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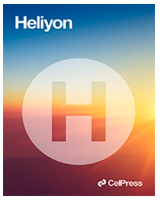

Research article

Genetic analysis and molecular validation of gene conferring petal spot phenotype in interspecific crosses of cotton

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ABSTRACT

Cotton (*Gossypium* species) has received considerable interest from the geneticists, cytologists and evolutionary biologists since the last more than a century. Here, we explore the genetics of petal spot in the interspecific derivatives involving tetraploid and diploid cottons; and confirm the location of gene governing petal spot phenotype on chromosome A7 by demonstrating co-segregation of SSR marker NAU 2186 with petal spot phenotype. The presence of petal spot was observed to be dominant over its absence. Petal spot inheritance showed significant deviation from the expected Mendelian ratio in all the segregating populations indicating segregation distortion. The distortion was biased towards the *hirsutum* parent which has important implications from introgression point of view. We also report a strong association between petal spot and petal margin coloration phenotypes. Extant American cotton varieties generally lack petal spot and margin coloration phenotypes. These petal characteristics can serve as morphological markers during germplasm characterization.

1. Introduction

Importance of cotton may be judged from the fact that it is cultivated in greater than 80 countries across the continents and economies of many countries are dependent directly or indirectly on cotton. During 2021–22, global acreage under cotton was estimated to be 32.636 million ha and three Asian countries (China, India and Pakistan) produced nearly 49 % of the world cotton [1]. Approximately 85 % of the global cotton farmers (24.2 million) belong to these Asian cotton producing countries [1]. Out of approximately 50 known *Gossypium* species, *G. hirsutum*, *G. barbadense*, *G. herbaceum* and *G. arboreum* were domesticated independently [2] for their fiber, though seed oil and proteins are other important by-products of cotton. The diploid cotton species ($2n = 2x = 26$) consist of any one of the A - G, and K genomes, whereas all seven tetraploid cottons ($2n = 4x = 52$) harbour AD genomes [3]. *G. raimondii* has been identified as the nearest living family member of D-genome progenitor of the tetraploid cottons [2,4,5]. Of the two A-genome diploid cotton species, *G. herbaceum* is most likely the progenitor [6].

Old World Cottons were once cultivated on sizeable area in the Indian sub-continent. However, these cotton species have largely been replaced by Upland cotton (*G. hirsutum*) due to its higher yield, superior fiber quality and better price. Currently, *G. hirsutum* is dominantly grown species worldwide covering greater than 98 % of cotton area [7]. *G. arboreum*, though cultivated on a limited area, has been reported to be excellent source of stress tolerance [8–14]. Given narrow genetic base of many crop plants including cotton

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[15,16] and the need to develop climate resilient cultivars, it has become all the more important to deploy useful traits/genes from related species into cultivated ones. However, several pre- and/or post-fertilization barriers are encountered during interspecific gene transfer especially when crosses are attempted between cotton species with different ploidy levels. The situation becomes more complex due to the occurrence of segregation distortion (deviation from expected Mendelian ratio) which affects estimation of recombination values, hence efficiency of genetic mapping. A trait of interest can be successfully utilized in a crop breeding programme if its inheritance is known.

Floral traits such as petal colour, presence/absence of petal spot, pollen colour etc. are important morphological traits for cotton germplasm characterisation. Petal spot, a prominent region on adaxial petal base is a characteristic of *Gossypium* species. Petal spots are absent in the modern Upland cotton cultivars [17] whereas, the primitive races possess the pigmented petal spots [18,19]. Petal spots are supposed to attract insects and aid in cross pollination thereby enhancing yield [20]. The major pigments accountable for petal colour in plants are flavonoids, anthocyanins and carotenoids. Based on transcriptome profiling and metabolic pathway analysis, a recent study [21] in cotton has demonstrated that flavonoids and anthocyanins (not the carotenoids) were responsible for the petal coloration. Among the anthocyanins, cyanidin and delphinidin derivatives were found to be predominant at the petal spot site in *G. arboreum* and constituted greater than 90 % of the total anthocyanins [22]. A single dominant locus present on chromosome A7 was observed to condition the petal spot phenotype and a GST-coding gene was identified as candidate gene for petal spot coloration in this study. Similarly, in Pima cotton (*G. barbadense*) *Gbar_A07G008330* gene located on short arm of chromosome A7 encoding a transcription factor R2R3MYB113 regulates petal spot development [20]. In this case also, the petal spot region contained higher levels of anthocyanins especially cyanidin and delphinidin. Light is the major environmental factor affecting anthocyanin biosynthesis [23]. Among various anthocyanin - related loci in cotton, *R2* locus is associated with red petal spots [20,24,25]. Genetic analyses showed

