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The Role of *KNOX* Genes in the Evolution of Morphological Novelty in Streptocarpus [W]

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The genus Streptocarpus comprises species with diverse body plans. Caulescent species produce leaves from a conventional shoot apical meristem (SAM), whereas acaulescent species lack a conventional SAM and produce only a single leaf (the unifoliate form) or clusters of leaves from the base of more mature leaves (the rosulate form). These distinct morphologies reflect fundamental differences in the role of the SAM and the process of leaf specification. A subfamily of *KNOTTED*-like homeobox (*KNOX*) genes are known to be important in regulating meristem function and leaf development in model species with conventional morphologies. To test the involvement of *KNOX* genes in Streptocarpus evolution, two parologous *KNOX* genes (*SSTM1* and *SSTM2*) were isolated from species with different growth forms. Their phylogenetic analysis suggested a gene duplication before the subgeneric split of Streptocarpus and resolved species relationships, supporting multiple evolutionary origins of the rosulate and unifoliate morphologies. In *S. saxorum*, a caulescent species with a conventional SAM, KNOX proteins were expressed in the SAM and transiently downregulated in incipient leaf primordia. The ability of acaulescent species to initiate leaves from existing leaves was found to correlate with *SSTM1* expression and KNOX protein accumulation in leaves and to reflect genetic differences at two loci. Neither locus corresponded to *SSTM1*, suggesting that *cis*-acting differences in *SSTM1* regulation were not responsible for evolution of the rosulate and unifoliate forms. However, the involvement of KNOX proteins in leaf formation in rosulate species suggests that they have played an indirect role in the development of morphological diversity in Streptocarpus.

INTRODUCTION

Morphological variation is a prerequisite of evolution, yet its underlying genetic basis is poorly understood. Related species are often distinguished by relatively minor variations in organ size and form. In Drosophila, subtle morphological differences have been attributed to changes in expression of regulatory transcription factor genes (e.g., Stern, 1998; Sucena et al., 2003). Variation in the activity of transcription factor genes also underlies some of the artificially selected morphological changes that accompanied the domestication of maize (*Zea mays*) (Doebley et al., 1998; Doebley and Lukens, 1998; Wang et al., 1999). In contrast with closely related species, higher order taxa tend to differ more dramatically in body plan, for example, in the number, identity, or relative positions of organs. Such evolutionary innovations may have also involved transcription factor genes because the changes sometimes correlate with altered transcription factor expression and can be mimicked by mutations in transcription factor genes in both plants and animals (Doebley and Lukens, 1998; Carroll, 2000).

One genus of plants that encompasses species with different body plans is Streptocarpus, the Cape primrose. Streptocarpus consists of ~140 African and Madagascan species that fall into two main clades on the basis of internal transcribed spacer (*ITS*) sequence phylogenies and chromosome numbers, and are broadly consistent with classical taxonomic subdivisions (Hilliard and Burtt, 1971; Möller and Cronk, 2001). Clade I consists of caulescent species that form stems and leaves conventionally from a shoot apical meristem (SAM; Figure 1B). Clade I corresponds to subgenus Streptocarpella with the genus Saintpaulia (African violets) nested within it (Möller and Cronk, 1997). Like most Gesneriaceae, caulescent Streptocarpus species favor warm, humid, and aseasonal habitats (Burtt, 1998). Clade II species, corresponding largely to subgenus Streptocarpus, lack a vegetative SAM and therefore have no erect vegetative stem. Some acaulescent species, termed unifoliate, have a single leafy organ (a phyllomorph) that arises by continued growth of one cotyledon after germination (Figure 1A; Jong, 1970, 1978; Jong and Burtt, 1975; Burtt, 1994). In other species, additional phyllomorphs are produced from existing phyllomorphs without involving a conventional SAM. Such plants are rosette like and are called rosulate (Figure 1C). Acaulescent species are able to abscise the distal part of the phyllomorph under drought conditions and hence colonize drier and more seasonal habitats than

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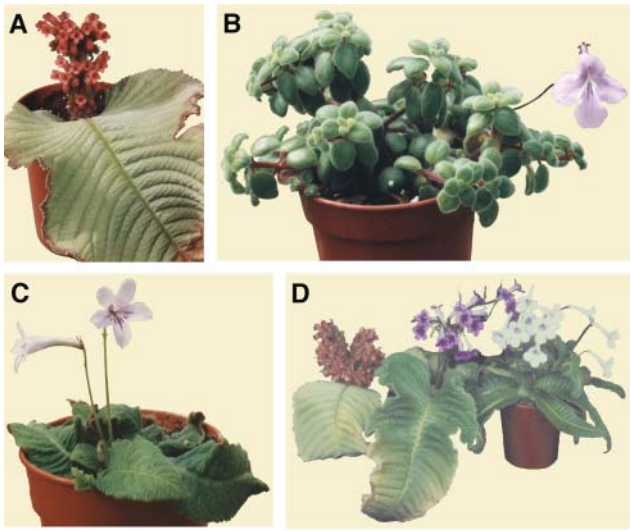

Figure 1. Streptocarpus Growth Forms.

(A) Unifoliate *S. dunnii*.
(B) Caulescent *S. saxorum*.
(C) Rosulate *S. rexii*.
(D) *S. dunnii* (left) and *S. rexii* (right). The *S. dunnii* × *S. rexii* F1 hybrid (center) has a rosulate arrangement of multiple, large phyllomorphs.

caulescent species (Burtt, 1998). Although lacking a conventional vegetative SAM, all acaulescent species form inflorescence meristems that arise from phyllomorph midribs and produce bracts and flowers conventionally (Figures 1A and 1C). Whereas unifoliate species are monocarpic and die after flowering, rosulate plants are perennial.

Species with different growth forms within Clade II can form fertile hybrids. In crosses between rosulate and unifoliate species, inheritance of the rosulate character was suggested to involve dominant alleles at two loci (Oehlkers, 1938, 1942, 1964). In some acaulescent species, application of gibberellic acid (GA) resulted in either the formation of extra phyllomorphs or induction of a vegetative shoot (Rosenblum and Basile, 1984).

Studies in model species have demonstrated the importance of Class I *KNOTTED*-like homeobox (*KNOX*) genes in SAM function and leaf development. Several of the effects of altered KNOX activity resemble the morphological differences among Streptocarpus species. For example, *Arabidopsis thaliana* mutants lacking activity of the *KNOX* gene *SHOOT MERISTEMLESS* (*STM*) do not form a SAM during embryogenesis (Barton and Poethig, 1993), as in acaulescent Streptocarpus species. In weaker *stm* mutants, leaves are formed from the base of cotyledons or existing leaves, apparently without the involvement of a SAM (Clark et al., 1996), resembling rosulate acaulescent Streptocarpus. Gain-of-function *KNOX* mutations that cause misexpression in leaves can have the opposite effect of conferring meristematic properties on the leaf. These properties are manifest as ectopic leaf outgrowths, and occasionally, SAMs (reviewed in Tsiantis and Hay, 2003), and therefore mimic the morphologies of acaulescent Streptocarpus. A further suggestion that altered *KNOX* activity might be involved in the novel morphology of Streptocarpus is that KNOX activity is able to repress GA biosynthesis (Sakamoto et al., 2001; Hay et al., 2002), and unifoliate Streptocarpus can phenocopy caulescent Streptocarpus when they are treated with GA, suggesting that acaulescent morphology might involve reduction of GA levels by KNOX activity (Rosenblum and Basile, 1984).

To further investigate the role of KNOX activity in Streptocarpus evolution, we isolated paralogous *STM*-like genes, *SSTM1* and *SSTM2*, from a range of Streptocarpus species. We found *SSTM1* expression in leaves of rosulate but not unifoliate species, suggesting that changes in *SSTM1* expression have been involved in evolution of novel morphologies. We were able to attribute rosulate and unifoliate morphologies to genetic differences at two loci, although neither locus was found to correspond to *SSTM1*. This suggested that mutations in *SSTM1* itself have not been responsible for altered gene expression or morphology.

