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Identification of novel sources of genetic variation for the improvement of cold germination ability in upland cotton (*Gossypium hirsutum*)

Junghyun Shim · Ritchel B. Gannaban · Benildo G. de los Reyes · Rosalyn B. Angeles-Shim

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Abstract Upland cotton (*Gossypium hirsutum*) is inherently susceptible to low temperature stress especially during the early seedling growth and boll maturation stages. The goal of the study is to identify novel sources of genetic variation that can be used to improve cold tolerance of cotton during seed germination. Genetic diversity analysis of thirty accessions from the core *Gossypium* Diversity Reference Set (GDRS) and twenty recombinant inbred lines derived from intercrossing cotton mutants with altered fatty acid content profiles established genetic variation in the test germplasm based on simple sequence repeat (SSR) genotyping. The mutants clustered in a single clade, whereas the GDRS accessions were separated into four different clades. Screening for germination ability at 12 °C and 15 °C showed that the fatty acid mutants had a significantly better overall germination compared to the GDRS accessions. Hydropriming improved the germination rate and uniformity of the GDRS accessions at 12 °C and 15 °C but not those of the fatty acid mutants, which recorded a better overall germination at 15 °C even without hydropriming. The tolerance of the FA mutants to cold stress during germination is proposed to be conferred by the higher proportion of unsaturated to saturated fatty acids in the mutants compared to the GDRS accessions. Principal component analysis established phenotypic patterns of variation that is consistent with the observed genotypic variation in the test germplasm. Results of the study indicate the potential of the mutants and select GDRS accessions as donors in breeding for cold germination ability.

Keywords *Gossypium hirsutum* · Cotton · Cold tolerance · Germination · Genetic diversity · Fatty acids

Introduction

Upland cotton (*Gossypium hirsutum*) is native to tropical and subtropical environments and grows best under long-season cultivation in warm climates. While maximum cotton production can be achieved when plants at the juvenile and adult vegetative phases are grown at a temperature range of 21–30 °C, optimum germination requires a higher temperature of

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Junghyun Shim and Ritchel B. Gannaban contributed equally to this work.

J. Shim · R. B. Gannaban · B. G. de los Reyes · R. B. Angeles-Shim (✉)
Department of Plant and Soil Science, College of Agricultural Sciences and Natural Resources, Texas Tech University, Lubbock, TX 79409-2122, USA
e-mail: rosalyn.shim@ttu.edu

28–30 °C (Lehman 1925; Ludwig 1932; Stanway 1960). Decrease in temperature below 15 °C concomitantly reduces germination percentage and induces chilling injuries that often leads to seedling malformations, taproot loss, reduced vigor, increased occurrence of seedling diseases, poor stand establishment and ultimately, reduced yields. Similarly, low temperature stress at the later stages of growth negatively impacts the crop by delaying fiber elongation and reducing cell wall thickening, resulting in low cellulose production and overall decline in crop productivity (Kittock et al. 1986; Speed et al. 1996; Krzyzanowski and Delouche 2011).

The innate susceptibility of cotton to low temperature stress severely limits the planting window for the crop particularly in temperate regions. While late season planting provides an optimum range of temperature for seed germination, it runs the danger of having the crop mature late in the fall when unexpected cold snaps below 15 °C are very likely to occur and negatively impact yield potential, as well as fiber and seed quality (Gipson et al. 1969). Conversely, early season planting to ensure maturation of the crop under warmer temperatures risks poor germination, emergence and stand establishment due to cold stress in the early spring (Christiansen and Thomas 1969; Pettigrew 2002; Buxton et al. 1977).

Chemical priming of seeds before planting has been proven to mitigate the negative impacts of cold stress on seed germination during early season planting. Exogenous applications of abscisic acid and mefluidide before planting early in the season effectively provided cold tolerance to germinating seeds (Rikin et al. 1979, 1984; Li 1994). Once the seedlings emerge, foliar spraying with the plant growth regulating chemicals, ethephon, mefluidide, and/or diethanolamine efficiently reduced seedling damage. Such strategies to mitigate the adverse effects of cold stress have increased the yield of cotton planted in the first week of April in the mid-Southern US by 10% (Pettigrew 2002).

Despite the benefits afforded by chemical mitigation strategies to the overall growth and agronomic performance of cotton planted in the early spring, the additional costs required for these inputs limit the economic productivity of the crop. In the long term, breeding and cultivation of cotton varieties with improved tolerance to low temperature stress during the germination and early seedling stages would be the most economical and efficient approach to establish production stability of early planted cotton. Identification of novel genetic variation that can be exploited to improve cold germination ability is a key step towards the development of cotton varieties with high seedling vigor characterized by high potential to germinate, emerge and establish quickly in the field at suboptimal temperatures.

Natural populations and mutant libraries of cotton hold a tremendous amount of genetic variation that can be tapped to create novel gene combinations that can increase crop productivity under a wide range of agricultural ecosystems. The US National Cotton Germplasm Collection holds more than 10,000 cotton accessions representing 45 *Gossypium* species (Campbell et al. 2010; Hinze et al. 2015). A subset of this collection makes up the *Gossypium* Diversity Reference Set (GDRS) which represents 70% of the genetic diversity present in the total cotton germplasm collection. Molecular genetic characterization of 1982 tetraploid accessions of the GDRS using 105 SSR markers indicate the presence of intra-species genetic variation that can be used for trait improvement (Hinze et al. 2015). A significant portion of this variation is also captured in the core GDRS accessions that represent a good spectrum of allelic haplotypes present across the entire diversity panel (Hinze et al. 2017). Based on the results of extensive phenotypic, physiological and biochemical profiling, the core panel also represents a large portion of phenotypic variation for salt and dehydration stress tolerance potential (B.G. de los Reyes, unpublished data). Thus, the core GDRS provides a good baseline for the initial assessment of both genetic and phenotypic variation relevant to seedling cold tolerance potential.

Alongside the natural variation provided by the core GDRS panel, induced genetic variations such as those generated by chemical mutagenesis provide an additional and valuable genetic resource (Auld et al. 1998, 2000; Bechere et al. 2009, 2012; Aslam et al. 2016) that can be utilized to broaden the genetic base of crops which has been severely limited by the bottleneck effects of domestication and artificial selection such as cotton (Shim et al. 2018). In this study, genetic and phenotypic variation for cold germination ability was evaluated across a panel comprised of a subset of the core GDRS and chemically-induced mutant lines generated from the breeding program at Texas Tech University (Auld et al.