Fig. 1. (a) Flower of *G. hirsutum* without petal spot (b) presence of margin coloration; (c) flower of *G. arboreum* displaying petal spot and no margin coloration (d) flower of interspecific F_1 hybrid.

that inheritance of petal spot phenotype is monogenic [4,20,26–29]. Segregation distortion for the petal spot phenotype has also been reported in cotton [30–33]. In this article, we report the inheritance of petal spot and confirm its chromosomal location using DNA markers; and the linkage relationship between petal spot and petal margin coloration in the interspecific derivatives obtained from Upland cotton and *desi* cotton crosses.

2. Materials and methods

2.1. Experimental plant material

Interspecific cotton hybrid was generated by crossing *G. hirsutum* (Acc. LH 2107) as female and *G. arboreum* variety LD 491 as male parent [14]. LH 2107 is an advance line with cream petals lacking petal spot (Fig. 1a) and possesses slightly pink coloration on the abaxial side of the petals (Fig. 1b), whereas LD 491, a commercial variety of *arboreum* cotton has white petals with prominent petal spot and without any pink petal coloration (Fig. 1c). Due to partial pollen fertility of the interspecific F_1 hybrid, it was crossed as the male parent with LH 2107 to generate first backcross (BC_1F_1) population. This *G. hirsutum* × *G. arboreum* F_1 was also hybridized with F 2164 (an Upland cotton variety) to generate another population. Since F 2164 and LH 2107 have the same petal morphology, the progeny was considered as BC_1F_1 population for genetic analysis. A single BC_1F_1 plant (LH 2107/LD 491/F 2164) bearing flowers having petal spot and petal margin coloration was self fertilized to produce BC_1F_2 population. Freshly opened flowers of individual plants of various populations and parental lines were examined for presence/absence of petal spot and petal margin coloration.

2.2. Statistical data analysis

Chi-square tests were used to study inheritance as well as to establish independence/linkage relationship between the floral traits.

2.3. Molecular analysis

For the validation of gene conferring petal spot phenotype, 43 cotton microsatellite (SSR) primer pairs specific to chromosome A7 were synthesized. These included NAU 0845, NAU 3654, NAU 2685, NAU 2863, NAU 2686, NAU 2657, NAU 3735, NAU 2887, NAU 3380, NAU 2002, NAU 2108, NAU 3028, NAU 1362, NAU 5491, NAU 4082, NAU 3582, NAU 2682 [34], NAU 2772, NAU 2820, NAU 4956, BNL 3308, JESPR 38, HAU 0775, MGHES 0075 [35], NAU 1043, NAU 2186, NAU 2432, CIR 169, MUSB0463, JESPR 0065 [27], DOW 078, CIR 0412, DPL 0136, DPL 0167, DC 30046, DC 20124 [36], BNL 1597, NAU 1048 [26], CIR 393. CIR 335 [37], NAU 1305, BNL 2634 [38], BNL 2646 [39]. Total genomic DNA from tender leaves of parents and individual plants of BC_1F_1 population (LH 2107/LD 491/LH 2107) was extracted following Cetyltrimethyl ammonium bromide (CTAB) method [40]. *In-vitro* amplification of DNA fragments was conducted in a 96-well thermal cycler. PCR volume of 12 μL included 3 μL of template DNA (20 ng/μL), 5 μL of master mix (2× premix), 2 μL of autoclaved water; and 1 μL of upstream and downstream primers (0.5 μM). Reaction protocol was 94 °C for 5 min; 94 °C for 1 min, 52–56 °C for 1 min, 72 °C for 0.5 min, 30 cycles, and final extension at 72 °C for 7 min. 3.5 % agarose gel was used for resolution of amplified PCR products.