RESULTS

Isolation of *SSTM1* Genes

To investigate the role of *KNOX* genes in morphological evolution, we isolated cDNA sequences of *STM* orthologs from three Streptocarpus species representing the main growth forms: caulescent *S. saxorum*, unifoliate *S. dunnii*, and rosulate *S. rexii*. Primers were designed to exon sequences conserved between *STM* and its orthologs from other species and used to amplify Streptocarpus *STM*-like cDNA sequences by rapid amplification of cDNA ends (RACE). Alignment of the inferred amino acid sequences of the Streptocarpus products (*SSTM1* genes) with other KNOX proteins showed that *SSTM1* had all typical features of a Class I *KNOX* gene, including regions encoding the MEINOX, ELK, and homeodomains (Figure 2A; Bürglin, 1997, 1998). Phylogenetic comparison of the *SSTM1* sequences with those of other *KNOX* genes placed them together in a well-supported clade with *STM* but no other Arabidopsis gene (Figure 2B). The *SSTM1* genes were therefore likely to be orthologs of Arabidopsis *STM*.

Streptocarpus Contains Two *STM*-Like Genes That Are Part of a Small Gene Family

DNA gel blot hybridization was used to determine the number of *STM*-like *KNOX* genes in Streptocarpus and to estimate the size of the Streptocarpus *KNOX* gene family. Genomic DNA from *S. dunnii*, *S. rexii*, and *S. saxorum* was digested with different restriction enzymes and probed either with the highly conserved *SSTM1* homeobox sequence (which is potentially able to detect all *KNOX* genes) or with fragments from the poorly conserved 5′ and 3′ regions of *SSTM1* to detect only *STM*-like genes.

High stringency hybridization with the homeobox probe detected two to three strongly hybridizing fragments in each species and 4 to 10 additional fragments that hybridized weakly (Figure 3A). At low stringency, two additional fragments were detected in *S. dunnii* and *S. rexii* (Figure 3B). This suggested that the *KNOX* gene family comprises 6 to 14 members in Streptocarpus. High stringency hybridization with either the 5′ region of *SSTM1* or its 3′ region detected a minimum of two fragments in

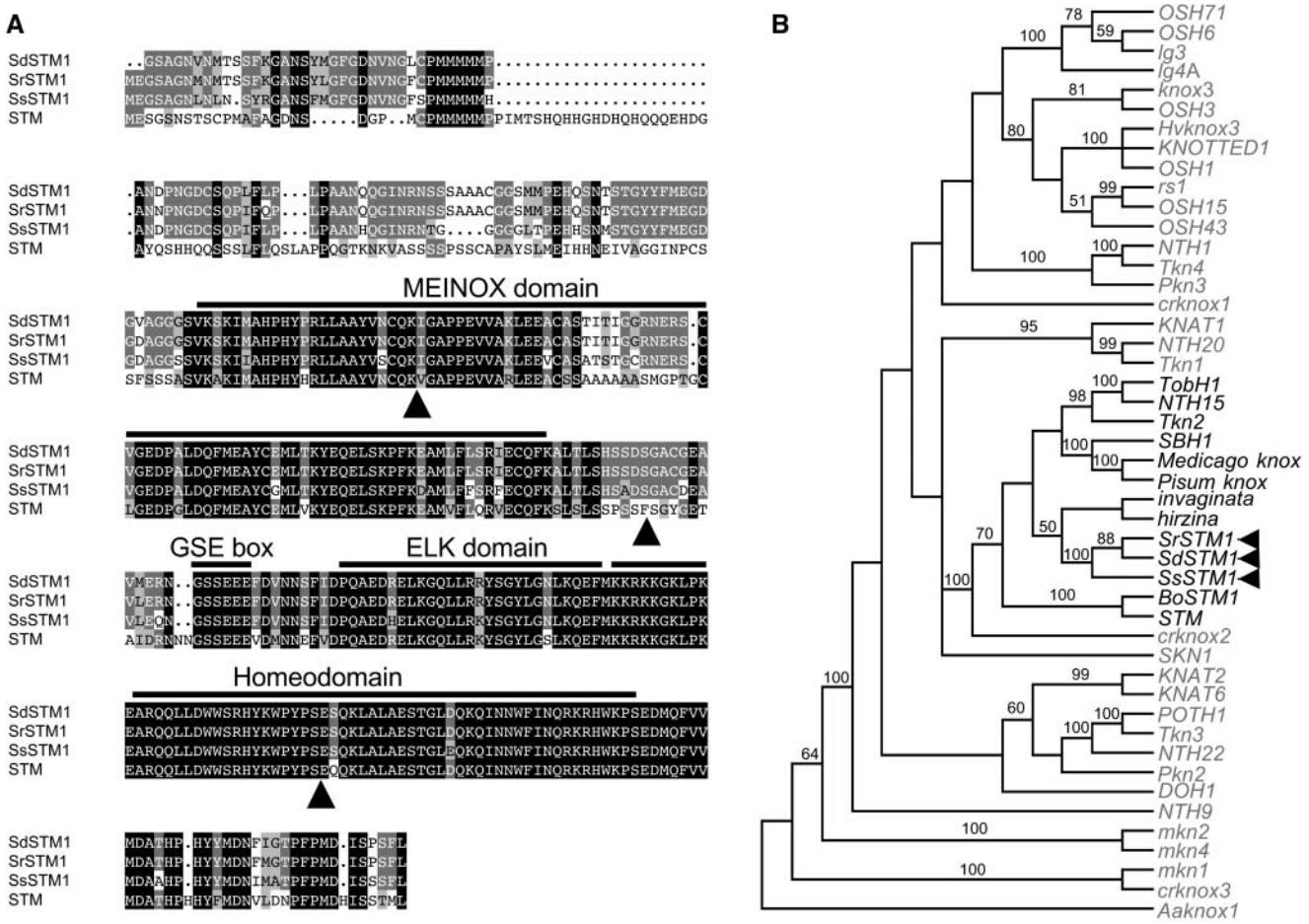

Figure 2. Structure and Phylogeny of *SSTM1* Genes.

(A) Alignment of inferred SSTM1 protein sequences from *S. dunnii* (SdSTM1), *S. rexii* (SrSTM1), and *S. saxorum* (SsSTM1) to Arabidopsis STM, showing shared conserved domains and intron positions (arrowheads). Black boxes highlight invariant amino acids, dark gray boxes amino acids conserved between Streptocarpus proteins, and light gray boxes conservative substitutions. Sequences were aligned using ClustalW.

(B) Strict consensus of the four most parsimonious *KNOX* trees using homeobox cDNA sequences. Dicot *STM*-like genes are highlighted in black and Streptocarpus genes with arrowheads. *Aaknox1*, *mkn1*, and *Crknox3* were included as outgroups and trees rooted on the algal sequence, *Aaknox1*. Bootstrap values are given above branches.

each species, supporting the existence of two *STM*-like genes in Streptocarpus (Figures 3C and 3D).

Streptocarpus *STM1* Genes Are Orthologous

To confirm that Streptocarpus *STM1* genes were likely orthologs of each other, we did a phylogenetic analysis. *STM*-like sequences were amplified from the genomic DNA of 26 species representing the variation within the genus. The amplified region contained parts of the last two exons, encoding the highly conserved homeodomain, and the divergent intervening third intron.

More than one sequence was amplified from many species, supporting the presence of two *STM*-like genes. Parsimony analysis of the highly conserved exon sequences did not fully resolve gene relationships (Figure 4A). Intron sequences from the *SSTM1* genes of three species—*S. saxorum*, *S. pallidiflorus*, and *S. glandulosissimus*—could not be included in the intron phylogeny because they contained direct imperfect repeats of ~100 nucleotides (see Supplemental Figure 1A online). However, analysis of aligned intron sequences from remaining species placed the genes into two clades (Figure 4B). This suggests that *STM*-like genes duplicated before the subgeneric split between Clades I and II Streptocarpus. One gene clade was identified as *SSTM1* (on the basis of exon sequence shared with cDNAs) and the second designated *SSTM2*.

