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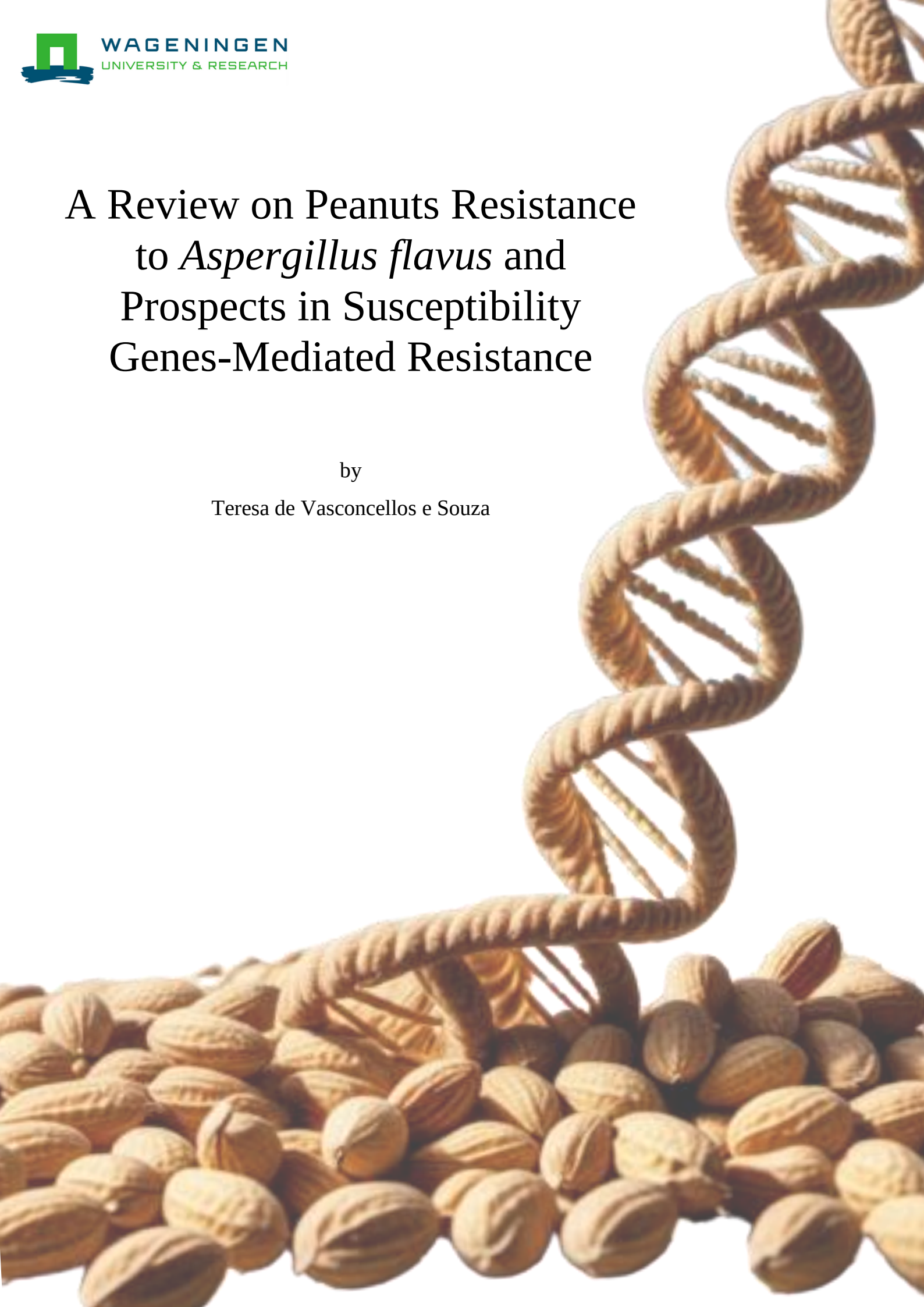

A Review on Peanuts Resistance to *Aspergillus flavus* and Prospects in Susceptibility Gene-Mediated Resistance

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Summary

This study encompasses a review of the existing literature on peanut resistance to *Aspergillus flavus* and explores the potential of manipulating susceptibility genes as a resistance breeding strategy. Peanut (*Arachis hypogaea L*.) ranks among the world's most economically significant oilseed crops. However, the profitability and safety of peanut production are severely hampered by aflatoxin contamination, caused by the fungal pathogen *Aspergillus flavus*. To address this issue, the paper commences with a chapter dedicated to the pathogen, covering aspects such as *A. flavus* life cycle, pathogenicity, factors influencing its growth, and aflatoxin contamination, along with suggested control strategies. So far, traditional methods for disease management and aflatoxin control have demonstrated limited success. Moreover, it features a section dedicated to the genomic regulation of the pathogen, including the regulation of aflatoxin biosynthesis.

The subsequent chapter explores peanut resistance, underlining the molecular mechanisms researchers have sought to decipher in the context of peanut resistance to *A. flavus* and aflatoxin contamination. Various methodologies have been explored in this regard, and consequently, different studies have identified three distinct mechanisms of resistance: 1) *in-vitro* seed colonization resistance (IVSC); 2) resistance to pre-harvest aflatoxin contamination (PAC); and 3) resistance to aflatoxin production following infection (AP). Thus far, the most feasible method of reducing or eliminating *A. flavus* infection and subsequent aflatoxin accumulation is by growing resistant peanut varieties, and while progress has been made in this matter, achieving complete immunity remains an ongoing challenge.

Furthermore, this paper provides an overview of six separate studies that collectively identified 43 quantitative trait loci (QTLs), including four major QTLs linked to *A. flavus* resistance and ten major QTLs associated with aflatoxin contamination. In addition, a concise summary is presented of studies examining single nucleotide polymorphisms (SNPs) and candidate genes associated with peanut resistance to the aflatoxin-producing fungus. Also, a section describing successful applications of host-induced gene silencing (HIGS), RNA interference (RNAi) and overexpression against the pathogenic fungi and aflatoxin is included.

Despite the progress achieved, further research is needed to better understand the genetic basis of resistance and to develop effective strategies for managing *A.flavus* and aflatoxin contamination. In this context, this study includes a chapter dedicated to the potential of manipulating susceptibility genes—a strategy that has gained growing attention as an alternative approach to conferring durable resistance and ensuring the security of peanut cultivation. However, the identification and exploitation of susceptibility genes in peanuts are still in the early stages, with limited available

knowledge, as elucidated in chapter (5.3). So far, various research proposals have been put forth regarding candidate susceptibility factors, including annexins, syntaxins, calmodulin, 9-cis-epoxy carotenoid dioxygenase and two *mildew resistance locus O* (*MLO*). However, there have been no instances of genetic manipulation studies and functional analysis conducted on these yet. Additionally, a recent study characterized two salicylic acid (SA) SA hydroxylase genes (*AhS5H1* and *AhS5H2*) as the first S genes in peanuts. Also, the Ethylene Responsive TF *ABR1*, a repressor of the ABA signaling pathway, has been reported to confer resistance against pre-harvest aflatoxin contamination. Based on the gathered information, it can be suggested that there is a potential for S gene-mediated resistance in peanuts. However, further research and genome-wide mining of susceptibility genes are warranted to fully understand and validate this approach.

1) Background

The Arachis genus, native to South America, is composed of around 80 diploid geocarpic species, characterized by the growth of aerial flowers and subterranean fruit, that allow them to thrive in harsh environments. These species are classified into nine taxonomic sections [1] [2] and among them, the allotetraploid (2n = 4x = 40) *Arachis hypogaea L*. is one of the most important oil and legume crops grown widely [3]. Commonly known as cultivated peanut or groundnut, this species with an AABB genomic constitution and a total genome size of~2.8 Gb [4], is believed to be derived from a recent hybridization event between two diploid wild progenitors, *Arachis duranensis* (AA genome) and *Arachis ipaensis* (BB genome) [5].

Aspergillus flavus is a saprophytic soil-borne fungus that belongs to the phylum Ascomycota and is placed under the family Trichocomaceae of the order Eurotiales within the class Eurotiomycetes [6]. *A. flavus* is closely related to *A. parasiticus,* and they often infect the same crops, including peanuts, maize, cotton and other oilseeds. During pathogenesis, both fungi produce potent mycotoxins named aflatoxins, that pose severe health risks to both humans and animals. There are four major types of aflatoxins (B_1, B_2, G_1 and G_2), however, toxigenic strains of *A. flavus* typically produce only types B1 and B2, whereas *Aspergillus parasiticus* can produce all four [7]. Moreover, aflatoxins are the product of a complex polyketide pathway, involving at least 27 enzymatic reactions that are encoded by the "aflatoxin biosynthesis gene cluster". In turn, the expression of these genes is coordinated by two cluster-specific regulators: *aflR* and *aflS*, in addition to external stimuli, as will be discussed later [8].

The pathogen thrives in hot and humid agro-ecological environments and can contaminate peanuts during pre-harvest and post-harvest stages. Additionally, the fungal infection and aflatoxin contamination are enhanced by abiotic and biotic stress

factors, soil type as well and inadequate pre- and post-harvest processes/conditions [9], [10]. To date, the prevention of *A. flavus* infection in peanuts has been regarded as the best solution towards aflatoxin contamination [11]. As a result, considerable research efforts have been directed toward the development of aflatoxin-resistant peanut cultivars for disease management [12]. Nevertheless, progress has been challenging due to the intricate genetic nature of resistance: resistance is quantitative in nature, highly influenced by the environment (G × E interaction), has low heritability and there's a lack of reliable phenotyping protocols [13]. Still, progress has been made through quantitative trait locus (QTL) mapping, which has enabled the discovery of numerous QTLs associated with resistance to *A. flavus* and aflatoxin. Likewise, various genomics, proteomics and metabolomics approaches have provided insights into a multitude of genes, proteins, and signalling molecules that play pivotal roles in peanuts' defence against *A. flavus* infection and aflatoxin contamination [13], [14].

1.1) Aspergillus flavus life cycle and infection

A. flavus persists in the soil, where it can endure for up to three years, in the form of either sclerotia or conidia, or it can reside within plant tissues as mycelia [15]. Soil movement and rain splash dispersal are the main contributors to below-ground infection. In this scenario, fungal spores infiltrate peanut seeds, initiating the establishment of mycelia that extend throughout the peanut shell, and seed coat, and eventually reach the cotyledon. The penetration process occurs through cracks on the seed coat and pod walls, often induced by mechanical injuries and/or abiotic stresses, such as heat and drought [16]. Furthermore, infection may also take place in the radicle and hypocotyl, which ultimately leads to the emerging seedlings' decay [17] and subsequent alterations in the plant's physiological responses, thereby diminishing the plant's inherent defence capacity against infection. Infected seedlings can be distinguishable by the presence of powdery clusters of yellow-green spores and shrivelled/ desiccated characteristics [18].

However, under favourable conditions, the sclerotia germinate to produce additional hyphae (later forms mycelium) or new conidia. The conidia serve as a new source of inocula that is dispersed into the environment through wind or insect-mediated pollination [9], increasing the population of *A. flavus* [15]. Once in contact with a new host plant, the pathogenic colonization is enhanced by favourable growth conditions, nutritional resources, and the presence of leaf-feeding insects, which either provide entry points or serve as vectors for the pathogen [9], [18]. Additional symptoms manifested in infected peanut plants include the development of necrotic lesions, chlorosis on the above-ground portions, as well as aflaroot – reduced/lack of development of secondary roots [18]. A visual representation of the *A. flavus* life cycle can be found in Figure 1.

