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RE-EVALUATION OF ANTHOCYANLESS TRAITS IN GOSSYPIUM HIRSUTUM AND G. BARBADENSE

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Abstract

Stocks of three *Gossypium* species were investigated for genes that expressed an anthocyanless trait. This research served as repetition of Rhyne-Carter non-pinking phenotype and genetic verification. Two diploid *G. arboreum* stocks had non-pinking phenotypes and a third was wild type, petal spot and pinking plant parts. The classical "ghost" petal spot on a yellow petal exhibited a white petal spot and non-pinking plant parts. The other non-pinking – white (ivory) petals and absence of white petal spot "ghost" – had to be confirmed by crossing with the pinking Garohill. The segregation was 3 red spot to 1 no spot; the derivative of basic spotless used by Gerstel and tracing to S5000. In Pima S6 *nene*, *G. barbadense*, a transfer from non-pinking by backcrossing segregated in F_2: ¼ non-pinking white petal spot on yellow petals. At the R_2 petal spot locus, it was comparable with the classic ghost expression and inheritance. On a yellow petal background in *G. hirsutum*, non-pinking exhibited the "white petal spot" and its non expression on standard cream petal. However the wild type typical of modern transgenics, spotless and pinking plant parts, segregated in F_2 and testcrosses at two active loci. The wild type $2r_2r_1$ made F_1 with red petal spot pinking and TC showed that a "ghost" gene at R_2 interacted with r_2 intragenomic and r_1 intergenomic. As $R_2R_2r_1r_1$ it persists as a standard petal spot. If there is no r_1, as PS 6 had, then 2 $R_2r_1^o$ is non- pinking. The wild *G. tomentosum* with yellow petals and spotless non-pinking plant parts differs from Rhyne-Carter non-pinking, as verified by numerous researchers, but not reported because of absence of petal spot.

Introduction

Harland (1939) indicated the lack of anthocyanin in two Asiatic and one Hawaiian species among the known *Gossypium* species. Wild *G. tomentosum* had yellow petals and no petal spot but the Asiatic's yellow petals had ivory petal spot. On the ivory petal the expression was called the "ghost". Yu and Chang (1948) developed basic spotless and Stephens used it and extracted S5000 from the N14 ghost among the stocks that Gerstel (1953) used. Silow (1941) showed ghost to be allelic to the ghost R_2 of *G. anomalum* that interacted with R_3 spotless to produce anthocyanin. Gerstel (1953) investigated chromosomal pairing of white spot *G. arboreum* $2A_2$ and *G. anomalum* $2B_1$; Gertsel and Phillips (1958) commented on the stability of amphdiploids of *G anomalum*.

The use of bridging 6X form of standard *G. hirsutum* X *G. anomalum* (2 AD B_1) produced a large red petal spot (Rhyne, 1951). Gerstel and Phillips used this marker in their studies (1958). Using Rhyne's $2A_2D_1$ and their amphidiploids, "ghost" was transferred from $2B_1D_1$ to $2r_2r_1$ wild type (Rhyne, 1965) and to M8 $2r_2r_1$ (V. Meyers, 1971). Later, the use of Rhyne's petal spot stock and a similar R_2 stock of Stephens (1948) produced a spotless form called non-pinking (*npnp*) by Rhyne and Carter (1991). This non-pinking, when crossed with wild-type $2r_2r_1$, produced F_1 hybrids with large red petal spot and pinking plant parts. This epistatic gene action was reported as R_2 interacting with r_1 intergenomically (Rhyne and Carter, 1991). Kohel, Stelly and Yu (2002) reported that their non-pinking did not involve the r_1 on chromosome 16. The purpose of this communication is to evaluate the genetics of non-pinking stocks of our nursery.

Materials & Methods

Not pinking stocks in three backgrounds were used: (1) two diploid *G. arboreum* obtained from Stewart at Arkansas, a pinking Garohill $2A_2$ from the Texas collection and also a recent wild type *G. davidsonii* $2D_3$ collected by Percival and Stewart; (2) a *G. barbadense* PS 6 *nene* multiple backcross derived from Rhyne-Carter (1991) *npnp* (the 1999 BC F_1 had been sent to the ARS USDA at Maricopa, AZ for preservation and/or confirmation); (3) an upgraded *npnp* stock of Rhyne-Carter *npnp*. The wild types $2r_2r_1$ of *G. hirsutum* stocks represented conventional and transgenic forms. Plant parts were inspected from emergence to frost in parents and families of crosses. Photographs were essential in the identification and verification of phenotypes.

