REVIEW ARTICLE

Biotechnological solutions for major cotton (*Gossypium hirsutum*) pathogens and pests

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Abstract   Cotton (*Gossypium* spp. L.) is the largest source of natural fibers in the world, with a planted area of more than 33 million hectares in 2019. Biotic stress caused by a variety of pathogens and pests has considerable negative impacts on cotton, and control measures increase global production costs. Among the most important diseases affecting cotton are bacteria and fungi that infect leaves, stems, roots and fruits. In addition, viruses, nematodes, insects and mites cause considerable losses. Here, we summarize the diversity of biotic stresses affecting the cotton crop and highlight present and future biotechnological solutions for disease control, including transgenes, RNAi, gene editing and bioagents. We demonstrate that ''Ag Biotech'' solutions help keep the cotton industry sustainable in cotton-producing countries.

Major cotton pathogens and pests

Cotton (*Gossypium* spp. L., Malvaceae) is the largest source of natural fibers in the world. In addition, cotton is one of the most important speculative annual crops, generating substantial economic returns. The cotton industrial chain involves approximately 150 countries and provides income for approximately 100 million families. The worldwide cotton planting area in 2019 occupies more than 33 million hectares, and the most important cotton-growing regions are in Central and East Asia, the southern United States (USA), the Brazilian Savanna and West Africa. The USA, along with Brazil, India, Uzbekistan and Australia, are the most important cotton fiber exporters. India is the greatest cotton producer, followed by China, the USA and Pakistan (USDA, 2019a).

As is the case for many major crops, biotic stress in cotton caused by pests and diseases contributes to annual losses worldwide of between 10 and 30%. Among the most important diseases affecting cotton are those caused by bacteria, such as bacterial blight, caused by *Xanthomonas citri* pv. *malvacearum* (Jalloul, Sayegh, Champion, & Nicole, 2015). Fungal pathogens are also of considerable importance, causing major diseases such as Fusarium wilt (caused by *Fusarium oxysporum* f. sp. *vasinfectum*) (Cox, Babilonia, Wheeler, He, & Shan, 2019), Verticillium

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wilt (*Verticillium dahliae*) (Shaban et al., 2018), anthracnose (*Colletotrichum gossypii*), ramulosis (*Colletotrichum gossypii* var. *cephalosporioides*) (Salustiano et al., 2014), ramularia gray mildew (*Mycosphaerella areola*) (Shete, Kasal, & Perane, 2018), root rots (*Sclerotium rolfsii* and *Rhizoctonia solani*) (Mehta et al., 2015), leaf blight (*Alternaria macrospora*) (Cia et al., 2016), leaf spot (*Cercospora gossypina*) (Rothrock, Woodward, & Kemerait, 2015) and target spot (*Corynespora cassiicola*) (Galbieri et al., 2014). The most commonly encountered viruses are those from the genus *Begomovirus*, which cause cotton leaf curl disease, cotton leaf crumple disease and mosaic disease (Naqvi et al., 2019), as well as those from *Polerovirus*, which cause cotton blue disease and atypical cotton blue disease (Correa et al., 2005; Silva et al., 2015). These viruses are generally associated with insect vectors (Naqvi et al., 2019).

In cotton systems worldwide, more than 1300 plant-feeding pests, including insects and mites, have been found. Less than 1% of these plant-feeding pests, however, cause critical economic losses. The most damaging pests include cotton bollworm (*Helicoverpa armigera*), pink bollworm (*Pectinophora gossypiella*), armyworm (*Spodoptera frugiperda*), leafworm (*Alabama argillacea*), boll weevil (*Anthonomus grandis*), aphids (*Aphis gossypii*), thrips (*Frankliniella* spp., *Thrips tabaci*, *Neohydatothrips variabilis* and *Scirtothrips dorsalis*), dusky cotton bug (*Oxycarenus hyalinipennis*), tarnished plant bug (*Lygus lineolaris*), cotton fleahopper (Pseudatomoscelis *seriatus*) and spider mites (Tetranychus urticae) (Allen, Luttrell, Sappington, Hesler, & Papiernik, 2018). During the seedling stage, thrips and cotton aphids are usually the most common insect pests. As cotton plants initiate flower bud formation, a complex of insects and spider mites become significant yield-limiting pests. In addition, losses in cotton can also occur due to high pressure from nematodes, such as root-knot nematodes (*Meloidogyne incognita*), reniform nematodes (*Rotylenchulus reniformis*), sting nematodes (*Belonolaimus longicaudatus*) (Weaver, 2015) and crazy cotton nematodes (*Aphelenchoides besseyi*).

