhance the coryledon fusion defect of stm single mutants (Belles-Boix et al., 2006). In rice, the loss-of-function phenotype for the kn1 ortholog, OSH1, is a milder version of the limited shoot observed in maize, with three leaves produced before terminating (Tsuda et al., 2011). The osh1 mutant can be rescued by regenerating plants from callus, in which case fully mature plants are recovered but exhibit an inflorescence defect, similar to the kn1 loss-of-function phenotype in permissive backgrounds. Rice plants carrying mutations in the rs1 ortholog, OSH15, show a short stature (Sato et al., 1999), similar to the double rs1kn1 mutants recovered when not fully introsgressed into B73. osh1osh15 double mutants initiate a coleoptile but fail to form a meristem (Tsuda et al., 2011), reminiscent of the rs1; kn1 double mutant in maize (Fig. 5). Thus, in both maize and rice, two homeobox genes are important for maintaining the SAM, yet one plays a more significant role. The similar relationship of the orthologous kn1-osh1 pair suggests evolutionary conservation of the knox hierarchy.

When analyzed in well-introgressed material, rs1; kn1 double mutants consistently had limited shoots. However, a small number of plants with limited shoots were homozygous for kn1 and heterozygous for rs1-mum1. This finding suggests that loss of one copy of rs1 was occasionally sufficient to tip the balance between making a complete shoot or termination of the shoot meristem. Yet, homozygous rs1-mum1 plants had no phenotype when heterozygous for kn1. Thus, one normal copy of kn1 was more effective at promoting shoot development than one normal copy of rs1. Indeed, rs1-mum1/+;kn1-e1/+ plants were always normal; thus, simply reducing the copy number of knox transcription factors does not affect development. While kn1 mRNA levels are approximately twice as high as rs1 in the SAM (Supplemental Fig. S1) and could explain the important role of kn1, a more likely explanation is that kn1 is expressed in the central and morphogenetic zone of the meristem, whereas rs1 is expressed only at the base of the SAM (Jackson et al., 1994).

A critical role for kn1 was also observed during reproductive development. A significant reduction in tassel branch number and spikelet density was observed when only one normal copy of rs1 was present in kn1 homozygous mutants. Interestingly, kn1 and rs1 are expressed at similar levels in tassel primordia (Supplemental Fig. S1), and contrary to the SAM, here both genes have similar expression patterns (Jackson et al., 1994). Taken as single mutants, only kn1 has a tassel phenotype, again highlighting the critical role of kn1 in the knox hierarchy. Given that rs1 is duplicated, mutations in both rs1 and gnt (Foster et al., 1999) may be necessary to obtain a phenotype in the presence of a functional kn1. However, the low expression level for gnt in most tissues may indicate that it plays a less important role.

Shared KNOX Targets

All knox gain-of-function mutants, Kn1, Rs1, Gn1, Lg3, and Lg4, share common defects. Leaves are darker green, plants are shorter, and the sheath-blade boundary is affected. Although the pattern of misexpression is not known for Lg3 and Lg4, Rs1 and Gn1 display misexpression at the base of leaf primordia (Schneebberger et al., 1995; Foster et al., 1999). This misexpression is responsible for the displaced sheath-blade boundary and perturbations to the ligule and sheath regions, respectively. Kn1 phenotypes depend on the localization of misexpression, which is allele dependent. When kn1 is misexpressed at the margin of the leaf blade, ectopic sheath/auricle prongs grow out (Ramirez et al., 2009). When kn1 is misexpressed along the veins of the blade, knots occur (Smith et al., 1992).

Phenotypic differences are likely due to the location and timing of misexpression, while the commonality of knox dominant phenotypes is likely due to shared targets. Indeed, several genes bound by KN1 in shoot apices are also bound by other KNOX proteins (Fig 3). One common target that contributes to the shorter stature and dark green leaves is the GA pathway. Using ChIP assays, we showed here that KNOX binding to the ga2ox1 gene occurs even in the absence of KN1.
indicating that this target is shared with other KNOX proteins. In addition, in vivo luciferase reporter assays revealed that both Rs1 and LG3 bind to the KN1-bound cis-regulatory element derived from the ga2ox1 gene (Fig. 4). Finally, ga2ox1 is up-regulated in the leaves of Rs1 and LG3 dominant mutants (Fig. 2).

