Heterogeneous Expression Patterns and Separate Roles of the \textit{SEPALLATA} Gene \textit{LEAFY HULL STERILE1} in Grasses

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\textit{SEPALLATA} (\textit{SEP}) genes exhibit distinct patterns of expression and function in the grass species rice (\textit{Oryza sativa}) and maize (\textit{Zea mays}), suggesting that the role of the genes has changed during the evolution of the family. Here, we examine expression of the \textit{SEP}-like gene \textit{LEAFY HULL STERILE1} (\textit{LHS1}) in phylogenetically disparate grasses, reconstruct the pattern of gene expression evolution within the family, and then use the expression patterns to test hypotheses of gene function. Our data support a general role for \textit{LHS1} in specifying determinacy of the spikelet meristem and also in determining the identity of lemmas and paleas; these two functions are separable, as is the role of the gene in specifying floret meristems. We find no evidence that \textit{LHS1} determines flower number; it is strongly expressed in all spikelet meristems even as they are producing flowers, and expression is not correlated with eventual flower number. \textit{LHS1} expression in only the upper flowers of the spikelet appears to be the ancestral state; expression in all flowers is derived in subfamily Pooidae. \textit{LHS1} expression in pistils, stamens, and lodicules varies among the cereals. We hypothesize that \textit{LHS1} may have affected morphological diversification of grass inflorescences by mediating the expression of different floral identity genes in different regions of the floret and spikelet.

INTRODUCTION

Grasses are a diverse family of >10,000 species (Clark et al., 1995), including the cereal crops rice (\textit{Oryza sativa}), barley (\textit{Hordeum vulgare}), oats (\textit{Avena sativa}), pearl millet (\textit{Pennisetum glaucum}), maize (\textit{Zea mays}), and sorghum (\textit{Sorghum bicolor}). Grass inflorescences are comprised of spikelets that, depending on the species, contain from one to forty florets (Clifford, 1987). At the base of the spikelet are two bracts: an inner and outer glume. Florets within the spikelet may mature from top to bottom (basipetally) or from bottom to top (acropetally) depending on the species. Each grass floret typically consists of a pistil, three stamens, and two lodicules (second whorl organs) subtended by a palea and lemma (outer whorl structures with similarities to prophylls and bracts, respectively); sexuality of the floret and the number of stamens and lodicules vary from species to species. For example, the rice floret is bisexual and consists of a pistil, six stamens, two lodicules, palea, and lemma. Although rice is occasionally described as having only a single flowered spikelet, we follow the interpretation of Stapf (1917) that the rice spikelet contains three flowers, one bisexual and fertile and two strongly reduced and sterile. The glumes are then the tiny flaps of tissue often known as rudimentary glumes in the rice literature. This interpretation is supported by the work of Prasad et al. (2001) and Komatsu et al. (2003) as well as data presented in this article. In contrast with rice, maize is monoecious, with male florets borne in tassel inflorescences at the apex of the plant and female florets borne in axillary ear inflorescences. Maize spikelets consist of two florets that develop basipetally. Whereas both florets are functional in tassel spikelets, only the upper floret is functional in ear spikelets. Florets in the tassel consist of three stamens, two lodicules, palea, and lemma, whereas florets in the ear consist of a pistil, palea, and lemma.

The great diversity of plant forms is presumably caused by differences in where, when, and how genes are expressed. Thus, the study of evolution of development involves identifying genes whose expression pattern differs among organisms. Much of the literature on developmental genetics, however, has focused on genes whose function (regulation, expression, and their pheno- typic consequences) is conserved rather than variable. Notable exceptions to this are the work of Kramer and Irish (1999), who showed variation in expression pattern of \textit{MADS} box genes among some basal dicots, and Kim et al. (2003), who showed that variation in leaf morphology correlated with differential expression of \textit{PHANTASTICA}-like genes.

\textit{SEPALLATA} (\textit{SEP}) genes play an essential role in floral development by interacting with, and mediating the expression of, floral identity genes (Honma and Goto, 2001; Pelaz et al., 2001b; Favaro et al., 2003; Ferrario et al., 2003; Vandenbussche et al., 2003). \textit{SEP} genes form a well-supported clade within the \textit{MADS} box gene phylogeny that can be further subdivided into three clades: a mixed eudicot and monocot clade containing the \textit{Arabidopsis thaliana} \textit{SEP3} gene, a eudicot clade containing the...
Arabidopsis SEP1, SEP2, and AGL3 genes, and a clade comprised solely of monocot sequences (Figure 1; S.T. Malcomber and E.A. Kellogg, unpublished data). Because of the unresolved relationships at the base of the SEP phylogeny, the monocot clade is not demonstrably homologous to either the SEP3 or the SEP1/SEP2/AGL3 clade. SEP genes are particularly diverse in grasses, with at least five genes in rice (Nam et al., 2004) and eight genes in maize (Münster et al., 2002), and current phylogenetic analyses suggest that most of this genetic diversity arose shortly after the evolution of the family (Theissen et al., 2000; Theissen, 2001).

