Aliso 22, pp. 107–125

PERIANTH DEVELOPMENT IN THE BASAL MONOCOT *TRIGLOCHIN MARITIMA* (JUNCAGINACEAE)

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ABSTRACT

Basal monocots exhibit considerable variation in inflorescence and floral structure. In some cases, such as *Triglochin maritima,* it is not clear whether the lateral and terminal structures of the inflorescence are flowers or pseudanthia, or where the limits between flowers and inflorescence lie. To address these questions, morphological studies were carried out, and the results show that in *T. maritima* both terminal and lateral structures are flowers, not pseudanthia. The terminal flower of *T. maritima* develops from the apical inflorescence meristem, suggesting that the apical meristem identity changes from "inflorescence" to "flower" during inflorescence development. In addition, distal flowers of *T. maritima* are reduced, and there is no distinct flower-subtending bract; instead, the perianth develops unidirectionally, resulting in an abaxial-median bract-like tepal and bilaterally symmetrical flowers, similar to those of other basal monocots, such as *Aponogeton* and *Acorus.* It is possible that the leaf primordium changes its positional homology from "flower-subtending bract" to "tepal." Therefore, in some basal angiosperms with abbreviated development of lateral flowers the demarcation of the flower vs. the inflorescence is ontogenetically ambiguous. In situ hybridization experiments show that a putative ortholog of the B-class gene *APETALA3/DEFICIENS* is expressed in developing stamens and carpels, and may also be expressed in the shoot axis of the very young inflorescence. This expression pattern seems to be consistent with the gradual transition between inflorescence and flower that was observed morphologically.

Key words: *APETALA3,* basal angiosperms, fading borders, gene expression pattern, Juncaginaceae, MADS-box gene, monocots, organ identity, *Triglochin.*

INTRODUCTION

Recent molecular phylogenetic investigations of flowering plants have revealed that the position of monocots remains uncertain; the monocots, Chloranthaceae, and magnoliid clade form a grade between the basalmost clades (Amborellaceae, Nymphaeaceae, and Austrobaileyales) and eudicots (e.g., Qiu et al. 2000; Borsch et al. 2003; Hilu et al. 2003). Within the monocots, the monogeneric Acoraceae, with three or four species, are sister to all other extant monocots in most analyses (Chase et al. 1993, 2000, 2006; Duvall et al. 1993*a*, *b*; Qiu et al. 1993, 2000; Nadot et al. 1995; Savolainen et al. 2000; Soltis et al. 2000; Borsch et al. 2003; Hilu et al. 2003), but occasionally placed within alismatids (Qiu et al. 2001; some trees in Zanis et al. 2002). Acoraceae exhibit little variation in floral morphology (Chen et al. 2002) and are characterized by a single, cone-shaped inflorescence with numerous small flowers in a dense arrangement (spadix), and the inflorescence elevated on a stalk together with a foliar leaf (spathe). The trimerous flowers consist of two whorls of inconspicuous, scale-shaped tepals (the outer median tepal is on the abaxial side of the flower and bract-like), two whorls of stamens with introrse anther dehiscence, and a synascidiate-symplicate trimerous gynoecium that lacks septal nectaries (Buzgo and Endress 2000; Buzgo 2001).

Following Acoraceae, Alismatales are sister to the remaining extant monocots (Fig. 1; Angiosperm Phylogeny Group II [APG II] 2003; Chase et al. 2006). Alismatales consist of four subclades that form a polytomy: (i) Tofieldiaceae, (ii) Najadaceae, Hydrocharitaceae, Butomaceae, Limnocharitaceae, Alismataceae, (iii) Cymodoceaceae, Ruppiaceae, Posidoniaceae, Potamogetonaceae, Zannichelliaceae, Zosteraceae, Juncaginaceae, Lilaeacae, Aponogetonaceae, Scheuchzeriaceae, and (iv) Araceae. Alismatales exhibit an impressive diversity of flower morphology. Within Alismatales, Tofieldiaceae (Fig. 1, i) have open, racemose inflorescences with small to medium-sized flowers with a moderately conspicuous or inconspicuous perianth and a calyculus (an annular collar around the pedicel, possibly corresponding to three congenitally fused bracts; Zomlefer 1997; Remizova and Sokoloff 2003; Remizowa et al. 2006). In some species of Tofieldiaceae the calyculus is close to the perianth, similar to a whorl of sepals. In other species, it is a basally fused whorl of bracts on the pedicel, or it may be adaxially open, representing the flower-subtending bract (Remizova and Sokoloff 2003; Remizowa et al. 2006). The five

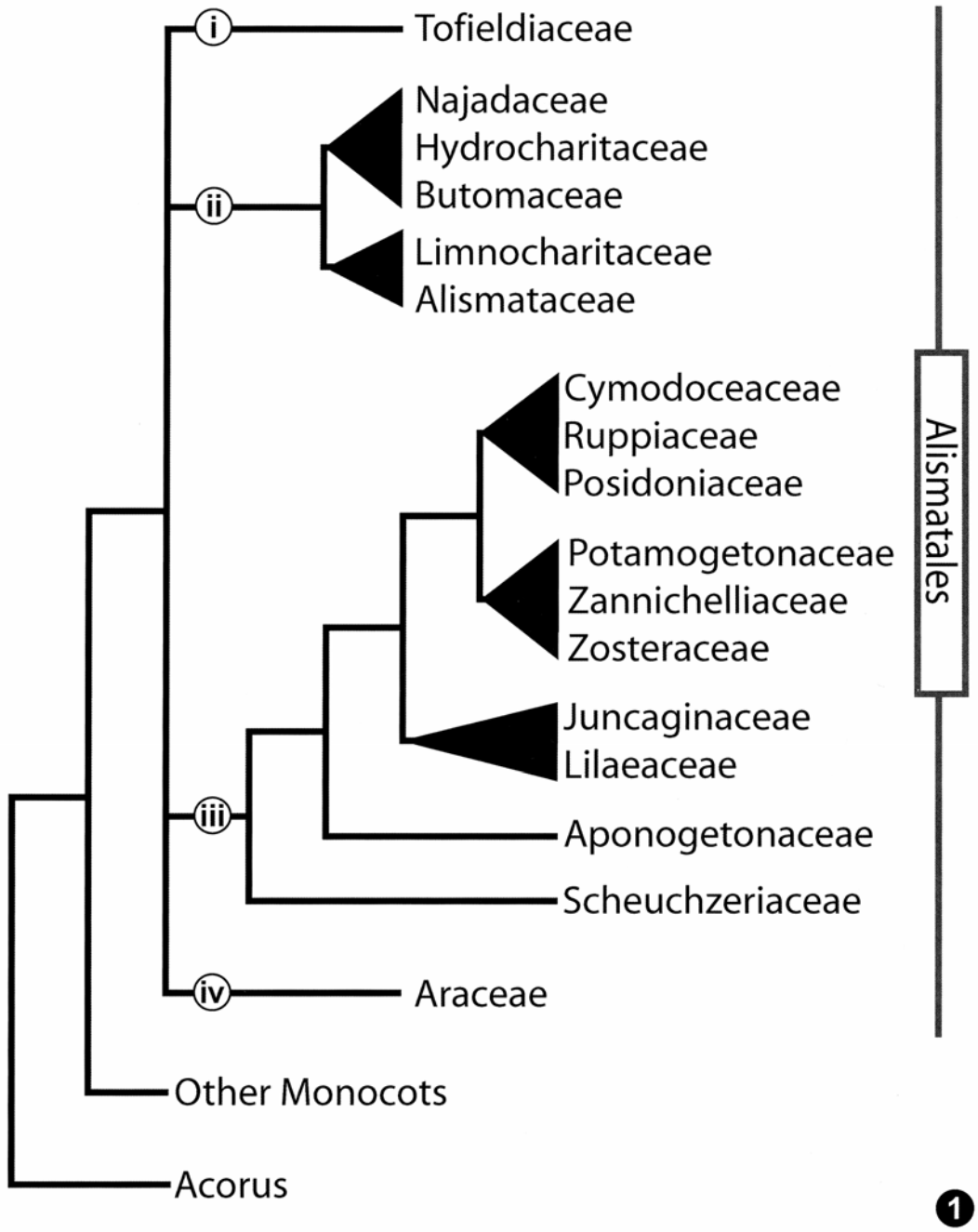

Fig. 1.—Phylogeny of Alismatales composed after Les et al. (1997) (within clades ii and iii) and APG II (2003) (Alismatales and outgroups).

families of clade ii (Fig. 1, ii), Butomaceae, Limnocharitaceae, Alismataceae, and to a lesser extent, Najadaceae and Hydrocharitaceae, generally exhibit expanded inflorescences with pedicellate flowers that are subtended by bracts. In addition, in these taxa the perianth is differentiated into sepals and petals; some produce conspicuous flowers (e.g., *Echinodorus* Rich. ex Engelm., *Sagittaria* L.) (Singh and Sattler 1972, 1973, 1974, 1977*a*; Charlton 1968, 1973, 1974, 1991, 1999*a*, *b*; Charlton and Ahmed 1973*a*, *b*; Sattler 1973; Sattler and Singh 1978; Erbar and Leins 1994; Haynes and Holm-Nielsen 1994; Cook 1995*a*, *b*, 1998*a*, *b*; Haynes et al. 1998*a*).

Scheuchzeria L. (Scheuchzeriaceae, Fig. 1, iii), the sister to other members of clade iii in Alismatales (Les et al. 1997; Chase et al. 2006), also possesses expanded inflorescences, but flowers are few, small, and inconspicuous with small perianth organs. In *Scheuchzeria,* flower-subtending leaves are the main protective organ for the flower (Uhl 1947; Posluszny 1983; Haynes et al. 1998*c*; Gupta et al. 1998). The inflorescence morphology in the remaining members of

clade iii (Aponogetonaceae, Juncaginaceae, Lilaeaceae [now included in Juncaginaceae, APG II 2003], Potamogetonaceae, Zannichelliaceae, Zosteraceae, Cymodoceaceae, Ruppiaceae, Posidoniaceae; Les et al. 1997, Chase et al. 2006) provides a strong contrast to that of the second clade. Typical for this third clade are small, inconspicuous floral units (term including flowers and pseudanthia, definition below) in a dense arrangement, often sessile, sub-sessile, or on a swollen inflorescence axis (spadix) and with a reduced perianth (Eber 1934; Uhl 1947; Hutchinson 1973; Sattler 1965, 1973; Haynes et al. 1998*b*). The reduction of the perianth in members of clade iii sometimes blurs the distinction between the flower-subtending bract and tepals (e.g., in *Aponogeton* L. f., Juncaginaceae, some Potamogetonaceae), similar to *Acorus* L. (Buzgo and Endress 2000) and the calyculus in Tofieldiaceae (Remizova and Sokoloff 2003; Remizowa et al. 2006). In contrast, in some other species of Potamogetonaceae, as well as taxa outside this clade with similarly reduced flowers in dense inflorescences (e.g., Araceae), the flower-subtending bract is not included in the perianth, although the bract may be suppressed. Correlated with this different pattern of floral reduction, flower-like terminal structures are absent from Potamogetonaceae and the fourth clade, Araceae (Fig. 1, iv; Buzgo 2001).

