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Review

Biotechnological advances for combating *Aspergillus flavus* and aflatoxin contamination in crops

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ABSTRACT

Aflatoxins are toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive byproducts of *Aspergillus* spp. that contaminate a wide range of crops such as maize, peanut, and cotton. Aflatoxin not only affects crop production but renders the produce unfit for consumption and harmful to human and livestock health, with stringent threshold limits of acceptability. In many crops, breeding for resistance is not a reliable option because of the limited availability of genotypes with durable resistance to *Aspergillus*. Understanding the fungal/crop/environment interactions involved in aflatoxin contamination is therefore essential in designing measures for its prevention and control. For a sustainable solution to aflatoxin contamination, research must be focused on identifying and improving knowledge of host–plant resistance factors to aflatoxin accumulation. Current advances in genetic transformation, proteomics, RNAi technology, and marker-assisted selection offer great potential in minimizing pre-harvest aflatoxin contamination in cultivated crop species. Moreover, developing effective phenotyping strategies for transgenic as well as precision breeding of resistance genes into commercial varieties is critical. While appropriate storage practices can generally minimize post-harvest aflatoxin contamination in crops, the use of biotechnology to interrupt the probability of pre-harvest infection and contamination has the potential to provide sustainable solution.

Contents

Abbreviations: FDA, Food and Drug Administration; ppb, parts per billion; IVSC, in vitro seed colonization; ICRISAT, International Crops Research Institute for the Semi-Arid Tropics; RAPs, resistance-associated proteins; RIPs, ribosome inactivating proteins.

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1. Introduction

Mycotoxins are toxic metabolites produced by fungi, particularly by saprophytic molds growing on agricultural products. These cause not only economic losses but also pose health hazards to humans and animals. Some of these mycotoxins, including aflatoxins, are potentially carcinogenic and mutagenic with harmful effects to humans, livestock, and poultry. Aflatoxin was first identified in 1960 following a severe outbreak of a disease called "Turkey 'X' Disease" in the UK that killed over 100,000 turkey birds.

Aflatoxins are a group of structurally related bis-furanocoumarin compounds that are toxic, mutagenic, carcinogenic, teratogenic, and immunosuppressive agents produced as secondary metabolites by the fungi *Aspergillus flavus* and *A. parasiticus* on a variety of food products [1]. *A. flavus* commonly produces B_1 and B_2 aflatoxins, while *A. parasiticus* produces two additional aflatoxins, G_1 and G_2. These toxins are largely associated with food commodities produced in the humid tropics and subtropics, such as cereals (maize, sorghum, pearl millet, rice, and wheat), oilseeds (peanut, soybean, sunflower, and cotton), spices (chillies, black pepper, turmeric, coriander, and ginger), nuts (almond, Brazil nut, pistachio, walnut, and coconut), and milk [2]. Aflatoxins are not digestible by animals and thus end up in the meat. They are also heat and freeze stable and thereby, remain indefinitely in the food. AFB_1 is the most toxic and potent carcinogen because of its association with hepatocellular carcinoma (liver cancer) [3].

A. flavus is a common fungus with a typical yellow green appearance. Its population increases during hot dry weather, drought stress, and extreme temperatures in the geocarposphere (area surrounding peanut pod that influences microbial complement of the soil). Insects and nematode vector infection, which enters through the lesions, not only compromises the plants' self-defense to fungal invasion, but also increases aflatoxin formation in the seeds [4–6]. The infections impair both plant growth and yield. The fungus continues to grow and produce toxin in poorly dried grain.

1.1. Nutrition, health, and economic considerations

Aflatoxins are potent immune suppressors interfering with activities of important cells that boost immunity in humans and animals [2]. While chronic sub-lethal doses may have nutritional and immunologic consequences, large doses can lead to acute intoxication resulting to direct liver damage and death. Nevertheless, both types of exposure have a cumulative effect on the risk of developing liver cancer. The extent of carcinogenicity in individuals is largely dependent on the dose and duration of exposure, categorized into (1) acute illness as a result of consumption of foods contaminated with very high levels of aflatoxin, and (2) chronic illnesses/cancers (especially liver cancers) as a result of exposure to low levels of toxin. The resulting liver toxicity can produce a cumulative effect over time and lead to diseases like hepatic fibrosis, cirrhosis, and fatty liver disease. Moreover, these metabolites are capable of binding to protein, DNA and RNA, which interferes with the normal cellular functions resulting to initiation of carcinogenesis, mutagenesis or necrosis of the liver, causing impairment of fetal growth and development leading to miscarriages [7]. Aflatoxins reduce fetal growth due to their interference with protein synthesis and deficiency of micronutrients like vitamins A, B_{12}, C, D, and E; and minerals like zinc, selenium, iron and calcium. Aflatoxins are also known to play an important role in slowing the recovery rate from protein malnutrition. There have been reports on accelerated HIV progress due to aflatoxin-related immune suppression and decline in nutritional status in patients, exacerbating the effects of HIV/AIDS, malaria and several nutrition-related illnesses such as kwashiorkor, a nutritional disorder caused by protein deficiency in humans [7].

Aflatoxin contamination of crops result in direct economic effects, such as loss of produce or market value, healthcare and associated costs, and indirect economic effects such as loss of animals, costs for food-borne disease surveillance and monitoring, etc. While the U.S. Food and Drug Administration (FDA) has imposed stringent regulations on levels of aflatoxin at 20 ppb (parts per billion) in food and feed, and 0.5 ppb in milk, the European Union (EU) has set the limit at 2–4 ppb. These regulatory guidelines (enforced within the U.S. as well as internationally) have put a tremendous economic burden of over US$932 million on agriculture globally due to crop losses caused by mycotoxigenic fungi including *A. flavus* [8,9]. Depending on the market, economic losses may reach 100% owing to complete rejection of the produce when aflatoxin levels are higher than acceptable standards. Africa alone loses over US$670 million annually due to requirements to comply with the EU standards for all food exports [10].

1.2. Factors contributing to aflatoxin contamination in crops

Aflatoxin contamination of foods and feeds occur at both pre- and post-harvest, which is highly dependent on biological (biotic) and environmental (abiotic) factors that lead to mold growth and toxin production. Aflatoxin production at post-harvest stage takes place when the crop is harvested either during floods or any unseasonal rains, followed by improper storage conditions. In addition to this, mechanical or insect and bird damage, drought stress, and excessive rainfall encourage pre-harvest mold growth and aflatoxin production. The incidence and levels of fungal infection and aflatoxin contamination vary markedly, both seasonally and geographically [11], which are dependent on variations in the fungal strains [12,13], interference by other organisms, moisture, temperature, soil conditions, etc. The fungal spores penetrate either due to sudden and extreme changes in weather which cause damage to the pod wall/kernels (in peanut), by insect or birds (in cotton), and during pollination (in maize). Plant stresses such as nutrient deficiency and a continued dry weather also increase levels of aflatoxin. Interactions between intrinsic and extrinsic factors also influence

A. flavus growth and aflatoxin production in stored commodities [14].

2. Strategies for pre-harvest elimination of *A. flavus* and aflatoxin

Different measures of prevention, elimination, and decontamination or inactivation to minimize the risk of mycotoxin contamination in pre-harvest, harvest and post-harvest conditions have been comprehensively reviewed [15]. Preventing infection by *Aspergillus* species at pre-harvest stage is an important step in reducing aflatoxin contamination. Biocontrol technologies using competitive exclusion for aflatoxin prevention have been developed to enhance genetic resistance and chemical control measures for long-term suppression of aflatoxin contamination [15]. The primary requisite for designing strategies for the reduction or elimination of aflatoxins is the knowledge about their fungal sources and growth, a complete understanding of the complex interrelationships during host plant–*A. flavus* interaction and aflatoxin contamination processes. The three main strategies essentially aimed at interrupting the mechanisms responsible for pre-harvest aflatoxin contamination have been broadly categorized as: (1) causing cell wall degradation (cellulases, pectinases, amylases, and proteinases); (2) impairing fungal development (cell wall synthesis and conidiophore and conidial formation); and (3) preventing aflatoxin biosynthesis [16,17]. These research efforts involve modern biotechnological tools and resources, requiring affordable and rapid screening, detection and quantification techniques. Integrated management practices are needed to facilitate the development of aflatoxin-resistant varieties [18].

