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Resistance Mechanisms to *Aspergillus flavus* Infection and Aflatoxin Contamination in Peanut (*Arachis hypogaea*)

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Abstract: Preharvest aflatoxin contamination in peanut is a serious and world-wide problem concerning food safety and human health. Aflatoxin contamination is known to be influenced by numerous factors. Drought and high temperatures are conducive to *Aspergillus flavus* infection and aflatoxin contamination. Plant-host resistance is a highly desirable tactic that can be used to manage this problem. Screening, identification and field evaluation of germplasm for resistant traits are important techniques. This review summarizes research progress in the area of host resistance mechanisms to aflatoxin contamination in peanut. Resistance mechanism in peanut to aflatoxin contamination has been studied for nearly 30 years. Some resistance mechanisms and resistant factors have been reported to contribute to the resistance to aflatoxin-producing fungi, but no effective efforts have been made to breed for these traits because the mechanisms to resistance traits might not be still fully understood. Future researches are expected to use advanced biotechnology to understand the comprehensive mechanisms governing the resistance pathways in order to use the information in breeding programs for crop improvement and control of preharvest aflatoxin contamination. The risk of aflatoxin contamination could be prevented before the contaminated grains get into the food chain.

Key words: Peanut, aflatoxin, food safety, resistance mechanism

INTRODUCTION

Aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus*, are associated with both acute and chronic toxicity in animals and humans including acute liver damage, liver cirrhosis, induction of tumor and teratogenic effects[1,2]. The most important aflatoxins are B_1, B_2, G_1 and G_2, distinguished by their fluorescent colors under ultraviolet light[3]. Aflatoxins occur naturally and have been found in a wide range of commodities and pose a serious threat to human and animal health, as well as to agricultural economics all over the world[4-6]. More than 50 countries have enforced or proposed aflatoxin regulations for foodstuffs. The maximum limits range from zero detectable to 50 μg kg^{-1}[7].

Peanut is one of the most susceptible host crops to *A. flavus* invasion and subsequently aflatoxin production. After identification of the peanut aflatoxin problem in 1963[8], many studies have been done in a number of peanut-producing countries. It is evident that peanut could be invaded by *A. flavus* and *A. parasiticus* and subsequently become contaminated with aflatoxin, before harvest and post harvest[9-12]. The extent of contamination varies with geographic location, agricultural and agronomic practices, storage and processing period. In some regions, contamination is predominantly preharvest while in others it is major postharvest[13,14].

Adopting some cultural practices, curing and drying and storage practices can minimize aflatoxin contamination. But these practices may not be suited to small-scale farming in developing countries, especially in tropical areas. Chemical control and removal of toxin have not yet been completely successful[15]. It was suggested that an effective solution to the problem would be the use of peanut varieties that are resistant to infection by the aflatoxin-producing fungi, or resistant to aflatoxin production if colonized by the fungus[15-17].

Systematic researches on host resistance mechanism of peanut resistant to aflatoxin did not begin until, Mixon and Rogers[18], reported two resistant Valencia-type

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genotypes (PI 337394F and PI 337409). This review will summarize research progress in the area of resistance mechanisms to aflatoxin contamination in peanut.

RESISTANCE-SCREENING METHODS

Much of the effort to study the host resistance to aflatoxin has been directed toward identifying peanut genotypes resistant to *A. flavus* infection and aflatoxin production. An effective screening technique would be helpful for identification of source of resistance and could aid in studying resistant mechanisms.

Screening for seeds resistance to *A. flavus* invasion: Mixon and Rogers[18] developed a laboratory inoculation method for screening peanut genotypes for resistance to *A. flavus* invasion and colonization of rehydrated seeds and identified two resistant Valencia-type genotypes (PI 337394F and PI 337409). Several researchers also identified some resistant genotypes[15,19,20].

The scientists at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) established a screening method on the basis of Mixon's method[21,22]. The sound, mature seeds from intact pods were selected and dried and stored for about 30 days. The kernels were surface sterilized with 0.1% mercuric chloride, rinsed in sterile deionized water, hydrated to 20% moisture content and surface inoculated with a conidial suspension of an aflatoxigenic strain of *A. flavus* and then incubated at 25°C for 7-8 days under 100% relative humidity. Genotypes with 15% or fewer seeds colonized were regarded as resistant.