1998, 2000; Bechere et al. 2009, 2012). The goal was to establish a baseline for future comparison of genetic and phenotypic variation across a larger germplasm collection towards the identification of potential genetic donors for cold germination improvement in cotton.

Materials and methods

Plant materials

A subset of the core GDRS (Hinze et al. 2015, 2017) composed of 30 *G. hirsutum* accessions from the National Cotton Germplasm Center, as well as 20 recombinant inbred lines (F_5 generation) that were derived from intercrossing the fatty acid mutants AFIS 1-1422-A5 and SCM3-7-3-A3 in the genetic background of Acala 1517-99 and SC 9023, respectively, (Thompson et al. 2019) were used in the study (Table 1). The mutant lines were generated at Texas Tech University from 1997 to 2008 by mutation induction of the Texas High Plains cotton cultivars SC 9023 and Acala 1517-99 using ethylmethanesulfonate (EMS). The EMS-induced mutants in the background of SC 9023 were selected primarily for tolerance to the herbicide, Imazamox, whereas those in the background of Acala 1517-99 were selected for fiber quality up to the M_5 generation before they were analyzed for fatty acid composition. The M_5 mutants AFIS 1-1422-A5 and SCM3-7-3-A3, which were identified to have lower palmitic acid (18.10–18.70%) and higher linoleic acid content (56.80–58.70%) compared to commercial cultivars, were crossed and advanced by single seed descent up to the F_5 generation. The selected F_5 lines have 17.30–19.60% palmitic acid, 1.90–3.00% stearic acid, 17.70–24.00% oleic acid and 58.20–61.80% linoleic acid. The inclusion of the fatty acid (FA) mutants in the study was based on reports of better and faster germination at cooler temperatures of seeds with higher proportion of unsaturated versus saturated fatty acids (Thompson et al. 2019; Linder 2000). On the other hand, the selected GDRS accessions have been proposed to represent a broad spectrum of variation for tolerance to abiotic stresses particularly salinity and drought. Both GDRS accessions and FA mutants were grown at the Horticultural Gardens of the Department of Plant and Soil Science in the winter of 2018 for seed multiplication and leaf sampling for DNA extraction.

Genetic diversity analysis using SSR markers

Genomic DNA was extracted from young leaf tissues of the experimental materials following the TPS method (Miura et al. 2009). A core panel of 105 SSR markers that are distributed across the 26 chromosomes of upland cotton and are known to discriminate among accessions within and among different *Gossypium* species (Supplementary Table 1) (Yu et al. 2012; Hinze et al. 2015) were used to genotype the experimental materials following a standard PCR protocol (Shim et al. 2015). The amplified PCR fragments were resolved in 3% agarose gel in 1X TBE and scored based on their molecular weight. SSRs are co-dominant markers and are not able to discriminate heterozygous alleles in polyploids. For this reason, each of the alleles identified by the SSRs were treated as independent loci and were scored as dominant markers. Allelic phenotypes based on the absence or presence of bands were scored as '1' and '0', respectively (López-Vinyallonga et al. 2015). Descriptive statistics for the SSR markers including the number of different alleles (Na), number of effective alleles (Ne) and expected heterozygosity (He) were generated using GenAlEx v 6.5b3 (Peakall and Smouse 2012). Polymorphism information content (PIC) of each individual SSR allele was calculated using the formula $PIC = \sum P_i^2$, where P_i is the frequency of the *i*th allele in the genotypes tested (Weir 1990). For dominant markers, this formula was simplified to $PIC = 2P_iQ_i$, where P_i is the frequency of the present band and Q_i is the frequency of the absent band (Tehrani et al. 2008). A genetic distance matrix based on the SSR scores was generated using GenAlEx v 6.5b3 and used to calculate similarity indices based on Jaccard's coefficient. Genetic divergence among the experimental materials was determined by clustering analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) subroutine in MEGA with 1000 bootstraps (Kumar et al. 2018).

Viability seed testing

Replicated warm germination tests were conducted to ensure that the observable differences in the

Table 1 GDRS accessions and fatty acid mutants used in the genetic diversity and cold germination ability analysis

No.	Inventory no.	Species	Accession name	Country of origin
1	SA-0002	*G. hirsutum*	Algerian Brown	Algeria
2	SA-0033	*G. hirsutum*	Hopi	USA
3	SA-0165[a]	*G. hirsutum*	M.U. 3 UA 7-41	Trinidad and Tobago
4	SA-0298	*G. hirsutum*	Wonder Wilt Wannamaker's	USA
5	SA-0300[a]	*G. hirsutum*	Rowden #2	USA
6	SA-0369[a]	*G. hirsutum*	D and PL 10-1	USA
7	SA-0582	*G. hirsutum*		USA
8	SA-0718	*G. hirsutum*	Arkansas 11 Nucala X Rowden 20-4	USA
9	SA-0857[a]	*G. hirsutum*	Acala Original	USA
10	SA-0881[b]	*G. hirsutum*	Missdel	USA
11	SA-1055[ab]	*G. hirsutum*	M 100	Uzbekistan
12	SA 1106[ab]	*G. hirsutum*		
13	SA 1156[a]	*G. hirsutum*		
14	SA-1232	*G. hirsutum*	AC 134 CB 4029	Pakistan
15	SA-1330[a]	*G. hirsutum*	Reba P 279 (Reba B-50 X Dpl Smo.)	Chad
16	SA-1406	*G. hirsutum*	S4727	Russia
17	SA-1412[a]	*G. hirsutum*	Chung Mein-Jue #7	China
18	SA-1512	*G. hirsutum*	Deltapine 50	USA
19	SA-1759	*G. hirsutum*	Chaco 510 Inta	Argentina
20	SA-1766	*G. hirsutum*	Ceix	El Salvador
21	SA-2580	*G. hirsutum*	Acala 1517-99	USA
22	SA-2895	*G. hirsutum*	Lambright 2020A	USA
23	SA-3284[a]	*G. hirsutum*	VIR-6654 SAC-24-4	Russia (Mexico)
24	SA-3403[a]	*G. hirsutum*	VIR-7137 Coker 201	Russia (USA)
25	SA-3781	*G. hirsutum*	Acala Royale	USA
26	TX-0307[ac]	*G. hirsutum*		Mexico
27	TEX 112[ac]	*G. hirsutum*		Guatemala
28	TEX 1556[ac]	*G. hirsutum*		Dominican Republic
29	TEX 1801[ac]	*G. hirsutum*		France
30	TEX 2150[abc]	*G. hirsutum*		Trinidad and Tobago
31	FA101-1	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
32	FA103-1	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
33	FA110-5	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
34	FA110-6	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
35	FA110-8	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
36	FA110-9	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
37	FA210-2	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
38	FA210-4	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
39	FA210-7	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
40	FA210-10A	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
41	FA301-1	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
42	FA301-3	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
43	FA302-3	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
44	FA303-1	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
45	FA303-3	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA

Table 1 continued

No.	Inventory no.	Species	Accession name	Country of origin
46	FA304-1	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
47	FA304-2	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
48	FA306-8	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
49	FA307-3	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA
50	FA309-3	*G. hirsutum*	EMS mutant intercross (F_5 generation)	USA

[a]GDRS accessions exhibiting 34–75% germination in replicated viability testing

[b]GDRS accessions that were excluded from genetic diversity analysis due to several missing data

[c]GDRS accessions identified as landraces. The rest of the GDRS accessions are cultivars

germination ability were due to the genetic variation for the trait and not due to poor viability of the seed lot or other post-harvest storage effects. Thirty seeds of each genotype were surface-sterilized, rolled in moist blotting paper, placed inside a sealed container and kept inside a growth chamber set at a constant temperature of 30 °C. Seed germination based on a 2 mm radicle protrusion was recorded after 4, 8 and 12 days in the growth chamber. Genotypes with $\geq$ 80% germination were considered viable and used for cold germination screening.

Cold germination screening

All experimental materials were screened for germination ability at the minimum cardinal temperature of 15 °C and at the critically low temperature of 12 °C following a modified method by Raphael et al. (2017). Briefly, 30 seeds of each of the GDRS accessions and FA mutants were surface-sterilized with 70% ethanol (v/v) for 1 min and with 20% bleach (v/v) for 10 min before rinsing them three times with sterile, distilled water. Surface-sterilized seeds were placed in petri dishes lined with two sheets of sterilized, qualitative filter paper. A third sheet of qualitative filter paper was laid over the seeds before spraying the plates with approximately 6 ml sterile distilled water. Three replicates for each of the cotton accessions and mutant lines were maintained for 14 days at 12 °C, 15 °C or 30 °C (control).

Additionally, the effects of hydropriming in enhancing cold germination ability in cotton were also determined in triplicate set-ups. A total of 30 seeds of each GDRS accession and FA mutant were surface-sterilized and plated as described above. The seeds were hydrated by spraying approximately 6 ml of sterile distilled water on to the plates before sealing them with parafilm. The plates were then incubated at 30 °C for 8 h to allow imbibition. Instead of drying the seeds after 8 h of hydration, the plates were directly transferred in growth chambers set at 12 °C, 15 °C or 30 °C. All plate set-ups, with or without hydropriming, were transferred at 30 °C after 14 days of low temperature treatment to determine seed recovery.

Germination parameters

The number of germinated seeds in all set-ups was recorded daily for 14 days. Seeds with 2 mm radicle protrusion were considered germinated. Data on germination percentage (GP), mean germination time (MGT), mean daily germination (MDG), peak value (PV) and germination index (GI) were calculated for all experimental lines. GP was derived from the total of number of seeds that germinated/total number of seeds $\times$ 100. MDG was calculated as the germination percentage at the end of the germination test/the number of days to the end of the germination test. PV was derived from the cumulative full-seed germination percentages on any day/the number of days to reach these percentages (Djavansher and Pourbeik 1976; Orchard 1977). MGT was calculated using the formula $\mathrm{MGT} = \sum f \cdot x / \sum f$ where f = seeds that germinated on day x. GI was established using the formula $\mathrm{GI} = (10 \times \mathrm{n1}) + (9 \times \mathrm{n2}) + \cdots + (1 \times \mathrm{n14})$, where n1, n2…n14 refer to the number of germinated seeds on the first, second, and subsequent days until the 14th day. The multipliers 10, 9 and 1 are weights given to the number of germinated seeds on the first, second and subsequent days, respectively (Scott et al. 1984; Bench et al. 1991).

Statistical analysis

A two-way analysis of variance (ANOVA) and a post hoc Tukey's test with significance level set at $p < 0.05$ was carried out to examine the effects of temperature, genotype and interaction of both factors on the cold germination ability of the experimental materials. Patterns of variations in the ability of the test germplasm to germinate at low temperatures, with or without hydropriming, was also established by principal component analysis (PCA) using RStudio v1.1.463 (RStudio Inc 2015). The mean values for GP, MGT, MDG, PV and GI of all the test materials were used as basis for the PCA.

Results and discussion

Descriptive statistics of SSR markers used in genetic diversity assessment

Of the 105 SSR markers used for genotyping, 51 did not amplify or did not give consistently clear bands in 50% of the materials tested and therefore were eliminated from the analysis. Several missing data also excluded the GDRS accessions SA-0881, SA-1106, SA-1055 and TX-2150 from the genetic diversity assessment.

Fifty-four SSR markers consistently amplified targets in the experimental materials, generating a total of 88 alleles. Of the 54 SSRs, 22 were polymorphic. The observed number of alleles per SSR ranged from 1 to 4, with an average of 1.62 alleles per marker. The calculated number of effective alleles (Ne) ranged from 1.02 to 2.00, with a mean Ne of 1.50 ± 0.05 (Table 2).

SSRs are co-dominant markers and can clearly distinguish between the homozygote and heterozygote forms of alleles when genotyping a diploid species. In genotyping an allopolyploid species such as cotton however, the ability of SSRs to discriminate heterozygous alleles is confounded by the amplification of duplicate loci in the homeologous genome or the presence of multiple alleles due to heterozygosity. To render our data suitable for analysis, each of the alleles generated by the 22 polymorphic SSRs were treated as an individual locus and scored as dominant markers. Following this convention, 42 independent loci were identified to be polymorphic across the experimental materials. Six loci that were generated by the SSR markers BNL2960, BNL1047, BNL1531, JESPR153, CIR218 and BNL1673 recorded a band frequency of $\leq 5\%$, indicating the presence of rare, informative bands that are unique to an accession or mutant line. The expected heterozygosity (He) of markers used across all accessions ranged from 0.02 to 0.49, with a mean He of 0.30 ± 0.02.

