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Posted Date: 10 March 2026

doi: 10.20944/preprints202603.0635.v2

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Article

The Black Cotton: Bt-Integrated Genetic Design of Naturally Colored Cotton Fibers — Black, Blue, Pink, Green, and Brown — The Five

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Abstract

What if cotton could grow already colored — eliminating the need for dyes altogether? Today nearly all cotton is harvested white and later dyed using chemical processes that account for roughly 17–20% of global industrial water pollution. Billions of liters of water and large quantities of synthetic chemicals are used each year simply to give fabrics their color. This work explores a transformative alternative: **cotton that produces its own colors while growing**. We present a unified biological design framework for cotton fibers capable of naturally producing six shades — the existing **brown** and **green**, along with engineered **pink**, **blue**, and, for the first time, **black cotton**. Instead of dyeing fabric after harvest, the plant itself is programmed to create pigments directly inside the fiber. A key innovation is a dual-pigment strategy that enables the production of black cotton by combining two natural pigment systems commonly found in plants and biological materials. By carefully activating these pathways only in the developing fiber, the plant can generate stable coloration without affecting normal growth. Beyond proposing the concept, this study provides a practical roadmap for turning naturally colored cotton into a real agricultural technology. The framework outlines the full journey from laboratory design to field deployment, including gene construction, plant transformation, greenhouse testing, field trials, regulatory approval, and large-scale seed production. Methods for combining color traits with existing pest-resistant cotton varieties are also discussed to ensure compatibility with modern farming. If successfully implemented, naturally colored cotton could dramatically reduce the environmental footprint of the textile industry by eliminating large portions of the dyeing process. In the long term, this approach points toward a future where the colors of clothing are not manufactured in factories but **grown directly in the field**.

Keywords: Bt-cotton; naturally colored cotton; black cotton; anthocyanin biosynthesis; melanin; proanthocyanidins; fiber pigmentation; genetic engineering; CRISPR/Cas9; lab-to-field translation; sustainable textiles; polyphenol oxidase; gene stacking

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1. Introduction

Cotton is a cornerstone of the global textile economy. Approximately 25 million tonnes of raw cotton lint are produced annually across more than 80 countries, with India, China, the United States, Brazil, and Pakistan accounting for over 75% of world output [1]. The fiber — derived from epidermal trichomes on the seed coat of *Gossypium* species — is spun into yarn and woven or knitted into fabrics for clothing, home furnishings, medical textiles, and industrial applications.

Two transformative developments have reshaped cotton agriculture. First, the introduction of **Bt-cotton** in 1996 (USA) and 2002 (India), providing built-in resistance against lepidopteran pests through expression of Cry proteins from *Bacillus thuringiensis* [2]. Second, a renewed scientific and commercial interest in **naturally colored cotton** — varieties whose fibers accumulate pigments during development, eliminating post-harvest dyeing [3].

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The environmental rationale is compelling. Textile dyeing accounts for an estimated 17–20% of global industrial water pollution and consumes ∼1.3 trillion liters of water annually [4]. Synthetic azo dyes, heavy metals, and finishing chemicals discharged into waterways cause ecological damage and health risks. A cotton plant whose fiber is already colored at harvest bypasses these polluting steps entirely.

Motivation: The modern textile dyeing industry is one of the largest sources of industrial water pollution worldwide. Conventional cotton fabrics are typically dyed after harvesting using synthetic dyes, chemical fixing agents, salts, and heavy-metal-containing additives. It is estimated that textile dyeing and finishing contribute nearly **17–20% of global industrial water pollution**, releasing large volumes of untreated wastewater into rivers and agricultural land. These effluents often contain azo dyes, sulfide compounds, chromium, copper, and other toxic chemicals that persist in soil and aquatic ecosystems. In addition to contaminating freshwater resources, dyeing processes require extremely large quantities of water — often more than **100–150 liters of water per kilogram of fabric**. The resulting wastewater can reduce oxygen levels in rivers, block sunlight penetration needed for aquatic life, and introduce carcinogenic compounds into the environment. Long-term discharge of such pollutants also degrades agricultural soils, affecting crop productivity and biodiversity. These environmental challenges provide a strong motivation for developing alternatives such as **naturally colored cotton**, where fibers are produced with inherent pigmentation during plant growth, thereby eliminating the need for chemical dyeing and significantly reducing water pollution. In this paper, we go further than previous reviews in several important ways. The specific novel contributions are detailed below.

1.1. Contributions of This Paper

This work makes four distinct contributions that, to the best of our knowledge, have not been previously reported together in the literature:

1. **Black cotton as a new design target.** We propose, for the first time, a *dual-pathway* genetic strategy to produce near-black cotton fiber by combining (a) eumelanin biosynthesis via heterologous expression of tyrosinase (*TYR*) from *Streptomyces antibioticus* and polyphenol oxidase (*PPO*) from *Vitis vinifera*, with (b) hyperaccumulation and laccase-catalyzed oxidative polymerization of proanthocyanidins in the fiber lumen. We specify the complete gene list, donor organisms, construct architecture (six transcription units in a Level 2 Golden Gate binary vector), and fiber-specific promoter assignments for this new target (Section 3.5, Table 2, Figure 3).
2. **Unified six-color design framework.** We present the first single paper that systematically covers the genetic design procedures for all six cotton fiber colors — white (reference), brown, green, pink, blue, and black — within one integrated biochemical pathway map (Figure 2), enabling direct comparison of gene requirements, donor organisms, and engineering complexity across the full color spectrum.
3. **Reproducible bench-level laboratory protocols.** Unlike previous reviews that describe strategies at a conceptual level, we provide step-by-step, experimentally reproducible protocols with specific reagents, concentrations, media compositions, instrument settings, and quantitative decision gates (Protocols 1.1–5.1). These cover: RNA-seq-based pathway profiling, codon-optimized gene synthesis, Golden Gate multigene assembly (Level 0 → Level 1 → Level 2), *Agrobacterium*-mediated transformation of cotton cv. Coker 312, tiered molecular screening (PCR, Southern blot, RT-qPCR, LC-MS/MS with diagnostic *m/z* values for each pigment class), and HVI fiber quality evaluation against defined threshold and target values.
4. **Complete 15-step lab-to-field translational pipeline.** We map the entire journey from T_0 transgenic plant to commercial farmer deployment through 15 explicit stages spanning five phases — Laboratory, Greenhouse, Contained Field Trials, Regulatory, and Commercial — including protocols for confined field trial design (RCBD, biosafety monitoring), multi-environment G×E analysis (AMMI/GGE biplot), regulatory dossier content (molecular characterization, substantial

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equivalence, toxicity, allergenicity, environmental risk), seed multiplication chain (breeder → foundation → certified seed), and farmer extension with insect resistance management (Figure 4, Protocols 6.1–8.1). This operational-level translational detail has not been previously consolidated in a single colored-cotton publication.

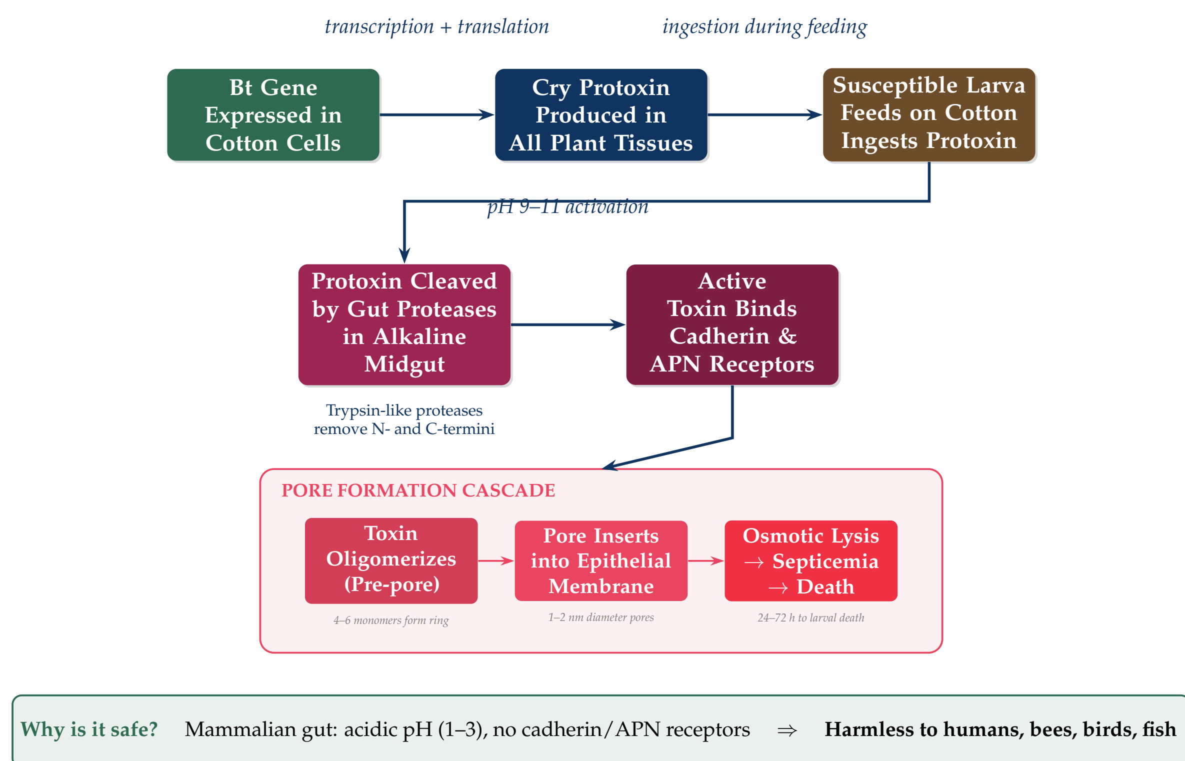
The remainder of this paper is organized as follows: Section 2 reviews Bt-cotton technology; Section 3 describes the biochemistry of fiber pigmentation for all six colors; Section 4 presents the environmental rationale; Sections 5–6 provide the laboratory protocols and translational pipeline; Sections 7–8 cover breeding techniques and Bt-color stacking strategies; Section 9 presents comprehensive fiber comparisons; Sections 10–12 discuss challenges, global research, and future directions; and Section 13 concludes.

2. Bt-Cotton: Mechanism, Generations, and Global Impact

2.1. Mechanism of Action

Bt-cotton expresses one or more crystalline (Cry) or vegetative insecticidal (Vip) proteins from *Bacillus thuringiensis*. When a susceptible lepidopteran larva — such as the American bollworm (*Helicoverpa armigera*) or pink bollworm (*Pectinophora gossypiella*) — feeds on Bt-cotton tissue, it ingests the Cry protoxin. In the insect's alkaline midgut (pH 9–11), the protoxin is cleaved by gut proteases into an active toxin fragment. This fragment binds to cadherin and aminopeptidase-N receptors on midgut epithelial cells, inducing pore formation. The resulting osmotic imbalance leads to cell lysis, feeding cessation, septicemia, and larval death within 24–72 hours [2].

The Cry protein is **highly specific**: it requires the alkaline pH and receptor proteins found only in target insect guts. Mammalian guts are acidic (pH 1–3) and lack these receptors, making Cry proteins harmless to humans, livestock, birds, fish, and beneficial insects [5].



Why is it safe?	Mammalian gut: acidic pH (1–3), no cadherin/APN receptors	⇒	**Harmless to humans, bees, birds, fish**

Figure 1. Mechanism of Bt toxin action in lepidopteran larvae. The Cry protoxin expressed in cotton tissues is ingested by the feeding larva, activated by alkaline gut proteases, and binds specific midgut receptors. The expanded pore formation cascade (highlighted box) shows oligomerization of toxin monomers into a pre-pore complex, insertion of 1–2 nm pores into the epithelial membrane, and the resulting osmotic lysis leading to septicemia and larval death within 24–72 hours. The mechanism is highly specific to target insects due to the requirement for alkaline pH and specific receptor proteins absent in mammals and beneficial insects.

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2.2. Generations of Bt Genes

Table 1. Evolution of Bt gene deployment in commercial cotton.

