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RESEARCH PAPER

# Is *LEAFY* a useful marker gene for the flower–inflorescence boundary in the *Euphorbia* cyathium?

**Gerhard Prenner[1,*], N. Ivalú Cacho[2,†], David Baum[2] and Paula J. Rudall[1]**

[1] Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK
[2] Department of Botany, University of Wisconsin, 430 Lincoln Drive, Madison, WI 53706-1381, USA

[†] Present address: Department of Evolution and Ecology, University of California-Davis, One Shields Avenue, Davis, CA 95616, USA.
[*] To whom correspondence should be addressed. E-mail: g.prenner@kew.org

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## Abstract

**The flower-like reproductive structure of *Euphorbia* s.l. (Euphorbiaceae) is widely believed to have evolved from an inflorescence, and is therefore interpreted as a special type of pseudanthium, termed a cyathium. However, fuzzy morphological boundaries between the inflorescence, individual flowers, and organs have fuelled the suggestion that the cyathium does not merely superficially resemble a flower but could actually share developmental genetic pathways with a conventional flower. To test this hypothesis, immunolocalizations of FLORICAULA/LEAFY (LFY), a protein associated with floral identity in many angiosperm species, were performed in developing cyathia of different species of *Euphorbia*. Expression of the LFY protein was found not only in individual floral primordia (as predicted from results in the model organisms *Arabidopsis* and *Anthirrhinum*), but also in the cyathium primordium and in the primordia of partial male inflorescences. These results provide further evidence that the evolution of floral traits in pseudanthial inflorescences often involves expression of floral development genes in the inflorescence apex. This finding blurs the conventional rigid distinction between flowers and inflorescences.**

**Key words:** Cyathium, *Euphorbia*, Euphorbiaceae, *FLORICAULA/LEAFY*, flower, inflorescence, Malpighiales, pseudanthium.

## Introduction

The hallmark feature of *Euphorbia* (including former segregate genera *Monadenium*, *Synadenium*, and, *Pedilanthus*) is its unique reproductive structure, the cyathium, which resembles a flower but is widely interpreted as a condensed inflorescence or pseudanthium (Prenner and Rudall, 2007). Following the pseudanthial interpretation, the cyathium consists of an involucre enclosing clusters of staminate flowers that surround a single terminal carpellate flower (Fig. 1A, D, H). Individual flowers are highly reduced, typically to a single organ, and their perianth is either entirely lost or reduced to lobes that are initiated late in ontogeny (cf. Rudall, 2010). However, the morphological identity of the cyathium has long been controversial. Payer (1857) and Baillon (1858) followed Linnaeus (1753) in describing it as a single flower, whereas Lamarck (1786), Jussieu (1789, 1824), and Brown (1814) preferred the pseudanthial hypothesis and considered it to be an inflorescence. Most subsequent anatomical work has tended to support the view that the cyathium evolved from an inflorescence (Hoppe and Uhlarz, 1982; Gilbert, 1994).

Some authors have favoured a more fuzzy interpretation of the cyathium. Corner (1958) speculated that it could share homology with both flowers and inflorescences. Recent detailed comparative ontogenetic and morphological investigations (Prenner and Rudall, 2007; Prenner *et al.*, 2008*a*, 2009) highlighted the indistinct boundary between the inflorescence, flower, and floral organs. Prenner and Rudall (2007) suggested that the apparently mixed identity of the cyathium could result from an overlap between expression of genes regulating flower and inflorescence development. Thus, the cyathium could be considered a special case that defies ready assignment to either a flower or an inflorescence (cf. Baum and Donoghue, 2002; Prenner *et al.*, 2009). Such morphological misfits are