The compact inflorescence and reduced perianth in some members of Alismatales make it difficult to ascertain the identities of particular structures. In their reviews of floral morphology in basal angiosperms, Eames (1961), Claßen-Bockhoff (1990), and Hay and Mabberley (1991) suggested a gradual transition of organ identities in some taxa. Although species of *Triglochin* L. (Juncaginaceae) have been well studied for their floral morphology (Cordemoy 1862; Uhl 1947; Eames 1961; Aston 1973, 1993*a*, *b*; Robb and Ladiges 1981; Ford and Ball 1988; Cooke and Davies 1990; Harden 1993; Endress 1995; Haynes et al. 1998*b*; Igersheim et al. 2001), interpretations of the "floral units" (definition below) are controversial. The "floral units" have been considered to represent either flowers (Hill 1900; Arber 1940; Eckardt 1957; Singh 1973; Serbanescu-Jitariu 1973; Lieu 1979; Charlton 1981; Endress 1995; Igersheim et al. 2001) or pseudanthia (Miki 1937; Uhl 1947; Eames 1961; Burger 1977). The definition of a pseudanthium, however, also differs among authors. According to Rudall and Bateman (2003) it is a structure that is neither a true flower nor a true inflorescence. This differs from the traditional definition of a pseudanthium as an inflorescence that imitates a flower, as a result of the aggregation of flowers (Eames 1961; Claßen-Bockhoff 1990; Endress 1994). This second definition neither implies nor excludes the loss of the distinction of meristem identity between flower and inflorescence. We follow this second, more commonly used terminology. For the structure that resembles a flower (actual flower or pseudanthium), Claßen-Bockhoff (1990) uses the term pollination unit, or blossom, whereas Rudall and Bateman (2003) use "floral unit." In this study we apply floral unit (Rudall and Bateman 2003), which includes flowers and pseudanthia. The term does not imply a function in animal pollination (as *Potamogeton* L. and *Triglochin* are both probably wind-pollinated), although most authors use "flower" in reference to *Triglochin* (Hill 1900; Lieu 1979; Charlton 1981; discussion below).

In angiosperms, lateral shoots (including lateral flowers) typically are subtended by a leaf. The subtending leaf is thereby considered an appendage of the main shoot (Troll 1937; Esau 1977; Hagemann 1963, 1970, 1984). Consequently, the flower-subtending leaf is considered extrafloral. In many species the flower-subtending leaf is reduced to a scale-shaped flower-subtending bract. Bracts and tepals are often similar and difficult to distinguish, especially in basal angiosperms (von Balthazar and Endress 2002; Buzgo et al. 2004*a*, *b*). Many taxa have no visible flower-subtending leaves (e.g., *Arabidopsis* Heynh.), and in these cases, the flower-subtending leaf or bract is not a universal morphological marker for an extrafloral position.

Although most authors do not explicitly differentiate between lateral and terminal floral units in *Triglochin,* they apparently refer to the lateral floral units (Miki 1937; Uhl 1947; Eames 1961; Rudall and Bateman 2003). In this study, we examine these two positions in the inflorescence separately: (i) to determine whether the floral units are flowers or pseudanthia, and (ii) to identify the limits between inflorescence and flower. We hypothesize (Hypothesis 1) that the lateral structures in *Triglochin* are pseudanthia (Miki 1937; Uhl 1947; Eames 1961; Rudall 2003). We predict that the answer is not absolute, but that the transition from inflorescence to flower is gradual. The following hypotheses specify those characteristics of a flower that concern the loss of flower delimitation.

Hypothesis 2: The primordium in the position of the flower-subtending leaf is not always extrafloral, but is sometimes involved in the perianth. The concept of the flower-subtending leaf as a marker for an extrafloral position is challenged by studies of some basal monocots (Burger 1977; Buzgo and Endress 2000; Remizova and Sokoloff 2003; Rudall 2003; Remizowa et al. 2006) and magnoliids (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996). Some taxa possess reduced flowers that develop unidirectionally (from abaxial to adaxial), in which the first organ of a lateral flower is on the abaxial side of the lateral shoot and could therefore be considered either a flower-subtending bract or a first abaxial tepal. Such situations occur in Saururaceae and *Acorus* (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996; Buzgo and Endress 2000). Here we discuss a similar phenomenon in *Triglochin maritima.*

Hypothesis 3: In *Triglochin maritima,* the terminal structure is composed of organs corresponding to several flower primordia, and therefore is a pseudanthium. This hypothesis corresponds to statements regarding (i) floral units in *Triglochin* in general (for lateral flowers; Miki 1937; Uhl 1947; Eames 1961; Rudall 2003) and (ii) terminal flower-like structures (Greek *pelōr* for "monster") in some taxa (Buzgo and Endress 2000; Buzgo 2001; Rudall and Bateman 2003). However, this hypothesis contradicts Miki (1937), Uhl (1947), and Charlton (1981), who considered the inflorescence to be indeterminate. Among basal monocots and magnoliids with dense inflorescences, unidirectional flower development, reduction of the adaxial floral organs, and formation of peloria at the apex of the inflorescence appear to be correlated (Buzgo and Endress 2000; Buzgo 2001). Strong initial floral bilateral symmetry and reduction on the

adaxial side of the flower can result in flowers represented by only a single organ (Burger 1977; Dahlgren et al. 1985; *Lilaea* Bonpl., Arber 1940; Posluszny et al. 1986), and ultimately the formation of a terminal pseudanthium.

Hypothesis 4: Genes that are considered strictly floral are transcribed in the inflorescence axis. That is, genetically, the inflorescence of *T. maritima* has features that are typically exclusive to the flower. The MADS-box orthologs *DEFICIENS* (*DEF*) and *APETALA3* (*AP3*) take part in B-class function, which is responsible for stamen and petal-like features in *Antirrhinum majus* L. and *Arabidopsis thaliana* (L.) Heynh., respectively (Coen and Meyerowitz 1991). *DEF/AP3* orthologs are regulated by genes that also control the induction of floral meristem identity (see Discussion for citations). Therefore, the presence of B-class mRNA is strong evidence for floral identity. Further, floral MADS-box genes have been intensively studied, offering a large literature for comparison of sequences and mRNA localization profiles. The MADS-domain is well conserved and suitable for screening for genes in a total RNA extraction. The C-terminal sequence is highly variable, which allows easy identification of different members of the MADS family. In addition, the C-terminal sequence can be used to construct RNA probes that are sufficiently specific to target genes exclusive to the *AP3* clade.

MATERIAL AND METHODS

Morphological Studies

Plants of *Triglochin maritima* were collected in March 2001 and January 2002 near Copenhagen, Denmark (Buzgo collection numbers: 1068, 1072, 1073); other taxa were collected at various times and locations (Table 1). Buds of *T. maritima* were removed by dissection and either used for RNA extraction (below) or fixed in FAA, involving a short application of vacuum (about 7 min) until no more bubbles appeared, and incubated for approximately 6 hr at 4°C, then transferred to 70% ethanol (RNase free), and dehydrated along an ethanol series. For scanning electron microscopy (SEM), samples were critical point-dehydrated, gold-sputtered, and observed in a Hitachi S-4000 FE-SEM at the University of Florida Biotechnology Program. For microtome sections, the samples were transferred to xylene and embedded in Paraplast, sectioned using a rotary microtome (10 μm thick), and placed onto Fisherbrand SuperFrost/Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Mounting was in Cytoseal 280 (Richard Allen Scientific, Kalamazoo, Michigan, USA). Observations were made using a Leica MZ12-5 dissection microscope and a Carl Zeiss compound microscope with transmitted light. Photographs were taken with a Nikon Coolpix 995 digital camera. Image editing included linear adjustment of contrast, color-temperature, frame, and resolution, using Adobe Photoshop vers. 7.0.

Isolation and Sequence Analysis of cDNA Clones for AP3 *Homologs*

Total RNA extraction from *T. maritima* was carried out using FastPrep120 (Bio101 Savant, Qbiogene, Irvine, California, USA) tissue homogenizer and the FastRNA Green kit (Bio 101). Total RNA concentration was estimated by 1% agarose gels and by spectrometry with an Eppendorf Bio Photometer. Reverse transcription was conducted using GeneAMP In Situ Core Kit (Perkin-Elmer Applied Biosystems, Wellesley, Massachusetts, USA), adding RNAguard RNase inhibitor (Human Placenta, Amersham Biotech, Piscataway, New Jersey, USA), MLV-M Reverse Transcriptase with Buffer II (Applied Biosystems, Foster City, California, USA), and a $T_{(16)}$-primer with an adapter ($T_{(16)}$-CCGAGAGTCGATCAGCTGC). The polymerase chain reaction (PCR) was carried out with Amplitaq Gold Polymerase (Applied Biosystems) and *Pfu* DNA polymerase (Promega, Madison, Wisconsin, USA), using intron-spanning primers for *AP3* homologs based on alignments of B-class MADS-box genes (Kramer et al. 1998; forward TA232 TGGAAGAACGAGTATGAGACC, *Tr.ma.AP3–191F* ACTGCACCCCAACTACAAATAC; reverse, *Tr.ma.AP3–498R* CTTCCACATTGCGCAGATCG) on a PTC-200 Peltier thermocycler (MJ Research, Waltham, Massachusetts, USA) and on a Robocycler Gradient 40 (Stratagene, La Jolla, California, USA).

The cDNA PCR products were cloned and selected using PCR-Script AMP Cloning Kit (Stratagene) and re-amplified by PCR using primers for *T7* and *T3* promoters in the vector according to the Stratagene PCR-script instruction manual. For full cDNA sequences, SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, California, USA) was employed, with the internal primers and the adapter (above). Sequencing used the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase, FS (Applied Biosystems). Sequence analysis was performed on an ABI 377 Prism DNA Sequencer (Applied Biosystems) with associated software. The sequences were analyzed for continuous open reading frames (GenDoc vers. 2.6). Blast searches (Blastp) were performed against GenBank, and sequences were aligned with genes annotated as MADS-box genes using GenDoc vers. 2.6 and manually. The sequences *Tr.ma.AP3–1* and *Tr.ma.AP3–2* were deposited in GenBank as accession number AY956349 and AY956348, respectively. To reconfirm the sequence homology of our probe templates with *AP3*-annotated amino acids, a preliminary maximum parsimony analysis and a bootstrap analysis were carried out, involving 427 nucleotide sequences representing all major clades of MADS-box genes (sequences from Becker and Theißen 2003, combined with sequences from Johansen et al. 2002), using PAUP* vers. 4.0b10 for 32-bit for Windows (Pentium 4 CPU 2.4 GHz PC, Win XP) and for unix (on a Dual 2 GHz PowerPC G5, OS X) (Swofford 1998). The specifications of the maximum parsimony analyses included simple taxon addition, using tree bisection-reconnection (TBR) branch swapping, and saving 100 most parsimonious trees. The bootstrap analysis included 100 replicates, using TBR branch swapping and saving 100 trees.