3. Results and discussion

The parental lines used in present investigation had contrasting floral phenotypes. The flowers of *G. hirsutum* accessions *viz.*, LH 2107 and F 2164 were devoid of petal spot but possessed pink petal margin coloration on the abaxial side. On the other hand, flowers of *G. arboreum* genotype LD 491 possessed a bright petal spot on adaxial side but no petal margin coloration.

Table 1
Chi-square test for petal spot phenotype in cotton.

Phenotype	Observed number	Expected number	$\chi^2 = (O-E)^2/E$
BC_1F_1 population: LH 2107/LD491/LH 2107			
Plants having petal spot	60	109	22.02
Plants lacking petal spot	158	109	22.02
Total	218		$\sum = 44.04$*
LH 2107/LD491/F 2164			
Plants having petal spot	38	82	23.609
Plants lacking petal spot	126	82	23.609
Total	164		$\sum = 47.218$*
BC_1F_2 population: LH 2107/LD491/F 2164			
Plants having petal spot	90	118.5	6.85
Plants lacking petal spot	68	39.5	20.56
Total	158		$\sum = 27.41$*

Note. *Significant at 5 % level.

3.1. Genetics of petal spot

Interspecific F_1 (LH 2107/LD 491) recorded the petal spot phenotype indicating dominant expression of this trait. Complete dominance of petal spot phenotype in cotton has been documented earlier [29,41–44]. In the present investigation, expression of petal spot phenotype in terms of size and intensity in the interspecific F_1 hybrid was mild (Fig. 1d) as compared to that of *arboreum* cotton parent LD 491 indicating incomplete/partial dominance. Similarly, a recent report [20] shows an intermediate size and shade of colour of petal spot in an interspecific cotton hybrid. The inheritance of petal spot phenotype is simple and the gene has been located on chromosome A7 in cotton [4,20,26–29]. Based on single gene model, the petal spot phenotype is expected to observe a ratio of 1:1 in BC_1F_1 population and 3:1 in F_2 population. In the present study, chi-squared value registered significant deviation from the expected Mendelian ratio in the backcross as well as F_2 populations suggesting segregation distortion for the petal spot phenotype (Table 1). Plants having petal spot phenotype were observed to be less in number than expected in all the segregating populations and vice versa. These results are consistent with the observations of Wilson [30] and Zhang et al. [31] recorded in the F_2 populations obtained from intraspecific and interspecific crosses of cotton, respectively. Recently, segregation distortion for petal spot phenotype in backcross derivatives obtained from Synthetic cotton polyploid (A2D1)/Upland cotton (AD1)/Upland cotton (AD1) cross has been reported [33]. In this case also, absence of petal spot was observed to be tilted in the direction of Upland cotton parent. On the contrary, plants with petal spot significantly exceeded the expected numbers in an interspecific F_2 population of cotton [32]. Results of these studies in cotton suggest that the region on chromosome A7 harboring petal spot gene is prone to segregation distortion. A number of mechanisms causing segregation distortion such as abortion of gametes (either male or female or both), preferential fertilization, competition of pollen tubes, selection of zygotes etc. have been reported. Also, physiological, cytoplasmic and environmental factors are known to affect segregation distortion. In the present study, different intensities of petal spot and margin coloration phenotypes were observed in various segregating populations (Fig. 3) which can be attributed to variable expressivity of these traits under the influence of modifiers. These results are supported by previous investigations of Harland [45] and Erpelding [46] which suggested the effect of modifiers on

