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A discussion on cotton transformation during the last decade (2010–2021); an update on present trends and future prospects

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Abstract

The introduction of genetically modified (GM) cotton in 1996 in the US and its worldwide spread later rejuvenated cotton production in many parts of the world. The evolution is continued since then and currently, the 3rd and fourth generation of same GM cotton is grown in many parts of the world. The GM cotton introduced in 1996 was simple Bt cotton that expressed a single *Cry1Ac* gene, the later generation carried multiple *Cry* genes along with the genes controlling herbicide tolerance. Current day GM cotton does not only give stable resistance against lepidopteran insects but also facilitates the farmers to spray broad-spectrum herbicides without harming the crop. The evolution of GM cotton is continued both on the basic and applied side and interventions have been introduced during the last decade. Earlier the cotton transformation was limited to Cocker strains which are getting possible in many other varieties, too. It is successful with both gene gun, and *Agrobacterium* and inplanta transformation has made it a routine activity. Apart from overexpression studies for various purposes including biotic, abiotic, and quality traits, RNAi and genome editing are explored vigorously. Through this review, we have tried to explore and discuss various interventions for improving transformation protocols, the applications of cotton transformation, and future strategies being developed to get maximum benefits from this technology during the last decade.

Keywords: Cotton transformation, Overexpression, Genome editing, Sustainable agriculture, RNAi

Introduction

The earliest appearance of cotton (*Gossypium hirsutum* L.) is documented to originate in the Yucatan peninsula (*G. hirsutum* var. *yucatanense*) in Mexico with its domestication occurring around 5 000 years ago. Domestication was initially carried out for its fibers and over time its significance has been increased for cottonseed and cottonseed oil (Renny-Byfield et al. 2016). In current years, cotton has, however, faced declining yields owing to various biotic and abiotic stresses, i.e., drought, salinity, the high temperature which in turn impact the patterns of attacks by lepidopterans, coleopterans, and weeds. Thus, the crop is affected by combinations of both abiotic and biotic stresses which need to be paid heed to. Conventional cross-breeding has resulted in the development of many high-yielding and stress-tolerant genotypes. This technique seems to fail when gene(s) responsible for trait improvement is only present in unrelated species and it is not possible to bring such genes through crossings. Genetic engineering provides one such possibility (bringing genes from unrelated species) whereby the focus is on improving various traits that lead to high yields, tolerance to salinity, drought, pests, weeds, and sustainable agricultural traits such as higher water use efficiency (WUE). Herbicide resistance and insecticide tolerance has been

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perfected whereas the stacking of traits is being achieved especially to combat abiotic and biotic stresses, individually and their combinations (Rao et al. 2016). An in-depth understanding of various combinations of stresses can be carried out by studying diverse signaling patterns which in turn would help to generate transgenic events that cater to complex combinations of stress. Transformation incidences can be improved by repeating several experimental attempts with diverse available parameters to obtain a wide array of optimum conditions to choose from.

Genetic engineering

Genetic engineering refers to the modification of an organism's genome. The modification may consist of a change, deletion or addition of a nucleotide base pair. It may, additionally, also include a transfer of gene segment from another organism into the host cell. Genetic transformation specifically refers to the techniques that are employed to obtain organisms that have been modified genetically, called the GMOs (genetically modified organisms). *G. hirsutum* is one of the first major crops whose transgenic varieties were introduced into the market globally. The crop finds basic application in consumer and industrial products such as in the textile industry. Cottonseed oil contributes to the food industry while seedcake is the major protein source in demand in the feed industry. Cotton exports generate billions in profit. Generally, the transformation of elite cultivars is preferred because they are adapted in farmers' fields for agronomic characters. Monsanto's Bollgard-I and Bollgard-II contained single and stacked *Cry* genes which were obtained from *Bacillus thuringenesis* that releases δ-endotoxins rendering the cultivars tolerant to pests (Qaim 2010). Similarly, competing weeds were eliminated by the use of herbicide-tolerant cotton cultivars (containing the cp4 EPSPS gene), such as Roundup Ready Flex (RF) cotton which was the first glyphosate-tolerant cotton (Puspito et al. 2015). Currently, various transformation methods have been employed to genetically-modified cotton. These methods include *Agrobacterium*-mediated transformation, protoplast treatment with PEG, particle bombardment, pollen tube pathway, and electroporation. Genetic engineering provides a promising alternative to genes that can otherwise be obtained from crop wild relatives or even unrelated sources like bacteria, fungi, animals, or plants.

Due to limited germplasm and the use of the same genetic sources, the conventional breeding methods no longer seem to be as promising to bring major uplift in cotton production. The development of stable transformation methods has paved the way for the improvement of the cotton genome. Since the development of the first transgenic cotton plant, back in 1987 many traits related to biotic and abiotic stress tolerance, and increase in cottonseed oil, fiber quality, and production have been explored. Thus, merging conventional breeding methods with genetic engineering contribute to improved yield and quality of cotton. The adoption of *Bt* cotton has changed the traditional breeding patterns including the use of agrochemicals with an observed improvement in the quality and quantity of the produce. The contribution of *Bt* cotton needs to be analyzed in detail considering global cotton cultivation. Various factors such as climate conditions, lack of quality germplasm, use of agrochemicals, and irrigated areas have forced the extensive adoption of *Bt* cotton by farmers around the globe. Thus, it is imperative to investigate the benefits of genetic engineering and its prospects (Bakhsh et al. 2015; Noman et al. 2016).

There is room for the improvement of abiotic and biotic stress tolerance since related determinants are unavailable within a crossable gene pool. Thus, employing genetic engineering seems inevitable for stable gene transformation. The last two decades resulted in more than 80% of global transgenic cotton (Juturu et al. 2015). Despite the huge success of transgenic cotton, various combinations of stresses keep on harming in-field cotton owing to the ever-changing environmental and climatic aspects. Resurgences of diseases are observed in recent times such as the appearance of bacterial blight and Fusarium wilt in the U. S. in 2017 that lead to a $45 million in yield loss (Cox et al. 2019).

