The extended auricle1 (eta1) Gene Is Essential for the Genetic Network Controlling Postinitiation Maize Leaf Development

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ABSTRACT

The maize leaf is composed of distinct regions with clear morphological boundaries. The ligule and auricle mark the boundary between distal blade and proximal sheath and are amenable to genetic study due to the array of mutants that affect their formation without severely affecting viability. Herein, we describe the novel maize gene extended auricle1 (eta1), which is essential for proper formation of the blade/sheath boundary. Homozygous eta1 individuals have a wavy overgrowth of auricle tissue and the blade/sheath boundary is diffuse. Double-mutant combinations of eta1 with genes in the knox and liguleless pathways result in synergistic and, in some cases, dosage-dependent interactions. While the phenotype of eta1 mutant individuals resembles that of dominant knox overexpression phenotypes, eta1 mutant leaves do not ectopically express knox genes. In addition, eta1 interacts synergistically with lg1 and lg2, but does not directly affect the transcription of either gene in leaf primordia. We present evidence based on genetic and molecular analyses that eta1 provides a downstream link between the knox and liguleless pathways.

IN plants, lateral organs such as leaves are born on the flanks of meristems (Steeves and Sussex 1989). This is a reiterative process, which originates with recruitment of segment initial cells to form a phytomer composed of leaf, node, internode, and axillary bud (Scanlon et al. 1996). A subset of these cells initiates the leaf and is termed the leaf founder cells (Poethig 1984). These founder cells divide, giving rise to leaf primordia, which undergo longitudinal differentiation that occurs basipetally (from tip to base such that blade differentiates prior to sheath) as well as laterally from the midrib to the margin (Harper and Freeling 1996b). The maize leaf provides a simple model for examining the genetic cues involved in organ regional identity and cell fate determination.

The ligule, a morphological feature of the grasses, is an epidermal fringe of tissue derived from the adaxial leaf surface. The ligule bisects the longitudinal, or proximo-distal, axis of the leaf into proximal sheath and distal blade (Figure 1). Along with the ligule, a pair of triangular-shaped auricles forms the blade/sheath boundary. Differentiation of auricle is first visible as a thin line of cells that separates the blade and sheath, which can be seen only after initiation of the ligule (Becraft et al. 1990). The auricle cells enlarge concomitantly with ligule outgrowth and then divide as the blade and sheath expand. After the leaf emerges, the auricle cells expand further, allowing the leaf blade to bend out horizontally from the main axis.

Our understanding of ligule and auricle development stems from analysis of leaf structures in wild-type plants as well as in mutant plants that show disruptions at the blade/sheath boundary. Aberrations in the auricle are often associated with a disrupted ligule, implying that their development is closely linked. Mutants that affect the auricle and/or the blade/sheath boundary include lg1, lg2, rs2, Rs1, Lg3, Kn1, and Gn1 (see below and Table 1). These mutants can be divided into two distinct groups. The first group is defined by recessive mutants that show altered ligule and auricle development resulting from absence of essential proteins during leaf primordial development. The second group is defined by mutants that affect proximodistal identity and ectopically express KNOX proteins in the leaf.

The first group of genes consists of liguleless (lg1) and liguleless2 (lg2). Recessive mutants of lg1 remove both the ligule and auricle, but a rudimentary ligule is formed in the upper leaves (Becraft et al. 1990; Sylvester et al. 1990). Recessive mutants of lg2 remove the ligule and auricle in the first one to three leaves. The ligule and auricle recover in later leaves but the blade/sheath boundary remains displaced. Double-mutant analysis of lg1 and lg2 revealed that these two genes interact in a dosage-dependent manner (Harper and Freeling 1996a). Double-mutant lg1 lg2 plants fail to form a ligule or auricle and the blade/sheath boundary is diffuse (Harper and Freeling 1996a). Reverse transcriptase (RT)-PCR analysis showed that LG2 expression precedes that of LG1 (Walsh et al. 1998). These genetic and molecular analyses have shown that lg1 and lg2 act...
The class 1 knotted1-like homeobox (knox) genes rough sheath 1 (rs1), gnarley1 (gn1), and liguleless3 (lg3) have also been defined by dominant mutants (Becraft and Freeling 1994; Fowler and Freeling 1996; Kerstetter et al. 1997; Foster et al. 1999). On the basis of loss-of-function phenotypes, the knox genes are thought to be required for meristem maintenance and to repress differentiation, although the precise function of these genes has not been determined (Kerstetter et al. 1997; Vollbrecht et al. 2000). In Arabidopsis, the knox genes SHOOT MERISTEMLESS (STM) and KNAT1 (KNOTTED1-LIKE IN ARABIDOPSIS THALIANA) may act redundantly to confer meristematic identity (Long et al. 1996; Byrne et al. 2002).

Another member of the second group has been defined by recessive mutants in rough sheath 2 (rs2). Mutants of rs2 have several phenotypic effects, including dwarfing, disruption of the blade sheath boundary, twisting of the leaves, loss of blade tissue, and disorganization of cell division resulting in leaf proximalization (Schneeberger et al. 1998). RS2 encodes a MYB-domain-containing factor with homology to the PHANTASICA (PHAN) gene from Antirrhinum and the ASYMMETRIC LEAVES1 (AS1) gene from Arabidopsis (Timmermans et al. 1999; Tsiantis et al. 1999; Byrne et al. 2000; Ori et al. 2000).

