The *aero2* (*aeromaculata2*) mutation in pea increases leaf flecking and complexity but, unlike *aero1*, does not promote flowering

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The *aero1* (*aeromaculata1*) mutation results in more extensive expression of the patches of silver flecking (aeromaculata) normally seen on pea leaves leading to a phenotype known as supaeromaculata (2, 5). While *aero1* mutants are characterized by increased leaf flecking, they also display a strong pleiotropic phenotype that includes shorter internodes, reduced stature and lower yield (10, 12). In addition, flowering in *aero2* plants is strongly promoted in terms of both node of flower initiation and time to first open flower; fewer leaves are produced before the onset of apical arrest and senescence; and the increases in leaf complexity that occur during ontogeny occur at lower nodes than in wild-type (WT) plants (12).

The heteroblastic increase in leaf complexity in pea has been examined extensively (4, 13, 14). Scale leaves occur at nodes 1 and 2. The first true leaf occurs at node 3 and consists of a pair of basal stipules and a rachis bearing a pair of opposite leaflets and a simple terminal tendril. Above node 3, leaf complexity increases with the addition of pairs of tendrils and leaflets. The leaves of adult WT plants possess two to three pairs of proximal leaflets, two to three pairs of distal simple tendrils, and a terminal simple tendril (13). The node at which the first leaf with 4 leaflets (C-4) occurs may be some 30-40 % lower in *aero1* mutants than in their WT isolines (12).

We have classified *aero1* as a heterochronic mutant and have argued that the broad spectrum of pleiotropic traits may all reflect the acceleration, relative to the WT, of various developmental steps during the course of ontogeny (12). Acceleration is obvious in the case of traits like earlier flowering, senescence and leaf change but less obvious in the case of increased leaf flecking. However, the silver flecks are underlain by sub-epidermal air spaces (11), and the more extensive flecking of *aero1* leaves may arise through an acceleration of certain anatomical or biochemical changes during development of the leaf (12).

Supaeromaculata mutants are not uncommon and at least ten *aero1* alleles are known (2, 10, 12). In this paper, we report on a new recessive supaeromaculata mutant Dr J.L. Weller obtained at Hobart in an M_2 population following EMS treatment of cv. Torsdag (HL107). We cleaned up the new mutant by several generations of single-plant selection and two generations of backcrossing to cv. Torsdag, before including it in the Hobart pea collection as HL303. F_2 and F_3 data from the two backcrosses confirmed single-gene recessive inheritance. The combined F_2 data of 80 WT and 34 mutant plants are in accordance with a 3:1 ratio ($P > 0.2$) and mutant F_2 plants bred true in F_3. There was evidence the WT allele was not fully dominant over the mutant allele. Visual separation of WT F_2 plants into two classes based on normal versus slightly elevated flecking intensity approximated a 1:2 ratio. However, there was no clear difference between the two groups and a few doubtful plants were arbitrarily assigned to one or other class. In contrast, mutant F_2 plants were visually distinct.

We made reciprocal crosses between the new *aero* mutant and mutants *aero1-1* and *aero1-10* . Six F_1 plants were grown from each cross. All 12 F_1 plants had a WT phenotype. We conclude the new mutant is not allelic with *aero1* and have named the new gene *AERO2* (*AEROMACULATA2*) with HL303 as the type line for mutant allele *aero2-1*.

The supaeromaculata phenotype of *aero2-1* is slightly weaker than that of *aero1-10*, and much weaker than the *aero1-1* phenotype (Fig. 1). In young seedlings, the increased flecking of *aero1-1* plants was clearly evident at day 10 from the stipules and leaflets of the first foliage leaf (node 3). In contrast, the increased

flecking of *aero1-10* plants was not fully evident until expansion of the stipules and leaflets at node 5 at around two weeks from sowing. For *aero2-1* plants, some increase in flecking could be seen on the stipules at node 5 but an obvious supaeromaculata phenotype was not fully apparent until expansion of the leaves at nodes 6 and 7.