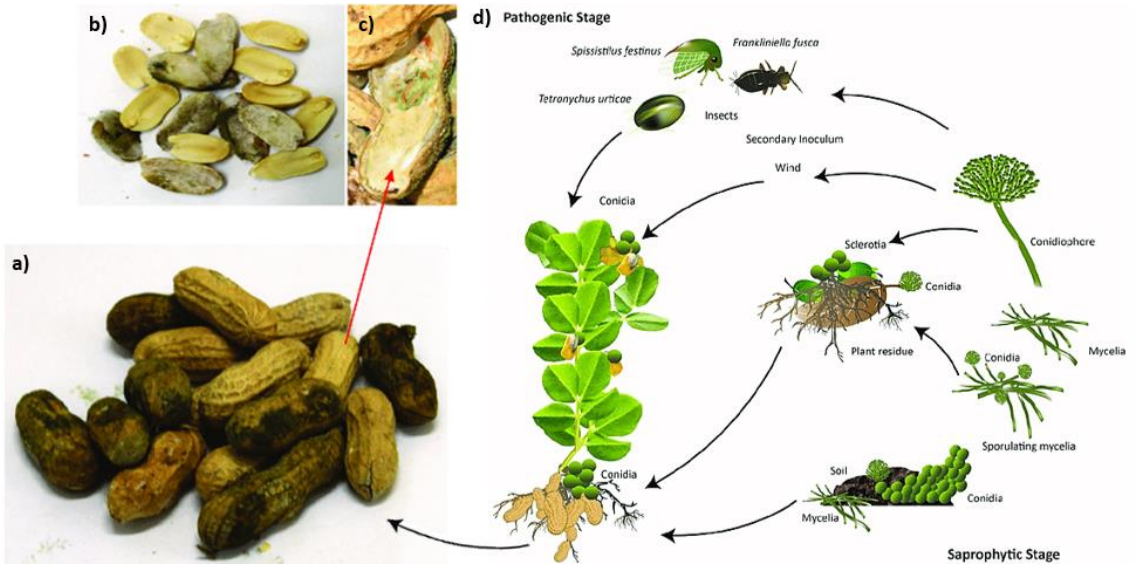

Figure 1. a) Closeup of pod shells (a) and peanut kernels (b) showing symptoms of infection. c) shows sporulation (spore production) on the surface of the peanut. d) the life cycle of Aspergillus flavus *in peanuts. Adapted from Alam, Tariq & Rustgi, Sachin & Anco, Daniel. (2020). Management of Aflatoxins in Peanut. Clemson University* [9]

1.2) Pathogenicity in other crops

As an opportunistic pathogen and saprophyte, *A. flavus* has a broad host range. These fungi are predominantly found in fat-containing crops (peanuts, tree nuts) and oilseeds (cotton, sunflower, soybean, sesame), but are also frequently found in cereals (sorghum, barely, maize, rice and wheat) along with various spices (turmeric, coriander, ginger, black pepper and chilli) [10]. More recently, reports have emerged regarding their presence in various fruits and vegetables [20]. The occurrence of *A. flavus* has been comprehensively studied. Explicitly, under favourable environmental conditions and inadequate storage conditions, the pathogen has been noted to thrive and produce aflatoxin in nearly all types of crop seeds. Plus, *A. flavus* is also known to cause opportunistic infections in immunocompromised humans and animals [6], [21]

In peanuts, *A. flavus* causes a seedling disease known as yellow mould of seedlings or aflaroot [22]. This disease leads to the deterioration of emerging seedlings, primarily caused by the rapid infection of the radicle and hypocotyl [17], [22], consequently reducing seed viability and germination rates [23]. While the infection can extend throughout the entire plant, it is noteworthy that the stems and roots, particularly those in close proximity to the soil, are more frequently affected than the leaves and petioles compared to the leaves and petioles [24].

In corn, *A. flavus* is responsible for one of the most common fungal diseases known as Aspergillus ear rot [25], [26]. The symptoms resemble the ones observed in peanuts, yellow-green mould patches that appear powdery on the kernels. *A. flavus* sclerotia in soil and mycelia act as primary inoculum for infection and are brought to the surface of

developing seeds by insects and wind, leading to infection of ears through the silks [27]. Once the conidia germinate in the ear, it rapidly progresses to the glumes until reaching and colonizing the kernels [28].

In cotton, the aflatoxigenic fungus causes boll rot, a yellow spot disease that affects the fibers affecting cotton quality [29]. The process through which *A. flavus* contaminates cottonseeds remains unclear and literature is scarce. It is suggested that initially, pathogenicity is associated with damages to developing bolls and partial suture opening; followed by mature cotton exposure to high humidity and temperature during pre- and post-harvest stages [30]. Moreover, experimental studies suggest that the fungus could enter seedlings, flowers and developing bolls early in the season and affect bolls maturing in late August/September [31].

Lastly, numerous researchers have documented instances of *A. flavus* infection and aflatoxin contamination in red chilli peppers worldwide [32]. These peppers are mainly cultivated in warm semi-arid regions characterized by high temperatures and humidity conditions that are conducive to fungal infections and subsequent aflatoxin contamination [33], which occurs in pre- and post-harvest stages [34]. Nevertheless, the precise mechanisms underlying aflatoxin contamination in chilli peppers have yet to be thoroughly explored, and more investigation is required to elucidate the infection process. Similar concerns extend to the contamination of fruits and vegetables.

1.3) Factors influencing *A.flavus* infection and Aflatoxin contaminations – control strategies

Before the **harvest**, the presence of *A. flavus* and aflatoxin contamination is subject to an array of factors, including meteorological conditions, soil composition and biome, agricultural practices, and the production of phytoalexins [35]–[37].

a. Environmental conditions

High temperatures coupled with drought have long been recognized as basic variables for aflatoxin contamination in peanuts [12], [38]–[40]. This set of factors is known to affect plants' physiology and subsequently make them more susceptible to infections. For instance, Dorner et al. (1989) noted that phytoalexins are inhibited by drought stress and that in immature peanuts, aflatoxin contamination did not occur until the antimicrobial compound production ceased in drought-stressed plants [41]. Additionally, drought stress increases proline production in plants [42], which has been reported to enhance aflatoxin occurrence [43].

Various studies have also demonstrated that both temperature and water activity levels exert an influence on the growth rate of *A. flavus* as well as the expression of genes involved in aflatoxin biosynthesis and regulation [44]–[46]. The occurrence of *A. flavus*

and aflatoxin contamination has been observed across a wide spectrum of temperatures (T) (ranging from 12 to 48°C) and water activity (a_w) values (ranging from 0.86 to 0.96) [47], [48]. However, fungal occurrence is not conducive at temperatures below 25 °C and above 37 °C, whereas at $a_w < 0.85$ their progression is slowed, and ceases at values between 0.70 and 0.75 a_w [49], [50]. Furthermore, Schmidt-Heydt et al., (2010) showed that T × a_w interactions influence the expression of *aflS*/*aflR* - two pivotal regulatory genes in the aflatoxin biosynthesis pathway. Namely, the ratio of expression of aflS:aflR was higher when high amounts of aflatoxin were produced. Subsequently, Yu et al., (2011) demonstrated that higher temperature or water activity levels were associated with the down-regulation of *aflS* and of *laeA.* In *A. flavus*, *laeA* is required to produce aflatoxin [21] and its deletion may result in the absence of sclerotia production [52].

b. agricultural practices

Improper agricultural practices play a vital role in the infection cycle of *Aspergillus* inoculum in the field [53].

1. Irrigation

Studies have shown that optimum irrigation will reduce fungal infection and aflatoxin contamination in peanuts, as well as in maize [39], [54]–[57]. As it was mentioned before, field contamination by aflatoxin is associated with high temperatures and drought stress, therefore, a well-timed irrigation system, which reduces drought stress and soil temperatures is recommended [9]. Plus, the water used for irrigation is suggested to be of suitable quality for the intended use [58]

2. Insect Management

As previously mentioned, the damage caused by insects to plant tissue is associated with the occurrence of *A. flavus* infection and aflatoxin contamination, as it provides entry points for the fungus or serves as a vector to infect healthy plants [18]. Hence, surveillance and the use of insecticides, pesticides and insect-resistant cultivars are advised to mitigate insect damage and control fungal growth and subsequent aflatoxin production [59].

It is worth noting that the use of bio- and chemical control agents poses the risk of generating fungal-resistant strains; potential contamination- affecting food safety; acting as a source of environmental pollution - toxic residues and undesirable biological effects; besides being time-consuming and costly [60], [61]. Nevertheless, various studies have reported their success in repressing aflatoxin contamination [62]–[64]. For instance, Bowen and Mack (1993) showed that peanuts treated with insecticide larvae of *Elasmopalpus lignosellus* had a lower level of *A. flavus* infection [65]. Likewise, peanuts treated with *A. flavus* NRRL 21882, a pesticide active ingredient, were shown to contain lower amounts of aflatoxin than untreated crops, via outcompeting and displacing *A. flavus* [66]. Lastly, transgenic peanuts containing the Bt (*Bacillus*

thuringiensis) gene exhibited lower levels of aflatoxin than non-Bt peanuts in preliminary analysis of log-changed information [18], [59].

3. Weed management and crop rotation

A.flavus has a wide range of host plants. Hence, an effective strategy to break the pathogen growth entails proper management of weeds (that may act as alternate hosts); disposal and management of crop residues; as well as selection of suitable non-host rotation crops, like sweet potato, to minimize the rate of between-season survival of pathogenic species [35], [67]. For instance, Abrahan et al. (2016) assessed the effect of crop rotation on groundnuts and showed its effectiveness in reducing aflatoxin contamination levels [68]. In addition, it is important to take into account the significance of planting conditions. For instance, in semi-arid environments favourable to *A. flavus*, the practice of crop rotation may have little to no impact on fungal activity [58].

c. Soil composition

As a repository of microorganisms, the soil stands as the primary source of infection for *A. flavus* [9]. The aflatoxigenic fungus has been isolated from soils across various major biomes and climate zones, but it exhibits a higher prevalence in regions characterized by warm temperatures [18]. Furthermore, the composition of the soil plays a pivotal role in *A. flavus* proliferation. Light sandy soils, particularly in dry conditions, have been observed to favour the growth of *A. flavus*, in contrast to heavier soils with greater water-holding capacity, which tend to reduce aflatoxin contamination [12], [53]. On top of that, the presence of other fungi in the soil, such as Penicillium and Fusarium species, has been shown to mitigate aflatoxin contamination, possibly due to competitive inhibition [69]. Lastly, the application of lime, calcium, farmyard manure and residues from cereal crops to the soil has been shown to reduce aflatoxin contamination in groundnuts by 50% to 90%. This reduction can be attributed to calcium's role in fortifying the peanut cell wall, creating a physical and biochemical barrier. Additionally, farmyard manure helps provide optimal growth conditions for various beneficial microorganisms, which play a crucial role in suppressing soil infections[35], [70].

During **harvest**, practices such as digging and threshing, which can result in mechanical damage to peanut pods, enhance the susceptibility to *A.flavus* contamination [35], [59]. Hence, safe harvesting procedures to avoid damage and contamination to peanuts should be implemented; and all the equipment and machinery employed in the harvesting process should undergo thorough cleaning and maintenance to ensure they do not serve as potential sources of contamination [58]. Moreover, harvesting before optimum maturity or a delay in harvesting also exerts an influence on A. flavus infection and aflatoxin contamination. Immature plants are predominantly more susceptible to the fungus infection, whereas a delay in harvest provides an opportunity for *A. flavus* to produce aflatoxin [6], [38], [39].

In turn, **post-harvest** management mainly focuses on mitigating aflatoxin contamination [35] as the compound is challenging to eliminate once *A.flavus* infection occurs [16], [35]. Therefore, post-harvest management strategies fall to create an environment that is non-conductive for infection and to avoid proliferation [9].

a. Separation of healthy/infected pods

Prior to storage, the removal of aflatoxin-contaminated seeds plays a pivotal role in minimizing aflatoxin levels during post-harvest phases [63]. This is achieved through either manual or electronic colour sorting, targeting discoloured peanuts, which are indicative of shelled peanuts infected by *A.flavus* [64], [71] as well as damaged, shrivelled, misshaped seeds/pods [9].

b. Storage and transportation conditions

Generally, contamination is largely due to improper handling of the drying, storage, and transport conditions that contribute to mould formation [6]. Namely, aflatoxin contamination occurs when pod moisture levels are above 8% and the temperature is over 25 °C. Hence, to keep an expansion in aflatoxin occurring during storage and transportation, it is essential to maintain moisture and temperature levels below that which will enhance growth [18]. For such, it is recommended to dry the pods to set moisture content and place them in clean non-contaminated storage containers [16], [58].

2) *A.flav*us genomics and Aflatoxin biosynthesis – Genetic Regulation

A. flavus genome has already been sequenced, and a complete genome microarray is available and has been used to study the genetic regulation of aflatoxin biosynthesis [72]. The genome size of the aflatoxin-producing fungus is expected to be approximately 36 Mega base pairs (Mb), organized in 8 chromosomes, with about 12,197 predicted genes. Moreover, the *A. flavus* genome has been described to be compacted with less duplicated sequences or multiple copies of genes [21], [49], [72].

As one of the most toxic and carcinogenic secondary metabolites, significant efforts have been made to better understand the biochemistry, genetics, and regulation of aflatoxin biosynthesis [8], [21], [72], [73]. Aflatoxins are produced by a complex and highly regulated polyketide pathway [74], that involves at least 27 enzymatic reactions with several bioconversion steps. Currently, the generally accepted AFB1 aflatoxin biosynthetic pathway is as follows: acetate → polyketide → norsolorinic acid (NOR) → averantin (AVN) → 5'-hydroxy-averantin (HAVN) → oxoaverantin (OAVN) → averufin (AVF) → hydroxyversicolorone (HVN) → versiconal hemiacetal acetate (VHA) → Versiconol acetate (VOAc) → versiconal (VOH) → versiconal (VAL) → versicolorin B

(VERB) → versicolorin A (VERA) → demethylsterigmatocystin (DMST) → sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) → 11- Hydroxy-O-methylsterigmatocystin (HOMST) → aflatoxin B1 (AFB1) [8]. For more information on the genes involved in the major conversion steps from early precursors to aflatoxins and their functions, please refer to Caceres et al. (2020). *Aflatoxin Biosynthesis and Genetic Regulation: A Review*. Toxins (Basel).