PIC values for each individual locus ranged from 0.04 to 0.50, with an average of 0.26 (Table 3). Twenty-one loci had PIC values of > 0.25, whereas five loci recorded lower PIC values between 0.12 and 0.25. The lowest PIC of 0.04 was shared by the six rare alleles that occurred at $\leq 5\%$ frequency. Despite the low PIC values, these alleles were not excluded from the genetic diversity assessment to ensure that the full extent of allelic variation available in the experimental materials are represented. Overall, 62% of the individual locus recorded high PIC values, indicating the suitability of the markers in assessing the genetic diversity present in the experimental materials.

Aside from establishing the informativeness of the SSR markers in differentiating among genotypes, PIC is also a function of the number of known alleles and their frequency distribution in a set of experimental materials (Botstein et al. 1980; Chesnokov and Artemyeva 2015) and is therefore reflective of the genetic diversity of the genotyped germplasm. The mean PIC value for the SSRs, which coincides with the mean He value, suggests high genetic variation among the genotypes tested.

The markers used in the study belong to a core set of SSRs that has been used to establish genetic diversity in a wide range of cotton germplasm (Yu et al. 2012; Hinze et al. 2015, 2016). Initial validation of the robustness of this core SSR set in establishing polymorphisms between and within species produced a PIC of 0.65/1 when differentiating between species and 0.29/1 when differentiating accessions within species (Yu et al. 2012). The use of the same core set of SSRs to define genetic diversity among 1541 *G. hirsutum* accessions produced a lower PIC value of 0.07 (Hinze et al. 2015). Discrepancies between the current and the previously reported PICs for the core SSRs are attributed to the differences in the number and composition of the test germplasm, as well as to the threshold used to establish the PIC values. Yu et al. (2012) scored the same set of SSRs as dominant markers with a maximum PIC of 1 to differentiate six

Table 2 Summary statistics of the SSR markers used to genotype the subset of GDRS accessions and FA mutants

Descriptive statistics	Value
Total no. of markers used	105
Total no. of markers that amplified in > 50% of the experimental materials	51
Average no. of observed alleles per SSR	1.6
Average no. of effective alleles (Ne)	1.5 ± 0.05
Average expected heterozygosity (He)	0.30 ± 0.02
No. of polymorphic SSR markers	22
No. of independent alleles amplified by polymorphic SSRs	88
No. of polymorphic alleles amplified by polymorphic SSRs	42
PIC range of alleles that were considered as independent markers	0.04–0.50
Average PIC of alleles that were considered as independent markers	0.26

G. hirsutum accessions. In the present study, the SSRs were used to genotype 20 FA mutants derived from intercrossing EMS-induced mutants in two different genetic backgrounds and 26 GDRS accessions that represent allelic haplotypes across the diversity panel of cotton. As in the previous report (Yu et al. 2012), the SSRs were scored as dominant markers capable of distinguishing only two alleles at a time. The maximum PIC value however, was pegged at 0.5 based on the formula $P_i = Q_i = 0.5$ (Chesnokov and Artemyeva 2015; Hinze et al. 2015). The different PIC threshold, combined with the genetic nature and number of the GDRS accessions genotyped, contributed to the higher PIC value obtained in the current study. Nonetheless, the results support the suitability of the core SSR set in distinguishing *G. hirsutum* accessions as previously reported.

Genetic diversity across the core GDRS accessions and EMS mutants

Based on SSR analysis, the calculated genetic distance across the test germplasm ranged from 0.01 to 0.26, with an average of 0.093. UPGMA clustering using Jaccard's coefficient grouped the test germplasm into five clusters i.e. clusters I, II, III, IV and V based on a minimum similarity threshold of approximately 38% (Fig. 1). The twenty FA mutants, along with the GDRS accessions SA-2580 and SA-1406 formed the biggest clade (cluster V), followed by the sixteen GDRS accessions that grouped into cluster IV with the TX-0307 landrace. The remaining GDRS accessions grouped into minor clades (clusters I, II and II) composed of 2–3 genotypes each.

Clustering of the FA mutants was based on a 50–100% genetic similarity. The FA mutants used in the study were recombinant inbred lines derived from crosses between EMS-induced mutants in the genetic background of SC 9023 and Acala 1517-99. Clustering of the FA mutants in a single group reflects the shared pedigree of the lines. Conversely, variation within the clade reflects the intrinsic genetic differences between the cultivars used to generate the mutants, in addition to the variations introduced by natural cross pollination of the FA mutants in the field. Outcrossing rates in upland cotton range from 5 to 50% in the US depending on the climatic conditions imposed by the geographic location, as well as on the cultivar-dependent, physio-anatomical structure of cotton flowers (Turner 1950; Moffet et al. 1980). The FA mutants were advanced to the M_5 generation, and the intercrosses up to the F_5 generation in the field. Possible natural outcrossing among the mutant lines and even with the different cotton cultivars planted in the field during the same time may have contributed to the variations observed within the FA mutant cluster.

The FA mutants 103-1 and 110-5 grouped together with SA-1406 and SA-2580, respectively at 70–80% similarity, suggesting a shared genetic lineage of the FA mutants with either GDRS accessions. SA-2580 is the cultivar Acala 1517-99 that was selected from progenies of a breeding line having an earlier version of the Acala cultivar in its pedigree (Cantrell et al. 2000). Acala was first cultivated in Texas in the early 1900s and since then has been widely utilized in breeding programs as a donor line for field selections and for the development of new cultivars with higher yield, better fiber quality and adaptability to the Texas High Plains conditions (Turner 1914). SA-2580 was

Table 3 Calculated polymorphism information content (PIC) values for independent SSR alleles that amplified polymorphic targets in the test germplasm

