Molecular monitoring of the introgression in *Gossypium hirsutum* L. of *G. sturtianum* Willis genes controlling the "glanded-plant and glandless-seed" trait.

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Abstract

One hundred mapped micro-satellites markers located on the chromosomes of subgenome A_h were used to monitor the introgression of DNA fragments coming from the Australian species *G. sturtianum* Willis in BC_1, BC_2, BC_2S_1, BC_2S_2, BC_2S_3, BC_3, BC_3S_1 and BC_3S_2 obtained from the *G. hirsutum* L. x *G. raimondii* Ulb. x *G. sturtianum* (HRS) trispecific hybrid. In these plants, the inhibition of the gossypol synthesis only in the seed seems to be linked to the substitution of fragments of linkage groups C2, C3, C4, C5, C6, C7 and C9 of *G. hirsutum* by homeologous segments of *G. sturtianum* chromosomes. The prospect to use these genetic stocks to develop commercial varieties is discussed according to the putative genetic determinism of the "low-gossypol seed and high-gossypol plant"-trait.

Introduction

Cotton is the world's most important textile fibre producing crop, the fifth leading vegetable oil crop and its seeds are a vital source of protein and calories in the cattle and dairy industries. One of the main traits characterizing the *Gossypium* genus is the presence of pigment glands containing terpenoid aldehydes throughout the plant (Altman et al., 1990). Among the 50 diploid and tetraploid species of *Gossypium*, the "glandless-seed and glanded plant" trait exists only in some Australian wild diploid species belonging to *Sturtia* and *Hibiscoidea* sections (Brubaker et al., 1996). Gossypol is the main terpenoid aldehyde found in cotton seeds. Its concentration varies from zero in the seed kernel of some Australian wild diploid species (Brubaker, 1996) to more than 9 % in *G. davidsonii* Kell. (Carter et al., 1966). Upland cotton seeds usually contain from 0.6 to 2% gossypol (Lusas & Jividin, 1987). As this triterpenoid is very toxic to humans and monogastric animals (Lusas & Jividin, 1987; Alford et al., 1996), its concentration in all food and feed products produced with cotton flour must be very low and has to be systematically controlled. The gossypol content of cotton (*Gossypium* spp.) is controlled by at least six independent loci, namely gl_1, gl_2, gl_3, gl_4, gl_5 and gl_6 (Pauly, 1979). The formation of gossypol glands in the cultivated upland cotton is controlled by two main alleles, Gl_2 and Gl_3 (Mc Michael, 1960), located on the homeologous chromosomes 12 (A_h-genome) and 26 (D_h-genome), respectively (Endrizzi et al., 1985, Samora et al., 1994). Seed gossypol content is controlled mainly by the Gl_2 allele (Lee, 1965; McCarty et al., 1996). In Australian wild species, the genes involved in gossypol gland formation appear to be controlled by a repressive mechanism which acts until the cotyledon open and young plantlets begin to form chlorophyll (Mergeai, 1992). Bridge crosses and recurrent backcrossings were used to introgress the seed gland elimination