To date, about 30 genes involved in aflatoxin biosynthesis have been identified which are grouped in the "aflatoxin biosynthesis gene cluster", within a 75-kilobyte (kB) region on chromosome 3's subtelomeric locus in the fungal genome [21], [75]. All these genes exhibit a consistent regulatory pattern, and their expression levels can directly affect the level of aflatoxin biosynthesis [73]. Moreover, these aflatoxin biosynthesis pathway genes are mainly symbolized by a three-letter code "afl" followed by a capital letter from "A" to "Y", characterizing each individual gene involvement [72]. Some notable examples of genes within the aflatoxin biosynthesis cluster include the *nor-1* and *ver-1* genes, responsible for the production of key intermediates. The *aflD (nor-1)* gene is responsible for the conversion of norsolorinic acid (NOR) to averantin (AVN), and the *ver-1 (aflE)* gene encodes versicolorin reductase, which catalyzes the conversion of versicolorin A to versicolorin B. Moreover, *aflP (omtA)* codes for an O-methyltransferase that methylates versicolorin B to produce demethylsterigmatocystin. Other genes, such as *aflK, aflN, aflM, aflX* and *aflL* are involved in the formation of different precursors during the polyketide synthesis, that serve as substrate reactions for aflatoxin synthesis [8], [75].

The expression of the aflatoxin biosynthesis pathway genes is coordinated by two cluster-specific regulators: *aflR* and *aflS.* These two genes, located adjacent to each other within the cluster, are divergently transcribed and have independent promoters. However, the region between the *aflR* and *aflS* is relatively short, and they may share binding sites for transcription factors or other regulatory elements [73], [76]. The *aflR* gene encodes a sequence-specific DNA-binding protein with a binuclear zinc cluster (Zn(II)2Cys6), required for the transcriptional activation of other genes involved in the biosynthetic pathway, thus acting as a positive regulator. Notably, overexpression of *aflR* has been demonstrated to result in a 50-fold increase in aflatoxin production [77], whereas deleting *aflR* in *A. flavus* suppresses the expression of aflatoxin pathway genes [78]. In addition, the application of different inhibitors of aflatoxin production has been shown to effectively reduce the expression of *aflR* [79]. As for the *aflS* gene, its precise role in aflatoxin biosynthesis remains unclear [73]. However, overexpression of *aflS* in *A. flavus* has been observed to result in higher levels of aflatoxin production, thus acting as an enhancer [80]. Besides, Chang P.K (2003) observed that a*flS* binds to *aflR* in *A. parasiticus* and argues that *aflS* modulates aflatoxin expression through its interaction with *aflR* [76].

Additionally, aflatoxin as a secondary metabolite, is co-regulated with fungal development. Numerous regulatory factors that govern fungal development have been identified, and some of them control genes involved in aflatoxin production. For instance, Cary et al. (2017) reported that the disruption of the *hbx1* gene resulted in the loss of production of sclerotia, conidiophores, conidia, and aflatoxin. Subsequently, down-regulation in the expression levels of aflatoxins biosynthetic genes (*aflM, aflD, aflC*), regulatory gene (*aflR*), conidiophores biogenesis genes (*flbA*, *flbC*, *flbD*, and *flbE*) and conidiation regulatory pathway genes (*brlA* and *wetA*) was observed [81] [82]. The transcription factors *NsdC* and *NsdD* have also been reported as essential for the production of asexual sclerotia, normal aflatoxin biosynthesis, and conidiophore development in *A. flavus* [83], [84]. Research conducted by Zu et al. (2020) found that *A. flavus cta1* deletion mutant resulted in a down-regulation of the expression levels of *aflQ*, aflC, and *aflD,* showing that *cta1* plays an important role in aflatoxin biosynthesis in *A. flavus* [85]. Moreover, two APSES transcription factors, *AfRafA* and *AfStuA*, have been recognized for their roles in aflatoxin synthesis, fungal development and consequently pathogenicity [86]. Additionally, Wang et al. (2020) suggested that basic leucine zipper (bZIP) transcription factor *AflRsmA* could regulate sclerotium formation, oxidative stress response, and aflatoxin biosynthesis in *A. flavus* [87]. The *AflSkn7* transcription factor has also been discovered to influence morphogenesis, pathogenicity, aflatoxin production, and stress response in *A. flavus* [88]. Hu et al. (2018) discovered that the plant homeodomain (PHD) transcription factor *Rum1* regulates morphogenesis and aflatoxin biosynthesis *A. flavus* [89]. In addition, Yuan et al. (2019) observed a notable decrease in aflatoxin, conidia, and conidiophore production with the deletion of the *hexA* gene, while the deletion of *sakA* resulted in reduced mycelial growth and increased sclerotia and aflatoxin production [90]. Another study discovered that deletion of *aflPex5* was found to cause defects in sporulation, sclerotia formation, aflatoxin biosynthesis, stress response, and crop infection [91]. Similarly, the nucleoside diphosphate kinase (*Aflndk*) role in sclerotia production and conidia development in *A. flavus* was also shown using gene-knockout [92]. It has also been revealed that gene knockout experiments of either *VeA* or *laeA* genes (Velvet complex) resulted in loss of aflatoxin and sclerotium production [93]–[95]. Plus, deletion of *hamF*, *hamG*, *hamH*, *hamI*, showed near identical phenotypes to laeA mutants - sclerotia loss and reduced aflatoxin synthesis [96]. And lastly, *Spc105*, interaction partner of *LaeA*, has also been shown to be required for normal conidiophore development and sclerotia production of *A. flavus* [97].

Finally, aflatoxin regulatory mechanisms are also triggered by exposure to environmental changes, nutrient sources, as well as oxidative stress response. Ultimately, these elements have the capacity to activate various cell signaling pathways, therefore modulating the expression of genes responsible for aflatoxin synthesis [8], [31]. Hence, a comprehensive understanding of the genetic mechanisms underlying

aflatoxin production and external stimuli enables researchers to devise effective strategies to control fungal toxicity.

3) Peanuts' Resistance to *A. flavus* Infection and Aflatoxin Contamination

Over the years, researchers have tried to understand the mechanisms underlying peanuts' resistance to *A.flavus* infection and the subsequent prevention of aflatoxin contamination. This resistance is a multifaceted trait that involves several aspects. Similarly to maize, peanuts' resistance to *A.flavus* and aflatoxin contamination is a sum of three different mechanisms: 1) *in-vitro* seed colonization resistance (IVSC); 2) resistance to pre-harvest aflatoxin contamination (PAC); and 3) resistance to aflatoxin production following infection (AP) [98], [99]. Overall, resistance to a pathogen is determined by a plant´s genetic makeup and it can be due to a single gene in the host plant (monogenic) or to more than one gene (polygenic). Peanuts 'resistance to *A. flavus* is quantitative in nature, meaning it involves the interaction of multiple genes, making it challenging to breed for. In addition, it is genotype-specific, hence, resistance can vary among different peanut genotypes [13]. Lastly, it is highly influenced by Gene-environment (G x E) interactions [48], [100]. Though the additive gene effect plays a more substantial role in resistance [48], the environment's influence on genotype performance leads to inconsistent phenotypic outcomes. This variability, in turn, limits the widespread utilization of germplasm [16]. On top of this, the absence of reliable phenotyping protocols also imposes a prevalent technical drawback that further complicates traditional peanut breeding efforts [101], [102].

3.1) Constitutive components in peanut shells and seed coats associated with resistance

The peanut shell and seed coat are key players in mitigating *A. flavus* infection [16]. Previous research has demonstrated that resistance to pod infection is primarily attributed to the structure of the pod shell [100], which serves as an initial physical defence mechanism against fungal infection [103]. As for the seed coat, being the outermost layer of peanut kernels, it acts as a physical and chemical barrier against *A. flavus* infection, [104] and its resistance is attributed to variations in its structure and composition [100]. In this context, various researchers have attempted to characterize features in peanut shells and seed coats relative to resistance. Respectively, LaPrade´s research group suggested that resistance might be attributed to seed coat thickness and permeability [105]. Taber et al. (1973) revealed that kernels of resistant genotypes exhibited smaller hilum and a more compact arrangement of a palisade-like layer of testa compared to susceptible genotypes [106]. Later, Guimarães et al. (2012) noted that in wild peanut germplasm species, the pod shell and seeds are rich in lignin,

protecting against mechanical, biotic and abiotic stressors [107]. In addition, Liang et al., (2003) also indicated the physical-barrier role of wax and cutin layers of peanut seed coats in resistance to *A. flavus* infection[108].

Beyond physical properties, the presence of biochemical compounds has also been reported to confer *A. flavus* resistance. Various phenolic compounds (such as p-coumaric acid, ferulic acid, hydroxybenzoic acid, and chlorogenic acid), flavonoid compounds (notably epicatechin), and other chemical substances (such as quercetin and resveratrol) have been demonstrated to be present in peanut seed coats using High-performance liquid chromatography (HPLC) analysis [109], [110]. The role of phenolic compounds in inhibiting *A. flavus* growth has been well-documented and examined in various studies [111]–[113]. In addition, Tannins are secondary metabolites with known antioxidant and antimicrobial properties, that are deposited in seed coat cell walls to protect the seed form invading pathogens [104]. Lindsey and Turner (1975) demonstrated that the presence of Tannins in peanut testa inhibits the growth of *A. flavus in vivo* [114]. Similarly, Liang et al. (2003) and Turner et al. (1975) reported on trypsin and 5,7-dimethoxy isoflavone association with *A. fl*avus resistance, respectively [115] [116]. More recently, Sharma et al. (2021) noted a strong association between pipecolic acid (Pip) accumulation with peanut seed resistance against *A.flavus* infection [117].

3.2) Induced defence mechanisms

When peanuts are infected, they activate their immune system, which involves two major layers of defence: pathogen-associate molecular patterns (PAMPs)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is initiated when cell-surface pattern recognition receptors (PRRs) are activated by pathogen-associated molecular patterns (PAMPs). This triggers early resistance responses, including activation of mitogen-activated protein kinase (MAPK) cascades, transcription of resistance-related genes, and reactive oxygen species (ROS) production, among others. Meanwhile, ETI is triggered by the recognition of pathogen effectors by plant intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs) and is often associated with localized programmed cell death [118], [119].

In a recent study by Cui et al. (2022), weighed gene co-expression network analysis (WGCNA) and comparative transcriptomics of peanut genotypes (J11, R x Zhongua-12, S) led to the identification of various genes involved in *A. flavus* defence response. Namely, two highly expressed MAP kinases (*arahy. L410JY* and *arahy. BC5GM2*), along with Cytochrome P450, were identified during *A. flavus*-induced defense responses. Pattern recognition receptors (PRR) such as *RPVOD7* were discovered to induce peanut PTI by the recognition of *A. flavus* PAMPs. In addition, six NBS-LRR genes were identified

using High-throughput sequencing, and shown to be upregulated during *A. flavus* infection, suggesting their role in ETI. Serine threonine kinase (STK) *arahy. D2YYPY*, which recognizes *A. flavus*-released effectors, was also identified and associated with ETI responses [120]. The plant recognition and infection by *A.flavus* have also been reported to be facilitated by fungal oxylipins and pathogen elicitor molecules, while host oxylipins inhibit aflatoxin biosynthesis [6]. Jasmonic acid (JA), salicylic acid (SA) and ethylene signalling pathways are mediated by a dynamic network of transcription factors (TFs), like bZIP, WRKY, ERF, MYB, MYC, and NAC, [121], [122]. These TFs further serve as crucial regulators in the production of reactive oxygen species (ROS), Pathogenesis-related (PR) proteins, and Phytoalexins accumulation, that play major roles in resistance after infection [99], [122]–[124]. Respectively, the peanut-defence mechanism involves oxidative homeostasis in response to the accumulation of ROS upon *A.flavus* infection and drought stress [102], [125]. As shown by Liang et al. (2006), the outburst of ROS is accompanied by an increase of LOX (lipoxygenase) activity, which may cause changes in lipid peroxidation (oxylipins synthase), cell wall strengthening, phytoalexin synthesis and hypersensitive cell death in peanut seeds [126]. Additionally, higher expression of LOX has also been reported to induce signal molecules like JA, methyl JA, fatty acids and secondary metabolites that target pathogens [102], [127], [128]. The plant defence response also involves various pathogenesis-related proteins (PR) proteins, which have been reported as predominantly expressed in peanut-resistant cultivars and associated with *A. flavus* resistance. For instance, Liang et al. (2001) and Dixon et al (2002) observed that Phenylalanine ammonia-lyase (PAL), and soluble glutathione transferases (GSTs), exhibited significantly higher levels in resistant peanut cultivars than in susceptible ones. Pal is a precursor of both lignin and phytoalexins, whereas GSTs under stress have peroxidase activity that protects the cells from oxidative injuries [129]. Similarly, Chitinase and β-1-3-glucanase were shown to exhibit higher levels in resistant peanut genotypes (GT-YY9 and GT-YY20) compared to susceptible genotypes (Georgia Green and A100) after *A. flavus* inoculation [130], [131]. β-1,3-glucanase in particular, can digest fungal cell walls and produce higher levels of elicitors both at transcript and protein level during *A.flavus* infection [99], [122]. Moreover, Phytoalexin like Resveratrol, are antibiotic secondary metabolite that have been shown to inhibit spore germination and hyphal extension of *A.flavus* [132]. Interestingly, under drought stress conditions, the levels of phytoalexin in peanut cultivars were observed to be reduced, which correlates with the increased susceptibility of drought-exposed peanuts to *A. flavus* infection [133]. Lastly, quercentin, an antioxidant flavonoid compound, has been shown to inhibit the proliferation of *A.flavus*, by repressing the expression of aflatoxin biosynthetic-related genes [134]. Please consult Table 2 for a concise overview of the transcriptomic and proteomic findings about important genes and pathways associated with aflatoxin contamination in peanuts.

