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Polyamine Levels in Petunia Genotypes with Normal and Abnormal Floral Morphologies[1]

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ABSTRACT

We characterized the polyamine pathway in *Petunia hybrida* genotypes that were either wild type or that had been identified as having altered floral morphology. Analysis of four normal morphology lines revealed two patterns of endogenous levels of putrescine and arginine decarboxylase: two with higher levels of putrescine, two with lower levels of putrescine. Analysis of F1 and backcross progeny between high putrescine and low putrescine strains is consistent with their differences being due to a dominant allele for low putrescine content and arginine decarboxylase activity. Four *Petunia* mutants with floral morphology changes were also screened. One of these mutants, *alf*, showed high levels of putrescine and high levels of arginine decarboxylase late in development; these high levels were found whether the *alf* line was present in either of the two types of normal morphology genetic backgrounds that had been characterized.

Changes in polyamine concentration and in the activity levels of the various polyamine biosynthetic enzymes have been correlated with a wide variety of plant growth and environmental response phenomena, including cell division (9), responses to stress such as acid (21), and heavy metal (20), hormone response (3), and floral development (11, 14). Polyamine-conjugates, covalently bound to phenolic acids, have also been found in high levels in plants, and may be the effectors of some of these phenomena (16).

In previous reports (12, 13) we have described the isolation of tobacco cell cultures resistant to MGBG[3] a potent inhibitor of SAMdc. All MGBG resistant cell lines that were successfully regenerated into whole plants revealed abnormal differentiation of the floral meristems (14). The floral phenotypes seen included stamenoid-ovules, stigmoid anthers, petaloid anthers, petaloid sepals, extra petals, shrunken anthers, long styles, and a shift in developmental timing resulting in what is referred to as 'puzzle box.' Many explanations of the correlation between MGBG resistance and floral abnormalities were possible, including regeneration artifacts and multiple mutations due to somaclonal variation or the use of a mutagen. One way of testing the association of MGBG resistance with the altered floral development would be simply to perform crosses and test for linkage of the two traits, but the developmental anamolies were generally so severe that the flowers were completely sterile. In two cases, *Mgr3* and *Mgr12* (15) we were able to obtain small numbers of F1 and backcross progeny. The phenotypes seen suggested both *Mgr3* and *Mgr12* were nuclear dominant traits, and the floral phenotypes were linked to the MGBG resistant phenotypes, although the total number of progeny screened was quite small in each case.

These results strongly suggested a correlation between altered polyamine synthesis and altered floral morphology, but only in the limited system of cell culture derived tobacco plants. In order to test further the hypothesis of the involvement of polyamine biosynthesis in the differentiation of floral meristems, we undertook to characterize the polyamine content of several *Petunia hybrida* lines, including some with abnormal floral development. These *Petunia* genotypes were derived from whole plant studies as part of a search for genetic mutations in floral pigmentation and floral morphology (see Gerats *et al.* [8] for a brief description). These plants thus had no prior relationship with either cell culture or polyamine selections.

MATERIALS AND METHODS

Stocks. Various *Petunia hybrida* genetic stocks were used in the experiments (Table I); each of which has been inbred for at least 10 generations, and are homozygous for all genes tested, although some level of heterozygosity of course persists. The *alf* mutation was isolated recently (4) and is presumed to have arisen following the insertion of a transposable element into the wild type allele. It arose in a strain that harbored an unstable *an1* allele. This particular *an1* allele gives rise to mutations in unlinked loci at a very high frequency; approximately 30% of these new mutations are unstable themselves. The *alf* mutation has a reversion frequency of about 1%.

Enzymes Assays. Leaves or floral tissues were homogenized in 2 ml/g of lysis buffer (100 mM Hepes [pH 7.5], 10 mM DTT, 10 mM EDTA, 0.04 mM pyridoxal-P). The extract was clarified by centrifugation at 10,000 rpm (12,000 *g*) for 15 min. The activity assays for ORNdc, ARGdc, and SAMdc were subsequently measured by $^{14}CO_2$ release from carboxyl labeled ARG, ORN, and SAM as previously described (2, 10, 18). One enzymic unit is equivalent to 1 pmol CO_2/h at room temperature. Protein concentration was determined using the Coomassie blue method (1).

