Stipule morphologies of the *sinuate leaf (sil)* mutants

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Form and development of the pinnately compound leaf of pea are genetically controlled by a number of known genes (6). Some genes appear to affect only blade development and others to affect only stipule development. The best known blade mutations are *afila (af)*, which transforms the proximal leaflets into branched tendrils; and, *tendrilless (tl)*, which transforms the distal tendrils into leaflets (4). A mutation that affects both blade and stipule, known as *sinuate leaf (sil)*, was originally recognized in an *af* line. *af/af sil/sil* plants possess "adventitious tendrils arising from a cleft in the distal tip of stipules" (Fig. 1; 5). Marx (5) originally described the phenotypes of *sil* mutants in *Af/Af, Af/af* and *af/af* backgrounds. He reported that *Af- sil* plants had sinuate or undulated leaflet and stipule margins but that only *af/af sil* plants had stipule tip modifications. He concluded that there were clear gene interactions between *Af* and *Sil*, but that anatomical or histological studies were necessary to develop a better understanding of them. In an attempt to address Marx's challenge, we recreated these three lines and started making observations of stipule form changes during shoot ontogeny in preparation for selecting material for anatomical preparations. Here we correct Marx's observations on stipular morphologies present on *Af/Af sil/sil* and *Af/af sil/sil* plants and provide a hypothesis to explain the interactions between *Af* and *Sil*.

**Materials and Methods**

Two *sil* lines, *W6 15137 (A Af b er ffs I pal sil)* and *W6 15110 (a af i sil)* were obtained from the Marx collection at the USDA Western Regional Plant Introduction Station, Pullman, Washington and were the parental lines in this study. *W6 15110* was used as the female and *W6 15137* was used as the male parent. The *F₃* plants were grown and allowed to self. *F₃* seedlings from individual *F₂* plants were phenotyped to identify the genotype of the parental line. Seed was separated into three genotypic categories: *Af/Af sil/sil, af/af sil/sil*, and *Af- sil/sil*. For observations and photography, plants were grown in a standard greenhouse in the fall (short days) when the stipular phenotypes were most dramatic. *F₄* seeds of each genotype were planted during the first week of October and developed under natural lighting conditions. Greenhouse temperatures were approximately 20° in the day and 15° at night.

**Results**

Distinct morphological features were characteristic of stipule tips on plants of each of the genotypes generated in this study. There was a progressive change in expression from node to node, up the plant. On adult flowering plants, the stipules at lower, or juvenile, nodes displayed nearly normal phenotypes, which
increased in severity up to the last two preflowering nodes, which typically displayed the most extreme phenotypes. The leaves subtending flowers had a normal stipule phenotype (Fig. 1b). For each of the genotypes listed below we describe phenotypes typical of lower, middle and upper (preflowering) nodes.

Af/af sil/sil plants - At lower and middle nodes, many stipules had a greener, diamond-shaped zone at their tips, which increased in size at successive nodes (Figs. 2a,b). At upper nodes, stipules had clefs or were deeply bisected due to the presence of adventitious leaflet-like structures (Fig. 2c). These central structures were deeper green than the surrounding stipule.

Af/af sil/sil plants - At lower to middle nodes, these stipules also had greener distal tips, or terminal clefs, often with leaflet-like structures present within them (Figs. 2d,e). The central leaf-like structure was darker green than the remainder of the stipule. At upper nodes, stipule clefs possessed terminal, adventitious wildtype leaves with leaflets and tendrils (Fig. 2f). Leaflet and tendril pairing on these adventitious leaves was irregular. The complexity (i.e. the number of leaflets and tendrils along the rachis) of these adventitious "leaves" increased at successive nodes. Cleft depth also increased with adventitious leaf complexity. The most complex leaf-like structures had their "petioles" positioned at the node on the shoot. This bisected the stipules into two unequal parts. Therefore it appeared that the stipule had been converted into a wildtype leaf with its own stipules.

af/af sil/sil plants - Some juvenile stipules were adaxially curled from puckered, contorted "knots" on the midrib about 1/2 to 2/3 of the way up (arrows, Fig. 2g). Some stipules were adaxially bent (*, Fig. 2g). These stipules typically also had greener tips. At middle nodes, the stipules possessed clefs with "adventitious tendrils" or "adventitious af leaves" emanating from the base of the cleft.