factor(s) from *G. sturtianum* Willis into *G. hirsutum* (Mergeai et al., 1998, Vroh Bi et al., 1999). Relatively small population sizes can be used to achieve progress from marker-assisted selection (MAS) (Yousef & Juvic, 2001). Therefore, incorporating DNA markers in traditional breeding programs with a phenotypic selection (PS) can reduce the time and efforts needed to achieve breeding goals. The recent development of molecular tools allows a more-precise approach to the structure and composition of the existing genomes in both diploid and polyploid species in cotton. Microsatellites, or simple sequence repeats (SSR), are useful codominant molecular markers because they are based on a simple polymerase chain reaction (PCR)-based technique, abundant, and randomly distributed throughout the cotton genome (Karaca et al., 2002). SSR loci tend to be both multiallelic and highly polymorphic for repeat number, which is easily scored and used in genotyping (Goldstein & Pollock 1997; Yu et al.,1999). They are also considered ideal markers for gene mapping and plant breeding (Peakall et al., 1998). Many SSR loci have already been assigned to specific chromosome arms (Liu et al, 2000).To date a SSR map of cotton is in construction (Lacape et al., 2003) which will offer a valuable new tool to analyse introgression in breeding programs. One of the major limitations in the genetic improvement of the "glanded plant-glandess seed" character is the paucity of information about gene(s) controlling the inhibition of the synthesis of gossypol in seed, the manner of inheritance, and the absence at the molecular level of a linkage map of DNA markers with Gl_2 and Gl_3, locis controlling the glanded character in upland cotton. It is then important as a first step to determine chromosomal segment(s) responsible of the expression of this character. To reach this goal we have combined phenotypic selection, based on gland density and seed gossypol content, with amplification of mapped SSR loci, in the analysis of progenies originating from wide recurrent crosses and selfing. The objectives followed in the present study are to (i) present the scheme of selection adopted in our work, (ii) report the expression of the inhibition of gossypol synthesis in seeds in HRS backcross derivatives, and (iii) determine chromosomal composition of HRS hybrids, and derivatives expressing a significant inhibition of the gossypol synthesis in the seed using mapped SSR.

Materials and methods

Plant materials

All the plants used for the creation of the trispecific hybrid HRS (*G. hirsutum* x *G. raimondii* x *G. sturtianum*, $[A_hD_hD_5C_1]$) are maintained in the cotton collection of the Gembloux Agricultural University. Two cultivars of *G. hirsutum* L. $2(A_hD_h)_1$ (NC8 and C2), selected in Democratic Republic of Congo, one accession of *G. raimondii* Ulbr. ($2D_5$) and one accession of *G. sturtianum* Will. ($2C_1$) were used for the creation of the HRS hybrid according to the pseudophyletic introgression method (Mergeai, see these proceedings). One cotton variety (Stamf) selected in Togo was used for backcrossing the HRS hybrid. The scheme to create the trispecific hybrid is detailed in Vroh Bi et al. (1998).

The BC_2S_1/09 and BC_3/09 plants issued from the HRS hybrid produced seeds with very different levels of gossypol glands and were chosen for their ability to give segregating progenies for this trait (Mergeai et al. 1998). These two plants were selfed and backcrossed to cultivar Stamf with or without application of a distilled water solution of growth regulators (100 $mg.l^{-1}$ naphtoxyacetic acid + 50 $mg.l^{-1}$ gibberellic acid) on the ovary just after pollination to produce BC_2S_2, BC_2S_3, BC_3S_1 and BC_3S_1 materials. Figure 1 shows the scheme followed to obtain the analysed materials. Only plants resulting from seeds having the lowest level of gossypol glands were retained each generation.

Assessment of gossypol content and external gossypol gland density of the seeds

In each generation, evaluations of the external gossypol gland density and seed gossypol content of the seed were carried out in order to select the genotypes which expressed most the "low-gossypol seed and high-gossypol plant" character. The gland density was assessed after removing seed integument on soaked kernels and according to a visual scale ranging from 0 for totally glandless to 10 for totally glanded seeds. The gossypol content was assessed seed by seed using the model developed by Benbouza et al. (2002) : $\%G = b_i\ (N/S)$; where $\%G$ is the content of gossypol in %, N is the number of gossypol glands per seed section, S is the area of the seed section expressed in mm^2, and b_i is a regression coefficient depending on the considered genotype. To obtain the data needed to calculate the seed gossypol content, each seed was cut in two longitudinal sections after removal of the teguments in order to assess its total number of glands N per section and its section area S in mm^2. These operations were carried out with a Nikon Eclipse E800 light and fluorescent microscope (Nikon, Tokyo, Japan) using a JVC-3-CCD colour video camera (JVC, Tokyo, Japan) and the Archive Plus program of Sony (Sony Electronics, NJ, Park Ridge, USA) to capture and analyse the images. The evolution of gland level in seedlings of backcrossed and selfed progenies derived from low-gossypol seeds was observed daily, considering the gland level in seedlings of *G. hirsutum* as controls.