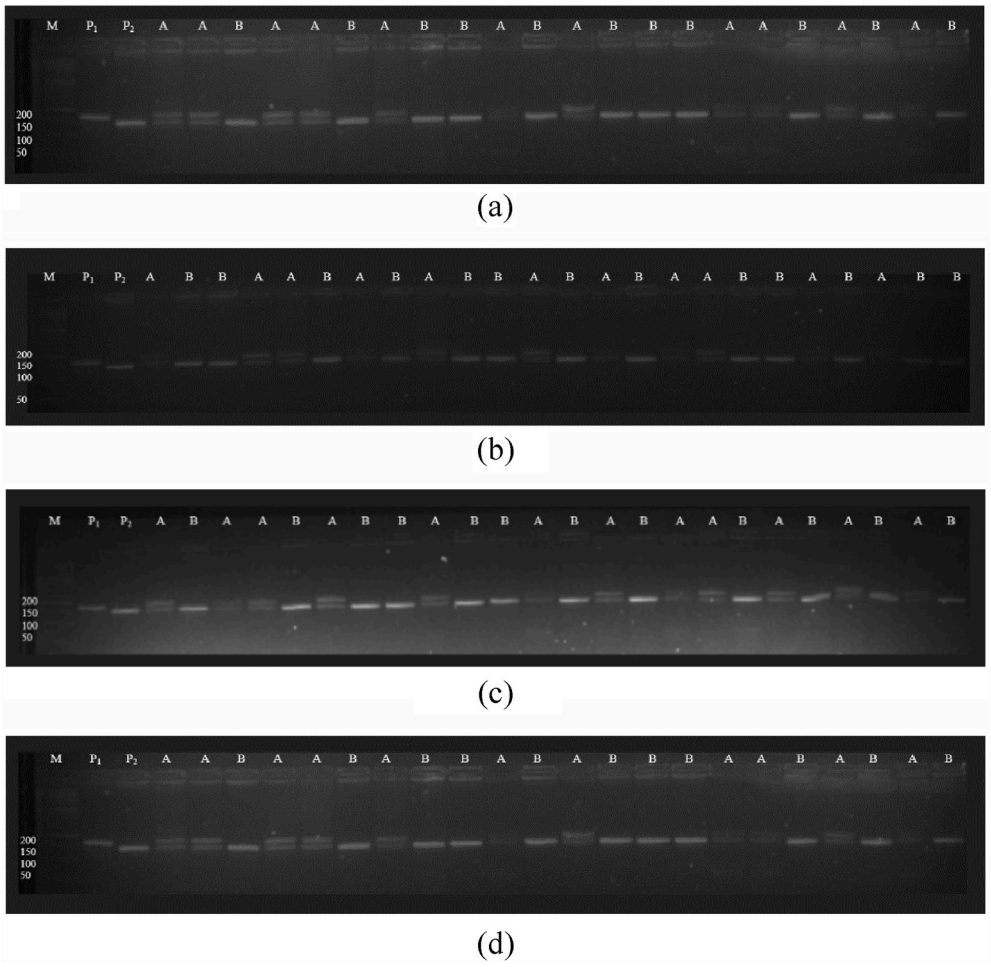

Fig. 2. Gel pictures (a–d) showing co-segregation of SSR marker NAU 2186 with petal spot phenotype. P_1, *G. arboreum*cv. LD 491; P_2,*G.hirsutum*Acc. LH 2107; A: Plant with petal spot; B: Plant without petal spot; M, 50-bp DNA ladder.

petal spot pigment development and corolla pigmentation in cotton, respectively.

3.2. Molecular validation of gene governing petal spot phenotype

As stated earlier, gene conferring petal spot phenotype has been mapped on chromosome A7 belonging to A-genome in cotton. In these studies, tetraploid × tetraploid or diploid × diploid cotton crosses were attempted to develop the mapping populations. In our investigation, we validated location of the gene governing petal spot phenotype onto chromosome A7 in an interspecific population developed from cross of Upland cotton (tetraploid) and diploid *desi* cotton (*G. arboreum*). Of 43 SSR primer pairs specific to chromosome A7, five namely NAU 2186, BNL 2646, NAU 2686, JESPR 0065 and NAU 2685 were observed to be polymorphic between the parental lines. SSR marker NAU 2186, which was reported to be 4 cM away from the R_2 locus [27] was used for further analysis. A total of 46 BC_1F_1 plants with petal spot phenotype and 47 plants lacking petal spot from the cross LH 2107/LD 491/LH 2107 along with parents were genotyped with NAU 2186. All the plants with petal spot phenotype were found to be heterozygotes carrying one allele from each of the *arboreum* and *hirsutum* parents, whereas only one *hirsutum* specific allele was amplified in plants without petal spot (Fig. 2). The results unambiguously validate the location of gene governing petal spot phenotype onto chromosome A7 in cotton.