Results and Discussion

Non-pinking in diploid 2A$_2$ Background

The two Arkansas stocks were light green with plant parts that did not turn pink. One had deep yellow petals Y_a and a white (ivory) spot at the inner base. It was the classic "ghost" phenotype of Harland, Silow, Gerstel and many Indian researchers. The second stock had similar plants parts, ivory petals y_a and no discernible spot. It also carried the nectariless condition of some A dipolid stocks. That it was a derivative of basic spotless (S5000) , no 'ghost' on ivory petal, had to be determined by segregation. So, the Garohill wild-type, sun red, pinking plants parts and full red spot on pale yellow petals (Y_a^P) was used. This version was present also because it putatively carried compatibility (*le*) with 2D$_3$ yellow petal Le^{da^v} (Silow, 1941; Lee, personal communication). If so, *le-Ne/Le ne* homeologic linkage would be present in these A$_2$ stocks.

The Garohill essentially was male sterile and was high-node sympodium with 11-13 seed per locule. The ivory petal (y_a) stock was low node, fertile in seed and flower (Gerstel, 1953), 5-7 ovules per locule and prolific. The F$_1$ was wild type with pale yellow petal. The F$_2$ and BC to ivory had discrete *Ne* vs. *nene*, wild type R_2 vs. spotless, pale vs. ivory. In late season no empty seed when challenged by the 2D$_3$. The S5000 challenged by diploids 2D$_1$, 2D$_5$, 2D$_3$ and 2AD had also failed to cross (Rhyne, unpublished). Garohill had crossed with 2D$_5$ (Endrizzi and Phillips, 1960) and on wild type $2r_2r_1$ (Gerstel, 1953). The diagnosis had to be basic spotless genotype of 2A$_2$. A result of these studies was an upgraded basic spotless stock with many ovules per locule, given to the DNA cotton program at Tifton, Georgia and the two non-pinking identified from Arkansas, but the stock present in 2004 was a basic spotless, upgraded, and *nene*.

Non-Pinking in Pima S6 *nene* – *G. barbadense*

A PS 6 *nene* received from Turcotte, USDA, ARS, Maricopa, AZ with a comment 'not quite PS6' after 10 BC to PS6 from a donor M8 nectariless. It was wild type $2R_2r_1$, full red spot on yellow petal. Its F$_1$ with *npnp* of Rhyne-Carter had a larger petal spot and less amount of pinking than PS 6 *nene*. The 1 BC to PS 6 F$_2$ segregated into three phenotypes (Table 1). As in the diploid Asiatic, red spot dominant, but in the F$_1$ the spot was larger and more intense. Some of the *npnp* phenotype had a white spot as Pima Y_1 was segregating and *G. hirsutum* y_1y_1 cream petal does not visibly show white petal spot. After several BC to PS 6 *nene*, using the F$_1$ type as donor, the 1999 BC F$_1$ was sent to Maricopa. A single seed of this F$_1$ remained in 2003 to be planted late in our nursery. The 2004 BC F$_2$ had 19 *npnp* each nectariless, yellow petal, with ivory spot at the expected ratio of ¼ (P>0.09). This phenotype is similar to the yellow flower ghost 2A$_2$ sent by Stewart. The segregation involved the R_2 locus only (Figure 1). The r_1, if active, were similar in each parent.

Table 1. Segregation in Pima S6 *nene* backcrosses.

	Pinking		Non-Pinking	
	Pima S6	F$_1$	*npnp*	
	+		> R.C.[a]	
1994 BC		>		
1995 BC F$_2$	19	52	31	
Expected: 1:2:1	(25.5)	(51)	(25.5)	P >0.30
1999 BC F$_1$		y-1		
2003 BC F$_1$		1		
2004 BC F$_2$			19[b]	
Expected (1/4)			(13)	P >0.09

[a] R.C. – *Gossypium hirsutum* background, non-pinking $\overline{ex}$ Rhyne_Carter
[b] Pictured in Fig 1c.

Figure 1. White petal spot on yellow petals.

