GENE EXPRESSION RELATED TO THE SEMIGAMY GENOTYPE IN COTTON (*GOSSYPIUM BARBADENSE*)

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Abstract

The original semigametic line, 57-4, was isolated as a doubled haploid found in the commercial cultivar Pima S-1. The two genotypes were considered to be isogenic for the comparative studies. Although the selfed 57-4 and its derived semigametic line (Sev7) with virescent gene (v7) were consistent in producing haploids in 1996 and 1997, ranging from 36.1% to 49.9%, variation did exist in haploid percentage among individual plant progenies. The semigametic lines produced a substantial number of smaller seed than normal cottons, resulting in greater seed weight variation. The seed weight and haploid percentage are significantly correlated, i.e. the smaller the seed, the more haploid plants produced. Seed less than 100 mg produced all haploid plants. In comparison with Pima S-1, with the use of mRNA differential display technique, thirty-one differentially displayed cDNA fragments were isolated, of which, some exhibited semigamy specific expression across ovules and/or anthers. So far six cDNAs have been cloned and sequenced, one of which showed high homology to a kinetochore protein gene. The comparative molecular studies between semigametic lines and their non-semigametic isolines may eventually lead to the isolation of the semigamy gene.

Introduction

Semigamy is a type of facultative apomixis in which the male gamete does not fuse with the female gamete after entering the embryo sac. Subsequent development can give rise to an embryo containing haploid chimeral tissues of paternal and maternal origins. It provides a potential system to study reproductive biology and an alternative way to generate haploids at will in plant breeding. As evidenced in histological, cytological or genetic studies, the natural occurrence of semigamy has been reported in five families and 13 species, including *Rudbeckia spp., Zephyranthes spp., Cooperia spp., Coix aquatia* and *Gossypium barbadense* (Battaglia,1945; Coe, 1953; Mousesyan, 1977; Rao and Narayana, 1978; Solntseva, 1974; Solntseva and Vorsobina, 1972; Turcotte and Feaster, 1963, 1967). Semigamy was also induced in *Arabidopsis thaliana* by irradiating ovules and subsequently pollinating with untreated pollen (Gerlach-Cruse, 1970).

In cotton, Turcotte and Feaster (1963) first reported a high frequency, haploid-producing Pima line, 57-4, which was a doubled haploid (DH) from a haploid mutant found in the commercial cultivar Pima S-1. Later they developed a *G. barbadense* semigametic marker strain Vsg-7 by combining the virescent -7 (v_7) gene with semigamy (Turcotte and Feaster, 1969, 1973). They reported that semigamy was a dominant mutant (Se) on one locus (Turcotte and Feaster, 1974, 1975). Lines with the semigamy gene can produce 30-60% haploids when selfed and about 0.7-1.0% androgenic haploids when they were used as female parents in crosses with normal non-semigametic cottons (Chaudhari, 1978, 1979; Turcotte and Feaster, 1976). In this way, DH lines have been developed from cultivars, and intra- and inter-specific hybrids between upland cotton (*G. hirsutum*) and *G. barbadense* (Barrow and Chaudhari, 1976; Chaudhari, 1979; Feaster and Turcotte, 1973; Jenkins et al., 1984; Mahill, 1982; Mahill et al., 1983, 1984a, b; Stokes and Sappenfield, 1981; Turcotte and Feaster, 1982). The semigamy has also been transferred into different cotton cytoplasms (Mahill, 1982; Stewart, 1990), which facilitates the rapid replacement of nuclei. Although the discovery of semigamy attracted a lot of attention, there have been no active haploid breeding programs using semigamy lines in the public or private sectors. There are several problems hindering the use of semigamy:

(a) chromosomal doubling techniques are not always successful.
(b) low frequency of paternal haploids produced.
(c) lack in early identification method of haploid seeds or seedlings. Stelly et al. (1988) proposed a scheme called hybrid elimination and haploid production (HEHP) using a strain with semigamy (Se), lethal gene (Le_2^{dav}), virescent (v_7), male sterility or glandless (gl_2gl_3). This system proved to be successful (Stelly and Rooney, 1989). However, it has not been used by others.
(d) stability of semigamy lines. 57-4 produced 32.1% to 43.3% haploids in the field over years and produced as high as 61% haploids in the greenhouse in Turcotte and Feaster's test. Chaudhari (1978) obtained 40% haploids in the S1 generation of Vsg-7. Researchers in China also found that semigamy was unstable where Zhang (1993) reported that haploid percentage varied from 0 to 58.3% among progeny-rows of Vsg-7 and concluded that the semigametic trait was lost in some plants. The reasons why there was such high variation in haploid production in the semigamy strains are not presently understood.
(e) little knowledge concerning the mechanism of semigamy.

