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Evolution of extreme specialization within a lineage of ectomycorrhizal epiparasites

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Monotropes (Monotropoideae, Ericaceae) are achlorophyllous, epiparasitic plants that receive all of their fixed carbon from green plants through a common ectomycorrhizal association rather than by a direct parasitic connection (Fig. 1)[1,2]. Using molecular identification methods we show that some monotropes are highly specific in their fungal associations and at least one species, *Pterospora andromedea*, is specialized on a single species group within the genus *Rhizopogon*. Phylogenetic analysis of the Monotropoideae shows that specialization has been derived through narrowing of fungal associations within the lineage containing *P. andromedea*. High specificity is contrary to past predictions for the Monotropoideae and for plant communities in general, raising many questions about the roles of mycorrhizal specificity in ecosystem function.

Ectomycorrhizae are mutualistic associations between a vascular plant root and a fungus; they are the dominant nutrient-gathering organs in most temperate forest ecosystems[3]. These mutualisms vary from general to specific, but when specificity occurs it is one-sided: fungal species may be specific to a single plant species or genus, but plants typically have dozens or even thousands of fungal associates[4,5]. Plant specificity has been assumed to inhibit the ability of establishing seedlings to form new mycorrhizal associations[6,7], and because monotropes are entirely dependent on these associations, it has been suggested that they should be generalists[6]. This view was supported by a previous survey of monotrope associates that was based primarily on proximity of these plants to fungal sporocarps[8].

To test the hypothesis that monotropes associate with many unrelated fungi, we used three different complementary polymerase chain reaction-based methods to identify fungal associates of four of the most common monotrope species: *Pterospora andromedea*, *Monotropa hypopithys*, *Monotropa uniflora*, and *Sarcodes sanguinea*. Results show a variety of fungal associates and levels of specificity within the monotropes (Table 1). *Sarcodes sanguinea* appears to be a generalist that associates with at least three unrelated families of fungi. Although our sampling of *Monotropa* species was more limited, our results indicate that both may be specialists. *Monotropa hypopithys* was associated only with suilloid fungi, a monophyletic group that includes *Rhizopogon* and *Suillus*[9,10], whereas *M. uniflora* individuals collected from widely divergent habitats were all associated with fungi in the Russulaceae. *Pterospora andromedea* was found to be an extreme specialist. All 31 individuals, collected from a broad geographic region, were associated with the *Rhizopogon subcaerulescens* species group (Table 1). These results indicate a higher level of ectomycorrhizal specificity for *P. andromedea* than has been reported for any ectomycorrhizal plant. In comparison, *Alnus rubra*, which is considered to be exceptionally specialized, associates with 30 fungal species, many of which are only distantly related to each other[11].

We can reject the idea that specificity of *P. andromedea* is based on a lack of opportunity to associate with other fungi for the following reasons. (1) Ectomycorrhizal fungal diversity is very high within small pine monocultures; even adjacent root tips are frequently colonized by different fungi[12]. (2) Suilloid fungi, though they produce numerous fruiting bodies in such forests, often account for a very low percentage of the colonized tree roots[13,14]. (3) In the five locations where *P. andromedea* occurred with *M. hypopithys* or *S. sanguinea*, the latter two species were associated with different fungi (Table 1). (4) We analysed host tree roots present within the root ball of one *P. andromedea* and adjacent tree roots within 0.5 metres of three other *P. andromedea* plants and found that *R. subcaerulescens*, though present on the

