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Introgression of QTL-Hotspot Region Enhances Drought Tolerance in Cotton Genotypes

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ABSTRACT

Drought tolerance is a complicated trait that primarily involves genetic and physiological characteristics. Quantitative trait loci (QTL)-hotspot regions from an upland cotton (*Gossypium hirsutum*) cultivar (CNH 28I) were introgressed into a Pima cotton (*G. barbadense*) cultivar (Suvin) with superior fiber properties to increase drought tolerance in Pima cotton. To screen the recombinant inbred lines (RILs) derived from CNH 28I×Suvin, QTL-hotspot regions on different chromosomes were selected for drought-tolerant traits: osmotic pressure (OP), canopy temperature (CT), carbon isotope ratio (CIR), relative water content (RWC), root weight (RW), root length (RL), root tip (RT), root surface area (RSA), and root volume (RV). The cotton genome's 157 simple sequence repeat markers related to drought QTL-hotspots were used to genotype the RILs. RIL population amplified polymerase chain reaction products of the same size as the drought-tolerant parent CNH 28I revealed introgression of QTL-hotspot regions. Sixteen QTLs generated polymorphism in the RIL population and parents, of which five (NAU2557, CIR143b, MUSB0818c, JESPR0205, and BNL1053) were associated with CIR, five (NAU2474, BNL3594b, BNL2884, BNL3259, and BNL1153b) with OP, four (JESPR230c, BNL3347, MUSS096a, CI061b) with RV, one (BNL3173b) with RWC, and one (BNL1705) with RL. Using the physiological metrics of RWC, CT, chlorophyll, and fiber length, six improved lines with drought-tolerant characteristics and fiber length were chosen by genotyping and phenotyping.

Cotton (*Gossypium* spp.), the most important natural fiber crop in the world, is the mainstay of the global textile industry. Drought is an abiotic factor that can negatively impact physiological and biochemical processes for agricultural yield and plant growth (Hou et al., 2018; Khan et al., 2018). An important aspect of understanding the genes governing physiological traits is the integration of quantitative trait loci (QTL) data, physiological knowledge, and gene expression data. Using F3 families from the interspecific cross between *Gossypium barbadense* L. cv. F-177 and *G. hirsutum* L. cv. Sivon, QTLs were found in water-stressed cotton (Levi et al., 2009a, b; Saranga et al., 2001, 2004). Osmotic potential (OP), carbon isotope ratio (CIR), total dry matter, boll weight, boll number, harvest index, seed cotton yield, and chlorophyll a and b are economically important traits associated with drought tolerance (DT) and were identified as DT QTLs (Saranga et al., 2001).

According to Babar et al. (2009), the OP QTLs BNL3259, BNL1153, and BNL2884 were located on chromosomes 14, 25, and 6, respectively. Near isogenic lines (NILs) were generated from the *G. barbadense* cv. F-177 and *G. hirsutum* cv. Sivon populations using marker-assisted selection (MAS), and physiological parameters such as OP, CIR, and chlorophyll a and b (Levi et al., 2011). Two QTLs were found for relative water content (RWC) and mapped on chromosomes 23 and 12 using the closest markers NAU2954 and NAU2715, respectively (Saeed et al., 2011). One QTL for excised leaf water loss on chromosome 23 and NAU2540 located on chromosome 12 were identified and mapped for DT traits in upland cotton (Saleem et al., 2015). Using 1,295 simple sequence repeat (SSR) markers, a genetic map was created that revealed 1,342 loci spread across 26 chromosomes covering 3,328.24 cM. Zheng et al. (2016) discovered 13 QTL clusters on nine chromosomes 2, 3, 5, 6, 9, 14, 15, 16, and 21. Six single nucleotide polymorphisms linked to leaf temperature, two QTLs on each of the chromosomes A05, A11, and D03, and one QTL on chromosome A01 were found in upland cotton seedlings under

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drought stress (Li et al., 2019). Abiotic stress-tolerant cotton cultivars can be created using MAS-based breeding programs using cotton with genetic resources of various attributes (Kushanov et al., 2021).