At present, pest and disease control depends largely on conventional pesticides, the most widely used method for crop protection. Their extensive application, however, has led to serious ecological problems, including hazards to human and animal health, the development of resistance in target pests and pathogens, and environmental pollution. As such, there is an urgent need to develop creative and environmentally friendly strategies to effectively control these cotton pests and pathogenic organisms to achieve agricultural sustainability (Alavanja, 2009). In this context, ''Ag Biotech'' solutions help keep the cotton industry sustainable and reduce the application of chemical compounds in producing countries.

Cotton genome

The genus Gossypium includes approximately 45 diploid ($2n = 2\times = 26$) and five tetraploid ($2n = 4\times = 52$) species. The diploid species aggregate into eight genomic groups (A–G and K). Groups A, B, E, and F occur naturally in Africa and Asia; C, G, and K are found in Australia, while D is indigenous to the Americas (Wang et al., 2019). All

allotetraploid species originated in the Americas from interspecific hybridization and subsequent polyploidization events between the A- and D-subgenomes. These two subgenomes reunited geographically by transoceanic dispersal approximately 1–2 million years ago. The probable related ancestral donors of the A genome are Gossypium herbaceum (A_1) and Gossypium arboreum (A_2), and the D genome donor is Gossypium raimondii (D_5) (Hu et al., 2019). The A-genome species are cultivated, whereas the D-genome species do not produce spinnable fiber. The widely cultivated cotton cultivars are allotetraploid species. More than 90% of the annual cotton crop worldwide is *Gossypium hirsutum* (AD_1), Upland or American cotton, and less than 10% is *Gossypium barbadense* (AD_2), extra-long staple or Pima cotton (USDA, 2019b). On the one hand, polyploidization improves fiber and disease resistance traits; on the other hand, the complex genome of allotetraploid cotton presents a challenge for cotton gene functional analyses and genetic improvement through transgenic approaches (Gao et al., 2017; Wang et al., 2018).

During the last decade, knowledge about cotton genetics has experienced a revolution, with tremendous progress in sequencing and assembly of the whole cotton genome due to the large amounts of data obtained from transcriptomic, proteomic and metabolomic studies (reviewed by Yan et al., 2016; Ashraf et al., 2018; Zaidi, Mansoor, & Paterson, 2018). The whole genomes of two diploid and two tetraploid cotton species and several *Gossypium* mitochondria and chloroplasts are now available. All the knowledge accumulated about cotton genetics from the genomics era is accelerating the emergence of new genetic modified (GM) and non-GM cotton cultivars that can face the most important cotton pathogens and pests and serve as a bridge between modern cotton breeding and comparative and functional genomics. However, the large knowledge gaps related to the molecular regulation of basic biological pathways in cotton require time to be filled due to the difficulty of identifying their key regulator targets as well as the absence of a fast, simple genetic transformation system for cotton.

Genetically modified cotton

Transgenic technologies are an important component of plant breeding pipelines today. There are three major methods for cotton transformation: the pollen tube pathway-mediated method, the biolistic particle delivery system and Agrobacterium-mediated genetic transformation. Among them, Agrobacterium-mediated genetic transformation is the most widely used in cotton genetic transformation (Zhang, Meng, Abid, & Zhao, 2019). The usual steps for developing a GM cotton comprise product concept, gene discovery, evaluation, event selection, variety development, regulatory processes, field production, and commercial release. All steps are accompanied by a quality control program called Stewardship to ensure the genetic integrity of the GM traits (ETS, 2019). Since the first commercialization of genetically modified (GM) crops in the late 1990s, despite criticisms, GM technology has gained the trust of farmers and consumers worldwide (Akbar et al., 2019). Currently, there are an estimated 191.7 million hectares of transgenic crops cultivated world-