Generation	Gene(s)	Protein(s)	Target Pests	Year
Bt-I (Bollgard I)	*cry1Ac*	Cry1Ac	*H. armigera*, *H. zea*	1996
Bt-II (Bollgard II)	*cry1Ac* + *cry2Ab*	Cry1Ac + Cry2Ab	Broader Heliothinae; delays resistance	2006
Bt-III (TwinLink+)	*cry1Ab* + *cry2Ae* + *vip3Aa*	Cry1Ab + Cry2Ae + Vip3Aa	Very broad; multi-mode action	2019
Stacked events	*cry1F* + *cry1Ac* + HT genes	Cry1F + Cry1Ac + herbicide tol.	Bollworms + herbicide tolerance	Various

2.3. Global Impact

Bt-cotton has been adopted across 15+ countries on over 25 million hectares. In India, where adoption exceeded 95% of the cotton area by 2014, Bt-cotton reduced insecticide use by ∼30–40% and increased lint yield by ∼30% [6]. Cumulative economic benefits to Indian farmers have been estimated at over USD 67 billion (2002–2020) [7]. Challenges including bollworm resistance evolution and secondary pest outbreaks remain active areas of management [8].

3. Biochemical Basis of Cotton Fiber Pigmentation

Cotton fiber color is determined by pigment molecules that accumulate in the secondary cell wall and/or the lumen of the fiber cell during boll maturation. We describe six target colors, including **black** as a new engineering goal.

3.1. Brown: Condensed Tannins (Proanthocyanidins)

Brown fiber results from condensed tannins (CTs) — oligomeric/polymeric flavonoids synthesized via the phenylpropanoid–flavonoid pathway. The branch-point enzymes are leucoanthocyanidin reductase (LAR, producing catechin) and anthocyanidin reductase (ANR, producing epicatechin). These monomers polymerize and oxidize to brown complexes in the fiber lumen. Shade depends on polymerization degree, catechin/epicatechin ratio, and oxidation state [9]. The TT2 (MYB), TT8 (bHLH), and TTG1 (WD40) transcription factors form the MBW complex that activates PA biosynthetic genes [10].

3.2. Green: Suberin–Caffeic Acid Complexes

Green fiber is *not* caused by chlorophyll. It results from suberin and wax deposits complexed with caffeic acid and hydroxycinnamic acids in the secondary cell wall [11]. The CYP86 cytochrome P450 family and fatty acid elongase (FAE) enzymes are key. Green fibers darken upon washing (exposure of deeper pigmented layers), unlike dyed fabrics that fade [12].

3.3. Pink / Red: Cyanidin-Type Anthocyanins

Pink/red hues derive from cyanidin-type anthocyanins produced by DFR acting on dihydroquercetin, followed by ANS and glycosyltransferases (3-GT). The fiber vacuolar pH (typically 4–5) shifts anthocyanins toward red; achieving stable pink requires co-pigmentation engineering and pH control [13].

3.4. Blue / Purple: Delphinidin-Type Anthocyanins

Blue requires delphinidin, which differs from cyanidin by an additional 5′-hydroxyl group on the B-ring. This hydroxylation requires **flavonoid 3',5'-hydroxylase (F3'5'H)**, absent from the cotton genome. A heterologous F3′5′H gene must be sourced from blue flowers (*Clitoria ternatea*, *Petunia*,

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Viola). Vacuolar pH must be raised above 6.0 (via Na^+/H^+ antiporters) for the blue quinoidal form [14,15].

3.5. Black: Melanin + Oxidized Proanthocyanidin Hyperaccumulation

Key Insight

Black cotton is a new design target not previously reported in the literature. We propose a dual-pathway strategy combining melanin biosynthesis with proanthocyanidin hyperaccumulation and enzymatic oxidation.

Black is the most technically ambitious target color. No naturally occurring cotton produces truly black fiber. We propose achieving black through a **dual-pathway strategy**:

Pathway 1: Melanin biosynthesis. Melanin is the pigment responsible for black/dark brown coloration in many organisms (human skin, squid ink, fungal spores). The pathway begins with tyrosine, which is oxidized to DOPA (3,4-dihydroxyphenylalanine) by **tyrosinase** (TYR), then to dopaquinone, which spontaneously polymerizes into eumelanin (black–brown) or pheomelanin (red–yellow). For black cotton, we target eumelanin production by introducing:

- **Tyrosinase gene** (*TYR*) from *Streptomyces antibioticus* (bacterial tyrosinase with high activity and thermal stability) or *Agaricus bisporus* (mushroom tyrosinase).
- **Polyphenol oxidase (PPO)** from *Vitis vinifera* (grape) or cotton's own *GhPPO* genes, overexpressed under a fiber-specific promoter to catalyze oxidative browning of phenolic substrates toward melanin precursors.
- **4-hydroxyphenylpyruvate dioxygenase (HPPD)** for enhanced tyrosine pool in fiber cells.

Pathway 2: Proanthocyanidin hyperaccumulation + oxidation. Simultaneously, the proanthocyanidin pathway is hyperactivated by overexpressing the MBW transcription factor complex (TT2+TT8+TTG1) to flood the fiber lumen with condensed tannins. A laccase gene (*LAC*) from *Trametes versicolor* (white-rot fungus) is co-expressed to catalyze oxidative polymerization of these tannins into high-molecular-weight, deeply dark complexes.

Combined effect: The co-deposition of eumelanin in the cell wall and heavily oxidized, high-MW proanthocyanidins in the lumen produces an opaque, near-black fiber. The two pigment systems operate independently, providing color redundancy and deepening.

Table 2. Gene requirements for black cotton: dual-pathway strategy.

Pathway	Gene	Source Organism	Function
Melanin	*TYR*	*S. antibioticus*	Tyrosinase: Tyr $\rightarrow$ DOPA $\rightarrow$ dopaquinone
Melanin	*PPO*	*V. vinifera / GhPPO*	Polyphenol oxidase: broad phenolic oxidation
Melanin	*HPPD*	*A. thaliana*	Boosts tyrosine precursor pool
PA accum.	*TT2+TT8+TTG1*	*A. thaliana / Medicago*	MBW complex: activates PA biosynthesis
PA oxidation	*LAC*	*T. versicolor*	Laccase: oxidative tannin polymerization

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3.6. Summary: Six Target Colors

Table 3. Complete pigment summary for all six cotton fiber color targets.

Color	Pigment Class	Key Genes	Natural?	Status
White	None (bleached)	—	Yes	Global standard
Brown	Condensed tannins	LAR, ANR, TT2/TT8	Yes	Commercial (niche)
Green	Suberin–caffeic acid	CYP86, FAE	Yes	Commercial (niche)
Pink	Cyanidin anthocyanins	DFR, ANS, 3-GT	Partial	Breeding / R&D
Blue	Delphinidin anthocyanins	F3′5′H, DFR, ANS, pH mod.	No	Transgenic R&D
Black	**Melanin + oxidized PAs**	**TYR, PPO, LAC, MBW**	**No**	**Proposed (this paper)**

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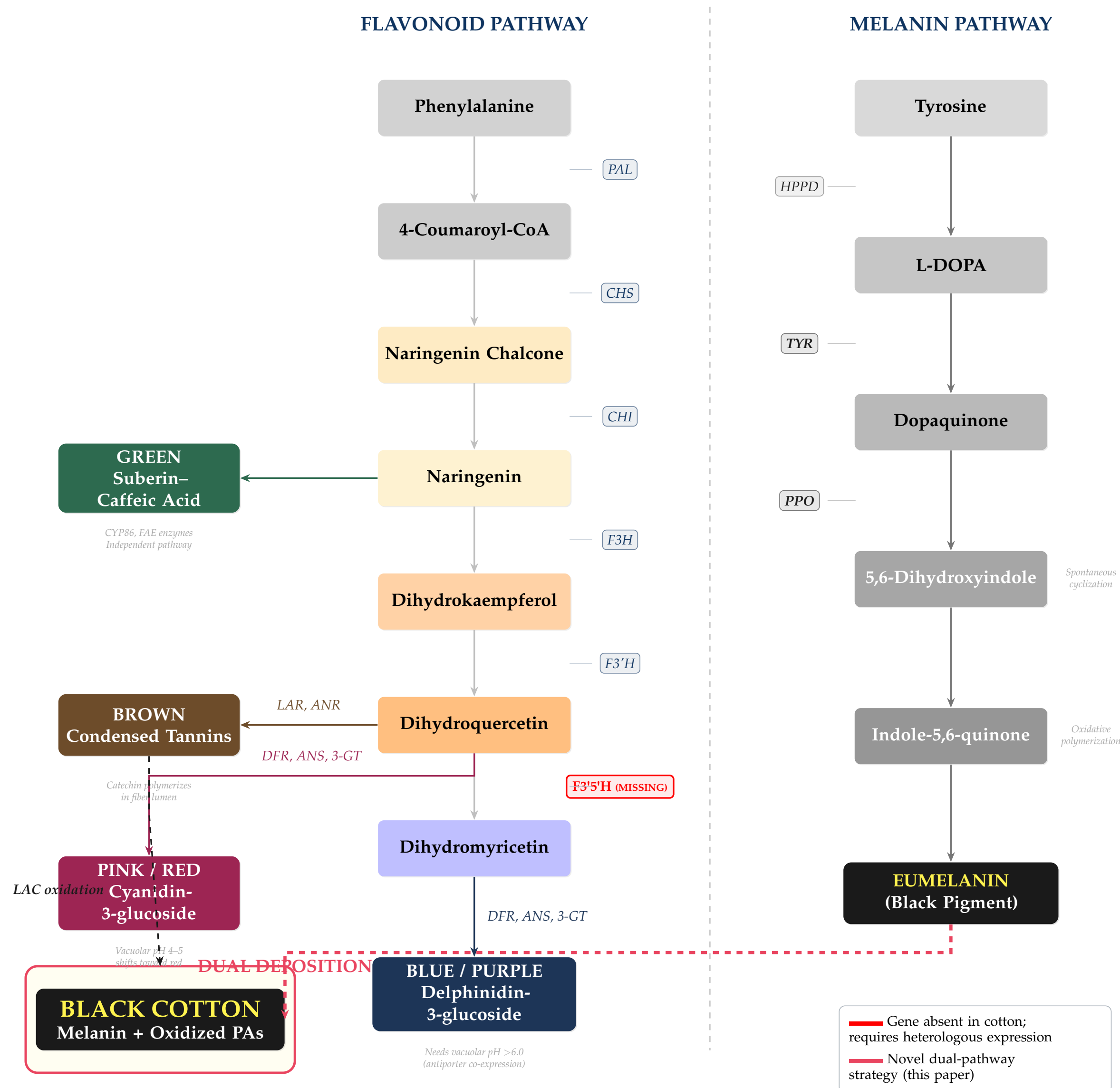

Figure 2. Unified biosynthetic pathway map for all six cotton fiber color targets. **Left:** The flavonoid pathway branches toward brown (tannins via LAR/ANR), pink (cyanidin via DFR/ANS), and blue (delphinidin via DFR/ANS, requiring heterologous F3′5′H, red label). Green arises independently. **Right:** The melanin pathway converts tyrosine to eumelanin. **Black cotton** (highlighted) uses dual deposition of eumelanin and laccase-oxidized proanthocyanidins. Abbreviations: PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H/F3′5′H, flavonoid hydroxylases; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3-GT, glucosyltransferase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; TYR, tyrosinase; PPO, polyphenol oxidase; HPPD, hydroxyphenylpyruvate dioxygenase; LAC, laccase.

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4. Environmental and Health Rationale

4.1. Environmental Impact of Textile Dyeing

Table 4. Environmental comparison: dyed white cotton vs. naturally colored cotton.

Parameter	White + Dyeing	Colored Cotton
Water use for coloring	100–150 L/kg fabric	0 L
Chemical discharge	Azo dyes, heavy metals, formaldehyde	None
Energy (coloring step)	High (90–100°C dye baths)	None
Worker health risk	Dermatitis, respiratory illness, cancer	Minimal
Color durability	Fades with washing	Stable or deepens
CO_2 footprint (coloring)	5–8 kg CO_2/kg fabric	∼0 kg

4.2. Health and Hypoallergenic Properties

Synthetic textile dyes — particularly disperse and azo dyes — are recognized contact allergens causing textile dermatitis in an estimated 1–3% of the population [16]. Naturally colored cotton is free of synthetic additives, making it suitable for infants, surgical patients, and individuals with atopic dermatitis [17].