Using the Cotton SNP63K array, QTLs linked to quality and productivity were identified in a recombinant inbred line (RIL) population under field drought circumstances. Two RIL populations were genotyped using 10 water stress deficiency characteristics (Ulloa et al., 2020). The multi-parent advanced generation inter-cross (MAGIC)-RILs, which were created using 11 parents in which tolerant and sensitive alleles recombined by transgressive segregation, revealed 18 QTLs for plant height and seven QTLs for dry shoot weight for abiotic stress tolerance (Abdelraheem et al., 2021). To address climate resilience, four QTLs for DT were found on chromosome A13 and three QTLs for DT on chromosome A01. These QTLs can be used for MAS to create cotton cultivars with DT (Abdelraheem et al., 2021). In 181 intraspecific RILs produced from *G. hirsutum* var. MCU5 × TCH1218, 53 QTLs for plant height, number of sympodial branches, boll number, and boll weight were found by QTL analysis under water stress and irrigated circumstances (Boopathi et al., 2022). Lal et al. (2022) verified molecular markers associated with fusarium wilt resistance (Foc 1) in RILs of chickpea (*Cicer arietinum* L.). The Cotton QTL database, Release 2.3 (January 2018), contains QTLs for DT that have been mapped by multiple authors. The SSR markers associated with the main DT traits, such as OP, CIR, canopy temperature (CT), root tip (RT), root weight (RW), RWC, root length (RL), root surface area (RSA), and root volume (RV) are used in this study for validation (Said et al., 2015).

In an interspecific cross between *G. barbadense* and *G. hirsutum*, 204 SSR markers revealed 261 segregating bands, resulting in 233 mapped loci in cotton (Kuang et al., 2022). In this study, to genetically improve the drought resilience of DT *G. hirsutum* cv. CNH 28I with improved fiber quality traits from *G. barbadense* cv. Suvin, interspecific crosses was made and RILs were developed having introgressed DT traits. For the crop improvement program, DT lines were developed using the QTLs assessed in this study.

MATERIALS AND METHODS

Plant Materials. *G. hirsutum* cv. CNH 28I, which contains QTLs for DT-related traits, and *G. barbadense* cv. Suvin, which has superior fiber properties, were crossed and the F1 was forwarded until F14 by the single seed descent method. Each year progeny were screened in drought conditions. After stabilization, progeny were phenotyped for DT traits for three consecutive years (2021 to 2023), representing the F12 to F14 generations. A total of 129 RILs derived from this interspecific cross were used in this study.

Phenotyping the RIL Population. For three consecutive years (2021 to 2023), the RIL population was phenotyped for physiological parameters: CT, chlorophyll content, RWC, and proline content. CT is measured remotely by an infrared thermometer (IRT). Canopies emit long-wave infrared radiation as a function of their temperature. The IRT senses this radiation and converts it into an electrical signal, which is displayed as the temperature being measured in the RIL population. Chlorophyll content was measured using a Soil Plant Analysis Development (SPAD)-502 meter, which is a hand-held device that is widely used for the rapid, accurate, and non-destructive measurement of leaf chlorophyll concentrations. RWC was calculated using the formulae described by Clarke and Townley-Smith (1986):

$$RWC=(\text{fresh weight-dry weight})\div(\text{turgid weight -dry weight})\times 100.$$

The proline content in the RIL population was determined in leaf samples (1 gm fresh weight) by measuring the quantity of the colored product of proline reaction with ninhydrin at 520 nm absorbance (Bates et al., 1973). The cotton seeds were grown in agar media supplemented with different polyethylene glycol (PEG)-8000 concentrations (0, 5, 10, and15%) in half Murashige and Skoog (MS) media (Murashige and Skoog, 1962) for each concentration. The seedlings were grown for one month then the shoot and root lengths were measured.