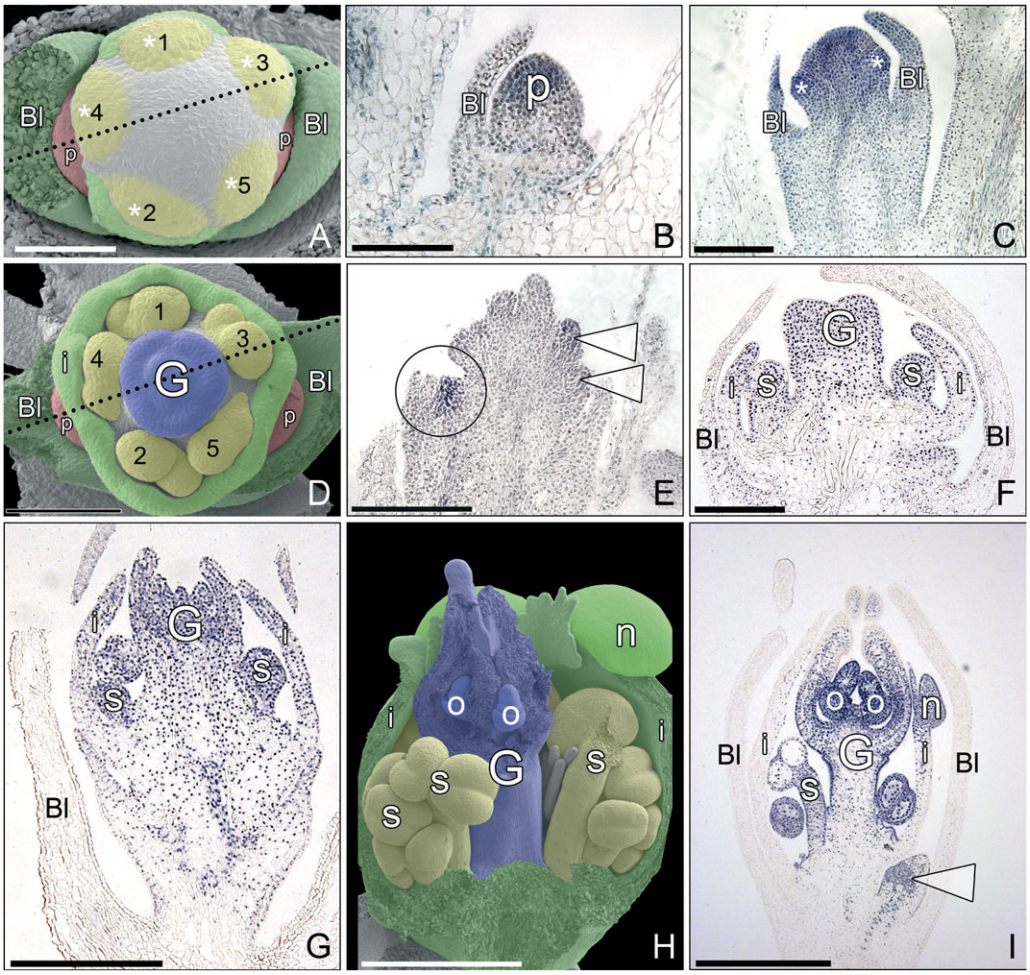


**Fig. 1.** Immunolocalization of LFY protein in different ontogenetic stages of the cyathium. (A, D, H) SEM images; all others longitudinal sections (LS). (A) *Euphorbia myrsinites*, SEM image of a young cyathium. Cyathium primordia in red, bracteoles and male inflorescence subtending bracts in green, male partial inflorescences in yellow. The dotted line shows the approximate position of the sections in (B) and (C). (B) *Euphorbia milli*. The cyathium primordium is subtended by a bract and shows strong expression of LFY protein. (C) *Euphorbia tithymaloides*, young cyathium showing expression in the male inflorescence primordia (asterisks). (D) *Euphorbia myrsinites*, SEM images of a somewhat older cyathium. Colours are the same as in (A). (E) *Euphorbia milli*, young cyathium showing expression of LFY protein in male flower primordia (arrowheads) and in a cyathial primordium (encircled). (F) *Euphorbia myrsinites*, young cyathium showing expression of LFY protein in young male flowers and in the gynoecium. (G) *Euphorbia myrsinites*, older cyathium with LFY expression in young male flowers and young gynoecium. (H) *Euphorbia pteroneura*, older cyathium, bracteoles removed, cyathium dissected to show the central gynoecium (i.e. naked and stalked female flower) flanked by two bundles of stamens (i.e. male flowers). The gynoecium is dissected to show two young ovules (note that the integuments are just starting to develop). At the rim of the involucre one nectary is visible. Colours are the same as in (A). (I) *Euphorbia nicaeensis*, similar developmental stage to that in (H), LFY expression in male flowers, ovules, nectary, and cyathial primordium (arrowhead). Bl, bracteoles; i, involucre; G, gynoecium/female flower; n, nectary; o, ovule; p, cyathial primordium; s, male flower/stamen. Scale bars, 100 μm in A, B; 200 μm in C–F; 500 μm in G–I.

frequently ignored in studies of inflorescence architecture (cf. Benlloch *et al.*, 2007; Prusinkiewicz *et al.*, 2007; Prenner *et al.*, 2009).