DNA isolation

DNA was isolated from young leaves using an improved version of the protocol developed by Vroh bi et al. (1996). DNA solution were quantified using fluoroscan and diluted when necessary in H_20 ddw (Merck) to a concentration of 10 ng/µl before being stored at –20C° until PCR amplification.

Microsatellite analysis

Simple sequence repeat (SSR) markers used to characterize the trispecific hybrid HRS and its progenies were derived from a repeat-enriched cotton genomic library developed by B.Burr at Brookhaven National Laboratory. Clone sequences used for primer definition are available at http://demeter.bio.bnl.gov/acecot.html. The SSR analysis conditions were as described in Risterucci et al. (2000), using a 5' end labelling of the forward primer with γ-[33P] ATP and a 55°C annealing temperature. Amplification was performed on MJ Research (Water Town, Mass., USA) PTC 100 thermal cycler. After the addition of 20µl of loading buffer (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol), the mixes were denatured at 92°C for 3 min, and 5 µl of each sample were loaded onto a 6% polyacrylamide gel with 7.5 M urea and electrophoresed in 0.5% TBE buffer at 110-120 W for 1h 15 min. The gel was dried for 30 min at 80°C and exposed to X-ray film (Kodak). Number of nucleotides of the amplification product was estimated from its mobility in the gel compared to a 30-330 bp size AFLPs standard. Sizes over 330 bp were visually estimated.

The mapped SSRs reported here were initially chosen on the "A-genome" of modern tetraploid cotton based on (i) The higher pairing affinity of the donor C chromosomes (large size) for A chromosomes (medium size) than for D chromosomes (small size) (Endrizzi et al, 1985); and (ii) The greater efficiency of the seed gossypol gland repression mechanism in the wild Australian species against "A" genome carrying the Gl_2 allele determining seed gossypol gland than the "D" genome (Mergeai, 1992). Considering these two factors, it was expected that the A_h chromosomes of *G. hirsutum* would be mostly confronted with C_1 chromosomes of *G. sturtianum*. The SSR on A_h subgenome were selected according to their mapping position on the tetraploid genetic map (Lacape et al., 2003) to cover the entire length of the chromosomes. Each of the thirteen A_h chromosomes of the map was screened by a minimum of four SSRs, except for the chromosome C12 (carrying loci gl_2), for which all

available SSR were used. Totally, 136 SSRs were tested on 17 DNAs including the HRS hybrid (*G. hirsutum* x *G. raimondii* x *G. sturtianum*), *G. sturtianum*, *G. raimondii*, as well as cultivars C2, NC8, Stamf, and TM1 standard of *G. hirsutum* and the selected BC_1, BC_2, BC_2S_1, BC_3, BC_2S_2, BC_2S_3, BC_3S_1 and BC_3S_2 materials.

Results

Production of introgressed materials

Table 1 presents the total number of BC_1, BC_2, BC_2S_1, BC_2S_2, BC_2S_3, BC_3, BC_3S_1 and BC_3S_2 genotypes for which the external gossypol gland density of the kernel wall was assessed, the number of plants that were cultivated from these genotypes and the number of individuals that were finally selected in each generation of the HRS trispecific backcross derivatives. Only plants obtained from seeds having a reduced external density of gossypol glands were retained. The number of selected plants in each family depended also on the observed segregation of the desired character in the progeny. Genotypes selected at BC_1, BC_2, BC_2S_1, BC_2S_2 and BC_3S_1 stages combined two factors for selection, low level of visible external gossypol glands on the kernel and good fertility. For next generations, the seed gossypol content assessed on part of the seeds produced by selfing (Table 2) and the introgression of chromosomal segments of *G. sturtianum* identified by using microsatellites (Table 3) were combined to select the genotypes expressing most the desired trait. Except the BC_1 plant, all the materials selected are self fertile and their level of fertility expressed by the number of seeds produced per plant and per year generally increased in advanced generations.