wide (ISAAA, 2018). Numerous benefits of GM crops have been documented, including reductions in the use of chemical insecticides, operational advantages that reduce labor needs, and increases in the biological control activity of natural enemies of crop pests, minimizing yield losses (Flachs, 2017). From 2017 to 2018, the global adoption of transgenic cotton increased by approximately 42%, and its adoption in the main producing countries was remarkable, such as Brazil (84%), India (93%), China (95%), the USA (96%) and Australia (100%) (ISAAA, 2018). Herbicide-tolerant (HT) GM cotton has been available since 1995, and its tolerance to potent herbicides such as glyphosate, glufosinate, and dicamba provides farmers with a broad variety of options for effective weed control. Insect resistance has been available since 1996, with GM cotton containing genes from the soil bacterium *Bacillus thuringiensis* (Bt) and producing insecticidal proteins. The adoption of stackedvarieties, which have both features or, in some cases, multiple HT and Bt traits, has accelerated in recent years (Chakravarthy, Reddy, Reddy, & Rao, 2014; Shehryar et al., 2019).

Over recent decades, scientists have made numerous attempts to produce insect-resistant cotton by inserting exogenous genes from different sources. Cry (crystal) proteins obtained from gram-positive spore-forming bacteria with entomopathogenic properties, such as *B. thuringiensis* Bt toxins, are currently the most important commercial traits for insect resistance. Cry toxins (δ-endotoxins) interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding, resulting in the formation of a prepore oligomeric structure that is insertion-competent. Cry proteins are specifically toxic to the insect orders Lepidoptera, Coleoptera, Hymenoptera and Diptera, as well as to nematodes (Bravo, Gill, & Soberón, 2007). Vegetative insecticidal proteins (Vips) from entomopathogenic bacteria, mainly *B. thuringiensis*, have also shown excellent insecticidal activity in *Gossypium* sp. (Saleem et al., 2010). The Vip1 and Vip2 proteins act as binary toxins and are toxic to some members of Coleoptera and Hemiptera. The Vip1 component is thought to bind to receptors in the membrane of the insect midgut. The Vip2 component enters the cell, where it displays its ADP-ribosyltransferase activity against actin, preventing microfilament formation. Vip3 is toxic to a wide variety of members of Lepidoptera and, to date, is the only commercially employed Vip protein in cotton. Its mode of action has been shown to resemble that of the Cry proteins in terms of proteolytic activation, binding to the midgut epithelial membrane, and pore formation, although Vip3A proteins do not share binding sites with Cry proteins (Saleem et al., 2010). Cotton with stacked Bt protein genes, such as Bollgard II® cotton that has both *cry1Ac* and *cry2Ab2* genes, Widestrike® (Cry1Ac + Cry1F proteins), Twinlink® (Cry1Ab + Cry2Ae proteins), Bollgard® 3 and TwinLink® Plus (*vip3A* gene), have a broader pest-control spectrum and a greater potential to prevent or delay the emergence of resistant insect populations compared to plants with single Bt genes (Singh, 2018).

The extensive use of *Bt* cotton has reduced the application of broad-spectrum insecticides worldwide and may be a major contributing factor in the recent triggering of outbreaks of secondary pest species (Gowda et al., 2016). In recent years, the *Lygus* complex has been the top-ranked pest complex in cotton in the USA in terms of economic losses, followed by thrips. The emerging resistance to key chemicals in *Lygus* and thrips are rendering insecticides less sustainable management options for these pests. A Bt protein (Cry51Aa2) provided a significant reduction in the number of *Lygus* nymphs and a subsequent yield increase, in addition to fewer thrips and minimal injury. As such, Cry51Aa2 may become a valuable additional tool for the management of *Lygus* and thrips in cotton in the coming years (Akbar et al., 2019). Another pest of economic importance in the Americas is the cotton boll weevil (*Anthonomus grandis*), which is capable of decimating 100% of a cotton field. In Brazil, where there is no official boll weevil eradication program, insecticide spraying has become economically inviable, with more than 30 sprays per season required for adequate control. Recently, Cry proteins such as Cry1Ba6 (Martins et al., 2010), Cry8Ka (Oliveira et al., 2011) and Cry1Ia12 (Grossi-de-sa et al., 2007) have been described as entomotoxic against this coleopteran pest. Until now, the most effective Cry toxin against boll weevils under experimental conditions has been from Cry10Aa. The expression of the *cry10Aa* gene under the control of the cotton ubiquitination-related promoter *uce*A1 in GM cotton plants resulted in a strong entomotoxic effect against boll weevils (Ribeiro et al., 2017).