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation refers to the genome transformation by utilizing the capability of the *Agrobacterium* to transfer foreign genes into plant host cells. It makes use of its Ti plasmid whereby the T-DNA is integrated into the host plant genome such that it is transferred to the offspring (Li et al. 2017b). A basic transformation protocol includes the application of a slight injury to plant tissue. This site is needed for exposure to the *Agrobacterium* carrying the desired gene construct. Transformation frequencies have been improved following several tested protocols including the use of super-binary vectors, vir gene inducers, ternary system, and modifications of the Ori of the vectors amongst others. A binary vector consisting of additional virulence genes from a Ti plasmid to enhance the transformation frequency is refered to as a super-binary vector (Komori and Komari 2011). Vir gene inducers refer to factors that facilitate the expression of vir genes which include, amongst others, acetosyringone, vanillin caffeic acid (Simon et al. 2015). A ternary system makes use of an accessory plasmid that is a virulence helper plasmid

to help carry additional virulence gene cluster. It is essentially a three accessory system consisting of disarmed Ti plasmid, a helper plasmid and an additional virulence helper plasmid (Anand et al. 2018). A broad host range origin of replication ensures a high plasmid copy number resulting in an increased T-DNA transfer. For instance, the pSa plasmid produce 2~4 copies per cell, RK2 and pVS1 plasmids are reported to maintain 3~12 copies per cell while 15~20 copies per cell are obtained by the repABC origin (Zhi et al. 2015; Vaghchhipawala et al. 2018).

The choice of *Agrobacterium* strains needs to be considered based on the choice of crop. Cho et al. (2014) reported AGL1 to be the most effective strain in maize in comparison to LBA4404, EHA105, and GV3101. Alternatively, for effective transformation in cotton, the use of EHA105 and LBA4404 strains seem promising (Zhang et al. 2014). Transformation frequencies of 33% by strain AGL1 and 10% by strain LBA4404 were observed in sorghum (Wu et al. 2014).

Important plant tissue components used in optimized combinations help increase transformation efficiency even in recalcitrant crops. For instance, Cho et al. (2014) were able to establish optimum combinations of glucose, cytokinin, and copper for co-cultivation, resting and selective media in maize. In addition, the choice of tissue to be transformed should also be taken in consideration. Meristem cells are preferred since the foreign gene can be easily accepted by these cells upon quick division. Such cells also tend to be less probable to have somaclonal variations and genetic mutations (Kalbande and Patil 2016). According to Rajasekaran (2019a), a variety of explants may be transformed through particle bombardment. For example, transformation of apical meristem would ultimately help in deriving transformed shoots, bypassing regeneration steps, and thus reduce the time to obtain adult plants. In an experiment demonstrating particle bombardment, Rajasekaran (2019b) bombarded gold particles coated with β-glucuronidase or *uidA* on isolated mature seeds. The L1 layer was observed to be stably transformed while a reduced transformation of germ line was obtained, i.e., up to 0.71%.

Many transformation processes are aided by the co-culture medium of the explants. Transformation frequency (TF) can be enhanced at this stage in the presence of a certain optimized temperature. Cotton meristem transformation was reported by Chen et al. (2014) to achieve the highest efficiency at 23 °C and even a slight increase in degree would reduce the transformation levels. On the contrary, the transformation of cotton embryogenic callus was most favorable at 19 °C. This indicates that different explants may require different temperatures for effective transgene integration. Transformation may also be influenced by the time of *Agrobatcerium* inoculation. The pistil drip inoculation was carried out by Chen et al. (2010), with the suspension of *Agrobacterium* in a 10% sucrose inoculation solution containing Silwet L-77 and acetosyringone for the transformation of *bar* gene. Inoculation in the evening yielded ~ 0.07% to 0.17% herbicide resistant plants. This efficiency was further increased to 0.46%~0.93% upon excision of stigma prior to inoculation. On the contrary, a low rate of transformation (0.04%~0.06%) was observed when inoculation was carried out in the morning. In stark contrast, to aforementioned example, morning inoculations were preferred by Mogali et al. (2013). Treatment of stigmatic surface by 5% sucrose solution amplified the success of pollination up to 23.5%. Additionally, rate of boll set was observed to marginally increase with the use of boron. Hence, a combined application of sucrose and boric acid yielded a 32.5% increase in boll set. Boll shedding is a definitive occurrence following *Agrobacterium* application. Though, a liquid agroculture resulted in less boll shedding than a solid agroculture (making use of agar medium) did.

In addition to the various mentioned factors, the choice of transformation methods also influence the success of transformation. In case of biolistic method of transformation, some of the factors upon which the success of bombardment depend include the pressure applied, the choice and distance of the tissues along with the metal used for the particles. Kharbikar et al. (2013) used embryonic axes of cotton cv. NH 545 for the transformation of *Cry1Ac* gene pBin Bt-3. Bombardment of gold particles at 900 pounds per square inch (psi) at 6 and 9 cm yielded an overall gene transfer efficiency of 3%. On the contrary, the use of explant by Khan et al. (2011) was the excised cotton embryos. Tungsten was used as the materials for micro-projectiles with a pre-optimized distance of 22 cm at a pressure of 4.13 bar. A transformation efficiency of 0.26% was obtained. A critical observation of both the described experiments revealed a huge difference in the pressure applied. A 4.13 bar pressure is around 60 psi which is quite low compared with the 900 psi applied by Kharbikar et al. (2013). Additionally, explants at 6 and 9 cm are expected to have a higher transformation probability (as observed by the results) than those at a greater distance of 22 cm.

On the contrary, *in planta* transformation of plants usually evades the laborious and often recalcitrant regeneration procedures experienced in *in vitro* plant transformation techniques. Some methods that came under umbrella of *in-planta* transformation included injecting plant tissues with *Agrobacterium*, vacuum infiltration, floral dip, spray, and pollen-tube pathway (Niazian et al. 2017). The pollen tube pathway, for instance, essentially

delivers the transgene into cotton embryo sacs for integration into cotton genome. Despite the apparent virtues, the transformation efficiency is low, i.e., about 0.5%~1.0% for majority transformations (Wang et al. 2013). Thus, in comparison to *in vitro* techniques, this method is independent on rigorous field bioassays before molecular characterization. Unlike co-cultivation that requires the application of an injury, the pollen tube pathway is pre-dominantly non-injurious, however, an absence of skilled handling and injection of increased volumes of DNA may damage the ovary. Moreover, the success of this technique is highly dependent on flower morphology (Ali et al. 2015). This is precisely why this method is not found to be favorable in soybean due to its small flowers with narrow and fragile staminal columns. Cotton, however, has large flowers can be easily pollinated.