4) Genetics and molecular bases of peanut resistance to *A.flavus*

The breeding of varieties with resistance to *A. flavus* infection has been a primary objective in peanut breeding programs and was recognized as the most cost-effective measure to mitigate aflatoxin contamination [135]. However, this pursuit often results in a bottleneck due to peanuts' narrow genetic diversity and understanding of genetics [11]. More precisely, the lack of desirable parental genotypes with stable and effective resistance levels [101]. Nonetheless, through screenings of peanut germplasms, breeders have found various genotypes with considerable resistance levels [13]. As mentioned previously, the resistance trait against *A. flavus* infection is genotype-specific, influenced by environmental conditions and quantitative in nature, making it challenging to breed for. Thus, resistance levels can be defined through a combination of methods and criteria. These include phenotypic assessment for signs of infection, as well as aflatoxin quantification and disease Incidence (percent seed infection index (PSII)) as described by Yu et al. (2019) [136]. In addition, researchers also make use of QTL analyses and Molecular markers to identify genomic regions and genes associated with resistance, followed up by functional genomic studies. This information not only provides valuable insights into the molecular mechanisms involved in peanut-pathogen interactions but also establishes a basis for more targeted and efficient breeding programs aimed at developing peanut varieties with enhanced resistance to *A. flavus* and aflatoxin production.

A well-known example of a peanut-resistant germplasm to *A. flavus* infection is the Indian commercial variety J11 [3]. J11 resistance was been reported to be related to drought stress, pod maturity [137] and seed coat integrity [138]. Through *in vitro* seed colonization (IVSC), J11 was first discovered by Mehan et al. (1981) to be resistant to *A.flavus*, and later to *Aspergillus parasiticus* by Kisyombe et al. (1985) [139], [140]. Subsequently, J11's stable resistance to *A.flavus* infection has been repeatedly proven in various studies [139], [141]. Transcriptomic and proteomic analyses led to the identification of 663 differentially expressed genes (DEGs) and 314 proteins (DEP) of peanut cultivar J11 as a result of *A. flavus* infection [142]. In another study, the RNA-seq approach was deployed to understand the host-pathogen interaction and 4,445 DEGs were identified in different combinations across four stages after inoculation in J11 and JL 24 (a susceptible genotype) [99]. Subsequently, genome-wide association (GWAS) studies along with quantitative trait locus (QTL) mapping have been conducted to pinpoint specific genes and genomic regions associated with resistance to *A. flavus* infection in J11. These findings will be detailed in the next section.

4.1) Quantitative Trait Locus (QTL) Mapping

Liang et al. (2009) identified six QTLs associated with *A. flavus* infection in three independent RIL mapping populations (Yueyou 13 x Zhenzhuhei, Yueyou13 x Fu95-5 and

Yueyou13 x J-11) using an SSR-based genetic linkage map [143]. The un-named QTLs were not specified in the article, however, three were located on chromosomes A01, A02 and B05, respectively, and showed a phenotypic variation explained (PVE) > 10 %.

Yu et al. (2019) identified individual QTLs for the percent seed infection index (PSII) and the contents of aflatoxin AFB1and AFB2 via a RIL population obtained from a cross of Zhonghua 10 (susceptible) × ICG 12625 (resistant), harvested from 3 environments. Transgressive segregation and continuous distribution in the RIL population for both PSII and aflatoxin contents suggest that both parents had favourable alleles for resistance to aflatoxin contamination. In total, 14 QTLs were identified and distributed on different chromosomes. The major QTL for pathogen infection –*qPSIIA10* - was mapped on LG A10 and identified as consistent - repeatedly detected in two environments and explained 11.32-13% PVE. The major QTLs for resistance to aflatoxin production *qAFB1A07* and *qAFB1B06*.1 were co-localized with *qAFB2A07* and *qAFB2B06* - exhibited major and stable effects across multiple environments with 9.32–21.02% PVE. Also, a strong interaction between resistance to the production of AFB_1 and AFB_2 was confirmed by conditional QTL mapping. Lastly, two major QTLS *qAFB2B05* and *qAFB2B07*, were further exclusively detected in a single environment with 11.05–14.45% PVE. Genotyping of RILs revealed that *qAFB1A07* and *qAFB1B06*.1 interacted additively to enhance the resistance to AFB1 and AFB2 accumulation. As a result, the author suggests the combination of the resistant alleles of these QTLs as an effective strategy for increasing peanut resistance against aflatoxin contamination [136]. Still, it is important to note that the loci responsible for aflatoxin resistance in peanuts have not been delimited in relatively small genomic intervals [144].

Meanwhile, Khan et al. (2020), identified two QTLs by utilizing SNP based genetic map using specific length amplified fragment sequencing (SLAF-Seq), on a RIL population obtained from a cross between Xinhuixiaoli (resistant) and Yueyou 92 (susceptible), during *in-vitro* seed colonization (IVSC). Respectively, major QTL *qRAF-3-1* was mapped within 1.67 cM and explained 17.17% of PVE, while minor QTL *qRAF-14-1* was located within 1.34 cM with 5.15% PVE (Table 3). QTL *qRAF-3-1* was located on A03 and had a positive additive effect, while *qRAF-14-1* on B04 was found with a negative additive effect for the trait of resistance to *A. flavus* infection. Showing opposite additive effects, the two QTLs are supposedly derived from different parents. Hence, Xinhuixiaoli is regarded as the donor of the main QTL *qRAF-3-1*. Additionally, wide segregation in the RIL population against *A. flavus* infection was detected, which demonstrates transgressive segregation in resistance. Lastly, 67 and 137 candidate functional genes were discovered within the genomic regions of *qRAF-3-1* and *qRAF-14-1*. Using microarray analysis, putative disease resistance RPP13-like protein 1 (RPP13), Pentatricopeptide repeat (PPR) proteins and LOX genes responded to *A. favus* infection and were proposed for further investigation towards A. *flavus* resistance in peanuts [145].

Additionally, Jiang et al. (2021) identified six QTLs for *A. flavus* infection resistance on chromosomes A05, A08, B01, B03, and B10 via a RIL population derived from a cross

between Zhonghua 16 (susceptible) × J11 (resistant). Major QTL *qPSIIA08* was detected in one environment with a PVE of 10.87%, whereas the stable QTL *qPSIIB10* was consistently detected in 4 years, showing 6.91–10.58% PVE. The positive additive effects of *qPSIIA05, qPSIIA08, qPSIIB01, qPSIIB03.a*, and *qPSIIB03.b* indicate that the favourable alleles were from J11 and confer resistance to infection. However, the favourable alleles of the stable QTL qPSIIB10 (with a negative additive effect), were from the susceptible parent Zhonghua 16. This suggests that susceptible germplasms might possess valuable loci for resistance against *A. flavus* infection, and so it is not the QTL, but its desirable allele, which needs to be selected for in breeding. Also, further analysis of the phenotypic effect of pyramiding QTLs was performed, and the results demonstrated that pyramiding all favourable alleles of the six major QTLs could significantly enhance resistance [3].

In continuity, Jin et al. (2023) detected eleven additive QTLs for aflatoxin production resistance via an RIL population with the same genotypes Zhonghua 16 (susceptible) × J11 (resistant). Three major QTLs on chromosomes A05, B05 and B06 were identified: *qAFTB05.2* was only detected in one year with 9.90% PVE (≈10 PVE hence it was regarded as a major QTL); *qAFTA05.1* was consistently detected in three years, showing 5.99–11.42% PVE; *qAFTB06.3* was repeatedly detected in two years with 8.23–10.63% PVE. In addition, conditional QTL analysis was performed with conditional phenotypic values for Aflatoxin content and percent seed infection index (ATC|PSII). The results revealed that the major QTL *qAFTB05.2* was not found in conditional mapping and the qAFTA05.1 decreased the additive effect compared to that of the unconditional QTL However, the additive effect of the major QTL *qAFTB06.3* was significantly higher than that of the unconditional QTL. Analysis of the phenotypic effect of pyramiding QTLs was also performed in this study, and the result suggests that pyramiding favourable alleles of major QTLs could decrease aflatoxin production. Respectively, the favourable alleles of *qAFTA05.1* and *qAFTB06.3* were from J11, whereas the favourable alleles of *qAFTB05.2* were from Zhonghua 16 [146].

Lastly, Yu et al. (2023) identified four QTLs on A02, A07 and B07 for aflatoxin production resistance via a RIL population RIL population derived from the cross between Xuhua13 (susceptible) and Zhonghua 6 (resistant). Among them, one major QTL *qAFTsA07.1* was stably detected across three environments and could explain 13.39% of PVE. Fine mapping later revealed that the allele in *qAFTsA07.1* from Zhonghua 6 has a negative effect on aflatoxin content and confers resistance to aflatoxin production [144].

For a comprehensive summary of the data collected, please refer to the supplementary material– Table 1: *Bi-parental QTL mapping of genomic regions controlling aflatoxin contamination in peanuts*

4.2) Functional genomics

Genome-wide association studies (GWAS) have emerged as a powerful tool to detect markers (SNPs) closely associated with peanut resistance to *Aspergillus flavus* and aflatoxin contamination. A recent study Yu et al., (2020) identified two resistant accessions (*Zh.h0551* and *Zh.h2150*) and 60 SNP (single nucleotide polymorphism) markers associated with aflatoxin production (AFB1 and AFB2) with 16.87%–31.70% PVE (Table 4). This was done through GWAS of 99 accessions of the Chinese peanut mini-mini core collection OCRI-CAAS, Wuhan, China. Interestingly, a total of 18 association peaks, distributed across 11 chromosomes were identified as associated with aflatoxin content in peanut seeds across different environments. Among these, 2 were linked to both AFB1 and AFB2, four were associated with AFB1 alone, and two were associated exclusively with AFB2. These findings suggest that the resistance to both AFB1 and AFB2 in peanut seeds largely share the same mechanism controlled by multiple genes. Moreover, two genes encoding a leucine-rich repeat (LRR) domain were identified in chromosome B01 in a 50 kb candidate genomic region of the SNP marker SNP19994. In the same genomic regions, also a gene encoding WRKY TF was identified. In plants, the LRR domain has been established to play a crucial role in the mechanism of plant disease resistance - serving as the initial line of defence against pathogens. Lastly, the SNP marker SNP02686, which exhibited the highest PVE for AFB2, also displayed an association with AFB1 in one environment (2017). This SNP was located 33.18 kb from the *Aradu.WAPPM* gene, which is predicted to encode ATP-citrate lyase (ACLY). This enzyme is responsible for generating cytosolic acetyl-CoA and oxaloacetate, key substrates of the aflatoxin biosynthetic pathway [101].