Expression of the "glanded-plant and glandless-seed" trait

Table 2 shows the distribution of the gossypol content in the seeds produced by selfing each of the HRS trispecific hybrid backcross derivatives selected in our work. These data are compared to the distribution of the gossypol content in a sample of seeds produced by *G. hirsutum* cultivar Stamf. The gossypol content of each seed considered in this table was assessed using the method of Benbouza *et al* (2002). A drastic diminution of the gossypol content of the seed kernels is observed for the selfed derivatives selected in the progeny of the BC_2 plant. This decrease is particularly notable in the two BC_2S_3 genotypes we selected. All these plants presented a normal density of gossypol glands on their aerial parts. The segregation of the inhibition of the gossypol synthesis only in the seed is much wider for the BC_3, BC_3S_1 and BC_3S_2 materials; totally or almost totally glandless seeds are however regularly produced by these plants. It must be noted that a proportion of produced glandless seed were deformed. This proportion of distorted seeds varied from 33% for ($BC2S_3$/b, $BC3S_2$/b) to 80% for BC_2S_3/a (Table 2).

Microsatellites analysis

Among one hundred thirty six SSR primers tested , 100 (73%) SSR loci were identified to be polymorphic (some primers pairs yielded more than one locus). Their number per chromosome varied from 4 on chromosomes C2 to 15 on chromosome C9 (Table 3). The sizes of amplified products varied from 50 to 510 bp. All microsatellites used covered the entire length of the chromosomes except for chromosome C2 in which only 77 cM, out of 172.3 cM, were covered with available microsatellites. Table 4 indicates the distance covered by the SSR used for the thirteen "A_h" chromosomes. In the case of the absence of polymorphic bands between *G. raimondii* and *G. sturtianum*, the two wild parents of the HRS hybrid, the introgression of SSR locis, mapped on "A_h" subgenome, was considered as *G. sturtianum* introgression, taking into account the much higher pairing

affinities existing between A_h and C_1 chromosomes in a $A_hC_1D_hD_5$ trispecific structure where D_h chromosomes find almost perfect D_5 homeologs to pair at metaphase I.

The distribution of the introgressed segments along all the chromosomes was random. Primers BNL1897 and BNL1727 amplified two loci. While BNL3255 and C195 amplified three loci; BNL1707 amplified the highest number of loci (5). Out of 100 polymorphic SSRs amplified on the analysed materials, 61 loci were introgressed from the wild species (*G. sturtianum*) into HRS and its derivatives (Table 3). DNA segments were introgressed from twelve linkage groups of the thirteen initial "A_h" chromosomes (Table 3). The 16 loci conserved in the two BC_2S_3 selected genotypes are localised on C2, C3, C4, C5, C6, C7 and C9 linkage groups. BC_3S_2 materials conserved 9 loci mapped on C3, C5, C6, C7 and C9 linkage groups. Figure 2 shows an example of introgression and conservation of BNL 3989 locus on C3. The introgression from *G. sturtianum* was of two types. In the most frequent case, SSR alleles characterizing the cultivated and wild cotton were present, and all progenies were heterozygous at the introgressed locus. In the second case, one of the *G. hirsutum* alleles was replaced by the co-allele of *G. sturtianum.* This last case, which is consistent with reciprocal recombination, was observed on chromosome 3 with the BNL 2443b and on chromosome 6 with the BNL3359b. Two cases of no amplification, of one of the duplicated SSR loci were observed on C4 (BNL3835a) and (CIR195z) on A01. Lack of amplification of an allele was observed for some individuals for some SSR; this can be explained, according to Smulders et al. (1997), by the result of divergence or deletion in the sequences flanking the microsatellite (Karp et al., 1998) creating a null allele. The production of an undetectable amount of PCR product is another explanation given by Smulders et al. (1997). Some individuals amplified new alleles; such as in the case of HRS hybrid on C7 (BNL 1694a) and BC_3 selfed derivatives on C9 (BNL 686a). This can be explained by the heterozygosity, at this SSR loci, of the individuals used for the creation of the trispecific hybrid HRS or in recurrent crosses This kind of amplification probably indicates the intensity of genetic recombination occurring in a complex and diversified genetic background, knowing that terminal introgression would require a single crossover, whereas an intertitial segment would require a minimum of two crossovers.