A combination of microinjection and 20 s of sonication was reported by Gurusaravanan et al. (2020), to obtain high transformation efficiency in *G. hirsutum* L. KC_3. Shoot apex explants of cotton were microinjected carefully to prevent damage followed by incubation for 1d in dark. On the next day, the explants were shifted to culture of *Agrobacterium* for sonication. The optimum shock time was 20 s beyond which the explant damage was increased greatly. Experiment was further optimized for best results with *Agrobacterium* cell density of 0.6 $OD_{600\ nm}$ and three microinjections. Maximum transformation efficiency was observed to be 20.25%.

A variation in the gene expression levels is observed for different genotypes despite following the same transformation under similar conditions. Lei et al. (2012) reported the integration of *SNC1* gene in Juanmian No. 1 and Zhong 35 cotton cultivars for resistance against Fusarium wilt. The disease incidence rates for Juanmian No. 1 controls and transformants were 66.7% and 37.5%, respectively, while for Zhong 35 controls and transformants were 50.0% and 22.2%, respectively. Such an observed variation in gene expression levels may be linked to a variation in the ease of transformation for both varieties.

To fully reap the benefits of transformation in cotton, it is incumbent to develop a cotton regeneration method whereby time can be saved. According to Bouchabke-Coussa et al. (2013), marker-free transgenic plants can be obtained with the use of *Agrobacterium* binary vector carrying *WUS* and the desired gene. It was observed that *in vitro* overexpression of *AtWUS* was synonymous with improved somatic embryogenesis and induced organogenesis on embryo-like structures in the absence of phytohormones. An increased fraction of explants leading to embryogenic tissues were obtained with the *AtWUS-GFP* (green fluorescent protein) which is probably due to the stability of the fusion product.

Various sugars and phenolic compounds are believed to induce vir genes for transformation. Acetosyringone is a prominent phenolic compound that contributes greatly to the effective transformation of *Agrobacterium*. Synergistic actions of various other compounds responsible for transformation may include vanillin, vanillic acid, caffeic acid, gallic acid, and coumarin as in the case of the microalga *Dunaliella salina* (Simon et al. 2015). Additionally, a down-regulation of o-methyltransferases has been reported to reduce the susceptibility of plants to agro-infection by lowering the virulence of *Agrobacterium* (Maury et al. 2010). The increased production of phenolic compounds may occur *in vitro* especially in the presence of certain C sources for certain plant species. For example, the presence of phenolic compounds is observable in cotton explants and is quite vivid in media containing sucrose. This is reported to hinder plant regeneration resulting in a reduced transformation frequency (Chen et al. 2014).

Instances of T-DNA transformation may further be increased with the removal of entities that may affect the plant-*Agrobacterium* interaction. This includes the removal of the gaseous phytohormone namely ethylene. This may be carried out by providing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity to *Agrobacterium* which helps in cleaving ACC (the ethylene precursor) to ammonia and α-ketobutyrate (Nonaka and Ezura 2014). In a comparison study (Someya et al. 2013), ACC deaminase gene driven by virD1 promoter showed an increased ACC deaminase activity than with the use of lac promoter. Further, salicylic acid is also known to suppress the transcription of repABC operon, vir genes, and genes related to quorum sensing thus impeding the ability of *Agrobacterium* to infect plants (Someya et al. 2013). *Agrobacterium*-plant interactions are also affected by gamma-aminobutyric acid (GABA) which occurs by degradation of quorum sensing signals leading to a reduction in horizontal gene transfer of Ti plasmid. A combination of the ACC deaminase and GABA transaminase activity yields a super-*Agrobacterium* ver. 4 that is known to exhibit increased transformation efficiency (Nonaka et al. 2019).

More work needs to be done on visual marker system to aid genetic engineering and breeding in cotton. For example, *Agrobacterium*-mediated transformation was used to introduce red fluorescent protein, DsRed2 (obtained from Discosoma sp.), to be expressed in two cultivars, JIN668 and YZ1. An early-stage selection tool for transgenic calli is provided by DsRed2 which can be visually observed in calli, somatic embryos, and various tissues and organs in mature plants. In order to analyze its stable heritability, Sun et al. (2018) crossed the Yz-2-DsRed2 transgenic line with different cultivars. The

progeny from a male parent showed stable inheritance with 100% expression in F_1 hybrids. A negative association was obtained between DNA methylation and DsRed2 transcription following a methylation-specific polymerase chain reaction(PCR) approach on CaMV35S promoter. Thus, DsRed2 was claimed to be a reporter gene for transformation and molecular breeding programs in cotton (Sun et al. 2018). Based on the increased sensitivity of upland cotton towards hygromycin, Bibi et al. (2013) claimed that hygromycin resistance should be the preferred choice for a marker over kanamycin resistance to screen mutant plants following a pollen tube mediated transformation.

Despite successful transformation events, the effect of transgenic crops may not have a pronounced effect in field. This discrepancy in the gene expression levels can be understood through spatio temporal studies of the crop. An in-depth explanation was provided by Bakhsh et al. (2012) whereby Cry2A toxin levels seem to fluctuate amid the crop growth duration. Additionally, different crop parts exhibit difference in toxicity levels, with the leaves having high toxicity than the reproductive parts. Moreover, the transgenic lines were observed to have a 100% mortality rate at 30 days age of plant which dropped to 60%~80% mortality by 90 days of crop age. Other factors that may influence transformation efficiency include the gene insertion point and its ultimate effect on the translation, inner environment of the cell based on the alterations in the outer environment and transgene copy number to name a few (Rao et al. 2011).