Screening for resistance to preharvest aflatoxin contamination: The *in vitro* seed inoculation screening in the laboratory do not correlate well with the field results[23,24], which has stimulated considerable research to develop field inoculation techniques for screening peanut genotypes for preharvest resistance.

Many approaches have been used to inoculate the peanut plant or the soil to increase the *A. flavus* population densities and to enhance the contact between *A. flavus* and the peanut pod[25]. Common methods used to delivery *A. flavus* inoculums are aqueous suspensions of *A. flavus* conidia, cracked and *A. flavus*-infected corn and organic-matrix infested with *A. flavus*[24,26].

Holbrook *et al.*[27] developed a large-scale field screening technique to directly measure field resistance to preharvest aflatoxin contamination that uses subsurface irrigation in a desert environment to allow an extended period of drought stress in the pod zone while keeping the plant alive. Without subsurface irrigation, peanut plants died and their seeds rapidly dehydrated in the soil before contamination could occur. Using this method, 15 accessions showed 70 to 90% reductions in aflatoxin contamination in comparison to susceptible accessions in three environments. Large-scale field screening for resistance to preharvest aflatoxin contamination was also enhanced by the development of a consistently successful and reproducible field inoculation technique[28].

Anderson *et al.*[29] developed a procedure for greenhouse screening of individual plants or pods from single plant for resistance to invasion by aflatoxigenic fungi and subsequent aflatoxin production by completely isolating the pods from the root zone and imposing drought-stress only on pegs and pods. High levels of fungal infection and high amounts of preharvest aflatoxin accumulation were observed by mid-bloom inoculating with *A. parasiticus*-contaminated cracked corn and imposing drought-stress period of 40-60 days.

CONSTITUTIVE COMPONENTS IN SEEDS RELATED TO RESISTANCE

Structure of seed coat: As seed coat is the outmost layer of peanut kernels, several researchers have attempted to characterize features relative to resistance[30-34]. LaPrade *et al.*[30] suggested that resistance could be attributed to seed coat thickness and/or permeability. In other experiments, Taber *et al.*[31] observed that kernels of resistant genotypes had smaller hila, more compact arrangement of palisade-like layer of testa and a thicker waxy surface compared to susceptible genotypes. Zambettackis and Bockelee-Morvan[32] also reported considerable diversity in the testa structure of peanut lines viewed with electron and light microscopes. A method of classifying these differences was given as a guide to measurement of resistance to *A. flavus*.

The role of wax and cutin layers in peanut seeds coat associated resistance to invasion and colonization by *A. flavus* was studied by Liang *et al.*[35]. The results showed that wax contents of resistant genotypes were significantly higher than susceptible cultivars. The resistant kernels had a thicker and coarser waxy deposit on seed coat surfaces than the susceptible genotypes under scanning electron microscope. Removal of wax with chloroform and removing of cutin with KOH and cutinase or both[36,37] can increase the susceptibility of peanut seeds. The bioassays of wax *in vitro* showed that there were no significant test-by-treatment interactions. These results indicated wax and cutin layers of peanut seed coat might only play a role of physical barrier in resistance to *A. flavus* invasion and colonization.

Polyphenol compounds: Considerably more extractable antibiotic phenols, especially tannins, have been reported to be present constitutively in peanut testa and thought to function as performed inhibitors to *A. flavus*[38,39]. Several researchers attempted to find the relationship between the tannins contents and *A. flavus* resistance and developed a chemical method of screening for *A. flavus* resistance in peanut.

Turner *et al.*[40] isolated 5-7-dimethoxyisoflavone from peanut and observed the inhibition of *A. flavus* growth. Using several genotypes, Sanders and Mixon[41] extracted tannin from the testa of peanut genotypes differing in seed colonization by *A. parasiticus*. They found correlation coefficients of 0.74 and 0.76 between percent seed colonization and mg of tannin/g of intact seed and per gram of seed coat, respectively. When potato dextrose agar (PDA) was amended with 2.5, 5.0, 7.5, 10, or 20% tannin (wt/vol) and incubated with the fungus for 52 h at 25°C, the mycelia growth on the agar plants was inhibited by 64, 78, 88, 89 and 96%, respectively. Lansden[42] examined the fungistatic properties of various tannin fractions from seed coats and determined their effect on aflatoxin production by *A. parastiticus*. Both the growth of *A. parasiticus* on PDA and production of aflatoxin in liquid culture were inhibited by these tannins. Karchesy and Hemingway[43] had identified various compounds that were present in peanut seed coats and determined the structures of some of the isolated polyphenolic compounds.