Crop resistance to *A. flavus* invasion and aflatoxin contamination is not only genotype-specific, but may also be attributed to several biochemical, environmental and physical factors, with drought playing an important role in host–pathogen interactions. Keeping this in mind, several strategies including the use of biocontrol agents, good agricultural practices, and plant breeding for resistance have been employed to prevent and/or reduce aflatoxin contamination at pre-harvest stage.

2.1. Using biocontrol agents

One of the potential options for *A. flavus* management is biocontrol in the field itself. Bacterial species such as *Bacillus subtilis*, *Lactobacillis* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp. inhibit fungal growth and consequent production of aflatoxins by *Aspergillus* in laboratory experiments [19]. Several strains of *B. subtilis* and *P. solanacearum* isolated from the non-rhizosphere of maize soil inhibit aflatoxin accumulation [20]. Unfortunately, these are less effective under field conditions [21]. Similarly, some saprophytic yeast species such as *Pichia anomala* and *Candida krusei* show potential as biocontrol agents against *A. flavus* under laboratory conditions [22]. Efforts have been made to develop other potent biocontrol agents including *Trichoderma* spp. (fungi), *Pseudomonas aeruginosa* (antagonistic bacteria), and *Streptomyces cavourensis* (actinomycetes) [23]. Field experimentation is necessary to test the efficacy of these agents for reducing aflatoxin contamination.

Most of the success in biological control of aflatoxin contamination in crops during both pre- and post-harvest has been achieved through application of competitive non-toxigenic strains of *A. flavus* and/or *A. parasiticus*. The effectiveness of biocontrol using atoxigenic *A. flavus* strains was based on the logic that these are predominantly asexual, genetically stable and aggressive as competitors coupled with their inability to recombine with native toxigenic strains [24,25]. Significant and consistent reductions in aflatoxin contamination (70–90%) have been observed in peanut and cotton field experiments using such non-toxigenic *Aspergillus* strains [26]. Two commercial products (afla-guard® and AF36®) based on atoxigenic *A. flavus* strains have been approved in the U.S. by the Environmental Protection Agency for biological prevention of aflatoxin contamination in peanut, maize, and cottonseed [27]. A few African atoxigenic strains of *A. flavus* have also been identified that competitively exclude toxigenic fungi in maize and peanut fields. These strains have been shown to reduce aflatoxin concentrations by 70–99% in both laboratory and field trials [28]. A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin (AflaSafe®) has gained provisional registration for determining its efficacy in on-farm tests in sub-Saharan Africa. In addition, field trials are currently being undertaken with locally selected candidate strains in Kenya and Senegal [29].

Nevertheless, available data suggest that biocontrol strains are capable of reducing aflatoxin-producing populations only by four- to five-folds [13]. Many short- and long-term challenges face this strategy because the population biology of *A. flavus* is still not well-understood owing to its diversity and ability to form heterokaryotic reproductive forms. Also, not all of the offspring that result from their out-crossing inherit the atoxigenic phenotype. *Aspergillus* spores survive for prolonged periods, thereby requiring repeated applications, which over many generations have the potential to increase the load of "super-competitors" with toxigenic populations [30]. Hence, there is an urgent need to select the biocontrol strains on basis of the environmental resilience, type of crop, and the soil into which they will be introduced. It is also critical to select the most flexible biocontrol strategy that can adapt to climate change and resultant changes in the soil nutrients and concomitant microbiome populations.

Several reports have indicated that the use of atoxigenic *A. flavus* could result in sexual recombination resulting in genetic and functional hyperdiversity in *A. flavus* [31]. There has been enough evidence of recombination within the aflatoxin gene clusters of *A. parasiticus* and *A. flavus* populations within the same field. This could result in the breakdown of effectiveness of using atoxigenic *A. flavus* for biocontrol due to the production of novel *A. flavus* phenotypes, resulting in greater diversity in the field [32].

The presence of high populations of *A. flavus* under a biocontrol strategy can result not only in increase in sexual reproduction, but also in re-assortment of genes which may further have consequences such as competitiveness between the strains and their aflatoxin producing capacity [33]. With the possibility of obtaining multiple vegetative compatibility groups (VCGs) in a single generation, sexual recombination may further increase the population of toxigenic strains, rendering the biocontrol strategy ineffective. A recent review critically described the current state and outlook of using atoxigenic isolates of *Aspergillus*. The report concluded that understanding genetic variations among *A. flavus* strains is critical for developing a robust biocontrol strategy, and it is unlikely that a "one size fits all" strategy will work for pre-harvest aflatoxin reduction [13].

2.2. Good agricultural practices

Management practices such as timely planting, maintaining optimal plant densities, avoiding drought stress, providing adequate plant nutrition and weed control, controlling plant pathogens and insect pests, and following proper harvesting practices should be employed to reduce the incidence of aflatoxin contamination in the field [23,34]. Crop rotation and management of crop residues also play an important role in controlling *A. flavus* infection in the field [23]. For maize and peanut, any action taken to interrupt the probability of kernel infection will reduce aflatoxin contamination substantially. Soil amendments like lime application, use of cereal crop residues, and farm yard manure have also been reported as

effective management practices in reducing *A. flavus* contamination as well as aflatoxin levels by 50–90% in peanut [35]. A calcium source such as lime has also been reported to thicken the peanut cell wall, accelerating pod filling, while manure facilitates growth of microorganisms that suppress soil infections [29,35].

2.3. Plant breeding

Two main requirements for developing cultivars resistant to pre-harvest *A. flavus* infection and aflatoxin contamination are the availability of genetic variability for resistance and accessibility to reliable and efficient screening techniques. Research methods have been developed for identifying indirect tools for selection for resistance to pre-harvest infection and contamination. These are aimed to facilitate breeding for the development of germplasm resistant to fungal growth and/or aflatoxin contamination and reducing the costs involved in screening of contaminated commodities [4,36].

The value of any resistant source depends upon the level and stability of its resistance. Breeding for resistance to *A. flavus* and *A. parasiticus* and/or aflatoxin production plays a significant role in preventing aflatoxin contamination. While genetics of resistance mechanisms for *A. flavus* and aflatoxin contamination have not yet been clearly elucidated, statistically significant genotype-environment interactions have been observed [37]. The allelic relationship among various sources for resistance traits that can help breeders to pyramid the non-allelic genes for each resistance mechanism is still unknown and may not exist. Similarly, no correlation has been observed between *A. flavus* resistance under laboratory conditions when compared to that under field conditions [38–40].

2.3.1. Peanut

The hyphae of *Aspergillus* penetrate the peanut pod walls and the seed coat reaching the nutrient-rich cotyledons. Desired resistance can be broadly classified to be at the level of pod infection (pod wall), seed invasion and colonization (seed coat), and resistance to aflatoxin production (cotyledons) [41]. The resistance to pod infection is attributed to pod-shell structure, which acts as a physical barrier. The resistance to seed invasion and colonization is due to the moisture content and heat stress that is correlated with density and thickness of palisade cell layers, fungistatic phenolic compounds, presence of wax layers, and absence of microscopic fissures and cavities [37]. Sources containing all the three types of resistance (pre-harvest seed infection, in vitro seed colonization or IVSC, and aflatoxin production by *A. flavus*) have been reported in cultivated peanut. Since the levels of *A. flavus* infection and aflatoxin contamination primarily relate to environmental conditions, especially to drought stress during pod maturation, seed infection cannot be directly correlated to aflatoxin production [6,38,39]. Each of these components is highly variable, independent and expressed at low levels, appearing to be governed by different genes with no significant relationships between them [42]. Until now, no direct correlation between fungal growth and aflatoxin production has been observed in studies with peanut genotypes. This suggests that the genotypes produce different amounts of aflatoxin per unit growth of the fungus depending on the fungal strain and environmental conditions.