In Situ Hybridization

RNA probes were generated from the DNA insert representing the sequence 3′ from position 191 (*Tr.ma.AP3–191F* forward primer) comprising the more specific K and C regions of the *AP3* homolog. PCR-amplified sequences were

inserted into pGEM vector (Riboprobe in vitro Transcription Systems, Promega, Technical Manual No. 016), and inserts of selected clones were sequenced for determination of the insert direction. Clones representing two insert directions were chosen: antisense as probe, and sense as negative control. From each construct, plasmid DNAs were prepared using E.Z.N.A. EaZy Nucleic Acid Isolation-Kit (Omega Biotek, Inc., Doraville, Georgia, USA). Plasmid DNAs were digested with *Hind* III (Promega), purified by phenol-chloroform extraction and a sodium-acetate ethanol precipitation, and checked on 1% agarose gel. Probe synthesis was by Riboprobe System-*SP6* (Promega) transcription kit, including Böhringer-Mannheim DIG RNA Labeling Mix (Roche Applied Science, Indianapolis, Indiana, USA), followed by a DNase digest (Promega), according to the transcription kit protocol. Hydrolysis of the RNA probe was in a mix of Na_2CO_3 (60 mM) and $NaHCO_3$ (40 mM), fragment length was evaluated on agarose gel, and the hydrolysate was precipitated in ethanol containing sodium-acetate, tRNA, and dithiothreitol (DTT).

Hybridization followed a modification of the protocol of the Meyerowitz lab (*http://www.its.caltech.edu/~plantlab/protocols/insitu.html* [Jan 2005]). Microtome slides with sections of *T. maritima* were deparaffinized and hydrated in a xylene-ethanol series, followed by a digest with Proteinase K (Promega), and an acetylation reaction. Hybridization was at 55°C. For background suppression, slides were incubated in RNAse A (Sigma-Aldrich, St. Louis, Missouri, USA, not boiled), then washed twice in 0.2× SSC in a gyratory agitator for one hour at 55°C, and pre-blocked in phosphate buffered saline buffer (PBS) with 1% BSA-c (New England BioLabs, Inc., Beverly, Massachusetts, USA, purified BSA #B90015). Sections were incubated with Böhringer-Mannheim Anti-Digoxigenin Fab Fragment solution according to the manufacturer's instructions. Signal detection was by alkaline phosphatase reaction with NBT/BCIP Tablets (Roche) according to the manufacturer's instructions. The signal was monitored in the dissection microscope and photographed as described above.

RESULTS

Morphological Development

The inflorescence of *Triglochin maritima* is initiated as a large coherent meristem at the apex of the shoot, which becomes conical as flowers are initiated (Fig. 2). The diameter of floral primordia is small in relation to the inflorescence, allowing several primordia to appear at one level around the inflorescence. Floral primordia appear in several parastichies (Fig. 2). Lateral meristems are initiated acropetally in fast succession along the inflorescence, above a short basal peduncle. As the distal diameter of the shoot apical meristem (SAM) of the inflorescence is reduced, the number of floral primordia progressively decreases, whereas the size of primordia at initiation is not reduced significantly. At the time when the inner tepals initiate, the inflorescence becomes deformed between the last foliar leaf of the main shoot and the prophyll of the continuation shoot. The side of the inflorescence facing the continuation shoot is flatter, while the side toward the last foliar leaf maintains its rounded surface. The two sides are separated by two rims longitudinally on the inflorescence, corresponding to the limit where the inflorescence touches the prophyll (Fig. 3).

At initiation, some "floral" primordia first exhibit a slight enlargement of the abaxial side (Fig. 4), but no distinct abaxial organ develops earlier than the rest of the floral meristem (Fig. 5). The abaxial median tepal and lateral outer tepals are initiated almost simultaneously, with a larger fraction of the floral meristem dedicated to the median abaxial tepal. As a result, most flowers develop with a slight bilateral symmetry: the abaxial tepal is slightly larger, the outer tepals do not form an isometric triangle (60°), and instead the lateral tepals slant toward the transverse orientation (Fig. 6). A flower-subtending bract is not initiated (Fig. 5, 6). The size of the inflorescence SAM reduces gradually as floral primordia emerge from it (Fig. 2). Finally, a short lag occurs after which the remainder of the SAM gives rise to a terminal structure. The lateral primordia across the lag abruptly change from flower to single floral organs, and the terminal structure is identical to a flower (below; Fig. 8–10). That is, the terminal flower is the last one to be initiated, and the inflorescence is a determinate raceme (Troll 1964; Weberling 1989).

In the flowers, the three inner tepals develop almost synchronously, followed shortly by two trimerous alternate whorls of stamens (Fig. 6–9). At this stage the outer abaxial tepal grows faster in most flowers and increasingly appears bract-like (Fig. 6). Distally in the inflorescence, the adaxial organs develop to a smaller size, and the position of the lateral outer tepals slants toward the adaxial side. Just below the apical flower, this adaxial inhibition affects even the median inner tepals and stamens; in some flowers these organs are absent (Fig. 8). However, on the two longitudinal rims of the inflorescence that meet in the terminal flower (Fig. 3), most flowers appear radially symmetric with equal outer tepals (Fig. 7, 9). At this stage, the constriction below the first abaxial organ elongates: the pedicel is formed, and the first abaxial organ is clearly identified as a floral organ (tepal).

During organ initiation, the floral center remains prominently convex: cell division at the floral SAM exceeds the formation of organ primordia, and a lateral expansion of the receptacle (below the outer tepals) is not observed (Fig. 6–9; however, it expands above the tepals. When the hemispherical carpel primordia initiate, the floral apex has risen above the stamens (Fig. 6, 7, 10). As a result of this meristem expansion, the carpels are initiated on the slope of the floral SAM and have a tilted base (Fig. 11, 12). The outer carpels alternate with the inner stamens and arise after them following a lag; the inner carpels appear after the outer carpels following a short plastochron (Fig. 6, 7, 11); that is, the plastochron between the two whorls of carpels is shorter than that between the inner stamens and outer carpels. At initiation of the inner carpels, each outer carpel develops a rim around a depression. The rim appears more like a torus than a horseshoe (as is typical for many other Alismatales; e.g., Sattler 1973; Sattler and Singh 1973, 1978), correlating with the meristem expansion of the adaxial carpel side and the elongation of the floral apex (Fig. 12). Within the three inner carpels, the apex of the flower remains plane to slightly convex (Fig. 6, 12).

In later development, the tepals elongate and overlap (Fig. 11, 13). Normally, the abaxial median tepal overlaps all oth-

Table 1. Material examined; vouchers deposited at Z + ZT.

Taxon	Collection voucher number M. Buzgo	Collection Date	Source
Triglochin bulbosa L.	*782*	09 May 1997	Switzerland, Botanic Garden, University of Basel, #2707/95 WP; Italy, Sardinia, Lanusei, Monte Tonneri, 680 m.a.s.l., *Bruno Matter 571*
Tetroncium magellanicum Willd.	*949*	09 Jun 1999	Chile, from the vicinity of Punto Arenas by an expedition "Patagonia '85" from Cambridge University, UK, by Charlton Nov 1985
	950	09 Jun 1999	Chile, from the vicinity of Punto Arenas by an expedition "Patagonia '85" from Cambridge University, UK, by Charlton Nov 1985
Triglochin maritima L.	*781*	09 May 1997	Switzerland, Botanic Garden, University of Basel, #2310/91 WS, *Bruno Matter 580*
	786	19 Apr 1999	Switzerland, Botanic Garden, University of Zurich
	1010	05 Mar 2000	UK, pond 5 m SW from the dike rim, at the dike foot, 20 m SE from the east end of Havengore Bridge, Great Wakering, Southend On Sea, Essex, 0°50′22″E, 51°33′15″N
	1011	17 Mar 2000	UK, Colchester, East Anglia
	1059	05 Jul 2000	UK, Balephetrish, Tiree, Inner Hebrides
	1068	18 Mar 2001	Denmark, Vestamager Kalvebod Faelled, Reservat, Copenhagen, 500 m from entrance, marshland meadow, close to a pond. Denmark, Botanic Garden University, Copenhagen, 70C, Nursery, RNA R10318
	1072	18 Mar 2001	Denmark, Vestamager Kalvebod Faelled, Reservat, Copenhagen, 500 m from entrance, marshland meadow, close to a pond
	1073	14 Jan 2002	Denmark, Vestamager Kalvebod Faelled, Reservat, Copenhagen, 500 m from entrance, marshland meadow, close to a pond, extensive sheep grazing, RNA R20112
Triglochin microtuberosa Aston	*s. n.*	10 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Newfoundland States Forestry, Dicky Creek Rd., Wolli Wolli River Bridge, Yellow Cuttings, chain of little ponds following a temporary creek or ditch, 29°55′45″S, 153°09′29″E, 38 m.a.s.l.
	907	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Yuragir NP, E of Pillar Valley, Collets Crossing Rd., Wanderer Creek, at Musician River, 29°50′11″S, 153°12′07″E, 3 m.a.s.l
	909	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Yuragir NP, E of Pillar Valley, Collets Crossing Rd., Wanderer Creek, at Musician River, 29°50′11″S, 153°12′07″E, 3 m.a.s.l.
	913	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, entrance to Newfoundland States Forestry, Barcoongere Way, Dicky Creek Rd., bog with ponds, 29°58′12″S, 153°10′26″E, 16 m.a.s.l.
	914	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, entrance to Newfoundland States Forestry, Barcoongere Way, Dicky Creek Rd., bog with ponds, 29°58′12″S, 153°10′26″E, 16 m.a.s.l.
	916	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, entrance to Newfoundland States Forestry, Barcoongere Way, Dicky Creek Rd., bog with ponds, 29°58′12″S, 153°10′26″E, 16 m.a.s.l.
	919	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Newfoundland States Forestry, Dicky Creek Rd., Wolli Wolli River Bridge, Yellow Cuttings, chain of little ponds following a temporary creek or ditch, 29°55′45″S, 153°09′29″E, 38 m.a.s.l.
Triglochin multifructa Aston	*908*	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Yuragir NP, E of Pillar Valley, Collets Crossing Rd., Wanderer Creek, at Musician River, 29°50′11″S, 153°12′07″E, 3 m.a.s.l.
	912	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, entrance to Newfoundland States Forestry, Barcoongere Way, Dicky Creek Rd., bog with ponds, 29°58′12°S, 153°10′26″E, 16 m.a.s.l.
	915	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, entrance to Newfoundland States Forestry, Barcoongere Way, Dicky Creek Rd., bog with ponds, 29°58′12″S, 153°10′26″E, 16 m.a.s.l.
	917	11 Oct 1998	Australia, New South Wales, N of Coffs Harbour, Newfoundland States Forestry, Dicky Creek Rd., Wolli Wolli River Bridge, Yellow Cuttings, chain of little ponds following a temporary creek or ditch, 29°55′45″S, 153°09′29″E, 38 m.a.s.l.

