COTIP: Cotton TILLING Platform, a Resource for Plant Improvement and Reverse Genetic Studies

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Cotton is cultivated worldwide for its white fiber, of which around 90% is tetraploid upland cotton (Gossypium hirsutum L.) carrying both A and D genome. Since centuries, yield increasing efforts for the cotton crop by conventional breeding approaches have caused an extensive erosion of natural genetic variability. Mutation based improvement strategies provide an effective way of creating new allelic variations. Targeting Induced Local Lesions IN Genomes (TILLING) provides a mutation based reverse genetic strategy to create and evaluate induced genetic variability at DNA level. Here, we report development and testing of TILLING populations of allotetraploid cotton (G. hirsutum) for functional genomic studies and mutation based enrichment of cotton genetic resources. Seed of two cotton cultivars “PB-899 and PB-900” were mutagenized with 0.3 and 0.2% (v/v) ethyl methanesulfonate, respectively. The phenotyping of M₁ and M₂ populations presented numerous mutants regarding the branching pattern, leaf morphology, disease resistance, photosynthetic lesions and flower sterility. Molecular screening for point mutations was performed by TILLING PCR aided CEL1 mismatch cleavage. To estimate the mutation frequency in the mutant genomes, five gene classes were TILLed in 8000 M₂ plants of each var. “PB-899” and “PB-900.” These include actin (GhACT), Pectin Methyl Esterase (GhPME), sucrose synthase (GhSUS), resistance gene analog, and defense response gene (DRGs). The var. PB-899 was harboring 47% higher mutation induction rate than PB-900. The highest rate of mutation frequency was identified for NAC-TF5 (EU706348) of DRGs class, ranging from 1/58 kb in PB-899 to 1/105 kb in PB-900. The mutation screening assay revealed the presence of significant proportion of induced mutations in cotton TILLING populations such as 1/153 kb and 1/326 kb in var. “PB-899” and “PB-900,” respectively. The establishment of a cotton TILLING platform (COTIP) and data obtained from the resource TILLING population suggest its effectiveness in widening the genetic bases of cotton for improvement and utilizing it for subsequent reverse genetic studies of various genes.

Keywords: Gossypium hirsutum, EMS mutagenesis, TILLING, point mutation, RGAs, fiber quality

Abbreviations: CLCuD, cotton leaf curl disease; CLCuV, cotton leaf curl virus; cm, centimeter; CODDLE, codons optimized to discover deleterious lesions; CTAB, cetyl trimethylammonium bromide; ddH₂O, double distilled deionized water; DRGs, defense response genes; EMS, ethyl methanesulfonate; Gb, giga base pairs; kb, kilo bases; NGS, next generation sequencing; RGAs, resistance gene analogs; RL, root length; SNP, single nucleotide polymorphism; SL, shoot length; TILLING, Targeting Induced Local Lesion IN Genomes.
INTRODUCTION

Cotton is primarily grown for its white fiber around the globe with an added advantage of its seeds as the major oil production source. Cotton belongs to the family Malvaceae. Its genus (Gossypium L.) comprises of 45 diploid species (2n = 2x = 26) and five allotetraploid species (2n = 4x = 52). Gossypium hirsutum L. is one of the tetraploids, possessing coverage of A (African ancestor; the herbaceum) and D (American ancestor; the raimondii) genome (Wendel and Cronn, 2003). It possesses most of the characteristic nature of both A and D genome minus the polyploidization anomalies in its 2.83 Gb nuclear genome (Grover et al., 2004; Comai, 2005; Hendrix and Stewart, 2005). According to the global econometrics, the polyploid AD genome of G. hirsutum contributes more than 90% to the world yarn production. Therefore, it is the best candidate in Gossypium genus to explore the silent variability related to its resistance mechanism and fiber quality. However, the continuous biased manipulation of available variability in cotton germplasm through conventional breeding approaches has resulted in the great loss of genetic potential added with increased vulnerability to many biological hazards. In this scenario, widening the genetic base by mutation can diversify and create novel changes in the functional genes. The mutagenesis of cotton genome can also help to have better understanding of gene function.

Various mutagenesis techniques have been routinely used in gene function studies like T-DNA insertional mutagenesis (Alonso et al., 2003) and gene silencing (Wesley et al., 2001), but proved impractical, time consuming and costly in cotton species owing to gene transformation barriers. In contrast, chemical mutagenesis provides a wide range of genetic alteration options owing to gene transformation barriers. For EMS treatment, 100,000 cottonseeds were de-linted with metallic ions which are usually present in cotton residue. The percent germination rate was recorded by Minitab 10.0 using Biplot analysis. The data for RL and SL measured in centimeter was analyzed for induced variation at seedling stage by Minitab 10.0 using Biplot analysis.