Fig. 2. sil phenotypes across various Af backgrounds, (a-c). Af sil genotype, a. stipule tip matches the greener color associated with leaflets (lower nodes); b. stipule tip colored and slightly elongated (middle nodes); c. stipule tip is deeply bisected with adventitious leaflet-like structure, (d-f). Af/af sil genotype, d. adventitious leaflet-like structure developing in apical stipule cleft (lower nodes); e. stipule partially converted into trifoliate structure (middle nodes); f. stipule converted to wildtype leaf, complete with own stipules (upper nodes), (g-i). af sil genotype, g. juvenile stipules showing contortion at tips (lower nodes); h. stipule converted into fully developed af leaf (upper nodes); i. expanding adult leaf showing conversion occurring at early stages of leaf development. Bars = 2.5 cm. B - leaf blade; L - leaflet; P - petiole; S - stipule.
Discussion

More leaf form mutants are known for pea than probably any other species of plant. In addition, these mutants span a phenomenal range of phenotypes. The range of phenotypic expression and extent of gene interactions associated with the sil mutants have not been appreciated in the past. The sil phenotype shows some similarity to another stipule mutant, cochleata. In light of evidence that A/ and Tl show gradients of expression in pea leaves (3,4,7) and that auxin plays important roles in pea leaf morphogenesis (2), we propose a new interpretation of Sil action and its interaction with A/.

Clearly Marx missed the fact that A/Af and A/af plants display characteristic sil stipule phenotypes. His omission may be because expression differs in different pea backgrounds, but more importantly we have noted that expression is much more evident in short day growing conditions than long day. A range of expression occurs both on a single plant (i.e. heteroblastic expression) and across the genotypes. Expression is greater for the A/af and af/af genotypes compared to AfAf, so the presence of at least one recessive af allele is necessary for strong expression. It is possible that sil is directly influenced by daylength, which has not been tested. However, we hypothesize that the increased expression in short days is related to the fact that under these conditions more vegetative leaves are produced per plant and that the last formed vegetative leaves show the greatest expression. Leaves associated with flowering do not show sil expression in any of these genotypes.

The range of sil expression across the genotypes can be categorized. The patterns of expression that occur in at least two genotypes are: (i) greener patches at the stipule tips, (ii) presence of "adventitious leaves" at the distal tip, and (iii) conversion of the stipule into a leaf. Two other observations made previously by Marx (5) support our interpretation that the novel structures produced in the latter two expression categories are leaves in the true sense. First, Marx (5) illustrated the phenotype of af sil tl plants and showed that the "adventitious leaves" are morphologically similar to the leaves on af tl plants, in that they possessed branched pinnae with miniature leaflets. These observations demonstrate that two leaf form mutations, af and tl, have the same effect in these novel stipule-derived structures on sil plants as they do in true leaves. Further, Marx (5) showed that in combination with two different wax mutants, wb and wlo, the amount of wax deposited on the tips vs. the base of sil stipules differed and that the amount of wax on the tips was more similar to that on leaflets for both wax mutants. In addition, we interpret the greener stipule tips on sil mutants to possess leaf blade identity, because leaflets have less wax on their surfaces than stipules and the observed color difference appears to be due to wax deposition. So we interpret the three categories of sil expression to represent a transition from stipule to leaf conversion. The stipules in the first two categories are mosaics of leaf blade and stipule identities and the final stage represents a complete conversion to a leaf. SEM and anatomical studies are in progress to test this hypothesis.

The sil mutant shares some phenotypic similarities and many differences with the cochleata (coch) mutant of pea. Both these mutants have compound stipules resembling true leaves at middle and upper, preflowering nodes. The compound stipules on coch leaves are conversions to leaf blades only, because they do not possess "stipules" of their own (8) as the sil mutants do. No interaction with Af is necessary for this conversion on coch plants, and no mosaic intermediates have been described, coch mutants have abnormal flowers with reduced fertility (8), whereas sil mutants have normal, fertile flowers.

Auxin has long been known to be produced in developing leaf primordia and to be transported basipetally through developing shoots. Cells respond to auxin and depending on their position and status, undergo cell division, elongation or differentiation. The tips and margins of developing leaves are thought to be sources of auxin (1). Since stipule tips and margins are most affected by sil, we predict that the combination of af and sil
causes excessive auxin accumulation, due to increased synthesis and/or defective transport. In this high auxin environment, the developing cells of the stipule perceive that they are leaf primordia instead of stipule primordia and follow an altered developmental path. We hope to test this hypothesis experimentally.

Conclusions
The sil mutants of pea are characterized by sinuate leaflet and stipule margins and, in certain genetic backgrounds, conversion of the stipule into a stipule/leaf blade mosaic or into a leaf. A distinct stipule tip phenotype for sil mutants is present in all Af/af backgrounds, and it shows a heteroblastic progression of severity for each genotype. Conversion can range from slight differences in wax deposition to complete conversion of the stipule into a compound leaf with its own stipules. The extent of this conversion depends on various factors including daylength and nodal position. More dramatic conversions are evident when plants are grown in short days. Due to the fact that the sil mutation primarily affects margin and tip growth, the Sil gene may be involved in auxin perception or transport.

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