Table 1. Continued.

Taxon	Collection voucher number M. Buzgo	Collection Date	Source
Triglochin palustris L.	*808*	31 Aug 1997	Switzerland, Döss da las Plattas, Fuorcla dal Fuorn, ct. Graubünden Grisons, slight N slope of Piz Daint, 10°16′E, 46°38′N, 2260 m.a.s.l.
	982	06 Jan 2000	UK, Royal Botanic Gardens, Kew, aquatic garden, #1969–19260
Triglochin procera R. Br.	*883*	06 Oct 1998	Australia, N New South Wales, Nambucca Heads, Hotel La Hacienda, flat pond of a horse meadow
	1017	02 May 2000	UK, Royal Botanic Gardens, Kew, aquatic garden
	816	23 Mar 1998	Australia, Vanchep, Perth, 10 Jan 1995 by Georgina Reiss
	817	23 Mar 1998	Switzerland, Botanic Garden, University of Zurich; Australia, Vanchep, Perth, 10 Jan 1995 by Georgina Reiss
Triglochin striata Ruiz & Pav.	*815*	23 Mar 1998	Switzerland, Botanic Garden, Zurich; Australia, Capel, Bunbury, Western Australia, 200 km S of Perth by Georgina Reiss
	882	6 Oct 1998	Australia, N New South Wales, Nambucca Heads, Hotel La Hacienda, flat wet meadow at a sea bay
Maundia triglochinoides F. Muell.	*905*	10 Oct 1998	Australia, New South Wales, N of Coffs Harbour, between Coffs Harbour & Pillar Valley, Chaffin Swamp, Tucabia, shady river on meadows below a bridge, 29°40′10″S, 153°07′17″, 7 m.a.s.l.

er organs. However, the outer abaxial tepal can be covered by the lateral tepals, because the inflorescence continues to be deformed as it grows between the continuation shoot and the last foliar leaf (Fig. 13). Along the longitudinal rim, more space is available on the lateral side of each flower than on the median side. This causes the lateral tepals to be lifted away from the flower above the abaxial tepal (Fig. 13), resulting in an asymmetric appearance. However, this is a secondary effect, and the flower is actually bilaterally symmetric. After all organs are initiated, the terminal structure is identical to a "flower"—completely radially symmetrical whorls of tepals, stamens, and carpels. Until anthesis, the terminal structure remains the largest "flower" of all on a prominent base lifted above the adjacent subterminal flowers.

Before anthesis, the inflorescence emerges from the foliar leaf sheaths, by elongation of the basal inflorescence axis (Fig. 14). The internodes between the flowers elongate later, separating the flowers from each other before anthesis (Fig. 15). The flowers are protogynous.

Morphological Studies of Triglochin procera, T. striata, *and* Maundia

For comparison, flowers of *Triglochin procera* (s.l., including *T. multifructa* and *T. microtuberosa*; Fig. 16–19), *T. striata* (Fig. 20–22), and *Maundia triglochinoides* (Juncaginaceae; Fig. 23) were examined. Whereas, *T. maritima* grows best above water level or only temporarily submerged, we found that the rhizome of the Australian *T. procera* complex is almost always submersed. *Triglochin procera* differs from *T. maritima* in having a much more robust growth form, with an inflorescence that can reach more than 1 m in length (instead of 40 cm in *T. maritima*), bearing flowers on its distal 25 cm. Flowers of *T. procera* are correspondingly larger, up to 1 cm in diameter (compared with 3–4 mm in *T. maritima*). The flowers of both *T. procera* and *T. maritima* are trimerous-hexacyclic, but the stigma of *T. procera* is more spreading and star-shaped; additionally, carpels are only basally fused and sometimes twisted. Because of the larger size of flowers of *T. procera,* we expected them to be more radially symmetric than those of *T. maritima.* Indeed, we found fewer indications of flower reduction, though some reduced flowers occur apically. We also had difficulty in distinguishing the terminal flower from lateral flowers. Nevertheless, flowers of *T. procera* also exhibit bilateral symmetry (Fig. 18, 19) and lack a subtending bract. Instead, the outer median tepal is abaxial and slightly prominent (Fig. 19), as in *T. maritima.* The inner median tepal on the adaxial side is smaller than the other inner tepals, but expands above the outer lateral tepals (Fig. 18, 19).

Triglochin striata from Australia was only observed in cultivation. It differs from both *T. procera* and *T. maritima* by being much smaller. The distal portions of the leaves are round in transverse section, and the entire slender inflorescences of *T. striata* reach only 30 cm (Fig. 20), with flowers of about 3 mm in diameter with only one whorl of carpels. Associated with smaller flower size, flower reduction within the inflorescence is much more frequent (we never found all whorls to consist of three organs). Particularly at the base

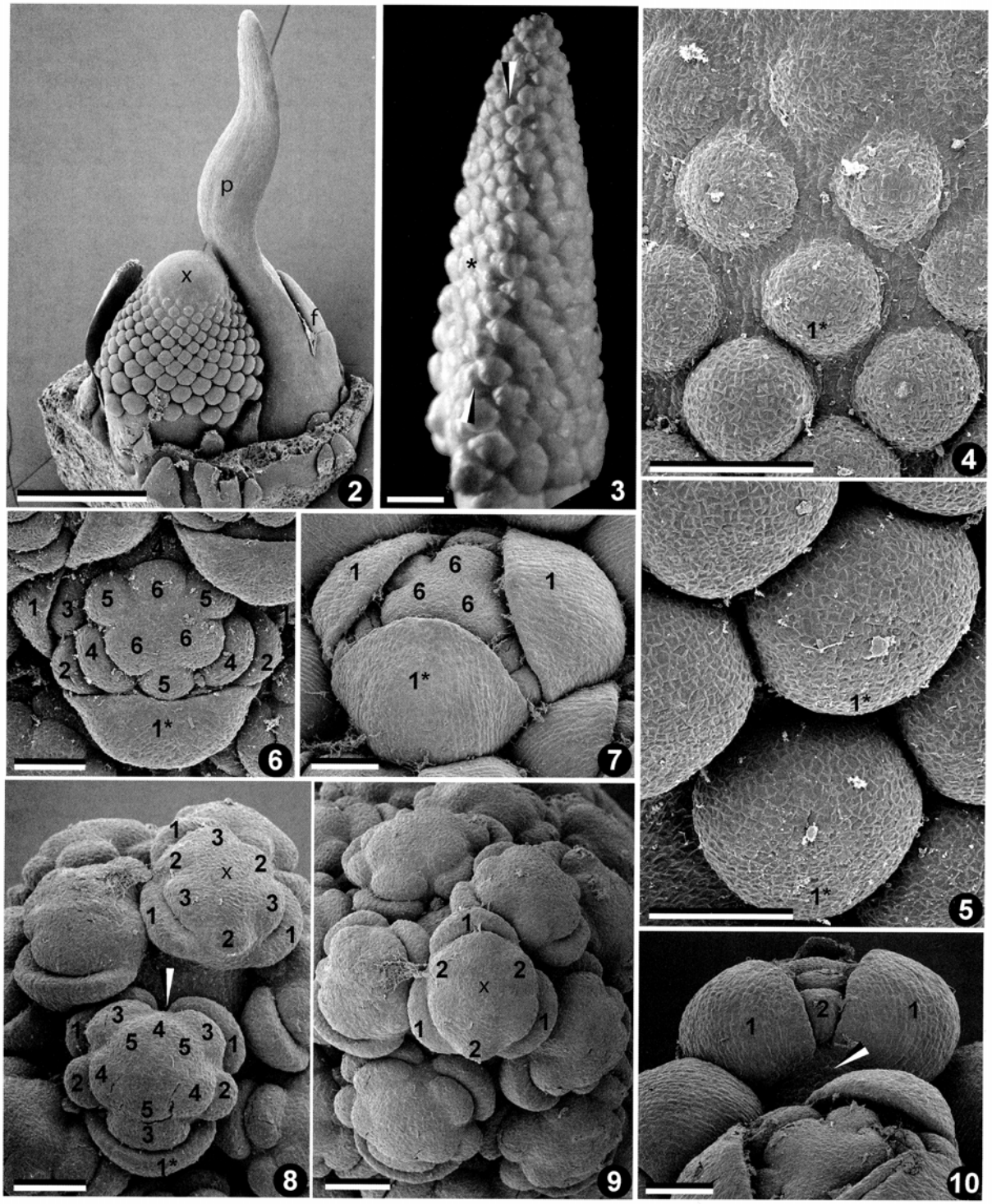

Fig. 2–10.—Early floral development of *Triglochin maritima,* all but Fig. 3 are SEMs.—2. Young inflorescence, side view, initiating lateral flowers, dome-shaped SAM (x), prophyll (p), foliar leaf (f) of the continuation shoot.—3. Young inflorescence, side view showing the side toward the prophyll (*) and the longitudinal rim (arrows).—4. Close up of flower primordia at initiation, apical view, the abaxial side is more pronounced (1*) than the adaxial side, and there is some space between the primordia.—5. Close up of flower primordia after initiation, apical view, the abaxial side (1*) is equal to the adaxial side as compared with Fig. 4, and there is almost no space between the primordia.—6. Young flower along the side of the inflorescence, apical view, outer tepals (1), the outer, abaxial median tepal is larger (1*), whereas inner tepals (2), outer and inner stamens (3, 4), outer and inner carpels (5, 6) all develop equally.—7. Young flower on longitudinal rim of the inflorescence, apical-abaxial view, outer tepals (1) are equal, including the abaxial tepal (1*), the inner carpels (6) are elevated on the flower center.—8. Young flowers, oblique-apical view, apex of terminal flower (x), abaxial median tepal enlarged in lateral flowers