The latest studies are finally having success in finding resistance to pre-harvest seed infection, IVSC, or aflatoxin production that is crucial for developing strategies to reduce aflatoxin contamination in peanut [37]. Several hundred breeding lines have been tested and identified for yield and IVSCAF-resistance. Researchers were successful in transferring IVSCAF-resistance to different genetic backgrounds and developed six breeding lines [43,44]. Over 472 peanut lines were evaluated for pre-harvest seedinfection and yield, and a few have been released as improved germplasm lines at different locations in West Africa [45]. Fourteen core accessions of the U.S. peanut germplasm collection were recently reported to have an average of 70% reduction in pre-harvest aflatoxin contamination, of which six accessions showed over 90% reduction in multiple years of testing [46]. These genotypes have been hybridized to combine resistance with acceptable agronomic performance [46].

2.3.2. Maize

Several screening tools such as the silk inoculation technique, pin-bar inoculation technique (for inoculating kernels through husks with *A. flavus* conidia), kernel screening assay (KSA), and infesting maize ears with insect larvae infected with *A. flavus* conidia have been developed and are regularly used in breeding programs for developing germplasm resistant to *A. flavus* growth and/or its toxin contamination [47]. Genetic engineering of *A. flavus* tester strains with a *GFP*-tagged gene construct linked to an *A. flavus* β-tubulin gene promoter have also been extensively used to quantify fungal biomass and to monitor fungal growth. These tools provide quick and economical ways not only to measure fungal growth but also to determine the effects of kernel resistance mechanisms on fungus or to predict the corresponding aflatoxin levels [48,49].

So far, several maize breeding lines and populations with high and consistent resistance under varying environments have been released [50–54]. However, these lines reportedly contained tropical germplasm in their background and were prone to lodging and lower yields than the commercial hybrids, besides posing difficulties in transferring the resistance from these lines into agronomically acceptable varieties. More recently, some of the newer released breeding lines have revealed highly quantitative nature of host–plant resistance to *A. flavus* infection and aflatoxin accumulation and much better plant types [55].

Similarly, several resistant inbred lines have been incorporated into an aflatoxin-resistance breeding program to improve and determine the inheritance of resistance in crosses with elite U.S. maize lines [56]. Several short-duration varieties were also reported to be useful for breeders seeking germplasm sources for *Aspergillus* ear rot and mycotoxin reduction due to their low grain aflatoxin contamination [57]. The Genetic Enhancement of Maize (GEM) program, following inclusion of aflatoxin accumulation resistance as characterization criteria, has identified resistant germplasm that is further being characterized [58,59]. An aflatoxin association-mapping panel containing 300 maize lines has recently been released, of which 30–40 lines displaying good resistance in multiple environments are available for use [60,61].

3. Biotechnology for aflatoxin elimination

Traditional methods used for studying host–plant resistance to *A. flavus* have not been very efficient in identifying the specific metabolites or components that have direct effect on aflatoxin biosynthesis. Non-availability of durable sources of resistance in germplasm of various crops even after decades of continuous research has led to concerns, firstly on improving knowledge on the fundamental biological mechanisms that are responsible for regulation of aflatoxin biosynthesis by the fungus, and secondly on the efficiency of host–plant resistance factors to aflatoxin accumulation in crops. The state of knowledge on biotechnological strategies is analyzed below at three different levels: knowledge of the fungus; environmental factors (drought stress); and host-plant resistance (Fig. 1).

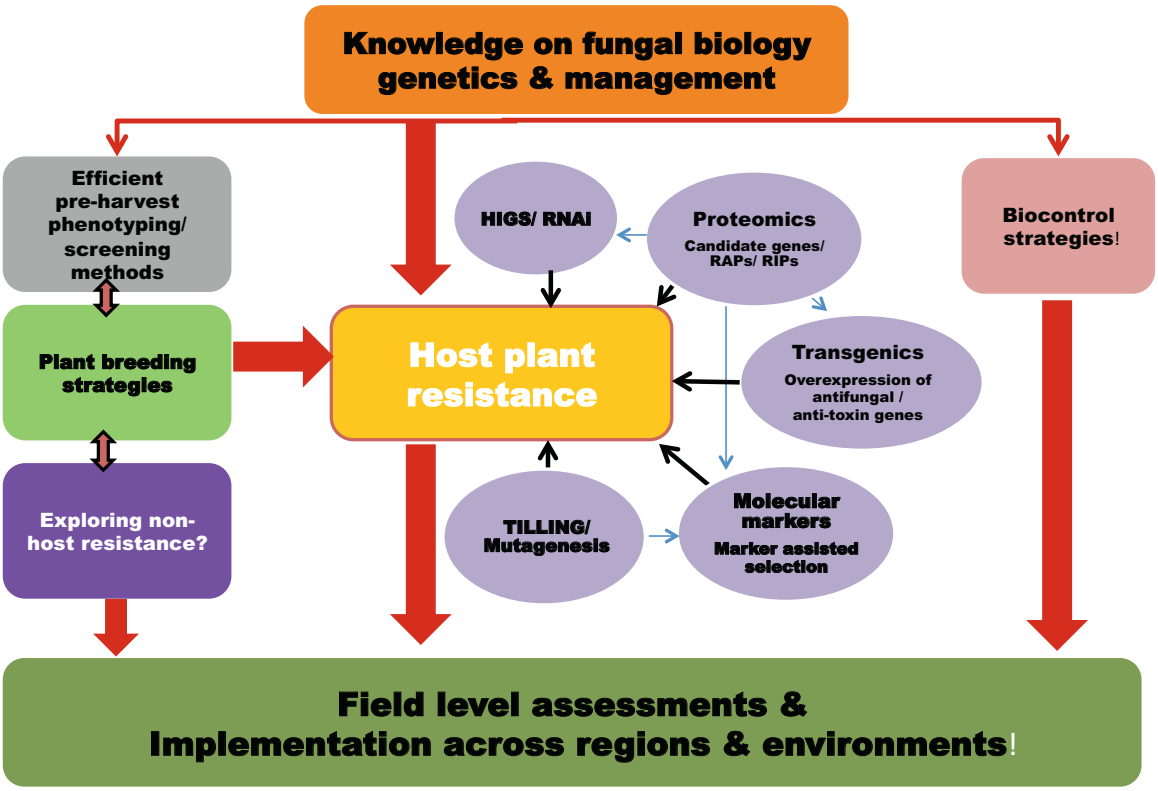

Fig. 1. Schematic representation of an integrated approach for pre-harvest aflatoxin management.

3.1. Knowledge of the fungus

The current status of research on aflatoxin management strategies and future possibilities has been reviewed comprehensively [62]. Significant progress has been made in explaining the aflatoxin biosynthesis pathway – the pathway intermediates, genes involved, corresponding enzymes, and regulatory mechanisms [63,64]. Gene manipulation studies have been extensively carried out for monitoring the molecular regulation of aflatoxin within the fungus. Significant success has been achieved in the identification of genes involved in aflatoxin biosynthesis and their subsequent cloning for use as "molecular tools" for identifying agents and compounds which may act as natural inhibitors in the aflatoxin biosynthesis pathway [17]. *A. flavus* and *A. parasiticus* have been mapped resulting in "pinning down" a 75 kilobase gene cluster (~30 genes) that governs the aflatoxin biosynthesis pathway [62,65]. This pathway is mainly controlled by the pathway-specific Cys6Zn2 DNA binding protein, AflR, along with a number of co-activators like AflJ, LaeA, VeA, VelB and VosA that fine-tune the timing of AflR's activity by forming a complex in the nucleus (Fig. 2). Nutritional stimuli such as carbon or nitrogen source, as well as environmental stimuli such as high temperature and pH activate global transcription factors and are especially important for the control of aflatoxin biosynthesis and induction of AflR expression [66]. Several global transcription factors such as CreA (for control of sugar utilization genes) and AreA (for control of nitrate utilization genes), PacC (involved in pH regulation of transcription) are involved in transmitting such signals for activation of the aflatoxin gene cluster through a signaling cascade involving cAMP-dependent protein kinase A [13]. This knowledge has opened the possibility of identifying resistance mechanisms which inhibit aflatoxin biosynthesis and fungal growth, apart from providing a robust and economical way of indirect measurements [13,48,49].