5. Genetic Design Procedures: Rigorous Laboratory Protocols

This section provides step-by-step protocols with sufficient detail for direct reproduction in a molecular biology laboratory.

5.1. Step 1: Target Pathway Identification

For each target color, determine the branch of metabolism to engineer (Table 3). Perform transcriptomic analysis (RNA-seq) of developing cotton fibers at 10, 15, 20, 25, 30, and 40 DPA (days post-anthesis) to establish baseline expression of flavonoid, phenylpropanoid, and melanin pathway genes. This identifies which endogenous genes are active and which must be supplied heterologously.

Cotton fiber development follows a well-defined timeline: 0–3 DPA (fiber initiation), 5–20 DPA (fiber elongation), 20–40 DPA (secondary cell wall synthesis and pigment deposition window), and 40–50 DPA (maturation and desiccation). Pigments must be deposited during 20–40 DPA to be physically trapped within the fiber.

Protocol 1.1: Fiber-Stage RNA Extraction — Detailed Procedure

Materials: Cotton plants (*G. hirsutum*) in greenhouse; liquid nitrogen in Dewar flask; fine-pointed forceps (Dumont #5); sterile scalpel blades (#11); Spectrum™ Plant Total RNA Kit (Sigma, cat. STRN250); DNase I RNase-free (Qiagen, cat. 79254); Agilent Bioanalyzer 2100 with RNA 6000 Nano chip; Illumina TruSeq Stranded mRNA Library Prep Kit; 2.0 mL nuclease-free tubes; dry ice; RNase-free water; 70% ethanol.

1. **Tag flowers on the day they open.** Walk through the greenhouse and identify flowers that opened that morning (anthesis day = 0 DPA). Tie a small colored string or paper tag around the peduncle (flower stalk) with the date written on it. *Visual cue:* Cotton flowers are cream-colored on Day 0; by Day 1 they turn pink; by Day 2–3 they are deep red and wilting. Tag only cream-colored flowers.
2. **Harvest developing bolls at 10, 15, 20, 25, 30, and 40 DPA.** Using sharp scissors, cut the tagged boll from the plant. **Immediately** (within 10 seconds) plunge the entire boll into liquid nitrogen. Store in a pre-labeled aluminum foil packet in a −80°C freezer. RNA degrades extremely rapidly once tissue is separated from the plant — have the liquid nitrogen Dewar within arm's reach *before* you cut.
3. **Dissect fibers from ovules on dry ice.** Working on a bed of dry ice in a cold room (4°C):
    (a) Remove the boll from the freezer and place on dry ice.

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   (b) Using a pre-chilled scalpel, slice the boll open (it will be frozen solid).
   (c) With fine forceps, peel fibers away from the ovule surface. The fibers appear as a white fuzzy layer coating each seed.
   (d) Collect ∼100 mg of fiber tissue into a pre-chilled, pre-labeled 2.0 mL tube. Keep on dry ice at all times.
   (e) Collect fibers from **at least 3 biological replicates** (3 different plants) per time point.

4. **Grind frozen fibers to fine powder.**
   (a) **Bead mill (preferred):** Drop one sterile stainless steel bead (5 mm) into each tube. Place in TissueLyser II (Qiagen) at 30 Hz for 30 s. Re-freeze in liquid nitrogen 1 min. Run second 30 s cycle. The fiber should now be a fine homogeneous powder.
   (b) **Mortar and pestle (alternative):** Pre-chill a porcelain mortar by filling with liquid nitrogen and letting it evaporate (∼5 min). Add liquid nitrogen + frozen fiber. Grind with firm circular pressure 2–3 min, adding liquid nitrogen as needed. Transfer powder to a pre-chilled tube.

5. **Extract total RNA.** Using the Spectrum™ Plant Total RNA Kit:
   (a) Add 500 μL Lysis Solution to frozen powder. Vortex 30 s.
   (b) Incubate 56°C for 5 min (denatures RNases).
   (c) Perform **on-column DNase treatment**: add 80 μL DNase mix (10 μL DNase I + 70 μL Buffer RDD) to spin column membrane; incubate room temperature 15 min. This removes genomic DNA that would confound RT-qPCR.
   (d) Elute RNA in 50 μL nuclease-free water. Elute again with 50 μL into same tube (total 100 μL).

6. **Assess RNA quality and quantity.**
   (a) Measure on NanoDrop: expect 50–500 ng/μL. A260/A280 should be 1.8–2.1 (pure RNA). A260/A230 should be >1.5 (minimal polysaccharide contamination — cotton is cellulose-rich). If A260/A230 is low, perform lithium chloride precipitation (add 0.25 volumes 10 M LiCl, −20°C overnight, centrifuge 16,000×g 30 min 4°C, wash pellet 70% ethanol).
   (b) **Verify RNA integrity**: Run on Agilent Bioanalyzer 2100 with RNA 6000 Nano chip. **RIN must be** $\geq$ **7.0** (10 = perfect; <7 = degraded — re-extract from new boll).

7. **Prepare strand-specific RNA-seq libraries.** Using Illumina TruSeq Stranded mRNA Kit:
   (a) Start with 500 ng–1 μg total RNA per sample.
   (b) Perform poly(A) selection with oligo(dT) magnetic beads (included). This selects mRNA and removes ribosomal RNA.
   (c) Fragment the mRNA by incubating at 94°C for 8 min in the fragmentation buffer provided (this produces ∼200–300 nt fragments).
   (d) Synthesize first-strand cDNA using random hexamer primers and SuperScript II reverse transcriptase (included), adding actinomycin D to prevent spurious second-strand synthesis during first-strand synthesis (preserves strand specificity).
   (e) Synthesize second-strand cDNA using DNA Polymerase I and RNase H, incorporating dUTP instead of dTTP. The dUTP marks the second strand for later degradation, ensuring strand specificity.
   (f) Ligate Illumina sequencing adapters to both ends of the double-stranded cDNA fragments.
   (g) PCR-amplify the adapter-ligated library for 10–12 cycles (use the minimum cycles that give sufficient yield, to minimize PCR duplicates and amplification bias).
   (h) Use unique dual index (UDI) adapters per sample for multiplexing.

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(i) Validate library size (expect 250–400 bp peak) on Bioanalyzer High Sensitivity DNA chip.
(j) Quantify by qPCR (KAPA Library Quantification Kit).

8. **Sequence.** Submit to a sequencing core facility. Request: 2 × 150 bp paired-end; ≥30M read pairs per sample; NovaSeq 6000 or equivalent.
9. **Bioinformatic analysis.**
   (a) Check quality with FastQC; trim adapters with Trimmomatic or fastp.
   (b) Map reads to *G. hirsutum* reference genome (TM-1 v2.1, from CottonFGD or NCBI) using HISAT2 or STAR.
   (c) Quantify expression as TPM using StringTie or featureCounts.
   (d) Identify candidates: **TPM** $<$ **1** in fiber = targets for heterologous expression; **TPM** $>$ **50** = candidates for promoter harvesting.

Expected outcomes: 3–5 highly active fiber-specific promoters (e.g., *GhExp1*, *E6*, *GhLTP3*) with TPM $>$ 100 during 15–40 DPA. Melanin pathway genes (*TYR*, *HPPD*) confirmed silent (TPM $<$ 1).
Timeline: 3–6 months. **Replicates:** ≥3 biological × 6 time points = 18 samples minimum.

5.2. Step 2: Gene Sourcing and Synthesis

Protocol 2.1: Gene Acquisition and Codon Optimization — Detailed Procedure

1. **Retrieve coding sequences from NCBI GenBank.** For each target gene (Table 5): go to https://www.ncbi.nlm.nih.gov/; search the "Nucleotide" database for the gene name and organism (e.g., "tyrosinase Streptomyces antibioticus"); find the complete CDS (verify start codon ATG and stop codon TAA/TAG/TGA); record the accession number; download in FASTA format.
2. **Codon-optimize each CDS for *G. hirsutum*.** Upload the native CDS to GenScript OptimumGene™ (https://www.genscript.com/codon-opt.html) or IDT Codon Optimization Tool. Select *Gossypium hirsutum* as target (or "Dicot plants" as proxy). Set **CAI target** ≥ **0.85** (Codon Adaptation Index; 1.0 = perfect match). The tool redesigns the DNA sequence to use cotton-preferred codons while encoding the identical protein. Download the optimized sequence.
3. **Remove problematic restriction sites.** Open the optimized sequence in Benchling (https://www.benchling.com, free academic), SnapGene Viewer (free, https://www.snapgene.com/snapgene-viewer), or NEBcutter (https://nc3.neb.com/NEBcutter/). Search for: **BsaI** (GGTCTC), **BpiI/BbsI** (GAAGAC), **EcoRI** (GAATTC), **HindIII** (AAGCTT), **BamHI** (GGATCC), **XbaI** (TCTAGA), **SacI** (GAGCTC). If found within the CDS, introduce a **silent mutation**: change one nucleotide to destroy the site while encoding the same amino acid. Example: BsaI site at codons GGT-CTC (Gly-Leu) → change CTC to CTG (both = Leu). Most codon optimization tools auto-remove these if specified in the "sites to avoid" field.
4. **Add flanking sequences for cloning.** For Golden Gate: add BsaI recognition sites with 4-bp fusion sites (AATG at start codon end, GCTT at stop codon end, following MoClo standard). For Gateway (alternative): add attB1 (5') and attB2 (3') recombination sites.
5. **Order gene synthesis.** Submit to: GenScript (https://www.genscript.com, 2–3 weeks, up to 3 kb); Twist Bioscience (https://www.twistbioscience.com, competitive pricing); or IDT gBlocks (<3 kb, 1–2 weeks). Order each gene in a holding vector (pUC57 or pTwist) with Golden Gate-compatible overhangs.
6. **Sequence-verify all synthetic fragments.** When the plasmid arrives:
   (a) **Resuspend** (if shipped dry): add 20 μL nuclease-free water to DNA spot; incubate 5 min RT.

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(b) **Transform into *E. coli* DH5α by heat shock:** Thaw 50 μL competent cells on ice 10 min. Add 1–2 μL plasmid DNA; flick tube gently 4–5× (do NOT pipette). Ice 30 min. **Heat shock**: 42°C water bath exactly 45 s. Ice 2 min. Add 250 μL pre-warmed SOC medium. Incubate 37°C, 220 rpm, 1 h. Plate 50–100 μL on LB agar + appropriate antibiotic (ampicillin 100 μg/mL for pUC57). Incubate inverted 37°C overnight. Expect 50–500 colonies.

(c) **Pick colonies:** Touch a single colony with a sterile pipette tip; drop into 3 mL LB + antibiotic in a 14 mL tube. Grow 37°C, 220 rpm, overnight. Pick 2–3 colonies per construct.

(d) **Miniprep:** Pellet 1.5 mL overnight culture (16,000×g, 1 min). Extract plasmid with QIAprep Spin Miniprep Kit (Qiagen). Elute in 50 μL. Typical yield: 5–15 μg.

(e) **Sanger sequencing:** Submit 500 ng DNA + sequencing primers (M13F/M13R for pUC57) to Eurofins Genomics, Genewiz/Azenta, or institutional core. Align reads to reference in Benchling or free tool FinchTV (https://digitalworldbiology.com/FinchTV). **Require 100% identity** across entire CDS.

Timeline: 4–8 weeks. **Cost:** USD 0.10–0.30/bp; complete black cotton gene set ≈ USD 3,000–10,000.

Table 5. Gene sourcing strategy for each target color.