Locus No.	SSR Markers	No. of alleles amplified	Total no. of alleles detected from the test germplasm	Total no. cotton lines genotyped	Frequency of the 'present' allele	PIC (2PiQi)
1	BNL3545	1	37	45	0.82	0.292
2		2	20	45	0.44	0.494
3	BNL4071	1	4	46	0.09	0.159
4	BNL2960	1	40	46	0.87	0.227
5		2	35	46	0.76	0.364
6		3	9	46	0.20	0.315
7		4	1	46	0.02	0.043
8	BNL0530	1	41	46	0.89	0.194
9		2	6	46	0.13	0.227
10	NAU2140	1	30	46	0.65	0.454
11		2	16	46	0.35	0.454
12	BNL3474	1	36	46	0.78	0.340
13		2	10	46	0.22	0.340
14	MUSB1015	1	43	46	0.93	0.122
15	DPL0541	1	41	46	0.89	0.194
16		2	44	46	0.96	0.083
17	BNL1673	1	2	46	0.04	0.083
18		2	2	46	0.04	0.083
19	BNL3090	1	44	46	0.96	0.083
20	BNL1495	1	28	46	0.61	0.476
21		2	18	46	0.39	0.476
22	BNL4061	2	26	46	0.57	0.491
23	DPL0135	1	10	46	0.22	0.340
24		2	36	46	0.78	0.340
25	JESPR220	1	21	46	0.46	0.496
26	BNL1521	1	43	46	0.93	0.122
27		2	3	46	0.07	0.122
28	BNL1047	1	45	46	0.98	0.043
29		2	1	46	0.02	0.043
30	JESPR065	1	25	46	0.54	0.496
31		2	21	46	0.46	0.496
32		3	36	46	0.78	0.340
33	BNL1531	1	45	46	0.98	0.043
34		2	1	46	0.02	0.043
35	BNL4030	1	26	46	0.57	0.491
36		2	3	46	0.07	0.122
37	JESPR119	1	10	46	0.22	0.340
38	CIR218	2	2	46	0.04	0.083
39	JESPR153	1	10	46	0.22	0.340
40		2	38	46	0.83	0.287
41		3	44	46	0.96	0.083
42		4	1	46	0.02	0.043

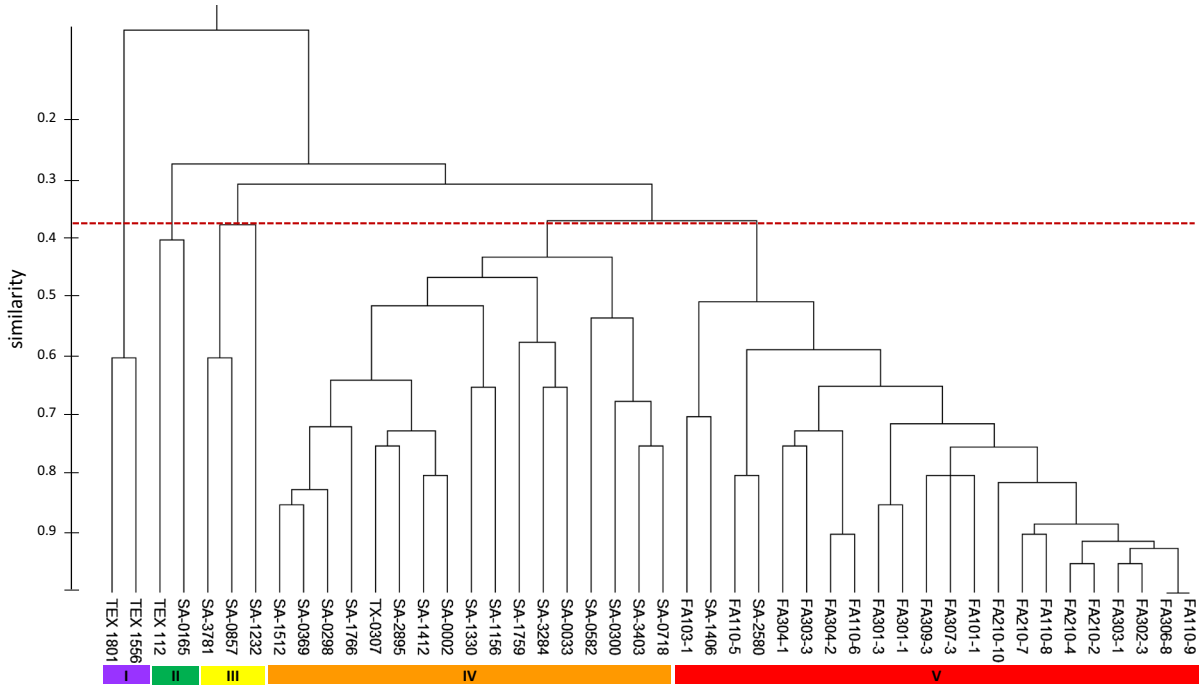


Fig. 1 UPGMA clustering of GDRS accessions and FA mutants based on Jaccard's similarity coefficient. Red broken line indicates genetic similarity threshold for the 5 major groupings as indicated by the colored bars labeled I, II, III, IV and V. (Color figure online)

one of the accessions used to generate the mutant populations and hence would naturally group with the FA mutants.

Relative to the FA cluster, accessions in cluster IV grouped together based on a lower genetic similarity ranging from approximately 42 to 85%. Cotton accessions comprising cluster IV originated from at least eight countries including the US, China, Mexico, Russia, Algeria, Chad, El Salvador and Argentina (Hinze et al. 2015). The tetraploid *G. hirsutum* is entirely Mesoamerican in origin despite having the ancestral genomes of cotton from Asia and Africa (A subgenome), as well as the Americas (D subgenome) (Wendel and Grover 2015). From the New World, *G. hirsutum* was introduced to over 50 countries across the globe (Wendel and Cronn 2003), including the eight countries where the GDRS accessions from cluster IV were collected. Clustering in a single clade underlines the shared genetic lineage of these GDRS accessions that possibly traces back to the ancestral *G. hirsutum* from Mesoamerica. Genetic divergence in members of this clade may be attributed to generations of adaptation to artificial selection for region-specific breeding targets, as well as to natural selection pressures that are unique to the environment where each germplasm was collected.

Narrower genetic similarities ranging from approximately 38 to 60% produced three clusters composed of 2–3 genotypes each. Clusters I and II included landraces (TEX 1801, TEX 1556 and TEX 112) from different geographic origins, whereas cluster III was composed of the genetically related Acala Original and Acala Royale from the US that grouped with cultivar AC 134 CB 4029 from Pakistan. Landraces are population complexes that are not necessarily high-yielding but are productive across different seasonal environments of a given locality (Mercer and Perales 2010; Frankel et al. 1998). Despite having adaptations to the edaphic and climatic conditions, as well as to traditional farming systems of a certain eco-geographical area, landraces lack formal crop improvement and are therefore genetically heterogeneous (Casañas et al. 2017). The higher degree of genetic variation in the landraces was reflected in the clustering of these accessions in smaller clades that were far removed from cultivars bred for productivity and quality traits.