3.2. Environmental factors

Drought has a direct effect on the suppression of biocompetitive (phytoalexins, antifungal proteins) or protective compounds (phenols) that not only influence the growth of *A. flavus* and aflatoxin synthesis but also prevents proper maturation of seeds [67]. This is a result of the reduced capacity of seeds to produce phytoalexins as the seed moisture content decreases during drought. This in turn leads to fungal infection that not only compromises defense but also exacerbate aflatoxin formation in maize, peanuts, and other crops, resulting in devastating economical loses [5]. While drought intensity increases aflatoxin contamination, drought tolerance does not seem to be sufficient in itself to reduce aflatoxin contamination in all crops [39]. Work is in progress in understanding drought–*A. flavus* interactions that will help in evaluating germplasm for sources of resistance under controlled micro sick-plots (refer to the section on phenotyping in this review). An understanding of mutual involvement of key enzymes such as chitinases, osmotins, peroxidases, and proteases in both fungal attacks and drought stress responses could potentially help in the identification of useful variations among genotypes, providing molecular tools for selection of resistant lines. The complex environmental interactions during abiotic stresses such as drought and heat indicate the need to understand the relationship between *A. flavus* colonization under such stresses, and oxidative stress that results in aflatoxin production [68]. Considering this, the potential of genetics, genomics and proteomics in understanding these relationships has been much emphasized [69,70].

3.3. Host–plant resistance

Researchers have been using conventional breeding to overcome the problem of aflatoxin contamination. This is done by combining the three kinds of resistances in one genetic background

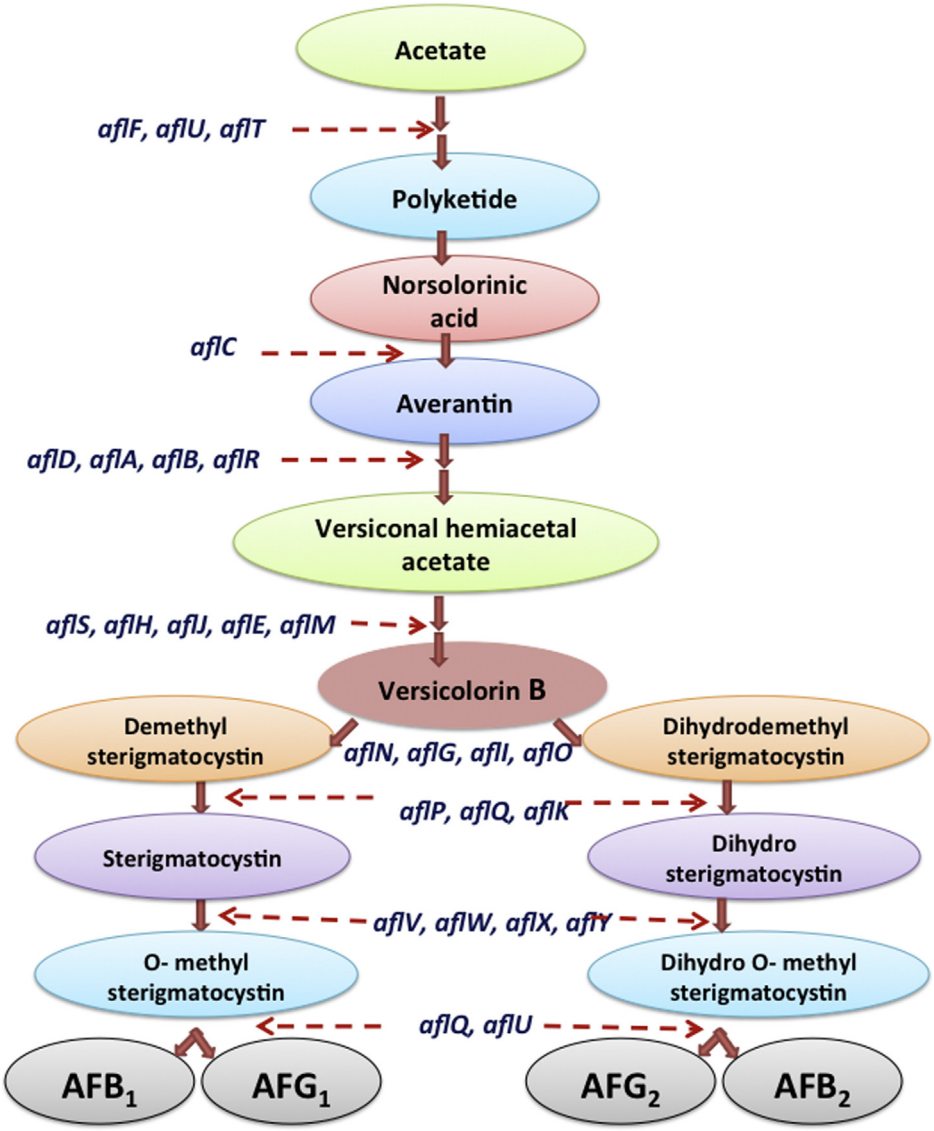

Fig. 2. Schematic representation of intermediates and key genes involved in aflatoxin biosynthetic pathway.

that could be further improved by pyramiding genes from different and diverse sources to contribute to resistance. However, the progress made so far has not met expectations, leading to increasing interest in using novel biotechnological tools to develop resistance to *A. flavus* infection and aflatoxin contamination. Genetic manipulations involved in host–pathogen interactions have been attempted which, with good post-harvest and crop husbandry practices, would be useful to alleviate aflatoxin contamination in food crops. Moreover, advances in genomics, marker development, and genetic engineering technology have the potential to improve food safety constraints related to aflatoxin contamination.

Research advances in microarrays, fungal expressed sequence tags (EST), and whole genome sequencing have led to discovery of several candidate genes responsible for the processes involved in host–plant interactions and aflatoxin contamination [17]. Similarly, plant factors that may influence fungal processes involved in invasion and aflatoxin contamination have been discovered with the available proteomic and natural product chemistry tools broadly divided into three categories: (1) seed proteins/inhibitors of fungal cell wall degrading enzymes; (2) seed/kernel natural products that may influence fungal growth and/or aflatoxin synthesis; and (3) plant stress-responsive proteins [17]. Key advances pertaining to different molecular strategies utilizing modern technologies like genomics, proteomics, and metabolomics have shown immense potential [71] in developing host-resistance against fungal invasion and aflatoxin contamination. Some of the research areas critical for the successful pre-harvest control of aflatoxin are discussed in detail below.

3.3.1. Candidate gene identification

Fungicides are often ineffective against *A. flavus* making it imperative to identify and select novel inhibitory compounds such as ribosome inhibiting proteins (RIP), lectins, small molecular weight polypeptides, hydrolases, cell-surface glycoproteins, and certain basic proteins, and characterize their respective genes before using them in plant genetic transformation [72].

In peanut, significant up-regulation of several resistance related genes has been observed using either cDNA libraries [69] or proteomic approaches [70] in two varieties following *Aspergillus* infection and subsequent aflatoxin contamination. Over 21,777 ESTs of 173,405 peanut ESTs were generated for identification of resistance-related genes involved in defense mechanisms against *Aspergillus* infection and subsequent aflatoxin contamination [69]. These have been used for marker development and

preparation of gene-based genetic maps, and also for transcript profiling to identify the candidate genes for traits of interest. The pod abundant transcripts in a high-density oligonucleotide microarray utilizing the available ESTs suggested the presence of distinct pathways involved in the generation of secondary metabolites apart from the accumulation of transcripts for storage and desiccation-related proteins [73]. The regulatory role of microRNAs (miRNAs) was also investigated to identify the ones that control gene expression at the post-transcriptional level. Over 100 putatively pod-specific/abundant genes, included those responsible for seed storage proteins and desiccation (e.g., late-embryogenesis abundant proteins, aquaporins, legumin B), oil production, and cellular defense. Gene expression profiling of resistant and susceptible peanut cultivars (infected with a mixture of *A. flavus* and *A. parasiticus* spores) identified 62 up-regulated genes in resistant cultivars in response to *Aspergillus* infection [74]. In addition, 22 putative *Aspergillus*-resistance genes were identified which were overexpressed in the resistant cultivar in comparison to the susceptible cultivar.

