Extending Marx's isogenic lines in search of *Uni* function

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Introduction

The backcross method of breeding is used widely to improve self-pollinating crop plants and to produce isogenic lines (1). Isogenic lines are inbred lines that are generally superior in quality but deficient in one or only a few specific traits. To produce isogenic lines, plants with an allele or alleles to be substituted are successively backcrossed to the superior parent (recurrent parent). This results in a stock with exactly, or nearly exactly the genotype of the recurrent parent except for the trait or traits of interest. Six rounds of backcrosses with the recurrent parent is the generally accepted standard in backcross breeding programs of self-pollinating species (1). Marx (13) produced a set of eight isogenic lines for three leaf form mutations (*afila - af*, *tendrilless - tl*, *stipuleless - st*, *afila tendrilless*, *afila stipuleless*, *tendrilless stipuleless*, *afila tendrilless stipuleless*, and *Afila Tendrilless Stipuleless - WT*). These lines are available from the Marx collection at the USDA Western Regional Plant Introduction Station (http://www.ars-grin.gov/ ars/PacWest/Pullman/GenStock/pea/MyHome.html). I report here that I have extended this set of isogenic lines by adding four lines incorporating the *tendrilled-acacia* (*uni-tac*) mutation to create *uni-tac*, *afila uni-tac*, *tendrilless uni-tac*, and *afila tendrilless uni-tac*. The leaf phenotypes of these lines are also described and compared.

Materials and Methods

Plant materials

The recurrent parents were W6 22593 (*WT*), W6 22594 (*tl*), W6 22597 (*af*), and W6 22598 (*af tl*). The donor parent was W6 15272 (*uni-tac*). This latter line was obtained from the Marx collection and the others were obtained from Cornell University before the collection was transferred to the USDA as part of the Marx collection.

Backcross breeding procedures

The donor parent was backcrossed to the recurrent parents. Twenty pollinations were done for each combination and the resulting F_1 seeds were pooled. F_2 plants with the *uni-tac* phenotype were selected from each backcross before repeating the pollinations. Six backcrosses were made in total for each combination. The *uni-tac* lines were used as the female parent for five of these backcrosses and as the male parent for one backcross for each combination to remove any maternal effects of the original donor parent. BC_6F_3 seeds from the sixth backcross for each new genotype were propagated.

Leaf form comparisons

Sixteen plants of each of the eight genotypes were grown in a standard greenhouse in the spring (long days). After flowering, the leaf from the last non-flowering node of each plant was analyzed and some of these were removed and photographed. Analysis consisted of counting, and identifying the number and form of each pinna in each pair on the leaf. Pinna position was numbered in order from lamina base to tip and only completely free pinnae were counted for the *uni-tac* genotypes as described in DeMason and Schmidt (5). Photographs of leaves were taken on a standard copy stand with a Canon Powershot G3 camera. Plates were assembled with Adobe Photoshop 6.

Means and standard errors of pinna pair numbers present on leaves of each genotype were calculated in Microsoft Excel XP and graphed with SigmaPlot 2000. T-tests were done between all relevant pairs of genotypes using Microsoft Excel XP.

Results

Leaf phenotypes

WT - All leaves on the last vegetative node were identical for all sixteen plants. Each had five lateral pinna pairs and a terminal tendril. The lateral pinna pairs consisted of two pairs of leaflets and three pairs of simple tendrils (Fig. 1a).

uni-tac - All leaves on this genotype had terminal leaflets. Thirteen leaves had two pairs of leaflets and one pair of tendrils (Fig. 1b). Two leaves had 2.5 lateral pairs and these were two pairs of leaflets and one lateral, unpaired tendril. One leaf had two pairs of lateral leaflets.

tl - All pinnae, both lateral and terminal, were leaflets (Fig. 1c). Eleven leaves had five lateral leaflet pairs, three had five pairs and one unpaired leaflet, and two had six pairs.

tl uni-tac - All pinnae on this genotype were also leaflets. The number of pinna pairs on the last vegetative leaf ranged from two to three. Many of the leaves that had only 2 pairs of lateral leaflets had bilobed terminal leaflets (Fig. 1d).

af – Leaf form on this genotype has been carefully described previously (4). Briefly, leaves on this genotype possess only tendrils, but there are two types: compound and simple. Adult leaves typically possess 2-3 pairs of compound tendrils in the proximal region and 3 pairs of lateral, simple tendrils in the distal region of the blade (Fig. 1e).