As1 is required for suppression of the Arabidopsis KNOX genes KNAT1, KNAT2, and KNAT6 in the leaf (Ori et al. 2000). Genetic evidence suggests that STM acts to suppress AS1 and AS2 in the meristem to prevent initiation of the leaf developmental program (Byrne et al. 2000, 2002). In addition, the PICKLE (PKL) gene, which encodes a chromatin-remodeling factor of the CHD class, enhances as1 and as2 mutants, indicating that PKL may have a general role in repression of KNOX genes in the leaf via changes in chromatin structure (Ori et al. 2000). Expression studies of rs2 mutants revealed the knox genes lg3, kn1, and rs1 are ectopically expressed in the leaf (Schneeberger et al. 1998). The rs2 gene is normally expressed in leaves whereas knox genes are expressed in the shoot apical meristem (Timmermans et al. 1999; Tsiantis et al. 1999). These molecular and biological studies have provided valuable insights into the complex regulatory mechanisms that govern leaf development.
genetic data suggest that the RS2/AS1/PHAN protein family functions to repress knox genes in the leaf.

Other components of the knox gene pathway include the maize mutant semaphore1 (sem1). sem1 acts to repress a subset of the knox genes in the maize leaf, mainly the genomic duplicates gn1 and rs1 (Scanlon et al. 2002). KNOX proteins appear to function through interactions with the BELI class of homeodomain proteins in both monocots and dicots (Bellaout et al. 2001; Mueller et al. 2001; Smith et al. 2002). Plant growth hormones are also regulated by knox gene action. KNOX proteins inhibit the expression of GA 20-oxidase in tobacco, tomato, and Arabidopsis, presumably inhibiting GA biosynthesis, while overexpression of knotted1 causes increased levels of cytokinin in tobacco (Li et al. 1992; Ori et al. 1999; Sakamoto et al. 2001; Hay et al. 2002).

We have identified a novel maize gene called extended auricule1 (eta1) on the basis of the behavior of a recessive mutant allele. Homozygous eta1 individuals have a wavy overgrowth of auricle tissue and the blade/sheath boundary is diffuse. However, one or two doses of eta1 mutant alleles result in synergism and enhancement of liguleless and knox phenotypes. We provide evidence based on genetic and molecular analyses with eta1 that the knox and liguleless pathways are linked in the genetic network controlling maize leaf development. These data also suggest that eta1 may function downstream of these pathways and show it is a key player in maize leaf development.

MATERIALS AND METHODS

Origin of the eta1-R allele: The eta1 reference allele, eta1-R, originated from an EMS screen of M2 segregating families performed by the Hollick lab (UC-Berkeley).

Mapping and introgression: The eta1-R mutation was introgressed five to seven times into the inbred lines Mo17, B73, W22, W23, and A188 via backcrossing and self-pollination. Individuals homozygous for eta1-R were pollinated by B-A and then self-pollinated. The formed on independent RNA pools at least three times for the spontaneous, MMC0081 forward and reverse, and umc1060 forward mutants in the Mo17 background. Heterozygous Mo17 for three generations. Homozygous eta1-R individuals in the Mo17 background were crossed to lg1-R and rs1-R homozygotes in the Mo17 background. The resultant F1 individuals were self-pollinated and scored in the F2 generation. The Lg3-O and Rs1-O alleles were obtained from the Maize Genetics Stock Center and backcrossed for seven generations into the Mo17 background. Individuals heterozygous for either Lg3-O or Rs1-O were crossed to eta1-R homozygous individuals in the Mo17 background. The resultant F1 individuals were either self-pollinated in the case of Rs1-0 or outcrossed to eta1-R homozygotes in the case of Lg3-0 (see Table 2).

PCR: DNA was isolated from 3-week-old seedlings in eta1-R segregating families from the resulting cDNAs. The PCR reactions were repeated with the primer sequences and amplification conditions refer to http://www.agron.missouri.edu). The RFLP csu 308 was also used in fine mapping the eta1-R mutation.