Because the grass SEP-like genes are not clearly orthologous to the eudicot SEP genes, it is impossible to make direct comparisons of function. Complicating the picture further, SEP genes have diverse patterns of expression in grasses, with several orthologous genes having expression patterns that differ in rice and maize. This heterogeneous pattern of expression strongly suggests that SEP genes in grasses are not functionally homogeneous and might have been important in the evolution of the complex inflorescence structures characteristic of the family (Theissen, 2001; Becker and Theissen, 2003).

O. sativa MADS1 (OsMADS1) is currently the best characterized SEP gene in grasses and is known to be LEAFY HULL STERILE1 (LHS1; Jeon et al., 2000a). Phylogenetic analyses have identified Z. mays MADS8 and Z. mays MADS14 as the maize homologs of OsMADS1 (Münster et al., 2002). Because the numbering of SEP genes in different species is confusing, we will refer to OsMADS1 and orthologous genes as LHS1; orthologous genes from different species will be identified by capitalized first letter of the genus name and lower case first letter of the species name. When multiple copies of the gene are present in a particular species, the different copies will be identified by a, b, c, etc. (see supplemental data online for additional information about the naming scheme). Thus, OsMADS1 will be referred to as OsLHS1, ZmM8 as ZmLHS1a, and ZmM14 as ZmLHS1b (Table 1).

OsLHS1 has a different pattern of expression than ZmLHS1a and ZmLHS1b. OsLHS1 is first expressed throughout the floral primordium of the fertile floret but is not expressed in the sterile lemmas (Prasad et al., 2001). In mature flowers, OsLHS1 is expressed strongly in the palea and lemma and weakly in the pistil (Prasad et al., 2001). Similar to rice, ZmLHS1a and ZmLHS1b are first expressed generally throughout the upper floret meristem, just as the primordium of the lower floret becomes visible (S.T. Malcomber and E.A. Kellogg, unpublished data; Cacharrón et al., 1999). Unlike rice, ZmLHS1a is expressed equally in all floral organs of the upper floret in mature spikelets (Cacharrón et al., 1999). ZmLHS1b is also expressed in all floral organs of the upper floret, but expression is more pronounced in the pistil than ZmLHS1a (Cacharrón et al., 1999). ZmLHS1a and ZmLHS1b were never detected in the lower floret. These different patterns in rice and maize indicate that LHS1 expression has diversified during the evolution of the grass family and that the genes may have different functions in different grass species.

The function of LHS1 genes is not clear, despite multiple studies in rice and maize based on mutations, overexpression, RNA interference (RNAi), and gene expression. Two nonsynonymous mutations at amino acid positions 24 and 27 in the MADS box of OsLHS1 are sufficient to generate the lhs1 mutant phenotype in rice (Jeon et al., 2000a), which has leafy palea and lemma, leafy lodicules that look similar to the palea and lemma, a decreased number of stamens, and, occasionally, an extra pistil or floret. However, lhs1 is a semidominant allele, so attempts to use the mutant phenotype as an indicator of OsLHS1 function are complicated without mutant gene expression patterns. The difference between the mutant and the wild-type gene is particularly evident when comparing the mutant phenotype and the wild-type gene expression; all four floral whorls are affected in lhs1 mutants, whereas OsLHS1 is only expressed in the palea, lemma, and pistil (Prasad et al., 2001). The RNAi data are similarly difficult to interpret. K. Prasad and U. Vijayraghavan (unpublished data) reduced levels of the OsLHS1 transcript using RNAi and observed that only the palea, lemma, and lodicules are affected in all flowers; this despite reports that OsLHS1 is not expressed in lodicules (Chung et al., 1994; Prasad et al., 2001). In other functional studies, a role for OsLHS1 in meristem determination was hypothesized after ectopically expressing the gene in rice and noting that some of the transformed flowers had either reduced or aborted inner floral organs (Prasad et al., 2001).

In addition to meristem determinacy, OsLHS1 may also be playing a role in palea and lemma identity because ectopic expression of the gene in rice was sufficient to transform the two sterile lemmas (often called sterile glumes in the rice literature) into palea/lemma-like structures (Prasad et al., 2001), and cosuppression of OsLHS1 using RNAi caused the palea and lemma

Figure 1. Summary of Relationships among SEP Genes.
Bayesian phylogenetic analysis of 76 SEP genes from GenBank using the general time reversible (GTR) model with some invariant sites and I-distributed rates (GTR+I+G) (S.T. Malcomber and E.A. Kellogg, unpublished data). Bayesian posterior probabilities are included above the branches. Orange, monocot sequences; blue, eudicot sequences.
to resemble sterile lemma-like structures (K. Prasad and U. Vijayraghavan, unpublished data). OsLHS1 also plays a role in shaping inflorescence morphology and, under certain environmental conditions, in the transition to flowering. Rice plants in which OsLHS1 was overexpressed had consistently shorter panicles and irregularly positioned branches (Jeon et al., 2000b; Prasad et al., 2001), and in one study (Jeon et al., 2000b), rice plants were significantly dwarfed and early flowering. However, Prasad et al. (2001) detected no reduction in plant size or flowering time between the transgenic and control plants, a difference from the Jeon et al. (2000b) study they attributed to variations in photoperiods between the two study sites.