Germination ability of the test germplasm under low temperatures stress

Viability testing resulted in a > 80% germination for all FA mutants and thirteen core-GDRS accessions, eliminating possible interference effects of seed lot quality and other post-harvest factors on the germination parameters observed for these lines. Seventeen core-GDRS accessions showed germination rates of 34 to 75% (Table 1). Re-testing the viability of these accessions using a different seed lot resulted in germination rates that were not significantly different from the initial results, suggesting an inherently lower germination ability of the 17 GDRS accessions under the specific conditions used in the experiment.

Germination is a quantitative developmental response of an individual seed that occurs in a specific time point under favorable physiological and environmental conditions. While two different seed genotypes may attain the same final germination percentage, one may germinate faster than the other (Orchard 1977; Scott et al. 1984; Kader et al. 1998). From a crop production standpoint, the ability of seeds to attain uniform germination in the shortest time possible is equally important to the ability of seeds to reach a maximum final germination percentage. For this reason, we used a time-to-event approach in evaluating cold germination ability of the test germplasm and considered the spread (MGT), duration (MDG) and high/low event (PV), alone or in combination with final germination percentage (GI) (Kader 2005).

Consistent with the empirically established effects of low temperature on the germination of cotton seeds (Krzyzanowski and Delouche 2011; Khetran et al. 2015; Cole and Wheeler 1974), the experimental materials recorded a general reduction in the overall germination, expressed in terms of mean GP, MGT, MDG, PV and GI, concomitant to decreasing temperatures from 30 to 12 °C (Table 4). The FA mutants recorded significantly higher mean values for GP, MDG, and GI at all temperature treatments, resulting in a higher, faster and a more uniform germination compared to both GDRS cultivars and landraces. At 15 °C and 30 °C, the GDRS landraces recorded a slightly but not significantly higher germination than the GDRS cultivars (Table 4; Supplementary

Table 4 Mean values of the germination parameters used to evaluate cold germination ability in the FA mutants, and GDRS cultivars and landraces

Germination parameter/group	Temperature treatment				
	12 °C	12 °C^{+}	15 °C	15 °C^{+}	30 °C
Germination percentage (GP; %)					
Fatty acid mutants	33.32a	30.18b	94.73a	–[c]	100.00a
GDRS cultivars	13.74b	53.05a	36.20b	92.40a	70.03b
GDRS landraces	11.13b	30.18b	42.87b	74.81b	76.65ab
Mean germination time (MGT)					
Fatty acid mutants	12.22a	11.33a	5.88a	–	1.90b
GDRS cultivars	12.42a	10.78a	5.09a	5.41a	5.16a
GDRS landraces	12.14a	11.33a	7.28a	6.82a	4.49a
Mean daily germination (MDG)					
Fatty acid mutants	0.07a	0.065a	0.81a	–	4.91a
GDRS cultivars	0.03b	0.082a	0.10b	0.31a	0.82b
GDRS landraces	0.03b	0.065a	0.14b	0.21a	0.51b
Peak value (PV)					
Fatty acid mutants	0.49a	0.46a	2.96a	–	8.14a
GDRS cultivars	0.20b	0.59a	0.68b	1.68a	3.03b
GDRS landraces	0.19b	0.46a	0.73b	1.27a	1.99b
Germination index (GI)					
Fatty acid mutants	0.57a	0.48b	4.81a	–	10.96a
GDRS cultivars	0.21b	0.78a	1.12b	4.39a	4.76b
GDRS landraces	0.21b	1.07a	0.98b	3.15a	4.84b

$^{+}$With hydropriming treatment

Values within a column followed by different letters indicate significant differences at $P < 0.05$

[c]No data. Effects of hydropriming not tested because of high GP obtained at 15 °C even without hydropriming

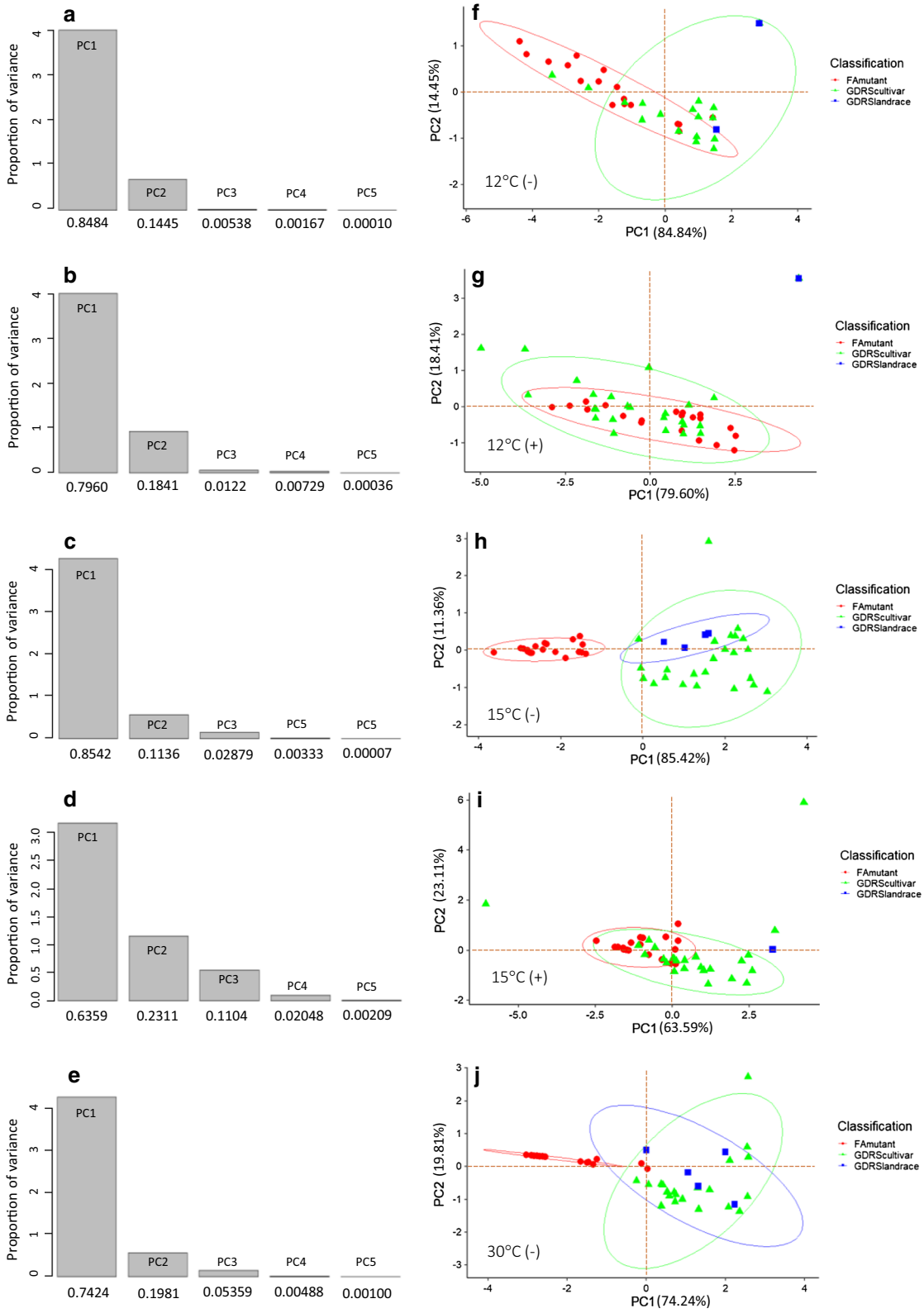
Table 2). Imbibition at 30 °C prior to cold exposure facilitated a more uniform and faster radicle emergence in both GDRS cultivars and landraces at both 12 °C and 15 °C, although germination of the landraces were more spread throughout the 14-day test period compared to the cultivars. In contrast, hydropriming did not improve the germination of the FA mutants at 12 °C. At 15 °C, the FA mutants already registered high mean values for germination and hence were no longer screened for the effects of hydropriming on germination at this temperature. Despite the benefits of hydropriming in the overall germination of the GDRS at 15 °C, the observed improvements were not at par with the germination ability of non-hydroprimed seeds of the FA mutants germinated at 15 °C (Table 4; Supplementary Table 2). Most of the seeds that did not germinate within 14 days at low temperatures were able to recover and germinate within 5–7 days at 30 °C. The FA mutants recorded the highest recovery at both low temperatures, with or without hydropriming, followed by the GDRS cultivars and the landraces. Significant effects of temperature, genotype and the interaction of both factors on all the germination parameters measured were established using a two-way ANOVA (Table 5).