Discussion

The application of a pressure of selection based on a reduced level of visible gossypol glands on the seed kernel wall every generation on the backcross and selfed derivatives of the HRS trispecific hybrid permitted the isolation of self fertile tetraploid cotton plants that produce variable proportions of seeds with very low gossypol content while they present a normal gossypol gland density on their aerial parts. The percentage of these seeds with very low gossypol content exceeds 50 % for the best selected BC_2S_3 material. All the BC_2S_3 and BC_3S_2 plants we isolated from low gossypol seed were introgressed by a rather large number of *G. sturtianum* chromosome segments. These segments are present respectively in six (C3, C4, C5, C6, C7 and C9) and seven (C2, C3, C4, C5, C6, C7 and C9) of the thirteen A_h linkage groups of the BC_3S_2 and BC_2S_3 materials. The probability to find all these alien chromosomic segments in a fortuitous way in a same genotype after so many generations of crossing and selfing is lower than 1/50.000. One can thus suppose that at least several of them play a major role in the expression of the "low-gossypol seed and high-gossypol plant" trait and that the determinism of this trait is controlled by more than two independent genes. If in the next step of our investigations the simultaneous segregation of the desired trait and these SSR markers is confirmed, their use will certainly facilitate the selection of commercial cotton genotypes expressing the "low-gossypol seed and

high-gossypol plant" character from the introgressed genetic stocks we developed. However, the rather high proportion of *G. sturtianum* heterozygous segments in the selected BC_2S_3 materials and the high proportion of deformed non viable glandless seed produced by these plants may be an indication of the presence among these segments of lethal factors that would cause the death of the embryo when homozygous.

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Table 1:Number of individuals produced and selected from HRS derivatives.

Generations	Total number of evaluated genotypes	Total number of plants cultivated	Total selected genotypes
BC1	51	16	1
BC2	17	4	1
BC2S1	15	10	1
BC2S2	4	4	2
BC2S3	35	2	2
BC3	13	10	1
BC3S1	13	13	2
BC3S2	47	18	2

Table 2: Distribution of the gossypol content in the seeds produced by selfing the HRS trispecific hybrid backcross derivatives

Parent genotype	% of gossypol in the kernel of the seeds produced by selfing of the parent genotypes																			Total No of evaluated seed
	0.000 0.049	0.050 0.099	0.110 0.149	0.150 0.199	0.200 0.249	0.250 0.299	0.300 0.349	0.350 0.399	0.400 0.449	0.450 0.499	0.500 0.549	0.550 0.599	0.600 0.649	0.650 0.699	0.700 0.749	0.750 0.799	0.800 0.849	0.850 0.899	0.900 0.949	0.950 1.000	
Stamf												3	6	11	7	10	8	4	3	1	53
BC_2S_1	4	1	2	13	11	15	12	4	2	2											66
BC_2S_2	1					6	7	8	15	4	1										42
BC_2S_3/a	28(11*)	6	4	4	6	2															50
BC_2S_3/b	9 (4*)	5	11	6	3	4	3														41
BC_3	1						1	1	6	6	5	9	3	7	5	1	1	1	2	2	51
BC_3S_1	2			2				1	1	1		4				1	1				13
BC_3S_2/a	5(4*)			1	1	1	2	5	3	3	3	5	4	5	8	3	2	1		2	54
BC_3S_2/b	3(1*)	1			1		3	7	8	5	7	1	2	5	5	2		1			51
Total No of evaluated seeds	53	13	17	26	22	28	28	26	35	21	16	19	9	17	18	7	4	3	2	4	421