In maize, ZmLHS1b is closely linked to *indeterminate floral apex1* (*ifa1*) on maize chromosome 1 (Cacharrón et al., 1999). *ifa1* mutants make extra spikelets and extra flowers in the spikelets and often have proliferating gynoecial tissue (Laudencia-Chingcuanco and Hake, 2002). This phenotype is consistent with *lhs1*, but whether a mutated ZmLHS1b causes *ifa1* has yet to be demonstrated. ZmLHS1a and ZmLHS1b are hypothesized to be acting as either selector genes or as upper floret meristem determinacy genes (Cacharrón et al., 1999). In the selector gene hypothesis, ZmLHS1a and ZmLHS1b are hypothesized to specify the identity of the upper floret primordium, distinguishing it from the lower one. This hypothesis predicts that LHS1 orthologs in other species are expressed only in the uppermost floret primordium of the developing spikelet and will not be evident in any of the lower florets (Cacharrón et al., 1999). In the upper floret meristem determinacy hypothesis, ZmLHS1a and ZmLHS1b are hypothesized to prevent back-transformation of the floret meristem to a spikelet meristem (Cacharrón et al., 1999). This hypothesis predicts that LHS1 orthologs are only being expressed in the uppermost floret primordium and are not detected in the spikelet meristem.

The authors cited in the previous paragraphs have thus suggested that *LHS1* is a gene that may (1) confer determinacy on the floret meristem, (2) specify lemma and palea identity, (3) tell the upper floret in the spikelet that it is the uppermost floret, and/or (4) determine floret number in the spikelet.

We have examined *LHS1* expression in several distantly related grass species, following the well-supported phylogeny of the grass family (Grass Phylogeny Working Group, 2001). We demonstrate that *LHS1* expression is heterogeneous among sampled grasses. These data and published reports support hypotheses that *LHS1* plays a role in meristem identity and in specifying palea and lemma identity, support the selector gene hypothesis for a subset of grass species, find no evidence for a role of *LHS1* in specifying floret number, and allow us to develop other testable hypotheses.

**RESULTS**

**LHS1 Phylogenetic Analysis**

We PCR amplified *LHS1* from cDNA of 13 distantly related taxa to supplement available sequences from *H. vulgare, Lolium perenne, O. sativa*, and *Z. mays* (Table 1). The PCR fragments ranged from 704 to 771 bp in length and represented 95% of

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Voucher information for new gene sequences from this study is included in GenBank.

*a* Gene not previously named.
the coding region of the gene (based on full-length sequences of Oryza and Loliu); all had a single open reading frame. Pairwise variation among LHS1 orthologs ranged from 5.2 to 23.0% (nucleotides) and 6.1 to 29.8% (amino acids) for the entire amplified fragment, with the vast majority of differences occurring in the C-terminal region. A protein translation of the 18 LHS1 nucleotide sequences showed that the two amino acid sites responsible for the lhs1 mutant phenotype—Arg24 and Gly27—were conserved among the sampled species. Sequence evidence thus suggests that the proteins are all functional. Bayesian phylogenetic analysis of the aligned LHS1 data set recovered a phylogeny very similar to that estimated by the Grass Phylogeny Working Group (2001) analysis (Figure 2). Differences between the LHS1 and Grass Phylogeny Working Group topologies were not strongly supported (<95% posterior probability) and probably represent sampling artifacts (Kellogg and Linder, 1995; Grass Phylogeny Working Group, 2001).

The sister relationship in the LHS1 phylogeny of ZmLHS1a and ZmLHS1b implies that these genes resulted from the maize tetraploidy event (Gaut and Doebley, 1997), which is quite recent relative to the other evolutionary divergences in this study. This observation is supported by the mapping of the two genes onto syntenic duplicated regions of maize chromosomes 1 and 9 (Cacharrón et al., 1999). DNA gel blot hybridizations using gene-specific probes on A. sativa, Chasmanthium latifolium, H. vulgare, O. sativa, P. glaucum, S. bicolor, and Z. mays suggested that all sampled species have a single LHS1-like gene, except maize, which has two, C. latifolium, which is a tetraploid (Gould, 1975) and has two, and A. sativa, which is a hexaploid (Koul and Gohil, 1987) and has three (Figure 3).

### LHS1 mRNA Expression Patterns

To determine organ-level pattern of LHS1 expression, we used RT-PCR with LHS1-specific primers on inflorescence, culm, leaf, and root cDNA from H. vulgare (Pooideae), O. sativa (Ehrhartioideae), P. miliaceum, S. italica, and S. bicolor (Panicoideae). This shows that the gene is largely restricted to inflorescence tissue with little or no expression in the leaf, culm, and root (Figure 4).

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**Figure 2.** Maximum Likelihood Phylogram of the LHS1 Data Set Recovered Using the GTR Model with Some Invariant Sites and G Distributed Rates (GTR + I + G).

Bayesian posterior probabilities are included above the branches, and subfamily abbreviations are included in parentheses (ARI, Aristidoideae; BAM, Bambusoideae; CEN, Centothecoideae; CHL, Chloridoideae; DAN, Danthonioideae; EHR, Ehrhartioideae; PAN, Panicoideae; POO, Pooideae).
To determine tissue-specific expression patterns of \textit{LHS1}, we conducted in situ hybridizations on several different grass species. As we had predicted, we found variation among species in the patterns of expression.