We also investigated the phylogenetic utility of the *SSTM* third intron by comparing our *SSTM* phylogeny with a phylogeny of published *ITS* sequences (see Supplemental Figure 1B online). This showed that the *SSTM* genes provide novel, low copy

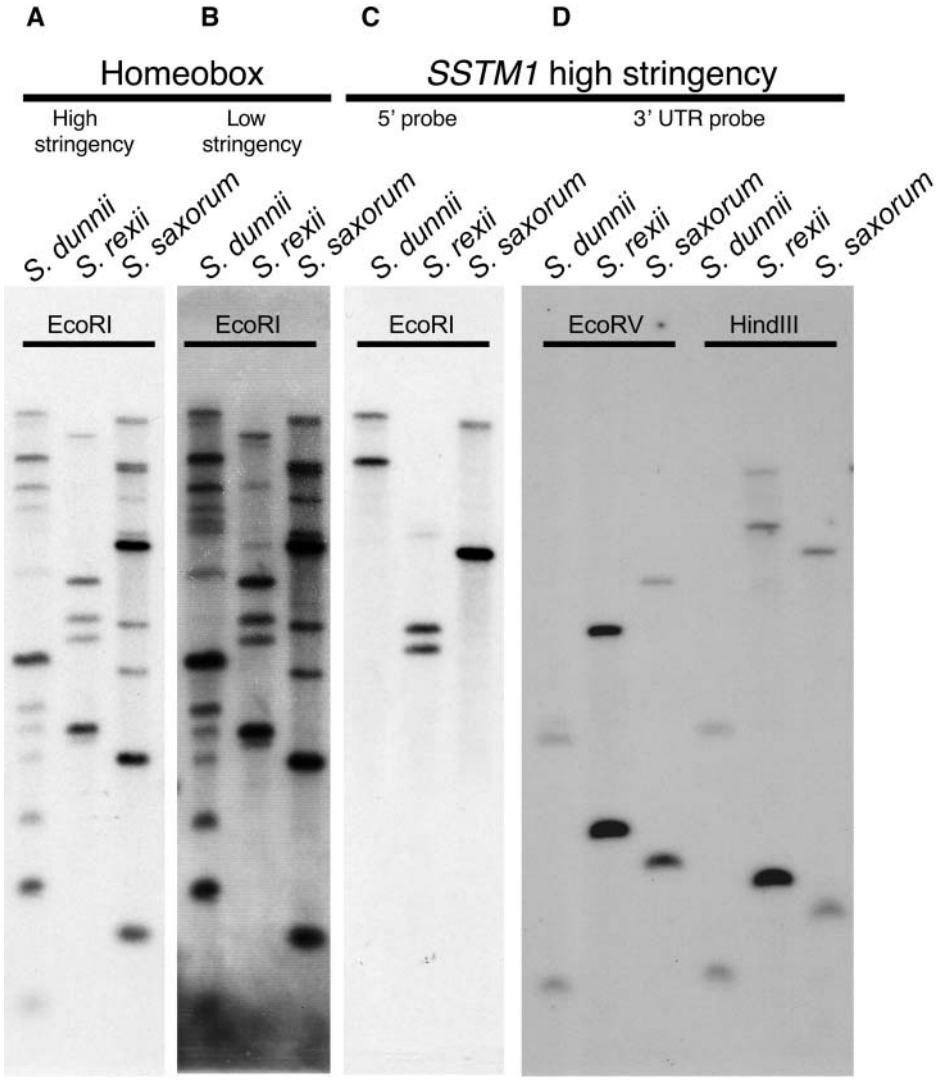

Figure 3. DNA Gel Blot Analysis of Streptocarpus *KNOX* Genes.

DNA gel blots of genomic DNA were probed at both high and low stringency with an *SSTM1* fragment that includes the homeobox (left) or at high stringency with the poorly conserved 5′ region of *SSTM1* or its 3′ untranslated region (right).

number markers for determining species level phylogeny in Streptocarpus that are likely to be useful in other plant groups.

KNOX Expression in Streptocarpus

To determine whether changes in KNOX activity may have been involved in the evolution of Streptocarpus, we compared *SSTM1* expression patterns by reverse transcription and PCR in different growth forms. In caulescent *S. saxorum*, expression was detected in apical tissues containing vegetative SAMs or inflorescence meristems and at a low level in the proximal leaf petiole but not in other parts of the leaf (Figure 5A). In unifoliate *S. dunnii*, no *SSTM1* expression was detected in leaves (plants had no inflorescences) or the petiolode (Figures 5B and 5D), although transcripts were detected in inflorescence apices using the same method (data not shown). In the rosulate *S. rexii*, *SSTM1* was expressed in the inflorescence apex but not in the leaf or distal midrib. However, in contrast with unifoliate *S. dunnii*, *SSTM1* expression was also detected in the *S. rexii* petiolode in the region from which further phyllomorphs were initiating (Figures 5C and 5D).

To further locate the sites of KNOX expression, tissue sections were challenged with antibodies raised against STM and able to recognize STM-like KNOX proteins in Arabidopsis (Lynn et al., 1999). The caulescent *S. saxorum* produces alternate whorls of three leaves from a flat SAM (Figure 6A). KNOX protein was detected in the SAM, visible as a triangular domain between the bases of existing leaf primordia in a transverse section (Figure 6B). In longitudinal sections, protein accumulation was seen in the SAM, axillary meristems, and provasculature extending into leaves but not at the sites of leaf initiation in the SAM (Figure 6D).

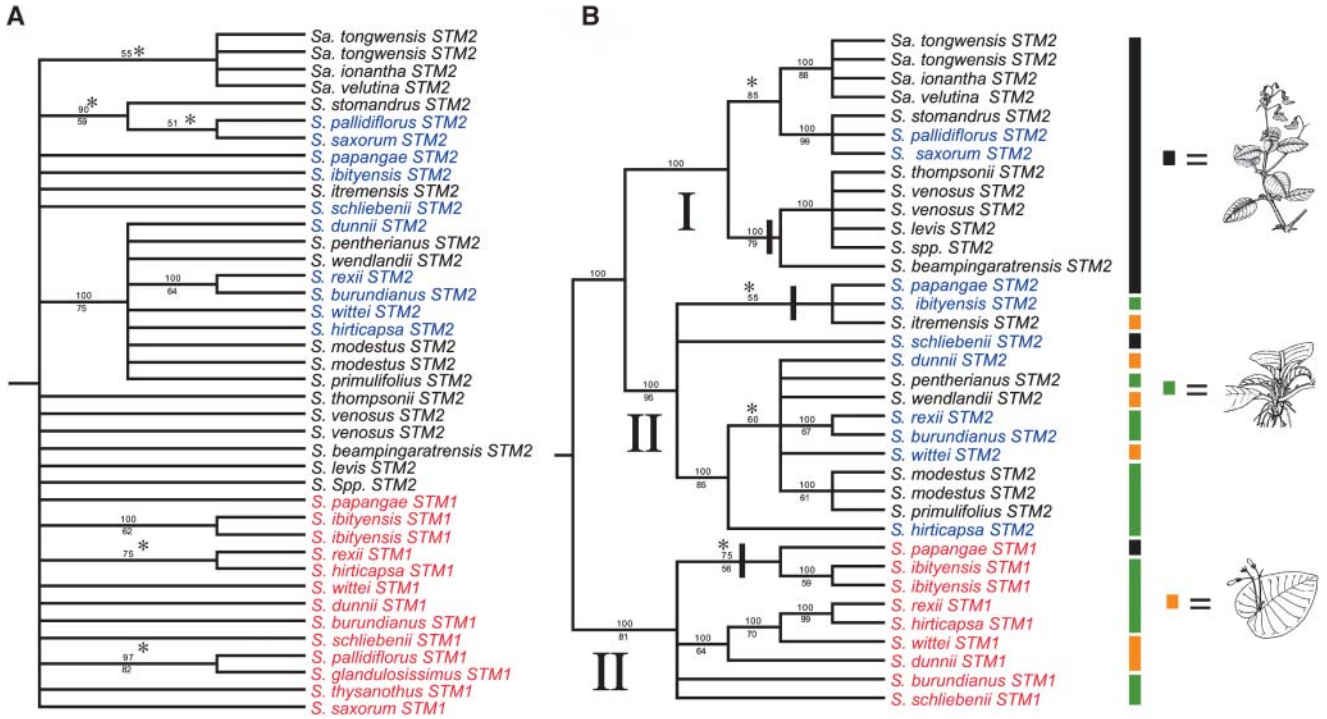

Figure 4. *SSTM* Phylogenies for Streptocarpus.