*: Number of deformed seeds

Table 3: Description of the SSR amplification in HRS backcross derivatives

Linkage groups	Total SSR tested	Poly - morphic SSR	Intro- gressed SSR	HRS	BC_1	BC_2	BC_2S_1	BC_2S_2	BC_2S_3/a	BC_2S_3/b	BC_3	BC_3S_1	BC_3S_2/a	BC_3S_2/b
C 1	9	4	3	3	1	1	0	0	0	0	0	0	0	0
C 2	4	3	3	3	2	2	2	0	1	1	0	0	0	0
C3	14	9	7	7	7	4	4	4	4	4	2	3	1	2
C4	11	8	6	5	3	3	2	2	1	1	2	1	0	1
C5 sup	8	7	5	5	1	0	0	0	0	0	0	0	0	0
C5	9	6	3	3	3	3	3	2	2	2	1	2	2	2
C6 sup	14	7	3	3	2	2	2	1	2	2	2	0	2	1
C7	6	6	3	3	1	1	2	0	2	2	1	1	1	1
C9	15	14	10	10	9	7	7	3	4	3	5	2	3	2
C10	6	3	3	3	1	0	0	0	0	0	0	0	0	0
C12	13	9	9	9	9	3	2	0	0	0	0	0	0	0
A01	9	6	2	2	1	1	1	0	0	0	1	0	0	0
A02	13	7	0	0	0	0	0	0	0	0	0	0	0	0
A03	12	11	3	3	1	0	0	0	0	0	0	0	0	0
Total	143*	100	60	59	41	27	25	12	16	15	14	9	9	9

***** five primers pairs amplified more than one loci .

Table 4 : Length (cM) covered by SSRs on A chromosomes.

Chromosomes	distance (cM) covered by SSRs and other DNA markers.	distance (cM) covered by the used SSR
C1	225.2	225.2
C2	172.3	77
C3	160.7	160.7
C4	97.5	88.3
C5	287.5	247.2
C6	289.5	268.3
C7	157.2	127.6
C9	270.2	264.7
C10	133.3	133.3
C12	180.7	164.4
A01	228.2	211.8
A02	246.9	226
A03	216.6	193.6