Genotyping RILs: DNA Extraction and PCR Amplification. Genomic DNA was extracted from young leaves of RILs along with the parental lines by following the cetyltrimethylammonium bromide method of DNA extraction (Paterson et al., 1993). The isolated DNA quality was checked by gel electrophoresis and quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA). QTL markers previously reported to link to DT (OP, CIR, CT, RT, RW, RWC, RL, RSA, and RV) were selected to validate among the parents. Polymerase chain reactions (PCR) were performed in a total reaction volume of 20 µl, which consisted of 2.0 µl of 10 PCR buffer,

2.0 µl of 2mM deoxynucleotide triphosphates, 2.0 µl of 25 mM MgCl2, 2.0 µl each of forward and reverse primer (3 µM), 3.0 µl of 10 ng/µl high-quality template DNA, 1.0 µl of 1 U/µl Taq DNA polymerase, and 6.0 µl of nuclease-free water. The PCR conditions were optimized and employed for amplification comprised of an initial denaturation of 94°C for 5 min followed by 35 cycles consisting of denaturation at 94°C for 40 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min. After 35 cycles all samples were kept for a final extension at 72°C for 10 min. The thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) was used for PCR amplification. The amplified products, along with 5µl of loading dye, were separated in 3.5% Agarose gel electrophoresis. The resolved PCR products were detected by ethidium bromide staining in agarose gels and documented using a gel documentation system (Bio-Rad, Hercules, CA).

RESULTS

Using RILs generated from *G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin, QTLs strongly associated with DT characteristics in cotton were screened. The 157 SSR markers that were chosen were first screened using genomic DNA samples from both parents to find polymorphisms. The OP hot-spot regions on chromosomes C1, C2, C6, and C25 were associated with eight QTLs. The CT hot-spot region on chromosome C6 was associated with four QTLs. The CIR hot-spot regions on chromosomes C15, C24, C25, and C21 were associated with 15 QTLs. Five QTLs on chromosomes C11 and C24 were associated with RW. Five QTLs associated with RL were found on chromosomes C5, C13, C14, and C21. Four QTLs on chromosomes C13, C19, and C21 were associated with RT. Four QTLs on chromosomes C1, C13, and C21 were associated with RSA. Five QTLs associated with the RV hot-spot region were found on chromosome C19. The cotton genome has two QTLs associated with RW on chromosome C20 and one QTL on each of chromosomes C13, C20, and C24. Sixteen SSR markers were discovered to be polymorphic between the parents (*G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin). Five QTLs are associated with CIR, five QTLs with OP, four QTLs with RV, one QTL with RWC, and one QTL with RL. The PCR results of interspecific crosses between *G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin and the list of QTLs associated with DT characteristics are provided in Table 1. The population progeny lines of QTLs screened using RILs have a banding pattern that is 99% comparable to that of

Table 1. Polymorphic SSR markers linked to drought tolerant traits in the cotton genome