MATERIALS AND METHODS

Growth Conditions of Pilot Scale TILLING Analysis

The cotton crop completes its life cycle in about 6 months, therefore, it was efficient to evaluate the selected EMS doses at seedlings stage. The SL and RL were selected as key parameters at seedling stage. Keeping growth conditions same, any changes in the SL and RL were obvious variations induced by EMS treatment. To achieve this goal, about 800 M2 seed samples (one boll/plant) were randomly selected from M1 populations of PB-899 and PB-900. The seeds were grown in a complete randomized design (CRD) in growth room on silica trays dimensioning 50 cm × 30 cm (W) with the mixture of sand: soil (1:1). The light and water conditions were kept same for all the grown seedlings. The germination rate, RL and SL was measured from 15 days old seedlings. The data for RL and SL measured in centimeter was analyzed for induced variation at seedling stage by Minitab 10.0 using Biplot analysis.

Plant Material and EMS Treatment

For EMS treatment, 100,000 cottonseeds were de-linted with conc. H2SO4 for each of PB-899 and PB-900. The percent solution of EMS (Sigma Aldrich Cat #: M0880) @ 0.2 and 0.3% (v/v) was prepared in ddH2O having an electrical conductivity (EC) of 10 µscm⁻¹. The EC was considered to avoid seed tissue damage from metallic ions which are usually present in non-deionized water. The de-linted seed completely dipped in aqueous solutions of EMS was kept at room temperature with continuous shaking of 50 RPM for 3 h. The mutagenized seed was washed twice with tap water, air dried and grown in the field conditions of the Postgraduate Agriculture Research Area (Faisalabad). The plant to plant and row to row distance was maintained as 30 and 90 cm, respectively. Fertilizers were applied right before sowing and pesticide treatments were applied twice at germination and squaring stage.
Phenotyping
The M₁ and M₂ populations were observed for phenotypic variations in field. The phenotypic observations for leaves were made at vegetative and reproductive phases to cover all the leaf developmental stages. The branching pattern observations were recorded after appearance of first monopodial branch till maturity. The floral modifications were recorded for open flowers on similar branches on same day in comparison to non-mutagenized plants. For boll opening category, bolls were observed on daily basis from 20 days after pollination (DAP) and continued up to 40 DAP. The CLCuV tolerance was observed during 45–120 days after sowing.

DNA Extraction, Normalization, and Pooling Setup
The young leaf tissue samples of 8000 M₂ plants were collected at squaring stage and DNA was extracted individually using modified CTAB method. For pilot analysis, DNA extraction was performed as duplets (equally weighed tissue form two seedlings, pooled as one sample). The DNA samples were then electrophoresed on 1% agarose gel along with HindIII/λ. DNA marker (Thermo Scientific, Gene Ruler 1 kb, Cat #: SM0311) and gel images were processed by “Image J 1.44p” image analysis software. The estimated concentrations were normalized to 20 ng/µl and used to prepare 4X DNA master pool plates. The normalization values (the amount of DNA from four samples to achieve accurate pooling) were obtained with the help of Harvard Hoechst DNA normalization user interface publicly available at http://dev.plasmid.med.harvard.edu/DNANORM/NormOnlyInput.jsp.

In previous TILLING studies of polyploid crops, mostly 4–6X DNA pooling approach was followed owing to their bigger genomes like wheat (Triticum aestivum L.) (Slade et al., 2005; Uauy et al., 2009). Similarly, for tetraploid cotton which contains far more complex genome than other cereal crops 4X DNA pooling design was followed to avoid background noise of mutations pollution. This also reduced the time taken by tedious analysis of mutation tracing in individual DNA samples. The 96 well 2.0 ml DNA plates were used for preparing Master Pool Plate. In single well of Master Pool Plate individual 4X DNA sample was prepared by combining four individual DNA samples from DNA source plate in same quantity and concentration. Each sample was prepared by combining four individual DNA samples. Plate. In single well of Master Pool Plate individual 4X DNA was achieved by taking 50 one individual plant, thus, equal volumes of normalized DNA master tube in DNA source plate contained normalized DNA of from DNA source plate in same quantity and concentration. Each sample was prepared by combining four individual DNA samples. Plate. In single well of Master Pool Plate individual 4X DNA was achieved by taking 50 one individual plant, thus, equal volumes of normalized DNA master tube in DNA source plate contained normalized DNA of from DNA source plate in same quantity and concentration. Each sample was prepared by combining four individual DNA samples.

Target Gene Amplification and CEL1 Digestion Setup
Considering low fiber quality and disease/pest epidemics on cultivated cotton in the sub-continent especially in Pakistan during last few decades, the genes related to fiber quality such as Sucrose Synthase (GhsSUS), Pectin Methyl Esterase (GhPME), and disease resistance genes such as RGAs belonging to NBS-LRR class and DRGs were targeted. Cotton TILLING PCR was carried out using 96 well PCR plates in 25 µl reaction volume/sample in Thermal cycler (C1000, Bio-Rad, USA) 1.25 U of Taq DNA polymerase (Thermo Scientific, Cat #: EP0402), 25 ng each of forward and reverse primer, 50 mM MgCl₂ and 5 mM dNTPs were used in 25 µl reaction volume. The PCR temperature profile followed was: initial denaturation at 95°C for 5 min, Loop 1 (eight cycles of touchdown): 94°C for 20 s, 63 to 55°C for 30 s (an increment rate of −1°C/cycle) and 72°C for 1 min, followed by Loop 2 (33 cycles): 94°C for 20 s, 50–55°C for 30–60 s depending on the gene, 72°C for 60 s (ramp to 72°C at 0.5°C/second) and a final extension step of 72°C for 5 min. These two loops were followed by denaturation step of 99°C for 7 min and Loop 3 (re-nature step of 70 cycles): 70°C for 20 s with an increment rate of 0.3°C per cycle to allow heteroduplexes formation in case of point mutation presence in the pooled DNA.