Non-pinking in *G. hirsutum* Background

In 2002, a single stock representing an upgraded Rhyne-Carter phenotype was challenged by the pollen of a $2r_2r_1$ carrier of transgenic Roundup tolerance. This netted a single nectariless, pinking, petal spot plant among the other F_1 nectaried, spotted plants. The nectaried F_1 were bulked and the F_2, were planted. When treated with herbicide, they segregated in a linear row of 500 plus plants with ¼ killed. The remaining ¾ tolerant plants formed a ratio of 4/16 wild type spotless, 11/16 petal spot pinking, 1/16 non-pinking spotless ($P > 0.05$). This *nene* F_1 was selfed, and the F_1 used as pollen donator on transgenic Bt DPL yellow leaf $2r_2r_1$, inbred since yellow leaf was observed in the transgenic variety release of 1997. The TC of 12 non-yellow plants showed 9 petal spot and 3 spotless. The self netted a small F_2 segregating for petal spot. Due to seed supply and nursery space, 3 of the 9 spotted TC and 1 spotless TC were advanced in 2004. The critical phenotype technique was employed in 2004 (Table 2). As in 2003, we grew the Bt yellow seedling inbred as a control. Each of the four families must segregate for yellow leaf. As each transgenic stock was wild type $2r_2r_1$ and the 2003 cross segregated in the ratio 4:11:1 *npnp*, if the upgraded stock was Rhyne-Carter non-pinking then: (a) r_2r_1 / $R_2r_1^o$ F_2 must segregate into 3 phenotypes – spotless pinking, petal spot pinking and *npnp*; (b) r_2r_1 / R_2r_1 F_2 must segregate standard ¾ R_2 petal spot to 1 wild type $r_2r_2r_1r_1$; (c) r_2r_1 / $r_2r_1^o$ F_2 has only spotless plants and $2r_2r_1^o$ is pinking; (d) r_2r_1 / r_2r_1 is wild type F_2. Table 2 shows two "b" families segregating yellow leaf and standard, 3 R_2 petal spot to 1 wild type, one "a" family with yellow leaf, petal spot, wild-type and *npnp*, and one "c" family spotless pinking with yellow leaf. With r_1r_1 'R_2' ghost became dominant red petal spot ($R_2R_2r_1r_1$ and $R_2r_2r_1r_1$ vs. $r_2r_2r_1r_1$ wild-type). The upgraded not pinking was the Rhyne-Carter genotype-phenotype.

Table 2. Segregation of non-pinking in transgenic *Gossypium hirsutum* using the critical phenotype method with "+" indicating the presence of the phenotype.

	Pinking		Non-Pinking	
	$2\ r_2r_1$	Petal spot	$2\ R_2r_1^o$	
Roundup Ready	>	F_1	+	
Bt yellow leaf TC	+	>		
2003 TC	3^c	9^{a+b}	0	
2004 TC				
$F_2 - 1_b$	+	+	0	$R_2r_2\ r_1r_1$
$F_2 - 2_b$	+	+	0	$R_2r_2\ r_1r_1$

$F_2 - 3^a$	+	+	+	$R_2r_2\ r_1r_1^o$
$F_2 - 4^c$	+	0	0	$r_2r_2\ r_1\_$

[a+b] Seed from the petal spot phenotype was utilized.
[c] Seed from the no petal spot phenotype were utilized.

White petal spot in non-pinking, yellow petal *G. hirsutum* . Our research on the recombination of R_2 Pima spot, normal sympodia and short branch cl_2cl_2 of "old" Pima and a need for verification of phenotypes, allowed Pima Y_1 to be present in *G. hirsutum* background (Table 3). The F_1 Pima S6 *nene* X *G. hirsutum* R_2^M stock backcrossed to R_2^M and the presumed recombinant, $R_2^MR_2^MCl_2cl_2$, was TC to *npnp*. The 9 plants of this one TC had red plant parts, large petal spot with Y_1y_1 segregation. Two *Y1y1* plants advanced to TC F_2. Table 3 contains segregation using the critical phenotype technique. TC-1 F_2 did not segregate for yellow-green *ygyg* but did for short-branch cl_2cl_2. The *P* for absences of $yg_2yg_2yg_1yg_1$ in ratio 15 *Yg* to 1 *yg* was acceptable; the cl_2cl_2 was present in parental $R_2^MR_2^M$ phenotype and absent in parental *npnp*. As in Rhyne-Carter (1991), the R_2 of *npnp* was tightly linked with normal sympodia Cl_2. The Y_1 had been present in the gamete with $R_2^Mcl_2yg_2Cl_1r_1^xYg_1$ (Ps_6). The 16 short-branch segregated 6 frego bracts (independent *fgfg*) and 4 y_1y_1. The yellow Y_1 had no petal spot. The 18 not-pinking plants had 4fgfg and 4 yellow petal plants with white petal spot -- Y_1Y_1 and $Y_1\_$ (Figure 1). The y_1y_1 plants were without a discernible petal spot (Figure 2). TC-2 F_2 also was homozygous for Yg_2Yg_2 and Cl_2Cl_2 with an acceptable *P*. Three of the 9 not-pinking plants had ghost on yellow petals, while the other phenotypes were spotless and the F_1 type had red spot.