How the dominant knox mutants perturb proximal/distal patterning is not known, but it is likely to result from genes bound and modulated in all dominant knox mutants. One candidate could be the yabby gene family. Four duplicated yabby genes belonging to the same clade as F1L (Juarez et al., 2004) are bound and positively modulated by KN1 (Bolduc et al., 2012). One of them, yabby15, is overexpressed in the leaves of Rs1 and LG3 dominant mutants (Fig. 2). This gene is normally expressed in the proximal part of the maize leaf (Juarez et al., 2004; Li et al., 2010), and its misregulation may contribute to the presence of proximal structures in the distal part of the leaf.

It is intriguing that lg3 is expressed in the proximal part of developing leaves. lg3 is also bound by KN1 (Bolduc et al., 2012) and likely bound by other KNOX proteins, suggesting that KN1 could contribute to the specification of the sheath and the sheath-blade boundary early in leaf development through the activation of lg3. However, lg3 loss-of-function mutants have no phenotype on their own and do not affect the phenotype of the dominant Rs1-O mutant (Tyers, 2000; Bauer et al., 2004). It is likely that, similar to rs1, understanding the true function of lg3 will require a higher order mutant combination.

We observed that compared with KN1 and RS1, LG3 promoted weaker luciferase activity increase in the tobacco (Nicotiana tabacum) leaf binding assay, yet misexpression of ga2ox1 and yabby15 was stronger in Lg3 than in Rs1 (Fig. 2). Several hypotheses could explain this discrepancy. First, the HD of LG3 differs from that of KN1 and RS1, and the cis-regulatory element used for the tobacco binding assay may not be ideal for LG3. RS1 and KN1 share 95% similarity through their 63-amino acid HD (88% identity), while LG3 and KN1 share 84% similarity (76% identity). LG3 could bind ga2ox1 through one of the other potential binding sites that yielded poor binding for KN1 (Bolduc and Hake, 2009) but was not tested for other KNOX proteins. Second, it is possible that in developing maize leaves, LG3 encounters binding partners (e.g., members of the BEL family, known to interact with KNOX proteins) not present in fully mature tobacco leaves, thus causing different transcriptional effects.

knox Modifiers

The recessive kn1 phenotype is very background dependent. The limited shoot phenotype is poorly penetrant in B73, occasionally visible in Mo17, 50% penetrant in W22, and up to 100% penetrant in W23, with an increase in penetrance at each generation of introgression (Vollbrecht et al., 2000; Lunde and Hake, 2009). We observed that many rs1;kn1 double mutants reached maturity after a limited number of introgressions into B73, while they did not maintain the SAM when they were very well introgressed (six backcrosses). These observations suggest that residual heterozygosity or a modifier from the stock that carried the rs1;mu1 allele rescues the limited shoot phenotype. This modifier could be different from the one that enhances the kn1 loss-of-function phenotype in W23. Identification of such modifiers may help highlight important players in meristem maintenance that have not yet been revealed.

CONCLUSION

We identified a loss-of-function mutation in rs1. While it has no phenotype on its own, loss of one or both copies enhances kn1 loss-of-function phenotypes, such as limited shoots and reduced tassel branch number. RS1 binds and modulates the expression of some of the genes regulated by KN1. These common targets are likely responsible for the shared phenotypes of gain-of-function knox mutants. The hierarchical redundancy we observed in maize is also mirrored by results in rice (Tsuda et al., 2011), demonstrating an evolutionary conservation.

MATERIALS AND METHODS

Plant Materials and Phenotypic Analysis

The kn1-c1 and kn1-r1 alleles of maize (Zea mays; Kerstetter et al., 1997; Vollbrecht et al., 2000) were introgressed six times into the B73 background. rs1-mum1 (previously referred to as rs1-872; Tyers, 2000) was obtained through the Trait Utility Service for Corn from Pioneer and backcrossed to B73, Mo17, and W23 for four to six generations before analysis or crossing to kn1 alleles. LG3-O and Rs1-1025 were obtained from the Maize Genetics Stock Center and introgressed four times into the B73 background before use in this study. Tissue for ChIP or qRT-PCR was dissected from plants grown in the greenhouse. For phenotypic analysis at the seedling stage, single and double mutants were grown in the greenhouse and inspected regularly within the first 10 d after planting. Analyses requiring mature plants were carried out in the Gill Tract summer field in Albany, California.