(A) Relationships between *SSTM* exon sequences flanking intron 3 derived from a midpoint-rooted majority rule consensus of 2480 most parsimonious trees. Exon sequences for *SSTM1* genes with repetitive introns are included. Percentage of the majority is given above branches with bootstrap support below. Branches that collapse in the strict consensus are indicated with an asterisk. Taxa represented more than once are highlighted in red and blue.
(B) Phylogeny of *SSTM* third intron shown as a midpoint rooted majority rule consensus of 9002 most parsimonious trees. For each paralogue (*SSTM1* or *SSTM2*) Clade I (I) and Clade II (II) species are grouped. Clades of Madagascan species are indicated by black bars across their bases. Morphological types are indicated at the right (illustrations redrawn from Hilliard and Burtt [1971] and Jong [1978]).

Signal was detected in leaves at later developmental stages (e.g., Figure 6C), suggesting that the absence of KNOX protein from the site of leaf initiation is transient. In newly initiated leaves, KNOX protein accumulated in two to three layers of cells abaxial to the prospective palisade mesophyll and persisted in these cells at the growing leaf margins and developing vasculature (Figures 6E to 6G) before becoming restricted to the vasculature (Figures 6H to 6J).

Unifoliate acaulescent species lack a conspicuous meristem until inflorescences arise from base of the midvein. We were unable to detect *SdSTM1* expression in plants without inflorescences (Figure 5B). We were also unable to detect KNOX proteins in juvenile *S. dunnii* plants (Figure 7C). Once plants started to make inflorescences, we detected KNOX proteins in the meristem (Figures 7A to 7D).

Rosulate species also lack a shoot, and new phyllomorphs are formed from the base of a mature phyllomorph on the petiolode, characterized by its cylindrical shape. Inflorescence meristems arise from the more distal laminar phyllomorph midrib. Because *SSTM1* was expressed in the petiolode of *S. rexii*, we examined KNOX protein expression in this region to determine whether phyllomorphs are initiated from regions with SAM-like properties. As expected, the STM antibody was able to detect KNOX protein in inflorescence meristems of *S. rexii* (Figures 7E and 7F). However, KNOX proteins were also observed in mounds of cells in the more proximal petiolode at a position from which new phyllomorphs would initiate (Figures 7G to 7I). The mounds of KNOX-expressing cells therefore resemble transient meristems that, on the basis of their positions, could each give rise to a single phyllomorph.

Two Loci Determine the Differences in Form in Clade II Streptocarpus

To examine the genetic basis for the differences in form in Clade II Streptocarpus, we crossed the rosulate *S. rexii* with two unifoliate species: *S. dunnii* or *S. wittei*. F1 hybrids, which were rosulate in form (Figure 1D), were backcrossed to the unifoliate parent. The forms of the *S. wittei* backcross progeny ($n = 112$) were scored in juvenile and adult plants, and the *S. dunnii* backcross progeny ($n = 16$) were scored only at maturity (Table 1; see supplemental data online). Plants with one major phyllomorph were classed as unifoliate and those with more than one as rosulate. Previous studies had suggested that the rosulate character was determined by a dominant allele at either of two loci: one promoting the rosulate character early in development, the other acting later (Oehlkers, 1938, 1942, 1964). If this were the case, the F1 plants should have been heterozygous at both

loci and therefore rosulate. Approximately half their backcross progeny should have inherited the early acting dominant allele and have developed the rosulate character early. Only 39% of the *S. wittei* backcross progeny (44/112) were scored as rosulate while juvenile, providing no support for the action of an early-acting dominant allele ($P = 0.023$ in a χ^2 test). If dominant alleles of two unlinked loci were responsible for the rosulate morphology at maturity, ~75% of the backcross progeny should have inherited at least one dominant allele sufficient to specify rosulate morphology. At maturity, 76% (98/128) of the *S. wittei* backcross progeny had rosulate morphology, consistent with the action of dominant alleles at two loci that come into effect gradually during development (Table 1; see supplemental data online). The frequency of rosulate plants in the small *S. dunnii* backcross population was also consistent with the action of two loci.

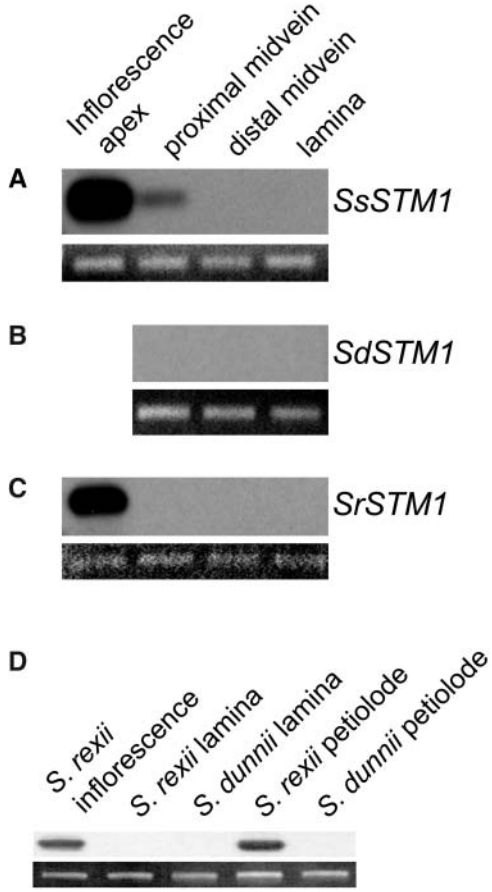

Figure 5. *SSTM1* Expression Patterns.

(A) to **(C)** The expression of *SSTM1* in inflorescence apices, proximal and distal midvein, and leaf lamina was compared by RT-PCR with a ubiquitously expressed 40S ribosomal subunit gene as a control.
(A) Caulescent *S. saxorum*.
(B) Unifoliate *S. dunnii*.
(C) Rosulate *S. rexii*.
(D) *SSTM1* expression was detected under the same conditions in the organogenic petiolode region of rosulate *S. rexii* but not in the petiolode of unifoliate *S. dunnii*.

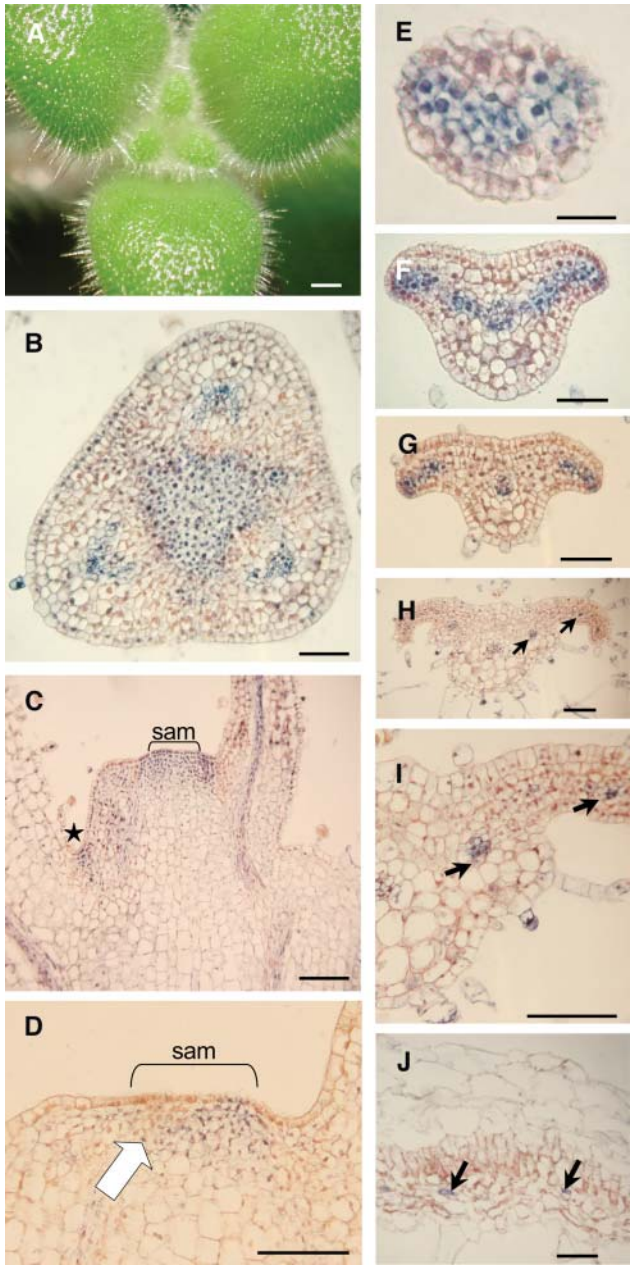

Figure 6. KNOX Immunolocalization in *S. saxorum*.

