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SHORT COMMUNICATION

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Altered expression of *GFLO*, the Gesneriaceae homologue of *FLORICAULA/LEAFY*, is associated with the transition to bulbil formation in *Titanotrichum oldhamii*

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Abstract *Titanotrichum oldhamii* inflorescences switch from flower to bulbil production at the end of the flowering season. The structure of the bulbiliferous shoots resembles the abnormal meristematic organization of the *Antirrhinum* mutant, *floricaula*. Gesneriaceae-*FLORICAULA* (*GFLO*) is thus a candidate gene in the regulation of bulbil formation. To investigate this hypothesis, part of the *GFLO* gene (between the second and third exon) was isolated using degenerate primers designed in regions conserved between *Antirrhinum*, *Nicotiana* and *Arabidopsis*, followed by genome walking to obtain the complete gene and flanking sequences. RT-PCR results showed that the *GFLO* homologue is strongly expressed in inflorescence apical meristems and young flowers. However, in meristems that had switched to bulbil formation, *GFLO* transcription was greatly reduced. The down-regulation of *GFLO* in bulbil primordia indicates that this gene is connected to, or part of, the bulbil-flower regulatory pathway. Phylogenetic analysis confirms the orthology of *GFLO* and *FLO*, and indicates that the gene may be useful for phylogenetic reconstruction at the genus or family level.

Keywords Bulbil · Gemma · Evolution of development · Floral induction · *GFLO* expression

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Introduction

The induction of flowering is one of the most important developmental transitions for sexually reproducing angiosperms. When plants enter the floral transition stage, shoot apices switch from vegetative to reproductive mode, often in response to environmental or endogenous signals (Simpson et al. 1999).

However, in *Titanotrichum*, a reversal of this transition, from flowering to bulbiliferous meristems, occurs at the end of the flowering season (Fig. 1). When plants are exposed to long-day (LD) conditions or in summer flowers are initiated, while under short-day (SD) conditions or in autumn bulbil primordia are formed, replacing all "floral" meristems at the top of the inflorescence. In addition, numerous new "bulbiliferous shoots" (newly formed shoots containing only bulbils) are initiated within the inflorescence (Wang and Cronk 2003). These bulbil shoots initiate in the axils of most bracts, and the existing inflorescence starts to branch vigorously. Usually, bulbils develop in clusters of 50–60 at each node of the inflorescence in the place of flowers. These clusters are reminiscent of a compressed lateral branch. Tens of thousands of V-shaped bulbils can thus be produced from a single plant. When bulbils start forming, pollinated flowers in the lower part of an existing inflorescence can still form viable seeds, but the seed set is generally low. A characteristic fertilization failure occurs in any residual flowers at the top of the inflorescence, possibly due to resource competition between bulbil and ovule development (Wang and Cronk 2003).

The transformation from floral meristems to bulbil primordia is an occasional phenomenon in flowering