Similarly, using the maize Unigene 1–1.05 arrays, 236 genes were identified with significant differences in their expression levels in a study comparing resistant and susceptible inbred maize lines during 48 h of post-*A. flavus* infection, of which 135 genes were up-regulated in the susceptible, while 112 genes were up-regulated in the resistant [75]. Multiple candidate genes involved in resistance to *A. flavus* infection were identified, several of which could be directly mapped to the maize chromosomes. Similarly, several maize genes involved in host plant responses under *A. flavus* kernel infection in two resistant and susceptible maize inbred lines were identified and characterized using a combination of microarray analysis, qRT-PCR analysis, and QTL mapping methods. An integrated database of candidate genes is being used in developing *Aspergillus*-resistant cultivars either through targeted marker-assisted breeding or by genetic engineering [76].

3.3.2. Targeting induced local lesions in genomes (TILLING)/mutagenesis

In addition to the available germplasm collections with naturally occurring variations, induced mutant collections for tetraploid peanut also are being developed. TILLING has been employed to screen for mutations in six genes in over 3400 mutant lines generated following ethylmethane sulfonate (EMS) treatment [77]. Targeted induced mutations of lipoxygenase (LOX) genes involved in the synthesis of hydroperoxide fatty acids reported to either promote or inhibit aflatoxin biosynthesis have also been explored in peanut [Rajasekaran, unpublished]. These TILLING populations are potentially useful for functional genomics studies, as well as to recover mutations of potential value for crop improvement.

3.3.3. Molecular breeding approaches

Identification of markers to facilitate the transfer of resistance traits into agronomically viable genetic backgrounds, while limiting the transfer of undesirable traits, is necessary due to the polygenic and complex resistance to aflatoxin contamination [78].

In peanut, molecular marker assays for aflatoxin resistance have detected little variation at the nucleic acid level, even though considerable variation has been detected in cultivated peanut varieties for agronomic and morphological traits [79]. Preliminary attempts to associate seed storage protein markers showing different electrophoretic profiles with recognized aflatoxin resistance have been unsuccessful [79]. One of the two amplified fragment length polymorphism (AFLP) markers reported to be linked with resistance to seed invasion was converted into a Sequence Characterized Amplified Region (SCAR) marker for more efficient breeding application [80]. Several peanut cultivars with improved productivity and possessing resistance to aflatoxin contamination are extensively used in developing such markers, of which one SCAR marker "AFs-412" is closely linked with resistance to *A. flavus* infection in peanut [80]. Several DNA markers, significantly associated with reduced accumulation of aflatoxin B_1, aflatoxin B_2, and total aflatoxin in screened germplasm lines were derived from an interspecific hybrid between *Arachis hypogaea* and a related diploid wild species, *A. cardenasii* [81]. Such studies indicate the possibilities of using molecular markers for improving the efficiency of selection when transferring the low aflatoxin production of the interspecific lines into elite peanut breeding materials in the future.

On the other hand, in maize a lot of progress in last few years has been made in combining traditional and molecular breeding methods to develop commercially acceptable maize varieties for imparting resistance to *A. flavus* infection and aflatoxin reduction. This includes various breeding techniques involving phenotypic screening and molecular markers associated with known antifungal genes to develop maize varieties with enhanced resistance to *A. flavus* and aflatoxin contamination [55,84]. Genes associated with host-plant resistance or susceptibility of maize to *A. flavus* infection and aflatoxin accumulation have been identified by a combination of qRT-PCR analysis, microarray analysis, and QTL mapping methods. Chromosomal regions associated with resistance to *Aspergillus* ear rot and aflatoxin inhibition were identified through RFLP analysis in three "resistant" lines, providing evidences for separate genetic controls in maize [76].

Many QTL have been reported on resistance to *A. flavus* infection and aflatoxin contamination in maize [56,82]. Promising QTL for low aflatoxin using SSR markers were detected in two populations [83] and were identified on chromosomes 2, 3 and 7 [56]. Over 22 QTL regions identified from two mapping populations were developed and evaluated for aflatoxin accumulation over a three-year period [84,85], of which six were most significant. Most of these studies reported multiple QTL, which have been found in only one environment, where a majority accounted for less than 5% of the phenotypic variation observed in the population and the environment in which it was measured [55]. Nevertheless, two QTL accounting for up to 20% of the phenotypic variation in multiple environments were identified on chromosome 4 [86].

Similarly, QTL studies have also been used to map resistance-associated protein (RAP) genes associated with maize aflatoxin resistance including, an embryo-specific protein, heat shock and glucanase genes [82] as well as a glucose dehydrogenase [86]. MpM1, the first gene-based marker specifically developed for resistance to aflatoxin accumulation in maize, has now been integrated into existing marker-assisted selection programs for incorporating resistance into elite maize breeding lines [87]. Several maize lines have been released with enhanced resistance to aflatoxin accumulation using marker-assisted breeding. This suggests that though difficult, it is possible to make progress in breeding for resistance to *Aspergillus* ear rot and aflatoxin accumulation [82,88]. Deciphering the underlying genetic and molecular information continues to be a major challenge due to large genomic regions containing these QTL. To address these obstacles, molecular markers closely linked to the QTL are needed to expedite the breeding process and to reduce the breeding cycle [89]. One such effort involved conducting a meta-analysis of *A. flavus*, aflatoxin, and ear rot resistance using all available data sets in maize through multiple QTL mapping populations to identify the conserved QTL, and to reduce the genomic region [90]. This revealed a 4.07/8 region of the maize genome containing more than twice the expected number of QTL for multiple diseases, indicating that this region contained a cluster of genes influencing the response to multiple pathogens [55]. The identified larger effect-QTL regions are being backcrossed to different maize lines to verify the stability effect in different genetic backgrounds [55].

To investigate aflatoxin resistance associated genes and their association with reported major QTL linked to aflatoxin resistance, an association mapping panel containing resistant germplasm has been developed [60] and genotyped via Genotype By Sequencing (GBS) approach [91]. One study using some of the lines from the association-mapping panel did not reveal any genetic sequences associated with aflatoxin accumulation [92]. However, another study using the entire panel found several gene sequences associated with maize grain aflatoxin levels [60,61]. Such studies on aflatoxin resistance associated genes and their association with major QTL, can potentially narrow down candidate genes for development of aflatoxin resistant crops.

Unlike maize and peanut, so far no known cotton varieties have been reported for enhanced resistance to *A. flavus* infection and aflatoxin contamination [72]. Nevertheless, volatile and non-volatile compounds that inhibit both fungal growth and aflatoxin production have been identified in cotton leaves that could be used as markers to enhance resistance against aflatoxin producing fungi through classical plant breeding and/or new molecular techniques.

3.3.4. Proteomics for studying host resistance mechanisms

Proteomics are being used as a novel tool in aflatoxin research [93] to identify RAPs and the candidate resistance genes associated with the resistance mechanisms among the resistant lines, in comparison with susceptible lines [69,70,94], as a potential strategy for controlling aflatoxin contamination in crops [95]. The discovery of storage and stress-related proteins as biomarkers for aflatoxin is potentially useful for breeders to find appropriate strategies to improve plant resistance and stress tolerance of host plants against fungal contamination.

A three- to four-folds increase in β-1,3-glucanase reported among resistant lines of peanut following infection with *A. flavus* indicated that the induction could be a part of general defense against fungal infection [96]. Differentially expressed seed protein profiles between a resistant cultivar and a susceptible cultivar revealed differences in 12 proteins including signaling components (CDK1, Oso7g0179400 and RIO kinase), SAP domain-containing protein, storage proteins (PII protein and iso-Ara h3), stress responsive proteins (L-ascorbate peroxidase, oxalate oxidase, heat shock protein precursor and trypsin inhibitor), 50 S ribosomal protein L22, and putative 30 S ribosomal S9 in the resistant cultivar challenged by *A. Flavus*–drought stress [70]. Efforts are ongoing for identifying RAPs/RAGs from the existing resistant peanut genotypes.

Proteome profiling in maize has enabled identification of several RAPs that have been categorized into three groups namely, stress-responsive proteins, storage proteins, and antifungal proteins, indicating that storage and stress-responsive proteins may play an important role in enhancing stress-tolerance of host plant [71,94]. Recently, constitutively expressed and inducible proteins have been shown to counter the function of other hydrolytic virulence proteins produced by *A. flavus*, one such group being of trypsin inhibitors [97,98] which inhibits the function of α-amylase used by the fungus for catabolism of complex carbohydrates [68].