Interestingly, *in vitro* salt stress was observed to increase transformation efficiency which was demonstrated by Barpete et al. (2016). Salt stress was applied to 2-day old germinated embryos—50 mmol·L^{-1} of NaCl, $CaCl_2$, KCL each which was followed by exposure to LBA4404 strain via co-cultivation. The highest transformation efficiency of 1.10% was achieved in embryos pre-treated with KCL, followed by NaCl and $CaCl_2$ both at 0.7%. The results show a pronounced effect on transformation when compared with an efficiency in embryos not pre-treated with salts that stood at 0.4%.

Lee et al. (2013) reported the development of plant-transformation-competent binary bacterial artificial chromosome (BIBAC) library along with comparative genome sequence analysis of upland cotton with its putative progenitor' *G. raimondii*. Both *Agrobacterium* and particle bombardment can be employed for the transformation of high molecular weight DNA vector. Digestion of DNA was carried out by *Bam*H1 in pCLD04541. About 76 800 clones, with an average size of 135 kb, were present in the library having a probability of obtaining at least one positive clone with a single-copy probe. The various genes contained in the library were related to fiber cellulose biosynthesis and its development, cotton-nematode interaction, seed fatty acid metabolism, and resistance to bacterial blight. Randomly about 10 000 BIBAC ends (BESs) were selected for sequencing to understand the relationship of the upland cotton genome with *G. raimondii*. A major constituent of transposable elements was retro-element *Gypsy/DIRS1* family in upland cotton. Both cultivars are greatly diverse at the genomic level.

Though transformation through *in vitro* techniques is deemed to be quite efficient, however, considering the reported transformation frequencies, this belief is debatable. The ease of the use of the various transformation methods are highly subjective depending on the optimized protocols and instrument availability in various labs around the world. For example, shoot apex transformation yielded 1.1% efficiency with PHYB gene upon 1 h co-cultivation with LBA4404 strain (Rao et al. 2011); a 1.19% efficiency with pyramiding of glyphosate resistance and *Bt* genes (Puspito et al. 2015); and 1.01% for the *CpTIP1* gene (Akhtar et al. 2014). Decent instances of transformation are obtained through *in planta* methods. Some of the reported transformation frequencies following the cotyledonary leaf bisection method are 6.89% for *At-NPR1* gene inducing resistance to *Alternaria alternata* (Kalbande and Patil 2016) and 2.27% for glyphosate tolerance up to 800~1500 mg·L^{-1} (Karthik et al. 2020). Manipulation of pollen tube pathway yields differing frequencies such as about 0.30% for induction of *Helicoverpa armigera* mortility (Mogali et al. 2013). Transformation through particle bombardment is believed to have slightly low frequencies and yield chimeras with more chances of epidermal transformants and co-suppression resulting due to multiple transgene copies with a greater probability of fragmented T-DNA (Chakravarthy et al. 2014). Reported transformation via particle bombardment include 0.71% efficiency with gold particles for β-glucuronidase (Rajasekaran 2019b), 3% efficiency with gold particles by Kharbikar et al. (2013) and 0.26% with tungsten particles by Khan et al. (2011). Despite the general belief, the transformation efficiencies are competitive.

Methods for confirmation of transformation

Verification of integration of desirable genes is important to the entire transformation process. Without this step the time and labor intensive techniques of genetic engineering are rendered inconsequential. The basic confirmation processes include molecular confirmation such as by Northern Blot Analysis, Southern Blot Analysis (Chen et al. 2010; Zhao et al. 2015; Zhang et al. 2014) in association with PCR carried out in thermal cycler (Sohrab et al. 2016). Variations of PCR may be employed as per the requirement of the experiment. These include reverse

transcription PCR (rt-PCR) and real time PCR (q-PCR) (Maqbool et al. 2010; Zhang et al. 2014). Western blot can be carried out alongside enzyme-linked immunosorbent assayELISA (de Oliveira et al. 2016). The NCBI BLAST Pair-wise Alignment algorithm programs are helpful in sequence analysis (Maqbool et al. 2010). Additional assays include phenotypic expressions such as GUS (β-Glucuronidase) assay (Maqbool et al. 2010; Bibi et al. 2013). Insect bioassays determine the level of toxicity against unwanted pests and larvae (Mogali et al. 2013; de Oliveira et al. 2016). Initial selection parameters are usually limited such as to selection pressure antibiotics, herbicide, etc., including kanamycin, phosphinothricin (Mogali et al. 2013; de Oliveira et al. 2016). Robust molecular assisted selection (MAS) would ensure early selection amongst putative transformants. The selection is quick and prevents frequent field inoculations. A notable example includes the tight linkage of microsatellite markers with the disease resistance genes. Pyramiding of resistance genes in desirable cultivars is possible since the marker expression is not obscured by the epistatic interactions amongst resistance genes (Marangoni et al. 2013).

A modification of the PCR included the use of loop-mediated isothermal amplification (LAMP)—an essay that is highly sensitive, rapid and efficient. Amplification of the gene of interest was carried in the presence of loop primers that greatly reduced the time, otherwise, utilized by conventional PCR process. A rapid DNA extraction followed by LAMP assay is claimed to take about 30 min (Rostamkhani et al. 2011) and the obtained products can be visually observed in reaction tube upon observing the turbidity. Surface plasmon resonance (SPR) is another biomolecule based detection system that can easily identify transgenic cotton within 5 min per sample. Zhao et al. (2013) used this method to detect transgenic *Cry1Ac* cotton individuals. In this protein based analysis, a CM5 sensor chip served as a base to immobilize monoclonal *Cry1Ac* antibodies against conventional cotton samples that were used as the detection threshold. Fluorescent multiplexed immunoassays (FMI) have gained importance for stacked GM traits. Yeaman et al. (2016) developed an FMI assays for major transgenic proteins including neomycin phosphotransferase II, β-glucuronidase, CP4-EPSPS, Cry1Ac and Cry2Ab2. Characterization requirements and results for FMI are like ELISA but has reduced time with a higher throughput.