Several SNP/InDel markers associated with aflatoxin resistance were also identified by GWAS. Ding et al. (2022) identified a total of 16 SNPs/InDels associated with three different resistances through GWAS using 99 accessions from the same Chinese peanut mini-mini core collection mentioned previously (Table 5). Namely, six SNPs/InDels associated with shell infection index (SLII) located on three chromosomes, five SNPs/InDels associated with seed infection index (SDII) distributed on four chromosomes, and five SNPs/InDels associated with aflatoxin content distributed on four chromosomes. The three resistances associated SNPs/InDels were distinctly located, which suggests that these traits might be controlled by different genes. Additionally, candidate genes *Arahy.J7VJ5I* and *Arahy.7ML2J7* were located at the downstream of SNP21021 and upstream of InDel21071, respectively, and code MYB transcription factors. This is noteworthy since MYB transcription factors (TFs) are known to play important roles in regulating lignin biosynthesis. Also, the candidate genes *Arahy.12GONV* and *Arahy.FTX6XU*, for the synthesis of glycosyltransferase, were identified in the candidate interval labelled by InDel21071. In some plants, glycosyltransferase function has been shown to promote lignification. Moreover, eighteen SDII-associated candidate genes were discovered for 2 SNPs and 2 InDels, while fifteen aflatoxin content associated candidate genes were

found for 2 SNPs and 2 InDels. *Arahy.R1ATPI* and *Arahy.1ZVJ53* genes were located at the downstream of SNP22577, which was related to effector receptor (NLR) - can regulate plant disease resistance by pathogenic recognition [147].

Transcriptomic and proteomic analyses have also been used to identify candidate genes involved in resistance response against *A. flavus* in peanuts.
Wang et al. (2010) reported twelve potentially differentially expressed proteins between peanut variety YJ-1 (resistant) and Yueyou 7 (suspectable) under well-watered conditions, drought stress, and *A. flavus* infection with drought stress. These proteins could be divided into four functional groups including defence response, signalling components, regulation of transcription and storage protein [148]. Moreover, Guo et al., (2011) conducted a peanut oligonucleotide microarray chip analysis and identified 62 genes with upregulated expression in the resistant cultivar, and 22 putative *Aspergillus*-resistance genes with high-level expression also in the resistant cultivar. The study used two peanut lines, Tifrunner (susceptible) and GT-C20 (resistant) and reported several important genes including lipoxygenase, late embryogenesis (LEA) proteins, defensins, and PR10, among others, in response to *A. flavus* infection [149] (Table 2). In addition, Wang et al., (2013) used RNA-seq for global transcriptome profiling of post-harvest seed of resistant (Zhonghua 6) and susceptible (Zhonghua 12) peanut genotypes under *A.flavus* infection and aflatoxin production stress, and obtained 128, 725 unigenes, of which 30, 143 were differentially expressed, and 842 are potential defence-related genes [150]. Similarly, Nayak et al. (2017) integrated IVSC and RNA-seq approach on J11 (resistant) and JL24 (susceptible) led to the identification of 4,445 DEGs involved in multiple pathways such as defence-related, PR or metabolic pathways targeting genes [99].
Korani et al. (2018) compared the seed transcriptome of resistant (ICG 1471) and susceptible (Florida-07) peanut cultivars and identified 4,272 DEGs associated with post-harvest resistance. In this study, the author highlighted the importance of WRKY TFs, heat shock proteins and TIR-NBS-LRR in providing resistance [122]. Further, Zhao et al. (2019) transcriptomic and proteomic analyses revealed 663 DEGs and 314 differentially expressed proteins during the infection of J11 peanut by *A. flavus* [142]. More recently, Jayaprakash et al. (2021) transcriptomic network study from publically available RNA-seq datasets of resistant and susceptible peanut varieties infected by *A. flavus* also revealed hub genes involved in peanut immune responses [151]. Cui et al. (2022) identified 18 genes positively associated with peanut resistance to *A. flavus* in two genotypes Zhonghua 12 (susceptible) and J11 (resistant), by comparative transcriptome and weighted gene coexpression network analysis (WGCNA) [152]. Wang et al. (2023) identified a total of 5768 DEGs and 349 differentially abundant metabolites between Zhonghua 6 and Yuanza 9102 during *A. flavus* infection, by transcriptomic and metabolomic analysis [153]. Collectively, these studies provide insight into the molecular mechanism of resistance to *A. flavus* and will contribute to the development of resistant peanut varieties.

4.3) Host-induced gene silencing, RNA interference and overexpression studies

Successful applications of host-induced gene silencing (HIGS), RNA interference (RNAi) and overexpression against the pathogenic fungi and aflatoxin have been reported. For instance, in a study by Sharma et al. (2017), HIGS of aflatoxin biosynthetic pathway regulatory genes *aflM* and *aflP*, was shown to inhibit aflatoxin production. This study also demonstrated that overexpression (OE) of antifungal plant defensins *MsDef1* and *MtDef4.2*, led to reduced *A.flavus* infection and aflatoxin production in susceptible peanut variety JL 24. The combined approach, with HIGS suppressing aflatoxin production upon infection and antifungal defensins boosting genetic resistance to *A. flavus* infection, resulted in durable resistance against various *Aspergillus flavus* morphotypes and nondetectable (or as low as 1–2 ppb) aflatoxin content in several peanut events/lines well [154]. Moreover, Prasad et al. (2023) described a HIGS strategy to simultaneously control *A.flavus* infection and aflatoxin contamination by multiplexed targeting *nsdC*, *veA*, *aflM*, and *aflR* genes, essential in fungal morphogenesis and aflatoxin biosynthesis. The HIGS plants exhibited lower infection and aflatoxin accumulation levels, and subsequent comparative proteomic profiling revealed a notable downregulation of several fungal proteins, such as the fungal sexual development regulator, *VelC*, and aflatoxin biosynthetic pathway proteins, including *AflC*, *AflL*, *AflM*, *AflQ*, *AflR*, *AflS*, *AflV*, *AflW*, *VeA*, and *AflJ*. These findings validate the suppressed growth of the fungus and the diminished levels of aflatoxin production, demonstrating the effectiveness of HIGS as a strategy for resistance in peanuts [155]. In turn, Arias et al. (2015) and Power et al. (2020) demonstrated that silencing five genes involved in aflatoxin biosynthesis - *aflR*, *aflS*, *aflC*, *pes1*, and *aflep* - by RNA interference (RNAi) in transformed peanuts, can also prevent aflatoxin accumulation following inoculation with *A. flavus* [156], [157]. Additionally, overexpression of a chitinase gene (*RChit)* from rice in transgenic peanuts was shown to enhance resistance to *A. flavus* infection [131]. Similarly, overexpression of the PR10 family putative resistant gene *ARAhPR10* reduced *A.flavus* infection and aflatoxin content in peanut transgenic lines [158]. Also, Sundaresha et al. (2009) reported that overexpression of a tobacco β 1–3 glucanase gene in peanuts increased its resistance towards *A.flavus* [159]. More recently, Liang et al. (2023) showed that overexpression of two peanut- susceptible hydroxylase genes (*AhS5H1* and *AhS5H2*) decreased salicylic acid content, increased 2,5-DHBA levels and increased susceptibility to *Pst DC3000*, a common bacterial pathogen. These results suggest that both enzymes had a similar role in planta which was confirmed *in vitro* and transgenic *Arabidopsis* plants. Additionally, transcript levels of defence-related genes suggested that the expression of *AhS5H1* and *AhS5H2* in peanuts is associated with defence mechanisms and tissue-specific [160].

Lastly, in a recent investigation by Yu et al. (2023), the gene named *AhAftr1* (*Arachis hypogaea* Aflatoxin resistance 1) was identified by RNA-Seq analysis on a RIL population derived from the cross between Xuhua13 (susceptible) and Zhonghua 6 (resistant). This gene, annotated as the "NB-LRRs protein gene" exhibited a structural variation (SV) in the LRRs domain, which was subsequently validated to confer aflatoxin production resistance via the ETI pathway in transgenic experiments [144]. These techniques have also been shown to successfully reduce *A.flavus* infection and aflatoxin contamination in maize by targeting the downregulation of the aflatoxin biosynthetic genes *aflR*, *aflM*, *aflC*; and *alk*, *amy1* and *p2c* of *A. flavus* [161]–[166].

5) The potential of Susceptibility genes in Resistance Breeding

For decades, resistant breeding has successfully relied on dominant resistance (R) genes to create resistant plants. However, resistance mediated by a single R gene often lacks durability due to the loss or mutation of the R gene's corresponding effector in pathogens. Hence, the approach of combining multiple R genes within a single genotype, referred to as "gene stacking," has been regarded as a more effective approach for maintaining crop resistance [167]. An alternative approach for acquiring resistant plants is the exploitation of disease susceptibility genes (S genes) [118], [168]. S genes can be defined as genes required to establish compatible plant-pathogen interactions and their impairment can lead to durable, recessively inherited, and potentially broad-spectrum resistance in various crops [118], [169].

S genes are part of various gene families and have a wide range of functions in plant-pathogen interaction through which they can be categorized into three types [170]: 1) genes involved in host entry. A notable example is the *Mildew resistance locus O (MLO)* involved in susceptibility to powdery mildews, where the inactivation of *MLO* prevents fungal penetration into host cells [169], [171]; respectively, 2) include genes that act as negative regulators of defence. For instance, in *Arabidopsis*, *Downy mildew resistance 6 (DMR6)* mutants express enhanced levels of the defence-associated hormone salicylic acid (SA), which results in the loss of susceptibility to *Hyaloperonospora parasitica* [172]; and lastly 3) genes that allow pathogen proliferation and sustain compatibility with the host. For example, the *Sugars Will Eventually Be Exported Transporter* (*SWEET*) genes, encoding putative sugar transporters, are induced by transcription activator-like effectors (TALEs) from rice-pathogenic *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). During infection, the efflux sugar transporter *OsSWEET11*, is upregulated and transports sugars into the apoplastic space, thereby providing nutrients to the pathogen [173], [174].

As research into S genes advances, it becomes increasingly clear that multiple functional categories of genes play a role in susceptibility. S genes not only encode host proteins

that contribute to pathogen establishment and sustenance by suppressing immune responses but also serve as negative plant immunity regulators, ultimately favouring disease development [118], [175]. Hence, disabling plant S genes, for example through loss of function, can restrict pathogenesis and confer resistance to plants. By understanding the genetic makeup of S genes, breeders can make use of wide-genome identification of candidate genes from different crop species and develop more targeted approaches based on comparative genetics for their exploitation [118], [169].

5.1) Identification and modification of plant susceptibility genes

The fundamental step in utilizing impaired S genes for any plant-pathogen interaction involves initially identifying these genes. Generally, two main strategies can be employed to identify S genes: forward and reverse genetics. To date, most S genes have been identified as naturally occurring variants or following mutagenesis through forward genetics screening. Yet, more recently, S genes have also been discovered through pathogen effectors and the host targets they manipulate [169], [176]. Respectively, forward genetic techniques entail screenings of wild germplasm or of a mutant population to identify plants that exhibit altered susceptibility to a given pathogen. Most studies make use of the model *Arabidopsis thaliana* to allow rapid identification of resistant cultivars via phenotypic observation [176]. In addition, researchers may also resort to reverse genetic techniques, which frequently use expression analyses followed by functional characterization to further investigate the genes as susceptibility factors. Candidate genes can be functionally characterized using various techniques, including gene-silencing or knockout techniques, such as Host-induced gene silencing (HIGS) and RNA interference (RNAi) [118]. As mentioned previously, it is also possible to identify S genes through pathogen effectors and host targets. This is because Effector targets are proteins in a host organism that pathogens manipulate to promote disease. As a result, they can be used as molecular probes to identify S genes. Also, these targets can be identified with a yeast two-hybrid assay [177]. Another possibility is to identify orthologues of known S genes across species. S gene orthologues are frequently conserved in various species, likely due to their involvement in various plant biological functions. This conservation trait is valuable in breeding since it makes it easier to identify and functionally characterize orthologs in different crops. In addition, the abundance of sequencing and transcriptomics data available for crops further aids these efforts via phylogenetic analyses [176], [178].

Furthermore, breeders can employ new breeding technologies (NBTs), such as genome editing techniques [179]. Such techniques involve the alteration of an organism´s genome sequences, by using engineered site-specific nucleases, such as zinc finger nucleases (ZFNs), transcription activators like effector nucleases (TALENs), and, recently, clustered regularly interspaced short palindromic repeat (CRISPR) systems with associated

protein 9 (Cas9) (CRISPR/Cas9) [180]–[182] (Figure 2). The nucleases, which create double-strand breaks (DSBs), can subsequently induce targeted changes in the genome, by deletion, replacement or insertion of specific sequences, and subsequently generate desired mutations and avoid associated fitness costs [179]. The applicability of these techniques has been shown in various crops and has revolutionized the field of plant disease resistance [175], [180], [182], [183]. Furthermore, the application of mutant S genes in breeding may also be achieved by Oligonucleotide-directed mutagenesis (ODM), another tool of genome editing. This technique employs a specific oligonucleotide (homologous except for 1bp) to introduce a mutation in the host genome, that disrupts the function of the target gene [182]. Similarly, homology-directed repair (HDR) is also used to introduce specific mutations into the susceptible background [176]. Lastly, 'Targeting Induced Local Lesions IN Genomes' (TILLING), is a nontransgenic technology that integrates chemical mutagenesis with a high-throughput detection method to identify mutations of interest [118].