Sr. No.	Name of Primer	Chrom. No.	Character[z]	Forward Sequence	Reverse Sequence	Polymorphic	Band size (bp)	
							P1 Suvin	P2 CNH-28I
1	NAU 2557	C15	CIR	F:CAACCATTCAGCTTCTTGTG	R:CGAGGACTCCTTTCATGTCT	P	50	60
2	CIR 143b	C15	CIR	F:AAGAAAGAAGAAACTTCCC	R:GCCATTAAGAAGGACAAA	P	120,290	120
3	MUSB 0818c	C15	CIR	F:ACTCCGCGAACCACAGTG	R:GTCGCCAGGCCGTGTAAC	P	260,350	260,400
4	JESPR 0205	C15	CIR	F:CCCAACTCTTTCCAAACTTGAG	R:GTACATATAGATGCCCTCGTG	P	110	90
5	BNL 1053	C21	CIR	F:AGGGTCTGTCATGGTTGGAG	R:CATGCATGCGTACGTGTGTA	P	180	180,200
6	NAU 2474	C1	OP	F:CTATTACCTCCGCCGTAGTG	R:CTGAGCTAATGCAAGAAGCA	P	190	200,230
7	BNL 3594b	C6	OP	F:AGGGATTTTGATTGTTGTGC	R:TGAATTCAAAACAAATGTTAGCC	P	200	190,230
8	BNL 2884	C6	OP	F:TCAACTCATACCAAATCAATTCC	R:CCCTGTTTTGTTCAATGGGT	P	190	180
9	BNL 3259	C14	OP	F:TTTTGAAATTCCAGCGAAGG	R:GTCAATACCTGCTTCTCCACG	P	240	200
10	BNL 1153b	C25	OP	F:CTTTATCCGGAGACGGAACA	R:CTAACTTTTGCTCACCCCCA	P	340,395	350
11	BNL 3173b	C1	RWC	F:AAGCTATAAAGAGAAGATGCAACG	R:TTTAACCATTGCGTGCAAAA	P	150	150,180
12	JESPR 230c	C19	RV	F:GGGACTAAAGAAGTAATTATGCC	R:GAAACCCTTGGCCATGAG	P	290	320
13	BNL 3347	C19	RV	F:AGACTGACATGCAGCTTCCA	R:ATCTTAATTTTGAGTATAGGATAGGGG	P	190	150
14	MUSS 096a	C20	RV	F:TCTGATAAACAGCGACAAAAGG	R:AAGAAATGAACTCTCACATGGC	P	250,320	255
15	CIR 061b	C24	RV	F:TTAGTCCTCTACATACCGAA	R:TCATAATAAAGGCGTGG	P	140	170
16	BNL1705	C21	RL	F:GCCAATTTAGTATAGGAAGCAAGT	R:CATGTATTATTTTCACCCCTCTCT	P	180	190

[z]CIR: carbon isotope ratio, OP: osmotic pressure, RWC: relative water content, RL: root length, RV: root volume

the DT parent CNH 28I, indicating that the DT trait has been introgressed into the genome.

Three replications of the *G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin RIL population were used to phenotype the RILs for physiological characteristics such as CT, proline content, RWC, and chlorophyll content. The phenotyping of these RILs revealed 8.15% CT fluctuation, 88.05% proline content variability, 11.95% RWC variability, and 4.64% chlorophyll content variability (Table 2). CT ranged from 27 to 31°C (Fig. 1) whereas chlorophyll concentration varied from 31 to 40 µg/cm (Fig. 2). Using the formula developed by Clark and Townley-Smith (1986), the RWC of the *G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin RIL population in three replicates was determined to be between 85 and 98% stable (Fig. 3). By measuring the amount of the colored result of the proline reaction with ninhydrin at 520 nm absorbance, the proline content of leaf samples (1 gm fresh weight) was shown to have increased in the RIL population and varied from 150 to 270 µmole/mg of tissue (Fig. 4).

The cotton seeds were cultured in MS media supplemented with varying concentrations of PEG-8000 (0, 5, 10, and 15%). Following PEG treatment, the phenotypes of plants displayed normal growth in the control and wilting leaves at 5% PEG concentration, wilting true leaves at 10% PEG concentration, and dry plant leaves at 15% PEG concentration (Fig. 5). The shoot and root lengths in the control were measured ranging from 4.8 to 16.6cm and 3.7 to 20.6cm, respectively. At the 5 and 10% PEG

Table 2. Descriptive values associated with drought related characters

Sr. No.	Physiological traits	Mean	Range	Standard Deviation	Standard Error	CV (%)
1	Proline Content (µmole/mg)	69.38	13.8-270.1	61.08	5.58	88.05
2	Relative Water Potential (%)	86.18	53.89-100	10.3	0.94	11.95
3	Canopy Temp. (°C)	29.27	26.2-32.2	1.36	0.12	4.64
4	Chlorophyll Content (µg/cm)	36.30	29.1-45.4	2.96	0.27	8.15

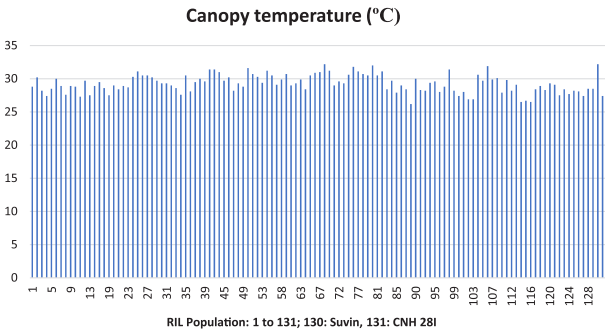

Figure 1. Frequency distribution of canopy temperature in recombinant inbred line population of cross *G. hirsutum* (28 I) X *G. barbadense* (Suvin).

