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Chapter 3

A New Approach in Aflatoxin Management in Africa: Targeting Aflatoxin/Sterigmatocystin Biosynthesis in Aspergillus Species by RNA Silencing Technique

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/51440

1. Introduction

Africa is faced with the challenge of merging its food crop production with its ever-increasing population in order to ensure food security of its people. The effort to meet Africa's food demand is however hampered by drought, crop diseases, insect pests, suitable storage facilities for various agricultural products, markets, lack of fertilizers, flooding, suitable seeds for various agro-ecological zones and poor rural infrastructure. The most limiting aspect and also ahealth concern is infestation of grains by fungal pathogens that also produce toxic fungal metabolites called mycotoxins [77, 25]. Though the fungi produce various mycotoxins, aflatoxins are a major concern in Africa [77]. This is partly because of the conducive weather for their accumulation in Africa (wet and humid climates and dry regions), their lethality on ingestion and widespread occurrence in maize (Zea mays) a main stable food crop grown in Africa by small-scale farmers for local consumption [28, 7].What this implies is that the main fungal genera and mycotoxin contaminant of maize in Africa is therefore Aspergillus species and aflatoxins respectively. Aspergillus species and aflatoxins not only attract worldwide attention but also are of great significance in Africa due to their negative impact on yield, human health, animal productivity and trade [54, 7, 77, 79, 28]. To exacerbate the problem, Sub Sahara Africa (SSA) experiences high temperatures and high relative humidity that predisposes many crops to fungal pathogens. In addition, majority of farmers in Africa are small scale hence rely on the consumption of homegrown crops. Therefore, irrespective of the quality considerations normally applied by some African governments to control aflatoxin contamination in food supply, aflatoxicoses will frequently occur in the continent.

The high temperatures and high relative humidity predisposes many crops to fungal and other pathogens. There is a significant correlation in aflatoxin levels in products after long storage in Agro-ecological zones with wet and humid climates and dry regions [28]. Maize is a staple food throughout the African continent but is highly colonized by Aspergillus species that produce aflatoxins [7] and the fungal contamination is of great concern. Peanuts (Arachishypogaea) are also grown in many African countries by small-scale farmers for local consumption and also export if food safety regulations are followed. Aflatoxin in peanuts seeds hamper international trade and also adversely affects health of consumers [54]. There should be reduction in food losses and maintenance of food quality. Due to malnutrition, there are approximately over 5million deaths in children under the age of 5 years in developing countries every year and aflatoxin contamination is suspected to be a factor in infant under-nutrition [38]. Some of the factors that contribute to aflatoxin contamination include; contact of product with soil during drying, high kernel moisture during storage, time of harvest [33]. Aflatoxins are mainly classified in B1 B2 G1 G2 M1 M2 based on chromatographic and fluorescent characteristics [42]. They occur in maize and other cereal crops, peanuts, cotton and oil seed crops. When Dairy cattle feed on commodities contaminated with Aflatoxin B1, the toxin is excreted in milk as aflatoxin M1 and can cause DNA damage, gene mutation and chromosomal abnormalities. Aflatoxins particularly B1 is confirmed a potential carcinogen [32]. In Kenya Aflatoxin M1 has been reported in milk [37] and in Gambia, Aflatoxin M1 has been detected in breast milk [87]. This leads to maternal exposure of aflatoxin M1 in breast milk to young children.

The failure of aflatoxin regulatory systems is therefore partly due to existing weather conditions, poor harvesting, transportation, marketing and processing conditions that favour proliferation of aflatoxin producing fungi [6, 62, 30, 4, 28, 67]. In addition, the Aspergillusspp have multiple infection courts that include;i) Mycelial growth on the silk kernels and cobs, ii) Kernel wounds created by insects and/or birds, iii) Soil debris and iv) Infected seed which predispose future maize crops to infection which makes it even harder to control [66, 50, 29]. This review summarises the current work on aflatoxins and their management in Africa. Furthermore it presents an argument based on the current knowledge on host and parasite macro and micromoleculartrafficking that suggeststhe possibility to circumvent the aflatoxin problem by use of cross species RNA interference. The aim is to arm maize with molecules that would shut down the aflatoxin biosynthesis upon infection with toxigenic fungi hence thwarting aflatoxin accumulation.