Color	Gene(s) Needed	Donor Organism	Function
Blue	F3′5′H; pH modifier	*Clitoria ternatea*; *Ipomoea nil*	5′-hydroxylation; vacuolar alkalinization
Pink	High-expression DFR, ANS	*Antirrhinum majus*; *Gerbera hybrida*	Anthocyanidin synthesis
Deep brown	TT2, TT8, TTG1	*Arabidopsis thaliana*; *Medicago truncatula*	MBW transcription factor complex
Green	CYP86A; FAE	Cotton native genes (promoter engineering)	Suberin biosynthesis enhancement
Black	**TYR, PPO, HPPD, MBW complex, LAC**	*S. antibioticus*; *V. vinifera*; *A. thaliana*; *T. versicolor*	Melanin + hyperaccumulated oxidized PAs

5.3. Step 3: Gene Cassette Construction

Protocol 3.1: Golden Gate Multigene Assembly — Detailed Procedure

Materials: Level 0/1/2 acceptor vectors (MoClo Plant Parts Kit, Addgene #1000000047); BpiI (NEB R0539S); BsaI-HFv2 (NEB R3733S); T4 DNA ligase (NEB M0202S); BSA 10 mg/mL; thermal cycler; *E. coli* DH5α/DH10B competent cells; LB agar + antibiotics; IPTG + X-gal.

1. **Level 0 parts:** Clone each element (promoter, CDS, terminator) into Level 0 acceptor vectors using BpiI (BbsI) sites.
Fiber-specific promoters: GhExp1 (elongation phase, 5–20 DPA); *E6* (secondary wall, 20–45 DPA); *GhLTP3* (early-to-mid, 10–30 DPA). *Terminators* (use diverse to minimize homology): *nos*ter, *ocs*ter, *35S*ter, *HSP*ter.
Level 0 reaction (per part): 40 ng acceptor vector + 40 ng insert + 1 μL BpiI (10 U/μL) + 1 μL T4 ligase (400 U/μL) + 1.5 μL 10× ligase buffer + 0.15 μL BSA + water to 15 μL.
Thermal cycling: 26 cycles of [37°C 3 min → 16°C 4 min]; 50°C 5 min; 80°C 5 min; hold 12°C.
Transform and screen: Add 5 μL reaction to 50 μL DH5α cells; heat-shock as in Protocol 2.1 Step 6(b). Plate on LB + spectinomycin (100 μg/mL) + IPTG + X-gal. **White** colonies = correct insert; **blue** = empty vector. Pick 2–4 white colonies; miniprep; verify by BpiI diagnostic digest and Sanger sequencing.

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2. **Level 1 transcription units (TUs):** Assemble verified Level 0 parts (promoter + CDS + terminator) into Level 1 position-specific vectors using BsaI.
   Level 1 reaction: 40 ng Level 1 acceptor + 40 ng each Level 0 part + 1 μL BsaI-HFv2 (20 U/μL) + 1 μL T4 ligase + 1.5 μL buffer + 0.15 μL BSA + water to 15 μL. Thermal cycling: same as Level 0 but **50 cycles**.
   Transform and screen: Heat-shock into DH5α. Plate on LB + appropriate antibiotic. Pick 2–4 colonies; miniprep; verify by BsaI diagnostic digest (correctly assembled TU should *not* be cut, since BsaI sites are consumed) and Sanger sequencing across all junctions.
3. **Level 2 multigene construct:** Combine all verified TUs into a single binary vector using BpiI. For black cotton:
   - TU1: *E6*pro::*TYR*::*nos*ter TU2: *GhLTP3*pro::*PPO*::*ocs*ter
   - TU3: *GhExp1*pro::*TT2*::*nos*ter TU4: *E6*pro::*TT8*-T2A-*TTG1*::*nos*ter
   - TU5: *E6*pro::*LAC*::*35S*ter TU6: *35S*pro::*nptII*::*nos*ter (loxP-flanked)

   Level 2 reaction: 40 ng Level 2 acceptor + 40 ng each of 6 TUs + 1 μL BpiI + 1 μL T4 ligase + 2 μL buffer + 0.15 μL BSA + water to 20 μL. Thermal cycling: 50 cycles.
   Transform: Use DH10B competent cells (preferred for large >15 kb constructs). Plate on LB + kanamycin (50 μg/mL) + IPTG + X-gal. Pick 4–8 white colonies. For large constructs, use midiprep (QIAGEN Plasmid Midi Kit) for higher yield.
4. **Verify the final construct** ($\sim$18–22 kb):
   (a) *Restriction digestion:* Digest 500 ng with EcoRI + HindIII (37°C, 1 h). Run on 0.8% agarose gel with 1 kb ladder. Compare fragment pattern to *in silico* prediction (Benchling or SnapGene).
   (b) *Full-insert sequencing:* Submit for Oxford Nanopore or PacBio long-read sequencing. Verify 100% accuracy across all junctions.
5. **Transform into *Agrobacterium tumefaciens*.**
   (a) *Prepare electrocompetent A. tumefaciens:* Inoculate a single colony of LBA4404 or AGL1 (disarmed strains retaining vir genes) into 5 mL LB + rifampicin (25 mg/L); grow overnight 28°C, 220 rpm. Transfer 1 mL to 100 mL fresh LB; grow to OD_{600} = 0.5–0.7 ($\sim$4–5 h). Chill on ice 30 min. Pellet cells at 4,000$\times$g, 4°C, 15 min. Wash pellet 3$\times$ with ice-cold sterile 10% glycerol (resuspend gently by swirling; do not vortex). After final wash, resuspend in 1 mL ice-cold 10% glycerol. Aliquot 40 μL into pre-chilled 1.5 mL tubes; snap-freeze in liquid nitrogen; store $-$80°C. Stable for $\geq$6 months.
   (b) *Electroporate:* Thaw one 40 μL aliquot on ice. Add 1 μL plasmid (100–500 ng). Mix by flicking. Transfer to a pre-chilled 1 mm electroporation cuvette. Pulse: **2.5 kV, 200 Ω, 25 μF** (time constant should read 4.5–5.0 ms on the electroporator display; if <4.0 ms, salt contamination in DNA — re-purify).
   (c) Add 1 mL SOC medium immediately; incubate 28°C, 200 rpm, **3 hours** (longer recovery than *E. coli* because *Agrobacterium* grows more slowly).
   (d) Plate 100 μL on LB + kanamycin (50 mg/L) + rifampicin (25 mg/L). Incubate 28°C for 2–3 days until colonies appear (slower than *E. coli*).
   (e) *Colony PCR:* Pick 8–12 colonies with sterile toothpicks; resuspend each in 20 μL water, boil 10 min, centrifuge briefly, use 2 μL supernatant as PCR template. Amplify 2–3 TUs (e.g., *TYR* and *LAC*) to confirm intact binary vector is present.

Timeline: 2–4 months for complete Level 0 $\rightarrow$ Level 1 $\rightarrow$ Level 2 + *Agrobacterium* verification.

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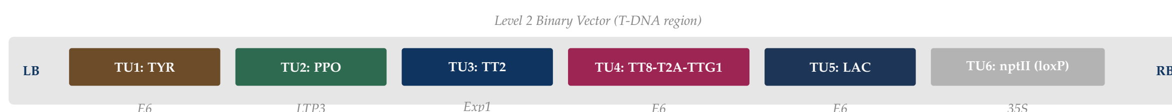

Figure 3. Schematic of the Level 2 multigene construct for black cotton showing six transcription units (TUs) within the T-DNA region. Each TU is driven by a fiber-specific promoter (labeled below). The selectable marker (*nptII*) is flanked by loxP sites for subsequent Cre-mediated excision.

5.4. Step 4: Cotton Transformation

Protocol 4.1: *Agrobacterium*-Mediated Cotton Transformation — Detailed Procedure

Media (prepare and autoclave in advance): Germination medium: $\frac{1}{2}$MS + 1.5% sucrose + 0.8% agar, pH 5.8. Co-cultivation medium: MS + 1 mg/L 2,4-D + 0.1 mg/L kinetin + 100 μM acetosyringone (add *after* autoclaving) + 0.8% agar. Callus induction medium (CIM): MS + 0.1 mg/L 2,4-D + 0.5 mg/L kinetin + 50 mg/L kanamycin + 250 mg/L cefotaxime + 0.8% agar. Embryogenesis medium (EM): MS + 1.9 g/L KNO_3 + 0.5 mg/L kinetin, **no auxin**, + 50 mg/L kanamycin + 0.8% agar. Rooting medium: $\frac{1}{2}$MS + 0.1 mg/L IBA + 0.8% agar. Add all antibiotics/hormones *after* autoclaving.

1. **Seed surface sterilization (Day 0).** Working in a laminar flow hood: First, remove cotton linters (fuzzy fibers) from Coker 312 seeds. *Option A — Acid delinting (perform in a fume hood with full PPE: acid-resistant gloves, face shield, lab coat):* Place seeds in a glass beaker, add concentrated H_2SO_4 to just cover the seeds, stir gently with a glass rod for exactly 2 min (the acid dissolves the linters but does not penetrate the seed coat in this time), quickly pour off the acid into a waste container, immediately rinse seeds 5× with large volumes of tap water, then rinse once with distilled water. *Option B — Use commercially delinted seed* (available from USDA Germplasm or collaborators; much simpler). Next, surface-sterilize: place 50–100 seeds in a sterile beaker in the laminar flow hood. Add 70% ethanol, swirl exactly 1 min, decant. Add 2.5% NaOCl (diluted from commercial bleach; check label for % NaOCl) + 0.05% Tween-20, swirl 20 min. Rinse 5× with sterile ddH_2O (1 min each rinse). *All subsequent steps must be aseptic — flame-sterilize instruments between uses; wear gloves sprayed with 70% ethanol; never pass hands over open plates.*
2. **Germination (Days 0–7).** Place seeds on germination medium, 10 seeds per dish, 2–3 cm apart. Seal with Parafilm. Incubate **in dark** at 28°C for 5–7 days. Hypocotyls should reach 3–5 cm, white/pale green.
3. **Hypocotyl excision (Day 7).** With a sterile scalpel, cut hypocotyl into 0.8–1.0 cm segments. Discard the top 0.5 cm (near cotyledons) and bottom 0.5 cm (near root). Expect 2–3 explants per seedling. Prepare 200–500 total explants (accounts for 1–5% efficiency).
4. ***Agrobacterium* culture (start Day 5).** Streak LBA4404 + binary vector on YEB + kanamycin 50 mg/L + rifampicin 25 mg/L agar; 28°C, 2 days. Pick one colony into 5 mL YEB + antibiotics; grow overnight 28°C, 220 rpm. Transfer 500 μL to 50 mL fresh YEB + antibiotics. Grow to OD_{600} = 0.6–0.8 (∼4–6 h; check hourly from hour 3). *OD 0.6–0.8 = mid-log phase = maximal virulence gene expression.*
5. **Infection (Day 7).** Pellet culture 5,000×g 10 min. Resuspend in liquid MS + 100 μM acetosyringone to OD_{600} = 0.5. Immerse hypocotyl explants 10–15 min with gentle shaking (50 rpm). *Acetosyringone mimics plant wound signals and activates Agrobacterium vir genes.*
6. **Co-cultivation (Days 7–9).** Blot explants gently on sterile filter paper (do not press hard). Place on co-cultivation medium. Incubate 25°C (slightly cool to slow bacterial overgrowth), **darkness**, 48–72 h. During this time *Agrobacterium* transfers the T-DNA into plant cell genomes.
7. **Callus induction and selection (Weeks 2–12).** Transfer to CIM. Kanamycin selects transformed cells; cefotaxime kills residual *Agrobacterium*. Subculture to fresh CIM every 3 weeks.

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Over 8–12 weeks, kanamycin-resistant calli grow from cut surfaces; non-transformed tissue yellows and dies. Expect callus on 10–30% of explants.