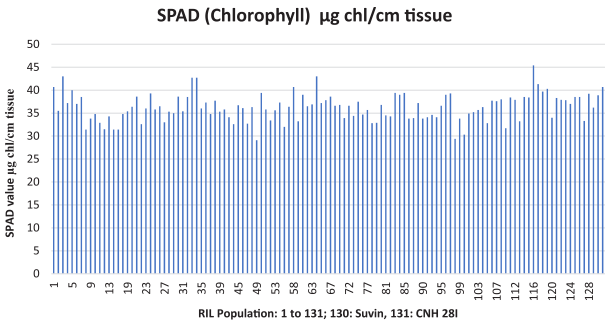

Figure 2. Frequency distribution of chlorophyll content in recombinant inbred line population of cross *G. hirsutum* (CNH 28 I) X *G. barbadense* (Suvin).

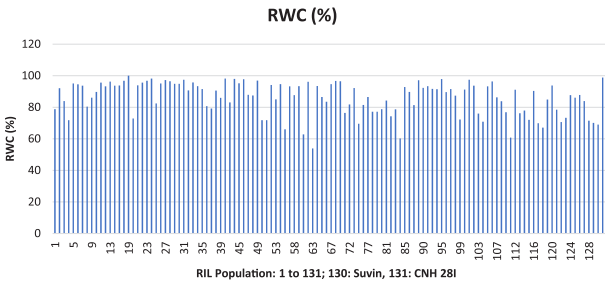

Figure 3. Frequency distribution of relative water content in recombinant inbred line population of cross *G. hirsutum* (CNH 28 I) X *G. barbadense* (Suvin).

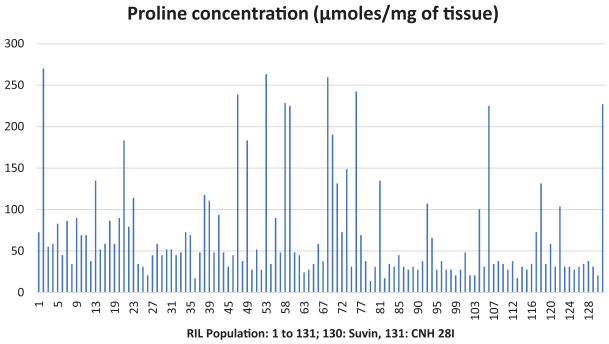

Figure 4. Frequency distribution for proline content in recombinant inbred line population of cross *G. hirsutum* (28 I) X *G. barbadense* (Suvin).

Figure 5. Phenotypes of plants after polyethylene glycol (PEG) treatment. From left to right: Control normal growth; wilting leaves at 5% PEG; wilting true leaves at 10% PEG; and dry plant at 15% PEG.

concentrations, a decrease in root length to 3.7 cm was observed, and decayed roots were observed in the 15% PEG concentration (data not shown) (Fig. 6). These phenotypic and genotypic data will be helpful to improve yield and yield components in cotton genotypes under water stress. In this study, high-yielding, DT lines were identified. Using the High Volume Instrument test, six DT lines suited for rainfed production were found to have good fiber length, strength, ginning outturn (%), and micronaire value (Table 3).

Figure 6. Phenotypes of plant roots after polyethylene glycol (PEG) treatment. From left to right: Control normal growth; wilting leaves androots at 5% PEG; wilting true leaves and roots at 10% PEG; decayed dry plant at 15% PEG.