\textit{S. bicolor} (Panicoideae) has an inflorescence comprised of pedicellate and sessile spikelets, both composed of two florets. Florets in the spikelet mature basipetally. The upper floret of the sessile spikelet is bisexual, with a pistil, three stamens, two lodicules, palea, and lemma. The upper floret of the pedicellate spikelet is staminate or sterile, with the pistil aborting early in development. The lower floret in both the sessile and pedicellate spikelets is reduced to a sterile lemma. \textit{S. bicolor} LHS1 (SbLHS1) is expressed throughout the meristem of the upper floret, before floral organ initiation but just after glume formation (Figure 5A). After floral organ initiation, SbLHS1 is localized in the palea, lemma, and pistil primordia of the upper floret, with no detectable expression in stamen primordia (Figure 5B). This expression pattern persists through floral development, with no detectable expression in the lodicules and stamens of either floret (Figures 5C and 5D). In the pistil, the gene is strongly expressed in the stigma and style (Figure 5E) and the inner integument of the ovule (Figures 5E and 5F). We find similar expression patterns in both the sessile and pedicellate spikelets (Figure 5D).

\textit{C. latifolium} (Centothecoideae) has a 4- to 24-flowered spikelet in which florets develop acropetally. The lower one to four florets at the base of the spikelet are sterile, whereas more apical ones are bisexual. Each bisexual floret consists of a pistil, one stamen, two lodicules, palea, and lemma. \textit{C. latifolium} LHS1 (CLHST1) is strongly expressed in the apical meristem of the developing spikelet, just after glume formation. No expression was detected in the four lower floret

![DNA Gel Blots Using the Gene-Specific C-Terminal Region of LHS1 cDNA as Probe.](image)

\textit{A. sativa} (As), \textit{C. latifolium} (Cl), \textit{H. vulgare} (Hv), \textit{O. sativa} (Os), \textit{P. glaucum} (Pg), \textit{S. bicolor} (Sb), and \textit{Z. mays} (Zm) genomic DNA each digested with \textit{BamH1} (B), \textit{EcoR1} (E), and \textit{HindIII} (H) restriction enzymes.

![LHS1 RT-PCR Expression Patterns in Inflorescence (INFL), Leaf, Culm, and Root Tissues of O. sativa (Oryza), H. vulgare (Hordeum), P. miliaceum (Panicum), P. glaucum (Pennisetum), and S. bicolor (Sorghum).](image)

Triose phosphate isomerase primers were used as a positive control.
meristems that we infer would have become sterile florets. 

*ClLHS1* expression was only detected in the upper florets that we assume would have developed into functional flowers (Figure 6A). In older spikelets, the gene is expressed throughout the spikelet meristem and all organs of the upper four florets (Figure 6B). In the fifth and sixth flowers below the apex, *ClLHS1* is expressed in all floral organs except the lemma (Figure 6B). In the seventh floret, expression is restricted to the anther sacs of the stamens and the style and ovule of the pistil (Figures 6B and 6C). In the oldest functional flowers, expression is restricted to the ovule (Figure 6D).

* A. sativa (Pooideae) has a two- to six-flowered spikelet with acropetal maturation of the florets. All florets in the spikelet are functional and bisexual, consisting of a pistil, three stamens, two
lodicules, palea, and lemma. *A. sativa LHS1 (AsLHS1)* is first expressed throughout the spikelet meristem and in floret primordia, just after glume formation (Figure 7A). We detected no difference in expression pattern among florets in different parts of the mature spikelet; *AsLHS1* is restricted to the palea, lemma, and pistil of all mature florets (Figures 7B and 7C).

*P. glaucum* (Panicoideae) has a two-flowered spikelet that matures basipetally. The upper floret is bisexual, whereas the lower floret is staminate or sterile. The upper floret consists of a pistil, three stamens, two lodicules, palea, and lemma. The pistil of the lower floret aborts early in development. In mature flowers, *P. glaucum LHS1 (PgLHS1)* expression is restricted to

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**Figure 6.** In Situ Analysis of LHS1 Expression in Different Tissues of *C. latifolium*.

(A) Developing spikelet, just after glume formation, expression in spikelet meristem with no expression detected in the four lower floret meristems (arrows).

(B) Older spikelet expression.

(B) Spikelet with eight developing florets (arrows) expression throughout the spikelet meristem and upper four florets; in the fifth and sixth florets, expression in all floral organs except the lemma; in the seventh floret, expression restricted to the pistil and the single stamen; in the oldest functional floret, expression only in the ovule.

(C) Expression restricted to anther sacs of the stamen and style and ovule of the pistil.

(D) Expression restricted to the ovule.

(E) Illustration depicting LHS1 expression (black) in spikelet.

*gl, glume; le, lemma; lo, lodicules; pa, palea; pi, pistil; sm, spikelet meristem; st, stamens. Bars = 100 μm.*
Figure 7. In Situ LHS1 Expression in Different Tissues of *A. sativa* and *P. glaucum*.

(A) to (D) *A. sativa*.
(E) to (H) *P. glaucum*.