Reverse transcriptase-PCR gel blot analysis: RNA was isolated from 3-week-old seedlings in eta1-R segregating families in the Mo17 and W22 backgrounds. The meristems and p1–p5 leaves from three individuals were pooled as were the p6–8 leaves at the ligule ridge stage. RNA isolation was performed using TRizol (Invitrogen, San Diego). RNA was treated with DNase and then reverse transcribed using Superscript II reverse transcriptase (RT) and an oligo(dT) primer (Invitrogen). Gene-specific primers for the liguleless1, ubiquitin (Mor et al. 1997), liguleless2 (Walsh et al. 1998), knotted1, gn1-1, roughsheath1, liguleless3, liguleless4, and liguleless4b genes were used to PCR amplify the corresponding genes from the resulting cDNAs. The PCR reactions were run for 20 cycles. The gene products were detected via Southern blot hybridization with gene-specific probes. RT-PCR was performed on independent RNA pools at least three times for each gene. RT-PCR reactions were repeated with the knox gene primers and amplified for 30 cycles to confirm expression patterns in the eta1-R segregating families. Primers for the knox genes were as follows: kn1-5: AGCTGCTGACGCAA GACTGTC, kn1-2: CATTAGCGATATGCTAGATGC AAC, gn1-1: TACGAGACCACACTCGACGGTGTCG, gn1-2: GGAAGACGAGACATGGATCCGAG, rs1-11464, TTCTGAAG, rs1-5: TCTGGTGCATGGTACCCGAC, and gn1-6: GCGATCATGGACCCGAGTCTG. The PCR reactions were run for 20 cycles. The gene products were detected via Southern blot hybridization with gene-specific probes. RT-PCR was performed on independent RNA pools at least three times for each gene. RT-PCR reactions were repeated with the knox gene primers and amplified for 30 cycles to confirm expression patterns in the eta1-R segregating families. Primers for the knox genes were as follows: kn1-5: AGCTGCTGACGCAA GACTGTC, kn1-2: CATTAGCGATATGCTAGATGC AAC, gn1-1: TACGAGACCACACTCGACGGTGTCG, gn1-2: GGAAGACGAGACATGGATCCGAG, rs1-11464, TTCTGAAG, rs1-5: TCTGGTGCATGGTACCCGAC, and gn1-6: GCGATCATGGACCCGAGTCTG. The PCR reactions were run for 20 cycles. The gene products were detected via Southern blot hybridization with gene-specific probes. RT-PCR was performed on independent RNA pools at least three times for each gene. RT-PCR reactions were repeated with the knox gene primers and amplified for 30 cycles to confirm expression patterns in the eta1-R segregating families. Primers for the knox genes were as follows: kn1-5: AGCTGCTGACGCAA GACTGTC, kn1-2: CATTAGCGATATGCTAGATGC AAC, gn1-1: TACGAGACCACACTCGACGGTGTCG, gn1-2: GGAAGACGAGACATGGATCCGAG, rs1-11464, TTCTGAAG, rs1-5: TCTGGTGCATGGTACCCGAC, and gn1-6: GCGATCATGGACCCGAGTCTG. The PCR reactions were run for 20 cycles. The gene products were detected via Southern blot hybridization with gene-specific probes. RT-PCR was performed on independent RNA pools at least three times for each gene. RT-PCR reactions were repeated with the knox gene primers and amplified for 30 cycles to confirm expression patterns in the eta1-R segregating families. Primers for the knox genes were as follows: kn1-5: AGCTGCTGACGCAA GACTGTC, kn1-2: CATTAGCGATATGCTAGATGC AAC, gn1-1: TACGAGACCACACTCGACGGTGTCG, gn1-2: GGAAGACGAGACATGGATCCGAG, rs1-11464, TTCTGAAG, rs1-5: TCTGGTGCATGGTACCCGAC, and gn1-6: GCGATCATGGACCCGAGTCTG. The PCR reactions were run for 20 cycles. The gene products were detected via Southern blot hybridization with gene-specific probes. RT-PCR was performed on independent RNA pools at least three times for each gene. RT-PCR reactions were repeated with the knox gene primers and amplified for 30 cycles to confirm expression patterns in the eta1-R segregating families. Primers for the knox genes were as follows: kn1-5: AGCTGCTGACGCAA GACTGTC, kn1-2: CATTAGCGATATGCTAGATGC AAC, gn1-1: TACGAGACCACACTCGACGGTGTCG, gn1-2: GGAAGACGAGACATGGATCCGAG, rs1-11464, TTCTGAAG, rs1-5: TCTGGTGCATGGTACCCGAC, and gn1-6: GCGATCATGGACCCGAGTCTG.
TABLE 2
Double-mutant analysis

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</tr>
<tr>
<td></td>
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<tr>
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<td>phenotypic classes</td>
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<tr>
<td>Rs2-R eta1-R‡</td>
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† Segregating families resulted from a self-pollination event of an F1 individual heterozygous for both alleles.
‡ Segregating families resulted from an outcross of heterozygous F1 individuals to individuals homozygous for the eta1-R allele.

RESULTS

Phenotypic analysis and effect of genetic background on eta1: The eta1-R mutation was originally isolated from an EMS mutagenesis screen. The eta1 phenotype segregates as a single recessive locus in F2 families. The most notable phenotype of eta1 is an overgrowth or extension of auricle tissue. The eta1 mutant is pleiotropic and displays a range of phenotypes, including displacement of the blade/sheath boundary, disruption of the ligule, reduction in internode spacing and overall plant height, and the production of smaller, more compact ear shoots (Figure 2, A–D).

To determine the developmental basis of the eta1 phenotype, ESEM analysis was used to compare wild-type and eta1 siblings (Figure 2, E–H). The blade/sheath boundary of eta1 mutants is severely displaced relative to wild type (Figure 2, E vs. F). The blade/sheath boundary normally runs perpendicular to the proximodistal axis of the leaf, thus forming a boundary between blade and sheath. However, the blade/sheath boundary of eta1 individuals runs nearly parallel to the proximodistal leaf axis (Figure 2F). In addition, the ligule fails to form completely in eta1 mutants. Although somewhat disorganized, morphologically recognizable blade, sheath, ligule, and auricle cells are visible in eta1 individuals. Examination of the auricle cells in eta1 mutants reveals some aberrant, disorganized divisions, but the auricle cell shape and size are comparable to those of wild type (Figure 2, G and H).