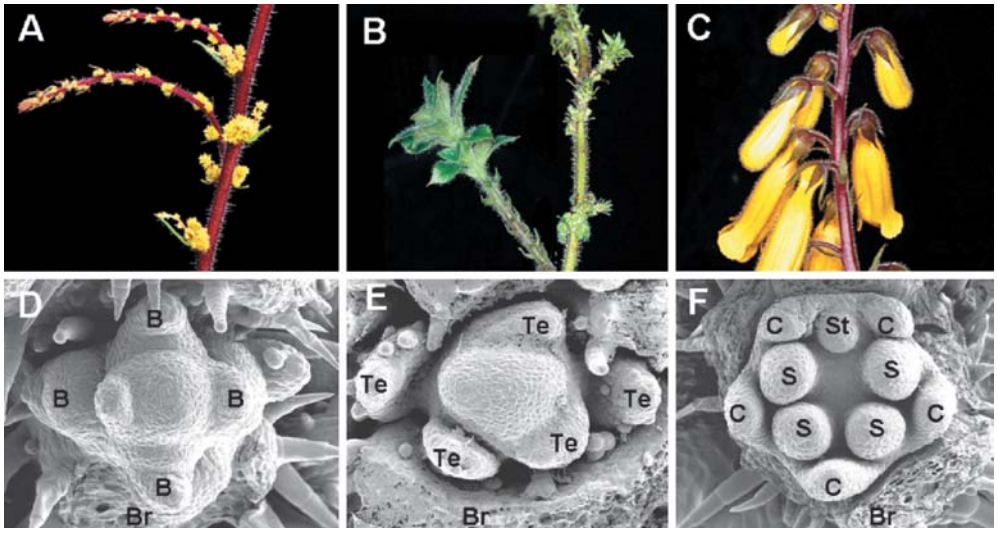

Fig. 1 Inflorescence transition in *Titanotrichum oldhamii* (**A**–**C**) and SEM pictures of the equivalent primordia shown below (**D**–**F**), after Wang and Cronk (2003). The bulbil shoot (**A**, **D**), or bracteose branching (**B**, **E**) develop from a floral inflorescence (**C**, **F**). In a low-light environment, bulbil primordia are generated in the axil of the bulbil shoot (**D**). Occasionally, a multi-bracteole unit is formed at the axil of inflorescence node (**E**). *B* Bulbil primordia, *Br* bract, *C* petals, *S* stamen, *St* staminode, *Te* bracteoles

plants (e.g. *Polygonum viviparum* and *Mimulus gemmiparus*; Diggle 1997; Moody et al. 1999) perhaps based on a common genetic mechanism in these plants. Bulbil production in such plants is often related to environmental conditions, as well as intrinsic factors such as position on the inflorescence (Diggle 1997). The inflorescence of *Titanotrichum* is an indeterminate raceme, as in *Antirrhinum* and *Arabidopsis*, and the shoot apical meristem (SAM) continues growing, with flowers formed in a spiral phyllotaxy until the apex eventually senesces. When shoot apices of inflorescences are removed, even in individuals growing under LD conditions, bulbil formation commences from all axillary meristems immediately after the physical manipulation of the SAM (Wang and Cronk 2003). Therefore, not only environmental conditions but also hormone regulation may be involved in bulbil initiation.

In *Antirrhinum*, the *floricaula* mutant (*flo*) results in the transformation of flowers into indeterminate axillary inflorescence shoots bearing a spiral of single bracts (Coen et al. 1990). A similar phenotype occurs in the *Arabidopsis* mutant *leafy* (*lfy*), in which most of the flowers are replaced by sepal-like structures bearing trichomes (Weigel et al. 1992). The bulbiliferous inflorescence and "bracteose branching" phenotype (an intermediate state between flowering and bulbiliferous inflorescence) in *Titanotrichum* are reminiscent of these mutant phenotypes (see Wang and Cronk 2003). Since the initiation of bulbils in *Titanotrichum* is day-length sensitive and involves the conversion of floral primordia into vegetative structures with no floral organ formation, the *FLO/LFY* homologue in *Titanotrichum* is a possible candidate gene for the regulation of the meristem transition.

Here we investigate the hypothesis that the Gesneriaceae *FLO/LFY* homologue (*GFLO*) is involved in the regulation of bulbil formation and describe the isolation of the *FLO/LFY* homologue from *Titanotrichum*. To test whether *GFLO* is expressed, an RNA transcript RT-PCR analysis was performed to check the expression pattern among stages of bulbil and flower development in *Titanotrichum*. The sequence of *GFLO* was also compared to several available *FLO/LFY* sequences from GenBank to investigate *GFLO* evolution. We hope this study may provide potential lines of investigation for further studies on the mechanism of bulbil development in plants.