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The objectives of this study are: (1) Characterization of semigamy in terms of variation in haploid production, seed weight variation and its relationships to germination and ploidy levels; (2) Gene expression related to semigamy using reverse transcription-PCR (RT-PCR) and differential display (DD).

Materials and Methods

Variation in Haploid Production in Selfed Progenies of Semigametic Strains

Two semigametic strains of cotton, Sev-7 (previously designated as Vsg-7 by Turcotte and Feaster) and 57-4, and a non-semigametic cultivar, Pima S-1, were used in this study. Sev-7 and 57-4 have been selfed for 10 years in Fayetteville, and Pima S-1 was kindly provided by Dr. R. G. Percy in 1996. The three genotypes were grown in the field and selfed in Fayetteville in 1996. The selfed seeds from Sev-7 and 57-4 were harvested on individual plant basis. In 1997, the selfed seeds were grown in peat pots in trays in the greenhouse and transplanted in the field. Each plant progeny was scored for haploidy based on morphological factors such as plant, leaf and boll size, boll-setting and branch thickness. Haploid percentage was calculated for each progeny line.

Seed Weight Distribution and Variation

Seeds of 57-4 and Sev-7, either selfed or open-pollinated, from 1990, 1991, 1993, 1995 and 1997 were weighed. T586 from 1995 and 1997 and Pima S-1 from 1997 were included as standards. Seeds from these strains are all naked and were not delinted. The seed weight average and variation (range, standard deviation, coefficient of variation) were calculated using SAS.

Correlation Between Seed Weight and Ploidy Levels

Each selfed seed from 57-4 and Sev-7 grown in 1995 was weighed and germinated in vermiculite. After seedlings emerged, chloroplasts in guard cells of the stomata on the abaxial surface of cotyledons were counted to determine ploidy level (Chaudhari and Barrow, 1975).

mRNA Differential Display

Total RNA from young leaves, ovules on the day of anthesis (DPA 0) and 1 day post-anthesis (DAP 1), and anthers 1 day pre-anthesis (DPA -1) were extracted (Nepomuceno et al., 1997; Wan and Wilkins, 1994). Differential display of reversely transcribed mRNA was performed essentially as described by Liang and Pardee (1992) and modified by Song et al. (1995). For this preliminary report one anchor primer, consisting of 9 dT plus 3' GC, and five decamer primers were used. The 10-mer primers were chosen as ones which would give high product numbers based on our previous experience.

cDNA Cloning and Sequencing

cDNA bands differentially displayed in the polyacrylamide gels were excised, reamplified, and cloned into pGEM-T vectors (Promega, Madison, WI). The cloned fragments were sequenced using an universal primer with an AutoRead™ 200 sequencing kit in an ALF™ DNA Sequencer (Pharmacia Biotech). DNA sequences were analysed and compared with Gen EMBL databases using the BLAST program.

Results and Analysis

Variation of Haploid Production in Sev-7 and 57-4

Sev-7 and 57-4 populations from selfed seeds in 1995 produced 43.2% and 46.3% haploid plants in 1996, respectively. Diploid plants selfed in 1996 produced 36.1% and 49.9% haploids in 1997, respectively. The haploid percentage varied among 57-4 plant progenies. Of 50 selfed parental plants, 14 produced more than 60% haploid progeny; 19 gave 50-60% haploids; 7 gave 40-50% haploids; 5 gave 30-40% haploids; 3 gave 20-30% haploids; and only 2 parental plants produced 14.3% and 16.7% haploids. In all, 90% of the progeny lines in 57-4 produced 30-73.3% haploids and only 4% had less than 20% haploids. As a population, 57-4 was stable in the production of haploids over years, although variation among plant progenies did exist. According to Turcotte and Feaster (1967), 57-4 produced 30% to 60% haploids. If this range is acceptable, there were only 3 plant progeny lines with fewer haploids than expected. In Sev-7, of 32 selfed plants, 22 (68.8%) produced 30-87.5% haploids; 6 (18.8%) produced less than 20% haploids. The low haploid percentage perhaps was due to one or more of the following factors: (a) The original population of 57-4 and Sev-7 might not have been homozygous for the semigamy genotype. The low haploid-producing plants in 1996 may have been heterozygous for the semigamy genotype (Sese). (b) Immature seeds and low germination of small seeds due to haploid embryos. This is especially true for the Sev-7 because of low plant vigor related to the virescent condition. (c) Developmental factors, such as flowering time and boll-setting positions, might affect haploid percentage.