Genes encoding various antifungal proteins, such as chitinases, have been expressed in cotton to confer resistance against various fungal pathogens, such as *Rhizoctonia solani*, *Alternaria alternata*, *Alternaria macrospora* and *Fusarium oxysporum* (Emani et al., 2003). Glucose oxidases conveyed resistance to the root pathogen *Verticillium dahliae* (Murray et al., 1999) and the harpin-encoding gene (*hpa1Xoo*) from *Xanthomonas oryzae* pv. *oryzae* has been used to enable resistance against various pathogens through a priming mechanism (Miao et al., 2010). Transgenic cotton plants expressing the antisense movement protein (*AV2*) and antisense coat protein (*ACP*) genes of CLCuV have also provided resistance to CLCuD (Amudha, Balasubramani, Malathi, Monga, & Kranthi, 2011).

The development of GM cotton is a long-term project that requires years of development and hundreds of millions of dollars of investment to ensure economic, social, ecological and toxicologic safety. Given that the development of transgenic cotton is costly and time-consuming, these traits will likely continue to be provided by only a few large companies.

RNAi

Plant-mediated RNAi technology for gene silencing is appropriate and efficient for both the control of diseases caused by pathogens and pests and the prevention of resistance development. RNAi-mediated resistance can be achieved by two approaches. The most ''canonical'' way is by the expression of a miRNA in a transgenic plant to silence a target pathogen or pest gene mRNA transcript. Another option is to use dsRNA molecules that can be sprayed over a plant to protect it from attack. This technology was successfully applied to achieve disease control in cotton through the induction of resistance against *V. dahliae* by the transgenic expression of miRNAs 159 and 166 (Zhang & Wang, 2015). This hemibiotrophic fungus causes vascular wilt disease and

is an important problem in several countries. Cotton vascular wilt is caused by the blockage of host xylem vessels by fungal conidia and microsclerotia (Gerik & Huisman, 1998). The cotton miRNAs upregulated during *V. dahliae* infection and their gene targets were identified by high-throughput sequencing (He et al., 2014; Zhang & Wang, 2015) During the fungal infection, miR159 and 166 are overexpressed and exported to the fungal hyphae, where they induce the silencing of two specific important fungal genes (Zhang, Wohlhueter, & Zhang, 2016, Zhang et al., 2016b). These results have prompted researchers to develop GM cotton wilt disease-resistant plants by overexpressing these two miRNAs. Interestingly, transgenic cotton plants that overexpress miR166 also show potential in reducing *Bemisia tabaci* populations and, more importantly, the spread of whitely-transmitted plant viruses (Wamiq & Khan, 2018).

Gene editing in cotton

The tools for genome editing have become more accessible and important for agricultural production over the last several years. All three primary targeted mutagenesis tools, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated (Cas) systems, can be designed to produce highly specific double-strand breaks in chromosomal DNA. These breaks are processed by nonhomologous end joining (NHEJ) and homologous recombination (HR) repair mechanisms, leading to localized mutations and intentional sequence replacements. Compared with ZFN and TALEN systems, the CRISPR/Cas system is easy to use and has high efficiency in generating targeted mutagenesis (Gao et al., 2017; Wang et al., 2019). The CRISPR/Cas system was developed from studies of the defense systems of archaea and bacteria that provide protection against invading plasmids and viruses. Class 2 CRISPR-Cas systems include types II, V, and VI, which are based on the Cas9, Cas 12, and Cas13 effectors, respectively. These programmable single-effector nucleases have enabled genome engineering and nucleic acid detection tools (Yan et al., 2019). Although CRISPR/Cpf1 (Cas12a) is the most effective system for cotton genome editing (Li et al., 2019), the CRISPR/Cas9 system has been tested the most. CRISPR/Cas9 comprises a CRISPR-associated Cas9 protein and an engineered single guide RNA (sgRNA) that specifies a target nucleic acid sequence. Cotton is an allotetraploid, and its genome is large and complicated, which makes it difficult to obtain materials that have a target gene mutation. The transformation of the CRISPR/Cas9 system into the cotton genome is an important step for research dealing with gene function and crop improvement (Chen et al., 2017; Li, Unver, & Zhang, 2017; Wang et al., 2018). One of the most efficient methods of delivering the CRISPR/Cas9 system into the cotton genome is using the Agrobacterium-mediated method with the shoot apexes as receptors (Chen et al., 2017). The CRISPR/Cas9 system has recently been used to confer molecular immunity against several eukaryotic viruses, including cotton DNA geminiviruses (Ali, Ali, Tashkandi, Zaidi, & Mahfouz, 2016), in addition to resistance to fungal diseases such as *Verticillium dahliae* (Zhang et al., 2018).