Materials and methods

Primer design

Part of *GFLO* (from the 3′ end of second exon to the middle of the third exon) was amplified with a pair of highly degenerate primers designed from several *FLO/LFY* homologues across angiosperms (M. Fröhlich, Natural History Museum, London, personal communication). The amplified products were then extensively cloned (to saturation) into vectors and more than 20 clones were sequenced according to procedures recommended by the manufacturer for the QIAGEN PCR CloningPlus kit and QIAGEN Spin Miniprep kit (Qiagen, Dorking, United Kingdom). To extend into the first, second and third exon regions of *GFLO*, we designed two pairs of degenerate primers using published sequences of *FLO* (GenBank no. M55525), *NFL1* (AH006598), *NFL2* (AH006599), *ALF* (AF030171) and *LFY* (M91208), and our first *GFLO* sequence fragment. The region between the end of exon 2 and the end of exon 1, including intron 1, was amplified using our newly designed primers LFY-F1 (forward: 5′-GCYCTTGAYGCTCTYTCHCAAGAA-3′) and LFY-Y1R (reverse: 5′-CTTRGYKGGRCATTTYTCRCC-3′). The region between the beginning of exon 3 and the 3′ end of the exon 3 was amplified using the newly designed primers LFY-WZF1 (forward: 5′ CCARGTGTTYAGRTACGCRAAG-

AA-3′) and LFY-Z1R (reverse: 5′-GRAGCYTGGTGGGSACAT-ACCA-3′). PCR profiles for the above two sets of primers started with an initial denaturing step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 3 min, and was terminated by a 10-min final extension step at 72°C. With the exception of the primers (MWG-Biotech, Milton Keynes, United Kingdom), all PCR reagents, including *Taq* polymerase, were obtained from Bioline (Bioline, London, United Kingdom). The PCR products were cloned into vectors and sequenced.

Genome walking

The genome walking protocol used (G. Ingram and K. Coenen, University of Edinburgh, personal communication) was adapted from the original of Silbert et al. (1995). First, 2.5 μg genomic DNA was digested with 6-bp blunt-end cutting restriction enzymes (e.g. *Eco*RV, *Pvu*II, *Pml*I and *Sma*I) in a 100-μl reaction according to the manufacturer's protocol (NEB Biolab, Hertfordshire, United Kingdom). Cleaned digests were ligated to an adapter (Silbert et al. 1995) using T4 DNA ligase at 16°C overnight (NEB Biolab, Hertfordshire, United Kingdom). Of the adapter-ligated DNA, 0.13–0.25 μg in 50-μl reactions was used to get the upstream and downstream flanking region of *GFLO* through nested PCRs; the first round of PCR utilized a newly designed gene-specific primer (LFY-WAG1: 5′-TTCCTGCTGCCTCCTTCTCTTGTTG-3′) and an adapter primer designed by G. Ingram at the University of Edinburgh (AP1: 5′-GGATCCTAATACGACTCACTATAGGGC-3′). A "step-down" PCR-based technique was applied to amplify desired products from the DNA fragment pool (Zhang and Gurr 2000). The second round of PCR also utilized a newly designed gene-specific primer (LFY-WAG2: 5′-CTAACCTTCTTGAGA-GAGAGCATCA-3′) and another adapter primer (AP2: 5′-AA-TAGGGCTCGAGCGGC-3′) using the first PCR product as template (1 in 50 dilution). The PCR conditions were identical to the first round except the temperature of annealing and extension started at 65°C. These products were then cloned and sequenced as before.

GFLO expression with RT-PCR

RNA extractions from tissue of inflorescence, bulbils and young leaves of *Titanotrichum* were performed using a QIAGEN RNeasy mini kit and cDNA was synthesized using a QIAGEN Ominiscript RT kit according to the manufacturer's recommendations. The resulting cDNA pools from these different tissues were then individually amplified using the LFY-F1 and LFY-Y1R primer pair. Since this primer pair spans the first intron, RT-PCR products are easily distinguished from genomic contamination. As a control, a 550-bp PCR fragment of the actin gene was amplified as positive control using a set of primers (forward: 5′-GCGATAATG-GAACTGGAATGG-3′ and reverse 5′-GACCTCACTGACTAC-CTTATG-3′ ;K. Coenen, personal communication). To check for over-saturated PCR products across different tissues, during the RT-PCR amplification (25-μl reaction), small aliquots of the PCR products were examined after 20, 25, and 30 cycles of amplification to determine the optimal cycles for comparison. The PCR products were separated on a 1% agarose gel and visualized with ethidium bromide staining (0.1 μg/ml). The gel intensity was calibrated with a quantitative DNA ladder using a Gene Genius image analysis system quantitatively (Syngene, Cambridge, United Kingdom) to estimate the level of gene expression.