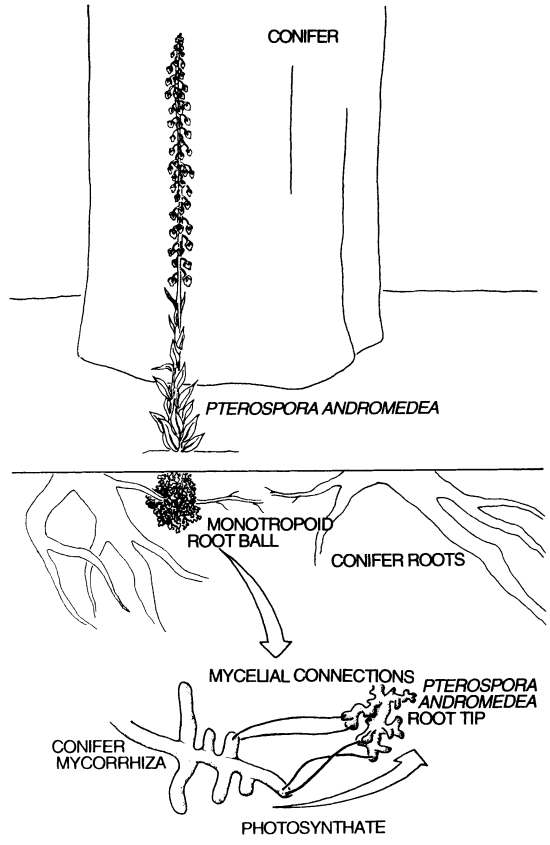

FIG. 1 Schematic summary of mycorrhizal epiparasitism in the Monotropoideae. Carbon fixed by the surrounding trees is transferred via a shared mycorrhizal fungus to the achlorophyllous monotrope: no direct connection between the two plants exists. The monotropes discussed in this paper have a compact root ball of mycorrhizae. The tree mycorrhizae, which are more diffuse in arrangement and more diverse in fungal associates, are often found within the root balls of the monotropes but are morphologically distinct.

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tree roots, was a minor component of the diverse fungal community present both within and near the rootballs. (5) At one site we collected fruit bodies of *Rhizopogon* species from the immediate vicinity of *P. andromedea*, and found that two ITS-RFLP patterns were represented in the three collections of *Rhizopogon* made. Neither of these patterns matched those from the *P. andromedea* samples even though one of these collections was made within 15 cm of a *P. andromedea* individual. Thus, *P. andromedea* clearly associates with a highly specific subset of the mycorrhizal fungal species present at a site.

Specificity is a common phenomenon in parasites, but detailed knowledge of how and why specificity evolves in parasitic lineages is often lacking[15,16]. In this case we have a good estimate of the phylogeny of the monotropes from previous molecular phylogenetic analysis; this shows that the genus *Monotropa* is clearly polyphyletic and that the closest relatives of the monotropes are likely to be chlorophyllous members of the genus *Arctostaphylos*[17] (Arbutoideae, Ericaceae). Overlaying the pattern of mycorrhizal associations onto the tree reveals that specificity is derived by a narrowing of mycorrhizal association from generalist, to suilloid specialist, and ultimately to specialization on *R. subcaerulescens* (Fig. 3). One could suggest that this resulted from fungal hosts evolving defences against the monotropes or by a gradual loss of host opportunity through ecological narrowing; in these scenarios, suilloids are the only fungi without an effective defense or the only ones common within the narrowed habitat. However, the rarity and diminutive size of these plants argues against them exerting significant selective pressure on the wide range of other common fungi. The frequency of other mycorrhizal fungi in these habitats also shows that monotrope specificity is not based on a lack of non-suilloid fungal associates.

We argue that specificity resulted from a one-sided selection on these parasitic plants to increase their fitness by optimizing carbon allocation, rather than from co-evolution in the sense of reciprocal selection[18]. Suilloid fungi are among the most host-specialized ectomycorrhizal associates of the Pinaceae, many being restricted to single genera or species groups[19,20]. Furthermore, although *Suillus* and *Rhizopogon* species can form mycorrhizae with non-host plants, these associations are usually aberrant in form, appear to have reduced function, and can result in preferential nutrient transfers from the 'non-host' to the specialized pinaceous host[20,21]. Thus, it is likely that physiological accommodation by the monotropes would be necessary in order for them to benefit fully from a suilloid association. But what is the benefit of this accommodation if the price is the loss of opportunity to form associations with other common fungi? The same question could be applied to the suilloids: what have these fungi gained by specializing on the Pinaceae that outweighs the loss of opportunity afforded by a generalist strategy? One obvious hypothesis is that the suilloids, through specialization, have become more efficient at obtaining photosynthate from their hosts, and the most widespread monotropes in these pinaceous ecosystems have in turn specialized on the most efficient conduit of fixed carbon.