RNAi-mediated gene silencing

In October 2018, US regulators approved the TAM66274 event whereby "Ultra-low gossypol cottonseed" (ULGCS) plants were developed. Cottonseeds contain elevated amounts of terpenoid gossypol which is detrimental for human and livestock consumption. With the help of RNAi mediated silencing, δ-cadinene synthase was knocked down in the presence of *α-globulin* promoter, resulting in reduced levels of gossypol in cottonseed (Hagenbucher et al. 2019; Rathore et al. 2020). However, gossypol provides defense to plants against leaf-feeding pests suggesting a decrease in defense following its knockdown. Thus, the method was used to analyze the insect resistance of ULGCS cotton plants against *Spodoptera littoralis* which is a causative agent of African cotton leaf-worm. It is interesting to note that ULGCS have a significantly high oil content of about 4%~8% (Palle et al. 2013). Surprisingly, the stability has also reported to be multi-generational as far as five amongst nine RNAi lines against certain pathogens (Rathore et al. 2012), though the gossypol was naturally found in cotton and has deleterious effects on the cotton pests. Likewise, gossypol is dangerous for animals where it goes in seedcake. Keeping in view the adverse effects of gossypol, CYP6AE14 transcript was transformed in cotton with a construct of 469 nucleotide while being assumed that the enzyme detoxifies gossypol. This transcript specifically interacts with the cotton bollworm cytochrome P450 CYP6AE14 upon consuming the transgenic cotton leaves leading to reduced bollworm larvae population (Zhang et al. 2017).

Alternatively, an intron hairpin (ihp) RNAi construct was developed that was able to express dsRNA homologous to CLCuRV intergenic region (IR) (Khatoon et al. 2016). The Narasimha cultivar was transformed with *Agrobacterium* containing the construct yielding nine independent transformed lines. Resistance to CLCuD was observed after 90 days of inoculation with viruliferous whiteflies. RNA interference (RNAi) has also been regulated to develop cotton resistant to bollworms (*H. armigera*) larvae. The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) gene plays a role in rate-limiting reaction of mevalonate pathway for juvenile hormone (JH) synthesis in cotton bollworm. This gene was allowed to be targeted by double-stranded RNAs (dsRNA). Feeding on leaves of transgenic cotton containing HMGR, its expression was significantly downregulated in larvae of *H. armigera*. The expression was as low as 80.68% compared with wild type. Additionally, an expression of vitellogenin was reduced up to 76.86% (Tian et al. 2015).

Silencing genes that code proteins essential for pests ensures safe and effective pest control strategy. RNAi can be used in combination with *Bt* toxins to achieve stronger durable pest resistance with an even reduced probability of pests becoming resistant (Tabashnik and Carrière 2017). Single Cry expression levels, at times, are low enough for the pests to survive the toxin. For example, *Pectinophora gossypiella*—a secondary cotton pest, is known to be tolerant to the low Cry toxin levels.

Thus, a selected combination of traits can also delay the occurrence of resistance in transgenic cotton such as pyramiding of *Bt* toxin with RNAi, referred to as the next generation transgenic development. One such instance involved dsRNA production from *Helicoverpa armigera* to interfere its juvenile hormone synthesis by targeting JH acid methyltransferase (JHAMT) coupled with *Bt* toxin (Ni et al. 2017).

CRISPR/Cas system for cotton improvement

Plant breeders have successfully been able to develop high yield cotton with superior quality of lint. Seed yield and fiber length of fiber have been increased as a result of traditional breeding techniques. Additional yield gains were obtained by using improved cotton husbandry techniques along with the use of wild relatives for traits related to abiotic and/or biotic stress tolerance. Such improved accessions may be further used for genetic manipulation of latest techniques, e.g., CRISPR/Cas for improved traits by eliminating undesirable genes (Rauf et al. 2019). CRISPR/Cas system is renowned for its accuracy, ease and increased efficiency (Mao et al. 2013; Feng et al. 2013; Chen et al. 2017). Though, it has been demonstrated to be successful in various crops, CRISPR/Cas based cotton genome editing needs to be researched upon. Cotton carries significance for its fiber, oil and protein for feed industry. Targeting both sets of homologous alleles of tetraploid cotton was difficult, thus a transgenic cotton genotypes having a single copy of green fluorescent protein (GFP) was used to understand the efficacy of CRISPR/Cas9 system (Janga et al. 2017). Targeted mutations were confirmed upon losing GFP fluorescence and presence of various indels making use of three separate sgRNAs. Hence, CRISPR/Cas9 is deemed useful to target various genes within cotton genome. The efficiency of CRISPR/Cas system for allotetraploid cotton was also demonstrated by Li et al. (2017a), who detected 50% truncated events in transgenic individuals. Additionally, no off-target mutations were also detected.

Cytidine deaminase fused with CRISPR/nCas9 (Cas9 nickase) or dCass9 (deactivated Cas9) was proved to be effective for creating point mutations (Qin et al. 2020). To create single base mutations in cotton genome, a base-editing system for the G. *hirsutum*-Base Editor 3 (GhBE3) was developed. The CRISPR/Cas9 plasmid (pRGEB32-GhU6.7) was inserted with a cytidine deaminase sequence in fusion with nCas9 and uracil glycosylase inhibitor (UGI). For both target genes *GhCLA* and *GhPEBP*, three target sites were chosen for test accuracy and efficiency of GhBE3. The editing efficiency of three target sites lies in 26.67%∼57.78%. C-T substitution efficiency was found between −17 bp and −12 bp from protospacer adjacent motif (PAM) sequence. Additionally, no off-target mutations were found amidst 1 500 potential off-target sites in the genome. The T_1 progeny was observed to inherit the edited bases.