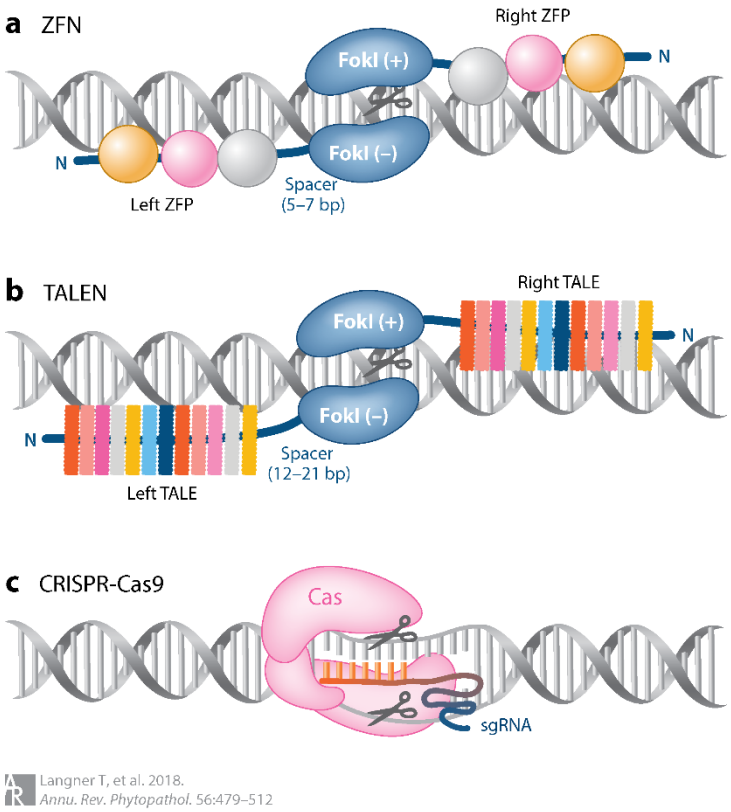

Figure 2- Figure 2 Schematic representation of three classes of programmable nucleases: a) of zinc-finger nuclease (ZFN); b) transcription-activator-like effector nuclease (TALEN), c) CRISPR-Cas9 nuclease Retrieved from Langner et al. (2018). *CRISPR Crops: Plant Genome Editing Toward Disease Resistance*. Annual Review of Phytopathology

5.2) Advantages and disadvantages

The impairment of Susceptibility genes has gained increasing attention for resistance breeding of crops for several reasons. Firstly, overcoming resistance conferred by impaired S genes is deemed more challenging for pathogens compared to resistance mediated by R genes. This is because R-gene-mediated resistance involves the recognition of pathogen effectors or invasion patterns (IP) by specific resistance genes in the host plant, leading to the activation of the defence mechanism. In turn, pathogens can adapt by losing or mutating their effectors or invasion patterns to avoid recognition. In turn, S-gene-mediated resistance relies on reducing or impairing susceptibility genes within the host plant, which affects the host's overall susceptibility to pathogens. Hence, the pathogen is required to gain a new manner to establish disease and counteract such genetic alterations, making it more challenging. Moreover, S-gene-mediated resistance is not as pathogen-specific as R-gene resistance, since it provides resistance to a broad range of pathogens, rather than just one specific race or strain. Lastly, orthologues of S genes are typically conserved in nature and have vital roles in plant growth and development, making them stable across species and resistant to rapid evolutionary changes. Subsequently, phylogenetic analyses of known S genes between species can facilitate the identification of S gene orthologs in different plant species [176] Therefore, resistance mediated by impaired S genes is commonly used in breeding for its potential to provide durable resistance against a broad spectrum of pathogens [118], [176].

A well-studied example of a long-lasting S-gene used in resistance breeding is the previously mentioned *Mildew Resistance Locus O (MLO1)* gene [127]. The *MLO* gene family encode proteins with seven transmembrane and C-terminal calmodulin-binding (CaMB) domains that are functionally important negative regulators for Powdery Mildew (PM) resistance [184], [185]. MLO-mediated resistance is based on the prevention of the fungus to penetrate the host cell during the early stages of invasion [169], [171]. To date, numerous *MLO* genes have been identified in several crop species, and inactivation of these genes has resulted in durable resistance to Powdery Mildew (PM) [171], [186]–[192]. Still, a significant drawback to the impairment of S genes encoding negative regulators of plant immunity is the often-accompanying adverse **pleiotropic effects**, deemed undesirable for breeding objectives. For instance, *MLO* mutants in barley (*HvMLO*) exhibited early senescence-like leaf chlorosis under certain environmental conditions [187]. Nevertheless, research has indicated that these effects can be mitigated through conventional breeding techniques employing suitable genotypes, or by selecting mild S alleles [169]. Furthermore, the extent of fitness cost is dependent on the plant species and the prevailing environmental conditions [118]. For example, silencing of *Defence No Death1* (*DND1)* by RNAi in tomato plants led to dwarfism (impediment of conidial germination and attachment, as well as hyphal growth) and spontaneous cell death without pathogen infection (autonecrosis). However, in potatoes, silencing of the orthologue caused only slight autonecrosis that is dependent on the plant

growing conditions [193]. Moreover, impairment of S genes can also influence other traits, such as sensitivity to abiotic stresses. E.g, in pepper plants, silencing *CaMLO1* gene, an ortholog of tomato *SlMLO1,* was demonstrated to induce resistance against *Xanthomonas campestris*, however, a side effect of this was a reduced tolerance to drought stress [194].

5.3) Candidate Susceptibility Genes

The identification and exploitation of susceptibility genes in peanuts are still in the early stages, and there is limited knowledge available. Just recently, Prasad et al. (2023) described a HIGS strategy to simultaneously control *A.flavus* infection and aflatoxin contamination by multiplexed targeting *nsdC*, *veA*, *aflM*, and *aflR* genes in peanuts. In this study, susceptibility-associated proteins (SAPs), such as annexins, syntaxins, calmodulin, and 9-cis-epoxy carotenoid dioxygenase and mildew resistance locus O (*MLO*), were significantly upregulated in susceptible WT controls plants compared to HIGS lines. Hence, the authors propose further investigation of these SAPs as potential targets in gene editing approaches for enhanced resistance in peanuts. [155]. Furthermore, in Traore et al. (2021) study, 25 *AhMLO* loci were identified and distributed in 14 of the 20 chromosomes of the cultivated peanut genome. Two of these were putative-specific genes that can be used as targets for loss of susceptibility studies and resistance breeding in peanuts. These two *AhMLO* loci were clustered with *MLO* gene members from other plant species responsible for the susceptibility in the clade V [168]. Plus, defense-responsive cis-regulatory elements (CREs) -TC-box and Thymine rich - were identified in the promoter region of the two *AhMLO* and presumed for PM susceptibility in different plant species [195], [196]. Additionally, Liang et al. (2023) identified two salicylic acid hydroxylase genes (AhS5H1 and AhS5H2) as candidate susceptibility genes in peanuts for the first time. Both hydroxylases were shown to share a similar role in planta, which was confirmed both *in vitro* and in transgenic *Arabidopsis* plants. Respectively, *Ah5H1* and *AhS5H2* overexpression lines displayed decreased SA content, increased 2,5-dihydroxybenzoic levels and were more susceptible to *Pst DC3000*, a common bacterial pathogen [160]. Specifically on resistance to *A.flavus* and aflatoxin contamination, Clevenger et al. (2016) pointed out ethylene responsive transcription factor *ABR1*, as a candidate susceptibility factor, due to its role as a repressor of the ABA signalling pathway that may play a role in permitting pre-harvest aflatoxin contamination in peanuts [197].

The findings of these studies offer valuable insights into the potential advancement of utilizing susceptibility genes in peanuts for the purpose of breeding resistance.

6) Discussion

The aim of this study was to 1) comprehensively review the existing literature on peanuts' resistance to *Aspergillus flavus* and aflatoxin contamination, and 2) to investigate the potential of manipulating susceptibility genes as a resistance breeding strategy against the aflatoxin-producing fungus.

The development of peanut varieties with resistance to both fungal infection and aflatoxin contamination remains a challenge. Traditional breeding efforts have focused on enhancing peanut resistance through the identification and incorporation of resistance genes [167]. However, recent attention has turned towards the potential of manipulating susceptibility genes as an effective strategy to render durable, recessively inherited, and potentially broad-spectrum resistance in peanuts [118], [169]. One notable advantage of targeting susceptibility genes lies in the complex and dynamic nature of plant-pathogen interactions. *A. flavus* fungi have evolved sophisticated mechanisms to exploit host plants, often relying on host susceptibility factors for successful infection and aflatoxin production. So, by disrupting these susceptibility factors, researchers can potentially limit pathogenesis, leading to enhanced resistance in peanuts [168].

To achieve this, breeders must initially seek to understand the molecular mechanics and genetic factors that contribute to peanut resistance and susceptibility. Significant breakthroughs have been achieved based on an extensive body of research. The resistance to *A. flavus* infection and aflatoxin contamination is acknowledged as a multifaceted trait influenced by environmental factors, encompassing three main mechanisms: 1) in-vitro seed colonization resistance (IVSC); 2) resistance to pre-harvest aflatoxin contamination (PAC); and 3) resistance to aflatoxin production following infection (AP) [98], [99]. As of today, there are no reports documenting the existence of all three resistance mechanisms within a singular genetic background [13]. Previous studies by Upadhyaya et al. (2002) and Utomo et al. (1990) suggested the independent inheritance of the three resistance mechanisms. Subsequently, studies on bi-parental QTL mapping by Yu et al. (2019), Khan et al. (2020), Jiang et al. (2021), Jin et al. (2023) and Yu et al. (20023) highlighted its polygenic nature and potential improvement through marker-assisted breeding. For instance, QTL mappings of J11 revealed that resistance to *A. flavus* infection is controlled by multiple QTLs and that the resistance-associated alleles in these QTLs were not stable across environments, thus demonstrating the influence of growth conditions on resistance. Moreover, the identification of multiple QTLs with relatively low levels of PVE (<10%) also suggests that minor QTLs contribute to resistance and that physiological and morphological traits contribute to the overall resistance. Another important finding was the discovery of the stable QTL *qPSIIB10*, with favourable alleles from the susceptible parent Zhonghua 16. By pyramiding favourable alleles of both J11 and Zhonghua 16, researchers managed to significantly improve resistance levels across environments, thus highlighting the importance of considering susceptible germplasms

in breeding efforts, since they might possess valuable loci for resistance to *A. flavus* infection [146]. A comprehensive summary of the identified QTLs associated with resistance can be seen in – Table 1: *Bi-parental QTL mapping of genomic regions associated with resistance against A.flavus infection and aflatoxin contamination.* Additionally, multiple SNP/InDel markers associated with aflatoxin resistance have been identified by GWAS, however, the loci responsible for aflatoxin resistance have yet to be delimited in smaller genomic intervals. Moreover, the integration of transcriptomic, proteomic and metabolomic analyses, has also been crucial in elucidating the molecular mechanisms involved in host-pathogen interaction and aflatoxin contamination. These omics approaches have supplemented information on candidate genes, pathways and networks contributing to peanut resistance and susceptibility (Tables 2, 3, 4 and 5), offering potential targets for genetic manipulation using biotechnological approaches in resistance breeding strategies.

The second part of this study focused on the potential of manipulating susceptibility genes as a resistance breeding strategy for peanuts against *Aspergillus flavus*. As mentioned, the impairment of susceptibility genes can confer durable, recessively inherited, and potentially broad-spectrum resistance [118]. Therefore, the utilization of S-gene-mediated resistance in peanuts holds significant promise. However, the current state of research on peanut susceptibility genes is scarce and reflects ongoing efforts to identify and characterize S- genes, as well as to understand their role in host-pathogen interactions. Thus far, various research proposals have been put forth regarding candidate susceptibility factors. For instance, in Prasad et al. (2023) study, the upregulation of SAPs (Annexins, Syntaxins, Calmodulin) in the susceptible plants suggests that these proteins may play a role in susceptibility, and thus be potential targets in gene editing approaches for enhanced resistance in peanut. Namely, Annexins are calcium-regulated phospholipid-binding proteins that mediate membrane fusion and play diverse roles in response to biotic and abiotic stresses. Previous studies have linked annexins with ROS production and the regulation of calcium signals, both of which are key components of plant stress responses. Therefore, a differential expression of annexins may potentially compromise the integrity of plant cell membrane and the overall defence response, resulting in an increased susceptibility [198], [199]. Syntaxins are another family of membrane proteins, which potentially play a role in penetration resistance to powdery mildew fungi [200] and were shown to confer resistance to Phytophthora in different crops via RNAi-mediated silencing [201], [202]. Moreover, Calmodulin (*CaM*), the primary calcium sensor in plants, interacts with calcium ions and regulates diverse cellular functions by modulating the activity of various target proteins in reaction to calcium signals. In tomatoes, silencing of the calmodulin-like proteins *SlCML55* was shown to inhibit *Phytophthora* infection [203]. Another candidate susceptibility gene is the 9-cis-epoxy carotenoid dioxygenase (NCED), a key enzyme involved in abscisic acid (ABA) biosynthesis. In Prasad et al. (2023) study, the NCED gene exhibited higher expression in the susceptible genotype,

indicating its potential role in susceptibility. [155]. ABA in known to be involved in various physiological processes, including stress/pathogen defence responses, and interplay with other signaling pathways, such as those responsible for salicylic acid (SA) or ethylene synthesis. Its influence on host immunity against pathogens varies based on the specific pathosystem – dual role: may contribute to defence response or enhance susceptibility [204]. For example, studies have shown that ABA can positively regulate defence mechanisms against powdery mildew in rubber trees, while in barley, ABA has been associated with increased susceptibility to *Magnaporthe oryzae* [205], [206]. Hence, modifying ABA levels may be explored as a way to enhance resistance to specific pathogens. Moreover, an interaction between Ca2+/calmodulin signalling components and ABA-based ROS defence responses has also been demonstrated in maize [207].