(A) Developing spikelet, just after glume formation, with five developing florets (arrows). Expression is in the spikelet meristem and all developing florets but not in the glumes.
(B) and (C) Mature spikelet.
(B) Expression in the palea, lemma, and pistil of all florets.
(C) Expression in the palea, lemma, and pistil.
(D) Illustration depicting LHS1 expression (black) in the spikelet.
(E) to (G) Mature floret with expression in the palea, lemma, and lodicules of the upper floret. No expression was detected in the pistil, stamens of the upper floret, or any organs of the lower floret.
(H) Illustration depicting LHS1 expression (black) in palea, lemma, and lodicules of the upper floret.

gl, glume; lf, lower floret; le, lemma; lo, lodicules; pa, palea; pi, pistil; sm, spikelet meristem; st, stamens; uf, upper floret. Bars = 100 μm.
the palea, lemma, and lodicules of the upper floret (Figures 7E to 7G). No expression was detected in the stamens or pistil of the upper floret, and no expression was detected in any organs of the lower floret (Figures 7E and 7F).

Evolution of LHS1 Gene Expression within Grasses

To investigate the evolution of LHS1 gene expression within grasses, we used maximum parsimony character reconstruction methods within the context of the Grass Phylogeny Working Group (2001) phylogeny. We supplemented our data in *S. bicolor*, *C. latifolium*, *P. glaucum*, and *A. sativa* with published expression patterns of OsLHS1 (Chung et al., 1994; Prasad et al., 2001), *H. vulgare* LHS1 (barley MADS7; Schmitz et al., 2000), and ZmLHS1a and ZmLHS1b (Cacharrón et al., 1999) (Table 2). Expression of the rice and maize genes was described above. *H. vulgare* has one floret per spikelet, and each floret is bisexual, consisting of a pistil, three stamens, two lodicules, palea, and lemma. HvLHS1 is expressed uniformly throughout the floral primordium and later becomes restricted to the palea, lemma, lodicules, and pistil (Schmitz et al., 2000).

All sampled species are similar early in development with expression throughout the spikelet meristem, just after glume formation but before floral meristem initiation; expression was never observed in glumes for any species. After this stage, LHS1 expression patterns diverged among the different species. Parsimony character reconstruction of the LHS1 expression pattern recovered an ancestral pattern of expression in the palea, lemma, and pistil and expression only in the upper floret(s) of the spikelet, with no expression in the sterile or staminate florets at the base (Figure 8). LHS1 expression throughout the spikelet is inferred to have evolved before the diversification of the Avena-Hordeum clade (subfamily Pooidae). Expression in the pistil was lost in the lineage leading to Pennisetum, and expression in stamens was gained in the lineages leading to Chasmanthium and *Z. mays*. Parsimony reconstruction of the ancestral expression in the lodicules was ambiguous. Assuming LHS1 was on in the ancestor suggests expression was lost in the Sorghum lineage and at the base of the Oryza, Hordeum, and Avena lineage and then subsequently gained in the Hordeum lineage. If LHS1 expression was off in the ancestor, then expression is inferred gained in the Hordeum lineage and at the base of the Chasmanthium, Pennisetum, Sorghum, and Zea lineage and subsequently lost in the Sorghum lineage (Figure 8).

**DISCUSSION**

The ABCDE model predicts how interactions among five classes of MADS-domain proteins specify the different floral organ whorls (Theissen, 2001). Class A genes are expressed in the outer and second floral whorls and specify sepals and petals. Class B genes are expressed in the second and third floral whorls and are involved in petal and stamen identity. Class C genes are expressed in the third and fourth floral whorls and regulate

### Table 2. Overview of LHS1 Expression Patterns

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</table>

+ , Gene expressed; --, gene not expressed; 0, organ absent.

Table 2. Overview of LHS1 Expression Patterns
stamen and carpel identity. Class D genes are only expressed in the pistil and are involved in ovule development (Theissen, 2001; Theissen and Saedler, 2001). Class E (or SEP) genes are expressed in all floral whorls in Arabidopsis (Rounsley et al., 1995; Pelaz et al., 2000); SEP proteins have been shown to interact with, and mediate the expression of, the other floral organ identity genes and play a role in floral meristem determinacy (Honma and Goto, 2001; Pelaz et al., 2001b; Favaro et al., 2003; Ferrario et al., 2003; Vandenbussche et al., 2003).

Although changes in the number, expression, and interaction of developmental genes have likely contributed to the evolution of plant form (Theissen et al., 2000), few studies in plants have demonstrated changes in gene expression that correlate with morphological evolution. For example, many aspects of the ABCDE model appear to be conserved among species such as Arabidopsis, petunia, maize, and rice (Coen and Meyerowitz, 1991; Schmidt and Ambrose, 1998; Ambrose et al., 2000; Kyozuka et al., 2000; Honma and Goto, 2001; Vandenbussche et al., 2003). However, several studies suggest that some SEP genes have divergent functions (Kotilainen et al., 2000; Yu and Goh, 2000; Pelaz et al., 2001a; Tzeng et al., 2003). This is particularly evident in grasses where SEP genes have complex patterns of expression within the floret (Chung et al., 1994; Kang and An, 1997; Cacharrón et al., 1999; Prasad et al., 2001;
Pelucci et al., 2002) and are considered unlikely to be functionally homogeneous (Theissen, 2001; Becker and Theissen, 2003). Given that divergence in SEP gene expression patterns might have been important in the evolution of inflorescence and floral diversity in grasses, we have begun investigation of the SEP genes in grasses, focusing initially on LHS1.