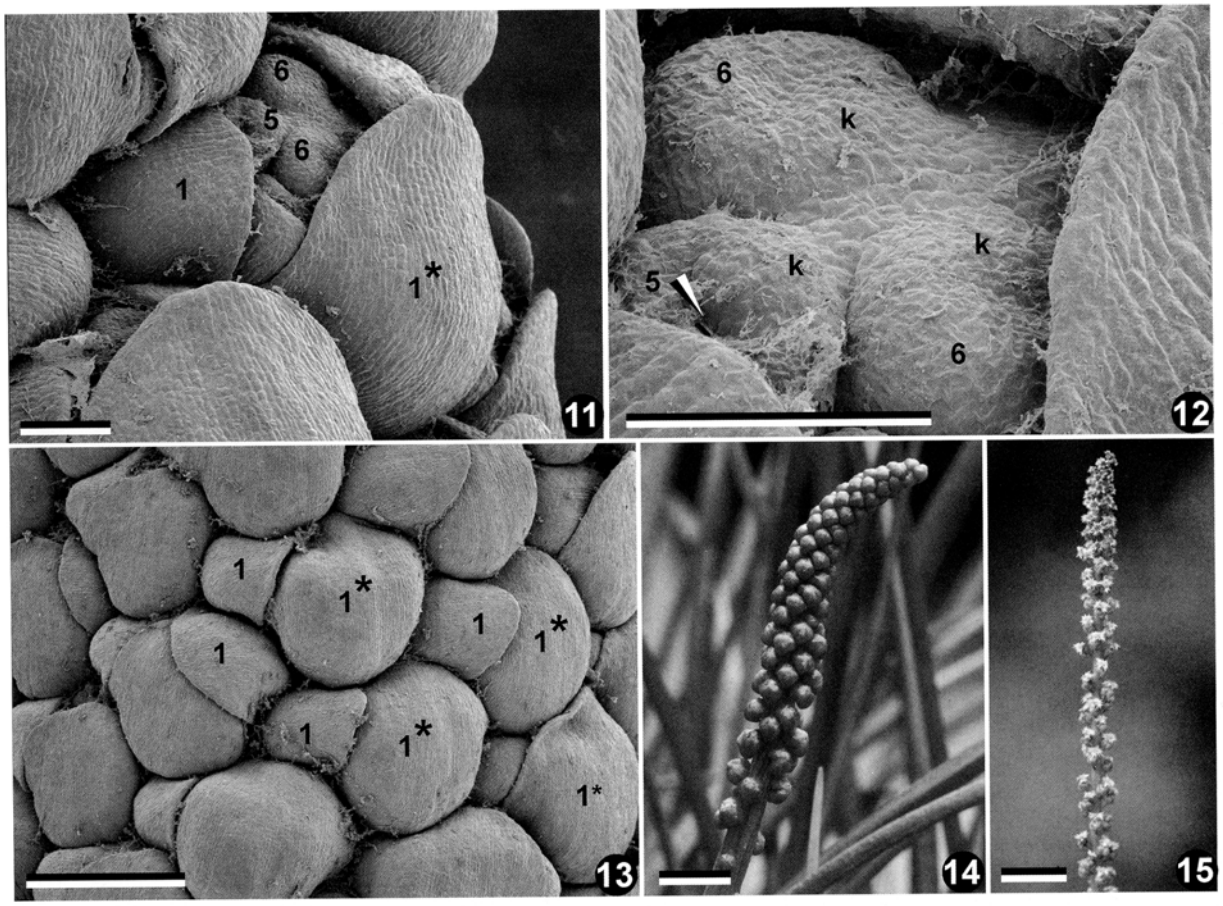

Fig. 11–15.—Later floral development of *Triglochin maritima.*—11. Young lateral flower shortly after initiation of inner carpels (6), SEM, side view.—12. Young gynoecium, SEM, oblique-apical view, carpels developing an adaxial cross meristem (k), outer carpels forming an ovary depression (arrow).—13. Flowers on the longitudinal rim of the inflorescence, SEM, apical view, the median abaxial tepals are overlapped by smaller lateral tepals (1), in flowers besides the rim, the abaxial median tepal (1*) overlaps the lateral tepals, as expected for unidirectional, bilaterally symmetrical development.—14. Young inflorescence, side view, the flowers are still densely arranged.—15. Inflorescence, side view, female stage of anthesis, stigma papillae exposed, internodes between flowers elongate. Outer tepals (1, 1* abaxial median tepal), carpels (5, 6), carpel cross meristem (k). Bars in Fig. 11, 12 = 0.1 mm, in Fig. 13 = 1 mm, in Fig. 14, 15 = 10 mm.

of the inflorescence, flowers appear irregular in symmetry and merosity. At mid-level of the inflorescence, the merosity of flowers may be reduced, resulting in apparently three tetramerous whorls (tricyclic) rather than six trimerous whorls, as in the two larger species described above (Fig. 21). No bracts were observed and the median abaxial tepal is prominent throughout the inflorescence, appearing bract-like (Fig. 21). Distally in the inflorescence, the adaxial side of the flower can be reduced to such an extent that the median abaxial tepal is the only sizable perianth organ and appears bract-like (Fig. 22). Terminal flowers were not observed in *T. striata,* because the slender inflorescences tended to abort at the tip.

Maundia triglochinoides, a monotypic Australian aquatic, appears similar to *T. procera* in gross morphology. The two reported differences between the species are the formation of stolons in *Maundia* F. Muell., and the merosity of the flower. *Maundia* has only two tepals, laterally on the abaxial side of the flower (Fig. 23), similar to the perianth in some Aponogetonaceae. In addition, the androecium consists of four to six stamens; the gynoecium of *Maundia* consists of four carpels (sometimes three or two distally in the inflorescence) with a prominent, plicate apex and is similar to female flowers of *Tetroncium* Willd. (Juncaginaceae, two or three tepals, three or four conically-elongate carpels with a large plicate proportion; pers. obs.). Due to the lack of material, terminal flowers and floral development could not be studied in detail in *Maundia* and *Tetroncium.*

←

(1*), adaxial tepals reduced in lateral flowers (arrow).—9. Young terminal flower, apical view, outer tepals (1), inner tepals (2), completely radially symmetrical.—10. Young terminal flower, side view, short elevation of the inflorescence between terminal flower and lateral flowers (arrow). SAM (x), foliar leaf (f), prophyll (p), outer tepals (1, 1* abaxial median tepal), inner tepals (2), stamens (3, 4), carpels (5, 6). Bars in Fig. 2–3 = 1 mm, in Fig. 4–10 = 0.1 mm.

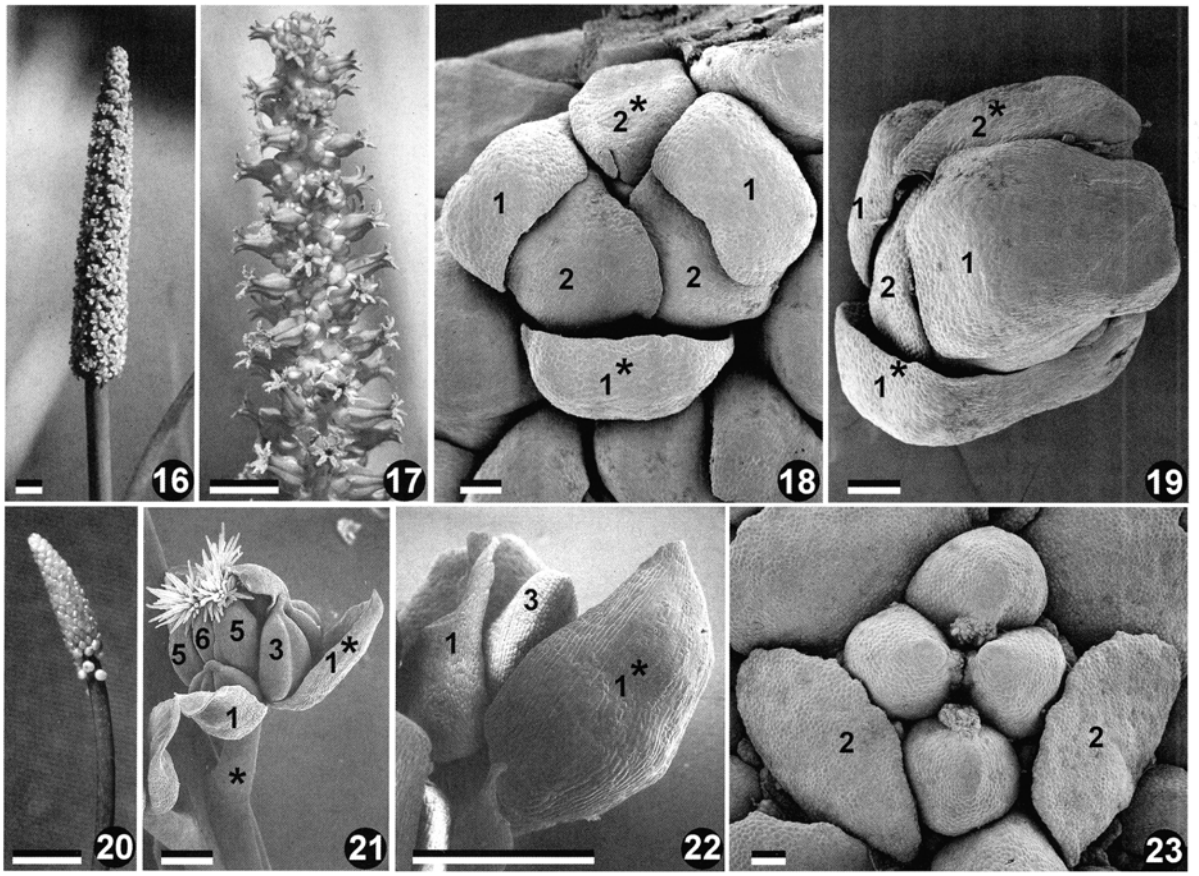

Fig. 16–23. Floral development in *Triglochin procera* (Fig. 16–19), *T. striata* (Fig. 20–22), and *Maundia triglochinoides* (Fig. 23).—16. Inflorescence, side view, female stage of anthesis.—17. Flowers, apical view, female stage of anthesis, just before stamens open.—18. Flower, SEM, apical view, before anthesis, the inner median tepal (2*) is smaller than the inner lateral tepals, and overlaps one of the outer lateral tepals.—19. Flower, SEM, side view, before anthesis, the inner median tepal (2*) is smaller than the inner lateral tepals, and overlaps the outer lateral tepals, the outer median tepal (1*) is prominent. Bars in Fig. 16, 17 = 1 cm, in Fig. 18, 19 = 0.2 mm.—20. Inflorescence, side view, before anthesis.—21. Flower, SEM, side view, female stage of anthesis, the outer median tepal is prominent, the lateral tepals are transverse (1), inner and outer whorl are not distinct (reduced perianth); also showing stamens (3), stigma papillae on top of carpels (5–6), and pedicel (*) without subtending leaf.—22. Flower, SEM, side view, before anthesis, the outer median tepal is much larger than the lateral one. Bars in Fig. 20 = 5 mm, in Fig. 21, 22 = 0.2 mm.—23. SEM, apical view, female stage of anthesis, there are only two abaxial lateral tepals (2). Outer tepals (1, 1* abaxial median tepal), inner tepals (2), outer stamen (3), carpels (5, 6). Bar in Fig. 23 = 0.2 mm.

Identification of APETALA3 cDNA Sequence and In Situ Hybridization

The *AP3* homolog sequences recovered (*Tr.ma.AP3–1, Tr.ma.AP3–2*) are nearly identical to each other and lack six amino acids at the 5′-end. The most similar DNA sequence found (Blastn) annotated for *AP3* was from Lauraceae (*AP3*-like of *Lindera erythrocarpa* Makino), not monocots. The best hits to monocots (*Oryza sativa* L. and *Asparagus officinalis* L.) have significantly lower blast scores, as do hits to model organisms (e.g., *DEFICIENS* A of *Antirrhinum majus*). The most similar amino acid sequences (Blastp) are from two monocots, *Asparagus officinalis* and a *Hemerocallis* L. hybrid cultivar; however, these two sequences are only annotated as MADS-box genes, not *AP3*-orthologs. The highest score for an *AP3*-annotated protein is from *Chloranthus spicatus* Makino of Chloranthaceae, a family that with monocots and magnoliids forms part of a polytomy after the basal grade of *Amborella* Baill., Nymphaeaceae, and Austrobaileyales (e.g., Soltis et al. 2000). The *Arabidopsis thaliana AP3* protein has a substantially lower score than the monocot and the Chloranthaceae sequences.