But a few years later, Jambunathan's[44] experiment showed there was no significant correlation between seed colonization and polyphenol contents by comparing eight resistant and five susceptible genotypes. Azaizeh *et al.*[45] obtained the same results after testing the relationship between the peanut genotypes and the influence of tannin extracts on *A. parasiticus* growth and aflatoxin production in twenty-three peanut genotypes.

Other chemicals: Amaya *et al.*[46] reported a possible relationship between resistance to *A. flavus* and total soluble amino compounds, arabinose content in peanut cultivars. Differences among cultivars were found in the elemental composition of the testae. The testae of resistant cultivar, PI 337409, contained intermediate levels of phosphorus, sulfur and a higher level of calcium[17].

Antifungal proteins: Plant seeds contain a number of proteins whose primary function is served as nitrogen storage required during germination and growth, but also contain other proteins which function to protect against infection of fungi during storage and germination. These antifungal proteins include enzyme inhibitors[47], nonspecific lipid transfer proteins[48], 2 S storage proteins[49], ribosome-inactivating-protein[50] and zeamatin[51]. Their localization and concentration within the kernels may play an important role in preventing fungal invasion[52].

Research on antifungal proteins related to aflatoxin contamination in corn revealed that several proteins including 14 kDa trypsin inhibitor[47], 18 kDa RIP and zeamatin[52], 28 and 100 kDa protein[53] might contribute to resistance to aflatoxin production. These proteins exhibit strong bioactivity against the growth of *A. flavus, A. parasiticus* and a morphologically diverse group of fungi[47,54].

Limited studies on the peanut antifungal proteins have been done. After comparing the protein profiles from resistant and susceptible peanut kernel, Pettit *et al.*[17], Szerszen and Pettit *et al.*[55] and Bianchi-Hall[56] found no distinct protein differences related to resistance to aflatoxin contamination. To search for anti-*A. flavus* protein in peanut kernel, Liang[57] conducted research to extract peanut seed proteins at different pH buffers and analyze these extracts with SDS-PAGE using five resistant genotypes and four susceptible genotypes. Consistent differences in protein profiles were detected only in proteins extracted at pH 2.8 buffer between resistant and susceptible genotypes. A protein of 38 kDa was unique or presented in higher concentration in resistant genotypes, whereas absent or in low concentration in susceptible genotypes. Protein extracted at pH 2.8 from seeds showed markedly antifungal activity against *A. flavus in vitro*. Furthermore, using $(NH)_4SO_4$ gradient precipitation and chromatography, Liang *et al.*[58] identified two biologically active fraction. Both can inhibit spore germination and extension of *A. flavus* hypha growth *in vitro*. The estimated molecular mass of these two purified proteins are approximately 14.2 and 26.3 kDa. Liang *et al.*[35] examined protein profiles of 15 peanut genotypes and revealed that the contents and activities of trypsin inhibitor were relative high in resistant genotypes. The concentration and activity of trypsin inhibitor in 8 resistant genotypes were significantly higher than that in 7 susceptible genotypes. The trypsin inhibitor in peanut kernel was purified by acetone fractionation, followed by passing through DEAE-Sephadex A50 ion-exchange column. The purified inhibitor in peanut kernel consists of two subunits with molecular weight of 10.3 and 17 kDs, respectively. The inhibition of germination and hypha growth of *A. flavus* was observed in protein concentration of 10 μg on V8 juice medium.

INDUCED DEFENSE MECHANISMS

Plants have several inducible defense mechanisms that act to limit fungus infection[51], including increased lignification and cell wall cross-linking, the production of small antibiotic molecules such as phytoalexins, host cell death at the site of infection such as hypersensitive response, the production of Active Oxygen (AO) and Pathogenesis-Related (PR) proteins synthesis such as enzymes involved in lignification and in the synthesis of phytoalexins, hydrolyses such as chitinase and β-1-3-glucanases[59-62].