To validate the functionality of *sgRNAs* to target three genes in cotton, Gao et al. (2017) analyzed the extent of CRISPR/Cas efficiency via a transient method. Mutations were generated in *GhCLA1* and in *GhPDS* and *GhEF1* at two target sites along with simultaneous editing of homeologous genes and deletions in polyploid cotton genome. Various *sgRNAs* expressing and targeting together indicated to highly efficient functionality with observation of 80.6% mutation frequency. A major composition of mutations included deletions of about 64% in cotyledonary tissues. The process is claimed to be accomplished in 3 days with the use of multiplex binary vector system (pYCLCRISPR/Cas9). Albino phenotypes were developed owing to targeting of *GhCLA1* gene (Wang et al. 2017). It was intended to develop transgenic lines that can also be developed for breeding purposes using CRISPR/Cas 9 system. For instance, cotton arginase gene (*GhARG*) was knocked out from R18 which is a transgenic acceptor variety obtained from Coker-312. Successful events of gene knock out from A and D genomes resulted in improved lateral root development irrespective of nitric conditions.

A heat inducible CRISPR/Cas12b editing system was utilized to generate mutants with the highest editing efficiency by Wang et al. (2020). Hypocotyls were exposed to *Agrobacterium* for 2 days and then shifted to callus induction medium. The transgenic cells harboring the AacCas12b gene were exposed to 42 °C, 45 °C, and 48 °C for varying eight incubation times (that spanned to hours and days). All of the hypocotyls treated at 48 °C for more than 2 days failed to develop hinting at the *in vitro* limiting temperature. The plant exposed to 42 °C for 4 days exhibited simultaneous editing from two target sites. The highest editing rate was observed at 45 °C for 4 days along with the least damaging effects on the callus. Off-target mutations were absent. Heritability was observed to be stable in T1 generation.

Applications of transformation for cotton improvement

Transformation is majorly directed to overcome damaging occurrences within the field. These include various climatic effects referred to as the abiotic stresses; damages through pests such as bacteria, fungi and insects are referred to as the biotic stresses. Additionally, transformation helps improve cotton quality by enhancing the fiber and cottonseed oil. These aspects are discussed below for a thorough understanding.

Abiotic stress tolerance

An application of *Agrobacterium*-mediated transformation involved the development of drought tolerant cotton. For example, in an experiment carried out in Texas and Arizona, USA, a combination of water deficit stress at differing timings given to transgenic cotton over-expressing isopentyl transferase gene (IPT) yielded drought resistant cotton. Success of the transformation was dependent upon the timing of drought stress. Less bolls were observed to be lost in IPT- transgenic cotton in reduced water stress especially in the earlier period of cotton growth. Thus, Zhu et al. (2018) have revealed that water deficit stress before flowering is preferred to reap the benefits of IPT-transgenic cotton. Delayed senescence in IPT transgenic cotton was achieved by Kuppu et al. (2013). Additional morphological changes included highly multiplied shoot and root biomass with high chlorophyll content leading to higher photosynthetic rates. Chen et al. (2019) reported the overexpression of *HUB2* gene from *Arabidopsis thaliana* in cotton to obtain increased boll number and plant height even under drought conditions. The overexpression of *GHSP26* gene in cotton resulted in elevated levels of tolerance to drought at three key plant growth stages, i.e., vegetative, squaring and boll formation (Maqbool et al. 2010; Shamim et al. 2013). Osmotic stress following a cell dehydration event requires an increased synthesis and activity of HSPs (Heat-shock proteins) for protection of various proteins.

In the event of *in planta* transformation involving the shoot apex, initially chimeras are generated. However, by the T_3 generation, Guo et al. (2018) observed the successful transmission of CP4-EPSPS transgene in a non-chimeric manner. The normal field sprays containing a 0.15% of glyphosate concentration whereas the transgenic individuals could tolerate up to 0.40% glyphosate concentration. Karthik et al. (2020) advocated a stringent two-level glyphosate screening of *in planta* transformants. First screening of the putative transformants was carried out at seedling stage of T_1 generation. In the presence of 1 000 $mg{\cdot}L^{-1}$ of glyphosate, 6% seedlings survived. The second screening yielded 2.27% of plants which were then allowed for molecular characterization. The modified CP4-EPSPS gene consisted of the chloroplast transit peptide (CTP) that aided the transgene to be targeted to chloroplast for tolerance of high glyphosate concentration.

Following the apical meristem targeted transformation of cotton embryos, Kesiraju et al. (2020) applied varying conditions to embryos to analyze the recovery of primary transformants. According to one setup, sterile water was used to wash the infected seedlings after transformation and shifted to autoclaved soilrite which were kept under diffused light resulting in less amount of recovered primary transformants. Alternatively, transformation was followed by shifting of seedlings to Petri plates containing water-soaked filter paper discs. These were placed in dark overnight resulting in speedy and increased embryo recovery of the primary transformants i.e. about 10–35%. The preliminary transformation efficiency using the GFP marker was around 28.6% which by the T1 generation was 26.6% in the presence of the stringent hygromycin screening. Of these, 3.31% advanced forth to the next generation.

Overexpression of AtLOS5 resulted in a series of reactions that lead to increased ABA production and ABA-induced physiological regulations in cotton cultivar Zhongmiansuo35. An elevated tolerance to drought conditions is demonstrated by a reduced transpiration water loss, improved membrane integrity under water scarce conditions and increase in fresh weight within the growth chambers compared with the controls (Yue et al. 2012). Transgenic cotton plants were also observed to accumulate increased levels of soluble sugars and proline with enhanced activity of antioxidants under drought stress conditions in transgenic cotton having TaMnSOD cDNA. An increase in biomass, root and leaf systems were observed in transgenic cotton (Zhang et al. 2014). To further reap the benefits of transformation, a crop can be introduced with different traits simultaneously. Such as the induction of tolerance to both drought and salinity in cotton by Yu et al. (2016) by the transformation of a homeodomain- START transcription factor from *A. thaliana* i.e. ENHANCED DROUGHT TOLERANCE 1/ HOMEODOMAIN GLABROUS 11 (*AtEDT1/HDG11*). In addition, to salt and drought tolerance, leaf stomatal density was reduced while leaf epidermal cell size was increased significantly.