3.3. Linkage analysis

As mentioned earlier, the parental lines differed with respect to some floral traits. *G. arboreum* line exhibited a bright petal spot and lacked margin coloration, whereas *G. hirsutum* parent was devoid of petal spot and had pink coloration on the petal margin. A chi-square test was applied to find out if margin coloration and petal spot characters were linked or not. Calculated value of the test was larger than the table values suggesting that petal margin coloration and petal spot traits were not assorting independently and were linked (Table 2). Linkage of petal spot and petal margin coloration has recently been demonstrated in backcross progenies derived from 'Synthetic cotton polyploid' and an Upland cotton cross [33]. A strong association between floral petal spots and corolla colour has been reported in *G. arboreum* and absence of recombination between these floral phenotypes has been attributed to a linkage block [46]. A previous study [47] in Asiatic cotton demonstrated that red pigmentation of flower and petal spot was under the control of closely linked genes. Similarly, it has been shown in the introgression lines developed from crosses between *G. hirsutum* × *G. bickii* that positions of genes conferring petal colour and petal spot are different but closely linked [48].

Some recent reports have provided deeper insights into the molecular mechanism of floral colour development in cotton [20–22,24,49,50]. Briefly, *Beauty Mark* (*BM*) gene has been implicated in the regulation of petal spot development in Pima cotton [20]. It encodes a transcription factor R2R3 MYB113 and regulates flavonoid level by directly targeting four flavonoid biosynthesis genes for petal spot formation. *GhTT19* gene has been linked to anthocyanin accumulation in red petals of cotton introgression lines [21]. *GhTT19* gene encodes a glutathione S-transferase (GST) protein made of 214 amino acids which is involved in anthocyanin transport. Transcriptional activity of *GhTT19* allele governing red petal phenotype was greatly enhanced through interaction with an MYB transcription factor GhPAP1. Further, it was shown that GhHY5-GhPAP1 module jointly regulated expression of *GhTT19* gene. Based on molecular mapping, transcriptome analysis and profiling of metabolites in *G. arboreum*, a gene designated *Gar07G08900* encoding GST (glutathione S-transferase) was identified for red petal spot phenotype [22]. Similarly, *GaPC* gene encoding an MYB transcription factor R2R3 governed petal colour in *G. arboreum* [49]. Genes/enzymes in anthocyanin and flavonoid biosynthetic pathways are activated by *GaPC* resulting in the development of petal colours. Several genes of anthocyanin biosynthesis pathway exhibited a strong linkage with a major pigment (cyanidin-3-O-glucoside) found in pink petals of American cotton [50]. Plant pigments are also known to play important role in imparting tolerance to various stresses. For instance, over-production of anthocyanins in transgenic cotton plants

Fig. 3. Variation in intensities of petal margin coloration and petal spot in the segregating populations.

Table 2
Chi-square test of independence for petal spot phenotype and petal margin coloration.

Phenotype	Observed number	Expected number	$\chi^2=(O-E)^2/E$
BC_1F_2 population: LH 2107/LD491/F 2164			
Petal spot and petal margin coloration absent	44	18.937	33.17
Petal spot absent and petal margin coloration present	24	49.06	12.8
Petal spot and petal margin coloration present	90	64.937	9.673
Petal spot present and margin coloration absent	0	25.063	25.063
Total	158		$\sum = 80.706$*

Note. *Significant at 5 % level.

caused enhanced resistance to certain insect pests [51]. Similarly, flavonoid accumulation in an Upland cotton mutant led to increase in resistance against a fungal wilt pathogen *Verticillium dahliae* [25].

4. Conclusion

The present investigation validates earlier reports of chromosomal location of gene conferring petal spot phenotype on chromosome A7 in *G. arboreum* as well as its orthologous in tetraploid cottons. Our results also corroborate other studies that chromosomal region involving petal spot gene is a hotspot for segregation distortion and has important implications *vis a vis* introgression of useful genes from related cotton species especially those having A-genome. Association between petal spot and margin coloration phenotypes has been established. The extant Upland cotton varieties typically lack petal spot and margin coloration. If these traits are introgressed in the Upland cotton cultivars, it would add to the existing set of morphological markers for germplasm characterization.

Data availability statement

Data recorded on individual plants may be shared on request.

CRediT authorship contribution statement

Salil Jindal: Writing – original draft, Investigation, Formal analysis, Data curation. **Dharminder Pathak:** Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Tanvir Dutt:** Investigation. **Pankaj Rathore:** Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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