GFLO and *FLO/LFY* evolution

In order to investigate the homology of *GFLO*, *Titanotrichum GFLO* nucleotides of all exons were aligned with several asterid FLO/LFY sequences. Phylogenetic trees were reconstructed using PAUP 4.0b8. Since the data matrix (12 taxa) is not extensive, we performed exhaustive searches to obtain the most parsimonious trees. To further evaluate the evolution of *Titanotrichum GFLO*, we amplified *GFLO* second exon sequences from two other Gesneriaceae species, *Besleria labiosa* (GenBank: AY523620) and *Streptocarpus rexii* (GenBank: AY523621).

Results and discussion

Sequence of *Titanotrichum GFLO*

The complete *FLO/LFY* homologue (together with 5′ and 3′ flanking region) was isolated from *Titanotrichum.* The gene (exons and introns) comprises a total of 1,687 bp (GenBank: AY523619). The degenerate primers used were capable of amplifying *FLO* and *LFY* in *Antirrhinum* and *Arabidopsis* (data not shown), and should thus amplify all *FLO/LFY* like genes present in Gesneriaceae. Extensive PCR and cloning (more than 30 clones of PCR products were examined) failed to detect any paralogous sequences, indicating that the gene is likely to be single copy in *Titanotrichum*. The *FLO/LFY* gene consists of three exons and two variable-length introns. The gene is also characterized by a proline-rich region near its 5′ N terminal and a highly acidic region in the second exon (Coen et al. 1990). Like *FLO/LFY*, *GFLO* consists of three exons and two introns, a proline-rich region in exon 1 and an acidic region in exon 2. Intron 1 and intron 2 were AT-rich. *GFLO* encodes a putative protein of 393 amino acids. The amino acid sequence in *GFLO* shows greatest similarities to *FLO* in *Antirrhinum* (90%; Fig. 2). Similarity to *NLF1* and *NLF2* was 87%, and to *LFY* 77%.

GFLO expression is linked to the bulbil development pathway

The RT-PCR results showed that *Titanotrichum GFLO* is expressed in inflorescence and bulbil primordia but not in vegetative tissues, such as leaves (Fig. 3). The expression is highest in young inflorescences (i.e. floral inflorescences) and unopened flowers, but is gradually down-regulated in bulbil primordia and young bulbils. As the RT-PCR was repeated three times with different RNA extractions with the same result, the down-regulation of *GFLO* is therefore considered to be a consistent feature of bulbiliferous shoots.

From our RT-PCR results, it was clear that *Titanotrichum GFLO* expression changes at the transition from flower to bulbil initiation and development (Fig. 3). This raises the question of whether *GFLO* might be a regulator of the transition or whether *GFLO* down-regulation is simply a downstream consequence of the transition. *FLO/LFY* homologues have been reported with various functions (e.g. *Impatiens ImpFLO* is expressed both in reproductive parts and in leaves after floral reversion; Pouteau et al. 1997). However, *Titanotrichum* is phylogenetically related to *Antirrhinum* in having a similar inflorescence morphology and *GFLO* is closely related to