2. Health effects associated with aflatoxins

Aspergillusflavus and Aspergillusparasiticus are of great concern due to production of aflatoxins and millions of people in Africa are chronically exposed to aflatoxins due to feeding on contaminated food. The aflatoxin problem is most serious in tropical and subtropical countries due to favorable climatic conditions for Aspergillusflavus and Aspergillusparasiticus. Human and animals are exposed to aflatoxin through diet [16, 7]. Animal feed is of concern due to contaminated animal feeds. It is estimated that about 25,200 – 155,000 people

http://dx.doi.org/10.5772/51440

worldwide have aflatoxin induced liver cancer. Of this population 40% occur in Africa [45]. There are economic losses that result from contamination of crops and animal feeds with aflatoxins and also public health problems that result from ingestion of products contaminated with aflatoxins [54, 7]. In many developed countries, there are stringent government regulations on aflatoxins than any other mycotoxins with very low threshold for tolerance [20]. Maximum limit of contamination with aflatoxin in peanuts in Brazil and USA is 20μg/kg while Canada and European Union have imposed a limit of 15μg/kg [24]. For animal feeds, European Commission has maximum level for aflatoxins in animal feeds at 0.02mg/kg [21]. A number of African countries still have to put in place regulatory mechanisms for aflatoxins. However, Kenya's limit for aflatoxin in products for human consumption is 20ppb [39]. The two general forms of effects of aflatoxins are acute and chronic toxicity.

a. Acute toxicity is caused by ingestion of large amount of aflatoxins from heavily contaminated food. This causes decreased liver function and could lead to blood clotting mechanism, jaundice, a decrease in serum proteins that are synthesized by the liver, edema, abdominal pain, vomiting and death of affected person. This was the case in Kenya in 2004 where they were 317 cases and 125 deaths reported due to consumption of maize contaminated with aflatoxins [17, 58] identified the S strain of Aspergillusflavus as the causal agent of the outbreak. Epidemiological, clinical and experimental studies have indicated that exposure to large doses of aflatoxin causes acute toxicity but exposure to small doses for prolonged periods of time is carcinogenic. The liver is adversely affected by aflatoxins that cause necrosis of liver cells and death [15].

b. Chronic toxicity is due to long time exposure to low aflatoxin concentration. The main symptoms are decreased growth rate that leads to stunted growth [26]. In Togo and Benin, children who are underweight as a result of aflatoxins are also at higher risk for infections and diarrhea [26]. Aflatoxin-albumin adducts (32.8pg/mg) were detected in 99% of children between 9 months – 5 years. Exposure to aflatoxin in children increases at weaning and this contributes to reduced growth [26]. Exposureof children to aflatoxin can be through contaminated milk containing Aflatoxin M1 that is a metabolite of AFB1. In domestic animals,aflatoxins cause lowered milk or egg production and immune suppression that is caused by reactivity of aflatoxin with T-cell and a decrease in vitamin K activities including decrease in phagocytic in macrophages. [61]. It has been reported that there is a high risk among people with Hepatitis B and Hepatitis C carriers to develop cancer due to consumption of food contaminated with aflatoxins [75]. Aflatoxins have also been linked to immune suppression [70] and higher prevalence of hepatocellular cancer has been reported in Africa [68].

3. Management strategies against aflatoxins

Aspergillus infection increase with high temperature, high humidity, insect damage and nitrogen deficiency. Temperature and humidity are therefore important in aflatoxin manage-

ment. A. flavus and A. parasiticus are unable to grow or produce aflatoxin at water activity of less than 0.7 (relative humidity below 70% or temperature below 100C, however under stress condition such as drought, aflatoxin contamination can be higher [17]. Various strategies have been suggested in management of aflatoxins. The strategies should adhere to the following: a) aflatoxin must be transformed to non-toxic products, b) fungal spores and mycelia should be destroyed to prevent formation of new toxins, c) the food or feed material should retain its nutritive value and palatability, d) the physical properties of raw material should not change significantly d) it must be cost efficient [5, 16, 64].

The Physical and chemical treatment of contaminated commodities include detoxification of aflatoxins using physical means such as removal of contaminated commodities or inactivation of the toxin in the commodity. These methods include mechanical sorting and separation, washing, density segregation, solvent extraction, irradiationand oxidation [5]. However,efficiency of these techniques will depend on level of contamination. Furthermore, results obtained are often uncertain and relatively costly and could remove or destroy essential nutrients in feed [41]. Also some of the methods have disadvantages such as nutritional loss, toxic, limited efficiency and high cost therefore limiting practical application. Various natural and synthetic agents could prevent growth of toxigenic fungi and formation of mycotoxins and these have been reviewed by Mahoneyet al. [47]. Chemical methods of deactivating mycotoxins in feeds and also clay products that could be used in deactivating mycotoxins have been extensively been reviewed by Kolosova and Stroka [41]. Management strategies can be divided into Pre-harvest and Post-harvest strategies.