Bioagents

Another promising approach for the management of biotic stresses in crops is based on the employment of ecologically friendly biopesticides that are specific to target organisms (Gupta & Dikshit, 2010). Biopesticides are natural pesticides derived from animals, plants, microorganisms and certain minerals. The active components of microbial pesticides include fungi, bacteria, viruses, protozoa or algae. While these compounds can have a broad spectrum of activity, bioagents specific to target pathogens or pests are also common (Lord, 2001).

Entomopathogenic fungi are insect parasites that can kill or seriously disable insect pests. *Beauveria bassiana* is one of the most commonly employed entomopathogenic fungi in biocontrol and is registered with the US Environmental Protection Agency (EPA) as a biocontrol agent for a wide range of insect pests (Salma, Ratul, & Jogen, 2011). Recently, Saad, Ghareeb, and Saeed (2019) showed the potential use of the entomopathogenic fungi *Cheilomenes lunata*, *Alternaria solani* and *A. alternata* against *Spodoptera littoralis*, also referred to as the African cotton leafworm or Egyptian leafworm. *S. littoralis* is a highly polyphagous organism that attacks cotton, feeding on leaves, flower buds, fruiting points and bolls and causing severe damage. *S. littoralis*-associated cotton damage is most prevalent in North Africa, especially in Egypt. The use of these fungi led to mortality in 60%, 40% and 33.3% of *S. littoralis* larvae, respectively.

Endophytic microorganisms have also been studied as a way to manage biotic stresses in cotton crops. Some endophytes may accelerate the growth of their host plants and increase nutrient absorption (Hashem, Abd-Allah, Alqarawi, Wirth, & Egamberdieva, 2016). Other endophytes protect their hosts by producing secondary metabolites that have the potential to kill or suppress the attack of pathogenic or herbivorous microorganisms (Carroll, 1988; Schulz, Boyle, Draeger, Römmert, & Krohn, 2002). The biocontrol activity of the endophytic fungi *Penicillium simplicissimum*, *Leptosphaeria* sp., *Talaromyces flavus* and *Acremonium* sp. isolated from cotton roots was evaluated against cotton wilt disease caused by a *V. dahliae* leafless strain in a greenhouse (Yuan, Feng, Wang, Li, & Shi, 2017). All treatments significantly reduced the incidence of the disease, with protective effects of 69.5%, 69.2%, 54.6% and 45.7%, respectively. Treatment with these endophytes not only delayed but also reduced wilting symptoms in cotton. The treated cotton plants showed increased transcription levels for phenylalanine ammonia lyase (PAL) and peroxidase (POD) genes, which lead to increased cotton defense reactions (Yuan et al., 2017).

Numerous bioagents have been identified for pathogen control, reducing populations in the rhizosphere and plant systems through the production of antimicrobial metabolites. Indirect mechanisms can also be involved, including increases in the population of other beneficial soil microbiota and modulation of host physical-biological pathways that trigger defense cascades within the plant system. Singh et al. (2016) demonstrated the efficacy of *Trichoderma harzianum* UBSTH-501 and *Pseudomonas fluorescens* PF-08 as bioagents for the promotion of plant growth in rice (*Oryza*

sativa). The plants exposed to the bioagents showed differential signaling that resulted in the increased uptake and translocation of essential mineral nutrients. The product Trichodel®, based on *Trichoderma spp.*, reduced the incidence and severity of *Fusarium oxysporum* f. sp., the causal agent of Fusarium vascular wilt in cotton, in treated cotton seeds (de Farias et al., 2019).