Resistance to *A. flavus* infection and aflatoxin accumulation in maize via rachis and silk tissue has also been studied using proteomics [99,100]. Differential expression of many stress/defense proteins using proteomic approaches has been carried out using available resistant and susceptible maize inbred lines during rachis juvenility, maturation, and post *A. flavus* challenge. Resistant rachis tissue contains abiotic stress-related proteins and proteins from phenylpropanoid metabolism and have constitutive defenses, while susceptible rachis contains pathogenesis-related proteins that are more dependent on inducible defenses [99]. These studies indicate that the degree of expression of distinct proteins during rachis development in maize lines determines its level of innate resistance against *A. flavus* infection and subsequent aflatoxin accumulation [99]. Comparative two-dimensional electrophoretic analysis of several resistant and susceptible inbred lines led to the identification of three PR proteins (PRm3 chitinase, chitinase I, and chitinase A), with higher antifungal activity in silks of resistant inbreds than in the susceptible lines [101]. Similarly, chitinase isolated from a resistant maize inbred inhibited the growth of *A. flavus* by 50% at a concentration of 20 μg/ml, suggesting that silk chitinases contribute to *A. flavus* resistance [102].

Proteome analysis and subtractive approaches have identified several proteins associated with resistance in maize, which have been characterized [95]. Proteomic comparisons of constitutive kernel embryo and endosperm proteins between susceptible and resistant genotypes not only shortened the time taken to identify RAPs but also enhanced the identification of unique or elevated levels of stress-related proteins/RAPs among aflatoxin-resistant lines [103]. Accumulation of such antifungal- and resistance-associated proteins contribute to the resistance observed in several maize lines along with morphological characteristics of resistant kernels such as thickened wax cuticles. Several resistance-associated and differentially expressed proteins were identified between resistant and susceptible lines using proteomics. The differentially expressed proteins were sequenced by electron spray ionization tandem mass spectrometry (ESIMS/MS) to obtain their peptide sequences in maize and soybean. These were later categorized into stress-related proteins and antifungal proteins, such as glyoxalase, peroxidase, trypsin inhibitor, and pathogenesis-related proteins based on their sequence homology [103,104]. In addition, β-1,3-glucanase, chitinases, pathogenesis-related proteins 10 and 10.1, ribosome inactivating proteins (RIPs), and zeamatin are involved in the resistance of maize against *A. flavus* [104]. Several constitutively expressed RAPs have facilitated the identification of sub-pericarp resistance mechanisms that were further evaluated for their potential involvement in resistance [72].

3.3.5. Genetic engineering of crop plants for resistance to A. flavus infection and aflatoxin production

Genetic engineering technology has the potential to incorporate beneficial plant traits, particularly the enhanced ability to withstand or resist attack by insects and pathogens toward increasing crop yields [105]. Development of transgenic varieties with antifungal traits that confer resistance to aflatoxin-producing fungi will be extremely valuable and will be an aid to breeding tools. Genome segments from plant pathogenic fungi have been widely used to make transgenic plants for increasing or decreasing the expression of several genes responsible for anti-fungal or anti-toxin activities [106]. Nevertheless, better knowledge of biochemical mechanisms involved in response to the fungal infection and its interaction with the environment is required, besides establishing the identity of plant mechanisms that inhibit aflatoxin production before incorporating specific anti-fungal genes into plant varieties for enhanced host–plant resistance. Moreover, the availability of efficient modern biotechnological tools which aid in the evaluation of plant–pathogen protein interactions, genomics and field ecology of the fungus has encouraged research on transgenic approaches to control invasion by *Aspergillus* for preventing aflatoxin contamination (Table 1).

Various antifungal proteins and peptides have been isolated from a wide range of plants and tested for their antifungal activity against several *Aspergillus* species that cause significant economic losses in crop production [107]. Different antifungal proteins and peptides used in genetic engineering of susceptible crop species to combat *A. flavus* infection and aflatoxin contamination include defensins, thionins, plant non-specific lipid transfer proteins (ns-LTPs), knottins, impatiens antimicrobial peptides, ribosome inactivating proteins (RIPs), lectins, and lectin-like peptides as previously reviewed [107,108]. More recently, newer research

Table 1
An update on the genetic transformation studies for resistance to *Aspergillus flavus* and aflatoxin contamination in crops.

Crop	Target trait	Gene	Source	Reference
Peanut	*A. flavus*	*Glucanase*	*Nicotiana tabaccum*	[105]
		mod1, D5C	*Zea mays*	[112,114]
		Anionic peroxidase synthetic peptide D4E1	*Solanum lycopersicum*	[111]
		Chitinase	*Oryza sativa*	[104]
		Defensins	*Medicago sativa*	Bhatnagar-Mathur et al. (unpublished)
		Forisomes	*Pisum sativum*	Bhatnagar-Mathur et al. (unpublished)
	A. flavus and aflatoxin biosynthesis	*Loxl*	*Glycine max*	[111]
		Nonheme chloroperoxidase gene (cpo)	*Pseudomonas pyrrocinia*	[115]
		Nonheme chloroperoxidase	Bacteria	[113]
		Lipoxygenase (PnLOX3)	*Arachis hypogaea*	Bhatnagar-Mathur et al. (unpublished)
		PR10 gene (ARAhPR10)	*Arachis hypogaea*	[116]
Maize	*A. flavus*	β-*1,3-Glucanase*	*Zea mays*	[119]
		Trypsin inhibitor protein	*Zea mays*	[87]
		mod1	*Zea mays*	[122]
	A. flavus and aflatoxin biosynthesis	*α-Amylase*	*Lablab purpureus*	[120]
		Glyoxalase I	*Zea mays*	[121]
		zhd101	*Zea mays*	[123]
		PR10 gene	*Zea mays*	[94]
Cotton	*A. flavus*	*Bacterial chloroperoxidases (CPO-P)*	*Pseudomonas pyrrocinia*	[127]
		Trypsin inhibitor protein	*Zea mays*	[87]
		Defensin D4E1 gene	*Medicago sativa*	[49]

strategies have been used for controlling the expression of many stress-responsive genes by engineering the regulatory machinery, using transcription factors and gene pyramiding instead of insertions of "single-action" genes.

Several researchers have been experimenting with different antifungal gene constructs often stacked with insect-resistant genes that potentially offer resistance to aflatoxigenic fungi in vitro, in situ, or in planta. Meanwhile, certain small lytic peptides such as D4E1 and D5C have demonstrated convincing inhibitory activity against *A. flavus* and show promise for transformation of plants to reduce infection of seed [72]. The problems associated with natural lytic peptides such as proteolytic degradation and non-specific toxicity to non-target organisms have been tackled by the rational synthesis of stable and target-specific peptides [109]. In addition to lytic peptide genes, a variety of other candidate antifungal genes from bacterial, plant, and mammalian sources have a good probability of being active against *A. flavus* upon transformation into plants. Transgenic plants expressing genes for synthetic analogs of cecropins and magainins have also demonstrated improved resistance to fungal invasion [110,111]. In vitro studies with leaf extracts of transgenic tobacco expressing the D4E1 gene not only reduced spore viability (colony-forming units) in *A. flavus* but also caused severe abnormal lytic effects on mycelial wall, cytoplasm, and nuclei [110,112].

In peanut, cuticular wax [113], tannin content, and chemical composition of the pericarps and embryos have an important role in the inhibition of fungal invasion by *A. flavus* and aflatoxin formation. Chitin, a linear polymer of *N*-acetyl glucosamine, is selectively degraded by chitinolytic organisms and used as a carbon source for their growth and multiplication. The physiological role of chitinases has not yet been documented in the general metabolism of higher plants because of the absence of its substrate chitin, and the lack of direct evidence supporting the hypothesis that plants produce chitinase as a protective mechanism against chitin-containing parasites. Several reports demonstrated the importance of chitinase as a key enzyme produced by several fungi and bacteria that is responsible for fungal cell/sclerotial wall lysis and degradation. Transgenic peanut expressing rice chitinase gene had enhanced resistance to *A. flavus* [114]. This supports a proposed in vivo role of these PR proteins as integral components of a general disease resistance mechanism that protect the host from invasion by fungal pathogens. Similarly, transgenic peanuts containing a glucanase gene from tobacco showed enhanced disease resistance to IVSC and no accumulation of aflatoxin [115]. More recently, lipoxygenases have been reported to have an important role in mediating plant host-pathogen interactions, catalyzing the oxidation of polyunsaturated fatty acids such as linoleic acid (18:2) and α-linolenic acid (18:3) to produce unsaturated fatty acid hydroperoxides. These oxylipin products have been shown to have a significant effect on differentiation processes in the mycotoxigenic seed pathogens *Aspergillus* spp. While 9*S*-HPODE stimulated mycotoxin production, 13*S*-HPODE inhibited it, presumably by structurally mimicking endogenous *Aspergillus* sporogenic factors such as *oxylipins* derived from oleic, linoleic, and linolenic acid [116–120].