Following recognition of the interesting and very strong pleiotropic phenotype of *aero1* (10, 12), we made a detailed phenotypic comparison between WT and *aero2* segregates in the F_2 of the first and second backcrosses between the mutant and cv. Torsdag. Both backcrosses gave consistent results. All plants were grown in the glasshouse at Hobart in individual 14-cm slimline pots under an 18-h photoperiod. Further details on growing conditions are as described earlier (12).

Fig. 1. Left to right: comparable leaves from a wild-type plant (cv. Virtus) and three supaeromaculata mutants aero1*-10 (MIII/122, ex Virtus),* aero1*-1 (JI2767, ex Torsdag) and* aero2*-1 (pre HL303, ex Torsdag).*

The *aero2* mutant also displayed a wide range of pleiotropic traits but with some major differences from the traits of *aero1* mutants.

The *aero2* mutation certainly accelerated increases in leaf complexity. The transition to 4- and 6-leaflet leaves occurred around 12% earlier in *aero2* than WT plants ($P < 0.001$, Table 1). All *aero2* plants attained 6-leaflet leaves and 38% went on to produce leaves with a supranormal state of 7 or 8 leaflets (Table 2). In contrast, only 90% of WT plants produced leaves with 6 leaflets, and not one produced leaves with 7 or 8 leaflets (Table 2).

In contrast to the *aero1* mutant, flowering was not promoted in *aero2* plants. The node of flower initiation was slightly higher (4% increase) and number of days to first open flower slightly increased (6%) in *aero2* plants compared with WT plants; both differences are significant ($P < 0.001$, Table 1). The rate of leaf appearance was 2% less ($P < 0.01$) in *aero2* plants contributing to the increase in flowering time. While these 2-6% differences are numerically small, they were statistically significant and very similar results were obtained in the second backcross (data not shown). Thus we believe they are a true effect of the *aero2* mutation.

Stem length between nodes 1 and 4 and 1 and 9 was around 25% less in *aero2* than WT plants (Table 1). The effect of the *aero2* mutation on internode length continued throughout the life of the plant. The average internode length of mature plants (excluding second growth) was around 20% less in *aero2* than WT plants in both backcross F_2 populations, and individual plots of this trait for *aero2* and WT plants did not overlap in either F_2. The anatomical basis for the 28% reduction in the length of internode 8 (between nodes 8 and 9) was examined using the epidermal strip technique of Arney and Mancinelli (1). The data in Table 1 are based on measurement of 10 epidermal cells per plant from 6 plants of each phenotype. They show an 18% reduction in cell number ($P < 0.01$) and an 11% reduction in cell length ($P > 0.05$) in *aero2*

plants. These results indicate reduced cell division as the major cause of the reduced internode length, although reduced cell elongation seems also to have contributed. The width of internode 8 was similar in WT and *aero2* plants (Table 1).

Table 1. Comparative data for several traits for wild-type and *aero2* segregates in the F_2 of the first backcross of the *aero2* mutant with initial line cv. Torsdag. Photoperiod 18 h. NS, not significant (P > 0.05)

	Wild type			*aero2*			Significance
Trait	Mean	SE	n	Mean	SE	n	of difference
Transition to 4 leaflets (node)	11.38	0.16	21	10.15	0.15	13	P < 0.001
Transition to 6 leaflets (node)	18.21	0.26	19	15.85	0.30	13	P < 0.001
Flower initiation (node)	16.38	0.11	21	17.00	0.00	13	P < 0.001
Flowering time (days)	35.29	0.23	21	37.54	0.22	13	P < 0.001
Flower/leaf relativity (nodes)	-0.39	0.05	21	-0.49	0.04	13	NS
Rate of leaf appearance (nodes per day)	0.476	0.003	21	0.467	0.003	13	P < 0.05
Stem length nodes 1-4 (cm)	4.94	0.12	21	3.70	0.11	13	P < 0.001
Stem length nodes 1-9 (cm)	39.20	0.83	21	29.56	0.67	13	P < 0.001
Length of internode 8 (mm)	93.2	3.8	6	67.4	1.6	6	P < 0.001
Width of internode 8 (mm)	3.46	0.09	6	3.58	0.04	6	NS
Epidermal cell length for internode 8 (microm)	331	14	6	294	14	6	NS
Epidermal cell number for internode 8	283	14	6	231	19	6	P < 0.01
Reproductive nodes	6.33	0.16	21	8.46	0.27	13	P < 0.001
Number of pods	10.05	0.33	21	9.77	0.30	13	NS
Number of seeds per plant	24.62	1.01	21	19.00	0.70	13	P < 0.001
Seed weight (mg)	248	3	21	216	3	13	P < 0.001
Pod depth (mm)	15.07	0.15	23	18.25	0.19	23	P < 0.001
Water congestion rating (0-4)	1.19	0.18	21	2.85	0.30	13	P < 0.001