af uni-tac - All leaves had terminal leaflets and most of these were abnormal in size and shape. They differed from typical leaflets on *WT* and *tl* plants in that they had elongated, tapering bases that lacked pulvini and were somewhat thigmotropic and had rounded or truncated leaflet tips (Fig. 1f, terminal pinna). The number of lateral pinna pairs on this genotype ranged from three to four. Fifteen out of sixteen leaves had compound pinnae at position 1. One leaf had a normal leaflet opposite a compound pinna at this position. Compound pinnae terminated with either a simple tendril or an abnormal leaflet. Seven leaves had compound pinna pairs at position 2; four had a normal leaflet opposite a compound pinna (Fig. 1f); and six had a leaflet pair. All of these leaflets were normal in size and shape. Pairs of simple tendrils were present at all distal, lateral pinna positions.

af tl – Leaf form on this genotype has been described previously (17). All leaves on these plants had 6 lateral pinna pairs. The terminal pinna was a leaflet on all leaves. The first five pinna pairs were all compound. On eleven leaves the last lateral pinna position consisted of a pair of leaflets (Fig. 1g). On three it was a leaflet opposite a compound pinna. One leaf had a pair of compound pinnae at this distal-most lateral position.

af tl uni-tac - All leaves on this genotype had terminal leaflets. All leaves except one (i.e. 15 out of 16) had a pair of compound pinnae at position 1. The odd leaf had a leaflet opposite a compound pinna. Fourteen leaves had a pair of leaflets at position 2 (Fig. 1h). Two leaves had a leaflet opposite a compound pinna at position 2. All other positions had leaflets on all leaves. The leaflets on this genotype were much larger than those on *af tl.*

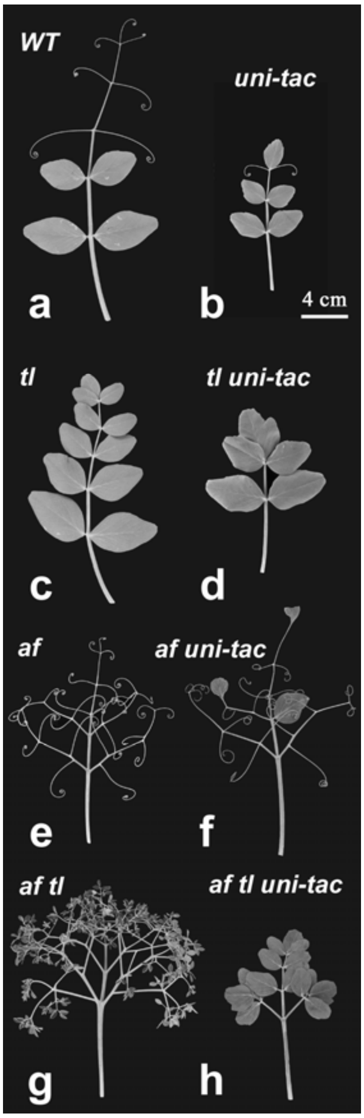

Fig. 1. Leaves from the last vegetative node of the eight isogenic lines. a. wildtype (WT). b. tendrilled acacia (uni-tac). c. tendrilless (tl). d. tl uni-tac double mutant. e. afila (af). f. af uni-tac double mutant. g. af tl double mutant. h. af tl uni-tac triple mutant.

Number of lateral pinna pairs

The mean number of lateral pinna pairs differed between these various genotypes (Fig. 2). *WT* had 5, whereas *af tl* had 6. *tl* and *af* each had means of 5.2. In t-test comparisons with *WT*, the only pair not found to be different was *WT* vs *af* (P=0.065). All the *uni-tac* lines had lower means compared to their closest genotypes. These were all significantly different at the 0.01 level. This reduction of the mean number of pinna pairs was 43% for *uni-tac* compared to *WT*. Reduction was less for the *af* genotypes: 30% and 34% for *af uni-tac* vs. *af* and *af tl uni-tac* vs. *af tl*, respectively. Finally, there was a 60% reduction in *tl uni-tac* vs. *tl*.

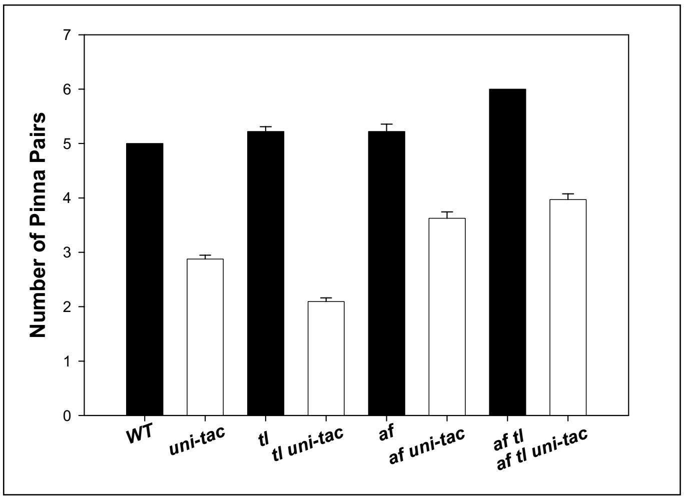

Fig. 2. Mean number of pinna pairs per leaf at last vegetative node of the eight isogenic lines. n = 16 and standard errors are shown in brackets. WT = *W6 22593*, tl = *W6 22594*, af = *W6 22597*, af tl =*W6 22598*, uni-tac = *W6 27606*, tl uni-tac = *W6 27608*, af uni-tac = *W6 27607*, af tl uni-tac = *W6 27609.*