Lignin and enzymes related to phenolic compounds: Several researches showed that the peanut seeds infected by *A. flavus* could accumulate more phenolic compounds such as cell wall-bound and some soluble phenolic compounds[63,64]. Liang *et al.*[65] compared the activities of Peroxides (PO), Polyphenoloxidase (PPO), Phenylalanine Ammonia Lyase (PAL) and the content of lignin in peanut genotypes with different resistance to *A. flavus* invasion after inoculation. The results showed that both PO and PPO activities and lignin content in resistant and susceptible genotypes increased after inoculation, but it appears earlier in time and higher in concentration in resistant genotypes. There was significant negative correlation between lignin content and infection rate at 7 days after inoculation. The activity of PAL, the key enzyme of metabolism of phenolic compounds (phytoalexin and lignin precursors) increased rapidly and reached their maximum levels in resistant genotypes at the first day after inoculation compared with the susceptible genotypes, which reached the maximum levels at the 4 days after inoculation. There was a significant negative correlation between PAL activity at the first day after inoculation and the seed infection rates.

Active oxygen and membrane lipid peroxidation: Liang *et al.*[58] systemically determined the changes of active oxygen species including superoxide anion (O_2^-), Hydrogen Peroxide (H_2O_2) and hydroxyl radial (OH^-), Lipoxygenase (LOX) activity and membrane lipid peroxidation levels in resistant and susceptible seeds after inoculation with *A. flavus*. In resistant genotypes, the level of Malondialdehyde (MDA) and the degree of parameter of membrane lipid peroxidation were significantly increased to 7-8 folds at 2-3 days after inoculation. Moreover, the generation rate of O_2^-, H_2O_2 and LOX were also increased markedly at early stage after inoculation. O_2^- and H_2O_2 were increased rapidly and reached to the maximums. However, no significant increase in the activities of superoxide dismutase (SOD) and catalase (CAT) was observed, which implied that there were not enough SOD and CAT to scavenge active oxygen. The accumulation of active oxygen and the increased activity of LOX can cause the changes in membrane lipid peroxidation, cell wall strengthening, synthesis of phytoalexin and hypersensitive cell death[59,66]. In contrast, the MDA level was increased at 5-6 days and the production rate of O_2^-, content of H_2O_2 and activity of LOX in susceptible genotypes were increased much later than in the resistant genotypes.

Phytoalexin accumulation: Phytoalexin are antibiotic secondary metabolites produced by plant in response to injury and invasion by some pathogens and appear to be involved in disease resistance[67]. Peanuts have been reported[68-70] to produce phytoalexins when sliced and exposed to their native microflora at room temperature in the dark. Peanut phytoalexins have been identified as isoprenylated stilbene derivatives closely related to 3,5,4'-trihydroxy-4-isopentenylstibene, Arachindin, Arachidin and £¬Arachidin[68,70] Wotton *et al.*[71] showed that the resistance to *A. flavus* infection in peanut seed is correlated with a potential for rapid accumulation of stilbene phytoalexin and the 4.9-12.8 μg mL^{-1} phytoalexin can inhibit the growth of *A. flavus in vitro*.

Resveratrol is a phytoalexin compound found in peanut seeds[72]. Liang[57] compared the synthesis capacity of resveratrol between the resistant and susceptible seeds after inoculation with *A. flavus*. The results showed that the accumulation of resveratrol in resistant genotypes was increased 30 folds at 3 days after inoculation, but the susceptible genotypes had the same levels until 4 days after inoculation. Cyclohexnomycin at a concentration of 100 μg mL^{-1} reduced 10% of total resveratrol production, while actinomycin D (100 μg mL^{-1}) reduced 93% of resveratrol content.

Induction of Pathogenesis-related proteins (PR-protein): Infection of plant with pathogens induces the accumulation of a group of proteins collective known as pathogenesis-related proteins (PR-proteins)[73]. The PR-proteins have certain characteristic properties, such as they are selectively extractable at low pH[74] and highly resistant to proteolytic enzymes[75].