(A) A vegetative apex of *S. saxorum* showing leaves in whorls of three. New leaves are initiated directly opposite a leaf in the preceding whorl (bar = 1 mm).
(B) Transverse section of apex. Nuclear-localized KNOX proteins can be seen in the central, triangular SAM and in leaf veins (bar = 50 μm).
(C) and **(D)** Longitudinal sections of different *S. saxorum* apices, showing staining in the central SAMs, SAMs in the axils of leaves (star), and in developing veins. An unstained region was detected at the site of leaf initiation from the SAM (arrow in **[D]**). Bar = 100 μm.
(E) to **(J)** Transverse sections through leaves at increasing stages of maturity. Arrows indicate vascular localization of KNOX proteins. Bars = 25 μm in **(E)**, 50 μm in **(F)** and **(G)**, and 100 μm in **(H)** to **(J)**.

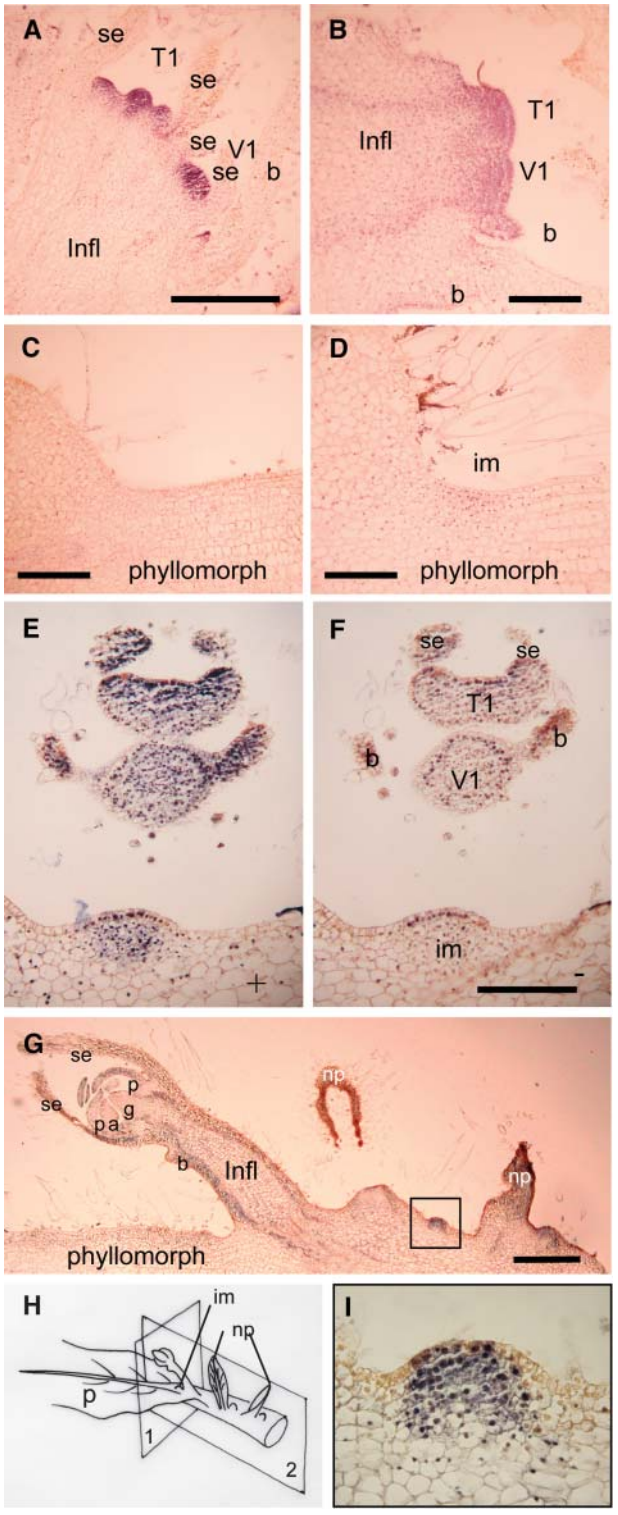

Figure 7. KNOX Immunolocalization in Acaulescent Species.

The inflorescence meristems of *S. rexii* and *S. dunnii* are formed in an acropetal sequence from the groove meristem at the proximal midrib. Each inflorescence meristem (im) gives rise to lateral bracts (b), a terminal older flower (T1) proximally, and a younger distal flower (V1) or repetitions of that unit (nomenclature according to Weber [1982]). se, sepal; p, petal; a, anther; g, gynoecium; np, new phyllomorph; Infl, inflorescence. Bars = 0.5 mm.

(A) to **(D)** *S. dunnii*.

(A) Two floral meristems in longitudinal section.

(B) A pair of floral meristems at an earlier stage in development, subtended by a bract.

(C) Longitudinal section through a juvenile phyllomorph in which we were unable to detect KNOX expression.

(D) Longitudinal section through a juvenile phyllomorph showing localized KNOX expression at its base.

(E) to **(H)** *S. rexii*. New phyllomorphs arise in two ranks from the more proximal petiolode.

(E) KNOX localization in a transverse section of an inflorescence, corresponding to plane 1 in **(H)**, and new inflorescence meristem using antibodies raised against Arabidopsis STM.

(F) An adjacent section to **(E)** probed only with secondary antibody (bar = 0.1 mm).

(G) A longitudinal section through a flowering phyllomorph corresponding to plane 2 in **(H)**.

(I) The inset region of **(G)** at higher magnification showing KNOX expression in a mound of cells at the prospective site of phyllomorph initiation.

SSTM1 Does Not Specify Rosulate Morphology

To determine whether *SSTM1* is one of the two loci directly responsible for morphological differences between unifoliate and rosulate species, and also whether *SSTM1* expression is *cis*-regulated, we followed segregation of *SSTM1* alleles in the two backcross populations.

If dominant alleles at two loci, *R1* and *R2*, determine rosulate morphology, the rosulate *S. rexii* parent will have been homozygous *R1*/*R1* *R2*/*R2* and the *S. dunnii* or *S. wittei* parent homozygous for the recessive alleles *r1* and *r2*. The F1 *S. rexii* × unifoliate hybrids will have been heterozygous at both loci and their backcross progeny composed of four different genotypes (*R1*/*r1* *R2*/*r2*, *R1*/*r1* *r2*/*r2*, *r1*/*r1* *R2*/*r2*, or *r1*/*r1* *r2*/*r2*) in equal proportions. Rosulate progeny would carry at least one dominant R_1 or R_2 allele.

SSTM1 genotypes of the backcross population were determined by amplification of an ~400-bp region of *SSTM1* followed by digestion with a restriction enzyme that cuts the *S. dunnii* or *S. wittei* products once and the *S. rexii* product twice to yield two smaller fragments (Figure 8). As expected, approximately half (62/128) of the backcross progeny were found to be homozygous for the *SSTM1* allele from the unifoliate parent, and the remainder were heterozygous (Table 1; see supplemental data online). If *SSTM1* is one of the *R* loci, unifoliate backcross progeny (*r1*/*r1* and *r2*/*r2*) should all be homozygous for the *SSTM1* allele from the unifoliate parent, and all plants carrying the *S. rexii SSTM1* allele should be rosulate. Neither of these criteria were fulfilled in the backcross populations (Figure 8, Table 1; see supplemental data online), suggesting that *SSTM1* is not one of the two loci that specifies the morphological differences between unifoliate and rosulate Streptocarpus.