DISCUSSION

Using genomic technologies to improve DT in different crops is crucial to reducing the effects of climate change (Cattivelli et al., 2008). The unified consensus linkage map of tetraploid cotton showed segregation distortion with a genome-wide bias to *G. hirsutum* alleles (parental genome ratio is 71/29) in the 140-line interspecific cotton RIL population, which was derived from a *G. hirsutum* × *G. barbadense* cross (Lacape et al., 2009). Marker validation involves testing a marker's effectiveness in determining a target phenotype across independent populations with varying genetic backgrounds.

There are two steps involved in MAS for DT. Finding molecular markers associated with DT and markers surrounding QTL that influence resistance is the first step. To assess the markers' efficacy in detecting the intended genotypes beforehand, those markers should be tested across several mapping populations and genotypes (Kushanov et al., 2021). To determine whether the previously published DT-linked markers can be used to create DT cultivars, the current work assesses them in a separate RIL mapping population. Through MAS, the QTLs for DT traits such as OP, CIR, and chlorophyll a and b were transferred to NILs derived from interspecific crosses between *G. barbadense* and *G. hirsutum*. These NILs introduced an extra QTL region, expressing higher CIR and lower expression of chlorophyll a and b, and osmotic adjustment (Levi et al., 2011, Saeed et al., 2011). The QTLs were also found and transferred to the RIL population, which was produced from the *G. hirsutum* cv. CNH 28I × *G. barbadense* cv. Suvin cross, in our study. Similarly, two RIL populations using the CottonSNP63K array showed drought resilience of cultivated upland cotton (*G. hirsutum*) under water stress (Ulloa et al., 2020). In cotton, molecular markers for sucking pest resistance were reported in two RILs derived from wide hybridization of wild relatives in cotton (Sridhar et al., 2017). Additionally, major QTL conferring resistance to a defoliating isolate of verticillium wilt was identified and mapped on chromosome 21 (Zhang et al., 2014). In contrast to our study, which validated molecular markers for DT traits, Lal et al. (2022) reported on the validation of molecular markers associated with biotic stress fusarium wilt resistance (Foc1) in RIL of chickpea (*Cicer arietinum*).

OP was discovered to be associated with the SSR primers NAU2474 and BNL1153, which were mapped on chromosomes 1 and 25, respectively, and BNL2884, BNL3259, and BNL3594, which were mapped on chromosome 6. CIR was discovered to be associated with the SSR primers NAU2557, CIR143, MUSB0818, and JESPR0205, which were mapped on chromosome 15, and BNL1053, which was mapped on chromosome 21. RV was discovered

Table 3. High Volume Instrument(HVI) test values of drought tolerant lines

Sr. No.	Upper Half Mean Fiber Length (mm)	Uniformity Index (%)	Micronaire	Fiber Strength Tenacity (g/tex)	Fiber Elongation (%)
1	29.5	79	3.1	27.3	5.6
2	29.0	80	3.4	28.1	5.6
3	28.7	80	3.9	28.6	6.0
4	28.3	79	3.1	25.3	5.6
5	28.2	79	3.2	27.9	5.9
6	28.2	79	3.8	29.6	6.1

to be associated with SSR primers JESPR230 and BNL3347, which were mapped on chromosome 24 as well as SSR primers MUSS096 and CIR061, which were mapped on chromosome 21. RWC was shown to be associated with SSR primer BNL3173, which was mapped on chromosome 1. In this study, the SSR markers were consistently associated with features related to DT. To create DT cultivars and transmit tolerant traits to high-yielding cultivars, MAS will employ the molecular markers assessed in this study. The RIL's higher levels of chlorophyll and osmotic solutes could help them resist drought. By adding PEG to the culture media, the water potential of the MS media is reduced, causing water stress that negatively impacts in vitro growth. PEG has been used to screen crop plants for dryness in vitro (Gopal and Iwama, 2007). According to our investigation, cotton seedlings exposed to a water deficit brought on by the PEG reaction exhibit drought resistance (Nepomuceno et al., 1998).

CONCLUSIONS

Breeding programs that aim to develop climate resilient cotton varieties will make use of the data produced by this study as well as the enhanced lines with fiber and DT features. The found markers will be used in breeding for MAS to create DT cultivars.

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