Seed Weight Distribution and Variation

During ginning we noticed that the semigametic lines had many unusually small seed. We speculated that these small seed may be related to the semigamy trait. Since 57-4 was a mutant from Pima S-1, they have the same genetic background and can be considered as isolines. We weighed the open-pollinated seed harvested in 1997 and compared their weight distribution (Figure 1). Seed weight of Pima S-1 exhibited a normal distribution, with non-significant skewness and kurtosis (both <1). Its seed size ranged from 90 to 180 mg with an average of 139 mg per seed. 57-4 showed skewed distribution with more small seed than Pima S-1. The skewness and kurtosis were both significant. The seed size ranged from 55 to 181 mg with an average of 142 mg per seed. There were 7.9% of seed with weight less than 90 mg. The seed weight variance in 57-4 (64112.4) was significantly higher than that of Pima S-1 (40275.2). The

standard deviation and coefficient of variation (CV) for 57-4 were also higher than those of Pima S-1.

The same tendency was observed with the seeds from Sev-7, 57-4 and T586 harvested in different years (Table 1). CV for normal cotton (T586 and Pima S-1) was in the normal range (10-15%), while the semigametic lines had CV's ranging from 19.4 to 45.7%, varying in different years. Interestingly, open-pollinated seed of 57-4 had lower CV than its selfed seed. In the natural out-crossing conditions in Fayetteville (20% out-crossing, Zhang and Stewart, unpublished), open pollination in 57-4 produces fewer small seeds and therefore lower variation in seed weight. The number of haploids from open pollinated seeds is also much lower than from selfed seeds.

Correlation Between Seed Weight and Ploidy Levels

The results are listed in Table 2. For 57-4, seed weight less than 100 mg had very low seed vigor (delayed germination) with 20% germination and small seedlings. These seedlings were all haploid or chimeras. Seed weight higher than 100 mg had normal germination. Seed weight between 100 mg and 135 mg also were all haploid or chimeras. Seed weight higher than 140 mg produced more diploid plants. The correlation between seed weight and ploidy levels was significantly positive ($r=0.6091$, $r_{0.05}=0.5824$). The haploid and chimeric plant percentage for all the seeds was 71.4%.

For Sev-7, seeds with weight lower than 100 mg had 15.8% germination, 100% of which were haploid plants or chimeras. Seed size between 100 mg and 110 mg also had high percentage haploid or chimeras. The diploid plant percentage became higher with increase in seed weight. The coefficient of correlation between seed size and ploidy levels was highly significant and positive ($r=0.4706$, $r_{0.05}=0.3370$, $r_{0.01}=0.4335$). The haploid and chimeric plant percentage accounted for 68.7% of seedlings. Most of the ungerminated seeds were smaller but looked normal and mature based on the dark seed coat color. When dissected, those seeds were either empty or with very small embryos or immature embryos with remnant endosperm.

Differential Display

For DDRT-PCR, roughly 100-150 bands per primer combination were displayed on the polyacrylamide gels, and 16-20 bands on the agrose gels. From our experience, although the low resolution agrose gel is not suitable for identifying genotype specific bands, it can be used to isolate tissue specific cDNAs. From only five primer combinations we were able to identify several anther specific or ovule specific cDNA bands.

After the initial inspection of the polyacrylamide gels, thirty-one bands were identified as being differentially displayed (Table 3). Those cDNA fragments can be classified into the following categories:

Type a, genotype specific. This type of fragment displayed in both the ovules and anthers in one genotype but not the other, e.g. SeZ-6 and SeZ-24.

Type b, genotype and ovule specific. Only displayed in ovules of one genotype but not the other, e.g. SeZ-7, SeZ-8, SeZ-15 and SeZ-20.

Type c, genotype and anther specific. Only displayed in anthers of one genotype but not the other, e.g. SeZ-16, SeZ-23, SeZ-25 and SeZ-30.

Type d, absent in DPA 1 ovules in 57-4, e.g. SeZ-9, SeZ-11, SeZ-12, SeZ-14 and SeZ-19.

Type e, displayed in DPA 0 ovules and DPA -1 anthers in 57-4, e.g. SeZ-26, SeZ-27 and SeZ-28.