To evaluate these alternative interpretations of the cyathium, the expression of FLORICAULA/LEAFY (LFY), a protein that is associated with floral meristem identity in most plant species studied to date, was examined. The LFY gene encodes a transcription factor that is present as one or very few copies in all angiosperms and has been found to be necessary and sufficient for the initiation of flowers in diverse species (e.g. Weigel *et al.*, 1992; Weigel and Nilsson, 1995; Blázquez *et al.*, 1997; Sessions *et al.*, 2000; reviews in Benlloch *et al.*, 2007; Moyroud *et al.*, 2009). In *Arabidopsis*, *LFY* is expressed in determinate flower primordia in the racemose inflorescence, but not in the indeterminate inflorescence apex, where the shoot meristem identity gene *TERMINAL FLOWER 1* (*TFL1*) is expressed instead (Bradley *et al.*, 1997). In *lfy* mutants, early developing flowers at the base of the inflorescence are transformed into inflorescence shoots, and later developing flowers show a mixture of flower and inflorescence traits (Schultz and Haughn, 1991; Weigel *et al.*, 1992).

In the majority of species studied to date, meristems destined to take on floral identity show strong expression of *LFY*. One exception, *Ionopsidium acaule* (Brassicaceae), showed abundant expression of *LFY* mRNA and LFY protein in the inflorescence meristem (Shu *et al.*, 2000; Bosch *et al.*, 2008). This exceptional case has been interpreted as indicating a specific role for *LFY* in the evolution of a compressed inflorescence axis in this species, analogous to the lack of internode elongation seen in a typical flower (Bosch *et al.*, 2008).

Expression of *LFY* offers the opportunity to evaluate the hypothesis that the cyathium is an inflorescence with partial floral identity. Put simply, if the flower-like attributes of the cyathium (determinate growth, compressed internodes) result from the superimposing of some aspects of floral identity on an inflorescence 'background', *LFY* should be expressed in the cyathium primordium (as well as in the developing flowers). Alternatively, if the flower-like features arose by means other than redeployment of the floral developmental programme, *LFY* expression should occur only in the structures that correspond to the reduced staminate and pistillate flowers, not in the young cyathium primordium itself.

## Materials and methods

Observations were made of cyathial development in *Euphorbia milli* (UW-Madison, s.n.), *E. myrsinites*, Royal Botanic Gardens, Kew (K) 1940-16401, *E. nicaeensis*, K 2001-1937, and *Pedilanthus tithymaloides* (UW-Madison, cultivated from Iltis 30229). Scanning electron micrographs were taken from *E. myrsinites*, K 1940-16401.

For scanning electron microscopy (SEM), samples were fixed in FAA (70% ethanol, formaldehyde, and glacial acetic acid, 85:10:5) immediately after collection, then transferred to 70% ethanol prior to dissection. Dissected plant material was dehydrated through an ethanol series to absolute ethanol and critical-point dried using a Tousimis® Autosamdri® 815B-Series A unit. Dried material was mounted onto specimen stubs using nail polish and was coated with platinum using an Emitech K550 sputter coater. SEM examination was performed with a Hitachi cold field emission SEM S-4700. Images were saved as TIFF files, and image processing was done using Adobe Photoshop CS.