Given the pleiotropic nature of the eta1-R allele, it was introgressed into five different maize inbred lines to determine the most expressive phenotypes and to help elucidate a precise eta1 function. As with many maize developmental mutants, eta1 displays background effects, but is fully penetrant in all inbred lines tested. Background effects have been previously documented with maize heterochronic mutants as well as with dominant knox mutants (Poethig 1988; Fowler and Freeling 1996). The auricle phenotype is most severe in Mo17 and W23, in which the auricles become highly elaborated, sometimes even forming a collar-like struc-
ture similar to the morphology of the blade/sheath boundary of rice (Figure 3A). The auricle phenotype is least severe in B73 with auricle extension only along the margin in adult leaves and little to no auricle extension in juvenile leaves (Figure 3B). In all of the genetic backgrounds tested, etal mutant plant height compared to wild-type siblings was similarly reduced (see Table 3). The most penetrant etal phenotypes were the reduction in plant height and the displacement of the blade/sheath boundary, which enabled consistent scoring even in weakly expressing backgrounds.

Mapping and dosage: The etal mutation was initially mapped using B-A translocation stocks, using standard procedures (Beckett 1994b). The translocation 5Ld uncovered the etal phenotype, indicating that the etal locus is distal to this chromosome breakpoint on the long arm of chromosome 5. The phenotype of etal-R hemizygotes is equivalent to etal-R homozygotes, suggesting that the etal-R allele is an amorph or has complete loss of function. SSR markers (Smith et al. 1997) were then used to fine map etal. Linkage was first found to the marker mmc0081, which maps to bin 5.05 (bin number is a positional designation along maize chromosomes; for example, chromosome 5 is divided into nine bins). SSRs in bins 5.04 and 5.05 were then tested for linkage. The SSR umc1221 located at position 329.5 was found to be closely linked to etal at a recombination distance of 1 cM. This marker was subsequently used for determination of etal genotype.

Double-mutant analysis

etal interacts synergistically with the liguleless1/liguleless2 pathway: Both the lg1 and lg2 genes function in formation of the blade/sheath boundary and elaboration of ligule and auricle. Mutants of lg1 and lg2 show a dosage-dependent genetic interaction, suggesting that they function in the same developmental network. To test whether etal may be involved in this network, we generated double mutants with etal-R and lg1-R or lg2-219.

A synergistic interaction was seen between lg1-R and etal-R (Figure 4). The lg1-R etal-R homozygotes show a marked displacement of the blade/sheath boundary, which is more severe than the displacement seen with either of the single mutants (Figure 4D). In addition, etal-R homozygotes form ligule tissue and lg1-R homozygotes form a rudimentary ligule, but the double mutant fails to form ligule or auricle. Unusual protrusions of undifferentiated tissue were observed on the abaxial leaf surface of double-mutant individuals (Figure 4E, arrows), which are not seen in either lg1-R or etal-R.

Figure 2.—Pleiotropic effects of the etal-R mutation. (A) Mo17 siblings in an etal-R-segregating family, wild type on the left and etal on the right. Note the compaction of internodes and auricle extension. (B) W22 siblings in an etal-R-segregating family, wild-type leaf on the left and etal on the right. Note the displacement of the blade/sheath boundary and the interrupted ligule line. Extension of auricle can be seen as lighter tissue extending up into the darker green blade. Arrows mark the auricle tissue in wild type and etal. (C) A188 siblings in an etal-R-segregating family, wild type on the left and etal on the right. Plant height is reduced to approximately one-half of wild type. (D) Ear shoots of an etal-R-segregating family in the Mo17 background, wild-type ear on the left and etal mutant ear on the right. The etal ear is more compact and husk leaves fail to form completely around the developing ear shoot. (E–H) ESEM images of the adaxial leaf surface of wild-type and etal individuals in a W23 background. The leaves imaged here are just extruding from the whorl, are the sixth leaf produced, and are approximately plastochron 10. (E) Wild-type leaf at the blade/sheath boundary. (F) etal leaf at the blade/sheath boundary. (G) Wild-type auricle cells. (H) etal auricle cells. Lg, ligule; sh, sheath; aur, auricle; bl, blade. Bar, 50 μm.
TABLE 3  

<table>
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<td>98 ($\pm$ 7)</td>
<td>56</td>
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</table>

$^a$ All height measurements are in centimeters and were taken at time of anthesis from the base of the plant to the tip of the tassel.

Figure 3.—The $eta1$-$R$ mutant displays background effects. (A) Leaf 10 in an $eta1$-$R$-segregating family in the W23 background, wild type on the left and $eta1$ on the right. Note displacement of the blade/sheath boundary and auricle extension (arrows). (B) Leaf 10 in an $eta1$-$R$-segregating family in the B73 background, wild type on the left and $eta1$ on the right. Note reduction of auricle extension (arrows) but blade/sheath boundary remains displaced.

single mutants. These protrusions did not coincide with venation and were localized to the proximal blade region in the presumptive auricle domain. The novel phenotype of $lg1$-$R eta1$-$R$ double mutants suggests that these two genes interact synergistically and not additively.

A synergistic interaction was also observed with $lg2$-$219$ and $eta1$-$R$, but surprisingly, our double-mutant analysis uncovered a dominant dosage effect of the $eta1$-$R$ allele (Figure 5). In the upper adult leaves of the $lg2$-$219$ mutants, ligule and auricle recover. However, $lg2$-$219$ homozygotes that were heterozygous for $eta1$-$R$ displayed extension of auricle in the upper adult leaves (Figure 5C). This dominant dosage effect was confirmed both genetically and molecularly. No notable phenotypic dosage effect was seen with $eta1$-$R$ homozygotes carrying a single copy of the $lg2$-$219$ allele (data not shown). Plants homozygous for both $lg2$-$219$ and $eta1$-$R$ were extremely short, twisted, and often infertile (Figure 5F). The blade/sheath boundary of double-mutant individuals was extremely displaced toward the distal portion of the leaf compared to either of the single mutants, but ligule outgrowth was still apparent. Taken together, the synergistic interaction of $lg1$-$R$ and $eta1$-$R$ and the synergistic dominant dosage effect of $eta1$-$R$ with $lg2$-$219$ place $eta1$ in the $liguleless1/2$ network of function.