Although the *aero2* plants produced two more reproductive nodes than WT plants in the first reproductive cycle ($P < 0.001$), both genotypes produced a similar number of pods ($P > 0.5$) (Table 1). The majority of WT plants (81%, 17/21) underwent normal monocarpic senescence at the end of the first reproductive cycle. However, the majority of *aero2* plants (92%, 12/13), after slowing to near zero growth, recommenced active growth and entered a second reproductive cycle. This difference is significant at $P < 0.001$. The failure of the *aero2* plants to undergo whole-plant senescence at the end of the first reproductive cycle may have resulted from their reduced reproductive load. While the *aero2* plants had the same number of pods as WT plants, they had 23% fewer seeds ($P < 0.001$) and the seeds weighed on average 13% less than seeds from WT plants ($P < 0.001$). The relationship between reduced reproductive load and failure to undergo whole-plant senescence after a single reproductive cycle was examined earlier in pea in respect of the *ar* and *n* mutants (7).

Table 2. Percentage of plants attaining 4, 6, and 7 or 8 leaflets at one or more nodes. Data are from the F_2 of the first backcross between the *aero2* mutant and initial line cv. Torsdag. There were 21 WT and 13 *aero2* segregates. Photoperiod 18 h.

Phenotype	Percentage of plants attaining		
	4 leaflets	6 leaflets	7 or 8 leaflets
Wild type	100	90	0
aero2	100	100	384

The depth of *aero2* pods exceeded that of WT pods by around 20 % in the first backcross F_2 (Table 1) and 10 % in the second backcross F_2 (data not shown). The *aero2* pods tended to be shorter than WT pods but pod length would certainly be influenced by the fact that *aero2* pods contained on average 20 % fewer seeds than the WT pods (Table 1).

A period of damp overcast weather during the growth of the first backcross F_2 resulted in some water congestion damage (see 8) to the plants. Water congestion damage was assessed on a subjective scale of 0 (no damage) to 4 (expansion of leaflet lamina fully suppressed). The *aero2* plants proved to be much more susceptible to water congestion than the WT plants (Table 1). For *aero2* plants the worst affected leaf was estimated, on average, to have lost about 75% of its potential lamina area compared with around 25% for WT plants. Any difference in susceptibility to water congestion was not exposed in the second backcross F_2 as all plants were free of damage.

In addition to the effects on leaf flecking, leaf complexity, and leaf susceptibility to water congestion damage, the *aero2* mutation may also have caused some unusual morphological features in one or more leaves starting at or just above the node of flower initiation. In the second backcross F_2, all four *aero2* plants had one or more leaflets where the leaflet base was rolled up into a very short conical funnel (Fig. 2), and two of the four *aero2* plants had one leaf where a leaflet appeared to be replaced by a short (around 5 mm), slender cylinder of translucent tissue, which, for want of a better term, we have referred to as a pin (Fig. 3). The funnel trait expressed in one or both of the first pair of leaflets. The pin trait expressed opposite a normal leaflet at the third pinna-pair position numbering from the proximal end of the rachis. These unusual leaf features were not observed in any of the 16 WT siblings. Unfortunately, we did not scan the much larger first backcross F_2 for these unusual features, so the observation rests on only four *aero2* F_2 plants. However, weak funnels have subsequently been observed in HL303 plants (HL303 is descended from one of these four *aero2* plants).