8. **Somatic embryogenesis (Months 4–10).** Transfer resistant calli to EM (no auxin — auxin removal triggers embryogenesis). Maintain 16h:8h light:dark, 28°C, 50–100 μmol m^{-2} s^{-1} light. Subculture every 3 weeks. Over months, some calli develop embryogenic structures (small, rounded, shiny, pale-green globules). Expect 5–20% of callus lines to produce somatic embryos.
9. **Embryo germination (Months 8–12).** Transfer individual somatic embryos (torpedo/cotyledon stage) to hormone-free MS + 3% sucrose. Under light, embryos germinate: root from radicle end, cotyledon leaves unfold. Takes 4–8 weeks; expect 30–60% conversion.
10. **Plantlet establishment and acclimatization (Months 10–14).** When plantlets have 3+ roots (>2 cm) and 2–3 true leaves: transfer to rooting medium 2 weeks; gently wash all agar from roots; plant in sterile peat:perlite (2:1) in 8 cm pot; place inside a clear plastic bag/humidity dome (>90% RH); growth chamber 28/22°C, 16h light. Over 2 weeks, gradually open bag (1 h Day 1, 2 h Day 3, 4 h Day 5, fully open Day 10–14). Transfer to 20 cm pots; grow to maturity.

Expected timeline: 8–14 months from explant to T_0 plant. **Expected efficiency:** 1–5 events per 100 explants (from 300–500 explants, recover 5–15 T_0 plants).

5.5. Step 5: Molecular Screening and Selection

Protocol 5.1: Tiered Molecular Screening — Detailed Procedure

1. **Tier 1 — PCR confirmation (eliminates non-transgenic escapees).**
   (a) Extract genomic DNA from a young leaf using the CTAB method: grind 100 mg leaf in liquid N_2; add 700 μL CTAB buffer (2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP-40); 65°C 30 min; add 700 μL chloroform:isoamyl alcohol (24:1), mix, centrifuge 12,000×g 10 min; transfer upper aqueous phase; add 0.7 vol isopropanol, centrifuge 12,000×g 10 min; wash pellet 70% ethanol; dissolve in 100 μL TE; treat with RNase A.
   (b) Design primers spanning a promoter–CDS junction (distinguishes genomic integration from residual *Agrobacterium*).
   (c) PCR: 50–100 ng DNA, 0.4 μM primers, Taq master mix. Cycle: 95°C 3 min; 35× [95°C 30 s, 58°C 30 s, 72°C 1 min]; 72°C 5 min. Include controls: positive (plasmid), negative (wild-type), water blank.
   (d) Run on 1.5% agarose gel. Score positive/negative. Expected: 60–80% of kanamycin-resistant plants are PCR-positive.
2. **Tier 2 — Copy number by Southern blot (identifies single-copy insertions).**
   (a) *Digest:* 15 μg genomic DNA + 50 U HindIII + 5 μL 10× CutSmart buffer + water to 50 μL; 37°C overnight. HindIII cuts once within T-DNA; each insertion produces one unique-sized fragment.
   (b) *Gel:* 0.8% agarose, large format, 25 V overnight for resolution of 5–20 kb fragments. Stain with ethidium bromide; photograph.
   (c) *Pre-transfer washes:* Soak gel in 0.25 M HCl 10 min (depurination); rinse; 0.5 M NaOH/1.5 M NaCl 30 min ×2 (denaturation); 0.5 M Tris pH 7.5/1.5 M NaCl 30 min ×2 (neutralization).
   (d) *Capillary transfer:* Build sandwich (bottom to top): tray of 20× SSC; glass plate bridge; Whatman paper wicks dipping into SSC; gel face-down; nylon membrane (Hybond-N+, cut to gel size, avoid air bubbles); 3 sheets dry Whatman paper; 10–15 cm stack paper towels; glass plate; 500 g weight. Leave 16–20 h.
   (e) *Fix DNA:* UV-crosslink membrane (Stratalinker, 1200×100 μJ/cm^2) or bake 80°C 2 h.

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(f) *Prepare DIG-labeled probe:* PCR-amplify ∼500 bp of *nptII* using DIG PCR Labeling Mix (Roche); verify on gel.

(g) *Hybridize:* Pre-hyb in DIG Easy Hyb 68°C 1 h; add denatured probe (boil 10 min, ice 5 min); hybridize 68°C overnight.

(h) *Wash and detect:* 2× SSC/0.1% SDS RT 5 min ×2; 0.5× SSC/0.1% SDS 68°C 15 min ×2; block 30 min; anti-DIG-AP antibody (1:10,000) 30 min; wash; CSPD substrate; expose to X-ray film 1–30 min.

(i) *Interpretation:* Each band = one insertion. **Select single-band plants.** Reject >3 copies.

3. **Tier 3 — Expression analysis (RT-qPCR).**

(a) Harvest developing fibers at 15, 20, and 25 DPA from T_0 plants (tag flowers as in Protocol 1.1 Step 1). Extract RNA as in Protocol 1.1 Steps 3–6; verify RIN ≥ 7.0.

(b) *Synthesize cDNA:* Use SuperScript IV First-Strand Synthesis Kit (Thermo Fisher, cat. 18091050). In a PCR tube: combine 1 μg total RNA + 1 μL oligo$(dT)_{20}$ primer (50 μM) + 1 μL dNTP mix (10 mM each) + water to 13 μL. Incubate 65°C 5 min (denatures RNA secondary structure); ice 1 min. Add 4 μL 5× RT buffer + 1 μL DTT (100 mM) + 1 μL RNaseOUT (40 U/μL) + 1 μL SuperScript IV (200 U/μL). Incubate: 23°C 10 min, 55°C 10 min, 80°C 10 min. The resulting cDNA can be stored at −20°C.

(c) *Design qPCR primers:* For each transgene, design primers producing 100–200 bp amplicons. Use Primer3 (https://primer3.ut.ee/) or NCBI Primer-BLAST. Avoid primers in repetitive regions. Also design primers for the reference gene *GhUBQ7* (ubiquitin 7, stably expressed in cotton fibers).

(d) *Run RT-qPCR:* Per well: 5 μL 2× PowerUp SYBR Green Master Mix (Applied Biosystems) + 0.5 μL forward primer (10 μM) + 0.5 μL reverse primer (10 μM) + 2 μL cDNA (diluted 1:5) + 2 μL water = 10 μL total. Run on QuantStudio 5 or Bio-Rad CFX96: 50°C 2 min, 95°C 2 min, then 40 cycles of [95°C 15 s, 60°C 1 min]. Include melt curve analysis (65–95°C) to confirm single amplicon. Run each sample in **technical triplicate**.

(e) *Analyze:* Calculate $\Delta C_t = C_t$(transgene) − C_t(*GhUBQ7*). Then $\Delta\Delta C_t = \Delta C_t$(transgenic) − ΔC_t(wild-type). Fold change = $2^{-\Delta\Delta C_t}$. **Select lines with expression** ≥ 10× wild-type background (i.e., fold change ≥ 10). For heterologous genes with zero background, use absolute quantification with a standard curve.

4. **Tier 4 — Metabolite confirmation (LC-MS/MS).** Harvest mature fiber (40–50 DPA, ∼200 mg). Grind in liquid N_2; add 1 mL methanol:water:formic acid (70:29:1); sonicate 15 min; centrifuge 16,000×g 10 min; filter 0.22 μm PVDF into LC vial. Analyze by UHPLC-ESI-MS/MS (Thermo Q Exactive), C18 column, gradient 5–95% acetonitrile, negative-ion mode. Confirm target pigments:

- Brown: catechin (m/z 289.07), PA dimers (m/z 577.13)
- Pink: cyanidin-3-glucoside (m/z 447.09)
- Blue: delphinidin-3-glucoside (m/z 463.09)
- Black: DOPA (m/z 196.06), eumelanin precursors, PA trimers (m/z 865.20), tetramers (m/z 1153.26)

5. **Decision gate:** Advance only lines that are: single-copy (Southern), high-expressing (>10× by RT-qPCR), pigment-positive (LC-MS/MS), and phenotypically normal (no stunting, wilting, or fertility issues). *Expected attrition:* from 10–15 T_0 events, expect 3–5 to pass all tiers.

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5.6. Step 6: Fiber Quality Evaluation

Table 6. Fiber quality parameters for colored cotton evaluation.

Parameter	Method / Unit	Minimum	Target
Upper half mean length (UHML)	HVI / mm	25	>28
Fiber strength (tenacity)	HVI / g/tex	20	>26
Micronaire (fineness)	HVI / Mic units	3.5–4.9	3.8–4.5
Uniformity index	HVI / %	>80	>83
Color intensity	Spectrophotometer (L*a*b*)	Visually distinct	Saturated, uniform
Wash fastness	ISO 105-C06 (grade 1–5)	Grade 3	Grade 4–5
Light fastness	ISO 105-B02 (grade 1–8)	Grade 4	Grade 6+

6. Lab to Field: The Complete Translational Pipeline

Key Insight

The journey from a T_0 transgenic plant in a greenhouse to a commercial cultivar in a farmer's field typically requires 8–15 years and passes through 15 distinct stages. This section maps each stage with specific actions, decision criteria, and timelines.

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Figure 4. Complete 15-step lab-to-field translational pipeline for colored Bt-cotton, showing five phases (Laboratory, Greenhouse, Field Trials, Regulatory, Commercial) with indicative timelines. Total duration: 8–15 years depending on regulatory jurisdiction and breeding complexity.

6.1. Phase I: Laboratory (Steps 1–4)

Covered in Section 5 (Protocols 1.1–5.1). Key output: 5–15 independent T_0 transgenic events confirmed by molecular screening.

6.2. Phase II: Greenhouse Advancement (Steps 5–8)

Protocol 6.1: T_0 to Homozygous Line (Greenhouse) — Detailed Procedure

1. **$T_0 \rightarrow T_1$: Self-pollination.** In the late afternoon (4–6 PM), identify flower buds that will open next morning (swollen, petals showing through calyx). Slide a glassine bag over the bud; secure with paper clip. Next morning (7–9 AM), the flower opens inside the bag. Using a small brush, transfer pollen from anthers to stigma. Re-bag; leave 2–3 days until petals wilt. Boll matures over 40–55 days. Harvest seed cotton at boll opening; gin; store T_1 seeds in paper envelopes at 15–20°C, $<$50% RH. Self-pollinate $\geq$10–15 flowers per T_0 plant (each boll = 25–35 seeds; need $\geq$200 T_1 seeds per event).
2. **T_1 segregation analysis.** Surface-sterilize 48 T_1 seeds (Protocol 4.1 Step 1). Place on MS + 50 mg/L kanamycin + 0.8% agar; 28°C, 16h:8h light. Score at 14 days: green = resistant (transgenic); yellow/bleached = sensitive (non-transgenic). **Chi-squared test for 3:1:** Expected for 48 seeds: 36 resistant, 12 sensitive. $\chi^2 = \frac{(O_R - E_R)^2}{E_R} + \frac{(O_S - E_S)^2}{E_S}$. Accept if $\chi^2 < 3.84$ ($p > 0.05$). *Example:* 38R, 10S $\Rightarrow \chi^2 = 0.111 + 0.333 = 0.444 < 3.84$ = accepted. Discard events with $\chi^2 > 3.84$ (multiple insertions or silencing).

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3. **$T_1 \rightarrow T_2$: Homozygous selection.** Grow 24 T_1 resistant plants to maturity; self-pollinate; harvest T_2 seed. For each, sow 24 T_2 seeds on kanamycin medium. **Homozygous T_1 parents** produce 100% resistant T_2 (24/24 survive); hemizygous parents segregate ∼18/24. Select non-segregating families. **Confirm by TaqMan qPCR:** duplex assay for *nptII* (FAM probe) vs. single-copy endogenous gene (VIC probe). Hemizygous = ratio 1.0; **homozygous = ratio 2.0**.
4. **Bt stacking.** Emasculate flower bud of colored line (female) in afternoon: remove all anthers with fine forceps before pollen shed; bag. Next morning, apply Bt-cotton (male) pollen to stigma; re-bag. Grow F_1; confirm hybridity by PCR for color genes (*TYR*, *LAC*) and Bt genes (*cry1Ac*, *cry2Ab*). Self-pollinate F_1. Screen ≥200 F_2 for double-homozygous (1/16 expected for independent loci); confirm by TaqMan qPCR at each locus. Advance F_3–F_5 by single-seed descent with marker confirmation each generation. *Note: commercial Bt events require a material transfer agreement from the IP holder.*
5. **Fiber quality and color evaluation.** Grow 10 plants per line; self-pollinate all flowers; harvest all bolls. **Gin:** separate fiber from seed (roller gin or lab saw gin); weigh lint and seed; calculate gin turnout = (lint weight / seed cotton weight) × 100; target ≥35%. **HVI:** send ≥25 g ginned lint to a fiber testing lab for UHML, tenacity, micronaire, uniformity. **Color (L*a*b*):** use spectrophotometer (e.g., Konica Minolta CM-700d); 5 readings per sample; report mean ± SD. Targets: black L*<25, pink a*>15, blue b*<−10. **Wash fastness (ISO 105-C06):** prepare 10×4 cm swatch; wash in Launder-Ometer at 60°C, 30 min, with 4 g/L ECE detergent and 10 steel balls; repeat 5 cycles; score on Grey Scale (1–5); minimum Grade 3, target Grade 4–5. Reject lines failing any minimum standard (Table 6).