For immunolocalization of LFY protein, two anti-LFY antibodies were used: one, here referred to as α-LFY, has been used in previous immunolocalization studies (Sessions *et al.*, 2000; Sliwinski *et al.*, 2007; Bosch *et al.*, 2008). The second antibody, here referred to as 123-LFY, was custom made by Alpha Diagnostic International Inc. (San Antonio, TX, USA) based on three LFY peptides (CRYAKKSGASYINKPKMRHY, CVQTIAKDRGEKCPTKVTNQ, and CEPGEVARGKKNGLDYLFH; with numbers 13528–13530). Cyathia of several *Euphorbia* species of various developmental stages were fixed in 4% paraformaldehyde [solid in 1× phosphate-buffered saline (PBS), pH 7.4] for 4 h under vacuum. The tissue was dehydrated in an ethanol series, moved to Histo-Clear, and embedded in paraffin (Paraplast Plus, Fisher). Embedded tissue was sectioned longitudinally using a rotary microtome (Reichert-Jung 2040; Leica). Sections (8 μm thick) were affixed to poly-lysine microscope slides. Slides were deparaffinized in Histo-Clear, rehydrated in an ethanol series, and treated as follows: 10 min in 20 μg $ml^{-1}$ proteinase K diluted in TE (0.1 M TRIS/0.05 M EDTA), 2× 5 min with PBS, and 30 min with BTX [100 mM TRIS-HCl pH 7.5, 400 mM NaCl, 1% bovine serum albumin (BSA), 0.3% Triton X-100]. Slides were transferred into a humid chamber and treated as follows: 3 h incubation in blocking solution (10% goat serum in BTX), 12 h incubation in a 1:300 dilution of anti-LFY antibody at 25 °C, 3× 15 min rinses in BTX, 60 min incubation in a 1:1500 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Promega), 3× 15 min rinses in BTX, and a 20 min incubation in detection buffer (100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$).

NBT/BCIP stock solution (Roche Diagnostics) was diluted in detection buffer (NBT, 0.15 mg $ml^{-1}$; BCIP, 0.075 mg $ml^{-1}$). Staining times varied between 30 min and 135 min. Staining was stopped with 1× TE, after which samples were dehydrated in an ethanol series, incubated in Histo-Clear, and mounted with DPX mounting medium. Sections were imaged using a Leitz Diaplan photomicroscope fitted with a Leica DC500 digital camera, using differential interference contrast for improved contrast.

## Results

Qualitatively similar results were obtained using both the α-LFY and 123-LFY antibodies. Since the α-LFY antibody has been validated previously (e.g. Sessions *et al.*, 2000; Bosch *et al.*, 2008), the correspondence between the patterns obtained with the two antibodies suggests that 123-LFY is also specific to LFY, as expected given the conservation of the three peptides upon which it was designed.

LFY protein was localized in three distinct sites, representing different stages of cyathium development (Fig. 1A, D, H), and also in other tissues as follows:

(i)Strong expression was detected in the primordium of the entire cyathium. This is the earliest recognizable developmental stage of the cyathium and represents the first undifferentiated bulge of cells on which the involucre, and male and female flowers later develop (Fig. 1B, E, I).

(ii)LFY expression appeared to be concentrated in the first set of primordia formed on the flanks of the cyathium primordium (asterisk in Fig. 1C, arrowheads in Fig. 1E). These primordia, which arise in a spiral pattern (Fig. 1A), are usually interpreted as male inflorescence primordia (Prenner and Rudall, 2007). They are subtended by bracts and later give rise to male flowers in a characteristic zigzag pattern (Prenner and Rudall, 2007).

(iii)LFY expression was also concentrated in individual male and female flower primordia and in young male and female flowers (Fig. 1D, F–I). Furthermore, distinct foci of expression were observed in young ovules, the base of the young gynoecium, and in young nectaries (Fig. 1I).

In contrast to expression in reproductive structures, LFY protein appeared to be absent or only weakly expressed at the base of the cyathium, in its subtending leaves, and in the involucre (i.e. the cup-shaped structure that is composed of the fused bracts of male inflorescences) (Fig. 1F, G, I).

## Discussion

A genetic overlap between flowers and inflorescences in many angiosperm taxa is indicated by a blurred inflorescence–flower

boundary, notably within the monocot order Pandanales (Rudall and Bateman, 2006, 2010). A similar lack of distinct floral units appears to occur in fasciated reproductive units, such as those of a species of Araliaceae, *Tupidanthus calyptratus* (Sokoloff *et al.*, 2007), or in flower-like terminal structures, such as those found in racemose inflorescences of a phylogenetically broad range of angiosperms (Sokoloff *et al.*, 2006). The present results suggest that blending of floral and inflorescence identity could also result in complex structures such as the cyathium of *Euphorbia*.