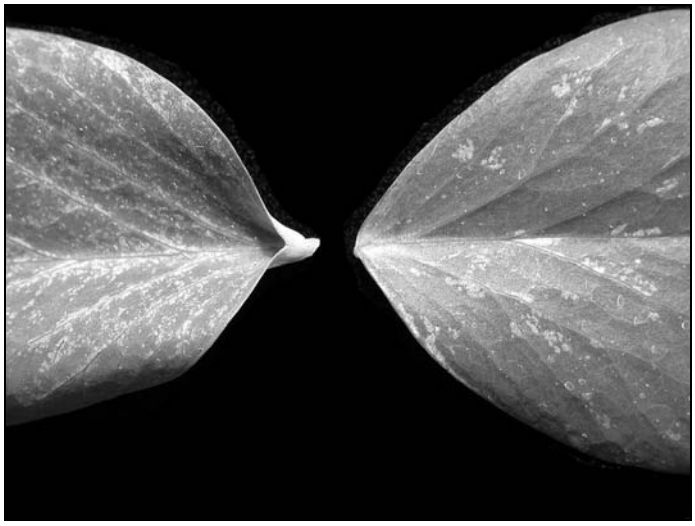

Fig. 2. Left, base of leaflet from the first position on leaf 18 of an aero2-*1 plant, which commenced flowering at node 17, showing up-rolling of the proximal section of the lamina to form a conical funnel. Right, base of a leaflet from the first position of leaf 17 from a WT plant (cv. Torsdag), which commenced flowering at node 16. Some whitish spray residue is present on both leaflets.*

The funnel and pin features give the impression that leaflet and lamina development has been partially or wholly truncated in these cases, which fits well with the idea that the *aero2* mutation is hastening the timing of various events during development of vegetative organs.

Funnel leaflets and pins/needles are the defining features of the *lld* (*leaflet development*) pea mutant (9). The strongest expression of the funnel-leaflet trait in *aero2* plants (Fig. 2) was approximately equal to the weakest expression of the funnel-leaflet trait illustrated for *lld* plants (Fig. 1B in 9). Interestingly, the peak expression of the *lld* phenotype occurred as the plants entered the reproductive phase (9), and expression of

funnels and/or pins in *aero2* plants commenced at or just above the node of flower initiation. Again, the strongest expression of the *lld* phenotype occurred at position 3 along the rachis (9) and that is where the pins (complete lamina suppression?) occurred in *aero2* plants (Fig. 3). Thus the leaf phenotypes of *lld* and *aero2* plants share certain similarities, both in morphological features expressed (funnels, pins) and in the timing and place of maximum expression. However, *LLD* has a more confined role in leaf development than *AERO2* as *lld* plants do not appear to have increased leaf flecking.

The *adt* (*air dots*) pea mutant has a phenotype that includes numerous tiny grey spots along the veins of leaflets and stipules, shorter and wider pods, and the terminal tendril of a leaf is often replaced by a leaflet (3). Thus the *aero2* and *adt* mutations both increase leaf flecking and alter pod dimensions but overall the two mutants are quite distinct.

Comparison of the list of phenotypic effects of the *aero1* and *aero2* mutations reveals a number of features in common. Both mutations appear to accelerate changes that affect vegetative traits like internode length and leaf complexity. The anatomical data (Table 1) show that reduced cell division is the major cause of the shorter internodes in the *aero2* mutant. This could indicate that cell division ceases earlier in the development of the *aero2* internodes than in the WT internodes.

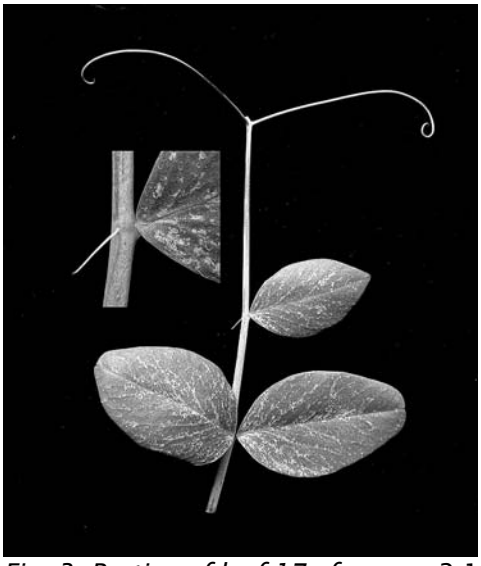

Fig. 3. Portion of leaf 17 of an aero2-1 *plant, which flowered at node 17, showing a pair of leaflets at the second position, a pin opposite a leaflet at position 3, and a pair of tendrils at position 4. The inset shows an enlarged view of the pin, which was 5-mm long x 0.15-mm wide.*