To further test whether differences in SSTM1 expression correlate with form, we used backcross plants with different forms in RT-PCR. This showed that as in *S. dunnii* there was no

Table 1. Data from Backcross Plants

Backcross Family Number and RBGE Accession Number	Qualifier	Genotype	Form during Juvenile Development	Form at Flowering	Summary of Data for Each Family during Juvenile Development	Summary of Data for Each Family at Flowering
Family 1	A	d/d	–	R		
(19980107)	C	d/r	–	R		
	E	d/d	–	R		
	F	d/r	–	R		
	G	d/d	–	R		
	H	d/d	–	U		
	J	d/r	–	R		
	K	d/r	–	U		
	M	d/r	–	R		
	P	d/r	–	R		
	Q	d/d	–	R		
	R	d/r	–	R		
	S	d/d	–	R		
	T	d/r	–	R		
	U	d/d	–	U		
	V	d/d	–	U	–	12R 4U
Family 2	B	w/r	R	R		
(19982603)	C	w/w	U	R		
	D	w/w	U	R		
	E	w/w	U	R		
	F	w/r	U	R		
	G	w/r	U	R		
	H	w/w	R	R		
	I	w/r	R	R	3R 5U	8R 0U
Family 3	B	w/r	U	R		
(19982606)	C	w/r	R	R		
	D	w/w	R	R		
	F	w/r	U	R		
	H	w/r	U	R		
	I	w/w	R	R		
	J	w/r	U	U		
	K	w/w	U	R		
	L	w/w	U	R		
	M	w/w	U	R	3R 7U	9R 1U
Family 4	A	w/w	U	R		
(19982607)	B	w/w	U	U		
	C	w/r	R	R		
	D	w/w	U	R		
	E	w/r	R	R		
	F	w/r	U	R		
	G	w/r	U	U		
	H	w/r	R	R		
	I	w/w	U	U		
	J	w/r	U	U		
	K	w/r	U	R	3R 8U	7R 4U
Family 5	B	w/w	U	U		
(19982610)	D	w/w	R	R		
	E	w/r	U	R		
	F	w/r	U	R		
	G	w/w	U	R		
	J	w/w	R	R		
	K	w/w	R	R		
	N	w/r	U	R		
	O	w/r	R	R		
	S	w/r	R	R	5R 5U	9R 1U

(Continued)

Table 1. (continued).

Backcross Family Number and RBGE Accession Number	Qualifier	Genotype	Form during Juvenile Development	Form at Flowering	Summary of Data for Each Family during Juvenile Development	Summary of Data for Each Family at Flowering
Family 7	A	w/r	U	R		
(19981619)	D	w/w	R	R		
	E	w/r	U	U		
	F	w/r	U	U		
	H	w/w	U	R		
	J	w/w	R	U		
	K	w/w	U	R	2R 5U	4R 3U
Family 8	A	w/r	R	R		
(19982620)	C	w/w	R	R		
	D	w/w	R	R		
	E	w/w	R	R		
	F	w/w	U	R		
	G	w/r	U	R		
	H	w/r	R	R		
	J	w/r	U	R		
	M	w/w	U	U		
	N	w/r	U	U		
	O	w/r	R	R		
	Q	w/r	R	R	7R 5U	10R 2U
Family 9	A	w/r	U	U		
(19982621)	B	w/r	R	R		
	C	w/r	U	U		
	D	w/r	R	R		
	F	w/r	U	R		
	G	w/w	U	R		
	H	w/r	U	U		
	I	w/w	R	R		
	J	w/r	U	R		
	L	w/r	R	R		
	M	w/w	R	R		
	N	w/w	R	R		
	P	w/w	R	R	7R 6U	10R 3U
Family 10	A	w/w	U	R		
(19982627)	B	w/r	U	R		
	C	w/r	R	R		
	D	w/r	U	U		
	E	w/r	U	R		
	F	w/w	R	R		
	H	w/w	U	R		
	I	w/r	U	R		
	J	w/r	U	U		
	K	w/w	R	U		
	L	w/r	R	R		
	M	w/r	R	U		
	N	w/w	R	R		
	O	w/w	U	U		
	P	w/r	U	R		
	Q	w/r	U	R		
	R	w/w	R	R		
	S	w/w	U	U	7R 11U	12R 6U
Family 11	A	w/w	U	R		
(19982623)	B	w/w	U	R		
	C	w/r	U	U		
	D	w/r	R	R		

(Continued)

Table 1. (continued).

Backcross Family Number and RBGE Accession Number	Qualifier	Genotype	Form during Juvenile Development	Form at Flowering	Summary of Data for Each Family during Juvenile Development	Summary of Data for Each Family at Flowering
	E	w/r	U	R		
	F	w/r	U	R		
	G	w/w	R	R		
	H	w/w	U	U		
	I	w/w	U	R		
	J	w/w	R	R		
	K	w/w	U	R		
	L	w/r	R	R	4R 8U	10R 2U
Family 12	A	w/r	U	U		
(19982624)	B	w/r	U	R		
	C	w/w	U	U		
	D	w/w	R	R		
	E	w/w	U	R		
	G	w/w	U	U		
	I	w/w	U	R		
	J	w/r	R	R		
	K	w/w	U	U		
	L	w/w	U	R		
	M	w/r	R	R	3R 8U	7R 4U

Genotypes of plants are represented as d/w (the *SSTM1* allele from the unifoliate recessive parent *S. dunnii*) and r (the *SSTM1* allele from the rosulate parent *S. wittei*). U denotes plants with unifoliate form, R denotes plants with rosulate form. RBGE, Royal Botanic Garden Edinburgh.

SSTM1 expression in the juvenile phylomorph of a unifoliate plant but that there was *SSTM1* expression in the rosulate plant (Figure 8C). Thus, although *SSTM1* does not cause morphological differences between forms, its expression does correlate with form.

DISCUSSION

Monophyly of the *SSTM1* and *SSTM2* Gene Clades

We identified two paralogous Class I *KNOX* genes, *SSTM1* and *SSTM2*, in Streptocarpus. Their highly conserved homeobox is interrupted by an intron that proved useful for phylogenetic reconstruction, giving comparable resolution to *ITS*. Our phylogenetic analyses showed that the *SSTM1* genes are likely to be orthologs and, thus, that it was appropriate to compare their function between species.

Changes in KNOX Activity Accompanied Evolution of Novel Morphologies

All Old World Gesneriaceae whose early growth has been examined have suppressed plumule development and unequal cotyledon development (anisocotyly). These traits are thought to give the seeds a boost in growth when they germinate, as there is little energy stored (Burtt, 1970, 1994). Taken to its extreme, this pattern of growth results in acaulescence, and the cotyledon gives rise to the first (rosulate species) or only phyllomorph (unifoliate species).

In Arabidopsis, caulescence requires *STM* to promote SAM activity and repress leaf fate (Barton and Poethig, 1993; Byrne et al., 2000). *STM* is expressed in cells of the SAM but not in cells that will form leaf primordia (Long et al., 1996)—a pattern of expression that is conserved in diverse seed plants (e.g., Jackson et al., 1994; Sundas-Larsson et al., 1998) and that makes *STM* expression a marker for SAM cell identity. In Arabidopsis, the domain of *STM* expression overlaps with those of other Class I *KNOX* genes that are largely restricted to the SAM (e.g., Lincoln et al., 1994; Chuck et al., 1996).

This pattern of KNOX protein expression was also seen in *S. saxorum*, suggesting conservation of KNOX function in promoting activity of the SAM in caulescent Streptocarpus. However, KNOX proteins also accumulated in *S. saxorum* leaf primordia after they had initiated from the SAM. Proteins were present in a layer of cells at the adaxial–abaxial boundary and later restricted to cells of the developing leaf vasculature. A very similar pattern of KNOX expression in leaves has been described for various taxa with compound leaf primordia, which form either compound leaves or derived simple leaves (Bharathan et al., 2002). A causal relationship between KNOX activity and compound leaf development has also been demonstrated in Arabidopsis and tobacco (*Nicotiana tabacum*), where ectopic *KNOX* expression in leaves causes compounding (e.g., Chuck et al., 1996; Hay et al., 2003), and in the compound leaves of tomato (*Lycopersicon esculentum*), where increased KNOX activity increases the degree of compounding (e.g., Hareven et al., 1996). *S. saxorum* provides an exception to the correlation between KNOX expression and compound leaf morphology because, like all caulescent

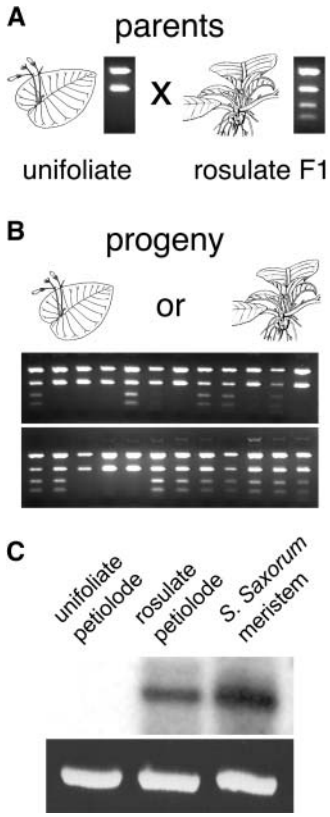

Figure 8. Inheritance of *SSTM1* Alleles and Expression in Different Growth Forms.