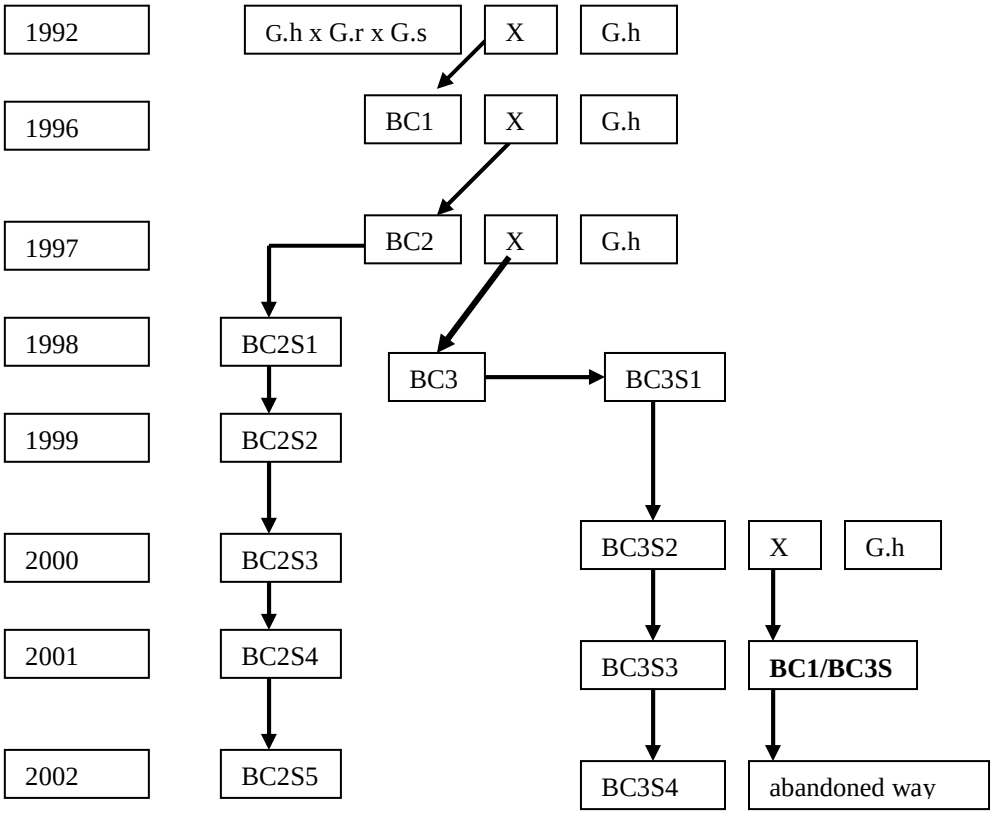

Figure 1: Development and exploitation of the HRS progenies.

G. h: *G. hirsutum;* G. r: *G. raimondii*; G. s: *G. sturtianum*

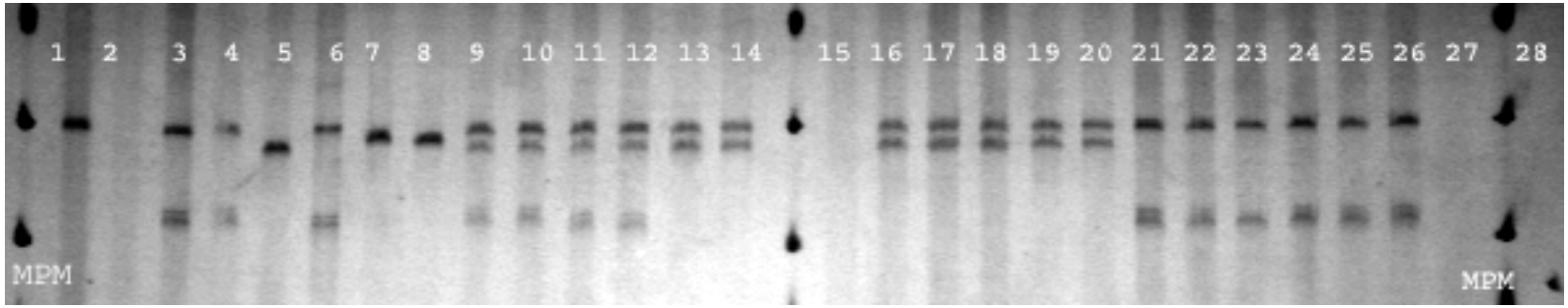

Figure 2: Example of segregation profiles (BNL3989) on HRS parents, HRS hybrid and its progenies.

1: *G. raimondii*; 2: *G. hirsutum* (var. Stamf), 3: *G. hirsutum* (var. NC8); 4: *G. hirsutum* (var.C2), 5:*G. sturtianum*; 6: *G. hirsutum* (var.TM1),
7, 8: HRS (*G. hirsutum* x *G. raimondii* x *G. sturtianum*); 9, 10: BC_1, 11, 12: BC_2; 13, 14: BC_2S_1.
15, 16: BC_2S_2; 17, 18: BC_2S_3/a, 19, 20: BC_2S_3/b; 21, 22: BC_3, 23, 24: BC_3S_1; 25, 26: BC_3S_2/a, 27, 28: BC_3S_2/b.