The ability of peanut seeds infested with *A. flavus* to synthesize PR protein was analyzed by Szerszen and Pettit[76]. Four new polypeptides (16.4, 18.1, 23.0 and 30.6 kDa with pI 7.95, 8.00, 7.90 and 7.55, respectively) were found in viable intact kernel 18-24 h following inoculation. The PR-proteins from resistant genotype (VRR245) and susceptible genotypes

(Yueyou 5) after inoculation with *A. flavus* were extracted at pH 2.8 buffer and were analyzed by native PAGE and SDS-PAGE[57]. In PAGE-gel, 3 new bands were detected at 3 days after inoculation in resistant genotype (VRR245) and at 5 days in susceptible genotype (Yueyou 5). The SDS-PAGE revealed the difference between resistant genotypes and susceptible genotypes. Compared to non-infected controls, resistant genotypes had more PR-proteins than susceptible genotypes and 3 new proteins (14.8,19.2 and 23.5 kDa) were detected in both resistant/susceptible genotypes after inoculation with *A. flavus*. Three new proteins (16.4, 17.3 and 18.4 kDa) and 4 new proteins (43.0, 47.0, 87.5 and 97.4 kDa) could be detected in VRR245 at the first day and 2-5 days after inoculation, respectively. These 4 proteins were only detected in VRR245 after inoculation with *A. flavus*. All these new proteins are resistant to digestion by trypsin.

Chitinase and β-1-3-glucanase are the important PR-proteins in defending the plant against pathogens. They can protect the plant from fungal infection by their direct lystic action on fungal cell wall or by releasing oligosaccharide signal molecules that can activate a variety of plant defenses[77,78]. Activities of Chitinase and β-1-3-glucanase have been reported to exist and inhibit the growth of *A. flavus* in mature corn kernels[79,80] and several isoforms of two hydrolytic enzymes appear in response to *A. flavus* infection[81]. However, limited information has been reported on the activities and isoforms of these two hydrolytic enzymes in peanut kernels. Liang *et al.*[62] reported the identification and characterization of β-1-3-glucanase in cultivated peanut.

MECHANISM OF DROUGHT INDUCED AFLATOXIN CONTAMINATION

Preharvest aflatoxin contamination of peanut is closely associated with severe late-season drought stress. Several studies have been conducted to understand the predisposition of peanuts to aflatoxin contamination after drought stress[82-85]. Further, invasion by *A. flavus* and aflatoxin contamination in peanuts subjected to water-stress usually occurs first and to a greater degree in small, immature peanuts[9-11,84,85]. It was suggested that in immature peanuts some seed resistance mechanism inhibit growth and aflatoxin production by *A. flavus* in response to water and soil temperature stress[84]. This resistance mechanism has been reported as production of phytoalexin[86-88]. Wooton and Strange[71] indicated that resistance of peanut seeds to invasion by *A. flavus* was correlated with their capacity to synthesize phytoalexin as an early response to invasion and conditions that promoted invasion of peanuts by *A. flavus* inhibited phytoalexin production. Phytoalexin accumulation in response to wounding was decreased if the seeds had a history of drought stress. Dorner *et al.*[11] demonstrated that as peanut seed water content decreased during drought and temperature stresses, the capacity of seeds to produce phytoalexins decreased, resulting in fungal activity and aflatoxin contamination.

Inheritance of resistance: Mixon[89] valuated the frequency of distribution for percentage seed colonization from F_1 and F_2 plants of reciprocal cross between PI 337409 (resistance) and PI 331326 (susceptible) and found a broad sense of heritability value of 78.5%. The inheritance of resistance to infection by *A. flavus* in peanut was studied following inoculation in progenies, F_1, BC_1, F_2 and F_3, which derived from the cross between the resistant parent Zhanqiu 48 and the susceptible parent Yueyou 92[90]. The resistant characters were controlled by a pair of major genes with additive value 0.38 and a pair of minor genes with additive value 0.12. The additive gene actions were important for resistant character and the percent of recombination between parent genotypes was estimated at 43.22%. Heritability based on major genes was estimated at 58%.