Polyamine Determination. Samples of leaves and flowers were homogenized in 7 ml of 5% perchloric acid per g of material. Polyamine concentrations were determined using HPLC (after benzoylation) or TLC (after dansylation) as described by Flores and Galston (7). Soluble polyamine-conjugate levels were determined as in Slocum and Galston (19) by boiling the extracts in 6 N HCl followed by measurement of the change in polyamine levels over basal concentrations.

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[3] Abbreviations: MGBG, methylglyoxal-bis(guanylhydrazone); ORNdc, ornithine decarboxylase; ARGdc, arginine decarboxylase; SAMdc, s-adenosylmethionine decarboxylase; put, putrescine; spd, spermidine.

Table I. *Description of the Strains Used*

The strains *V23*, *R27*, *M65*, and *W62* differ from each other in genetic constitution for the flavonoid genes.

R51	homozygous recessive (h.r.) for the gene *B1* (blind); the mature flower lacks the corolla, whereas the tube is normally developed.
R100	h.r. for *Gp* (green petals); the mature flower has two rings of sepals instead of the normal sepals + petals.
R129	h.r. for *Px* (phoenix); the tube of the flower is compressed. A new flower develops through the tube of a dying flower in young plants.
alf	h.r. for *alf* (aberrant leaves and flower); has to be kept in culture in heterozygous condition; floral tissues are formed in a very disordered way.

Table II. *Putrescine to Spermidine Ratios in Young Leaves of Wild Type and Floral Mutant Strains of Petunia hybrida*

The standard errors are based on 3 to 5 repetitions of the measurements. The floral mutant strains are designated by *.

	put	spd	put/spd
	nmol/g fresh wt ± SE		*ratio*
R27	112 ± 75	102 ± 49	1.0 ± .22
R100*	142 ± 12	134 ± 24	1.1 ± .04
R129*	68 ± 15	68 ± 14	1.0 ± .07
R51*	99 ± 21	103 ± 29	0.98 ± .16
V23	105 ± 42	230 ± 76	0.46 ± .05
W62	77 ± 17	177 ± 30	0.43 ± .03

RESULTS

Genotypes with Normal Morphology May Have Differing put to spd Ratios. The *Petunia hybrida* genotypes *V23*, *R27*, and *W62* have normal floral morphology; although *V23* and *W62* leaves and flowers are more brittle than leaves and flowers of the other strains. The polyamine contents in these lines were checked to obtain a wild type standard for comparison with floral developmental mutants (Table II). We measured the put, spd, and spermine contents after benzoylation and analysis on an HPLC reverse phase C18 column, as well as by dansylation and TLC analysis (7). Results from these experiments and from analysis of the various mutant strains indicated that the polyamine content per g of leaf or floral tissue was somewhat variable but the ratio of put to spd was constant across the various analytical runs. Unlike tobacco, where the polyamine-conjugate content of flowers is particularly high, the polyamine-conjugate levels of the *Petunia* lines tested here were quite low, and did not show significant changes during comparisons of the various lines (results not shown). The normal morphology stains fell into two groups, some with a low put to spd ratio of about 0.4, and some with a higher ratio of about 1.0.