Biotic stress tolerance

Biotic stresses refer to the impact of living organisms on crop yields and other physiological mechanisms. These organisms include insect pests, nematodes, worms, weevils, aphids, whiteflies and arthropods belonging to various orders of Hemiptera, Orthoptera, Lepidoptera, Coleoptera, Hymenoptera, and many others (Dhillon and Sharma 2010). Adoption of *Bt* cotton on a large scale has ensured an overall reduction of pests in the vicinity of the transgenic cotton field. Apparent benefits include reduction in costs of insecticide sprays and associated environmental contamination alongside elevated crop yields. A reduction of as much as 4%~28% in the Environmental Impact Quotient (EIQ) is observed in countries such as India, Australia, USA, and China. Use of *Bt* cotton has reported to result in a 41% decrease of costs on insecticide sprays in India.

Unlike the traditional insecticide sprays, *Bt* cotton only renders toxicity in pests that are directly exposed to plant tissue via ingestion. Pollinator population is not observed to be negatively affected by transgenic cotton which is why farmers get to keep their beehives to obtain honey (James 2011; Dhillon et al. 2011). Shi et al. (2012) reported that the overexpression of *GhDIR* gene resulted not only in the enhancement of lignin content but also observed to have a delayed spread of *V. dahliae* in transformants.

Apoptosis inhibitor genes from baculovirus may also help make cotton resistant to *V. dahliae*. The overexpression of both *p35* and *op-iap* genes prevented programmed cell death (PCD) induced from VD-toxin (Tian et al. 2010). Resistance to both *Helicoverpa armigera* and *Aphis gossypii* Glover was introduced together in cotton elite cv. Sumian 16 by Liu et al. (2019). *Galanthus nivalis* agglutinin (*GNA*) resistance gene resulted in the control of bollworm larvae and a decrease in aphid populations. In a similar experiment, simultaneous induction of resistance against *Spodoptera frugiperda* and *Anthonomus grandis* was achieved by de Oliveira et al. (2016) by expressing *Cry1Ia12* gene. Field bioassay revealed 40% mortality rate and delayed the development for *S. frugiperda* larvae while a 60% reduction in the population of *A. grandis*.

Sohrab et al. (2016) transformed Coker-310 with βC1 gene and T1 plants showed 60%~70% resistance to cotton leaf curl virus (CLCuV) disease. Akmal et al. (2017) found that *in silico* prediction hinted at the multiplicity of miRNA targeting such as the open reading frames C1, C4, V1 (CLCuMV) and βC1 (CLCuMB) which according was a good sign for antiviral defense.

In another study overexpression vector pBI-121-ghr-MIR166b in cotton was used to target ATP synthase within *B. tabaci.* The rate of insect mortality was found to be proportional to the ghr-miR166b expression levels (Wamiq and Khan 2018). Latif et al. (2015) introduced a synthetic codon optimized *EPSPS* gene with the help of pCAMBIA 1301. The variety CEMB-02 was chosen which already contained Cry1Ac and Cry2A. A 100% insect mortality was observed in field with plant tolerance to spray concentration of glyphosate at 1 900 mL per acre.

Various unconventional methods have also been employed to obtain insect resistant mutants. For instance, pollen mediated methods can overcome some of the limitations posed by conventional methods such as low transformation rate, and need for skilled labor and sophisticated equipment. The pollen magnetofection technology used positively charged polyethyleneimine-coated Fe_3O_4 magnetic nanoparticles (MNPs). These MNPs were bound with the electric negative DNA (pBI35SBTΔαCPTI plasmid) forming MNP-DNA complexes. A magnetic field was applied to ensure the uptake of these complexes by the pollen prior to pollination. Kanamycin screening was employed for confirmation of transformants that were resistant to insects. Magnetofection was observed to have very minimal damaging effects on the pollen, demonstrating an 80% viability (Zhao et al. 2017).

Improving oil yield and quality

A functional transcription factor GhDof1, belonging to a large family of DNA-binding with one finger (DOF), has been useful in improving cottonseed oil content by 16.28% along with tolerance to abiotic stresses (salt and cold). The cultivar 'Sumian 20' was transformed with Pro35S: GhDof1 vector via *Agrobacterium*. Resistance to kanamycin had helped screen putative transgenic plants. Additional impacts may include an increase in overall proline content as a result of upregulation in *GhP5CS* gene expression (Su et al. 2017).

Improving fiber yield and quality

Cotton is an important industrial crop species as it is largest renewable textile fiber source. Various genes are implicated in formation of high quality fiber. For instance, Wu et al. (2018) reported two MIXTA genes, namely N_2 and Li_3 encode for fuzz and lint fiber, respectively. Fuzz fibers do not elongate enough as lint fibers and usually impart a fuzzy appearance to cottonseeds. On the contrary, lint is spinnable fiber and needed for textile purposes. Both fibers are, however, important because breeders have reported that non-fuzzy phenotype usually leads to low quality lint (Wan et al. 2016). Another gene required for the development of fiber in cotton is PROTODERMAL FACTOR1 (*GhPDF1*) gene. A retardation in fiber initiation was observed by Deng et al. (2012) upon silencing of *GhPDF1* gene. This was accompanied with accumulation of hydrogen peroxide at elevated levels with a simultaneous reduction in the expression of genes related to ethylene and pectin synthesis. The cellulose synthase (CESA) family of genes have also been reported to be involved in fiber development, but more studies need to be carried out for this subfamily (Li et al. 2013). *GhCESA2* is claimed to be important for secondary cell wall synthesis while *GhCESA3* is observed to have continuous contribution in the development of fibers. During the stage of secondary wall deposition, GhMYBL1—a R2R3-MYB transcription factor is specifically expressed in cotton fibers. The expression is associated with enhanced level of lignin and cellulose biosynthesis (Sun et al. 2015). Similarly, overexpression of epidermal specific *GhHD-1* is linked with the increase in the number of fiber initials (Walford et al. 2012). In

two parallel studies, the variety NIAB-846 was used for the introduction of fiber genes from *Calotropis procera*. This cultivar was chosen for its low fiber quality and high germination rate of seeds. The fiber gene *CpTIP1* was the gene of interest for Akhtar et al. (2014) while the *CpEXPA3* gene was the gene of interest for Bajwa et al. (2013). Of the 50 putative transgenic plants, 5 plants were detected positive for the *CpTIP1* gene (Akhtar et al. 2014) while 4 plants were detected positive for the *CpEXPA3* gene (Bajwa et al. 2013). A 2.5-to-5.2-fold increase was observed for T1 and T2 generation of the *CpTIP1* gene. The third generation can be seen with an increase of 1.5 folds which jumped up to 3 and 3.5 folds in T4 and T5, respectively. An improved fiber strength and increased micronaire value (8%~10%) was observed for the *CpEXPA3* positive plants up till the third generation in comparison to the controls.