Regardless of whether this hypothesis is correct, these results demonstrate that an extreme in specialization on the plant side of ectomycorrhizae does exist, that it exists precisely where it was not

TABLE 1 Molecular identifications of fungal associates

Monotrope taxa	Number of plants	Fungi identified	Methods	Monotrope taxa	Number of plants	Fungi identified	Methods
P. andromedea (31)	3	suilloid gr.	Mt-LrRNA seq.	*M. uniflora* (6)	6	Russulaceae	Mt-LrRNA seq.
	30	suilloid gr.	TSOP	*S. sanguinea* (12)	6	suilloid gr.	TSOP and Mt-LrRNA seq.
	30	*Rhizopogon* or close relatives	TSOP				
	31	*R. subcaerulescens* gr.	ITS-RFLP and ITS seq.		4	*Rhizopogon* or close relatives	TSOP
					2	*Suillus*	TSOP
Monotropa hypopithys (9)	9	suilloid gr.	Mt-LrRNA seq.		5	Cantharellaceae	Mt-LrRNA seq.
	2	*Rhizopogon* or close relatives	TSOP		1	non-suilloid unknown fungi	Mt-LrRNA seq., TSOP
	1	*Suillus*	TSOP				
	6	suilloid gr. - genus not clear	TSOP				

Identification methods. *Mt-LrRNA seq* was based on sequence determination of the ML5/ML6 portion of the mitochondrial large subunit gene followed by phylogenetic placement of the unknown sequences (Fig. 2); Results of this method allowed us to identify most of the fungi to the level of family or subfamily. *TSOP* is based on taxon-specific oligonucelotide probing of the adjacent ML7/ML8 region of the same gene[22]. The five probes used specifically identify the Suilloid group (probe US1) as whole, which is a monophyletic lineage consisting of *Rhizopogon*, *Suillus*, *Truncocolumella*, *Alpova*, *Melanogaster* and the Gomphidiaceae, and the following taxonomic groups within it: portions of the Gomphidiaceae (probes G1 and G2), *Rhizopogon* and close hypogeous relatives (probe R1), and *Suillus* (probe S1). Identification to the suilloid group only, as in some *Monotropa hypopithys* samples, was based on strong hybridizations to the US1 probe and an absence of hybridization to the more specific probes. *Suillus tomentosus*, the most common species of *Suillus* in the Yellowstone area, would produce such a result, as it does not hybridize to the S1 probe[22]. Sequences, exact specificities and hybridization conditions have been described previously[22]. Fungal symbionts of *P. andromedea* were further identified to species by analysis of the internal transcribed spacer region (ITS), which is a nuclear encoded portion of the rDNA repeat unit[23]. We used restriction fragment length polymorphisms (RFLPs) of the ITS region to screen all *P. andromedea* root samples and to compare these samples to identified fungal samples collected from the same sites. The enzymes *Alu*I, *Hin*f I, and *Mbo*I were used as described elsewhere[23]. A total of six different RFLP patterns was found. One of these was an exact match to that derived from a *R. subcaerulescens* collection from the Oregon site; the others appeared to differ only slightly from this pattern. We then sequenced the ITS region from representatives of each different ITS-RFLP pattern and found that sequence differences among the patterns varied from 0.3 to 2%. Because this level of variation is consistent with that found within other basidiomycete species or species groups[24–26] we conclude that all *P. andromedea* root samples examined were associated with a member of the *R. subcaerulescens* species group. Note that this result is also consistent with the TSOP and Mt-LrRNA tests. **Sampling.** Total numbers of plant individuals sampled are given in parentheses following the monotrope species; numbers from this sample that were tested and identified with each diagnostic method are given in column two. Except for *M. hypopithys*, which was sampled only from Yellowstone Park, samples were taken from diverse habitats representing the full spectrum of environmental preferences of each species, and 1–3 individuals were taken from each site sampled. Collection locations follow: *P. andromedea*, California Sierra Nevada Mountains (mixed conifer, 10 sites in 3 counties); Oregon (*Pseudotsuga* dominated forest, 1 site), Yellowstone National park, Wyoming (*Pinus contorta* dominated forest, 6 sites, one of which had soils modified by geyser activity); *M. hypopithys*, Yellowstone National Park, Wyoming (*Pinus contorta* forest, 6 sites); *M. uniflora*; near Corvallis Oregon (mixed conifer/deciduous forest, 2 sites), Washington/Oregon border (sand dune ecosystem, 2 sites) and Minnesota (mixed conifer/hardwood, 2 sites); *S. sanguinea*, central Sierra Nevada mountains of California (mixed conifer forest, 6 sites in 3 counties).