The line *R51* contains the *b1* mutation (see below) but does not differ from its corresponding wild type strain in polyamine content. *V23* differs from *R51* (and its wild type) in having a lower ratio of put to spd (0.4:1 *versus* 1:1) and a lower ratio of the corresponding biosynthetic enzymes, ARGdc to SAMdc (approximately 1:10 *versus* 1:5), particularly in younger leaves (Fig. 1). This difference in put to spd ratio was further analyzed in F1 hybrids between these two lines (Fig. 1), and in backcross progeny with *R51* (Fig. 2). Comparison of the panels in Figure 1 shows that the *R51* × *V23* hybrid has a profile similar to *V23*. The results suggest that *V23* and *R51* differ in a heritable factor(s) that is dominant for low put and ARGdc decarboxylase levels. In the backcross, a 17:22 segregation of high and low put levels was seen, roughly consistent with the 1:1 segregation pattern expected for a single locus. The analysis in Figure 2 is for 14 randomly chosen plants of the backcross generation. A polymorphism for put to spd ratio thus exists in some normal morphology

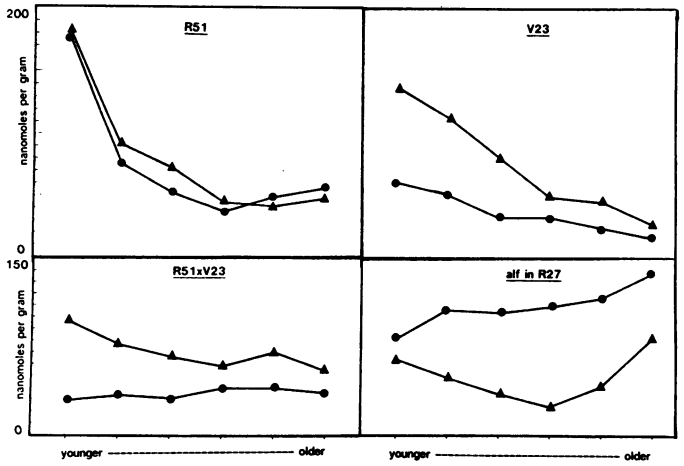

FIG. 1. Putrescine (●) and spermidine (▲) contents of petunia leaves from various strains, expressed in nmol/g fresh weight. The upper left panel is *R51*; the upper right panel is *V23*; the lower left panel is the F1 hybrid of *R51* and *V23* showing dominance of the low putrescine pattern; the lower right panel is the *alf* mutation in the *R27* genetic background, showing its characteristic elevated putrescine levels. Flowering plants, 4 to 6 months old, were analyzed. Young leaves were those that grew at the base of the most recently developing flower along the main axis; leaves were sampled at regular intervals down the main axis with those at the base scored as older leaves. The data shown is from a single representative experiment among several that were performed.

Petunia lines.

One Mutant with Abnormal Flowers Has a Developmentally Regulated, Altered put to spd Ratio, but Three Other Lines with Odd Flowers Do Not. Four different Petunia genotypes that had abnormal floral structures were screened: *bl/bl* lacks a corolla, *gp/gp* has green petals, and *px/px* generates new flowers with an existing flower. The homozygous *alf/alf* mutation has altered leaf and floral morphologies, including many floral parts out of place and partially turned into other parts; it is thought to be the result of insertion of a transposable element (4). Young, nonflowering *alf/alf* plants are indistinguishable from comparable wild type plants, but just before floral initiation, the leaves become smaller and branching increases. The sepals of the would-be flower are separately placed on the pedicel, while all other floral tissues are only partly developed, with a confused appearance.

Analysis of these lines revealed that the *alf* mutation has an elevated put to spd ratio and ARGdc to SAMdc ratio in old leaves (Fig. 1; Tables III and IV); the other three lines showed no significant changes of polyamine biosynthesis. The elevated put to spd ratio has a component of developmental regulation, being found in older leaves and in flowers, but not in younger leaves. The *alf* mutation was originally isolated in the *R27* background, then crossed and backcrossed once into the *V23* genetic background. The presence of the normal morphology—low putrescine polymorphism can be seen in this *alf* in *V23* plant by comparison of the put to spd ratios in the young leaves, where the *alf* mutation has no effect. In old leaves and flowers, the developmentally regulated elevated put and ARGdc levels characteristic of *alf* are seen in both genetic backgrounds. The *alf* locus evidently conditions a 2- to 3-fold increase in the putrescine to spermidine ratio, that is found only in flowers and older leaves of the plant, and that is independent of other genetic background effects on put and spd content.