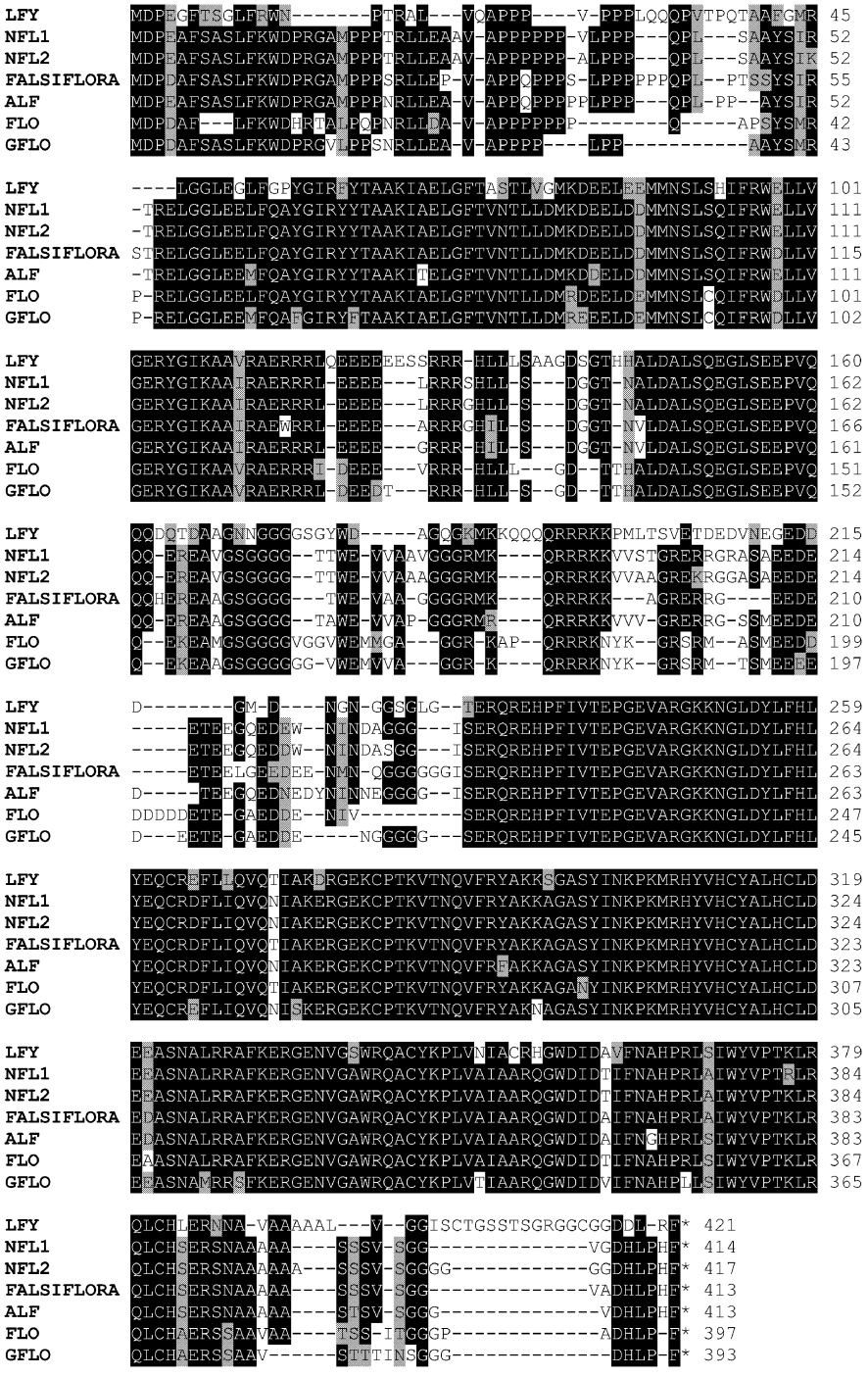

Fig. 2 Aligned amino acid sequences showing the relationship between *Titanotrichum GFLO* (Gesneriaceae-*FLORICAULA*) and sequences from tobacco (*NFL1* and *NFL2*), *Petunia* (*ALF*), *Antirrhinum* (*FLO*) and *Arabidopsis* (*LFY*). Regions of sequence conservation are marked in *black*, while regions of similar residues are marked in *gray*. The *GFLO* sequence shows most similarity to *FLO*

FLO. Thus the *GFLO* function is likely to be similar to that in *Antirrhinum*, where loss of function leads to inflorescence branching (i.e. vegetative meristem function). Given that, it is possible that determination of floral fate in *Titanotrichum* is promoted by *GFLO* expression and that *GFLO* down-regulation may release inflorescence meristems for vegetative growth. A similar response was observed in rice where the *FLO/LFY* homologue (*RFL*; Kyozuka et al. 1998) was found to be down-regulated in panicle branch initiation. *RFL* was shown to control panicle branch initiation but had little effect on floral development (Kyozuka et al. 1998).