4. Pre- harvest strategies

These include;

a. Good agricultural practices (GAP) that involve adequate fertilizer application and crop rotation with non-host.

b. Management of insect pests that predispose crops to fungal infection through availability of infection channels such as wounds and other entry points.

c. Optimal harvest time so that crops are not left in the field exposed to environmental factors that predispose crops to pathogen infection. Harvesting immediately after physiological maturity is recommended since aflatoxin level can increase with delayed harvest interval [35].

d. Suitable management of crop residues as they harbor pathogens that are able to survive saprophytically [5].

e. Management with fungicides has challenges due to environmental pollution and also emergence of resistant pathogen populations and also chemical residue in food products. Of fundamental valueare environmentally friendly strategies. Polysaccharides and glycoproteins particularly β-glucans from basidiomycetes Lentinulaedodes (edible

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mushroom) is known to promote health effects in animals and human and have ability to inhibit aflatoxin biosynthesis by stimulating the antioxidant defence of the toxigenic fungus. Oxidative stress induced using paraquatenhanced the expression of β-glucan synthase gene and stimulated effect of β-glucans production that leads to a higher aflatoxin inhibiting capacity. Efficient inhibition could be due to higher content of β-glucans [60]. Utilization of microorganisms or their enzymatic metabolites to detoxify mycotoxins in food and feed has advantages such as mild reaction conditions, target specificity, efficiency and environmental friendly.

f. Resistant hybrids could be very promising but commercial hybrids are not always available [1]. However, availability of resistant varieties is the best solution for farmers so long as they are available and affordable. Some high yielding yellow maize varieties with good resistance to Aspergillus have been identified. This includes AO901-25 that has a grain yield of 7115kg/h and low aflatoxin level (IITA). However there is still a lot to be done in order to consider consumer prevalence as most people in Africa have prevalence to white maize. Furthermore, reduction in aflatoxin level is still required. Menkiret al. [51] registered tropical maize germplasm with resistance to aflatoxins. These varieties have been distributed to National programs for the development of locally adapted hybrids.

g. Biological control is use of one microorganism to control another microorganism such as Pseudomonas strains [55]. It has been noted that Aspergillusflavus strains differ in aflatoxin production and this influences crop contamination. There are strains that produce a lot of aflatoxins and also produce numerous small sclerotia (<400μm). These are the 'S' strains (toxigenic strain). Another strain the 'L' strain produces low aflatoxin levels and a few large sclerotia that are about >400μm and are atoxigenic [18]. There is competitive exclusion when one strain competes to exclude another in the environment. This implies that a shift of strain profile from toxigenic to atoxigenic is a viable biological control strategy. Atoxigenic strains of A. flavus from Nigeria have been combined as a bio-control product and registered as AflaSafe. It is used on sorghum as a carrier at the rate of 10kg/ha applied 2-3 weeks before flowering. Native strains have been identified and are being used in African countries. In Diourbel (Senegal) peanuts treated with AflaSafe had aflatoxin level of 1.9ng/g while control had 29.7ng/g giving a reduction in aflatoxin level of 93%. In Ibadan (Nigeria), crops in treated plots had Aflatoxin level of 11ppb while control had 42ppb giving 73% in reduction of aflatoxin. Stored products had 105ppb in treated samples while untreated samples had 2408ppb giving a reduction of 96% in Aflatoxin level [7, 18]. Due to good performance of atoxigenic strains, peanut producers in Senegal and Gambia are willing to adopt competitive exclusion technology for aflatoxin control in peanuts.

5. Post-harvest management

Reduction of moisture in grains is very important. There are a number of technologies that could be used to dry maize fast. Such technologies have extensively been reviewed by Lut-

fyet al., [46]. These technologies are expensive and most African farmers may not be able to acquire them. However some of the post-harvest strategies that could be used in Africa include the following: Rapid and proper drying of maize to moisture level of 13% or below. This will halt growth of fungi in the product. Products stored with high moisture increase growth of fungi in the stored product and this leads to increase of aflatoxin in the product [27]. Post-harvest insect control can prevent damage to maize. Clays such as Novasil could bind to aflatoxin in animal feeds [36]. Other control strategies have been reviewed by Kerstin and Mutegi, [40]. Quality management systems for Hazard Analysis Critical Control Point (HACCP) should be employed for management of mycotoxins [65].