The maximum parsimony analysis included a total of 854 aligned amino acids, 494 of which were parsimony-informative. The strict consensus of the 100 most parsimonious trees that were retained placed *Tr.ma.AP3–1* and *Tr.ma.AP3–2* in a clade of *AP3* sequences, separate from a clade of *PI* homologs. The bootstrap support for the clade exclusively including all *DEF–AP3* transcription factors and *Tr.ma.AP3–1* and *Tr.ma.AP3–2* was 89%. These results support that *Tr.ma.AP3–1* and *Tr.ma.AP3–2* are orthologs of the *DEF–AP3* transcription factors.

Using *AP3* probes, mRNA localization was determined by in situ hybridization in inflorescences of two stages. In the younger stage examined (corresponding to stamen initiation; Fig. 6–10), *AP3* mRNA was detected throughout the entire inflorescence, as well as in leaves (Fig. 24, 26, 27). It is

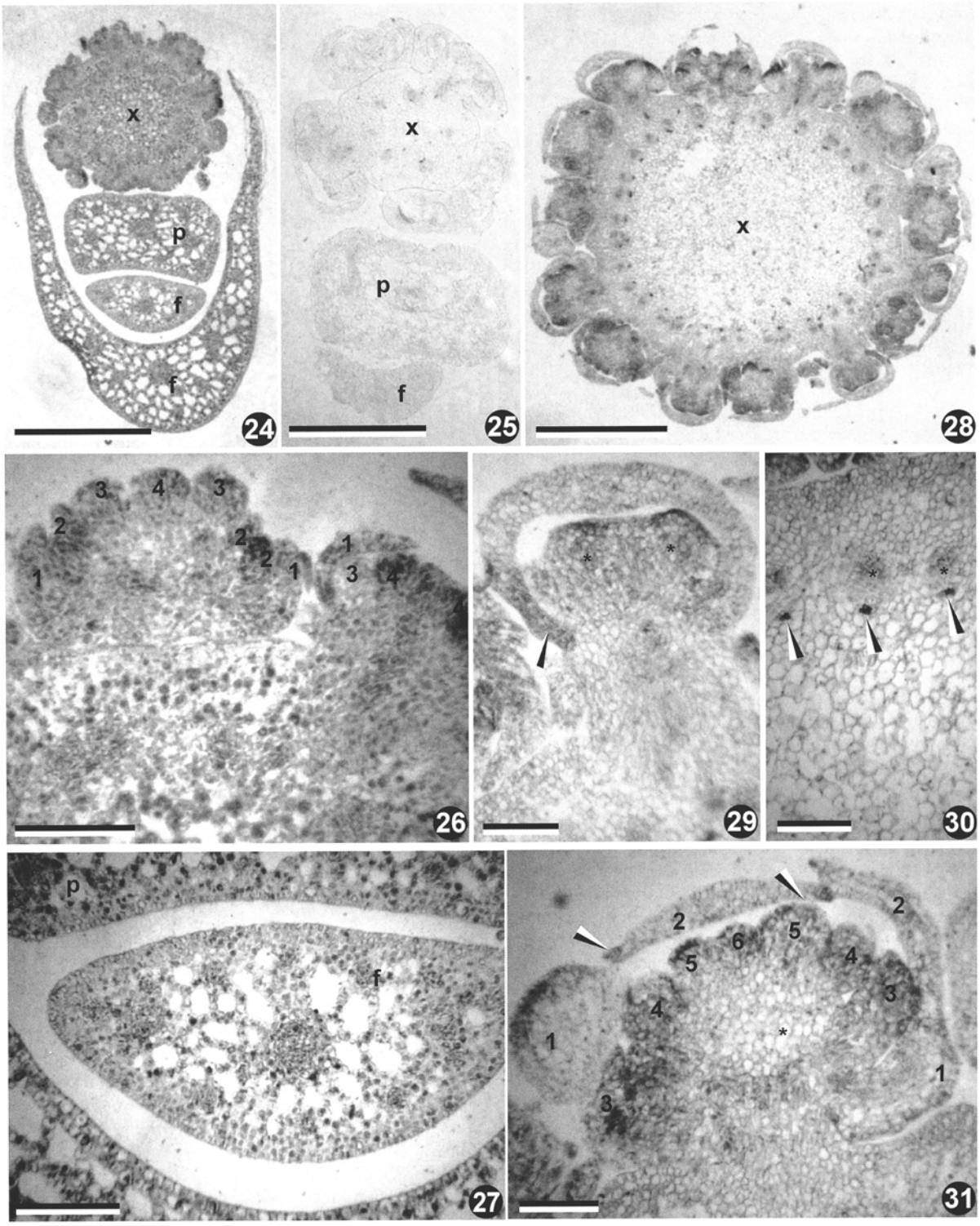

Fig. 24–31.—In situ hybridization of *Triglochin maritima AP3*-ortholog mRNA, all antisense except Fig. 25.—24. Transverse section of young inflorescence (x) at initiation of inner stamens.—25. Transverse section of young inflorescence (x), sense negative probe showing no signal (contrast enhanced linearily).—26. Longitudinal section of young flowers at initiation of stamens (3, 4).—27. Transverse section of young prophyll (p) and foliar leaf (f).—28. Transverse section of young inflorescence (x) at initiation of inner carpels.—29. Tangential longitudinal section of young stamen, thecae (*), and tips of tepals (arrow) show distinct signal.—30. Transverse section of young inflorescence: vascular bundles (*) and sclerenchyma (arrows) show a distinct signal.—31. Longitudinal section of a young flower at initiation of carpels. Signal occurs at the tip of tepals (arrows), and carpels (5 and 6), but is absent from the central tissue (receptacle, *). Inflorescence shoot center (x), prophyll (p), foliar leaves of continuation shoot (f), outer tepals (1), inner tepals (2), stamens (3, 4), carpels (5, 6). Bars in Fig. 24, 25, 28 = 1 mm, in Fig. 27, 29–31 = 0.1 mm.

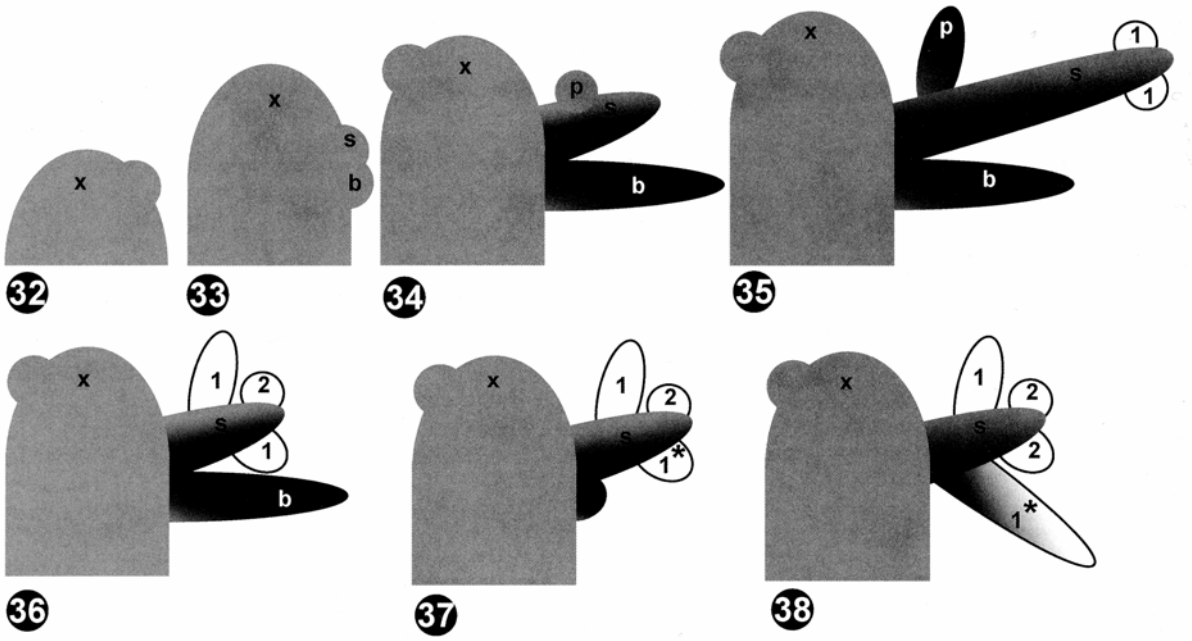

Fig. 32–38.—Schematic concept of initial bilateral symmetry of lateral shoots, unidirectional floral development as response to abbreviation of the axillary shoot and inhibition by subtending bract, and transition of the flower-subtending bracts into the perianth.—32. A lateral meristem forms below the SAM (x).—33. Lateral meristem subdivides into subtending leaf (b) and its axillary floral shoot meristem (s).—34. Leaf and axillary shoot obtain their organ identity distinction, the abaxial side of the axillary shoot is inhibited by the leaf (black as the leaf), the first organ of the lateral shoot (prophyll) develops on the adaxial side.—35. The lateral floral shoot grows beyond the inhibition of the subtending leaf (shoot tip without black), first floral organs (1) are initiated simultaneously.—36. The axillary floral shoot remains short, its first floral organ (1) corresponds to the prophyll (followed by the abaxial organ, 2).—37. The axillary floral shoot remains short, but the subtending leaf is suppressed; it still inhibits the axillary shoot, the first floral organ corresponds to the prophyll.—38. The axillary floral shoot remains short, but the subtending leaf is recaulescent with the lateral shoot by intercalary growth of the common base; the organ corresponding to the subtending leaf becomes the first floral organ. Gray = shoot axis, black = flower-subtending leaf, white = floral organ; main shoot center (x), prophyll (p), lateral floral shoot (s), flower-subtending leaf (b), outer tepals (1, abaxial tepal 1*), inner tepals (2).

unlikely that this signal reflects nonspecific hybridization with mRNAs in young tissues, because the negative control (sense) yielded much lower levels of background (Fig. 25). The older stage (corresponding to the initiation of carpels) shows a clear differentiation of signal (Fig. 28–31). *AP3* signal is strongest in newly initiating thecae, carpels (Fig. 29, 31), procambial tissue (Fig. 30), and tepal tips. Weak expression was detected in future inflorescence parenchyma, epidermal cells, tepal bases, and in the center of the flower (the terminating apex, rather than the carpels).