(A) and **(B)** *SSTM1* alleles were amplified from the F1 (*S. rexii* × unifoliate) parent of the backcross, from the unifoliate parent, and from a selection of their progeny and distinguished by restriction site polymorphisms after digestion. No correlation is apparent between *SSTM1* genotype and morphology.

(C) *SSTM1* expression correlates with plant form in backcross plants.

Streptocarpus, it produces simple leaves from simple primordia. This suggests that downstream targets of KNOX regulation needed for compounding in other taxa were either unresponsive or lost in Streptocarpus. Known targets of KNOX repression include genes needed for synthesis of GA phytohormones (e.g., Sakamoto et al., 2001). GA signaling is needed for compound leaf development in response to ectopic *KNOX* expression in Arabidopsis and in tomato leaves that normally express *KNOX* genes (Hay et al., 2002). Uncoupling of GA biosynthesis from KNOX control could explain why *S. saxorum* leaf development appears insensitive to KNOX expression.

Previous histological studies have suggested that acaulescent Streptocarpus might retain a vegetative SAM, albeit in an unconventional position (Jong and Burtt, 1975). Cells between the embryonic cotyledons of acaulescent species show the layered arrangement of conventional SAMs but do not contribute to growth after germination. Instead, a SAM-like structure subsequently appears in the petiole of the dominant cotyledon and later gives rise to inflorescence meristems as the groove meristem. This lead to the suggestion that the groove meristem represents a vegetative SAM displaced into the cotyledon petiole (termed the petiolode in this case; Jong and Burtt, 1975).

Our expression analyses with *S. dunnii*, a unifoliate acaulescent species, suggest that this is not the case because we detected no *SdSTM1* expression in juvenile plants. Our immunolocalizations corroborated this result in that we did not always detect KNOX protein accumulation in plants lacking conspicuous inflorescences. However, we did detect protein accumulation in inflorescence meristems. Thus, our results suggest that the groove (inflorescence) meristem initiates de novo in *S. dunnii* and is therefore not equivalent to a conventional SAM. This implies that the vegetative SAM was lost in evolution of the unifoliate growth form. The loss of *KNOX* expression needed for SAM formation in other angiosperms is consistent with the lack of a vegetative SAM in unifoliate Streptocarpus. *SSTM1* expression initiates as the inflorescence meristem initiates, suggesting that a change in the pattern of *SSTM1* expression, rather than a complete loss of *SSTM1* function, accompanied evolution of the unifoliate growth form.

Although the rosulate *S. rexii* also appears to lack a vegetative SAM, it expresses *SSTM1* and possibly other Class I *KNOX* genes in the mounds of petiolode cells from which phyllomorphs initiate. These mounds have also been suggested to arise from the groove meristem (Jong, 1978), although we have no direct evidence for this origin, and they may initiate de novo. They therefore resemble conventional SAMs in their structure, expression of *SSTM1*, and ability to form organs but may be unable to produce organs reiteratively (indeterminacy) and might

Table 2. Streptocarpus and Saintpaulia Species Used in This Study and Their RBGE Accession Numbers

Species	Authority	RBGE Accession Number
S. beampingaratrensis	Humbert	19972887
S. burundianus	Hilliard and Burtt	Herbarium S137a
S. dunnii	Hooker	19972909
S. glandulosissimus	Engler	–
S. hirticapsa	Burtt	19932793
S. ibityensis	Humbert	19932867
S. itremensis	Burtt	19972889
S. levis	Burtt	19982242
S. modestus	Britten	19943058
S. pallidiflorus	Clarke	19691211
S. papangae	Humbert	19972886
S. pentherianus	Fritsch	19972034
S. primulifolius	Gandoger	19912192
S. rexii	(Hook.) Lindl.	19870333
S. saxorum	Engler	19711885
S. schliebenii	Mansfeld	Herbarium S136a
Streptocarpus spp	–	19972893
S. stomandrus	Burtt	19711392
S. thompsonii	Brown	19941334
S. thysanotus	Hilliard and Burtt	No. 18 S129a
S. venosus	Burtt	19982247
S. wendlandii	Sprenger	19982266
S. wittei	Hilliard and Burtt	19981673
Sa. ionantha	Wendl.	19970092
Sa. tongwensis	Burtt	19970096
Sa. velutina	Burtt	19872179

therefore result from redeployment of only part of the mechanism needed for SAM formation and function. The full mechanism is presumably employed on formation of inflorescence meristems from the groove meristem.

In Streptocarpus, we found that the difference in *SSTM1* between a unifoliate and rosulate species correlated with the formation of additional phyllomorphs from SAM-like structures. Crosses between rosulate and unifoliate Streptocarpus indicated that the rosulate character, and hence vegetative *SSTM1* expression, was specified by dominant alleles at two loci. Neither locus corresponded to *SSTM1*. This suggested (1) that vegetative *SSTM1* expression was not sufficient to specify the SAM-like structures of rosulate Streptocarpus and (2) that the differences in *SSTM1* expression were not the result of differences in *SSTM1* alleles themselves but in the genetic prepattern of other factors that regulate *SSTM1* expression.

These conclusions are consistent with the findings from Arabidopsis and other model angiosperms that have shown that *STM* is only one of several genes required for normal SAM formation and function (reviewed in Veit, 2004). Other genes include *WUSCHEL* (*WUS*), which is expressed independently of *STM* and also required for SAM indeterminacy (Mayer et al., 1998). WUS or STM activity alone is insufficient for SAM formation, but WUS and STM together specify at least transient SAMs (Gallois et al., 2002). Expression of cofactors like WUS might therefore be required for the vegetative SAM-like structures of rosulate Streptocarpus. Other genes are known to promote *STM* and *WUS* expression, including the *CUP-SHAPED COTYLEDON* (*CUC*) genes that are both necessary and sufficient for *STM* expression and SAM formation in Arabidopsis (Takada et al., 2001). Therefore, differences in regulatory genes like *CUC* might underlie the evolutionary differences between unifoliate and rosulate Streptocarpus and between caulescent and acaulescent species.

Table 3. GenBank Accession Numbers of Genes Isolated in This Study

Species	Gene	Accession Number
S. beampingaratrensis	*S. beampingaratrensis STM2*	AY662116
S. burundianus	*S. burundianus STM1*	AY662123
	S. burundianus STM2	AY662108
S. dunnii	*S. dunnii STM1*	AY655752
	S. dunnii STM2	AY662103
S. glandulosissimus	*S. glandulosissimus STM1*	AY662126
S. hirticapsa	*S. hirticapsa STM1*	AY662122
	S. hirticapsa STM2	AY662109
S. ibityensis	*S. ibityensis STM1a*	AY662120
	S. ibityensis STM1b	AY662121
	S. ibityensis STM2	AY662100
S. itremensis	*S. itremensis STM2*	AY662101
S. levis	*S. levis STM2*	AY662117
S. modestus	*S. modestus STM2a*	AY662110
	S. modestus STM2b	AY662111
S. pallidiflorus	*S. pallidiflorus STM1*	AY662125
	S. pallidiflorus STM2	AY662097
S. papangae	*S. papangae STM1*	AY662119
	S. papangae STM2	AY662099
S. pentherianus	*S. pentherianus STM2*	AY662104
S. primulifolius	*S. primulifolius STM2*	AY662112
S. rexii	*S. rexii STM1*	AY655753
	S. rexii STM2	AY662106
	40S	AY796341
S. saxorum	*S. saxorum STM1*	AY655754
	S. saxorum STM2	AY662098
S. schliebenii	*S. schliebenii STM1*	AY662124
	S. schliebenii STM2	AY662102
Streptocarpus spp	*Streptocarpus* spp *STM2*	AY662118
S. stomandrus	*S. stomandrus STM2*	AY662096
S. thompsonii	*S. thompsonii STM2*	AY662113
S. thysanotus	*S. thysanotus STM1*	AY662127
S. venosus	*S. venosus STM2a*	AY662114
	S. venosus STM2b	AY662115
S. wendlandii	*S. wendlandii STM2*	AY662105
S. wittei	*S. wittei STM1*	AY655755
	S. wittei STM2	AY662107
Sa. ionantha	*Sa. ionantha STM2*	AY662094
Sa. tongwensis	*Sa. tongwensis STM2a*	AY662092
	Sa. tongwensis STM2b	AY662093
Sa. velutina	*Sa. velutina STM2*	AY662095