There has been sufficient evidence of *Aspergillus* sp. activating the seed lipid pools for sporulation and mycotoxin development and hence transgenic strategies using lipoxygenases have been developed in several crop species. Transgenic peanut overexpressing soybean *loxl* gene under the control of embryo specific promoter from carrot had reduced aflatoxin content [121]. Efforts are underway to generate peanut transgenics that overexpress peanut lipoxygenase gene (*PnLOX3*) for combating aflatoxin contamination. Greenhouse studies indicated that these transgenic events had a similar pre-harvest *A. flavus* infection comparable to their untransformed counterparts, but there was a significant reduction in aflatoxin accumulation. This indicated that different mechanisms of resistances are responsible for *A. flavus* infection and aflatoxin accumulation in peanut [Bhatnagar-Mathur et al., unpublished results].

When expressed in peanut, another antifungal gene, *Mod-1*, a synthetic version of maize ribosome inactivating protein gene (a proteolytically-activated form of RIP-1), had enhanced resistance to *A. flavus* and reduced aflatoxin contamination in the infected cotyledons [122]. Interestingly, insect-resistant peanut lines expressing *cryIA*(c) when evaluated for *A. flavus* infection [123] revealed a positive correlation between insect damage and aflatoxin contamination. Other antifungal genes such as D5C [124], tomato anionic peroxidase (tap 1), and synthetic peptide D4E have also been

evaluated in transgenic peanut for antifungal activity against *A. flavus* [121]. While transgenic peanut callus expressing the antifungal peptide D5C showed poor plant recovery attributed to its possible phytotoxicity, pure D5C showed strong activity against *A. flavus* in in vitro studies [124]. Bacterial chloroperoxidase genes when expressed in peanut also showed antifungal activity causing a significant reduction ($P < 0.05$) in *A. flavus* colony growth [125]. *A. flavus* infection and aflatoxin content was significantly lowered in transgenic peanut lines constitutively expressing PR10 family putative resistant gene (*ARAhPR*10) [126]. Activities are ongoing on exploring other antifungal genes to develop peanut transgenics by using gene encoding defensins such as *MtDef1EC* and *MtDef4EC* targeting the extracellular region, *MtDef4ER* targeting the endoplasmic reticulum, and *MtDef4VC* targeting the vacuolar region [Bhatnagar-Mathur et al., unpublished results]. Similarly, there have been efforts on using pea (*Pisum sativum*) forisome gene for developing peanut transgenics for resistance to *A. flavus* [Bhatnagar-Mathur et al., unpublished results].

Several maize hybrids have been developed with acceptable level of resistance either to *Aspergillus* or aflatoxin, but these tend to have lower levels of aflatoxin than their counterparts [127]. Since complete resistance is unlikely, use of genetic engineering has become potentially important in this crop. Several specific proteins with antifungal activities were identified in maize kernels during germination [128]. These include PR proteins such as hydrolases (chitinases and β-1,3-glucanases) which degrade structural polysaccharides of the fungal cell wall, ribosome inactivating proteins (RIPs) which modify and inactivate foreign ribosomes, and zeamatin which increases permeability of fungal cell membranes. The resistance of a maize hybrid to *A. flavus* infection also correlated with an elevated level of β-1,3-glucanase in transgenic cells [129]. A major role of the PR10 gene was highlighted in maize aflatoxin resistance based on a significant negative correlation between its expression (at either transcript or protein level) and kernel aflatoxin production [104]. Maize transformed with α-amylase inhibitors of Hyacinth bean (*Lablab purpureus*) blocked the α-amylase activity of *A. flavus*, and inhibit spore germination and fungal growth as well, which also resulted in reduction in aflatoxin contamination [130]. RNAi gene silencing of PR10 in mature kernels revealed a significant increase in *A. flavus* colonization and aflatoxin production when compared with the non-silenced controls. Decreased expression of another antifungal protein, maize trypsin inhibitor (TI) also correlated with kernel resistance to *A. flavus* infection in maize [97]. While a stress-related protein, glyoxalase I from maize, has also shown potential to directly inhibit aflatoxin accumulation [131], a synthetic version of a maize RIP gene (*mod1*) also efficiently controlled *A. flavus* [132]. Expression of detoxification genes such as *zhd101* reduced mycotoxin contamination (zearalenone) in maize kernels [133]. This has led to interest in identifying aflatoxin detoxification genes from *Trachyspermum ammi* for use in crop plants to develop transgenic resistance to aflatoxin contamination [134].

In cotton, bollworm or insect injury to cotton bolls has been thought to be an entry point for *A. flavus* spores, although concrete evidence is not yet available [135]. Aflatoxin contamination in cotton is not directly correlated with pink bollworm damage and contamination may occur in the absence of damage [136]. Transgenic interventions using bacterial chloroperoxidases such as CPO-P conferred antifungal activity against *Aspergillus* species and other phytopathogens in transgenic cotton [137]. Transgenic cotton balls expressing the maize kernel trypsin inhibitor protein (TIP) when inoculated with *A. flavus* showed no significant difference in fungal colonization when compared with the untransformed controls. This indicates that high expression levels of this antifungal protein are required to control *A. flavus* [49,97]. Nevertheless, expression of a defensin D4E1 gene imparted resistance to *A. flavus* by causing severe abnormal lytic effects on mycelial wall, nuclei, and cytoplasm, and inhibited the germination of *A. flavus* spores, as evident from in situ inoculation of cotton seed and in planta boll inoculation assays [49].

3.3.6. Host-induced gene silencing (HIGS)

Most of the transgenic approaches used potential native antifungal peptides/proteins that are expressed either constitutively or in response to fungal attack such as chitinases, β-1,3-glucanase, protease inhibitors, thionins, RIPs, lectins, and polygalacturonase inhibitor proteins [72]. More recently, host-induced gene silencing (HIGS) is emerging as a promising technology in which the pathogen is directed by the host plant to down-regulate the expression of its own genes. HIGS is a promising technology in which the pathogen is directed by the host plant to down-regulate the expression of its own genes, without requiring the host plant to express a foreign protein. Similar to the genomes of other eukaryotes, the genomes of filamentous fungi encode conserved components, such as RNA-dependent RNA polymerases that are involved in the RNAi process [138]. Studies have demonstrated that some *Aspergillus* mycoviruses are capable of RNA silencing suppression in *Aspergillus*, besides being the targets for degradation by *Aspergillus* machinery. Considering these recent descriptions of RNA interference and the interaction of *Aspergillus* mycoviruses with their host via RNA interference [139], gene silencing approach may offer the most feasible solution for incorporating resistance to aflatoxins in elite breeding lines in a relatively short time.

Recent studies indicate that a dsRNA virus from *Penicillium chrysogenum* can degrade the transcripts of aflatoxin genes by the RNA interference mechanism. This study proposed that suppression of *veA* gene by *Penicillium chrysogenum* virus-induced siRNAs eventually blocked aflatoxin biosynthesis in the virus transfected *A. flavus*, but might be nonspecific because of its effect on genes involved in both morphogenesis and secondary metabolism [140]. Similarly, silencing the expression of the trypsin inhibitor (TI) gene in maize increased the susceptibility of maize kernel tissue to *A. flavus* infection and aflatoxin contamination [98]. RNA interference studies were undertaken in maize to investigate the importance of pathogenesis-related protein (PR10) and study its negative correlation with the kernel resistance against *A. flavus* infection [72,98,104]. Reduction of PR10 expression by 65% in transgenic callus lines not only showed increased sensitivity to heat stress but also showed a significant increase both in the fungal growth and its toxin production in transgenic kernels compared to their non-silenced control counterparts [104]. More recently, three synthetic siRNA sequences (Nor-Ia, Nor-Ib, Nor-Ic) aimed at the two key genes of the aflatoxin biosynthetic pathway, *aflR* (a regulatory gene) and *aflD* (a structural gene), were used to control aflatoxin production and study their effects on aflatoxin G_1 (AFG_1) and aflatoxin B_1 (AFB_1) [141]. The observed correlations between changes in a*flR* and *aflD* transcript abundance indicate that there is a relationship between the expressions of these regulatory and structural genes, which suggests that *aflD* could be a potential target gene for effective reduction in aflatoxin [141].