Determination of Genotypes

The kn1-r1 genotype used primers E39 (5'-GCAGGTGACCACCGGTGCT-3') and E47 (5'-GAGATACCTACAAACTTTGG-3'), allowing the amplification of a PCR fragment 8 bp longer in the mutant allele compared with the wild-type allele. The genotype of kn1-r1 was confirmed with the gene-specific primers ES0 (5'-TGGATTGTAAGAAGCCG-3') and ER2 (5'-ATAGGACCTACCAATGG-3'), together or in combination with a primer detecting the nearby rD1 transposon (5'-CAAGGCTTACCTCAATA-3'). For rs1-872, we used gene-specific primers localized in the introns flanking exon 2, together or in combination with a Mu-specific primer (5'-AGAGAAGCCAACGCCAATGG-3').

ChIP and Immunodetection

For ChIP, shoot apices were dissected from 3-week-old seedlings showing four to five leaves (about 20 plants per biological replicate). Chromatin isolation and ChIP were performed as described (Bolduc and Hake, 2009). For immunodetection, standard procedures were followed (Ausubel et al., 1987). We detected the KNOX proteins in the same chromatin extracts that were used for the ChIP experiments. For KNOX detection after ChIP, 20% of the unbound fractions were used. KN1 antibodies used in this study were raised in rabbits against a full-length recombinant protein and further purified against
the N-terminal region of KN1 (amino acids 1–103) or the full-length KN1 (for the KNOX antibodies). We used the same serum that has been used in previous studies (Bolduc and Hake, 2009; Nowak et al., 2011; Bolduc et al., 2012).

**Real-Time Quantitative PCR**

Quantification of enrichment for KN1-bound or KNOX-bound loci after ChIP was performed by quantitative PCR on ChIP DNA essentially as described before (Morohashi et al., 2009). Ratio enrichments were calculated by normalizing to the total input of each sample and then the ratio target:untarget (tubulin promoter; primers NB149 and NB150) was calculated. Two biological replicates per genotype and per tissue were analyzed, with two technical replicates per sample. Primers are listed in Supplemental Table S5.

Quantification of mRNA accumulation by qRT-PCR was performed essentially as described (Bolduc and Hake, 2009). For each biological replicate, two technical replicates were assayed on a Bio-Rad CFX instrument. Normalization against the ribosomal RNA gene and relative fold enrichment were calculated using the Normalized Expression mode integrated in the instrument software. Primers and gene identifiers are listed in Supplemental Table S6.

**Transient in Planta Binding Assay**

The luciferase reporter assay was performed as described before (Bolduc and Hake, 2009) and used the same FlLuc reporter plasmids carrying two fiLuc reporter plasmids carrying two technical replicates per sample. Primers are listed in Supplemental Table S5.

**Statistical Analysis**

Statistical analysis of differences between samples was performed using a two-sample Student’s t test.

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Relative abundance of knox genes in meristematic tissues.
- **Supplemental Figure S2.** rs1-mum1 and kn1-e1 tassels.
- **Supplemental Figure S3.** lg3 is expressed in developing sheath.
- **Supplemental Figure S4.** KN1-specific or KNOX ChIP efficiency.
- **Supplemental Figure S5.** Alignment of the KN1, RS1, and LG3 homeodomain.
- **Supplemental Figure S6.** Comparison of tassels in family segregating kn1-r1 and rs1-mum1.
- **Supplemental Figure S7.** Accumulation of kn1, rs1, and lg3 in W23 embryos.
- **Supplemental Table S1.** Comparison between rs1-mum1 tassels and wild-type siblings.
- **Supplemental Table S2.** rs1-mum1 and kn1-e1 shootless in the Mo17 background.
- **Supplemental Table S3.** rs1-mum1 and kn1-e1 shootless in the B73 background.
- **Supplemental Table S4.** Height of rs1-kn1 double mutants after three backcrosses to B73.
- **Supplemental Table S5.** List of primers used for ChIP-qPCR.
- **Supplemental Table S6.** List of primers used for qRT-PCR.

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