Type f, only displayed in DPA 0 ovules in 57-4, e.g. SeZ-17, SeZ-18 and SeZ-21.

Others were quantitatively differentially displayed.

Thus far, six cDNA fragments specific to the semigametic line (57-4) have been cloned and sequenced. The sequence similarity was compared with known sequences based on the deduced amino acid sequences. While 5 cDNAs showed no significant similarities with published sequences, the deduced amino acid sequence of SeZ-4 revealed that this cDNA had regions with high homology to several proteins, including kinetochore protein, Corti protein, and RNA polymerase II elongation factor. Kinetochore protein, isolated in budding yeast and *Arabidopsis thaliana*, is evolutionarily conserved and required for cell cycle progression. Since kinetochore proteins are expected to be present in all higher organisms, we are unsure at this point whether the display represents a false positive or a mutated form of the protein in 57-4 which is related to semigamy expression during the fertilization and embryogenesis process. Any potential role of this protein in the expression of the semigamy trait would be pure speculation at this point. We are continuing to perform DDRT-PCR and isolate more putatively differentially displayed fragments. These fragments are being sequenced and selected as probes for Northern and Southern hybridizations, and for screening cDNA libraries. Ultimately, full length semigamy-specific gene transcripts are expected to be isolated and sequenced.

Differential display of mRNA transcripts and fingerprinting analysis have been successfully applied for identifying regulatory genes involved in complex processes of plants, such as self-incompatibility (Li and Gary, 1997), apomixis (Oliver et al., 1997; Vielle-Calzada et al., 1996), endosperm and embryo development (Chen et al., 1995; Heck et al., 1995; Opasahl-ferstad et al., 1997), fruit ripening (Wilkinson et al., 1995), seed dormancy (Johnson et al., 1995), fiber development (Song and Allen, 1997), environmental stress (such as sulfur starvation, water

deficit, ozone pollination) (van der Knaap and Kende, 1995; Nepomuceno et al., 1997; Truesdell and Dickman, 1997), tissue culture (Torelli et al., 1996; Tseng et al., 1995), senescence (Callard et al., 1996; Kleber-Janke and Krupinska, 1997), heat shock proteins (Joshi and Nguyen, 1996; Joshi et al., 1996) and tissue specific genes in roots (Brigham et al., 1995; Woo et al., 1995).The genetic basis of semigamy in cotton suggests that the isolation of the semigamy gene might be feasible. However, at present, there is no active transposable element found in cotton species for transposon tagging, and no saturated molecular map available for map-based cloning in cotton. The comparative molecular studies between semigametic lines and their non-semigametic isolines offers an alternative way, which may eventually lead to the isolation of the semigamy gene.

Conclusions

1. The two semigametic lines, 57-4 and Sev-7 were consistent in producing haploids in 1996 (46.3% and 43.2%, respectively) and 1997 (49.9% and 36.1%, respectively). However, variation existed in haploid percentage among individual plant progenies, which suggests that selfing and progeny test are needed to isolate the homozygous semigamy genotype.

2. The semigamy lines produce a substantial number of small seed with greater seed size variation than that of normal cottons (Pima S-1 and T586).

3. In 57-4 and Sev-7, seed less than 100 mg produced all haploid plants. The larger the seed, the more diploid plants are produced. The seed weight and ploidy level are significantly positively correlated.

4. In DDRT-PCR, agrose gels can be used to isolate tissue specific genes.

5. Thirty-one differentially displayed cDNA bands have been isolated, of which, some exhibited semigamy specific expression across ovules and/or anthers.

6. Six cDNA fragments were cloned and sequenced, one of which showed high similarity with known sequences in GeneBank.

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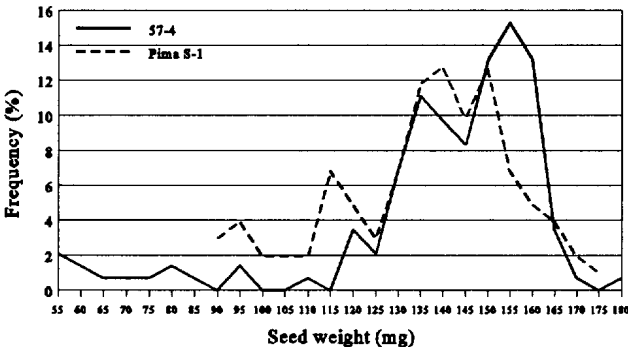

Figure 1. Seed weight distribution.