$eta1$ enhances regional identity phenotypes: The $eta1$ mutant phenotype is remarkably similar to that of proximodistal regional identity mutants, which cause disruption of the blade/sheath boundary and formation of proximal structures more distally. Of the regional identity mutants, we tested the interaction of $eta1$-$R$ with two of the semidominant class 1 $knox$ mutants, $Kn1$-$N$ and $Lg3$-$O$. To simplify the analysis, only one dose of $Kn1$-$N$ and $Lg3$-$O$ was used. The $eta1$-$R$ mutant enhanced the $Kn1$-$N$ phenotype (Figure 6). Individuals homozygous for $eta1$-$R$ and heterozygous for $Kn1$-$N$ displayed an increase in the number and size of knots, an increase in prominent venation, and an increase in ectopic patches of ligule compared to heterozygous $Kn1$-$N$ siblings (compare Figure 6B with 6D). There is no increase in auricle extension or in displacement of the blade/sheath boundary in the $Kn1$-$N/^+$ $eta1$-$R$ mutant individuals, suggesting that $eta1$-$R$ is enhancing the $Kn1$-$N$ phenotype.

Similarly, $eta1$-$R$ enhances the $Lg3$-$O$ mutant phenotype (Figure 7). Individuals homozygous for $eta1$-$R$ and heterozygous for $Lg3$-$O$ phenocopied $Lg3$-$O$ homozygotes. Again, there was no significant increase in the amount of auricle tissue in double-mutant individuals, but the leaves were severely proximalized. Double-mutant phenotypes included severe displacement of the blade/sheath boundary, ectopic ligule along the midrib, twisting of the midrib, and alteration in leaf attitude (Figure 7, D and E).

We also tested the interaction of $eta1$ with a fully dominant class 1 $knox$ gene, $Gnarley1$ (Foster et al. 1999). $eta1$-$R$ shows a dosage effect with $Gn1$-$R$ as seen in Figure 8. Plants homozygous for $eta1$-$R$ with one copy of $Gn1$-$R$ display an increase in leaf width, an increase in extension of auricle tissue into blade along the margin, and an increase in auricle proliferation at the blade/sheath boundary (compare Figure 8C with 8D).
The Role of *eta1* in Maize Leaf Development

In families segregating *Gn1-R* alone, *Gn1-R* homozygotes are indistinguishable from heterozygotes (Foster *et al.* 1999). However, when a second dose of *Gn1-R* is added in families segregating for *eta1-R*, the phenotype is severely enhanced (Figure 8, E and F). The blade/sheath boundary is severely distorted and the width of blade and sheath is increased. In addition, the leaf is increasingly proximalized with increasing dosage of *Gn1-R*, as sheath and auricle are displaced distally into blade, especially in the region flanking the midrib (Figure 8F). In other words, the *eta1-R* background permits a *Gn1-R* dosage effect that is not evident in a wild-type background.

Both *rs2-R* and *Rs1-O* show a strong synergistic interaction with *eta1-R* (Figure 9). RS2 has homology to MYB-like transcription factors and RS1 is a class 1 *knox* transcription factor. One of the functions of RS2 is to downregulate RS1 in the leaves (Schneebberger *et al.* 1998). When combined with *eta1-R*, *rs2-R* shows a synergistic interaction such that the *eta1-R* phenotype is exacerbated as well as the *rs2-R* phenotype (Figure 9). The double-mutant individuals show a reduction in height, excessively rough sheath, reduction in sheath length, increased auricle extension, and an overall increase in the proximalization of the leaf compared to *eta1-R* and *rs2-R* single-mutant siblings (compare Figure 9B and 9C with 9D). However, the synergistic interaction of *Rs1-O* and *eta1-R* is subtly different. The overall plant height of *Rs1-O* *eta1-R* double-mutant individuals is greatly reduced and the sheath length is reduced as is seen with the *rs2-R* *eta1-R* double mutants. The focus of the ectopic *knox* gene action is shifted distally so that the phenotype is most severe at the auricle region, and the sheath is not particularly rough compared to the *Rs1-O* single.

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**Figure 4.**—Synergistic interaction of *lg1-R* and *eta1-R* in the Mo17 background. Adaxial views of leaf 10: (A) wild type, (B) *eta1-R/eta1-R*, (C) *lg1-R/lg1-R*, and (D) *eta1-R/eta1-R lg1-R/lg1-R*. Note the increased displacement of the ligule/auricle line in the double mutant vs. that in either of the single mutants (solid arrows). (E) *lg1-R/lg1-R eta1-R/eta1-R* individual. Open arrows point to ectopic protrusions on the adaxial leaf surface just distal to the blade/sheath boundary.