DISCUSSION

Bilateral Symmetry and Flexibility of the Bract

We suggest that every lateral shoot starts with an inherent bilateral symmetry due to the subtending leaf, and that the putative flower-subtending bract is not always extrafloral, but is sometimes involved in the perianth (Hypothesis 2). These issues are closely linked. The leaf and its axillary shoot develop from a common meristem (Fig. 32, 33; Troll 1937; Hagemann 1963, 1970, 1984; Esau 1977); thus, both symmetry and the production of a flower-subtending bract depend on how abrupt the transition is between leaf and axillary shoot at the base of both organs. If the transition is gradual, then the upper (adaxial) side of the leaf and the lower (abaxial) side of the axillary shoot may mutually affect one another. For example, the meristem dedicated to the subtending leaf is absent on the abaxial side of the lateral SAM. As a result, the first leaf of the lateral shoot initiates on the adaxial side, opposite the subtending leaf. Indeed, in monocots, the first leaf on the axillary shoot is a single prophyll on the adaxial side of the axillary shoot, alternating with the subtending leaf, corresponding to a distichous phyllotaxy resulting from the abaxial inhibition by the subtending leaf (Fig. 34). Inhibition could be due to the lack of auxin, which was proposed to affect the radial position and size of lateral organs in tomato and *Arabidopsis* (Reinhardt et al. 2000). Although lateral shoots initiate with an inherent bilateral symmetry, this bilateral symmetry is lost as the lateral shoot grows. In a lateral flower with a significant pedicel, a prophyll, and possibly additional bracts, the SAM of the lateral shoot has time to equalize its sides: abaxial inhibition by the subtending leaf is countered by inhibition by the prophyll, the SAM becomes radially symmetrical, and whorled floral organs develop simultaneously (Fig. 35).

If floral development is abbreviated, no intermediate bracts are formed along the floral shoot, and the first organs initiated are already part of the perianth. Nonetheless, due to abaxial inhibition, the first floral organs still develop on the adaxial side, in the position of the prophyll. This results

in unidirectional flower development from adaxial to abaxial (Fig. 36), as in *Neuwiedia* Blume (Kocyan and Endress 2001). A flower-subtending leaf might be suppressed, as suggested for Nymphaeaceae (Cutter 1957*a*, *b*; Moseley 1972), the basal monocot family Araceae, and some Potamogetonaceae (Eber 1934; Posluszny and Sattler 1973, 1974; Tomlinson 1974; Posluszny 1981; Buzgo 2001). In Araceae and some Potamogetonaceae no median organ develops on the abaxial side or the outermost whorl, as if there was still inhibition by the flower-suppressed leaf (Fig. 37). Suppression of the flower-subtending bract has also been shown in *Arabidopsis* and other Brassicaceae (Saunders 1923; Troll 1937; Hagemann 1963, 1970, 1984; Esau 1977; Shu et al. 2000; Heisler et al. 2005). However, in *Arabidopsis,* the abaxial median sepal is larger during early flower development (Smyth et al. 1990), and later adjusts its growth to equal the size of the other three sepals. The result is similar to those cases in which the subtending bract is involved in the perianth (*Triglochin, Acorus*; Fig. 38). In the inflorescence of *Triglochin* there is no distinct flower-subtending bract, but the flower initiates with an abaxial organ that shares features of both the subtending bract and the tepal. This is a frequent phenomenon, especially in flowers that are small and initiate in fast succession, as has been discussed for *Acorus* (Buzgo and Endress 2000; Buzgo 2001). If floral shoot development is abbreviated even further, then the lateral meristem comprising subtending bract and axillary shoot does not subdivide before the meristem identity for the flower is determined. The result is a lack of inhibition by an extrafloral flower-subtending bract and a direct transition of the lateral shoot into the perianth zone, without forming any bracts (Fig. 38).

Meristem identity of the flower is based on the expression of specific genes (Coen et al. 1990; Schwarz-Sommer et al. 1990, 1992; Coen and Carpenter 1992; Huala and Sussex 1992; Singer et al. 1992; Weigel et al. 1992; Weigel and Nilsson 1995; Blázquez et al. 1997; Hempel et al. 1997; Lee et al. 1997; Ma 1997, 1998; Parcy et al. 1998; Weigel 1998; Wagner et al. 1999; Berleth et al. 2000; Ferrándiz et al. 2000; Frohlich and Parker 2000; Yu et al. 2000; Araki 2001; Coen and Langdale 2001; Pena et al. 2001; Soltis et al. 2002). By slightly altering gene expression levels, the first abaxial organ of the lateral structure (leaf and axillary shoot) might be turned into a floral organ (bract-like tepal). This would result in unidirectional development from abaxial to adaxial, as observed in *Acorus, Aponogeton,* and *Triglochin* (Fig. 37). Intercalary elongation within the common base of subtending leaf and axillary shoot results in a recaulescence of both organs: by intercalary growth, the subtending leaf is lifted away from the main shoot, along with the axillary shoot. This occurs in *Triglochin* and *Arabidopsis,* where a distinct pedicel is present. In *Triglochin maritima,* this feature is intermediate between the situation in *Arabidopsis* and *Acorus.* In *Arabidopsis,* the abaxial sepal is not much larger than the other sepals in later development. By contrast, in *Acorus* the bract-like appearance persists throughout development. In *Lilaea scilloides* (Poir.) Hauman (Juncaginaceae, sensu APG II [2003]), the perianth is reduced to a single median bract-like organ (Posluszny et al. 1986; but a bract according to Uhl 1947), similar to that of Saururaceae (see below). Strong reduction is also found in Aponogetonaceae. The Australian species *Aponogeton hexatepalus* H. Bruggen has two trimerous perianth whorls. Most other species of *Aponogeton* have only one trimerous perianth whorl (representing the inner whorl) with an adaxially median organ (Singh and Sattler 1977*b*; van Bruggen 1985, 1990, 1998; Hellquist and Jacobs 1998) that is often reduced, resulting in a flower like that of *Maundia.* Finally, *Aponogeton distachyus* L. f. possesses only one bract-like organ. As a result, the flower-subtending bract can appear as the abaxial median tepal of lateral flowers. This reflects a change of organ identity and of corresponding shoot order (from being an attribute of the flower as lateral shoot to an attribute of the inflorescence as main shoot).

In the magnoliid family Saururaceae, a bract-like leaf appears at the abaxial side of the otherwise perianthless flower (Tucker 1975, 1979). In some genera, this leaf is conspicuously petaloid (*Houttuynia* Thunb., *Anemopsis* Hook. & Arn.), whereas in others it is on a common stalk and shares vasculature with the rest of the flower at the pedicel (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996); no axillary shoots have been reported in association with this abnormal leaf, apart from the flower. Therefore, this median abaxial leaf meets the expectations of a perianth organ (sterile phyllome on a floral shoot, position on a receptacle, with short subsequent internodes, no axillary meristem; Buzgo et al. 2004*a*, *b*). Its interpretation as a flower-subtending bract lacks developmental support, and is historically based on earlier studies of the closely related family Piperaceae, which possess a more scale-like median abaxial organ inserted strictly on the inflorescence main axis (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996).

We, therefore, conclude that in some taxa with dense inflorescences, the delimitation between inflorescence and flower is less clear than classical morphology implies. The data indicate that in some taxa the organ initiated in the position of an extrafloral flower-subtending bract may become involved in the perianth as the median abaxial tepal.

Is There a Pseudanthium in Triglochin maritima*?*

Miki (1937) proposed a link between flowers of Potamogetonaceae to those of Pandanales based on: (i) the position of the tepals ("bracts" associated with stamens) on a common elevation with the stamens in Potamogetonaceae, and (ii) the assumption that floral reduction from Alismatales-like flowers is "not probable." No feature was given by Miki (1937) to differentiate "bracts" from tepals (axillary shoots, phyllotaxy) or to indicate that the floral units of *Potamogeton* were composed of several flowers, instead of representing single flowers lacking a perianth. In addition, no developmental data were provided. Only *Najas* L. and *Potamogeton* were considered by Miki (1937). The most significant data are provided by Uhl (1947), who concluded that the floral units of Scheuchzeriaceae, Aponogetonaceae, Juncaginaceae, and Potamogetonaceae were composed of radial "staminate units," and one to several central pistillate flowers. The staminate units consisted of a single stamen representing an entire reduced flower subtended by a bract (the tepal, in this study). The "floral unit" of all of these taxa

was considered to be composed of highly reduced inflorescences (staminate units) and therefore to represent a pseudanthium in the commonly used sense (see Introduction). This pseudanthial concept (Uhl 1947) is based on three observations: (1) the vasculature of the "staminate unit" leaves the rest of the floral vasculature as one strand, which then divides into two; (2) the staminate unit is often supported by a common elevated base (*Potamogeton*), or in some taxa (*Triglochin* subgen. *Cycnogeton* (Endl.) Buchenau & Hieron., *Scheuchzeria*; Uhl 1947) the inner whorl of staminate units inserts distally of the stamens of the outer whorl (see also Rudall 2003) and are shed as a unit (stamen and tepal together as "staminate unit" in *Triglochin*; Uhl 1947); and (3) reductions of flowers often involve merosity of all whorls (sectors consisting of tepal, stamen, and carpel). The study by Uhl (1947) included a diverse array of taxa, and its conclusions were based almost entirely on vasculature of mature stages. However, no developmental data were provided, and series of organ initiation were not presented. Uhl (1947) did not consider the possibility of unequal intercalary growth or unidirectional flower development.

In *Potamogeton* and *Triglochin* the initiation sequence of the organs on the floral units corresponds perfectly with that of flowers consisting of whorls of outer tepals, inner tepals, outer stamens, inner stamens, outer carpels, inner carpels (Charlton 1981; Posluszny 1981; this study). Any position of outer tepals seemingly distal from outer stamens can be explained by unequal intercalary elongation and unilateral flower development, which also can confuse the recognition of whorls in other taxa (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996; Buzgo and Endress 2000).

Our data support the hypothesis that each floral unit in *Triglochin* represents a distinct flower, not an inflorescence, in accordance with Hill (1900), Arber (1940), Eckardt (1957), Singh (1973), Serbanescu-Jitariu (1973), Lieu (1979), Charlton (1981), Posluszny et al. (1986), Endress (1995), and Igersheim et al. (2001), but in contrast to Miki (1937), Uhl (1947), Eames (1961), and Rudall (2003); there is no flower-like structure that is composed of several flowers (Endress 1994), and therefore no pseudanthium.

Terminal Peloria and Pseudanthia

In *T. maritima* and other species of *Triglochin,* a flower-like terminal structure occurs, which is considered a terminal flower by most authors (Hill 1900; Aston 1973, 1993*a*, *b*; Lieu 1979; Posluszny et al. 1986; Harden 1993), but this structure is considered absent by Uhl (1947) and Charlton (1981). The terminal structure is larger than lateral flowers, probably because it is formed by a larger primordium (the inflorescence SAM) than lateral flowers. The terminal structure is radially symmetrical (this study), but not aberrant, and therefore the term peloria may be inaccurate. For example, the terminal structure is initiated with a distinct lag in development after the lateral distalmost flowers of the inflorescence, causing a gap between the insertions of lateral flowers and the first organs of the terminal structure. Consequently, there is an abrupt transition from lateral floral primordia to floral organs toward the apex, although the subapical flowers show reduction on the adaxial side, as in *Houttuynia* and *Acorus* (Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996; Buzgo and Endress 2000).