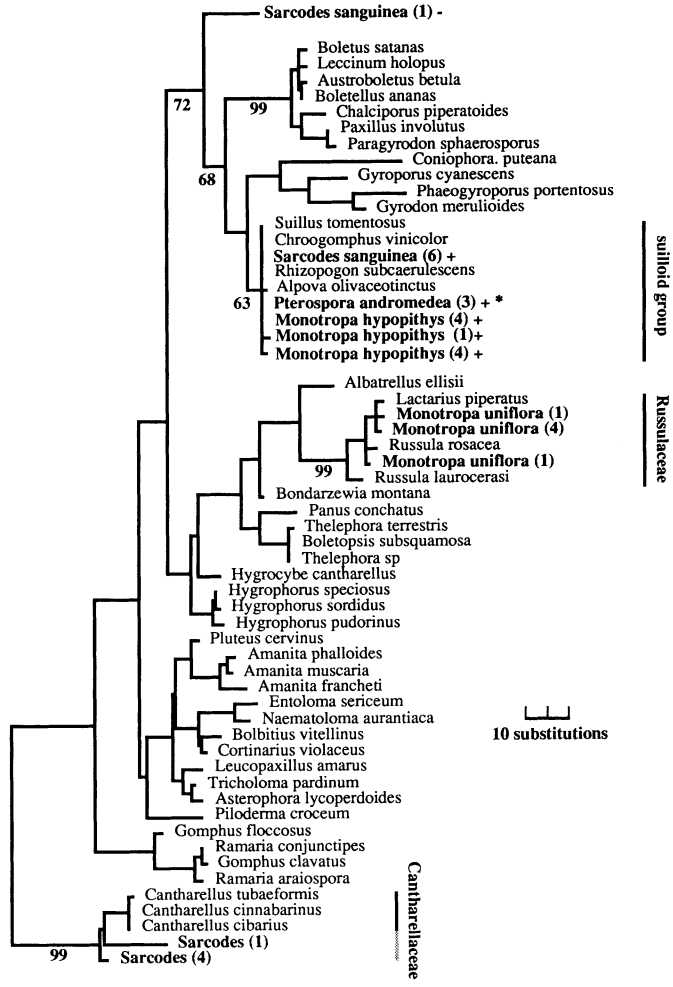

FIG. 2. Phylogenetic placement of ectomycorrhizal fungi associated with the Monotropoideae based on sequence analysis of a 350-base-pair region of the mitochondrial LrRNA gene. Known fungi are shown in light type; unknown fungi are shown in bold and named by the monotrope species with which they were associated. The small size and conserved nature of the sequence limits the phylogenetic resolution of this tree. However, placement of the unknowns within tight terminal clusters such as the Russulaceae and the Cantharellaceae are strongly supported by bootstrap analysis; numbers at key branches are based on 500 bootstrap replicates. In the case of the suilloid group, unknown sequences differ from known suilloid sequences by 0 to 0.5%, and are confirmed as suilloid (+) within the cluster and as non-suilloid (−) outside it by oligonucleotide probes targeted at other regions (Table 1). *Pterospora andromedea* samples are further confirmed as suilloid by ITS RFLP and sequence analysis that place them within the *R. subcaerulescens* species group (*) (Table 1). The tree shown is a subset of a larger neighbour-joining analysis of Kimura two parameter distances for 103 taxa of identified basidiomycetes representing 42 genera in 12 families. The pruned taxa are additional members of clades already well represented in the above tree and their presence or absence does not affect the placement of the unknown sequences. Gaps introduced for alignment purposes were treated as missing data. Bootstrapping, Kimura distances, and tree construction were based on the SEQBOOT, DNADIST and NEIGHBOR programs of the PHYLIP 3.5c package[27] complied for a Sun Sparc station. Parsimony analysis based on PAUP 3.1.1 (ref. 28) on a MacIntosh Quadra yielded the same placement of unknowns within the groups indicated when the subset of taxa shown was used; the full data set, however was too large for parsimony analysis with our computers. The root of the tree shown (the Cantharallaceae) was arbitrarily chosen for convenience of display, but it is near the midpoint by parsimony criteria. Horizontal distances correspond to the minimum number of inferred substitutions (that is, parsimony criteria); vertical distance is arbitrary. Details concerning this data set will be reported in more detail elsewhere (T. D. B. *et al.*, manuscript in preparation)