Table 3. Segregation of non-pinking in conventional *Gossypium hirsutum.*

	Pinking		Non-Pinking
	Non-petal spot	Petal spot	*npnp*
$R_2^MYg_xcl_2$ TC-1 F_2	$R_2^MR_2^Mr_1r_1$ > 16 $cl_2cl_2Yg_1Yg_1$ 6 *fg* $4y_1y_1$	F_1 0 cl_2cl_2	$R_2R_2r_1^or_1^{o\,a}$ + 18 $Cl_2Cl_2Yg_2Yg_2$ 4 *fgfg* 4 Y_1Y_1 – Ghost [b]
$R_2^MYg_2cl_2$ TC-2 F_2	All $Cl_2Cl_2\ Yg_2$		9 Cl_2Cl_2 3 Y_1Y_1 Ghost [b]

[a] Cream petal background $Cl_2Cl_2Yg_2Yg_2Yg_1Yg_1\ Cl_1Cl_1$
[b] Critical phenotype: yellow petal

Figure 2. White petals with no discernible petal spot.

Conclusions

The not-pinking of our research behaved genetically and expressed phenotypically as the Rhyne-Carter. In the earlier report the R_2 was placed on the A_h genome and in the linkage with short-branch, by the R.J. Kohel stock marked as $2r_2r_1$; cl_2cl_2. R_2 Cl_2 was tightly linked. Pima S6 sent by Turcotte had segregation at the R_2 locus as its kindred PS 6 *nene* of our study. In the *G. hirsutum* stocks each study showed a 4:11:1 non pinking ratio, indicating r_2r_2 as a spotless-pinking gene. The spotless of Asiatic A genomes is a counterpoint. The gene action in *G. hirsutum*, where interaction of various R_2 alleles is indicated, parallels the comparable action in the Asiatic diploids. The crosses of 'white' A_2 petal spot with $2r_2r_1$ produced the red spot (Gerstel, 1953; Gerstel and Phillips, 1958; Meyer, personal communication; Rhyne, unpublished). In Silow's analysis of anthocyanins of *G. anomalum* $2B_1$, he conjectured the interaction of the R_2 ghost and another, R_3. He transferred R_2 ghost to $2A_2$, showing in photographs a yellow petal with a large white spot; inside the white was a smaller standard red. The research was repeated at the diploid level and the large ghost was recovered but not the R_3 (Rhyne, unpublished). Rhyne (1951) transferred to r_2 the R_2 of $2B_2$, showing substituted R_2 continued to be a large red petal spot and also showed it interacting with r_1 and R_1. The B_1 gene disturbed brown lint Lc_1 but not N_1. Rhyne reported the tight linkage of B_1 (Yg_2 R_2) and disturbed Lc_1 (1951 and 1965). The *np* R_2 in PS 6 *nene* 1BC F_2 was associated with a brown lint; the 1999 (2003 F_1) had the darker brown lint (Table 1). The association could not be handled in 2004 because three hurricanes, wind and rain scattered the linted seed of the non-storm-proof Pima and terminal growth ceased. Rhyne (1965) reported using and confirmed Meyer (1971) having R_2R_2 in her M8 $2r_2r_1$ by transference from $2B_1D_1$ directly to *G. hirsutum* M8. Meyer used $2B_1D_1$ as cytoplasmic parent and Rhyne used her F_1 as pollen parent. By lineage, the Rhyne-Carter *npnp*, derived from Gerstel (1953) $2B_1D_1$, persisted as $2R_2r_1$. Table 2 indicated that our *npnp* stock reverted by interaction to 2 R_2 r_1 on the Bt yellow leaf transgenic.

Origin of Non-Pinking

Rhyne and Carter reported that some F_1 of apparent *G. hirsutum* segregated at the R_2 locus only. Among these was a stock sent by Endrizzi with a comment he had received and used it without alternation. Stephens (1961) inserted short-branch cl_2 into *G. hirsutum* r_2 yg_2Lc_1. Other stocks were derivates of Lee (1982) 15-4 that had Pima and Ecuadorian *G. barbadense* parentage. When F_2 ($2R_2r_1$ x $2R_2$ r_1^o) segregated a spotless plant this was significant (Gerstel and Phillips, 1958). A challenge of this exception with McNair $2r_2r_1$ commercial variety produced the F_1 with petal spot and the F_2 that segregated the exceptional *npnp*. The $2R_2r_1$ ovule parent had been crossed also with a derivative of Texas Multiple having $2R_2R_1$ as Gerstel and Phillips reported (1958) and the F_2 was the usual $3R_1$:r_1. The r_1 of male parent was unknown, but traced possibly to *G. barbadense*. The inference that alteration had occurred at R_2 was offset by the results of Turcotte's PS 6 inbred X non-pinking that segregated at R_2 in the 3 Petal spot: 1 non-pinking F_2. The r_1 of each was the inactive r_1^o. The r_1 of wild type $2r_2r_1$ is active, therefore the 4:11:1 *npnp* of *G. hirsutum* and the appearance of non-pinking in F_2 yellow-leaf Bt family in Table 2.

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