Our observations support a correlation between smaller inflorescences, proportionally stronger reduction of the adaxial organs in distal flowers, and the formation of terminal flowers that differ from the lateral ones, as suggested previously by Buzgo and Endress (2000) and Buzgo (2001) for *Acorus.* Members of the *Triglochin procera* group (Aston 1973, 1993*a*, *b*) grow vigorously, forming inflorescences in which the flower-bearing portion is up to 30 cm long, with flowers more than 8 mm in diameter with distinct pedicels, and the terminal flower resembles the lateral flowers. *Triglochin palustris* and *T. striata* have much smaller inflorescences than *T. maritima.* Flowers of *T. striata* possess distinct pedicels. However, in many cases not all floral organ whorls are trimerous, and whorls are sometimes difficult to distinguish. In the distal portion of the inflorescence, flowers are strongly reduced on the adaxial side (*T. striata*), sometimes leaving only one median tepal, which is bract-like.

The Australian group of annual species (*T. turrifera* Ewart, *T. centrocarpum* Hook., *T. hexagona* J. M. Black, *T. calcitrapum* Hook.) (Aston 1973; Harden 1993; K. Meney pers. comm.) has been reported to have extremely small inflorescences. In at least some of these species the lateral flowers are unisexual, with only the terminal flower being bisexual. This "completeness" of the terminal flower may result from a larger meristem as compared with the lateral primordia (as in *T. maritima*), and thus represents a distinct difference between terminal and lateral flowers, similar to that of the larger peloria in *Acorus* and Saururaceae. All Juncaginaceae and Aponogetonaceae may be affected by a convergent tendency of adaxial flower reduction, leading to similar transitions between bracts and tepals, between inflorescence and flower. Understanding the transition of inflorescence and flower in alismatids is crucial for elucidation of floral evolution in early monocots, and even for basal angiosperms, in general, because similar features also appear in magnoliids (Saururaceae; Tucker 1979, 1981, 1985; Liang and Tucker 1989, 1990; Tucker et al. 1993; Tucker and Douglas 1996) and basal eudicots (Buxaceae; von Balthazar and Endress 2002).

Several authors mention the loss of a sharp distinction between flower and inflorescence (Eames 1961) and consider a homeotic transition from flower to inflorescence in basal angiosperms and monocots (Sattler 1965; Posluszny et al. 1986; pseudanthic recapitulation, neotenic inflorescences, "paedomorphic trend," reviewed by Claßen-Bockhoff 1990; "metaflower," Charlton and Posluszny 1991; Hay and Mabberley 1991; Albert et al. 1998; Buzgo 2001; Rudall 2003; Rudall and Bateman 2003). Specifically for alismatids, Rudall (2003) suggests that the reproductive structures may represent neither flowers nor inflorescences in the proper sense. We agree that the limit between flower and inflorescence is unclear. However, there is an apparent hierarchy of reproductive shoots even in *Triglochin* and *Potamogeton,* which involves flowers, be they reduced or not (Hill 1900; Arber 1940; Eckardt 1957; Singh 1973; Serbanescu-Jitariu 1973; Lieu 1979; Charlton 1981; Posluszny et al. 1986; Endress 1995; Igersheim et al. 2001). Therefore, the term "inflorescence" is sufficiently accurate for the overall structure

(Eames 1961; Troll 1964; Claßen-Bockhoff 1990; Endress 1994).

In *Triglochin maritima,* we can recognize the terminal flower. Claßen-Bockhoff (1990) suggests a "paedomorphic trend," in which the progressive reduction of the inflorescence SAM results in the abbreviation (heterochrony) of the developmental process of lateral primordia, rendering them floral organs and resulting in an aberrant flower (peloria) at the inflorescence apex. This abbreviation reflects the "specific predisposition of the taxa concerned" required by Claßen-Bockhoff (1990) for the convergent evolution of pseudanthia. This requirement is met in *Acorus* and some Saururaceae (above). However, in the clade comprising Juncaginaceae and Potamogetonaceae, this predisposition is only represented by the reduction of the lateral floral units (flowers); we find no signs or intermediate cases indicating the reduction and rearrangement of floral units to lateral pseudanthia. Yet, this extension of floral characteristics may be represented in the partial extension activity of genes responsible for the determination of flower meristem identity, i.e., upstream from B-class genes.

Molecular Genetic Perspective

The lateral flowers of *Triglochin* apparently are not defined by a flower-subtending bract. The inflorescence starts development as one large meristem and the apex of this meristem turns into a flower. How far does floral identity reach out into the inflorescence? When does the transition of the inflorescence apical meristem to a flower primordium occur? How far is the assumption of a homeotic change of flower features into the supporting inflorescence shoot supported by concepts or data of molecular development? A test for floral features in inflorescence development is provided by genes that are considered strictly floral (Hypothesis 4). The gene we used to test this hypothesis is an ortholog of *Antirrhinum* L. *DEFICIENS* (*DEF*) and *Arabidopsis APETALA3* (*AP3*), a member of the B-class MADS-box gene family (e.g., Bowman et al. 1989; Sommer et al. 1990; Coen and Meyerowitz 1991; Soltis et al. 2002; Kramer et al. 2003; Stellari et al. 2004). Orthologs of *AP3* are strictly regulated downstream of *LEAFY* and A-class genes, both of which are required for the conversion of a shoot into a flower (Coen et al. 1990; Schwarz-Sommer et al. 1990; Coen and Carpenter 1992; Huala and Sussex 1992; Singer et al. 1992; Weigel et al. 1992; Weigel and Nilsson 1995; Blázquez et al. 1997; Hempel et al. 1997; Lee et al. 1997; Ma 1997, 1998; Parcy et al. 1998; Weigel 1998; Wagner et al. 1999; Berleth et al. 2000; Ferrándiz et al. 2000; Frohlich and Parker 2000; Yu et al. 2000; Araki 2001; Coen and Langdale 2001; Pena et al. 2001; Soltis et al. 2002). Because *AP3* is only transcribed after a flower-specific developmental pathway has been activated, the significant occurrence of its mRNA is a conservative indicator of floral meristem identity.

The "sliding boundaries" concept of the ABC-class model (Kramer et al. 2003) predicts that in *Triglochin maritima,* B-class genes would be expressed only in stamens, but not in either whorl of sepaloid tepals, bracts, or inflorescence main shoot (although Kramer et al. 2003 specify that in *Aquilegia* L. one of the three copies of *AP3* is the major factor for petaloid features, while the other two have expression patterns that are less correlated with petaloid features). For older developmental stages of *T. maritima,* our results generally correspond to this concept, although *AP3* is also weakly transcribed in the tips of tepals, very young carpels, and vascular strands. These expression patterns are in greater agreement with the concept of "fading borders" of gene expression described for basal angiosperms (Buzgo et al. 2004). "Fading borders" suggests that in basal angiosperms the functions of floral transcription factors are not restricted to only one zone or whorl of organs, but exhibit a gradual transition from the periphery to the center of the flower. Corresponding to the often spiral or irregular floral phyllotaxy in basal angiosperms (instead of a few distinct whorls of floral parts, as in eudicots), "fading borders" explains the gradual transition of morphological features, such as features commonly associated with stamens or petals (e.g., papillae, thickening, secretion, color). The concept does not specify how the gradual transition in gene function is achieved (duration of gradual expression, diversified function of gene copies [Stellari et al. 2004], transcription rate, post-transcriptional modification, or protein-affinities). Although "fading borders" was developed with a focus on B-class genes, other genes may exhibit a similar transition in expression pattern. The hypothesis of "fading borders" is supported by studies employing relative-quantitative gene expression (Kim et al. 2003, 2005). In particular, B-class genes are expressed in tepals, stamens, and carpels of several basal angiosperms that exhibit gradual transitions between adjacent floral organs.

For very young inflorescences, our expression results are puzzling in that the mRNA of *AP3* appears to be present not only in stamens, but also throughout the entire inflorescence (and even in leaves). The absence of signal from the negative controls (sense probes) supports the interpretation that the apparent expression is a true signal. One explanation could be that B-class genes are expressed in other meristems as well, for example, in procambial strands. B-class gene transcripts have been reported from procambial strands in other studies (e.g., Skipper 2002) and also occur in the procambial strands of older inflorescences of *Triglochin maritima* (this study). However, the future parenchyma of the leaves and inflorescence also stains strongly in leaves, even at a stage where the intercellular spaces have begun to form. Based on our results, it appears as if *AP3* is more widely expressed in the inflorescence of *T. maritima* than in other plants examined to date. Because of upstream regulation by floral meristem identity genes (see above), this broad expression of *AP3* suggests that at early stages of development the axis of the inflorescence may share some identity with that of a flower. This is in accordance with the transition of the inflorescence SAM into a flower: the identity of the entire young inflorescence is "floral" and the restriction of this identity to lateral meristems only occurs later. This pattern is consistent with reports of transcription of *SEPALLATA* in inflorescences of *Oryza sativa* (Malcomber and Kellogg 2004) and could explain similar phenomena in other monocots and basal angiosperms. If our interpretation of this pattern of *AP3* expression is correct, our results would expand the concept of "fading borders" beyond the limits of the flower to the inflorescence.

ACKNOWLEDGMENTS

We would like to thank Martin Skipper, Signe Frederiksen, Charlotte Hansen, and Lisbeth Knudsen (Department of Evolutionary Biology, University of Copenhagen, Denmark) for helping with RNA extraction and isolation of the gene sequence. We thank Peter K. Endress (University of Zurich, Switzerland), Paula J. Rudall, Mark W. Chase (Royal Botanic Gardens, Kew, UK), and Ole Seberg (Department of Evolutionary Biology, University of Copenhagen, Denmark) for permission to collect material at their departments, and for use of their facilities. Our understanding of the diversity of Juncaginaceae would not have been possible without the contributions of Bruno Matter (Botanic Garden, University of Basel, Switzerland), Mike Fay (Royal Botanic Gardens, Kew, UK), Alec Floyd (Coffs Harbour, Australia), Georgina Reiss and Kathy Meney (Perth, Australia), Miriam Herrmann (Stans, Switzerland), W. Alan Charlton (University of Manchester, UK), Usher Posluzny (University of Guelph, Canada), Gwen Harden and Alistair Hay (New South Wales National Herbarium Royal Botanic Gardens, Sydney, Australia), and Ian Black (English Nature, UK). We thank Günter Theißen (University of Jena, Germany) and Annette Becker (Monash University, Melbourne, Australia) for sending us sequences of MADS-box genes previously analyzed. We thank Matthew Gitzendanner, Greg Erdos, Karen Kelley, and Fred Bennett for technical support and instructions. We thank Peter Endress and Paula Rudall for the helpful comments on the manuscript. This study was funded by Swiss National Science Foundation grant 83EU-059822 and National Science Foundation grants PGR-0115684, MCB-0092075, and IBN-0077832.

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