4. Phenotyping as a critical component

Aflatoxin contamination in almost all crops is a result of complex fungus–environment interactions. Increased aflatoxin contamination in the field has been reported when drought and hot weather conditions occur during the growing season [142]. Irrigating maize was shown to reduce fungal infection and aflatoxin contamination, especially when applied during drought stress [143,144]. Similarly, increased aflatoxin contamination was observed in drought-stressed peanuts with increased soil

temperatures, besides affecting pre-harvest infection [5,143,145]. Most of the genotypes reported as resistant in vitro have not proven to possess significant level of pre-harvest aflatoxin resistance under field studies, necessitating the need to develop high throughput phenotyping assays that mimic field-like conditions [40,146,147].

In maize, field screening methods include inoculating the primary ears on all plants via a pinboard method, injection of inoculum through husk leaves into the side of the ear, or injection of inoculum down the silk channel varying from location to location. Significant genotype × environment interaction (GEI) has been observed supporting the observation that aflatoxin concentration among genotypes is affected significantly by environment. Similarly, despite several field-screening techniques available for peanut and cotton, these did not give conclusive and consistent results. This might be due to spatial variabilities inherent with most soil-borne fungi, variable environmental conditions, difficulty to induce drought stress, and high soil temperatures which are conducive for *A. flavus* growth. The associated variabilities in aflatoxin contamination even among the reported resistance sources have challenged researchers to develop more reliable and efficient screening techniques that may prevent escapes. Moreover, lack of robust screening methodologies may not only provide misleading conclusions pertaining to the level of resistance in genotypes, but also result in noisy gene expression analysis leading to flawed candidate gene identification.

Hence, it is imperative to develop effective selection methods that would provide strategies to improve genetic resistance to aflatoxin contamination by considering genotype × environment interactions critical for such a complicated trait. Simulating environments that exacerbate fungal growth and toxin accumulation becomes critical in designing effective strategies for phenotyping, in order to breed and precisely integrate resistance genes into commercial varieties through marker-assisted breeding or genetic engineering.

Developing robust pre-field screening assays for peanut: Efficient screening techniques controlling late season drought and elevated soil temperature in pegging zone are critically required to identify sources of resistance to *A. flavus* and subsequent aflatoxin contamination. These factors favor fungal colonization leading to pre-harvest aflatoxin contamination. In general, drought and high soil temperatures of 29–31 °C in the pod zone enhance aflatoxin contamination in peanut during the final 3–6 weeks of the growing season. Several efforts have been made in the past for large-scale pre harvest infection screening by completely isolating the pod zone and restricting the moisture to the root zone using polysterene barrier, subsurface irrigation, and rainout shelters, while maintaining the high soil temperatures using subsurface heating cables.

Simulating *A. flavus* infested micro-sick plots have been designed that allow water stress imposition in the pod zone immediately after peg formation until harvest, while keeping the root zone irrigated [Bhatnagar-Mathur et al., unpublished results]. These structures allow independent control over soil water in both pod and root zones, not only ensuring plant survival but also maintaining high soil temperature of 28–30 °C in the pod zone that favors optimum *A. flavus* invasion and aflatoxin contamination. The *A. flavus* population densities were measured in the soil at different stages of plant growth including planting, flowering and harvest. This facilitated a comprehensive evaluation of pre-harvest infection, IVSC and aflatoxin content in the peanut kernels, and reduced data variability that ensured higher and consistent contamination. This method not only has widened the approach of germplasm screening to identify sources of resistance to *Aspergillus* colonization and aflatoxin contamination for candidate gene identification, but also simulated field conditions for effective and stringent evaluation of the available transgenic events [Bhatnagar-Mathur et al., unpublished results].

5. Conclusions and the way forward

Several effective physical, biological, and chemical methods have been reported for the prevention and control of fungi and resulting mycotoxins. However, developing fungal resistant hybrids/crops to combat pre-harvest infection and resulting contamination has remained a challenge. Due to their inherent risks, post-harvest treatments such as alkalization, ammonization, heat or gamma radiation are not effectively used by farmers. While many biocontrol organisms have been investigated for their potential to reduce aflatoxin contamination of crops, only atoxigenic *A. flavus* strains have been used commercially. Considering the possible genetic recombination in *A. flavus*, precautions are needed to not only have stable biocontrol strains but also guard against inadvertent introduction of *A. flavus* strains that could impose an additional burden on food safety and quality. Considering these, the most critical factor for a better understanding of the mechanisms of gene regulation in aflatoxin biosynthesis is identifying natural inhibitors of *Aspergillus* growth and toxin formation. Rather than focusing only on identifying organisms that are antagonistic to *A. flavus*, it is important to shift the gears and invest efforts and resources in identifying the secondary metabolites of potential anti-fungal agents, which inhibit aflatoxin biosynthesis. Critical investigations on the efficacy of extracellular metabolites of actinomycetes (*Streptomyces hygroscopicus*, *S. diataticus*, and others) and antibiotic producing strains of *Pseudomonas fluorescens* can potentially reduce aflatoxin contamination. In addition, identification of novel promoter and enhancer elements will be crucial for achieving efficacious expression of anti-fungal/anti-mycotoxin genes for genetic engineering options.

While significant progress has been made in alleviating aflatoxin contamination through conventional plant breeding, these have not resulted in its complete eradication. The semi-arid environments are especially conducive to pre-harvest aflatoxin contamination due to end-of-season drought, whereas, in the wet and humid areas post-harvest contamination is more prevalent. Appropriate drying, curing and storage practices can minimize post-harvest aflatoxin contamination, but these will work only if the produce is free from pre-harvest aflatoxin, making this a major objective in breeding programs. Since available/reported traditional breeding methods are only partially effective, novel biotechnological methods for controlling aflatoxin are needed to develop pre-harvest host-plant resistance to aflatoxin accumulation. Over the years, biotechnology has emerged as a promising tool in significantly enhancing knowledge on the mechanisms of aflatoxin production, crop-fungus interactions, and pathogenicity of the fungus. Biotechnology has also been vital in providing insights into available genetic and genomic resources enabling design of effective and novel strategies for a nutritious, aflatoxin-free, safer, and sustainable food and feed supply. Nevertheless, a complete understanding of the host resistance mechanisms in crops is critical to enable the use of conventional and modern breeding tools to achieve cumulative or complementary benefits.

Hence, current advances in tissue culture techniques, genetic transformation and marker-assisted selection along with advances in the powerful new “omics” technologies offer great potential in developing crops resistant to *A. flavus* infection and aflatoxin production. While, identification of resistance traits in aflatoxin-susceptible crops through marker-assisted breeding can facilitate the development of resistant, commercially useful germplasm, transgenic approaches rapidly assess the efficacy of potent antifungal proteins or peptides and eventually transfer them to susceptible ones for enhancing their resistance. This, in effect, could lead to achieving sustainable food security, poverty reduction, and environmental protection faster than when traditional breeding is used.

Moreover, since it is still unknown what causes *Aspergillus* to produce aflatoxin in only certain plant species, and what makes crops such as soybean and wheat resistant to aflatoxin contamination in the field, identifying and exploiting non-host resistance (NHR) mechanisms in these crops holds tremendous potential in providing host crops with broad-spectrum and durable resistance against *A. flavus* and aflatoxin contamination in changing environments. This could lay the foundation for the development of improved germplasm with enhanced resistance to *A. flavus* and open new challenges and methods for combating *A. flavus* infection and aflatoxin contamination. Development and deployment of such improved germplasm in an effective manner will also be an important prerequisite for sustainable use of biotechnology for crop improvement and food safety.

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