Decision gate: Advance ≤3 elite events: single-copy, homozygous, Bt-stacked, fiber quality meeting standards, uniform color. **Timeline:** 18–44 months.

6.3. Phase III: Contained and Multi-Environment Field Trials (Steps 9–11)

Protocol 7.1: Confined Field Trial (CFT) — Year 1 — Detailed Procedure

1. **Regulatory pre-approval.** Contact national biosafety authority (GEAC India, USDA-APHIS USA, EFSA EU, CTNBio Brazil, OGTR Australia). Submit: gene construct map with all gene names/sources/promoters; molecular characterization data (PCR, Southern, RT-qPCR, LC-MS/MS); proposed trial GPS coordinates and maps; containment plan (isolation, border rows, destruction protocol); risk assessment (gene flow, non-target effects). Allow 3–6 months for review. **Do not plant until written approval is received.**
2. **Site selection and preparation.** Criteria: ≥500 m from commercial cotton; ≥200 m from wild *Gossypium*; fenced; road-accessible; representative soil/climate. Plant 10 m border of non-transgenic cotton around entire perimeter (pollen trap). Prepare land: disc plow, bed formation, pre-plant irrigation and fertilizer per local soil test.
3. **Trial design.** RCBD with 3 replications. Treatments: 1–3 transgenic colored events + controls (non-transgenic isogenic white, Bt-cotton Bollgard II, naturally colored parent if available). Plot: 4 rows × 6 m; row spacing 90–100 cm; plant spacing 15–20 cm within row. Sow at 2–3 cm depth; irrigate.
4. **Data collection.**
   - *Agronomic (at maturity):* stand count (3 wk), plant height (10 plants/plot), boll number (10 tagged plants), boll weight (20 random bolls), seed cotton yield (harvest entire plot), gin turnout.
   - *Fiber:* HVI (UHML, tenacity, micronaire, uniformity), color (L*a*b*), wash and light fastness.

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- *Pest resistance:* weekly bollworm larval counts (20 plants/plot); damaged boll % (50 bolls/plot at harvest); Bt protein ELISA on young leaves at ∼25 DPA (μg Cry/g fresh wt; minimum >1 μg/g for efficacy).
- *Biosafety:* pollen traps (petroleum jelly–coated slides at 50, 100, 200, 500 m downwind, weekly during flowering; count pollen grains under microscope); soil 16S rRNA sequencing (0–15 cm, 5 cores/plot composited, pre-plant + post-harvest); non-target insect surveys (pitfall traps + sweep nets at 0, 100, 500 m, weekly).

5. **Post-harvest destruction.** Shred all stalks (flail mower); deep-plow residue (≥20 cm); apply glyphosate 1.5 L/ha. Monitor for volunteer plants monthly for 2 subsequent seasons; destroy any cotton plants that emerge. Retain all harvested seed/fiber in locked storage; do not allow into commercial channels.

Timeline: 5–6 months (planting to harvest) + 2 seasons volunteer monitoring.

Protocol 7.2: Multi-Environment Trials (METs) — Detailed Procedure

1. **Site selection:** ≥10 sites spanning target production regions covering: water regime (rainfed vs. irrigated), climate (tropical, subtropical, temperate), soil (clay, loam, sandy), pest pressure (high vs. low bollworm).
2. **Design:** RCBD, 3–4 reps per site, same genotype set, ≥2 consecutive years.
3. **Data:** All CFT parameters plus **fiber processing trials**: ship ≥5 kg ginned lint to pilot textile mill for carding → drawing → spinning (record yarn count Ne, twist/inch) → knitting or weaving a swatch. Evaluate yarn strength, evenness (Uster tester), fabric hand.
4. **G×E analysis:** Compile data into genotypes × environments matrix. Run AMMI analysis (partitions variation into genotype, environment, and G×E interaction) and/or GGE biplots (graphically identifies best genotypes per environment). Software: R packages `agricolae` and `GGEBiplots`, or free tool GGEbiplot (https://www.ggebiplot.com).
5. **Select:** 1–2 lines with highest mean, lowest G×E interaction, acceptable fiber/color at all sites. Produce ≥500 kg seed for breeder seed stock.

Timeline: 2–4 years.

6.4. Phase IV: Regulatory Approval (Steps 12–13)

Protocol 7.3: Regulatory Dossier Preparation — Detailed Procedure

1. **Molecular characterization:** T-DNA insertion site by genome walking (TAIL-PCR or adapter ligation-mediated PCR) or WGS (≥1 kb flanking each side); copy number by Southern blot + digital droplet PCR (ddPCR, gold standard for regulatory submissions); absence of backbone integration (PCR with backbone-specific primers, confirm negative); expression profiling across tissues (leaf, root, pollen, fiber, seed) and stages by RT-qPCR + Western/ELISA; transgene stability across ≥5 generations.
2. **Compositional analysis (substantial equivalence):** Proximate composition of transgenic vs. control cottonseed (protein, fat, fiber, ash, moisture); fatty acid profile; amino acid profile; gossypol (free and total) and cyclopropenoid fatty acids. Analyze from ≥3 sites × 3 reps. Compare by ANOVA or equivalence testing; values within ILSI/OECD natural range = equivalent.
3. **Toxicity and allergenicity:** Bioinformatic screening of all novel proteins (TYR, PPO, HPPD, TT2, TT8, TTG1, LAC) against AllergenOnline/FARRP databases (flag >35% identity over 80 aa). Pepsin digestibility assay (SGF pH 1.2, 37°C; degradation within 30 s = low risk). Acute oral toxicity (mouse, ≥2000 mg/kg single dose, 14-day observation). 90-day subchronic feeding study (rat, transgenic cottonseed meal at 5%, 15%, 33% of diet vs. control; body weight, hematology, clinical chemistry, organ weights, histopathology; OECD TG 408).

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4. **Environmental risk:** Gene flow modeling from pollen dispersal data; non-target organism assessment (compare beneficial insect data from CFT/MET); weediness evaluation (seed dormancy, germination, vigor vs. control); biodiversity impact.
5. **Fiber safety:** Dermal sensitization (guinea pig maximization test OECD 406, or *in vitro* KeratinoSens™). Cytotoxicity of fiber extracts on L929 fibroblasts (ISO 10993-5; confirm viability >70%).

Timeline: Preparation 2–4 years (overlaps with MET). Review after submission: 2–5 years. **Cost:** USD 15–30M per event.

6.5. Phase V: Seed Multiplication and Commercial Release (Steps 14–15)

Protocol 8.1: Seed Systems and Farmer Deployment — Detailed Procedure

1. **Breeder seed (BS):** Produced by originating institution; isolation ≥1000 m. Rogue off-type plants during growing season. Verify genetic purity: grow-out test (sow 400 seeds, score every plant for color/morphology; <0.1% off-types required) + molecular marker test (PCR 96 random seeds for color + Bt transgenes, all must be positive). Harvest, gin, delint, treat with fungicide (carboxin + thiram). Volume: 50–100 kg.
2. **Foundation seed (FS):** Produced by certified agencies from BS. Isolation ≥500 m. Field inspected by seed certification authority ≥3 times (vegetative, flowering, boll-opening stages). Genetic purity: <0.5% off-types. Germination ≥65%. Volume: 5,000–10,000 kg.
3. **Certified seed (CS):** Produced by licensed seed companies from FS. Isolation ≥200 m. Seed treatment: imidacloprid + fungicide coating. Package in branded bags with QR code linking to variety info, planting guide, stewardship requirements. Volume: 100,000+ kg.
4. **Extension, training, and stewardship:**
   - *Farmer training workshops:* conduct field-day demos covering: why colored cotton must be grown separately from white; spatial isolation ≥500 m (or temporal isolation — plant 2–3 weeks offset so flowering doesn't overlap); insect resistance management (≥20% non-Bt refuge).
   - *Dedicated ginning:* contract local gins for separate colored cotton processing days/stands (even small colored fiber admixture downgrades white bales).
   - *Textile mill partnerships:* establish off-take agreements guaranteeing premium price ≥30% above white cotton; organize growers into cooperatives for collective bargaining and brand marketing.
5. **Post-release monitoring (annual):** Survey 20+ farms per region for: Bt resistance (discriminating-dose bioassays on collected larvae); gene flow (PCR wild *Gossypium* populations near fields); color stability (L*a*b* measurements on farmer-produced fiber across years); farmer adoption and satisfaction surveys. Report annually to regulatory authority.

Timeline: 2–4 years from breeder seed to full commercial availability.

7. Breeding Techniques for Colored Cotton

Genetic engineering (Sections 5–6) introduces novel color traits, but breeding is essential to combine those traits with high yield, fiber quality, and agronomic adaptation. This section describes the four principal breeding strategies applicable to colored cotton development, with sufficient detail for standalone implementation.

7.1. Conventional Hybridization and Pedigree Selection

Principle. The simplest and oldest approach: cross a naturally colored cotton donor line (typically low-yielding, with short/weak fiber) with a high-yielding elite white cultivar. The resulting F_1 hybrid

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is self-pollinated, and segregating F_2 progeny are evaluated over 6–8 generations of single-plant selection (pedigree method).

Procedure.

1. **Year 1:** Cross colored donor (P_1) × elite white (P_2). Harvest F_1 seed.
2. **Year 2:** Grow F_1 plants. All will be white (white is dominant over brown/green in most crosses). Self-pollinate.
3. **Year 3:** Grow F_2 population ($n \geq 2000$ plants). Select colored individuals with visually acceptable fiber, good plant vigor, and boll load. Expected colored frequency: ∼25% for single-gene colors (brown, green).
4. **Years 4–7 (F_3–F_6):** Grow progeny rows from selected F_2 plants. Evaluate lint yield (kg/ha), fiber quality (HVI: UHML, tenacity, micronaire), and color uniformity. Discard rows with poor yield (<70% of elite check), short fiber (<25 mm), or uneven color. Select the best 5–10% of rows each generation.
5. **Year 8 (F_7–F_8):** Lines are essentially homozygous. Conduct replicated yield trials (RCBD, 3 reps, 3+ locations) against check cultivars. Identify candidate varieties for release.

Strengths: No GMO regulatory requirements; inexpensive; proven methodology.

Limitations: Slow (6–10 years); limited to colors already in germplasm (brown, green); difficult to simultaneously improve color intensity and fiber quality due to negative genetic correlations between these traits [18,26].

7.2. Marker-Assisted Selection (MAS)

Principle. DNA markers tightly linked to fiber color loci allow breeders to identify colored genotypes at the seedling stage (2–3 weeks old) without waiting for bolls to mature (5–6 months). This eliminates one growing season per selection cycle.

Key mapped loci.

- **Brown fiber:** The Lc_1 locus on chromosome A07 is the primary determinant. Additional QTLs on chromosomes D11 and A09 modulate intensity. SSR markers BNL3590 and NAU3377 are within 2 cM of Lc_1 [19].
- **Green fiber:** The Gc_1 locus and flanking SSR markers have been identified. Green is inherited as an incomplete dominant trait.
- **Color intensity QTLs:** Multiple minor QTLs control shade depth. These are best captured using the CottonSNP63K or CottonSNP80K arrays for simultaneous multi-locus selection.

Procedure.