Discussion

The *"tac"* allele and its phenotype were first identified and described by Sharma (14). This mutant was produced by diethyl sulfate treatment. According to Sharma, the leaf phenotype possesses lateral and terminal leaflets and the presence or absence of a pair of subterminal, lateral, simple tendrils. Marx (11) determined that the mutation known as *unifoliata* (*uni*) and "*tac*" (hence *uni-tac*) are allelic and both he and Sharma (15) crossed *uni-tac* with other foliar mutants to observe gene interactions. Their descriptions were rather cursory and the lines were not isogenic. However, Marx (12) concluded that *uni-tac* affects the distal lateral and terminal pinna positions and tends to reduce "ramifications" of the pea leaf and Sharma (15) concluded that the *uni-tac* allele converts the terminal pinna into a full-sized leaflet and suppresses differentiation of lateral tendrils. Also, using non-isogenic lines, DeMason and Schmidt (5) did a careful morphological analysis of leaf form and used the SEM to observe the development of *uni*, and *uni-tac* leaves compared to *WT*. They concluded that the lateral pinnae on these genotypes correspond to the proximal-most pinna pairs on *WT* leaves; whereas the terminal leaflet corresponds to the missing distal tendril pairs and the terminal tendril on *WT* leaves. *uni* and *uni-tac* leaves have a truncated period of leaf development due to precocious differentiation of the terminal leaflet, which explains the reduced number of pinna pairs (5). Therefore, the role of the *Uni* gene is to promote continued tip growth of pea leaf primordia to allow for a longer period of pinna initiation and the production of larger and more complex compound leaves.

Hofer *et al.* (9) determined that the *Uni* gene in pea is the ortholog of the *Floricaula*(*Flo*)/*LEAFY*(*LFY*) gene and obtained the sequence of two *uni* alleles. Both of these have defects in the coding sequence. DeMason and Schmidt (5) sequenced the *uni-tac* allele from the Marx collection and found a normal coding sequence but extremely low transcript levels. Using semi-quantitative RT-PCR, DeMason and Chawla (3, 4) showed that shoot tips of the various leaf form mutants have differential expression of *Uni*. *Uni* mRNA levels are much more abundant in *af* (4.7X) and *af tl* (10X) but less abundant in *uni-tac* (0.15X) and *uni* (0.6X) than in *WT* (1X) (3). Further, *af uni-tac* and *af tl uni-tac* have reduced *Uni* mRNA levels compared to *af* and *af tl* (4). Therefore, for *uni-tac*, reduced *Uni* mRNA levels correlates with leaf form simplifications.

Uni is the most important gene for controlling the compound nature of pea leaves. Busch and Gleissberg (13) have proposed that two redundant pathways for leaf dissection (*KNOX1* and *FLO*/*LFY*/*UNI*) characterize the ancestral condition in eudicots with compound leaves. KNOX over-expression increases the branching in tomato leaves. Both mechanisms have been retained in some lineages, whereas one or the other has been lost in others.

Pea provides the best model system for studying the role of *FLO/LFY/UNI* in the development of compound leaves.

The goal of this project was to create isogenic lines of *uni-tac* to extend the set already constructed by Marx (13). Using these genetic resources, researchers can better determine gene interactions underlying the resulting leaf phenotypes and study the underlying molecular regulation. I also provide a careful analytical comparison of the adult leaves in the eight isogenic lines.

Leaf forms of the *uni-tac* and *tl uni-tac* genotypes in this new set of isolines are as expected. There are fewer lateral pinna pairs present and the ones present are similar in form and size to the lower-most ones on *WT* and on *tl*. And the terminal pinna is a leaflet. However, leaf forms of the *af uni-tac* and *af tl uni-tac* have some novel characteristics. The *af uni-tac* genotype has two types of leaflets, neither of which occurs on *af*. Leaflets that form directly on the rachis have normal sizes and shapes, but those at the leaf tip or the tips of compound pinnae have abnormal characteristics. The bases of these leaves are similar to tendrils in that they are elongate, lack pulvini and are slightly thigmotropic; whereas the tips are flattened and expanded like leaflets. Terminal leaflets fused to lateral tendrils on *uni-tac* plants have a similar morphology (pers. observ.). I interpret these abnormal leaves on the *af uni-tac* plants to be an intermediate between tendrils and leaflets: the bases are more tendril-like and the tips are more leaflet-like. These intermediates are quite different in form from the intermediates present on *Tl/tl* heterozygotes which have flattened tendrils or narrow leaflets as described previously (10, 17). The position and number of leaflets (both types) present on adult leaves of the *af uni-tac* genotype are quite variable from leaf to leaf. The number of leaflets present on the leaves varies at different stages of plant ontogeny as well. The leaves at the lower, juvenile nodes have relatively more leaflets than do adult leaves, or those subtending axillary inflorescences. As the plants get older the leaves they produce are more like those on *af* plants. This feature of *uni-tac* plant ontogeny was observed previously (7).