In this study, we have found broad variation in the expression pattern of the SEP gene LHS1 in phylogenetically disparate grasses. Expression of LHS1 orthologs is largely restricted to inflorescence tissue, but varies in organ expression within the floret and among florets in different regions of the spikelet. In formulating hypotheses of LHS1 function in grasses, we assume that localization of LHS1 RNA demonstrated in our work and in the literature represents localization of the protein, an assumption consistent with the observations of most floral organ identity MADS box genes (Theissen and Saedler, 1995; but see Tzeng and Yang, 2001). Our expression data support the selector gene hypothesis in all species with basipetal maturation of florets within the spikelet but not in species with acropetal maturation. Predictions of the upper floret meristem determinacy hypothesis are not compatible with our expression data. In sum, our expression data suggest several functions for LHS1 in grasses, including roles in maintaining meristem function, determining lemma/palea identity, and specifying the upper floret in certain species. These different functions of the gene may reflect changes in both timing and position of expression.

**Expression Evolution of LHS1 in Grasses**

We find conservation of expression pattern at the level of the spikelet meristem. All species have similar patterns of expression early in spikelet development, with LHS1 being expressed throughout the spikelet meristem but apparently excluded from the glumes. Later in development, however, LHS1 is restricted to the upper florets of the spikelet (remaining on in the spikelet meristem itself) in all grasses except oats and barley, here representative of the subfamily Pooidae. This taxonomic distribution leads us to hypothesize that LHS1 will also be expressed in all florets of Lolium, wheat, fescue, and all other pooid grasses. Thus, expression in lower florets appears to be a uniquely derived character for the subfamily.

In all flowers where it is expressed, LHS1 RNA appears in the palea and lemma; this is true for all grasses investigated to date (Table 2). Expression in whorls two (lodicules), three (stamens), and four (pistil), however, varies and does not correlate with the family-level taxonomy. The gene is on in the lodicules of Hordeum, Chasmanthium, Pennisetum, and maize, in the stamens of Chasmanthium and maize, and in the pistil of all sampled species except Pennisetum. Our phylogenetic analysis suggests that expression restricted to the palea, lemma, and pistil in the upper florets is ancestral within the family. Loss of expression in the pistil has occurred at least once in the Pennisetum lineage, and gain of expression in the stamens has occurred on at least two separate occasions, in the Chasmanthium and Zea lineages. Reconstruction of the lodicule expression pattern is ambiguous, but loss/gain of expression in the lodicules has occurred on at least three separate occasions.

A. sativa is a hexaploid (Koul and Gohil, 1987), and C. latifolium is reported to be tetraploid (Gould, 1975). Two of the three oat genomes cannot be differentiated using repetitive probes (Ananiev et al., 2002), whereas the third genome is somewhat divergent. Nothing is known of the two Chasmanthium genomes. The C-terminal probes used in this study do not differentiate among the oat genomes or between the Chasmanthium genomes, as indicated by the DNA gel blots in Figure 3. It is formally possible that the LHS1 copies in these two species have diverged in expression despite the high similarity in sequence and that the patterns we see are the sum of disparate patterns of individual genes. We think that this possibility is unlikely; even in maize, which is known to be an ancient tetraploid, the duplicate genes have near-identical expression patterns (Cacharrón et al., 1999). However, even if the gene copies have divergent patterns, it would suggest that at least one of the copies must have changed its role such that it is expressed in nonterminal flowers. Therefore, whether the gene copies have divergent or identical expression patterns in Avena and Chasmanthium, LHS1 appears to have different roles in acropetal and basipetal species, supporting our general conclusion that expression, and by inference function, of the gene has been modified in evolutionary time. Future work, of course, will have to test our general conclusions by looking at multiple diploid relatives of the species investigated here, and these will need to be coupled with functional studies in multiple systems in addition to maize and rice.

In the remainder of this article, we describe some trends in morphology and gene expression and generate hypotheses of gene function.

**LHS1 Expression Is Consistent with the Selector Gene Hypothesis Only in Species with Basipetal Maturation of Florets in the Spikelet but Does Not Determine Floret Number**

The selector gene hypothesis proposes that LHS1 orthologs specify the upper floret of a spikelet (Cacharrón et al., 1999). Our analysis supports this hypothesis in all species with basipetal maturation of florets within the spikelet (Oryza, Pennisetum, Sorghum, and Zea) but not in species with acropetal maturation (Avena and Chasmanthium). In the latter species, the gene is expressed either in several of the upper florets (Chasmanthium) or in all florets of the spikelet (Avena), suggesting either that LHS1 has a different function or has roles in addition to that proposed by the selector gene hypothesis in basipetal species.