APPLICATION OF FUNCTIONAL GENOMICS TECHNOLOGY

Functional genomics technology based on expressed sequence tags (ESTs) and microarray has been used widely in the research of plant disease resistance mechanism in recent years[91-93]. In peanut, the resistance trait against *Aspergilus flavus* infection is quantitative trait[94] and also effected by environment factors such as drought stress. To further illustrate the resistance mechanism in peanut against *A. flavus* and identify resistance genes, functional genomics technologies have been used to analyze expressed genes and to identify pathways involved in the resistance mechanisms[95-97]. Using peanut microarray chips, Luo *et al.*[97] compared the gene expression of peanut resistant line infected with *Aspergillus* under drought stress with drought stress alone and identified several genes regulated by fungal infection or drought stress. Functional genomics technology has advantages compared with traditional techniques especially in the interaction between plant, pathogen and environment. By using this technology, genome-wise global view of gene expression could be acquired. The comparison of gene expression in different cultivars and treatments could be revealed in one hybridization reaction and the expression data from different treatments can be compared for specific gene functions[97].

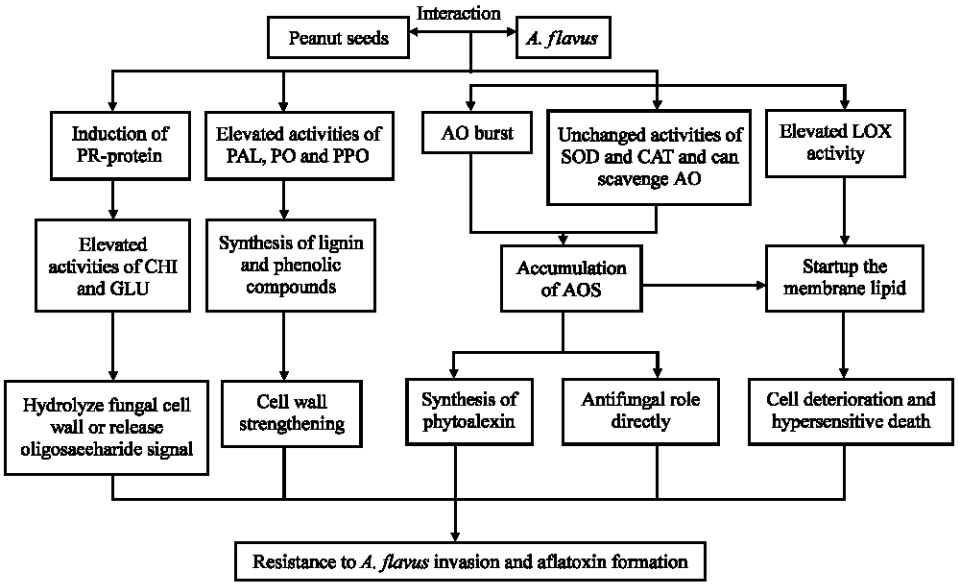

Fig. 1: Schematic drawing of defense responses activated by the interaction of peanut-*Aspergillus flavus*. PR: Pathogenesis-related Protein, PAL: Phenylalanine Ammonia Lyase, PO: Polyphenol Oxidase, AO: Active Oxygen, SOD: Superoxide Dismutase, CAT: Catalase, LOX: Lipoxygenase, CHI: Chitinase, PPO: Polyphenoloxidase, AOS: Active Oxygen Species, GLU: β-1-3-glucanase

Because of the complexity of the *Aspergillus*-plant interactions, better understanding of the genetic mechanisms of resistance will be needed using both conventional and molecular breeding for crop improvement and control of preharvest aflatoxin contamination[98]. Genetic improvement of crop resistance to drought stress is one component and will provide a good perspective on the efficacy of control strategy through genetic improvement. Although this technology is still in its infancy, microarray has been used widely for genome-wide gene expression analyses. This technology has been applied to characterize transcript abundance, complexity and stability and to identify novel target genes and pathways that are associated with biological process, to determine the regulatory elements of genes and pathways, to characterize gene functions by profiling genetic mutants and to profile gene expression pattern as a marker to predict biological performance. The lack of genomic information for peanut has hindered the progress in developing genetic and genomic tools and information for breeding and genetic enhancement.

CONCLUSIONS

Resistance mechanism in peanut to aflatoxin contamination has been studied for nearly 30 years. Some resistance mechanisms and resistant factors have been reported to contribute to the resistance to *A. flavus* (Fig. 1), but no effective efforts have been made to breed for these traits. This information on the mechanisms to resistance traits might not be fully understandable or they are highly influenced by environmental variation. As limited progress has been made in the molecular aspects, future researches are expected to provide a comprehensive understanding of the functions of peanut genes using gene expression analysis tools. The resistance mechanisms will be elucidated with advanced molecular biotechnology.

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