The accelerated transition to 4- and 6-leaflet leaves certainly categorizes *aero2* as a heterochronic mutant as the timing of these events is brought forward relative to the situation in the WT ancestor. The effect of the *aero2* mutation on leaf complexity is further emphasized by the occurrence of supranormal 8-leaflet leaves on some *aero2* plants. WT plants (with normal leaf flecking) do sometimes attain 8-leaflet leaves when grown under short-day conditions that allow vigorous growth and a prolonged vegetative phase but we have never observed 8-leaflet leaves on WT plants grown under the 18-h conditions used in this study.

The accelerated transition to 4- and 6-leaflet leaves was only about one-third as strong in *aero2-1* (Table 1) as in the *aero1-1* and *aero1-10* mutants (12). However, the *aero1* data were based on comparison of homozygous WT and mutant isolines, where as the WT plants in the *aero2* study were comprised of a mixture of homozygous and heterozygous plants. Any degree of partial dominance would diminish the observed difference. The effect on leaf flecking was also weaker in *aero2-1* than in the *aero1* mutants (Fig. 1). Our ability to comment further on the weaker action of *aero2-1* is limited by the fact that only one *aero2* mutant is currently available and we do not know whether *aero2-1* is a null or leaky allele.

The *aero1* mutation significantly promotes flowering as shown by several measures: there is a major reduction in the node of flower initiation and the time to first open flower; the size of the quantitative response to photoperiod is substantially diminished in *aero1* plants compared with WT plants; and development of the flower bud is accelerated relative to leaf development as evidenced by the higher flower/leaf relativity values (see 6) in *aero1* plants (12). In contrast, the *aero2* mutation does not promote flowering by any measure. On the contrary, we found a small but significant and repeatable delay in flower initiation and flowering time, and a lower flower/leaf relativity value (not significant) in *aero2* plants (Table 1). There was also no evidence that the photoperiod response was reduced in *aero2* plants (data not shown).

The difference between the effects of the *aero1* and a*ero2* mutations is interesting. The *AERO1* gene seems to be part of a basic timing or clock mechanism where mutation leads to a general acceleration of a

diverse range of developmental processes including flowering (12). In contrast, the effects of the *aero2* mutation are more limited and do not extend to acceleration of flowering, a key developmental process. This may mean that *AERO2* acts downstream of *AERO1* and with a role in the timing of a more limited range of developmental events.

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1. Arney, S.E. and Mancinelli, P. 1966. New Phytol. 65: 161-175.
2. Blixt, S. 1962. Agri Hort. Genet. 20: 95-110.
3. Gorel, F.L., Berdnikov, V.A. and Kosterin, O.E. 2002. Pisum Genetics 34: 1-2.
4. Lu, B., Villani, P.J., Watson, J.C., DeMason, D.A. and Cooke, T.J. 1996. Intl. J. Plant Sci. 157: 659-673.
5. Marx, G.A. 1986. Pisum Newslett. 18: 42-44.
6. Murfet, I.C. 1982. Crop Sci. 22: 923-926.
7. Murfet, I.C. 1985. Ann. Bot. 55: 675-685.
8. Murfet, I.C. 1990. Pisum Newslett. 22: 32-37.
9. Prajapati, S. and Kumar, S. 2001. J. Biosci. 26: 607-625.
10. Sidorova, K.K. and Uzhintseva, L.P. 1995. Russ. J. Genet. 31: 1008-1009.
11. Sifton, H.B. 1945. Bot. Rev. 11: 108-143.
12. Taylor, S.A. and Murfet, I.C. 2003. Physiol. Plant. 117: 100-107.
13. Villani, P.J. and DeMason, D.A. 2000. Ann. Bot. 85: 123-135.
14. Young, J.P.W. 1983. Ann. Bot. 52: 311-316.