PCA analysis based on the observed mean GP, MGT, MDG, PV and GI values for each genotype established phenotypic patterns of variation that is consistent with the observed genotypic variation in the test germplasm based on SSR marker profiles. More than 85% of the observed phenotypic variability in response to different temperature treatments was explained by principal component (PC) 1 (63.59–85.42%) and PC 2 (11.36–23.11%) (Fig. 2a–e). The FA mutants, and GDRS cultivars and landraces clustered separately from each other when germinated at 12 °C, 15 °C and 30 °C without hydropriming, although overlaps in the performance of the FA mutants and GDRS cultivars were observed at 12 °C (Fig. 2f, h, j). The more scattered distribution of both GDRS cultivars and landraces in the PCA biplot reflects the wider variability in the germination of the genotypes at these three temperatures. With hydropriming, the variability in the germination of the test germplasm at 12 °C was significantly reduced as indicated by the overlapping distribution of the genotypes in the PCA biplot (Fig. 2g). Similarly, hydropriming narrowed the phenotypic variation

Table 5 Two-way ANOVA of the germination parameters used to evaluate cold germination ability of the FA mutants, and GDRS cultivars and landraces

Source	DF	Sum of squares	Mean squares	F
Germination percentage (GP; %)				
Genotype	2	6604.71	3302.35	8.937*
Temperature	4	103688.45	25922.11	70.16*
Genotype × temperature	7	47160.231	6737.18	18.23*
Mean germination time (MGT)				
Genotype	2	96.98	48.49	72.25*
Temperature	4	2001.48	500.37	745.52*
Genotype × temperature	7	462.61	66.09	98.46*
Mean daily germination (MDG)				
Genotype	2	71.56	35.78	98.65*
Temperature	4	185.53	46.38	127.88*
Genotype × temperature	7	135.60	19.37	53.41*
Peak value (PV)				
Genotype	2	162.28	81.14	136.29*
Temperature	4	616.57	154.14	258.92*
Genotype × temperature	7	238.60	34.09	57.26*
Germination index (GI)				
Genotype	2	197.03	98.51	312.62*
Temperature	4	1376.12	344.03	1091.72*
Genotype × temperature	7	449.69	56.21	178.38*

*Significant at $P < 0.001$


a
Proportion of variance
PC1
PC2
PC3
PC4
PC5
0.8484
0.1445
0.00538
0.00167
0.00010
f
PC2 (14.45%)
PC1 (84.84%)
12°C (-)
Classification
FAmutant
GDRScultivar
GDRSlandrace
b
0.7960
0.1841
0.0122
0.00729
0.00036
g
PC2 (18.41%)
PC1 (79.60%)
12°C (+)
c
0.8542
0.1136
0.02879
0.00333
0.00007
h
PC2 (11.36%)
PC1 (85.42%)
15°C (-)
d
0.6359
0.2311
0.1104
0.02048
0.00209
i
PC2 (23.11%)
PC1 (63.59%)
15°C (+)
e
0.7424
0.1981
0.05359
0.00488
0.00100
j
PC2 (19.81%)
PC1 (74.24%)
30°C (-)

◀**Fig. 2** Patterns of variations in the cold germination ability of the test germplasm established using PCA based on the mean values of GP, MGT, MDG, PV and GI. Histogram representation of the proportion of variance contributed by each principal component (PC), with PC1 and PC2 accounting for most of the phenotypic variation observed in the test germplasm (**a–e**). Clustering of the FA mutants (red circle), GDRS cultivars (green triangle) and landraces (blue square) at 12 °C without imbibition (**f**) and with imbibition (**g**), at 15 °C without imbibition (**h**) and with imbibition (**i**) and at 30 °C (**j**). Clustering of the FA mutants in **i** is based on the mean values obtained for the all parameters measured from non-hydroprimed seeds. (Color figure online)

between the GDRS cultivars and non-hydroprimed FA mutants germinated at 15 °C (Fig. 2i).

Overall, the observed differentiation of the FA mutants from the GDRS cultivars and landraces based on phenotypic measurements for cold germination ability coincides with the clustering of the test germplasm into separate clades based on genetic similarities that was established by SSR marker profiles. Particularly, the observed degree of germination ability of the FA mutants and GDRS accessions in response to low temperature stress correlates with the degree of genetic variability established for the test germplasm.