One of the most intriguing targets proposed was the *mildew resistance locus O* (*MLO*). As previously mentioned, the specific *MLO* loci associated with PM susceptibility function as a negative regulator of hypersensitive response upon PM infection. Loss of function of these genes has been linked to confer a broad-spectrum and durable disease resistance in various crops. Accordingly, genome-wide identification of susceptibility genes is a prerequisite for loss of function studies [171], [186]–[192]. Hence, the recent discovery by Traore et al. (2021) of two putative-specific *AhMLO* loci grouped in clade V, provide a foundation for further research on the potential use of susceptibility gene-mediated resistance in peanut. Respectively, these two *AhMLO* loci can be targets for loss of susceptibility studies and their manipulation may offer a promising approach to enhance resistance in peanuts [168].

Particularly on resistance to aflatoxin, Clevenger et al. (2016) suggest a potential susceptibility factor, the Ethylene Responsive TF *ABR1* - a repressor of ABA signaling that may play a role in permitting pre-harvest aflatoxin contamination in peanuts. The disruption of *ABR1* has been shown to increase ABA expression, which aligns with the observation that ABA signalling is decreased in contaminated peanut seeds where the expression of homologues of *ABR1* is up-regulated [197]. Additionally, in *Arabidopsis*, *ABR1* has been characterized as a susceptibility hub that interacts with multiple *Pseudomonas syringae* effectors, suggesting that it may be targeted by the pathogen to promote susceptibility [208].

Furthermore, no S genes were previously known to confer resistance to pathogens in peanuts. However, a groundbreaking discovery was made in the study by Liang et al. (2023) regarding susceptible genes in peanuts. In this study, two salicylate hydroxylase genes (*AhS5H1* and *AhS5H2*) were successfully predicted and identified within the peanut genome [160]. These genes are involved in the salicylic acid (SA) signalling pathway, which is a key signalling pathway in plant defence responses, required for pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) in local tissue, as well as systemic acquired resistance (SAR) [209], [210]. Moreover, S genes are typically conserved across plant species, and phylogenetic analysis indicated that AhS5H1 is highly similar to SA *3-hydroxylase* (*S3H*), while *AhS5H2* resembles SA *5-hydroxylase* (*S5H*). *S5H*

and *S3H*, identified as *downy mildew resistant* (*DMR6*) and its close homolog *DLO1* (*DMR6-like oxygenase1*), respectively, function as partially redundant but distinct suppressors of immunity [211], [212]. Orthologs of the *DMR6* and *DLO* genes have been identified and proved to be S genes in several crops, conferring broad-spectrum resistance [to different pathogens] [213]–[215]. In Liang et al.´s study, the salicylate hydroxylases' functionality was verified both *in vitro* and in transgenic *Arabidopsis* plants, supporting the significance of the candidate S genes *AhS5H1* and *AhS5H2* in disease resistance to specific pathogens, including *A. flavus*. This discovery represents a significant advancement in the potential use of S-gene-mediated resistance in peanuts.

To conclude, the manipulation of peanuts susceptibility-genes to gain resistance against pathogens is an area of active research. Even though there are currently no examples of successful manipulation of peanut S- genes that confer resistance to *A.flavus*, the referenced studies discuss the identification of different candidate susceptibility factors, laying the groundwork for potential manipulation studies. Also, these findings not only offer valuable insights into peanut - *A.flavus* interactions but also suggest that employing S Genes-mediated resistance holds promise as an effective strategy for peanut resistance breeding. However, further research and genome-wide mining of susceptibility genes are warranted.

Proposed Research Framework

Despite challenges associated with utilizing S-genes in breeding, such as potential adverse pleiotropic effects, there is an optimistic outlook that the development of new genetic engineering tools will address some of these issues. Accordingly, to further study peanut S-genes, I suggest using CRISPR, a versatile tool for both forward and reverse genetic studies. The CRISPR/Cas9 gene-editing technology allows scientists to modify specific genes of interest (including Susceptibility genes) while sparing all others, to enhance resistance in crops. While this technology has proven successful in numerous crops, its application in peanuts has been limited, potentially due to the scarcity of suitable constructs and protocols. Nonetheless, a recent study by Neelakandan et al. (2022) has addressed this gap by developing two constructs that can be used for gene editing (loss-of-function) studies in peanuts [216]. Furthermore, the integration of base editors with the CRISPR/Cas9 system has also been employed to induce single-base alterations in plants [217]. Hence, CRISPR-based targeted mutagenesis holds significant potential in breeding to prevent fitness costs by making precise modifications, like introducing single-nucleotide polymorphisms (SNPs) in susceptibility (S) genes; or directly replacing functional S genes in cultivars by utilizing natural mutants present in the germplasm; or by synthetically generating mutant alleles for integration.

The following proposed research frameworks for identifying and characterizing S genes in peanuts are formulated based on the existing literature:

1) Select genetically diverse peanut cultivars with contrasting resistance levels to *Aspergillus flavus* and aflatoxin contamination. Cross these cultivars to generate a mapping population for subsequent genetic analyses.
   - Peanuts 'resistance to *A. flavus* is genotype-specific and highly influenced by the environment, which may cause inconsistent phenotypic outcomes. Hence, research is suggested to be performed under controlled conditions.
2) Assess the phenotypic response of the mapping population for signs of infection, as well as aflatoxin quantification and disease Incidence.
   - described by Yu et al. (2019) [136].
3) Conduct Genome-Wide Association (GWAS) studies along with Quantitative Trait Locus (QTL) mapping to identify genetic loci and candidate genes associated with resistance to *A. flavus* infection and aflatoxin contamination.
4) Conduct transcriptomic and proteomic analyses to identify differentially expressed genes (DEGs) and proteins associated with resistance and susceptibility.
5) Perform functional analyses to validate the candidate genes as susceptibility factors via gene-silencing or knockout techniques.

A different approach implies the identification of orthologues of known S genes. For such, make use of existing knowledge on susceptibility genes involved in responses to *A.flavus* infection and aflatoxin in other crop species.

1) Perform sequence analysis to identify putative orthologues of known S genes in the peanut genome. Make use of available genomic and transcriptomic data to pinpoint homologous sequences and conserved domains.
2) Conduct phylogenetic analyses to assess the genetic relationship and potential functional conservation.
3) Conduct Genomic Loci Identification of the orthologous sequences in the peanut genome - High-Density Genetic Linkage Map Construction Using Whole-Genome Resequencing for Mapping QTLs
4) Analyze the expression patterns of the orthologous genes in response to *A. flavus* infection, followed by functional analyze to validate their role.

Lastly, it is also possible to identify S genes through pathogen effectors and host targets. This is because effector targets are proteins in a host organism that pathogens manipulate to promote disease. As a result, they can be used as molecular probes to identify S genes.

1) Search existing literature to identify effector proteins associated with *Aspergillus flavus*, involved in manipulating host cellular responses to promote disease.
2) Conduct effector-target interaction analysis to identify putative host-targets in peanuts that are manipulated by *A.flavus.*
3) Perform sequence analysis to identify orthologues of known effector targets in the peanut genome, followed by phylogenetic analyses.
4) Subsequent genomic loci identification and functional validation.

These approaches not only provide valuable insights into the molecular mechanisms involved in peanut-pathogen interactions but also establish a basis for a more targeted and efficient breeding program aimed at developing peanut varieties with enhanced resistance to pathogen *A. flavus* and aflatoxin production.

7) Supplement material

Table 1 Bi-parental QTL mapping of genomic regions associated with resistance against A.flavus infection and aflatoxin contamination. Major QTLs in grey (PVE > 10%). The designated aabbcc and AABBCC do not reflect dominance or recessive - distinction between parental and maternal alleles, in studied that assessed which alleles contribute to the phenotype.

Genotype	Mapping population	QTL	LG	Position (cM)	Marker Interval	PVE %	LOD	Additive value	Resistance	Reference
Male parent J11: aabbcc ICRISAT, Hyderabad, India. Female parent Zhonghua 16: AABBCC OCRI-CAAS, Wuhan, China.	J11 (R) X Zhonghua 16 (S) RIL population	*qPSIIA05*	A05	56.5–57.5	c05b092-c05b093	5.50	3.17	2.41	*A.flavus* infection	[3]
		qPSIIA08	A08	53.5–54.5	c08b121-c08b122	**10.87**	5.97	3.39		
		qPSIIB01	B01	43.5–44.5	c11b078-c11b079	6.16	2.63	3.74		
		qPSIIB03.a	B03	52.5–53.5	c13b091-c13b092	9.16	5.16	3.07		
						9.23	3.91	4.58		
		qPSIIB03.b		36.5–37.5	c13b049-c13b050	5.03	2.74	2.68		
						5.75	2.75	2.87		
		qPSIIB10	B10	32.5–33.5	c20b057-c20b058	**10.10**	5.39	-3.23		
						9.94	5.28	-3.79		
						10.58	4.92	-3.92		
						6.91	2.84	-3.99		
Male parent Xinhuixiaoli: ? Landrace. CCGRIS. Guangdong, China. (Zh.h0341, *A. hypogaea var. fastigiata).* Female parent Yueyou 92:? GDAAS, Guangdong, China. (*A. hypogaea var. vulgaris*)	Xinhuixiaoli (R) × Yueyou 92 (S) RIL population	***qRAF-3-1***	A03	111.902–113.575	Marker8555604-8633509	**19.04**	10.54	7.94	*A.flavus infection*	[145]
		qRAF-14-1	B04	29.006–34.460	Marker4154940-4158241	5.15	2.85	-4.13		
Male parent ICG 12625: aabbdd ICRISAT, Hyderabad, India. (PI 497597, *A. hypogaea var. aequatoriana)* Female parent Zhonghua 10:	ICG 12625 (R) X Zhonghua 10 (S) RIL population	qPSIIA03	A03	28.50–30.20	AHGS2058 -AGGS0052	7.96	3.06	−2.62	*A. flavus* infection	[136]
		qPSIIA10	A10	43.50–44.70	AGGS1425 – ARS710	**13.00**	5.00	5.27		
				43.70–44.30		**11.32**	4.40	3.08		
		qAFB2A03	A03	50.19–55.08	AGGS1139 - AHGS2025	8.32	3.45	3.44		
		qAFB1A05	A05	51.10–55.70	AHGS1245 - AGGS0876	7.98	3.17	36.02		
		qAFB1A07	A07	83.40–99.20	ARS734 - GM2156	**14.57**	36.02	49.00		
				80.30–91.00		**17.87**	5.98	35.96		