Table 1. Seed weight variation

Genotype	Year	No. Seed	Range (mg)	Mean (mg)	S	CV(%)
57-4 selfed	1990	91	18.3-168.0	126.1	37.5	29.7
57-4 selfed	1991	148	33.2-173.1	141.5	27.5	19.4
57-4 selfed	1995	32	23.7-165.2	100.8	46.1	45.7
57-4 op	1995	59	28.8-159.9	133.8	28.3	21.1
57-4 op	1997	140	55.0-181.4	142.0	25.3	21.4
Sev7 selfed	1990	109	20.3-151.2	93.5	26.6	28.4
Sev7 selfed	1995	102	18.8-140.6	90.1	35.3	39.2
Sev7 op	1997	546	30.9-130.9	106.1	26.5	25.0
T586 op	1995	52	97.4-147.2	125.5	19.7	15.7
T586 op	1997	102	75.7-146.0	122.6	13.5	11.0
Pima S-1 op	1997	102	90.4-179.5	138.5	20.1	14.5

S- standard deviation; CV- coefficient of variation

Table 2. Seed weight, germination and ploidy level

Seed weight range (mg)	Average (mg)	Seed (no.)	Germination (%)	Haploid (%)
57-4 selfed, 1995				
<125	64.1	15	20.0	100.0
125-130	127.1	3	100.0	100.0
130-135	132.0	2	50.0	100.0
135-140	na	na	na	na
140-145	142.6	2	100.0	0
145-150	145.9	1	100.0	100.0
150-155	150.8	1	100.0	100.0
155-160	157.2	2	0	na
160-165	163.2	1	100.0	0
165-170	165.5	2	100.0	50.0
Sev7 selfed, 1995				
<100	56.4	38	15.8	100.0
100-105	102.4	6	83.3	80.0
105-110	107.2	5	60.0	100.0
110-115	112.6	4	75.0	66.7
115-120	117.7	5	80.0	50.0
120-125	122.2	6	100.0	50.0
125-130	127.0	3	100.0	33.3
130-135	130.5	2	100.0	50.0
>135	141.1	1	100.0	0

na-no seed available

Table 3. Differentially displayed cDNA fragments

cDNA	Primer combination		DPA +1		DPA 0		DPA -1	
			PS-1	57-4	PS-1	57-4	PS-1	57-4
SeZ-6	Poly T-GC + 173	#1	+	-	+	-	+	-
SeZ-24	Poly T-GC + 132	#1	-	+	-	+	-	+
SeZ-7	Poly T-GC + 173	#2	-	+	-	+	-	-
SeZ-8	Poly T-GC + 173	#3	-	+	-	+	-	-
SeZ-15	Poly T-GC + 149	#7	+	-	+	-	-	-
SeZ-20	Poly T-GC + 147	#2	+	-	+	-	-	-
SeZ-16	Poly T-GC + 149	#8	-	-	-	-	+	-
SeZ-23	Poly T-GC + 147	#5	-	-	-	-	-	+
SeZ-25	Poly T-GC + 132	#2	-	-	-	-	-	+
SeZ-29	Poly T-GC + B1	#1	-	-	-	-	+	-
SeZ-9	Poly T-GC + 149	#1	+	-	+	+	+	+
SeZ-11	Poly T-GC + 149	#3	+	-	+	+	+	+
SeZ-12	Poly T-GC + 149	#4	+	-	+	+	+	+
SeZ-14	Poly T-GC + 149	#6	+	-	+	+	+	+
SeZ-19	Poly T-GC + 147	#1	+	-	+	+	+	+
SeZ-26	Poly T-GC + 132	#3	-	-	-	+	-	+
SeZ-27	Poly T-GC + 132	#4	-	-	-	+	-	+
SeZ-28	Poly T-GC + 132	#5	-	-	-	+	-	+
SeZ-17	Poly T-GC + 149	#9	-	-	-	+	-	-
SeZ-18	Poly T-GC + 149 #10		-	-	-	+	-	-
SeZ-21	Poly T-GC + 147	#3	-	-	-	+	-	-
SeZ-10	Poly T-GC + 149	#2	++	+	+	+	+	+
SeZ-13	Poly T-GC + 149	#5	++	+	+	+	+	+
SeZ-22	Poly T-GC + 147	#4	-	-	-	-	+	++
SeZ-30	Poly T-GC + B1	#2	+	+	+	+	+	-

++ strongly expressed; + expressed; -absent.