DISCUSSION

This research was inspired by the idea that analysis of various floral morphology mutations might reveal the extent to which

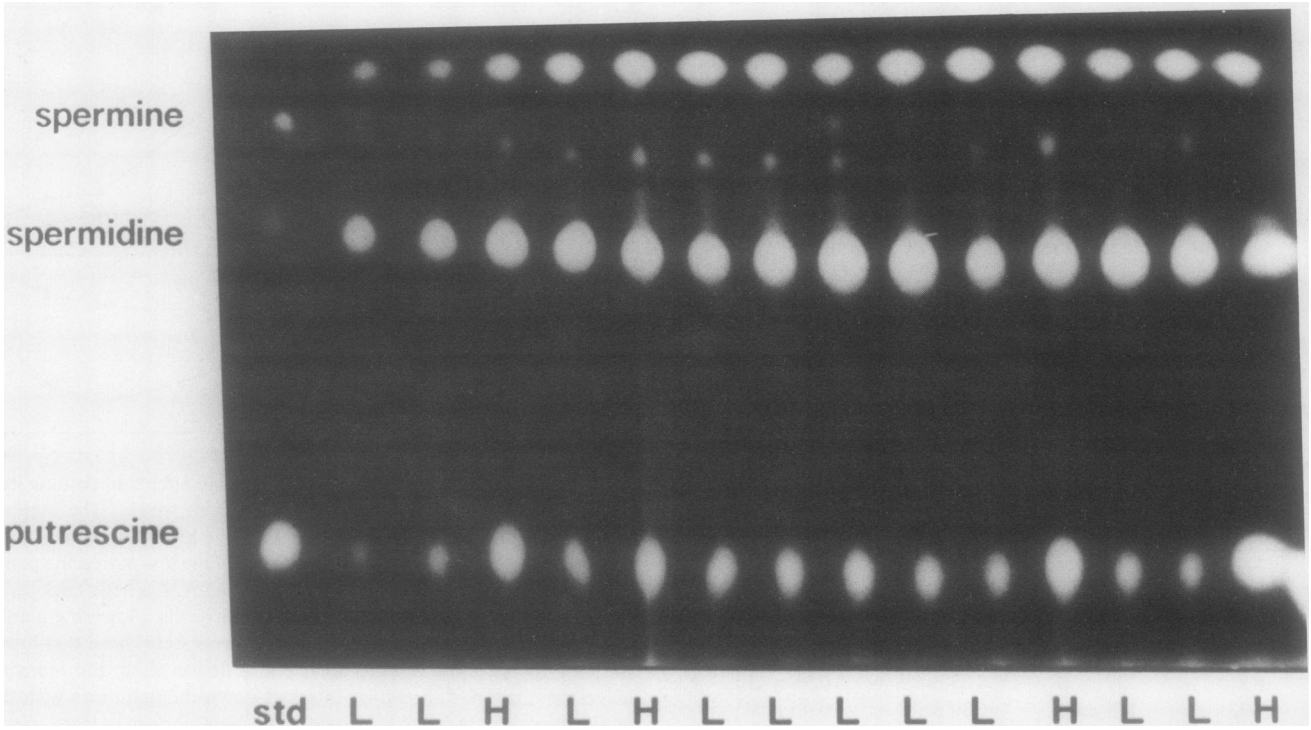

FIG. 2. A typical TLC plate showing high and low putrescine contents of backcross progeny, where the F1 hybrid (*R51* × *V23*) has been crossed to *R51*. For this figure, 14 plants were chosen at random and then analyzed. The lanes are marked L or H to indicate our scoring of the relative putrescine content.

Table III. *Polyamine Levels and Ratios in Wild Type and alf/alf*

The young leaves are the first leaves below a mature flower, the old leaves are leaves at the base of the plant. The standard errors are based on four measurements each.