The TILLING PCR product was treated with 10 µl of CEL1 digestion mixture containing 1 µl (0.125 U) of CEL1 enzyme, 3.5 µl 10X CEL1 digestion buffer (0.1 M Tris-HCl pH 7.7, 0.5 M KCl, 0.01% Triton X-100 and 100 µM PMSF) and 5.5 µl dH₂O in a final volume of 35 µl. The digestion reaction was carried out at 45°C for 45 min and reaction was immediately stopped by adding 10 µl 0.15 M EDTA to each well of PCR plate at 70°C for 5 min. The digested TILLING PCR mixture was added with 5 µl of DNA loading buffer (1:1, Xylene Cyanol: Bromophenol blue) and analyzed on 2.5% agarose gel containing 0.55% EtBr running in 1X TBE buffer at 100 V for 1 h. The
agarose gel width was maintained to 3–4 mm thickness. The gel images were captured and analyzed on UVP Photo Doc IT 65.

The mutation frequency of the target genes was individually calculated using the formula elaborated below and cumulative estimates were recorded for both PB-899 and PB-900 M2 cotton TILLING populations.

Mutational Frequency
\[
\text{Mutational Frequency} = \frac{\text{No. of samples screened} \times \text{Genomic region studied (kb)}}{\text{No. of mutations identified}}
\]

DNA Sequencing Scheme for SNP analysis

All the 4X pooled DNA samples which were positive for SNPs were traced back for individual DNA samples in the source DNA plate for verification by sequencing. The selected samples were tested again for previously identified point mutations by gene specific amplifications using duplex DNA (2X) pooling. The samples with real mutations were sequenced by Sanger sequencing method to verify the actual mutations. The heteroduplex formation between wild type and mutant DNA amplification strands produced overlapping peaks at the mutant positions which we considered true point mutation.

RESULTS AND DISCUSSION

Pilot Scale TILLING Analysis Satisfy the Use of Optimized EMS Doses

Based on our previously described kill curve analysis of EMS mutagenesis in Gossypium species, 0.3% (v/v) and 0.2% (v/v) aqueous solution of EMS were estimated as effective doses for seed treatment of var. "PB-899" and "PB-900" respectively (Aslam et al., 2013). The germination percentage test used to calculate EMS LD_{50} is shown in Supplementary Figure S1. The behavior of mutagens varied between species and among cultivars within a species, therefore, to estimate the maximum likelihood of effectiveness of EMS mutagenesis, we selected two closely related cotton cultivars of cotton. The other reasons of their selection include the commercial cultivation in the region and bulk seed availability in the cotton production farm of UAF. Before the development of actual TILLING populations, a pilot scale experiment was conducted to evaluate the mutation density and effectiveness of EMS lethal doses in growth room. Root length (RL) and shoot length (SL) were observed in response of EMS doses as they are obvious indicators of tissue injury at seedling stage. Significant induced variations were observed for both genotypes (Figures 1A,B,D–F).

Out of 400 plants, 376 mutant plants were selected for PB-900 to see their seedling performance. In biplot analysis, the mutants scattered adjacent to vertices of the polygon, e.g., mutant no. 48 was found best fit for SL from normal distribution of wild type measurement (Figure 1A) and no. 247 and 289 were found best for RL (Figure 1B). In contrast, mutant no. 214 and 215 showed poor performance for RL. Biplot analysis also provided the information of mutants with grouped performance of RL and SL, e.g., no. 267 was found significant combine performer while no. 174 was found non-significant performer (Figure 1C). The frequency distribution chart also confirmed the best performing genotypes for RL and SL. Such results revealed that biplot analysis is helpful in explaining the interrelationships between individual traits and also provided an independent selection criteria based on the combined performance of several traits (Yan and Rajcan, 2002).

The angle observed for RL and SL was greater than 90° (Figure 1C). The trait vectors for this biplot suggested a positive correlation between the RL and SL in PB-900. The scatter plot diagram shows that mutants have significant values compared to wild type phenotype. This suggested that the EMS treatment @ 0.3% has significant induced variations.

For PB-899, 424 plants were selected to study their performance of seedling parameters, i.e., RL and SL. The angle observed for RL and SL was more than 90°. The mutants scattered near the vertices of the polygon, i.e., mutant no. 415 were found to be best fit for SL (Figure 1D) and no. 19, 160, and 322 were found best fit for RL (Figure 1E). In the PB-900 mutant no. 49 was found best for the combined performance of RL and SL (Figure 1F).