Potential significance of mutations that affect the germination of FA mutants

The proportion of stored unsaturated to the saturated fatty acid content in oilseeds have been proposed to significantly influence seed germination. Studies on *Helianthus* species from Texas and Canada showed that accessions with lower proportions of saturated fatty acids had higher and/or faster germination at low temperatures (4 °C/10 °C) compared to accessions with higher proportions of saturated fatty acids (Linder 2000; Meadows 2012). Similarly, cotton seeds that have been genetically engineered to produce higher palmitic acid content recorded a high germination rate at 28 °C that significantly decreased at18 °C (Liu et al. 2017).

The effects of fatty acid composition on the germination ability of oilseeds are based on the established principles of energetics of seed oil synthesis and oxidation. Saturated fatty acids store more energy per carbon and have higher melting points compared to unsaturated fatty acids. For example, the saturated, 16-carbon palmitic acid has a melting point of 62.9 °C, whereas the di-unsaturated, 18-carbon linoleic acid has a melting point of − 5 °C (Linder 2000). At optimum temperatures (28–30 °C), β-oxidation of the single-bonded carbons of saturated fatty acids generates a larger amount of energy than the *cis* double bonds of unsaturated fatty acids, providing the germinating seeds with maximum chemical energy for growth. At lower temperatures however, the intermolecular bonds that closely pack the polar molecules in saturated fatty acids, reduces the rate at which energy is produced (Lehninger 1993; Linder 2000), resulting in slower seed germination.

Conventional cotton seeds contain approximately 50% linoleic acid, 22–26% palmitic acid, 16–20% oleic and 2–3% stearic acid (Dowd et al. 2010; Dowd 2015; Liu et al. 2009). The FA mutants used in the study have been identified to have lower proportions of palmitic acid (17.30–19.60%) and higher proportions of linoleic acid (58.20–61.80%). Given the significant difference in the melting points of the two fatty acid chains, the reduced proportion of palmitic acid chain alone is expected to effectively lower the cumulative melting temperature of the fatty acids in the mutant seeds, facilitating faster catabolism of the lipid reserves at 12 °C and 15 °C, and providing the seeds the required energy to germinate faster and at a higher rate compared to the GDRS accessions.

Additionally, the higher proportion of unsaturated to saturated fatty acids in the FA mutants may account for the non-responsiveness of FA mutants to the effects of hydropriming when germinated at 12 °C. Germination is a highly regulated process that starts with the imbibition of water by the mature, dry seed. Water influx during imbibition triggers reorganization of the cell membrane from the hexagonal to the lamellar phase. During reorganization, the cell membrane becomes highly permeable causing solutes and low molecular weight metabolites to leak out of the cell. Low temperature during water imbibition exacerbates this cytoplasmic leakage, resulting in cold-induced, structural damages in the seeds, and therefore poor germination (Liu et al. 2017; Noblet et al. 2017; Plazek et al. 2018; Yu et al. 2015).

Membrane unsaturation has been established to reduce the permeability of the cell membrane by increasing its flexibility during reorganization in water-imbibing seeds (Plazek et al. 2018; Upchurch 2008). Recent phospholipidomic studies in corn show that incorporation of unsaturated linoleic acid in the

membrane lipids increased cell membrane fluidity, facilitating the faster reorganization of the membranes from a gel to a crystalline liquid state even at a low temperature of 10 °C. The faster membrane remodeling significantly reduces electrolyte leakage during imbibition and rapidly restores cellular function, thereby facilitating faster and higher seed germination (Noblet et al. 2017). Conversely, earlier studies showing a significant decline in the germination of canola seeds engineered to have higher proportions (30–40%) of stearic acid compared to conventional cultivars have proposed the accumulation of saturated stearic acid in the membrane as the cause of poor seed germination at even optimum temperature (Thompson and Li, 1997).

In the current study, the higher proportion of linoleic acid in the FA mutants may have contributed to the unsaturation of the cell membrane, giving it more flexibility during reorganization even at the critically low temperature of 12 °C. The enhanced fluidity of the cell membrane due to membrane unsaturation allowed the FA seeds to rapidly restore normal cellular function, attaining their maximum germination potential at 12 °C without the need for hydropriming. Differences in the maximum germination potential of the FA mutants at 12 °C and 15 °C may be attributed as inherent genetic response to lower temperatures.

While the presumed uniqueness in the fatty acid profiles of these mutants seem to explain why they were able to sustain germination at low temperature in the context of cellular energetics (i.e. FA catabolism), the precise role of membrane properties as conferred by the FA profiles remains purely speculative, and needs to be validated with further studies.

Conclusion

Identification of sources of genetic variation that can be utilized to improve traits of agronomic importance is requisite for the development of an effective breeding strategy for any crop. An important component of this exercise is the purposeful characterization of genetic resources.

In the present study, we sought to evaluate the genotypic and phenotypic variation in the cold germination ability of a minimal set of cotton germplasm to identify sources of variation that can be used to improve germination of cotton under low temperature stress. Combined genetic and phenotypic screening established a wide range of genetic variation in the experimental materials that can provide the foundation for cold germination breeding in upland cotton.

At the minimum cardinal temperature of 15 °C, the FA mutants were identified as robust germinators, recording a higher overall germination of un-imbibed seeds compared to the imbibed seeds of the GDRS accessions. Even at the critically low temperature of 12 °C, the FA mutants outperformed the GDRS cultivars and landraces in terms of all the germination parameters measured, without requiring prior water imbibition treatment.

Generally, the GDRS cultivars were also identified as robust germinators at 15 °C but only when allowed to imbibe water at higher temperatures (28–30 °C) prior to cold germination. At the critically low temperature of 12 °C, the cultivars required prior imbibition at higher temperatures to achieve a minimum of 50% germination.

Lastly, the GDRS landraces were identified as moderate germinators that strictly require prior imbibition for an optimum mean germination of $\geq$ 74% at the minimum cardinal temperature of 15 °C. Like the GDRS cultivars, the landraces positively and significantly responded to hydropriming even at the critically low temperature of 12 °C. Improvement in the germination ability of the landraces however, was not at par with that of imbibed GDRS cultivars.

Based on controlled environment screening, the FA mutants constitute better donors of cold germination ability in cotton compared to the GDRS cultivars and landraces, although the unique and non-overlapping physiological properties of all the test germplasm make them ideal for potential use in genetic studies, particularly in understanding the molecular mechanisms underlying cold germination ability in cotton as affected by the fatty acid profiles in the seed. Screening for cold germination ability under actual field conditions is currently underway to validate the performance of the test germplasm under controlled conditions and establish their practical use in breeding programs.

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