**Figure 5.**—Dosage effect of *lg2-219* and *eta1-R*. Adaxial view of leaf 12 in a *lg2-219* *eta1-R* segregating family in the Mo17/W23 background. (A) Wild type; (B) *lg2-219/lg2-219*; (C) *lg2-219/lg2-219 eta1-R/+*; (D) *eta1-R/eta1-R*; (E) *lg2-219/lg2-219 eta1-R/eta1-R* (F) whole-plant view of the *lg2-219/lg2-219 eta1-R/eta1-R* double mutant. Arrows mark the auricle extension.
early p1–5 leaf primordia but can be seen only in p6–8 leaves (Walsh et al. 1998). We isolated mRNA from either meristem and p1–5 or p6–8 leaf primordia and then subjected it to RT-PCR with lg1- and lg2-specific primers. No difference in mRNA expression of either LG2 or LG1 was found in leaves of eta1-R mutants vs. their wild-type siblings (Figure 10A). On the basis of these data, eta1 is not likely to function upstream of lg1 or lg2.

Leaves of eta1 mutant plants do not ectopically express knox genes: The phenotype of eta1 mutants resembles a number of maize mutants where the molecular cause of the mutant phenotype is ectopic expression of knox genes. Because of this, KNOX-mRNA expression was assayed via RT-PCR with primers specific for lg3, lg4a, lg4b, rs1, gn1, or kn1. No notable differences were seen in eta1 individuals vs. their wild-type siblings with any of these probes. For example, KN1-mRNA expression was detected in meristems of both wild-type and eta1 mutant individuals, but not in developing leaves (Figure 10B). The same expression pattern was seen with RS1 and GN1 (data not shown). Even after 30 cycles of PCR, we detected no ectopic KN1, GN1, or RS1 expression in eta1 or wild-type developing leaves (data not shown). LG3-mRNA expression was detected at high levels in meristems and at low levels in developing leaves of wild-type and eta1 mutant individuals (Figure 10B). Both LG4A and LG4B were expressed in the same pattern as LG3 (data not shown). These results suggest that eta1 may act downstream of the ectopic knox pathway since it does not cause ectopic knox gene expression.

Northern blot analysis: The severity of the Kn1 pheno-
The Role of \textit{eta1} in Maize Leaf Development

Figure 8.—The \textit{eta1-R} mutation shows a dosage effect with \textit{Gn1-R}. Adaxial view of adult leaves from a family segregating \textit{eta1-R/Gn1-R} in the W22 background is shown. This family resulted from self-pollination of a plant heterozygous for both \textit{Gn1-R} and \textit{eta1-R}. (A) Wild type; (B) \textit{eta1-R/eta1-R}; (C) \textit{Gn1-R/+; } (D) \textit{Gn1-R/+ eta1-R/eta1-R}; (E) \textit{Gn1-R/Gn1-R eta1-R/eta1-R}; (F) whole-plant view of a \textit{Gn1-R/Gn1-R eta1-R/eta1-R} individual.

type has been correlated with increased KN1-mRNA expression in the leaves (Smith \textit{et al.} 1992). We wanted to determine if the severe \textit{Kn1-N/eta1-R} double-mutant phenotype could be attributed to increased KN1-mRNA levels or if mRNA levels were unchanged. KN1-mRNA is abundant and is easily visualized using Northern blot analysis, which is optimal for direct comparison of RNA concentrations. Figure 10C shows the results of a Northern blot analysis with a \textit{Kn1-N/eta1-R} segregating family in the W22 background. No KN1-mRNA can be detected in \textit{eta1-R} mutant leaves, which is consistent with our RT-PCR findings. Levels of KN1-mRNA did not differ significantly between \textit{Kn1-N/+} and \textit{Kn1-N/+ eta1-R/eta1-R} leaves (lane 4 vs. 8). These results further suggest that \textit{eta1} functions downstream of the ectopic \textit{knox} pathway.

**DISCUSSION**

Much effort in recent years has focused on identifying novel loci in the developmental genetic network controlling proximodistal patterning in the maize leaf. We describe a recessive mutation of the \textit{eta1} gene, \textit{eta1-R}, which affects proximodistal patterning in the maize leaf. To date there are only two published recessive mutations in maize, \textit{rs2} and \textit{sem1}, that are implicated in proximodistal patterning in the leaf and both act to repress \textit{knox} genes (Timmermans \textit{et al.} 1999; Tsiantis \textit{et al.} 1999; Scanlon \textit{et al.} 2002). While \textit{eta1} is involved in proximodistal patterning in the leaf, \textit{eta1} is unique in that it is not involved in regulating \textit{knox} gene expression. In addition, \textit{eta1} enhances the phenotypes of all known mutants affecting proximodistal patterning in the maize leaf. Thus, our findings implicate \textit{eta1} as a novel and essential component of the developmental genetic network controlling maize leaf development.

**The nature of the \textit{eta1-R} mutation and background effects:** Genetic evidence from B-A translocations reported here suggests that the \textit{eta1-R} allele is a complete loss-of-function mutation. This amorphous is essential when inferring a functional role for \textit{eta1}. The effect of background on the \textit{eta1} phenotype is not surprising given that background effects are well documented in maize. However, it is intriguing that \textit{eta1-R} displays background expressivities similar to \textit{Lg3-O} (Poethig 1988; Fowler and Freeling 1996), being most severe in Mo17 and least severe in B73. This could indicate that similar modifiers are involved in either enhancing or suppressing the aspects of proximodistal patterning in leaf development controlled by \textit{eta1} and disrupted by \textit{Lg3}.