expected[6,8], and that it occurs with fungal taxa that were thought to be nearly exclusive specialists of members of the Pinaceae. Furthermore, it means that key species of fungi are essential for the existence of these plants. This point is in contrast to the commonly espoused view that microbial species are functionally redundant.

Although the Monotropoideae are rare plants and their specialization may represent an extreme, it is clearly an extreme in a continuum that has close evolutionary connections with *Arctostaphylos*[17] (Fig. 3), one of the most common genera of understory shrubs in pinaceous ecosystems of western North America. Thus, specialization by these epiparasites demands that we re-evaluate our view of the evolution and role of specificity within the larger realm of ectomycorrhizal community structure and ecosystem function. □

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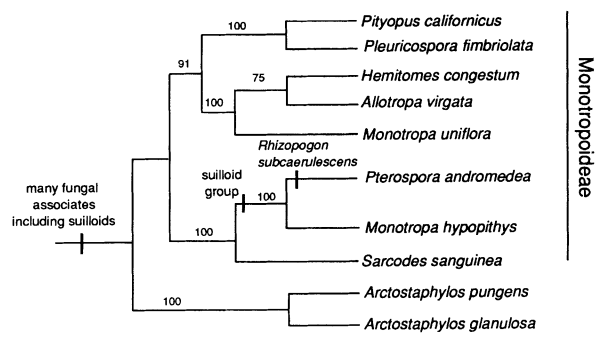

FIG. 3 Evolution of extreme specialization within a clade of the Monotropoideae. Cladogram is taken from a previous study and is based on parsimony analysis of partial 28S rRNA gene sequences[17]. Numbers at nodes are bootstrap indices of support based on a sample of a 1,000 replicates. Bars represent inferred location of changes in host association within the *P. andromedea*, *M. hypopithys*, and *S. sanguinea* clade. *Arctostaphylos* spp. of the Arbutoideae (Ericaceae), the subfamily most closely related to the Monotropoideae[17], are common in the understorey of coniferous forests, and are known to be generalists that associate with many fungi including *Rhizopogon* species[19,29]. *Sarcodes sanguinea* is a generalist with a similar, though possibly narrower range of associates that frequently includes suilloid fungi. *Monotropa hypopithys*, the closest relative of *P. andromedea*, appears to be restricted to associations with suilloid fungi, while *P. andromedea* associates exclusively with the *R. subcaerulescens* species group across its western geographic range. The clade containing *P. andromedea*, *M. hypopithys*, and *S. sanguinea* is strongly supported as a monophyletic group (bootstrap > 99%). The association of *M. uniflora* with the Russulaceae is not indicated with a bar because we do not know the fungal associates of other members of its clade. Note that the genus *Monotropa* as represented by this tree is polyphyletic. This conclusion is also corroborated by biochemical and morphological character sets[17]. Furthermore, the polyphyletic nature of this genus is supported by the finding that the *M. hypopithys* lineage associate specifically with suilloid fungi while *M. uniflora* associates only with fungi in the Russulaceae.

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A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*

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THE *KNOTTED* class of plant genes encodes homeodomain proteins[1,2]. These genes have been found in all plant species where they have been sought[3–5] and, where examined, show expression patterns that suggest they play an important role in shoot meristem function[5–7]. Until now, all mutant phenotypes associated with these genes have been due to gain-of-function mutations[4,5,8,9], making it difficult to deduce their wild-type function. Here we present evidence that the *Arabidopsis SHOOTMERISTEMLESS* (*STM*) gene, required for shoot apical meristem formation during embryogenesis, encodes a class I KNOTTED-like protein. We also describe the expression pattern of this gene in the wild-type plant. To our knowledge, *STM* is the first gene shown to mark a specific pattern element in the developing plant embryo both phenotypically and molecularly.