1. Extract DNA from cotyledons of F_2 seedlings (CTAB mini-prep, 96-well format).
2. Genotype with Lc_1/Gc_1-linked markers (SSR-PCR or KASP assays).
3. Retain only seedlings carrying homozygous favorable alleles (∼25% of population).
4. Transplant selected seedlings to field; evaluate agronomic traits as in conventional breeding.
5. Combine with foreground selection (color markers) and background selection (genome-wide SNPs for recovery of elite parent genome) in backcross programs.

Impact: MAS reduces the breeding cycle by 2–3 years and increases selection accuracy from ∼70% (visual phenotyping) to >95% (marker genotyping) [19].

7.3. Mutation Breeding

Principle. Chemical or physical mutagenesis of cotton seeds activates dormant pigment pathway genes or creates novel allelic variants. This approach can produce color shades not found in existing germplasm.

Mutagenesis protocols.

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- **Chemical (EMS):** Soak 500 seeds in 0.2–0.5% ethyl methanesulfonate (EMS) in phosphate buffer (pH 7.0) for 12–16 h at 25°C with gentle agitation. Rinse 5× with distilled water. Plant as M_1 generation. Expected mutation frequency: 1 per 100–300 kb.
- **Physical (gamma radiation):** Irradiate dry seeds at 200–350 Gy (^{60}Co source, dose rate ∼10 Gy/min). The LD_{50} for cotton is approximately 300 Gy.

Screening. M_1 plants are chimeric; harvest M_2 seed from individual M_1 plants. Screen ≥5000 M_2 plants visually for fiber color variants. Confirm heritability in M_3. Chinese researchers have produced intensified brown and novel greenish-yellow fibers using this approach [20].

Regulatory advantage: Mutant varieties are exempt from GMO regulations in most jurisdictions (including EU Directive 2001/18/EC, which explicitly excludes mutagenesis).

7.4. Genomic Selection and Speed Breeding

Genomic selection (GS). Whole-genome resequencing or dense SNP genotyping (≥10,000 markers) of a training population (500+ diverse cotton accessions phenotyped for color, fiber quality, and yield) enables construction of genomic prediction models (GBLUP, BayesB, or machine learning). These models predict the genetic merit of unphenotyped seedlings from their genotype alone, with prediction accuracies of 0.5–0.7 for fiber quality traits in cotton [21].

Speed breeding. Controlled-environment growth chambers with extended photoperiod (18–22 h), optimal temperature (28/22°C day/night), supplemental LED lighting (400–500 μmol m^{-2} s^{-1}), and early seed harvest (remove bolls at 35 DPA, force-dry, and germinate immediately) achieve 4–6 generations per year in cotton, compared to 1–1.5 under field conditions.

Combined GS + speed breeding workflow.

1. Genotype F_2 seedlings at 14 days with the CottonSNP63K array.
2. Apply genomic prediction model to rank seedlings by predicted color + quality + yield merit.
3. Advance the top 10% by single-seed descent under speed breeding conditions.
4. Re-genotype and re-predict at F_4; select top 5% for field evaluation.
5. Conduct replicated yield trials in F_5–F_6 under normal field conditions.

Timeline compression: From 10+ years (conventional) to 3–4 years (GS + speed breeding) for colors already in germplasm; 5–6 years for introgression of transgenic color into elite backgrounds.

8. Strategies for Combining Bt and Color Traits

The ultimate commercial product is cotton that simultaneously resists insect pests (Bt) and produces naturally colored fiber. This section describes four strategies for achieving this trait combination, with detailed workflows and decision criteria for selecting the optimal approach.

8.1. Strategy A: Multi-Gene Construct (Gene Stacking)

Principle. All genes needed for both pest resistance (e.g., *cry1Ac* + *vip3Aa*) and fiber color (e.g., F3′5′H + DFR + ANS + pH modifier for blue; or TYR + PPO + MBW + LAC for black) are assembled into a single multigene binary vector and co-transformed into cotton as one event.

Advantages.

- All traits segregate as a single Mendelian locus — no need for complex multi-locus breeding.
- Guarantees co-inheritance: every plant carrying the transgene has both Bt and color.
- Single regulatory event to deregulate (one insertion site, one molecular characterization).

Technical considerations.

- Construct size: a full Bt + black cotton stack requires ∼25–35 kb of T-DNA. Large T-DNA constructs (>20 kb) have reduced *Agrobacterium* transfer efficiency (typically 30–50% lower than 5–10 kb constructs).
- **Gene silencing risk:** Multiple transgenes driven by identical or homologous promoters can trigger transcriptional gene silencing (TGS) via repeat-induced methylation. **Mitigation:** use

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diverse promoters (e.g., *E6*, *GhExp1*, *GhLTP3*, *35S*) and terminators (*nos*, *ocs*, *35S*ter, *HSP*ter) to minimize sequence homology between TUs [22].
- **Position effects:** Transgene expression varies with chromosomal insertion site. Screening ≥20 independent events and selecting the best performer is essential.
- **Recommended for:** Colors requiring ≤3 genes (pink, enhanced brown) where total construct size remains manageable (<20 kb including Bt genes).

8.2. Strategy B: Marker-Assisted Backcross Introgression

Principle. Two established lines — a deregulated Bt-cotton event and a homozygous colored cotton line (transgenic or naturally colored) — are crossed conventionally. Molecular markers track both transgene loci through backcross generations to combine both traits in a single genotype while recovering the agronomic performance of the elite recurrent parent.

Detailed workflow.

1. **Cross:** Bt-cotton (recurrent parent, RP) × colored line (donor parent, DP). The RP is chosen for its yield, fiber quality, and disease resistance; the DP carries the color trait.
2. **F_1:** Confirm hybridity by heterozygosity at both Bt and color marker loci. Backcross to RP.
3. **BC_1:** Screen ≥200 BC_1 plants by PCR for the Bt transgene and by color-linked markers (foreground selection). Simultaneously, genotype with ≥200 background SNP markers (CottonSNP63K) to select individuals with maximum RP genome recovery. Target: ≥75% RP genome at BC_1.
4. **BC_2–BC_4:** Repeat foreground + background selection. Expected RP genome recovery: ∼87.5% (BC_2), ∼93.75% (BC_3), ∼96.9% (BC_4). With marker-assisted background selection, 96–99% recovery can be achieved by BC_3 [23].
5. **BC_3F_2 or BC_4F_2:** Self-pollinate the best BC line. Screen F_2 for plants homozygous at both Bt and color loci (expected frequency: 1/16 for two independent loci). Confirm by zygosity qPCR.
6. **Evaluation:** Conduct replicated yield trials and HVI fiber analysis of the homozygous stacked line versus the RP and DP checks.

Timeline: 4–6 years (conventional); 2–3 years with speed breeding (Section 7).

Advantages: Uses already-deregulated Bt events; regulatory burden is lower (may qualify as a conventional breeding product if the color trait is non-transgenic). Ideal for combining Bt with naturally colored (brown/green) varieties.

Limitations: Two independent loci segregate; ∼6% of progeny will lose one or both traits in each generation unless both loci are tightly linked or on the same chromosome. Linkage drag from the DP genome may introduce unfavorable alleles for fiber quality [23].

8.3. Strategy C: Retransformation of Bt Lines

Principle. An already-deregulated commercial Bt-cotton event (e.g., Bollgard II, MON15985) serves as the recipient genotype, and the color gene cassette is introduced by a second round of *Agrobacterium*-mediated transformation.

Procedure.

1. Obtain seed of the Bt-cotton event (requires material transfer agreement from the technology provider).
2. Transform hypocotyl explants with the color gene cassette using a **different selectable marker** (e.g., *bar* for phosphinothricin resistance, if the Bt event already uses *nptII*).
3. Screen regenerated plants for the presence of both the original Bt transgene (PCR) and the new color transgene (PCR + Southern blot).
4. Confirm that the two insertion events are on **different chromosomes** (FISH or genome walking) to ensure independent segregation, or on the **same chromosome** (linked) for guaranteed co-inheritance.
5. Advance to homozygosity and evaluate fiber quality and color.

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Advantages: Builds on the existing agronomic, fiber quality, and safety profile of the Bt event. The Bt trait's environmental and food safety data can be leveraged.

Limitations: Requires a new regulatory assessment for the stacked event (the combination is a new "transformation event" even though the Bt component is already approved). The Bt event's intellectual property holder must consent to retransformation. Total regulatory cost: USD 10–20M additional [24].

8.4. Strategy D: CRISPR-Based Pathway Activation (CRISPRa)

Principle. Instead of introducing foreign color genes, use a catalytically inactive Cas9 (dCas9) fused to a transcriptional activation domain (VP64, p65, Rta, or SunTag) to upregulate cotton's own endogenous flavonoid pathway genes. The guide RNAs (sgRNAs) are designed to target the promoter regions of native *GhDFR*, *GhANS*, *GhCHS*, and *GhTT2* genes, boosting their expression specifically in fiber cells when combined with a fiber-specific promoter driving the dCas9-VP64 cassette.

Key advantages.

- **No foreign coding DNA:** Only regulatory elements (dCas9, sgRNAs) are introduced; the pigment enzymes are the plant's own. In jurisdictions where gene-edited crops without foreign protein-coding sequences are exempt from GMO regulation (USA USDA-APHIS "Am I Regulated?" process; Brazil CTNBio Resolution 16/2018; Japan MAFF notification system; Argentina Resolution 173/2015; Australia OGTR SDN-1 exemption), this approach **bypasses the entire transgenic regulatory pipeline**, saving 5–8 years and USD 15–30M per event [25].
- **Stackable with Bt:** CRISPRa can be applied directly to existing Bt-cotton varieties. If the Bt event is already deregulated and the CRISPRa modification is non-transgenic under local law, the combined product may not require new biosafety assessment.
- **Multiplexable:** Multiple sgRNAs can simultaneously activate several pathway genes from a single polycistronic tRNA-sgRNA cassette.

Limitations.

- Cannot produce colors requiring genes absent from the cotton genome (notably F3′5′H for blue, TYR for black). For blue and black, a hybrid approach is needed: minimal transgenic insertion of the missing gene(s) + CRISPRa activation of endogenous supporting genes.
- Activation levels achievable by dCas9-VP64 ($\sim$5–50$\times$ baseline) may be insufficient for intense color. Newer activator systems (dCas9-SunTag-VP64, dCas9-VPR) achieve 100–1000$\times$ activation but are larger constructs.
- The regulatory status of CRISPRa (where a dCas9 protein IS expressed but does not cut DNA) remains ambiguous in some jurisdictions (EU, India, China). Pre-submission regulatory consultation is essential.

8.5. Decision Framework: Selecting the Optimal Strategy

The choice among strategies A–D depends on the target color, regulatory environment, intellectual property constraints, and available resources. Table 7 summarizes the recommended strategy for each scenario.

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Table 7. Decision framework for selecting the optimal Bt-color stacking strategy.

Color Target	Recommended Strategy	Est. Timeline	Rationale
Brown (natural)	B: Backcross introgression	3–5 years	No transgene needed for color; cross with Bt line
Green (natural)	B: Backcross introgression	3–5 years	Same as brown; Gc_1 locus is non-transgenic
Brown/green (intensified)	D: CRISPRa	2–4 years	Activate endogenous TT2/ANR; may avoid GMO regulation
Pink	D: CRISPRa + A (minimal)	4–7 years	CRISPRa for DFR/ANS; may need small transgene for pH
Blue	C: Retransformation + D	6–10 years	F3′5′H transgene required; CRISPRa for supporting genes
Black	**A: Gene stacking or C + D**	**8–12 years**	TYR and LAC transgenes essential; CRISPRa for MBW/PPO

9. Comprehensive Fiber Comparison

Table 8. Fiber comparison across all six cotton types. Asterisks (*) denote target values for engineered varieties. TBD = to be determined.