The significant variability in seedling traits of PB-900 and PB-899 suggested the effectiveness of EMS doses used for the development of M1 cotton populations.

Development of Cotton TILLING Populations

Using tested and verified EMS doses in destructive pilot experiment, large scale cotton TILLING populations of var. "PB-899" and "PB-900" were raised at the Postgraduate Agriculture Research Station (PARS) of the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad (UAF).

Initially about 100,000 cottonseeds were mutagenized with previously described 0.3 and 0.2% EMS doses for PB-899 and PB-900 respectively to raise M1 cotton population (Aslam et al., 2013). From almost 50% germinated plants, nearly 10% were able to produce bolls and set viable seeds. Finally, we obtained ~5000 samples as one boll per plant to raise M2 progenies. Here, progeny means M2 plants grown from seeds of single boll harvested from M1 plant. Out of total M2 plant progenies [No. of progenies (4000) × No. of plants/progeny (5) = 20,000 × No. of seeds (4) = 80,000 expected plants], 20,000 M2 plants were raised for each of M2 PB-899 and PB-900 populations. The germination rate was observed as 55 and 60% for PB-899 and PB-900, respectively. Further mortality and sterility in M2 population were observed during later growth stages and lastly 8000 plants were selected for mutation screening in each of PB-899 and PB-900 population and M3 seeds were harvested for future analysis. During the development stages of M1 and M2 cotton populations, beginning from germination till maturity, various phenotypic observations were recorded regularly and some anomalous phenotypes were cataloged (Figure 2).
Phenotyping Revealed a Diverse Range of Variant Trait Classes

In cotton TILLING platform, the phenotypic variability was observed both in M₁ and M₂ populations of PB-899 and PB-900. Numerous phenotypes were recorded in both cultivars possessing genetic lesions. Seven traits were observed which showed several variant classes. The most number of variable traits were observed in plant stature and leaf shape, each of which are sub-categorized into four classes (Table 1.). The most commonly observed phenotypes were related to plant stature, fruit type and CLCuV disease resistance (Table 1.). Some examples of the mutated phenotypes correspond to the branching pattern, flower sterility, boll maturity and CLCuV tolerance can be seen in Figure 3. The CLCuV tolerance was estimated by characteristic leaf symptoms of the virus infection shown in Figure 3k.

Gene CODDLE Analysis and the Primer Designing

To design highly specific and efficient primer pairs for target genes, nucleotide sequences of genomic DNA and CDS were retrieved from the NCBI database. To determine the effective region of target genes with maximum likelihood to be functionally altered by EMS mutagenesis, we use web accessible tool CODDLE¹ for sequence analysis. Several isoforms of each gene were aligned, their sequences were analyzed for conserved blocks and primers were designed by PRIMER3 accordingly covering the CODDLE approved functionally affective gene regions. Various primer set were designed for each gene during the cotton genetic screen (Supplementary Table S1). EMS induces GC to AT transition mutations and CODDLE process this information using the respective CDS statement of the gene to analyze the input genomic region of a target gene. Based on the selected amplicon window size, the CODDLE scoring matrix calculation generated a graph depicting the probable effectively mutagenized region with an expected mutation rate of all possible mutation kinds such as truncation, missense and nonsense or silent mutations (Figure 4). The Figure 4 demonstrates the CODDLE analyzed the region of G. hirsutum actin gene

¹http://www.proweb.org/coddle
FIGURE 2 | Establishment scheme of EMS induced cotton mutant library. 100,000 cottonseed were EMS mutagenized that produces overall on average 20,000 M₁ plant progenies. One boll per plant selection was made from fertile plants. For each M₁ plant, 4–5 plants were raised as a M₂ progeny. A total of 5000 M₂ progenies were raised. Only those plants from each progeny were tested for targeted gene TILLING which produced viable bolls. DNA was extracted from plants of each of the progeny labeled (A–E). Leftover seed was secured for future corresponding analysis and phenotyping. (This figure was drawn by Usman Aslam)

(GhACT). The highest peak values of ACTIN window scoring matrix are the regions of maximum likelihood of desirable alterations. The genes possessing larger introns were processed in smaller sections to cover the maximum exonic regions and primers were designed in the introns. To confirm the primer efficacy for single and pooled DNA samples, the target genes were amplified using the respective DNA samples and their amplicons were analyzed by gel electrophoresis.