AABBDD OCRI-CAAS, Wuhan, China. (*A. hypogaea var. vulgaris*)				83.80–98.20		**10.62**	4.70	25.68	Aflatoxin contamination	
		qAFB2A07		74.30–84.40	AHGS1454 - HAS1360	**10.84**	3.96	2.95		
				83.50–98.20	ARS734 - GM2156	**12.19**	5.10	2.20		
		qAFB2B05	B05	45.40–50.40	AGGS0979 - TC19E1	**11.05**	4.90	−3.49		
		qAFB1B06.1	B06	42.50–52.90	AGGS1515 - AGGS1587	**16.33**	6.40	−52.07		
				45.70–52.70	AGGS2069 - AGGS1587	9.52	3.90	−26.31		
		qAFB1B06.2		69.50–77.60	AHGS1464 - HAS0969	7.78	3.11	−19.13		
		qAFB2B06		43.10–50.10	GM2444 - AHGA335472	9.32	3.80	− 3.53		
				43.20–58.30	GM2444 - AGGS0983	**21.02**	8.80	−4.11		
		qAFB1B07.1	B07	39.20–51.70	AGGS1581 - GM2067	8.48	3.60	−40.35		
		qAFB1B07.2		86.00–86.50	TC3B4 - AHGS2233	7.30	3.10	−36.16		
		qAFB1B07.3		103.70–104.30	AGGS1081 - AhTE0615	7.46	3.20	−22.55		
		qAFB2B07		80.80–86.50	TC3B4 - AHGS2233	**14.45**	5.30	−4.48		
Male parent J11: aabbcc ICRISAT, Hyderabad, India. Female parent Zhonghua 16: AABBCC OCRI-CAAS, Wuhan, China.	J11 (R) X Zhonghua 16 (S) RIL population	***qAFTA05.1***	A05	27.41–35.52	c05b050–c05b062	7.95	4.44	12.77	Aflatoxin contamination	[146]
				25.19–38.08	c05b046–c05b066	**11.42**	6.35	18.09		
				22.02–35.80	c05b041–c05b063	5.99	3.29	10.64		
		qAFTA05.2		57.49–59.07	c05b093–c05b098	5.49	3.12	11.06		
		qAFTA05.3		63.71–70.01	c05b109–c05b115	5.83	3.11	11.07		
		qAFTA08	A08	25.15–25.91	c08b048–c08b050	4.99	2.82	−9.79		
		qAFTB05.1	B05	8.30–8.55	c15b022–c15b023	5.2	2.73	−9.91		
		qAFTB05.2		11.05–26.25	c15b031–c15b068	**9.90**	5.37	−13.79		
		qAFTB06.1	B06	39.06–39.56	c16b081–c16b083	4.83	2.65	9.95		
		qAFTB06.2		39.81–44.65	c16b084–c16b101	7.52	4.54	14.86		
		qAFTB06.3		46.04–56.26	c16b105–c16b137	8.23	4.64	12.91		
				46.04–57.03	c16b105–c16b139	**10.63**	6.58	17.6		
		qAFTB09.1	B09	39.57–41.65	c19b079–c19b086	4.61	2.86	11.58		
		qAFTB09.2		47.11–49.96	c19b104–c19b112	5.8	3.63	12.93		
male Parents: Zhenzhuhei, Virginia Fu 95-5, Spain J11: aabbcc ICRISAT, Hyderabad, India. female parent: Yueyou 13 Spain	three independent RIL populations: Zhenzhuhei (R) x Yueyou 13 (S) Fu95-5 (R) x Yueyou13 (S) J-11 (R) x Yueyou13 (S)	*N/A*	A01	20.35	TC11H06–TC4H07	22.7	4.30	0.209	*A.flavus infection*	[143], [218]
			A02	9.31	gi-716–TC1E05	**11.2**	2.26	-0.144		
			A03	5.31	pPGSseq18E7–Seq4E08	6.2	2.60	-0.047		
			A04	12.76	pPGPseq2H8–PM3	6.6	2.1	-0.039		
			B05	25.01	pPGPseq7G2–TC5A06	**10.5**	2.91	0.162		
			B08	6.78	TC11A04–PM137	7.3	2.4	0.015		

Male parent Xuhua13: aabb OCRI-CAAS, Wuhan, China. Female parent Zhonghua 6: AABB OCRI-CAAS, Wuhan, China.	Zhonghua 6 (R) x Xuhua13 (S) RIL population	***qAFTsA07.1***	A07	0.01-31.79	TIF.17:118381-PA11:2103429	**13.39**	6.94	17.51	Aflatoxin contamination	[144]
						11.03	5.26	21.15		
						8,48	3.98	13.47		
		qAFTsA07.2	A07	59.11-62.40	TIF.07:58988620-TIF.07:62491911	5.99	3,21	-11,73		
		qAFTsA02	A02	8.63-10.73	TIF.02:1722668-TIF.02:2765688	7.07	3,81	-17,24		
		qAFTsB07	B07	48.97-63.16	TIF.17:121175430-TIF.02:129143935	7.25	4,42	13,42		
				56.69-58.59	TIF.17:127579080-TIF.02:128477759	6.72	3,46	12,20		

Molecular Basis - Omic technologies

Table 2 A summary of transcriptomic and proteomic discoveries of key genes and pathways involved in peanut response to A.flavus and aflatoxin contamination. Aflatoxin Contamination (AP); Pre-Harvest Aflatoxin Contamination (PAC); Post Harvest Aflatoxin Contamination (PHAC); In vitro seed colonization (IVSC); salicylic acid (SA); jasmonic acid (JA); methyl jasmonate (Methyl-JA).

Genes	Function	Resistance Mechanism	Reference
Transcription factors			
WRKY	Plant defence and development. Transcriptional regulator of pathogenesis and antioxidant-related genes	AP	[99], [121], [122], [219], [220]
bZIP NAC MYB MYC ERF	Plant defence - Regulate the expression of biotic and abiotic stress genes. Involved in JA, SA and Ethylene signalling pathways	AP, PAC and PHAC	
Fatty acids			
TIR-NBS-LRR	Disease resistance. ETI response – Cell wall signalling and PAMPs perception.	AP and PHAC	[99], [120], [122], [150], [221][222]
lipoxygenases (LOX)	JA and methyl-JA signalling pathway.	AP, PAC PHAC	[145], [149], [223]
9s-lipoxygenase (9s-LOX)	lipid peroxidases metabolism.		
13s- lipoxygenases (13s-LOX)	lipid peroxidases metabolism.		
Oxylipins	Inhibit Aflatoxin biosynthesis. Development of cuticular elements. Involved in JA signalling pathway.	AP and PAC	[6], [102]
WRINKLED1	TF. Controls Fatty acid biosynthesis.	PAC	[197], [220]
secondary metabolites biosynthetic related genes involved in plant defence response.			
Phenylalanine ammonia-lyase (PAL)	Phytoalexins Biosynthesis and lignin precursor.	PHAC	[99], [122], [150]
Resveratrol synthase (RS)	Stilbene type-phytoalexins Biosynthesis	IVSC and PHAC	[99], [122], [150], [225]
Chalcone synthase (CHS)	Flavonoid biosynthesis pathway. SA signalling pathway.	IVSC and PHAC	[99], [122], [150], [197], [220], [226]
Deoxy-chalcone synthase	Flavonoid biosynthesis pathway		
Chalcone-flavanone isomerase (CHI)	Flavonoid biosynthesis pathway.	PHAC	[99], [220], [224]
cinnamate 4-hydroxylase (C4H)	Phenylpropanoid biosynthesis.	PHAC	[150], [227]
4-coumarateCoA ligase (4CL)	Phenylpropanoid biosynthesis.	PHAC	[150], [227]
UDP-glucose flavonoid 3 -O-glucosyltransferase 3	Anthocyanin biosynthesis and phenylpropanoid pathway.	PAC	[220]
SAM-dependent isoflavone 7-O-methyltransferase	Phytoalexins Biosynthesis.	PHAC	[228]

Pathogenesis-related proteins (PR)				
PR-10		Disease resistance. Stress signalling proteins.	PAC	[142], [150], [152], [197], [220]
PR2-like	β-1,3-glucanase	Disease Resistance PR-2. Fungal cell wall degradation-hydrolysis.		[20], [99], [122], [130], [142], [197], [229]
Chitinases		Disease Resistance PR. Modulate immune response. Chitin degradation.	AP, PHAC and IVSC	[99], [122], [131], [142], [150]
Peroxidases		Defence response. Regulates antioxidant metabolism.	AP and PAC	[99], [142], [148], [155]
Defensins	*DEF1*	Defence response. Antifungal activities.	AP, PHAC and IVSC	[99], [154], [224]
	DEF2			
Heat shock protein (HSP)	*HSP2*	Plant defence. Regulate heat shock factors. Cellular immunity and protein folding	AP, PAC and PHAC	[122], [230]–[232]
	Le-HSP17.6			
	HSP70			
	HSTF A-2			
Others				
Late embryogenesis abundant (LEA)	*LEA2*	Stress-responsive.	PAC	[149], [152], [231]
	LEA4	Stress-responsive. Seed maturation.		
Polygalacturonase inhibitor proteins (PGIPs)		Defence response.	AP	[150], [233]
BIG		Auxin signalling and transport.	PAC	[197]
Cu/Zn superoxide dismutase II		Antioxidant defensive protein.	PAC	[231]
Kunitz-type trypsin inhibitor		Defence response. Antifungal compound.	PAC	[99], [234]
Hevamine-A		Defence response. Chitinase activity.	PAC	[142]
ABA-responsive genes		Regulates stress-responsive genes. Modulates host immunity.	AP and PAC	[127], [197], [220]
ABR1		Ethylene-responsive TF. ABA signalling pathway repressor and susceptibility factor.	AP and PAC	[197], [235]
Serine/threonine kinase (STK)		disease resistance proteins. LRR receptor.ETI.	IVSC	[120], [152], [228]
RPVOD7		Pattern recognition receptor. PAMP perception. PTI.	IVSC	[104]
1-aminocyclopropane-1-carboxylate oxidase (ACO1)		ethylene signalling pathway.	IVSC	[152]
cytochrome P450		Disease resistance. Degradation of toxins.	PHAC	[13], [152], [228]

Table 3 In silico analysis of gene expression in genomic regions of the two QTLS – represents the upregulated candidate genes found in the genomic regions.

QTL	Chr	Start Position	End Position	mRNAID	Annotation
qRAF-3-1	A03	123903926	123906658	AH03G39640.1	Probable WRKY mRNA ion factor 51
		124037466	124044132	AH03G39710.1	Shikimate kinase 1%2C chloroplastic
		124211379	124235650	AH03G39780.1	ABC2 homolog 13
		124242653	124244987	AH03G39810.1	Cytochrome P450 71B34
		124252378	124257039	AH03G39830.1	Transcription factor TGA4
		124324761	124328850	AH03G39870.1	RING/FYVE/PHD zinc finger superfamily protein
		124907514	124910602	AH03G40150.1	Ethylene-responsive mRNAion factor ERF060
		125097218	125101788	AH03G40210.1	CDPK-related kinase 5
		125120689	125122471	AH03G40240.1	Lipase/lipooxygenase 2C PLAT/LH2 family protein
qRAF-14-1	B07	9339128	9343726	AH14G07090.1	myb-like HTH mRNAional regulator family protein
		9479021	9481005	AH14G07190.1	**Putative disease resistance RPP13-like protein 1**
		10475429	10483052	AH14G07740.1	LRR receptor-like serine/threonine-protein kinase GSO1
		10513772	10514962	AH14G07770.1	Leucine-rich repeat transmembrane protein kinase family protein
		10635976	10641199	AH14G07850.1	Peroxidase 53
		10696179	10700196	AH14G07860.1	Peroxidase 54
		10717448	10722950	AH14G07870.1	Peroxidase 54
		8821254	8825198	AH14G06690.1	Copper transport protein family
		8879603	8882894	AH14G06750.1	Calcineurin B-like protein 3

Table 4 Peak SNP markers associated with both Aflatoxin B1 (AFB1) and Aflatoxin B2 (AFB2) content. Retrieved from Yu et al. (2020).

Chromosome	Marker	Position	PVE% AFB1	PVE% AFB2
A01	SNP00539	34209850	26.41%	25.90%
A02	SNP02428	32402181	28.83%	31.15%
	SNP02686	46706823	22.96%	31.70%
A04	SNP05994	5449452	27.21%	26.45%
	SNP06730	41670901	26.48%	26.35%
	SNP07247	69306120	25.89%	25.41%
A06	SNP11095	44574792	27.15%	25.92%
	SNP11310	57503242	28.37%	26.66%
A08	SNP13363	855373	25.60%	25.10%
	SNP13464	12628002	27.68%	26.93%
B02	SNP20417	23772874	25.60%	25.10%
B08	SNP32483	100913450	28.49%	27.35%

Table 5 Significant markers associated with SLII, SDII and aflatoxin content. Retrieved from Ding et al. (2022).

Resistance	Chromosome	Marker	Position	PVE%
Shell Infection Index	A01	SNP00688	40368467	38.64%
	B02	InDel21071	9357931	24.80%
		SNP21070	9277537	33.74%
		SNP21188	14933055	31.72%
		SNP21021	6844,062	27.69%
	B10	InDel43846	112260595	26.99%
Seed Infection Index	B02	SNP22555	100237479	27.28%
		SNP22420	93852971	26.47%
	B08	InDel36369	38548488	27.62%
	B10	SNP42459	593672	25.82%
		SNP43163	53492328	23.33%
Aflatoxin content (ug/g)	A09	InDel15210	14197913	30.94%
	B10	InDel17247	23667814	36.76%
	B08	SNP37276	80753727	30.41%
		SNP38307	129415431	33.63%
	B02	SNP22577	101526304	34.91%

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