GM cotton for improving input use efficiency for sustainable agriculture

Transgenic events especially *Bt* cotton has contributed to international development goals by alleviating poverty due to reduced agricultural inputs. Moreover, transgenic crops are observed to grow well even in no-till soils. Such soils are lesser prone to erosion meaning the soil nutrients are kept intact so costs of nutrient inputs are bound to reduce. Transgenic cotton has also been reported to be involved in a reduced environmental impact quotient as compared with non-transgenic cotton. This has helped increase nutrient use efficiency and harvest index alongside a decrease in pesticide application and plant protection costs. Protection of forests and biodiversity is also attributed to transgenic cotton, a strategy referred to as "sustainable intensification" (Raymond Park et al. 2011; Singh 2017).

Another benefit with adoption of *Bt* cotton is the feasibility of sowing intercrops between the wide rows of cotton plants. For example, intercropping with groundnut ensures a reduction in the costly import of groundnut oil and prevention of nutrient depletion in cotton fields (Singh and Ahlawat 2011). *Bt* cotton is known to help improve crop productivity as a result of soil health due to conserved tillage systems. Reduced tillage treatments also observed a decrease in weed biomass. With the inclusion of *in situ* green manure, improved yield of cotton seed was observed (Blaise 2011). WUE is also a prominent yardstick for sustainable agriculture. Transgenic lines have been observed to have reduced water loss. An example involves the over-expression of *GhNAC2* in the presence of CaMV35S promoter generates transgenic cotton plants that show a reduction in both wilting and leaf abscission (Gunapati et al. 2016). Transgenic lines have also been reported to show less stressed phenotypes under drought conditions compared with Coker-312. Long cotton fibers obtained from stressed conditions were spun into yarn that was observed to be uniform and have more strength (Mittal et al. 2015). For instance, improved phosphorus acquisition in cotton was obtained by Liu et al. (2011a) via *Agrobacterium*-mediated transformation and particle bombardment (Liu et al. 2011b) with phytase gene (*phy A*) obtained from *Aspergillus ficuum*. Generally, amount of potassium found in soil is not sufficient to meet the needs of plant growth. Moreover, the available potassium is found usually in organic form while plant readily uptakes the inorganic form. Phytase activity of up to sixfold was observed in transgenic cotton when phytate was the only applied source in growth medium.

Integrated pest management and GM cotton

A decision support system that is a complete strategy to manage pests and involves the selection and use of a suitable tactic to control pests is referred to as integrated pest management (IPM). The selection is dependent upon the costs and the effects on the producers, the society and the environment. IPM includes both preventative and prescriptive measures. This means that techniques those can combat pests with a greatly reduced use of pesticides would help with IPM (Naranjo 2011; Trapero et al. 2016). In this regard, one of the first insect resistant varieties were adopted in Australia called Ingard® having a single Cry toxin protein. With a plantation on 30% of cotton area, a 50% reduction in insecticide application was observed. However, with the introduction of the Bollgard II®, a 100% area cultivation of the variety was able to be carried out resulting in a further reduction of insecticide spray. This is especially helpful to prevent occurrence of resistance to insecticides in non-pest insects. Additionally, secondary pest outbreak may also be avoided by preventing the reduction in the population of certain sucking pests (Wilson et al. 2013). Cotton genotypes having high phenol content and low amount of tannins are less susceptible to jassids (Shinde et al. 2014). Crop refuges may also help in reducing the occurrence of resistance in pests by retaining pest vulnerability. However, a combination of previously stacked varieties has led to a reduction in refuge requirement as much as by 30%. Monsanto's Bollgard II® Genuity™ and Dow AgroSciences' WideStrike® employ the 'natural refuge' option where non-cotton hosts of the vicinity have replaced the non-*Bt* cotton refuge (Que et al. 2010).

Future aspects

An in-depth understanding of plastid transformation in cotton would cater to concerns related to transgene escape while promoting acceptance amongst the public

with regards to GMOs (Yu et al. 2020). Tissue specific protein expressions would help overcome incidences where a reduction in the protein expression is observed as the plant matures (Bakhsh et al. 2012; Singh et al. 2016). Simultaneous suppression of two major cotton pests (*Spodoptera litura* and cotton bollworm) has been generated by stacking *Cry9C* with *Cry2A* or *Cry1Ac* (Li et al. 2014). Thoughtful stacking of traits in combination with suitable transformation and breeding methods would help generate transgenic lines to attain pragmatic strategies that efficiently combat multiple pests as the resultant genotypes are deemed to build upon novel insect resistant traits. Dlugosz et al. (2016) demonstrated the use of robotic platform for the isolation of protoplast from "Bright Yellow-2" tobacco suspension culture and their transformation. It can be inferred from their work that automation can be furthered to minimize human participation in steps that require high precision. For instance, direct DNA drop onto stigma or pollen tube pathway can be mechanized to avoid minimal injury to the ovary. A small sized CRISPR/Cas system needs to be developed to cater to the limited size of viral vectors. Moreover, Cas9 is able to recognize the PAM site after every 8~16 bp thus limiting its application. Avoidance of off-target effects is desirable. Effort needs to be put in to develop a tissue culture free CRISPR/Cas system for crops that are difficult to regenerate (Manghwar et al. 2019).

Authors' contributions

Qandeel-E-Arsh and Rana IA perceived the idea, Qandeel-E-Arsh and Azhar MT explored literature, Atif RM, Khan AI, Israr M helped in improving the article, Khalid S critically rephrased and rearrange the literature and Rana IA edited the literature to give final shape to article. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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