The present observations of LFY protein localization in putative male and female flower primordia of *Euphorbia* is consistent with the hypothesis that the cyathium evolved from a reduced thyrsoid inflorescence with a central female flower surrounded by 4–5 cymose bundles of male flowers (Prenner and Rudall, 2007; Prenner *et al.*, 2008*b*). However, additional localization of LFY protein in the primordium of the entire cyathium suggests that while the cyathium could be an inflorescence composed of multiple reduced flowers, it also has a degree of floral identity, as first hypothesized by Corner (1958). Future work would benefit from examination of LFY expression patterns in the closest relatives of *Euphorbia* (*Anthostema*, *Calycopeplus*, *Dichostemma*, and *Neoguillauminia*), as well as in Euphorbiaceae with non-cyathial inflorescences. Other tests of floral identity can also be conceived. One of the authors (NIC) is currently examining whether the zygomorphic cyathium of the *Pedilanthus* clade of *Euphorbia* (Cacho *et al.*, 2010) is caused by asymmetric expression of TCP-like genes, as in true flowers with zygomorphic symmetry (e.g. Coen *et al.*, 1995; Busch and Zachgo, 2007; Preston and Hileman, 2009).

LFY expression in the cyathial primordium is interpreted as indicating that this structure has some degree of floral identity. An alternative explanation is that the difference in expression between *Arabidopsis* and *Euphorbia* results from their different inflorescence morphology: indeterminate (racemose) in *Arabidopsis* but determinate (cymose) in *Euphorbia* (cf. Prenner *et al.*, 2009). Under this view, expression in the cyathial primordium is associated with the (reduced) terminal, pistillate flower that will eventually be produced. However, the fact that cyathial LFY expression occurs as soon as the cyathium begins to form demonstrates that LFY protein is present throughout cyathium development and is capable of influencing its development.

Sliwinski *et al.* (2007) activated *LFY* in established inflorescence meristems in *Arabidopsis*, resulting in the production of condensed multiflowered structures that could be interpreted as pseudanthia. To interpret a similar result, Baum and Donoghue (2002) invoked Corner's (1958) concept of 'transference of function', which refers to situations where physiological functions are transferred from one structure (e.g. a single flower) to another (e.g. an inflorescence). Localization of the LFY protein in both the cyathial primordium and individual male inflorescence primordia could be interpreted as such heterotopy, because a genetic programme that was formerly expressed in the ancestral location (i.e. the flower primordium) is now additionally expressed in a derived location (i.e. the primordium of the flower-like cyathium and the primordium of the male partial inflorescences).

In grasses, Bomblies *et al.* (2003) found expression of the maize *LFY* homologue (*ZFL*; Zea *FLO*/*LFY*) in initials of both single spikelets and spikelet pairs (see also Bomblies and Doebley, 2005, 2006). The *ZFL* gene was also expressed in single florets, early glumes, stamens, and lodicules, but a distinct *ZFL*-free zone was present at the tip of the entire inflorescence. Rao *et al.* (2008) found a similar pattern in rice, in which the *LFY* homologue *RFL* (Rice *FLO*/*LFY*) is expressed in both young spikelet meristems and the very young panicle apex. Furthermore, Rao *et al.* (2008) demonstrated *RFL* expression in vegetative axillary meristems. These broad patterns of *RFL* and *ZFL* expression resemble the present results for *Euphorbia* s.l., an interesting comparison because the grass spikelet can also be interpreted as a pseudanthium.

Another example of *LFY* expression at different hierarchical levels within the same plant (individual organs and primordia of reproductive units) was reported in Hydatellaceae (Rudall *et al.*, 2009). In this early-divergent angiosperm family, the reproductive units can be interpreted as either single flowers or strongly condensed inflorescences with highly reduced male and female flowers (see also Rudall *et al.*, 2007).

Ma *et al.* (2008) studied expression of *DFL*, the *LFY* homologue in *Dendranthema lavandulifolium* (Asteraceae). *Dendranthema* possesses typical asteraceous pseudanthial inflorescences with radially symmetric disc florets surrounded by monosymmetric ray florets. *DFL* is expressed in the vegetative shoot apical meristem, in both the inflorescence primordium and individual floral primordia, and also in bract primordia and at lower concentrations in petal primordia.

Overall, these data on a limited but phylogenetically broad range of angiosperms indicate a common pattern in which *LFY* is expressed in the putative inflorescence meristem of multiple independently evolved pseudanthia. The pattern suggests that expression of *LFY* homologues in inflorescences occurs whenever selection favours the production of flower-like properties at the level of the inflorescence. These results also demonstrate that *LFY* expression alone is insufficient to distinguish between an inflorescence and a flower, especially in cases where key architectural markers such as flower-subtending bracts and prophylls are absent.

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