Parameter	White	Brown	Green	Pink*	Blue*	Black*
Pigment source	None	Tannins	Suberin–caffeic	Cyanidin	Delphinidin	Melanin + PAs
Dyeing needed?	Yes	No	No	No	No	No
UHML (mm)	28–34	22–28	20–26	25–30*	25–30*	24–28*
Tenacity (g/tex)	28–32	20–25	18–24	>25*	>25*	>22*
Micronaire	3.5–4.9	3.5–5.2	3.8–5.5	3.5–4.9*	3.5–4.9*	3.5–5.0*
Yield (kg/ha)	1500–2500	800–1200	600–1000	TBD	TBD	TBD
Wash fastness	Varies	Good	Good	Testing	Testing	Testing
H_2O saved	—	100–150 L/kg	100–150 L/kg	100–150*	100–150*	100–150*
Availability	Global	Niche	Niche	R&D	R&D	Proposed

10. Challenges and Limitations

Fiber quality gap. Colored varieties have shorter, weaker fibers than elite white cultivars, limiting use in fine-count yarns (>40s Ne). Closing this gap requires sustained breeding investment [26].

Color uniformity. Natural pigment levels vary with environment (temperature, light, nutrition) and between bolls. Batch-to-batch consistency remains challenging [27].

Regulatory burden. Transgenic events require full biosafety assessment per country (5–10 years, USD 15–30M per event). CRISPR-based approaches may offer faster paths where gene-editing regulations are favorable [28].

Contamination of white cotton. Cotton outcrosses at 5–25% via insects. Even small colored fiber admixture severely downgrades white cotton bales. Isolation distances of 500–1000 m are required [29].

Supply chain inertia. The global cotton supply chain is optimized for white fiber. Colored cotton requires dedicated processing lines, keeping prices 30–60% higher [30].

Black cotton specific challenges. The melanin pathway involves reactive quinone intermediates that could potentially affect fiber cell viability. Careful promoter timing (late secondary wall phase, >25 DPA, after fiber elongation is complete) is critical to avoid premature cell death. Eumelanin is highly stable but may alter fiber surface chemistry, potentially affecting spinning performance.

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11. Global Research Landscape

FoxFibre, USA: Sally Fox's brown and green varieties (Coyote, Buffalo, Palo Verde) proved commercial viability in the 1990s. Adopted by Levi Strauss and Esprit for limited-edition lines [31].

China's national program: CAAS and Xinjiang Agricultural University have released 20+ cultivars. Xinjiang produced ∼15,000 tonnes of colored fiber in 2023 for Japanese and European organic markets [32].

CSIRO, Australia: Pioneering transgenic blue cotton using *Clitoria ternatea* F3′5′H under the E6 promoter. Greenhouse trials produced pale blue-lavender fibers; color intensification remains the challenge [33].

CICR, India: Released CICR-HB-I (brown) for tropical conditions. Crossing colored lines with Bollgard II hybrids. QTL mapping on chromosomes A07 and D11 [34].

EMBRAPA, Brazil: BRS Safira (brown) and BRS Verde (green) for semiarid northeast. Smallholder cooperatives achieve premium pricing in European fair-trade markets [35].

12. Future Directions

- **CRISPRa for non-GMO colored cotton:** Transcriptional activation of endogenous pigment genes may qualify for non-GMO treatment, dramatically reducing regulatory timelines [25].
- **Synthetic biology for novel colors:** Directed evolution of flavonoid enzymes could produce orange, violet, or true black pigments beyond natural chemistry [36].
- **Policy drivers:** EU Sustainable Textiles Strategy (2022) and emerging regulations limiting hazardous dye chemicals will push industry toward naturally colored fibers [37].
- **Speed breeding + genomic selection:** 4–6 generations/year with whole-genome prediction compresses breeding timelines from 10+ to 3–4 years [21].
- **Black cotton for premium markets:** Black is the single most demanded textile color after white. Naturally black cotton would displace sulfur and reactive black dyes, among the most toxic in the industry. The premium pricing potential (estimated 40–80% above white) provides strong economic incentive.
- **Bt-color-quality pyramids:** Genomic selection for simultaneous improvement of pest resistance, fiber color, and fiber quality in a single breeding program.

13. Accelerating the Timeline: Strategies for Rapid Achievement of Colored Cotton

The conventional timeline from laboratory gene construct to commercial colored cotton cultivar spans 8–15 years (Figure 4). This section presents concrete strategies to compress each phase, with color-specific acceleration paths summarized in Table 9.

13.1. Speed Breeding and Rapid Generation Advancement

Conventional breeding advances one generation per year under field conditions. Speed breeding in controlled-environment chambers (18–22 h photoperiod, 22/17°C day/night, LED supplementation at 400–500 μmol m^{-2} s^{-1}) can produce 4–6 generations per year in cotton [21]. Applied to colored cotton:

- T_0 to homozygous T_3 line selection is compressed from 3 years to 8–10 months.
- Bt × color backcross introgression (BC_1–BC_4) is compressed from 4–5 years to 12–16 months.
- Pedigree selection in conventional colored cotton breeding is compressed from 8–10 years to 2–3 years.

13.2. Genomic Selection for Multi-Trait Prediction

Whole-genome prediction models trained on genotyped diversity panels (≥500 accessions, CottonSNP63K array) can predict fiber color intensity, staple length, tenacity, and yield simultaneously from seedling genotype alone, eliminating the need to wait for boll maturity to phenotype. When combined with speed breeding, genomic selection reduces the complete breeding cycle from 10+ years

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to **3–4 years** for colors already present in germplasm (brown, green) and to **5–6 years** for backcross introgression of transgenic color into elite backgrounds [21].

13.3. CRISPR-Based Pathway Activation: Bypassing the Transgene Bottleneck

The single greatest time bottleneck for engineered colors (pink, blue, black) is regulatory approval of transgenic events (5–10 years, USD 15–30M per event). CRISPR-based transcriptional activation (CRISPRa) using dCas9-VP64 or dCas9-SunTag systems can upregulate endogenous flavonoid pathway genes without inserting foreign coding sequences. In jurisdictions where gene-edited crops without foreign DNA are exempt from GMO regulation (USA, Brazil, Japan, Australia, Argentina), this approach bypasses the transgenic regulatory pipeline entirely, potentially saving **5–8 years and USD 15–30M** per event [25].

Specific CRISPRa targets for each color:

- **Brown (intensification):** Activate endogenous *GhTT2* and *GhANR* promoters to boost proanthocyanidin flux. Could produce deeper, more uniform brown within 2–3 years using speed breeding.
- **Pink:** Activate endogenous *GhDFR* and *GhANS* while suppressing *GhLAR*/*GhANR* (redirect flux from tannins to anthocyanins) using CRISPRi (dCas9-KRAB). Timeline: 3–5 years.
- **Blue:** CRISPRa alone is insufficient because F3′5′H is absent from the cotton genome. However, a minimal transgenic approach (single-gene insertion of F3′5′H + CRISPRa of endogenous DFR/ANS) reduces the regulatory burden compared to a full multigene construct. Timeline: 5–8 years.
- **Black:** Requires heterologous TYR and LAC genes (not present in cotton). CRISPRa can activate endogenous MBW complex and PPO genes, but a hybrid approach (transgenic TYR + LAC, CRISPRa for PA pathway) is needed. Timeline: 7–10 years.

13.4. Parallel Processing of Regulatory and Breeding Pipelines

Conventionally, regulatory dossier preparation begins after field trials complete. A **parallel processing** strategy initiates regulatory engagement early:

- Begin molecular characterization (flanking sequence, copy number, expression profiling) at T_2 stage, concurrent with greenhouse advancement — saving 1–2 years.
- Initiate pre-submission consultations with regulatory authorities (GEAC, EPA, EFSA) during confined field trial Year 1 to clarify data requirements — avoiding costly protocol redesigns.
- Conduct food/feed safety studies (compositional analysis, acute toxicity, subchronic feeding) in parallel with multi-environment field trials — saving 2–3 years.
- For stacked Bt + color events, leverage existing Bt event safety data (already deregulated) to reduce the novel data requirements for the stacked event.

13.5. Doubled Haploid Technology

Doubled haploid (DH) production via anther culture or gynogenesis achieves homozygosity in a single generation rather than 6–8 generations of selfing. While DH protocols for cotton are less efficient than for cereals, recent advances using colchicine-treated anther culture in *G. hirsutum* have achieved 2–5% embryogenesis rates. Applied to colored cotton, DH can compress $T_0 \rightarrow$ homozygous line from 2–3 years to 6–8 months.

13.6. Synthetic Biology: Cell-Free Prototyping

Before committing to plant transformation (8–14 months), candidate gene combinations can be prototyped in *in vitro* cell-free transcription-translation systems or transiently expressed in *Nicotiana benthamiana* leaves (agro-infiltration, results in 4–5 days). This allows rapid screening of 10–50 gene/promoter combinations for pigment production before selecting the optimal construct for stable cotton transformation, avoiding 1–2 years of wasted effort on suboptimal designs.

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doi:10.20944/preprints202603.0635.v2

13.7. Consolidated Acceleration Timeline

Table 9. Accelerated timelines for each color target, comparing conventional vs. optimized approaches.

Color	Conventional Timeline	Accelerated Timeline	Key Acceleration Strategies
Brown (improved)	6–8 years	**2–3 years**	Genomic selection + speed breeding (4–6 gen/yr); CRISPRa intensification of endogenous TT2/ANR
Green (improved)	6–8 years	**2–3 years**	Genomic selection + speed breeding; MAS for Gc_1 locus
Pink	10–12 years	**4–6 years**	CRISPRa/CRISPRi (non-GMO regulatory path); speed breeding; cell-free prototyping
Blue	12–15 years	**6–9 years**	Minimal transgenic (F3′5′H only) + CRISPRa of DFR/ANS; parallel regulatory processing; speed breeding
Black	**15–18 years**	**8–12 years**	Hybrid approach (transgenic TYR/LAC + CRISPRa of MBW/PPO); cell-free prototyping of 50+ combinations; parallel processing; DH technology

13.8. Integrated Fast-Track Workflow

Combining all acceleration strategies yields a generalizable **fast-track workflow**: (1) cell-free or *N. benthamiana* prototyping of gene combinations (2–3 months); (2) stable transformation of the optimal construct into Coker 312 (8–12 months); (3) speed breeding to homozygous T_3 (8–10 months); (4) Bt stacking by speed-bred backcross (12–16 months); (5) confined field trials with parallel regulatory dossier preparation (2 years); (6) multi-environment trials with concurrent food/feed safety studies (2 years); (7) regulatory review leveraging existing Bt safety data (1–3 years); (8) seed multiplication and release (1–2 years). Total elapsed time for the most complex target (black): approximately **8–12 years**, compared to 15–18 years conventionally — a compression of 40–50%.

14. Conclusion

Naturally colored cotton represents a powerful shift in how textiles could be produced in the future. Instead of relying on large-scale chemical dyeing — one of the major sources of water pollution in the textile industry — cotton plants themselves could grow fibers that already carry color. By removing much of the dyeing process, this approach could significantly reduce toxic wastewater, lower water consumption, and prevent harmful chemicals from entering rivers and soils.

The environmental benefits extend beyond water and soil. When fewer dye chemicals are released into the environment, ecosystems surrounding cotton farms become healthier. Pollinators such as **bees and butterflies**, which are highly sensitive to chemical pollution, would benefit from cleaner water, safer plant surfaces, and more stable habitats. Healthier pollinator populations are essential not only for cotton ecosystems but also for the broader agricultural landscape, as many crops depend on them for pollination.

In this sense, naturally colored cotton is more than a textile innovation. It represents a step toward a more sustainable relationship between agriculture, industry, and nature — where the colors of our clothing grow directly from the plant while supporting cleaner environments and healthier ecosystems for pollinators and other living species.

In this work, we contributed four key elements to advance the field of colored cotton research. First, we proposed the design concept of **black cotton**, presenting a systematic biological strategy to achieve naturally dark fibers that could eliminate the need for some of the most polluting textile dyes. Second, we introduced a **unified six-color framework** that integrates the genetic and biochemical pathways for white, brown, green, pink, blue, and black cotton within a single design map. Third, we provided **practical laboratory guidelines** that enable researchers to experimentally implement these concepts in plant molecular biology laboratories. Finally, we outlined a **complete pathway**

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from laboratory research to agricultural deployment, describing the steps required for greenhouse testing, field trials, regulatory approval, seed multiplication, and farmer adoption. Together, these contributions provide a practical roadmap for transforming naturally colored cotton from a scientific idea into a sustainable agricultural technology.

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