Standardization of DNA Extraction Procedure and Pooling Set Up
Normalization and selection of appropriate DNA pooling level has a direct impact on the efficiency of mutation screening in huge populations which also have an ultimate link with DNA quality, therefore, to avoid erroneous and false positive results in downstream TILLING PCR analysis, good quality DNA is a crucial requirement. Young green leaves were used for DNA isolation from M₂ plants as older leaves contain high phenolic compounds and polysaccharides. The DNA of individual plant samples were extracted using the modified CTAB method. To obtain pure and good quality DNA, traces of phenolics and polysaccharides were removed using 2% β-mecraptoethanol in CTAB. To avoid DNA hydrolysis during long storage, precipitated DNA was washed with absolute alcohol instead of 70% ethanol and DNA was stored in 1X TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA pH 8.0) added with 3.2 μg/ml RNase,) instead of dH₂O. The long storage of DNA in dH₂O was observed
TABLE 1 | Occurrence of induced phenotypic variation in important traits of the mutant populations.

| Trait                  | Variation             | No. of M2 plants |
|------------------------|-----------------------|------------------|
| 1 Branching pattern    | Spiral shaped         | 4                |
|                        | Bowl shaped           | 15               |
| 2 Plant stature        | Bunchy top            | 3                |
|                        | Umbrella shaped       | 7                |
|                        | Dwarf                 | 157              |
|                        | Tall                  | 8                |
| 3 Boll Type and shape  | Oval tiny             | 33               |
|                        | Green big             | 6                |
|                        | Purple color          | 1                |
| 4 Leaf shape           | Large palm            | 8                |
|                        | Fuse lobed shaped     | 113              |
|                        | Variegated            | 3                |
|                        | Irregular lobe symmetry| 7              |
| 5 Fruit type           | Un-opened mature boll | 340              |
|                        | Tri-loculed           | 730              |
|                        | Opened infertile bolls| 870              |
| 6 Anther               | Inflated hollow       | 22               |
|                        | Infertile             | 56               |
| 7 CLCuD tolerance      | Partial resistant     | 230              |
|                        | Resistant             | 86               |
|                        | Susceptible           | 150              |

Testing of CEL1 Mismatch Cleavage Activity

The most crucial and difficult step in mutation analysis is the identifications of particular site of point mutations. The
mismatch specificity of CEL1 has enabled us the development of highly effective and user-friendly mutation detection methodology. The CEL1 enzyme is named after its source plant celery (A. graveolens L.) a close relative of Ajwain, as both belonging to the parsley family Apiaceae. It possesses a novel endonuclease CEL1 having molecular weight of 43 kDa that can detect destabilized regions of double stranded DNA helices, such as mismatches, insertions and deletions and most specifically base pair substitutions (Pimkin et al., 2007). To deal with such a large scale screening for mutation identification, CEL1 extraction protocol was established at the Plant Genetic Resource Lab, PBG, UAF, Pakistan (Supplementary Figure S2). To verify the accurate extraction of CEL1 endonuclease, testing of its precise mismatch activity and standardizing its working concentration for cleavage assay, two strategies were followed.

In one scheme, genomic DNA of two cotton cultivars with different genetic makeup were used, considering that they may possess huge number of SNPs compared to each other. The genomic DNA of an exotic upland cotton genotype Acala and a commercial mutant cultivar “NIAB-78” were taken in equal amount, denatured at high temperature and then re-natured gradually to low temperature with the principal that both the genotypes being the tetraploid having nearly similar genome with SNPs and deletions/additions at countless sites. When the re-natured DNA was treated with CEL1 digestion mixture it showed light smear on agarose gel indicating DNA cleavage compared to control DNA (re-natured mixture of Acala and NIAB-78 genomic DNA without CEL1 treatment) showed bright long smear intense near higher molecular mass position (Supplementary Figure S3A). This indicated the presence of numerous dissimilarities of genetic makeup leading to inefficient re-annealing.

In second scheme, PCR products of NAC-Transcription Factor was used as template in CEL1 digestion assay that considered to have SNP in mutant cotton cultivar NIAB-78 compared to exotic cultivar Acala which was considered abiotic stress susceptible genotype. Additionally, PCR product of a house keeping gene “actin” was used as an internal in digestion assay. The actin gene revealed no SNPs giving no restriction bands in CEL1 treatment, however, in case of NAC-TF gene, the re-natured product showed multiple bands after CEL1 treatment as compared to control treatments indicating SNPs at this locus in two genotypes (Supplementary Figures 3B,C). Multiple concentrations were tested for mutation detection in cotton TILLING populations. Each concentration was observed
FIGURE 5 | Schematic representation of cotton TILLING results. (A) Estimation of DNA concentration by Image J software and normalization. Left part showing band intensity measurement, right part is showing construction of reference equation (second order polynomial) and DNA normalization. (B) Schematic diagram of 4X DNA pooling plan. Each color is indicating individual DNA samples in a single column. R1C1 to R1C4 in the left side is indicating the code name of individual pool sample in master pool plate. RC1 in the upper side is the code name of 4X pooled DNA from single row of source DNA plate. Normalized equal concentrations are indicated by four small circles a big circle. Color is indicating respective samples in a row. (C) Agarose gel analysis of point mutations detection in TILLed genes. (C-i) Optimization of TILLING PCR on normalized DNA stored in 1xTE buffer. (C-ii) PCR amplification from DNA after 3 month storage in ddH$_2$O. arrows indicate weak amplification (C-iii) Mutation detection by CEL1 mismatch cleavage assay in (iii) GhACT, (C-iv) GhSUS, (C-v) GhPME, (C-vi) GhRGA (C-vii,viii) GhDRGs; first lane is in each gel image indicate wild type amplicon band and mutant cleavage fragments are pointed by arrows and respective size. TILLING PCR product was digested with CEL1 enzyme and electrophoresed on 1–3% agarose gel depending on the size of digested PCR products. (D) Verification of point mutations by sequencing analysis. (i) C to T substitution is indicated by an arrow in GhMIC-3 sequence analysis. WT indicate wild type sequence with no sequence error, mutant indicate the mutated sequence (ii) G to A substitution is indicated by an arrow in GhCHI sequence analysis. WT indicate wild type sequence without mutation, mutant indicate presence of point mutation in the sequence.