**Interaction with the \textit{liguleless} pathway:** Previous work with \textit{lg1} and \textit{lg2} suggests that this phenotype is saturated. For example, 18 independent \textit{lg1} alleles and 9 independent \textit{lg2} alleles have been identified, some in genetic
screens to identify other genes in the liguleless pathway, but no novel genes have been discovered (D. Braun and J. Walsh, personal communication). It was proposed that other factors in the liguleless pathway would be either pleiotropic or lethal (Harper and Freeling 1999). On the basis of genetic interactions of eta1 with lg1 and lg2 and the eta1 phenotype, eta1 can be considered a pleiotropic factor in the liguleless network of function. However, given the pleiotropic nature of eta1, it is unlikely eta1 is exclusively functioning in this pathway. RT-PCR gel blot analyses show eta1 does not affect lg1 or lg2 expression. However, eta1 may act on lg1 and/or lg2 at the protein level or perhaps could affect the spatial distribution of LG-mRNAs, which is difficult to test given that the expression patterns of lg1 and lg2 have not been precisely discerned. It is likely that eta1 function is necessary along with lg1 and lg2 during early leaf development for correct formation and differentiation of the blade/sheath boundary. lg1 function seems to be specific to ligule and auricle induction while lg2 function is specific to regional organ transitions (Walsh and Freeling 1999). lg2 is involved in early establishment of the blade/sheath boundary during early vegetative stages and in the transition from vegetative to floral branching in the apical tassel meristem (Walsh and Freeling 1999).

Consequently, the synergistic interaction of eta1 with lg1 indicates that eta1 plays a role in the formation of the blade/sheath boundary and in the elaboration of the ligule. Notably, the lg1 single mutant fails to develop ligule and auricle in the lower leaves but a rudimentary ligule is formed in upper leaves. In contrast, the eta1 lg1 double mutants do not form rudimentary ligule and the blade/sheath boundary is displaced over the midrib (Figure 4D). This double-mutant phenotype indicates eta1 is involved in formation of the rudimentary ligule in the absence of lg1. This is similar to the dosage-dependent synergistic interaction seen between lg1 and lg2. Double mutants of dominant Lg3 and Lg4 alleles with lg1 and lg2 also fail to form a rudimentary ligule, but do not enhance the ectopic knox phenotypes (Fowler and Freeling 1996). Plants homozygous for

Figure 9.—The eta1-R mutation interacts synergistically with rs2-R and Rs1-O. (A–D) Abaxial view of leaf 12 from a family segregating rs2-R eta1-R in the Mo17 background. This family resulted from a self-pollination of an individual heterozygous for both rs2-R and eta1-R. (A) Wild type; (B) eta1-R/eta1-R; (C) rs2-R/rs2-R; (D) rs2-R/rs2-R eta1-R/eta1-R. (E–H) Abaxial view of leaf 12 from a family segregating Rs1-O and eta1-R in the Mo17 background. (E) Wild type; (F) eta1-R/eta1-R; (G) Rs1-O/Rs1-O; (H) Rs1-O/Rs1-O eta1-R/eta1-R.
**Figure 10.**—(A) RT-PCR gel blot analysis of LG1 and LG2 expression in an *eta1*-regenerating family. Lane 1, wild-type meristems and p1–5 leaves; lane 2, wild-type p6–8 leaves; lane 3, *eta1*-R meristems and p1–5 leaves; lane 4, *eta1*-R p6–8 leaves. *Ubiquitin* was used as a loading control. (B) RT-PCR gel blot analysis of *kn1* and *lg3* in an *eta1*-regenerating family. Lane 1, wild-type meristems and p1–5 leaves; lane 2, wild-type p6–8 leaves; lane 3, *eta1*-R meristems and p1–5 leaves; lane 4, *eta1*-R p6–8 leaves. *Ubiquitin* was used as a loading control. (C) Northern blot analysis of a *Kn1-N/*+ *eta1/eta1*-regenerating family. Lane 1, wild-type meristems and p1–5 leaves; lane 2, wild-type p6–8 leaves; lane 3, *Kn1* meristems and p1–5 leaves; lane 4, *Kn1* p6–8 leaves; lane 5, *eta1* meristems and p1–5 leaves; lane 6, *eta1* p6–8 leaves; lane 7, *eta1* *Kn1* meristems and p1–5 leaves; lane 8, *eta1* *Kn1* p6–8 leaves. Lanes 1 and 2 are from a different Northern blot than lanes 3–8; however, they represent RNA from the same *Kn1-N* *eta1/eta1*-regenerating family.

*lg1-R* and heterozygous and/or homozygous for *lg2-R* failed to form the rudimentary ligule and had a displaced blade/sheath boundary (Harper and Freeling 1996a). These data indicate *eta1* functions in the same genetic network as *lg1*.

In addition to *lg1* interactions, *eta1* has a directional dosage-dependent interaction with *lg2*. The *eta1*-R allele behaves dominantly to extend auricle tissue in the upper leaves of *lg2*-219 homozygous plants. The *lg2* *eta1* double mutants also showed extreme displacement of the blade/sheath boundary relative to *eta1* and *lg2* homozygotes. The observation that the double-mutant plants were able to produce ligule suggests that neither of these genes is specifically involved in ligule induction, but may be involved prior to that in establishment of the blade/sheath boundary. These data indicate that *lg2* and *eta1* have partially overlapping functions in properly delineating the blade/sheath boundary.