Our results show that LHS1 is not responsible for determining the number of flowers in a spikelet (referred to as determinacy in some parts of the literature). In most grasses, the spikelet meristem is obvious as an extension or bump beyond the uppermost floret. In all species in which we have been able to observe this meristem, LHS1 is strongly expressed, independent of the number of flowers. Therefore, contrary to the suggestion that LHS1 converts the spikelet meristem to a floral meristem (Cacharrón et al., 1999), we find that LHS1 is strongly expressed in spikelet meristems as they are producing flowers, and some species (e.g., Chasmanthium) produce large numbers of flowers while maintaining LHS1 expression in the spikelet meristem.
**LHS1 Expression May Correlate with Floret Morphology and Sex Expression**

Within a spikelet, the flowers that express LHS1 are morphologically similar to each other. In many species, the LHS1 expressing florets are morphologically distinguishable from flowers not expressing the gene. In Pennisetum, for example, where LHS1 is expressed in the palea, lemma, and lodicules of the upper floret only, the lemma of the lower floret is similar in texture to the glumes rather than to the upper lemma, and the lower palea is membranous rather than thick and papery like the upper one. Likewise in rice, where LHS1 expression is restricted to the palea, lemma, and pistil of the fertile, upper floret, the lower flowers (LHS1 nonexpressing) are morphologically distinct, so distinct that they are often misdescribed as glumes. When LHS1 is overexpressed, then the lower flowers become LHS1 expressing and become morphologically similar to the upper palea/lemma (Prasad et al., 2001). In Avena, all flowers are LHS1 expressing and are all morphologically similar. Hordeum only has a single flower, so is uninformative. In Sorghum, lemmas of both flowers are similar in texture, but the lemma of the upper, LHS1-expressing flower bears an awn. Exceptions to the foregoing appear in Chasmanthium and Zea, in which the lemma and palea of the lower, LHS1 nonexpressing flowers are morphologically similar (at least superficially) to the upper, LHS1-expressing ones.

LHS1 expression may also correlate with the sex of the flower. In rice, Sorghum, Pennisetum, and Chasmanthium, the nonexpressing flowers are staminate or sterile, whereas all flowers that express the gene are bisexual. In staminate flowers (non-LHS1 expressing) of Sorghum and Pennisetum, the pistil is initiated but aborts, whereas in sterile flowers (non-LHS1 expressing) of rice and Chasmanthium, the pistil is not initiated. Exceptions are maize, in which the upper flower of male tassel spikelets and female ear spikelets both express the gene throughout, and the pedicellate spikelet of Sorghum, in which the upper floret expresses the gene despite being staminate.

**LHS1 May Have Multiple Interacting Partners and Multiple Roles in Spikelet Development**

We suggest that LHS1 has several functions in grasses, as do the related SEP genes FBP2 in petunia (Petunia hybrida) (Angenent et al., 1994), TMS in tomato (Lycopersicon esculentum) (Pnueli et al., 1994), and SaMADS D in Sinapis alba (Bonhomme et al., 1997). Multiple roles for LHS1 could be generated in at least two ways, and these are not mutually exclusive. First, we predict that LHS1 may have distinct regulatory elements that affect its expression in different organs and/or at different times in development, such that regulation by genes specifying lemma/palea identity will be independent of regulation by genes specifying meristem function. Second, we predict that LHS1 interacts with different MADS box genes in different organisms to form distinct heterodimers and tetramers. We know that the grasses have more such genes than Arabidopsis or most known eudicots (S.T. Malcomber and E.A. Kellogg, unpublished data; Münster et al., 2002; Nam et al., 2004), and this creates the possibility of many more interacting proteins. Larger numbers of possible combinations are hypothesized to lead to more phenotypic diversity (Theissen et al., 2000).

Expression of SEP3 orthologs in rice (OsMADS24 (=OsMADS8) and OsMADS45 (=OsMADS7)) and sorghum (SbMADS1) is restricted to the inner three floral whorls (Greco et al., 1997; Pelucchi et al., 2002). By contrast, expression patterns of other grass SEP genes are overlapping, but highly variable. Amongst the grasses, a complete SEP gene expression profile has only been published thus far for rice. OsLHS1 is expressed in the first and fourth floral whorls, OsMADS3 is expressed in the third and fourth whorls, and OsMADS4 (=OsMADS19) is expressed throughout the plant but is turned off in the second and fourth whorls late in development (Chung et al., 1994; Kang and An, 1997; Prasad et al., 2001; Pelucchi et al., 2002). In sum, these expression patterns suggest that rice has two SEP genes expressed in the first whorl, three SEP genes in the second whorl, at least three SEP genes in the third whorl, and at least four SEP genes in the fourth whorl. The expression of multiple SEP genes in each of the floral whorls suggests either redundancy of function, partitioning of function within each of the whorls, or a combination of both. Using the pattern of SEP gene expression in rice as a guide, we can hypothesize that the complex pattern of LHS1 expression we have detected in grasses is probably complemented by similarly complex patterns of SEP gene expression evolution among other grasses.

In conclusion, this study has demonstrated that much can be gained from using a comparative approach to infer where and when particular genes diversified and how these patterns of gene expression are correlated with morphological evolution. Using this approach, we have been able to test functional hypotheses from model species, help tease apart multiple roles for a gene product, and provide testable hypotheses for further investigation. Only such an approach can start to unravel the complexity of genetic mechanisms that have generated the morphological diversity in groups such as the grasses.