**SNP Analysis and Mutation Frequency Estimation in Cotton TILLING Populations**

The chemical mutagens are effective sources of mutation induction and widely used in reverse genetic studies. The EMS induces GC = AT transitions mutations in target organisms (Till et al., 2004, 2007a). In our experiment, the induced point

effective in DNA digestion but, 0.125 and 0.02 U showed higher rate of digestion. Moderate digestion was observed with 0.005 and 0.008 Units. In case of PCR product digestion analysis, all treatments showed similar digestion activity, which is the indication of good efficiency of CEL1 endonuclease. In cotton TILLING analysis, 0.005 units of CEL1 extract was used per 25 µL reaction for point mutation detection to avoid false positives (Supplementary Figure S3).
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**TABLE 2 | Mutation frequency estimation in EMS mutagenized cotton populations of var. “PB-899” and “PB-900.”**

| Sr. # | Name of target tilled genes | Gene locus number | Genome size per gene (bp) | Number of M\_2 progenies screened | Mutations identified (PB-899) | No. of mutations | Genome size of identified mutation (PB-899) | Mutation frequency (PB-899) | No. of mutations | Genome size of identified mutation (PB-900) | Mutation frequency (PB-900) |
|-------|----------------------------|------------------|---------------------------|--------------------------------|-------------------------------|-----------------|-------------------------------------------|---------------------------|-----------------|-------------------------------------------|---------------------------|
| 1     | Actin (GhAct)              | AF059484         | 968                       | 8,000                         | 30                            | 1/150 kb         | 1/180 kb                                   | 1/258 kb                   | 14              | 1/153 kb                                  | 1/178 kb                   |
| 2     | Sucrose synthase (GhSUS)   | FB742816         | 780                       | 8,000                         | 33                            | 1/189 kb         | 1/2.65 mb                                   | 1/346 kb                   | 17              | 1/3.9 mb                                  | 1/314 kb                   |
| 3     | Pectin methyl esterase (GhPME) | JX003001.1    | 995                       | 8,000                         | 3                             | 1/2.3 mb         | 1/150 kb                                   | 1/2.2 mb                   | 0               | 1/3.6 mb                                  | 1/134 kb                   |
| 4     | Resistance gene analogs (GhRGAs) | G. hirsutum I   | 924                       | 8,000                         | 49                            | 1/150 kb         | 1/150 kb                                   | 1/150 kb                   | 14              | 1/153 kb                                  | 1/153 kb                   |
| 5     | GHCHI                      | JX551527         | 924                       | 8,000                         | 51                            | 1/150 kb         | 1/150 kb                                   | 1/150 kb                   | 17              | 1/153 kb                                  | 1/153 kb                   |
| 6     | GhNAC5                     | Z68152           | 974                       | 8,000                         | 51                            | 1/150 kb         | 1/150 kb                                   | 1/150 kb                   | 17              | 1/153 kb                                  | 1/153 kb                   |
| 7     | GhNBS-LRR-RGAs             | Z68152           | 974                       | 8,000                         | 51                            | 1/150 kb         | 1/150 kb                                   | 1/150 kb                   | 17              | 1/153 kb                                  | 1/153 kb                   |
| 8     | GhMIC-3                    | GQ231919         | 511                       | 8,000                         | 45                            | 1/150 kb         | 1/150 kb                                   | 1/150 kb                   | 17              | 1/153 kb                                  | 1/153 kb                   |

**Total** 7264 379 1/153 kb 178 1/326 kb

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Mutation frequency was estimated by TILLING of eight genes on M\_2 cotton populations of PB-899 and PB-900 using agarose gel aided CELI mismatch cleavage detection system (Raghavan et al., 2007).