The *af tl uni-tac* genotype also has an unexpected feature. Although essentially all lateral pinnae on *af tl* leaves are compound, only the lowermost pinna pair on *af tl uni-tac* is consistently compound. Therefore, the pinna type at position 2 of this genotype is not similar to that of *af tl*. The two *af* genotypes used in this study have higher levels of *Uni* mRNA in their shoot tips because the developing compound pinnae express the gene, whereas no other pinna primordia do (8). In both the *af uni-tac* and the *af tl uni-tac* genotypes, there is a tendency for the pinnae at position 2 to be simple instead of compound as they are on adult leaves of *af* and *af tl*. This suggests that when the *uni-tac* mutation is added, pinnae at that position are more prone to loss of *Uni* expression in their primordia than those at position 1.

A comparison between the adult leaves of these eight genotypes shows that the mean number of pinna pairs varies. Among the original Marx lines, *WT* has the lowest mean and *af tl* has the highest. This correlates well with the relative amount of *Uni* mRNA present in the shoot tips (9). Adding the *uni-tac* mutation to each of these genotypes results in a reduction in the number of pinna pairs produced on the leaves. These reductions are not uniform across the board. The reduction of *tl uni-tac* compared to *tl* is more drastic than *WT* vs. *uni-tac* and those resulting from addition of this mutation to the *af* genotypes are less so. This is evidence of differential interactions between *Uni* and *Tl* and *Af*. These results are consistent with the gradient effects previously reported for *Tl* and *Af* on pinna form and anatomy in the various hetero-zygotes (6, 17). For example, *Af* weakly represses pinna length of the distal pinnae and strongly represses it in the

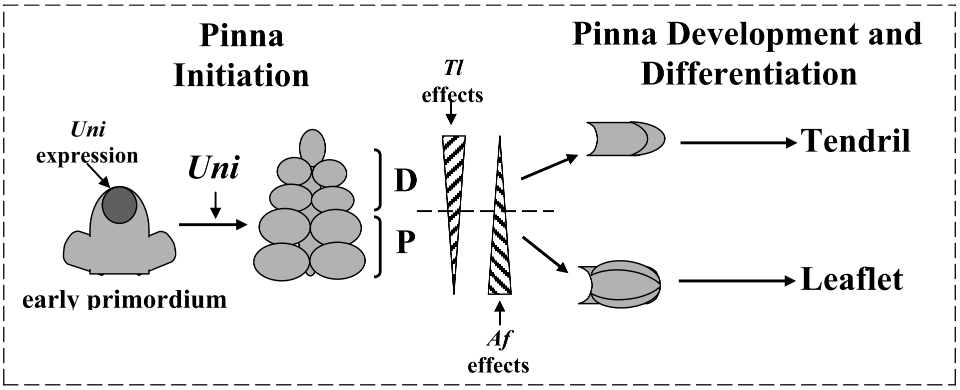

Fig. 3. Model illustrating roles for Uni, Tl and Af during morphogenesis of the WT leaf. D – distal pinna primordia, P – proximal pinna primordia.

proximal pinnae; whereas, *Tl* strongly increases pinna length in the distal pinnae and weakly increases it in the proximal. These gradient effects are presented in a model (6, Fig. 3).

Uni expression is concentrated in the growing leaf tip. Pinna primordia are initiated as long as this expression is maintained, and cease being initiated when it ceases. At this point, the terminal pinna starts to differentiate. This happens precociously in *uni* and *uni-tac* mutants. As pinnae are initiated, they determine their position along the leaf axis. Proximal pinnae are more responsive to *Af* and become leaflets, whereas distal ones are more responsive to *Tl* and become tendrils. The model illustrates how decreasing the duration of *Uni* expression in the leaf tip would affect distal pinnae more than proximal pinnae and the *tl* genotype (i.e. loss of both *Tl* and *Uni* function) more than *af* genotypes.

Seeds for each of these new lines have been deposited in the Marx collection at the USDA Western Regional Plant Introduction Station. The link to these accessions is http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?173. The lines are identified as: *uni-tac* – W6 27606; *af uni-tac* – W6 27607; *tl uni-tac* W6 27608; *afila tl uni-tac* – W6 27609.

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