**METHODS**

**Plant Materials**

Material from 13 species was collected from plants growing at Missouri Botanical Garden or from plants grown at University of Missouri-St. Louis from USDA seed stocks (Table 1). Plants were grown under standard greenhouse conditions.

**Isolation of cDNA, PCR, Subcloning, and Sequencing**

Total RNA was extracted from young inflorescences 0.1 to 4 cm in size using RNAwiz solution (Ambion, Austin, TX) according to the manufacturer’s instructions. cDNA was generated from the extracted total RNA using Superscript III (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions with a (T)15 or polyT with adaptor primer (S’-CCGGATCCTC-TAGAAGGGCG-3’) and sorghum (SbMADS19) primers (SaMADS D, LHS1, SEP3). Exons four and five of triose phosphate isomerase were PCR amplified to check the cDNA quality using degenerate primers TPX4F and TPX6R (Strand et al., 1997).

Double-stranded LHS1 homologs were PCR amplified from cDNA using the degenerate primers MADS1F (5’-ATGGGTMGSGGSAAGCGTG-3’) and either LHS1-633R (5’-TATCCCAACCTGCAGGCGGGAAGGG-3’) or LHS1-715R (5’-TATCCCAACCTGCAGGCGGGAAGGG-3’).
AGACGATCCACGATGTCCACCAGG-3’. PCR fragments were purified and subcloned into the pGEM-T easy vector (Promega, Madison, WI). Plasmid DNA was cleaned using an alkaline lysis/polyethylene glycol precipitation protocol (Sambrook et al., 1989) before sequencing using plasmid primers T7 and sp6. Dideoxy sequencing was conducted using the Big Dye 3.0 or 3.1 terminator cycle sequencing protocol (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on an ABI 377 automated DNA sequencer (Applied Biosystems). Base calling within the chromatograms was checked and confidence scores assigned using Phred (Ewing et al., 1998). Only nucleotide sequences with phred scores >20 were used in subsequent analyses. Contiguous alignments were edited using Seqman II (DNASTAR, Madison, WI). All sequences were submitted to GenBank (AY597511 to AY597522).

DNA Gel Blots

Approximately 10 micrograms of total DNA was digested with either BamHI, EcoRI, or HindIII, separated on a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with a [32P]dCTP-labeled C-terminal probe for 16 h at 65°C following Laurie et al. (1993). After hybridization, blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)/0.5× SDS at 65°C and twice in 0.1× SSC/0.1× SDS at 65°C.

In Situ Hybridization

Tissue was dissected at various developmental stages and vacuum infiltrated in FAA (47.5% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formaldehyde; Sigma, St. Louis, MO) for 10 min on ice, and then the fixative was renewed with fresh FAA and left to fix at 4°C overnight. Fixed material was dehydrated, cleared, and embedded in wax (Para- plast Plus; Oxford Labware, St. Louis, MO) as described by Jackson (1991). Ribbons of 8- to 10-µm sections were cut, mounted on Probe-On-Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and left to dry overnight.

LHS1-specific cDNA probe templates were generated by PCR amplifying the C terminus of LHS1 orthologs using LHS1-409F (5’-ATCAGCT-CAARAAAGAAYCA-3’) and either LHS1-633R or LHS1-715R. The amplified fragments ranged from 292 to 346 bp and were 34.2 to 36.6% different (nucleotides) from LHS1 orthologs (the most similar gene to LHS1). These fragments were subcloned into pGEM-T easy vector and sequenced to check for orientation. Sense and antisense riboprobes were generated using sp6 and T7 Megascript in vitro transcription kits (Ambion) with digoxigenin-labeled UTP (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Probe hybridization was followed Jackson (1991).

Probe hybridization, post-hybridization washing, and immunolocalization were as described in Jackson et al. (1994), except the RNase incubation step during the post-hybridization washes was omitted. Western Blue (Promega) was used for colorometric detection. Photographs were taken after 1 to 3 days of staining using a Nikon 995 digital camera (Tokyo, Japan) on a Zeiss Axioskop microscope (Jena, Germany) with bright-field illumination. Hybridizations with antisense probes were repeated at least three times on independently fixed and embedded material to verify expression patterns. No signal was detected in any of the control hybridizations with sense probes, except for a very faint, nonspecific background. Brightness, contrast, and color balance were adjusted using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY597511 to AY597522.

ACKNOWLEDGMENTS

We thank Missouri Botanical Garden and the USDA for access to plant materials and Andrew Doust, Michael Zanis, Jill Preston, Paula McSteen, and Dave Jackson for help with the expression studies. We also thank Andrew Doust, Neelima Sinha, Bob Schmidt, Sarah Hake, and three anonymous reviewers for comments on earlier versions of the manuscript. Kalika Prasad and Usha Vijayraghavan shared unpublished RNAi data in rice. This work was supported by National Science Foundation Grant DBI-0110189 (to E.A.K).

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Heterogeneous Expression Patterns and Separate Roles of the SEPALLATA Gene LEAFY HULL STERILE1 in Grasses

Simon T. Malcomber and Elizabeth A. Kellogg

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