The genes screened for point mutations analysis in *G. hirsutum* var. “PB-899” and “PB-900” include actin (GhACT), Pectin Methyl Esterase (GhPME), sucrose synthase (GhSUS), NBS-LRR RGAs and DRGs. The GhPME and GhSUS play key role in fiber elongation in cotton. Pectin is a major component of fiber related primary cell wall and pectin modification enzymes such as PME help regulate fiber elongation. In longer fiber chromosome introgressed lines (CSILs), PME was found upregulated during early stages of fiber development (Fang et al., 2014). Similarly, sucrose is a form of sugar molecule which is involved in synthesis of cell wall precursors and also required for cell homeostasis. Sucrose synthase is a major sucrolytic enzyme which reversibly converts sucrose into fructose and UDP-Glucose in plants (Geigenberger and Stitt, 1993). SUS plays important role in fiber development and specifically localizes in fiber initials (Ruan and Chourey, 1998; Ruan et al., 2003). Keeping in view these facts, we hypothesize that TILLING of PME and SUS genes could be more probable to produce fiber related mutants. In disease resistance category, RGAs are key markers for R-genes (disease resistance) that play important roles in disease resistance mechanisms and are conserved among plant species, predominantly with NBS-LRR domain which constitutes largest R gene family (Khan et al., 2016). In DRGs class, total four genes were tested, among them, two were pathogen related [Chitinase Gene (GhCHI) and meloidogyne-induced cotton-3 gene (GhMIC-3)] and two abiotic stress associated [class III peroxidase (Ghpod5) and NAC transcription factor 5 (GhNAC5)]. The GhNAC5 was screened in two sections, i.e., GhNAC5-1 and GhNAC5-2. Out of eight genes, GhACT, GhSUS and all members of GhDRGs were more prone to EMS induced point mutation while GhPME and GhNBS-LRR-RGAs showed least mutation rate, possibly due to out of reach of the mutation induction hot spots in cotton genome (Figure 5C; Table 2). In the M\_2 genetic screen of *G. hirsutum*, numerous mutants were identified, which showed the success of TILLING approach in the creation of genetic variability in tetraploid genomes.

The defense related gene GhNAC5 showed the highest mutation density per unit genome in both M\_2 cotton TILLING populations (Figures 5Cvii,viii; Table 2), however, the cumulative mutation frequency was estimated as one mutation per 153 and 326 kb for PB-899 and PB-900, respectively, which is higher than Sunflower (Sabetta et al., 2011; Kumar et al., 2013). The PB-899 showed 1.5–3.5 fold higher mutation density than other crop species, for example rice, pea, tomato, soybean and maize which possess diploid genomes perhaps this frequency figure is far less than that of other polyploid genomes such as wheat and brassica (Till et al., 2004, 2007b; Cooper et al., 2008; Uauy et al., 2009; Stephenson et al., 2010; Knoll et al., 2011; Chen et al., 2012). This variable response of two different cotton cultivars might be dose dependent or could be due to the differences in their genetic makeup. Because the sterile plants were not used in mutation analysis,
this could also be the result of the EMS characteristic effect of sterility (Greene et al., 2003; Perry et al., 2003) that make a plant species more vulnerable to lethal mutations resulted in a higher rate of infertility, i.e., 22% in M2 of PB-900 (Table 3). These results suggested that EMS dose is not only species specific but also genotype specific and depends upon mutagenic conditions and type of plant materials used for mutagenesis. These results also suggest rough estimates of *G. hirsutum* strength to withstand particular doses of EMS due to multiple gene redundancies in its tetraploid genome (Dalmais et al., 2008).

The actin is one of the universal housekeeping gene in plants and animals. It plays a crucial role in the plant cytoskeleton dynamics and carryout various processes in cell division, cell expansion and cell shape maintenance (Staiger and Schlöwa, 1987; Staiger and Canadé, 1991). The purpose of targeting actin was to estimate the effectiveness of EMS lethality in a constitutive gene expression of conserved genetic pathways in the cotton plant. In cotton genetic screen, a total of 43 point mutations were identified in PB-899 cotton genotype for actin while 30 were identified in PB-899 cotton genotype for G*ACT* (Table 2). The higher mutation rate in PB-899, i.e., 1 mutation/180 kb than that of PB-900 (1/258 kb) suggested higher penetration of EMS induced variability in PB-899, however, PB-900 was observed less prone to mutation induction. The overall data suggested that the effectiveness of EMS mutagenesis is not only species dependent but also genotype dependent within a single species (Aslam et al., 2013).

G*hPME* showed least alterations in response to EMS mutation induction. In M2 genetic screen of cotton, only three point mutations were identified in PB-899 and 2 in PB-900 cotton genotypes. Similar results were found for cotton RGAs (Table 2). In DRG class, all genes showed considerable mutation density with NAC-5 presenting highest mutation frequency in var. “PB-899,” i.e., 1 mutation/58 kb. These results coincide with the phenotypic performance of two cultivars which presented considerable variations in CCLCuD incidence level, suggesting the possible role of NAC-5 against CCLCuV resistance in cotton.

The point mutations detected by CELI mismatch cleavage assay were confirmed by re-sequencing. The DNA samples for which mutations were identified were 2X pooled, PCR amplified and after verifying by CELI cleavage, samples were used for sequencing. In sequencing data, the overlapping signal peaks with base pair substitution verify the actual mutation site (Figure 5D). Overall, the mutation density data of five gene classes per genotype demonstrates the success rate of development of these resource TILLING populations, however, due to a limited number of genes selection for point mutation analysis, further screening will help to reveal mutation enrichment in cotton TILLING populations.