Table 4. Gene Accession Numbers for *KNOX* Phylogeny

Species	Gene	Accession Number	Species	Gene	Accession Number
Oryza sativa	*OSH71*	AB028885	*Medicago trunculata*	*M. trunculata knox*	AF308454
	OSH6	AB028883	*A. thaliana*	*KNAT1*	U14174
	OSH3	AB028882		*STM*	U32344
	OSH1	D16507		*KNAT2*	U14175
	OSH15	AB016071		*KNAT6*	AB072361
	OSH43	AB028884	*Antirrhinum majus*	*hirzina*	AY072735
Zea mays	*lg3*	AF100455		*invaginata*	AY072736
	*lg4*A	AF457121	*Nicotiana tabacum*	*NTH1*	AB025573
	*Knox*3	BQ486722		*NTH20*	AB025714
	KNOTTED1	X61308		*TobH1*	AY169493
	rs1	L44133		*NTH15*	AB004785
Acetabularia acetabulum	*Aaknox1*	AF170172		*NTH9*	AB025713
Ceratopteris richardii	*Crknox1*	AB043955		*NTH22*	AB025715
	Crknox2	AB043956	*Lycopersicon esculentum*	*Tkn1*	U32247
	Crknox3	AB043957		*Tkn3*	U76408
Physcomitrella patens	*mkn1*	AF285148		*Tkn4*	AF533597
	mkn2	AF285147		*Tkn2*	U76407
	mkn4	AF28417			
Ipomoea nil	*Pkn3*	AB016002	*Picea mariana*	*SKN1*	U90091
	Pkn2	AB016000	*Solanum tuberosum*	*POTH1*	U65648
			Dendrobium grex	*DOH1*	AJ276389
Brassica oleracea	*BoSTM1*	AF193813	*Hordeum vulgare*	*Hvknox3*	X83518
Pisum sativum	*P. sativum knox*	AF063307	*Glycine max*	*SBH1*	L13663

METHODS

Twenty-six different species were used in this study (Table 2; see supplemental data online). Tissue was obtained from glasshouse-grown

Table 5. Tree Statistics for Phylogenies

	KNOX Gene Family Phylogeny	*ITS*	*SSTM* Coding Phylogeny	*SSTM* Intron Phylogeny
Length of aligned sequence (bp)	564	630	57	169
Number of variable characters	508	276	13	88
Number of parsimony informative characters	456	156	9	68
Percentage of informative characters	80.8	24.7	22.81	40.2
Number of trees	4	32	2480	9002
Length of trees	6151	556	25	98
CI	0.195	0.678	0.8	0.776
RI	0.498	0.648	0.932	0.924

plants or herbarium specimens at the Royal Botanic Garden Edinburgh. Genomic DNA for PCR was extracted as by Doyle and Doyle (1987) and for DNA gel blot hybridizations using the method of Chen and Dellaporta (1994).

STM-like sequences were amplified from genomic DNA using primers Fc (5′-TGGAGCCGCCACTACAAATG-3′) and GR22 (5′-TGAACCAGTTGTTGATYTGCTT-3′) designed to homeobox sequences conserved between Class I *KNOX* genes of *Arabidopsis thaliana* and *Antirrhinum majus* by Ian Oliver and Michael Möller. These were used in 50 μL of PCR with 20 ng of genomic DNA, 2 mM $MgCl_2$, and 1 mM primers in 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (1 min). PCR products were gel purified and either sequenced directly or after cloning into a plasmid. Sequences have been deposited in GenBank, and their accession numbers are given in Table 3 (see supplemental data online).

RNA was extracted using Trizol reagent (Gibco BRL, Paisley, UK). For 3′ RACE, reverse transcription from 1 μg of total DNAase-treated RNA was primed using Qt primer (Frohman, 1995). Nested amplification was performed using primers PHRILL (5′-CCRCACTACCCTCGCCTCTT-3′) and Qo (Frohman, 1995), then MEINOX1 (5′-CGCCTGAAGTKGTGGCAAAG-3′) and Qi (Frohman, 1995). The same PCR conditions were used as for amplification from genomic DNA, except that annealing was performed at 55°C with primers at a final concentration of 0.2 mM.

For 5′ RACE, reverse transcription was primed with ELKup (5′-GCTTGGGGRTCAATGAAACTGT-3′) and cDNA products ligated to an adaptor (Siebert et al., 1995). DNA was amplified using primers MEINOX2 (5′-AAGGGCTTTGAACTGRCAYTC-3′) and LAPCRT3 (Siebert et al., 1995).

To test for the presence of transcripts in different tissues, total RNA was treated with DNase and used in reverse transcription with Qt primer. Multiplex PCR was then performed with two *SSTM1* primers, MegsD (5′-CACGCAGTAGTGTGTGTAATGGAG-3′) and ELKup, and two primers for a constitutively expressed Streptocarpus gene, ribosomal up (5′-CCTGCAACTTGGTGGTACGGTA-3′) and ribosomal down (5′-CGACACACCCCTGGTACTTT-3′) as a control. Both primer pairs span introns. *SSTM1* products were detected by DNA gel blot hybridization with a DIG-labeled probe from the conserved region of *S. rexii SSTM1* (see below).

For DNA gel blot hybridization, 5 μg of genomic DNA was digested with *Eco*RI, *Eco*RV, or *Hin*dIII. Fragments were separated, blotted, and hybridized as by Langdale et al. (1991). High stringency hybridization was performed in 3× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and low stringency hybridization in 5× SSC at 58°C.

Immunolocalizations were performed essentially according to Donlin et al. (1995) using paraffin embedded tissue. The anti-STM antibody (a gift from Kathy Barton) was used at 1:500 dilution and detected with an alkaline-phosphatase conjugated secondary antibody. Nonspecific binding was blocked with 10% horse serum in PBS containing 0.1% BSA.

Sequence Alignment and Phylogenetic Analysis

SSTM1 and *SSTM2* intron sequences were aligned manually using Se-Al v2.0a11 (Rambaut, 1996). *ITS* sequences were aligned as discussed previously (Möller and Cronk, 2001), except that gaps no longer necessary in the reduced data set were removed. Sequences for the placement of *SSTM1* within the *KNOX* gene family were aligned using ClustalW and adjusted manually using Se-Al v2.0a11. Positions that were not unambiguously alignable were excluded from analyses. The *KNOX* phylogeny was rooted on *Aaknox1* (an algal *KNOX* gene that shows features of Class I and Class II *KNOX* genes). To verify that Class I *KNOX* genes fell in a separate clade from Class II genes, two Class II genes (*mkn1* from a moss and *Crknox3* from a fern) were also included. Accession numbers of *KNOX* sequences are given in Table 4 (see supplemental data online), and alignments have been submitted to TreeBASE (www.treebase.org/treebase/).

PAUP version 4.0b10 (Swofford, 1998) was used to generate Streptocarpus phylogenies. For all analyses, heuristic searches were performed using parsimony. Trees were started using stepwise addition, followed by 5000 random addition sequence replicates and then swapping on best trees. Branch swapping used tree bisection and reconnection with the MULTREES option on. For each addition sequence replicate, two trees of score ≥5 were saved. Tree statistics are shown in Table 5.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY655752 to AY655755 and AY662092 to AY662127.

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