The angiosperm shoot apical meristem (SAM) forms during embryogenesis and acts to generate leaves, stem and floral organs throughout the lifetime of the plant. *Arabidopsis* seedlings homozygous for recessive mutations induced by ethylmethane sulphonate (EMS) at the *SHOOTMERISTEMLESS* (*STM*) locus fail to develop a SAM during embryogenesis[10] (Fig. 1). The converse phenotype, the development of many, ectopic SAMs, has been observed in tobacco plants that constitutively express the wild-type maize *KNOTTED1* gene[11]). Because gain-of-function and loss-of-function mutations in regulatory genes often result in opposite phenotypes, we hypothesized that the *stm* mutations were the result of loss-of-function mutations in an *Arabidopsis KNOTTED*-like gene. An *Arabidopsis KNOTTED* homologue, the meriHB1 cDNA, has been isolated (C. Granger *et al.*, manuscript submitted) that is located near *STM* on chromosome I. No recombination was found between *stm-1* and a restriction-fragment length polymorphism (RFLP) detected by meriHB1 (0/74 chromosomes tested), indicating that *stm-1* is within 5 centimorgans (cM) of meriHB1 (95% confidence interval).

The genomic DNA sequence corresponding to meriHB1 was determined for two mutant alleles of *STM*; both *stm-1* and *stm-3* harbour mutations predicted to reduce gene function dramatically (Fig. 2). The *stm-1* allele carries a nonsense mutation upstream of the homeodomain. The *stm-3* allele carries a mutation at the 3′ splice acceptor site of the first intron. Similar mutations greatly reduce the use of this splice acceptor site in a variety of species[12–14]. Based on the close linkage of *STM* to the meriHB1 gene and the existence of deleterious mutations in the *stm-1* and *stm-3* alleles, we conclude that the *STM* locus encodes an *Arabidopsis KNOTTED* homologue and thus is likely to promote SAM formation through transcriptional regulation.

The predicted STM protein is 382 amino acids long and is most similar to the *SBH1* gene from soybean[3] (67% identity). It is roughly equally similar to maize *KN1*[1] (47% identity), *Arabidopsis KNAT1*[5] (47% identity) and *Arabidopsis KNAT2*[5] (44% identity). In the homeodomain, *STM* is again most similar to the soybean homologue (95% identity) and somewhat less similar to *KN1*, *KNAT1* and *KNAT2* (92, 89 and 83% identity, respectively). *STM* is a class 1 *KNOTTED*-like gene based both on its high degree of similarity to *KNOTTED* in the homeodomain and on its expression pattern in the plant (see below). Three blocks of conserved

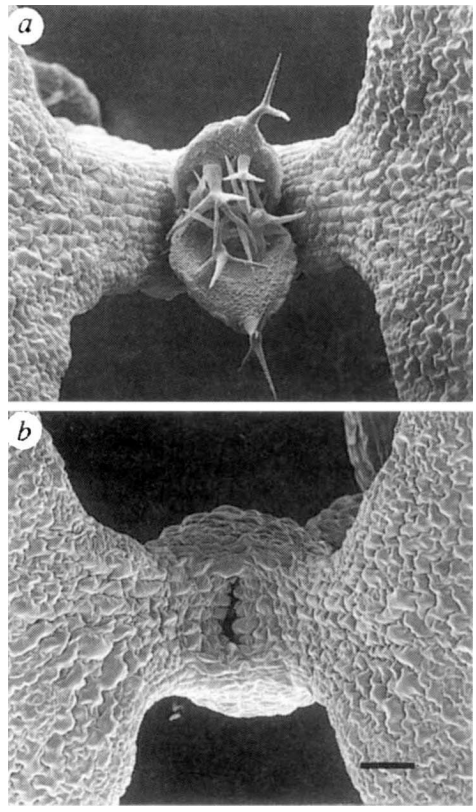

FIG. 1 Scanning electron micrographs of ~1-week-old wild-type (*a*) and homozygous *stm-1* (*b*) seedlings. Wild-type seedlings have a functional SAM as determined by the presence of two leaves at the bases of the cotyledons. Seedlings homozygous for the strong *stm-1* allele do not develop a shoot apical meristem. Scale bar, 100 μm. Seedlings were prepared and visualized by scanning electron microscopy as described in ref. 17

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