### Seed Maintenance

The seeds of PB-899 and PB-900 plants were harvested from M1 populations in one boll/plant fashion. The harvested bolls were ginned and cottonseeds were packed in craft paper seed storage bags with numerical numberings for example M1-PB-899-1, M1-PB-899-2, M1-PB-899-3, M1-PB-899-4, and so on. Similarly for PB-900, M1-PB-900-1, M1-PB-900-2, M1-PB-900-3, M1-PB-900-4, and so on. The M2 plants were raised from seeds of single boll/plant in boll to row progenies fashion. The harvested seed was packed in craft paper seed storage bags and labeled as following.

M2-PB-899-1a, M2-PB-899-1b, M2-PB-899-1c, M2-PB-899-1d, M2-PB-899-2a, M2-PB-899-2b, M2-PB-899-2c, M2-PB-899-2d, M2-PB-899-3a, M2-PB-899-3b, M1-PB-899-3c, and so on. Here, numeral 1, 2, 3 represents progeny number and small alphabets a, b, c, d represents plants in single progeny. The M1 and M2 seeds packages were deposited in seed storage facility (Temperature: 4°C, humidity < 30% without light) of Department of Plant Breeding and Genetics, University of Agriculture Faisalabad (UAF), Pakistan.

### CONCLUSION

The need for crop improvement to combat with biotic and abiotic problems in agriculture is increasing with the changing environmental perspectives. TILLING is a useful reverse genetic tool, used to create and identify mutations in various organisms especially in plants and successfully manipulated in several crop species. Furthermore, as EMS generates an allelic series of targeted genes, it is possible to explore the role of desired genes that are otherwise not likely to be recovered in genetic screens, based on insertional mutagenesis/transformation. Cotton is the most important fiber crop and the pillar of Pakistan’s economy. It is combating a long list of diseases and physiological problems, right from the germination to maturity, that endanger its yield to a great extent. Although, the conventional breeding approaches contributed a lot to cotton improvement, but simultaneously it also resulted in narrowing down of the genetic background and elimination of useful qualitative and quantitative alleles.

### TABLE 3 | Growth effects of EMS mutagenesis on var. PB-899 and PB-900 cultivars.

| Calculated EMS dose to develop TILLING population | 0% EMS M0 seed | 0.3% (v/v) [PB-899] | 0.2% (v/v) [PB-900] |
|-----------------------------------------------|----------------|---------------------|---------------------|
| M1 seeds grown in the field | 1000 each | 100,000 | 100,000 |
| Percentage of germinated M1 plants | 80% (PB-899), 75% (PB-900) | 45% | 55% |
| Percentage of plants having viable fertile bolls | 80% (PB-899), 75% (PB-900) | 10% | 13% |
| Number of M2 plants grown | 800 each, [boll descent progenies] | 20,000 | 20,000 |
| Percentage of germinated M2 plants | 85% (PB-899), 75% (PB-900), [boll descent progenies] | 55% | 60% |
| Percentage of plants produced infertile bolls | 0% | 15% | 22% |
from cotton. Therefore, the creation of a new allelic resource population for crop improvement is vital. The cotton TILLING is the very first step in Pakistan taking conventional mutagenesis to more advanced and sophisticated form and open the new doors to perform gene function analysis. The TILLING analysis of cotton M₂ population at a large scale represents the effective induction of point mutations in studied genomic region. Out of eight genes tested in cotton, DRGs found more effective to mutagenesis by EMS. This could be due to the presence of DRG locus in mutation hot spot regions of cotton genome. The higher mutation rate in DRGs suggested the usefulness of cotton TILLING populations in disease resistance research especially for CLCuD. TILLING analysis was performed on very little genomic region of cotton, therefore further screening of more genes is in progress. With the availability of these resource populations to cotton breeders and researchers, we are hopeful to prove its worth in cotton plant improvement research.

**Future Perspectives**

We are working on the development of *in silico* database for all population individuals, which is necessary for all possible collection of mutants. It will cover the recording of all types of plant morphologies appeared in M₃ population. This all data will be stored in a web accessible cotton TILLING database for information about mutant types. Secondly, expression behavior of mutated genes at the whole plant level will be performed to identify potential mutants with desirable traits that can be used in ideotype breeding. Furthermore, the developed TILLING populations will be screened for abiotic stress related genes in an effort of uncovering the left over induced variability.

**AUTHOR CONTRIBUTIONS**

The research is conducted, planned and supervised by Project Investigator AAK and Co-PI HMNC. UA optimized EMS mutagenic doses, perform the cotton seed mutagenesis, look after the M₁ and M₂ field populations and conducted the phenotyping. The pilot experiment was performed by SA, DNA extraction, TILLING PCR and CEL1 digestion assay was optimized and performed by UA. Data analysis was done by UA. WM helps in layout and management of field and lab experiments. The manuscript was written by UA, contributed by AAK and HMNC. Figures and tables were taken and designed by UA, biplot graphs were designed by SA. Logistics were provided by IAK.

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