Zhang et al. (2019) reported that *Paenibacillus polymyxa* ShX301, applied as a biocontrol agent in cotton plants against *Verticillium dahliae,* retarded foliar symptoms, significantly reduced disease incidence and severity, and strongly promoted the growth of cotton seedlings. In other studies on cotton, *P. polymyxa* Sb3-1 reduced the growth of *Verticillium longisporum* through the release of specific antimicrobial volatiles, such as 2-nonanone and 3-hydroxy-2-butane (Rybakova et al., 2016 and 2017).

Other promising potential bioagents include the growth-promoting rhizobacteria (PGPR) that are already associated with the suppression of various diseases (Sharma, Sindhu, & Sindhu, 2018). Different PGPR genera, such as *Bacillus, Streptomyces, Pseudomonas* and *Agrobacterium,* have been reported as effective biological control agents against numerous plant diseases (Jangir, Pathak, Sharma, & Sharma, 2018). These bacteria control disease through mechanisms such as the production of antibiotics and siderophores, which lead to the activation of systemic resistance (Mhlongo, Piater, Madala, Labuschagne, & Dubery, 2018). Adrees, Haider, Anjum, and Akram (2019) compared the effects of five rhizobacteria that significantly controlled charcoal root rot disease in cotton caused by *Macrophomina phaseolina*. This pathogen has been reported in cotton in the southeastern area of the USA and in Oklahoma and Texas (Watkins, 1981). The strongest biocontrol of this pathogen was observed using *Bacillus megaterium* ZMR-4 and *Bacillus subtilis* IAGS-174, with treated plants showing reductions in disease severity of 41.16 and 36.36%, respectively.

A recent study revealed that under field conditions, soil application and foliar spraying of *Bacillus amyloliquefaciens* resulted in a 52% reduction in the incidence of tobacco streak virus (TSV) in cotton (Vinodkumar, Nakkeeran, Renukadevi, & Mohankumar, 2018). In addition, *B. amyloliquefaciens* application improved cotton seed yield by up to 149.45 g/plant compared to the control. Thus, *B. amyloliquefaciens* can be explored as an efficient antagonist for the management of TSV in cotton crops.

Resistance pathways such as systemic acquired resistance (SAR) can be activated simultaneously in a plant after contact with elicitors of defense responses (Kruger, Szabo, & Zeyen, 2003). Inducer compounds belong to a wide range of different classes without any common chemical structure, including peptides, proteins, oligosaccharides, glycoproteins and lipids (Montesano, Brader, & Palva, 2003). Plant resistance induced by such protein inducers has attracted considerable attention and research efforts in recent years for the development of alternative, novel and ecologically friendly plant protection methods (Mishra, Sharma, & Misra, 2012; Wiesel et al., 2014). One example in cotton research is the use of PevD1, an important *V. dahliae* effector. Bu, Qiu, and Zeng (2014) demonstrated that *Escherichia coli* expressing the PevD1 protein can be used as an inducing bioagent and acts as an effector in the interaction with cotton. The induced resistance responses have included the positive reg-

ulation of genes related to secondary metabolites, cell wall modification and lignin accumulation.

The integration of biomolecules from microbial agents and biological control with optimized microbiomes represents a relatively unexplored area. However, recent research results indicate that it deserves significant research efforts. It represents a promising tool for improving yields and controlling diseases and pests in cotton as well as other crops, and it has the advantage of addressing food safety in an environmentally friendly and sustainable manner.

In conclusion, while cotton is susceptible to numerous pathogens and pests, cotton production today relies upon numerous biotechnological solutions for the prevention of losses. These biotechnology tools both minimize production costs and provide ecologically friendly control systems for the market and for global production. Worldwide trust in the cultivation of Ag Biotech cotton is growing, and it is now employed in production areas of the most important cotton-producing countries, including Brazil.

The continuous development of cotton genome sequencing and genome editing technologies associated with the improvement of transgenic technology and the use of marker-assisted selection will certainly bring new stress-resistant/tolerant cotton germplasms in the next few years. The use of these new tolerant germplasms in combination with the improvement of effective bioagents can revolutionize current phytopathogen sanitation practices, thereby reducing losses and benefiting the environment.

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