**Interaction with proximodistal axis regional identity mutants:** While the mutant *eta1* phenotype resembles that of *rs2* and the dominant *knox* mutants, we found that the *eta1* mutation does not ectopically express any of the class 1 *knox* genes (Figure 10). However, proximodistal regional identity mutants including class 1 *knox* genes interact genetically with *eta1*. Interactions between the dominant *knox* mutants have been previously documented (Fowler and Freeling 1996). Interestingly, *eta1* can phenocopy dominant *knox* mutations in double-mutant combination. For example, the double-mutant phenotype of *Lg3*-O *eta1*-R (Figure 7) is nearly identical to the double-mutant phenotype of *Lg3*-O *Lg4*-O (Fowler and Freeling 1996). This is also true of the *Kn1-N* *eta1*-R (Figure 6) double-mutant phenotype, which mimics that of *Kn1-O* *Rs1*-O (Fowler and Freeling 1996). Since the severity of *Kn1-N* *eta1*-R double-mutant individuals is not a direct result of increased KN1-mRNA levels and *eta1*-R in double-mutant combination with *knox* genes results in severe phenotypes, *eta1*-R likely functions either downstream of the pathway perturbed by *knox* neomorphs or in a parallel but convergent pathway that promotes differentiation.

In the *Rs1*-O mutant, the area of ectopic RS1-mRNA expression is greater than one would expect given the phenotypic consequences of that expression (Schneebberger et al. 1995). Specifically, ectopic RS1-mRNA expression can be detected in most cell types but only the blade and the ligular region display developmental defects. The authors propose that this could be due to competency of cells to respond to ectopic RS1 expression. Perhaps *eta1* is essential in competency and without its normal function cells are increasingly sensitive or responsive to ectopic *knox* gene expression. This could be one explanation for the change in focus of ectopic RS1 action in the *Rs1*-O *eta1*-R double mutants.

Interestingly, the phenotype of *rs2*-R *eta1*-R double mutants is slightly different from that of the *Rs1*-O *eta1*-R double mutants. Both double-mutant analyses were carried out in the same genetic background, which cannot account for the differences observed. It is possible that the difference in the synergistic interaction of *rs2*-R *eta1*-R compared to *Rs1*-O *eta1*-R can be attributed to as yet unidentified downstream *rs2* targets, which are likely to be misexpressed. In addition, the spatial and temporal expression of *knox* genes is likely to differ in *Rs1*-O and *rs2*-R. For example, in Arabidopsis, the rounded-leaf phenotype of *asl* mutants differs from that of the lobed-leaf phenotype of *35S:KNAT1* plants (Ori et al. 2000; Theodoris et al. 2003).

More surprising is the *eta1* *Gn1* dosage effect. *Gn1*-R acts as a true dominant and therefore *Gn1* heterozygotes cannot be distinguished from homozygotes. *rs1* and *gn1* are duplicate genes (Foster et al. 1999); therefore, it is interesting that we see a dosage effect only with *Gn1*-R and *eta1*-R and not with *Rs1*-O and *eta1*-R. There are multiple explanations for this. One possibility is that the dosage interaction with *eta1*-R and *Gn1*-R is due to allelic differences between *Gn1*-R and *Rs1*-O. Another possibility is that there is a modifier in W22 that confers dosage sensitivity with *Gn1*-R *eta1*-R and this modifier is not present in Mo17, the background in which the *Rs1*-O *eta1*-R double-mutant analysis was performed. A third possibility is that although *Rs1* and *Gn1* are thought to be duplicate genes, over evolutionary time they have
evolved slightly disparate functions and thus we found proximodistal identity to be more sensitive to dosage of Gn1 when Eta1+ function is lost. These data uncover a threshold of responsiveness to ectopic Gn1 that is seen when Eta1+ function is lost, which is consistent with etal playing a role in competency.

Alternative modes of etal action: Our current understanding of genetic control of patterning in simple leaves comes primarily from work with maize, Arabidopsis, and Antirrhinum, and in leaves it is clear that the KNOX genes to their gene targets, while as1 confers specificity for repression of the KNOX genes (Ori et al. 2000). Interestingly, the phenotype of pkl as1 double mutants mimics that of 35S:KNAT1 overexpression lines (Ori et al. 2000), much like etal in double-mutant combination with know genes mimics dominant knox double mutants. Ori et al. (2000) propose that PKL may play a role in restricting competency of cells to respond to morphogenetic factors. Given the pleiotropic phenotype of etal and its many genetic interactors, it is possible etal may have a general function in altering chromatin states and therefore competency similar to pkl.

An alternative possibility is that etal functions simply in restricting cell proliferation at the blade/sheath boundary. This seems unlikely given the effects of etal in double-mutant combination with know and lg2 genes mimics dominant know double mutants. Ori et al. (2000) propose that PKL may play a role in restricting competency of cells to respond to morphogenetic factors. Given the pleiotropic phenotype of etal and its many genetic interactors, it is possible etal may have a general function in altering chromatin states and therefore competency similar to pkl.

A dramatic observation is that etal does not ectopically express know genes on its own (Ori et al. 2000). These authors propose that PKL functions to limit accessibility of KNOX genes to their gene targets, while as1 confers specificity for repression of the KNOX genes (Ori et al. 2000). Interestingly, the phenotype of pkl as1 double mutants mimics that of 35S:KNAT1 overexpression lines (Ori et al. 2000), much like etal in double-mutant combination with know genes mimics dominant know double mutants. Ori et al. (2000) propose that PKL may play a role in restricting competency of cells to respond to morphogenetic factors. Given the pleiotropic phenotype of etal and its many genetic interactors, it is possible etal may have a general function in altering chromatin states and therefore competency similar to pkl.

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