LFY has been found to be involved in two of three major flower induction pathways, the day-length-dependent and day-length-independent processes, but not in the autonomous/vernalization process (Blázquez and Weigel 2000; Devlin and Kay 2000). Transformation of the flowering inflorescence to bulbilifery in *Titanotrichum* is promoted by short days and by excision of shoot apices (Wang and Cronk 2003). *LEAFY* is known to integrate day-length and endogenous (gibberellin) signals in the

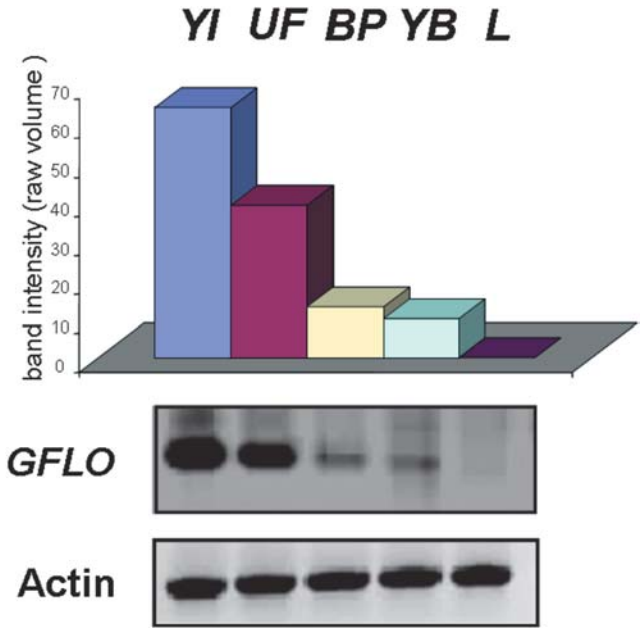

Fig. 3 Expression of *Titanotrichum GFLO* during inflorescence and bulbil development. RNA transcripts (*gel*) from different stages of young inflorescences (*YI*), unopened flowers (*UF*), bulbil primordial (*BP*), young bulbils (*YB*), and leaves (*L*), and estimated amount of transcript (*graph*: band intensity converted raw volume). There is a striking down-regulation in *GFLO* transcription associated with the transition from floral to bulbiliferous shoots. The gel image of actin transcripts (control) is shown *below*