	Old Leaves			Young Leaves			Flowers		
	put	spd	put/spd	put	spd	put/spd	put	spd	put/spd
	nmol/g fresh wt ± *SE*		*ratio*	*nmol/g fresh wt* ± *SE*		*ratio*	*nmol/g fresh wt* ± *SE*		*ratio*
R27	45 ± 1	40 ± 1	1.2 ± .02	114 ± 22	87 ± 20	1.5 ± .1	100 ± 16	90 ± 17	1.1 ± .05
alf in *R27*	96 ± 29	35 ± 10	2.3 ± .2	78 ± 20	66 ± 19	1.3 ± .1	175 ± 50	54 ± 13	2.7 ± .2
V23	19 ± 1	33 ± 3	0.6 ± .04	59 ± 13	144 ± 25	0.4 ± .02	111 ± 16	142 ± 19	0.7 ± .02
alf in *V23*	44 ± 9	41 ± 8	1.0 ± .02	59 ± 14	228 ± 57	0.3 ± .01	123 ± 35	50 ± 13	2.1 ± .12

Table IV. *Enzyme Activities in Old Leaves of alf/alf and Wild Type*

	Activity/mg Protein		SAMdc/ARGdc
	ARGdc	SAMdc	
			ratio
R27	76 ± 3	774 ± 50	10.2
alf/alf	252 ± 7	456 ± 9	1.8

polyamines are involved in this developmental process. Two results were obtained: first, that normal morphology strains of *Petunia* can differ in their putrescine content; second, that one out of the four lines tested with altered floral development also showed elevated putrescine content, while three of the four did not.

These examples provide two instances where high put and high ARGdc levels are recessive genetic traits. This can be seen in the analysis of the F1 and backcross progeny of *V23* and *R51* strains, and also in the analysis of the *alf* mutation in both genetic backgrounds. The biochemical genetic mechanisms that underlie the changes in put content are likely to be different for the put to spd ratio polymorphism in wild type strains, and the changes associated with the *alf* mutation. The *alf* mutation has the same effect of elevating put levels in both of the genetic backgrounds, and it has a component of developmental regulation (older leaves and flowers *versus* younger leaves) not found in the differences between normal genotypes. The data in Table III, for example, clearly show the superimposition of the *alf* developmental profile on top of the putrescine variability caused by the strain difference. The effects of the *alf* mutation may be described as indepdent of, or additive with respect to the difference between the normal genotypes, rather than being epistatic or interactive with it.

The research described here provides one example of altered put levels with no morphological consequences, and one example of altered putrescine levels correlated with significant morphological alterations. Thus, changes in put levels by themselves cannot be the only critical factor in whatever role polyamines have in floral development.

The *alf* mutation differs from the other three mutant genotypes tested in that its effects on floral development are more chaotic and variable. In contrast, *bl*, *gp*, and *px*, have sharply defined predictable changes in floral morphology. It can therefore be speculated that changes in polyamine titer might be associated with only certain types of developmentally unstable mutants.

Other researchers (5, 6, 16, 17) have found correlations between levels of polyamine-phenolic conjugates and aspects of floral development in maize and tobacco. In one case (17) male sterile lines of maize were found to have near zero levels of hydroxy-cinnamic acid amide levels, while significant levels were detectable in normal fertile plants and in restorer allele fertile plants. In tobacco (5, 6) a nonflowering line (RMB7) was compared to a normal variety (Xanthi); low levels of aromatic amine conjugates were found in the nonflowering line. This result is equivocal in the RMB7 line is not isogenic with Xanthi, and as we have demonstrated in this report, varietal differences in polyamine content may be found. In the *Petunia* lines we tested here, the levels of polyamine-conjugates were very low, and no significant differences were found between wild type and mutants.

The clearest way to pursue the possibility of a physiological link between flowering and the polyamines or polyamine-conjugates will be by additional characterization of mutants whose primary defect is in the polyamine pathway, and by continued characterization of plants with altered flowers in isogenic backgrounds.

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