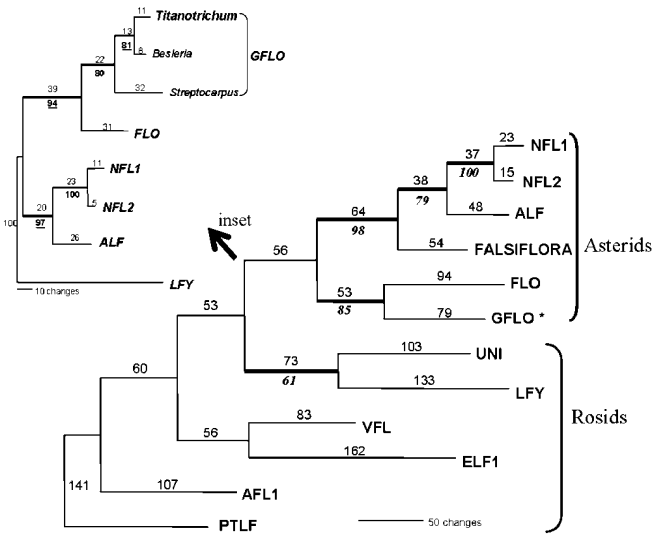

Fig. 4 The reconstructed phylogeny of *GFLO* and *FLO/LFY* evolution. The single most parsimonious tree based on DNA sequences (all exons) of *Titanotrichum GFLO* and *FLO/LFY* homologues shows the close sister relationship between *FLO* and *GFLO*. *FLO* (GenBank no. M55525), *FALSIFLORA* (AF197936), *NFL1* (AH006598), *NFL2* (AH006599), *ALF* (AF030171), *UNI* (AF035163), *LFY* (M912308), *VFL* (AF450278), *ELF1* (AF034806), *AFL1* (AB056158) and *PTLF* (U93196). *Values above branches* are branch lengths, and *numbers below branches* are bootstrap values (10,000 replicates). Branches with high bootstrap support (>60%) are *highlighted*. The *inset* tree shows the single parsimonious tree of the *GFLO* phylogenetic analysis result based on second exon DNA sequences only, rooted on *Arabidopsis*. The three Gesneriaceae *GFLO* sequences (*Streptocarpus*, *Besleria* and *Titanotrichum*) group closely together and are sister to *FLO*

regulation of the transition to flowering (Blázquez and Weigel 2000). The homologue *GFLO* is thus a possible candidate for integrating environmental and endogenous signals in *Titanotrichum* and determining the transition between floral and bulbiliferous shoots. It would thus be interesting to search for genes negatively regulated by *GFLO*, which, when released in response to *GFLO* down-regulation, might drive the bulbil developmental pathway. Although there is no report available yet about *FLO/LFY* function in other bulbiliferous plants, given the similar environmental trigger of bulbil initiation they share (i.e. short days; *Polygonum viviparum*; Diggle 1997), it would be interesting to examine the function of *FLO/LFY* in other bulbiliferous systems.

GFLO and *FLO/LFY* evolution

The reconstructed *FLO/LFY* DNA phylogeny supports *Titanotrichum GFLO* as the orthologue of *FLO* (Fig. 4). In the single most parsimonious tree obtained (CI =0.60; RI =0.41; tree length =1,532), *FLO* and *GFLO* were placed in their own clade with good branch support (bootstrap value, BS =85%). The four Solanaceae *LFY* homologues (*NFL1*, *NFL2*, *FALSIFLORA* and *ALF*) also grouped together with high branch support (BS =98%). These six homologues formed an Asterid clade, while the Rosid sequences *UNI*, *LFY*, *VFL*, *ELF1*, *AFL1* and *PTLF* formed an outgroup.

At a lower taxonomic level we analyzed the *GFLO* second exon data. The two *GFLO* homologues, *Besleria labiosa GFLO* and *Streptocarpus rexii GFLO*, aligned well with *Titanotrichum GFLO*, with only 5.8% and 14% of pairwise nucleotide differences to *Titanotrichum*, respectively. In the resulting single most parsimonious tree the three *GFLO* homologues grouped together forming a well supported clade, sister to *FLO* (CI =0.82; RI =0.68; tree length =341; BS =89%; inset of Fig. 4).

Although *FLO/LFY* evolution has been investigated in several large studies across green plants (Fröhlich and Meyerowitz 1997; Himi et al. 2001), *FLO/LFY* coding regions have seldom been evaluated at the family level or below. Although more than one copy of *FLO/LFY* has been isolated from some angiosperms (e.g. *NFL1* and *NFL2* in *Nicotiana*), these copies seem to be recent paralogues within genera rather than divergent ones (i.e. *NFL1* and *NFL2* have 97% identity in their coding region at amino acid level, while in apple *AFL1* and *AFL2* have 90% sequence identity). Given that *FLO/LFY* is often single copy in angiosperms (Fröhlich and Parker 2000), it is potentially easier to amplify and sequence compared to other, multi-copy, floral identity genes. *GFLO* sequences are easily alignable with little ambiguity at genus level.

Even in our lower taxonomic level analysis on the second exon data, the proportion of informative sites was high (around 10%, 45 informative characters in a total of 456 characters).

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