6. Cost effectiveness of aflatoxin reduction strategy in Africa

It is important to consider economic impacts of food contaminants such as aflatoxins as it imposes enormous socio-economic cost to human society. Wu and Khlangwiset [80] analyzed two potential aflatoxin control strategies in Africa, 1) pre-harvest control using atoxigenic strains of Aspergillusflavus competitively to exclude toxigenic strains in maize and 2) post-harvest intervention in a package to reduce aflatoxin contamination in peanuts in Guinea. Health benefit was gained from each intervention in terms of fewer aflatoxin-induced cases compared to cost of implementing the intervention. Both interventions were found to be cost-effective if applied widely in Africa. The monetary value of life saved and quality of life gained by reducing aflatoxin induced hepatocellular carcinoma exceeds the cost of either bio-control or post-harvest intervention package. The estimated cost-effectiveness ratio (CER: gross domestic product multiplied by disability adjusted life years saved per unit cost) for bio-control in Nigerian maize ranged from 5.10 - 24.8 while estimated CER for post-harvest intervention package in Guinea peanut ranged from 0.21 - 2.08. Any intervention with a CER >1 is considered by world Health Organization (WHO) to be very cost effective while intervention with CER > 0.33 is considered cost effective [80]. The way forward with toxigenic strains of Aspergillusflavusis therefore:-

1. Each African country should identify local non-toxigenic strains and develop a package for legal registration for use in aflatoxin management and develop capacity for manufacturing the strains.
2. There should be extensive awareness programmes in each country since some African countries exchange agricultural products across the border without strict control. Awareness of aflatoxin problem and management strategies should be extended to Medical Practitioners, religious leaders, herbalists and Private Sector.
3. Efficacy of non-toxigenic strains should be demonstrated through farmers Schools, Non-Governmental Organizations (NGO), extension staff, outreach programmes and Women groups involved in agricultural services. This will enhance adoption by farmers.
4. Government should provide incentives to resource poor farmers to access non-toxigenic strains that should be available in small packages.

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5. There should be surveillance of aflatoxin testing in food and feed products
6. Government officials should be sensitized on aflatoxins and advantages of using local non-toxigenic strains. This will assist in formulation of appropriate policies.

7. Current status of aflatoxins in Africa.

In 2010 the level of aflatoxin in maize stored by farmers in Kenya were found to be 1776ppb while in the markets the concentration was 1632ppb [49]. These levels are likely to cause acute toxicity if contaminated products are consumed. In 2011, 40% of samples that were taken from farmers' fields in Eastern and Western Kenya were found with aflatoxin level of >10ppb. In Mali between 2009- 2010 aflatoxin level in peanuts were found to be >10ppb in 35-61 % of samples from farmers' fields and 39-91% samples from farmers stores [73]. Peanut paste in Mali had high aflatoxin level of >300ppb. Apparently the levels of aflatoxins in West Africa have been quite high. Maize in Benin had 4,000ng/g, In Ghana aflatoxin level in peanuts was reported to be 216ng/g while peanut paste had 3,278ng/g and peanut sauce 943ng/g, cashew paste, 366ng/g. In Nigeria Peanut oil had 500ng/g while yam flour had 7600ng/g [7]. This an indication that Ghana urgently needs intervention strategies to mitigate the aflatoxin challenges. In Kenya,aflatoxin M1 has been reported in milk [37]. There have been re-occurrence of outbreaks of acute aflatoxicoses in Eastern province that causes various deaths [57, 58].The S strain morphotype of A.flavus was identified as the cause of aflatoxicoses in 2004 and 2006 [57]. Apparently the high incidence of S strain of A. flavushighly correlated with acute aflatoxicosis in Eastern region of Kenya [56, 58, 57]. A simple test for Aflatoxin in maize kernels is the Bright greenish-yellow fluorescence (BGYF) or the black light test. Kernels are viewed under UV lamp (365 nm) for characteristic BGYF. This indicates a possible presence of aflatoxin producing fungi or mycotoxin itself [84] Laboratories in Africa should be able to perform these tests during surveillance survey.

8. RNA interference Strategy and its mechanisms

RNA interference (RNAi) refers to post-transcriptional gene silencing mediated by either degradation or translation arrest. This mechanism was first discovered in plants where transgene and viral RNAs guide DNA methylation [74, 34, 52]. The process is a naturally occurring biological process that is highly conserved among multicellular organisms including plants. The process is mediated by small interfering RNAs (siRNAs) that are produced from long dsRNA of exogenous or endogenous origin by an endonuclease (an enzyme) called a dicer. The resulting siRNAs are about 21-24 nucleotides long with 2 nucleotide single stranded 3' end overhangs on each strand. The siRNAs are then incorporated into a nuclease complex called the RNA-induced silencing complex (RISC), which then targets and cleaves mRNA that is complementary to the siRNA [86].

In plants, RNAi plays a role in cellular defense, protecting the cell from inappropriate expression of repetitive sequences, transposable elements and viral infections [43]. RNAi has

proved to have ability to regulate the expression of genes involved in a variety of cell processes such as proliferation, apoptosis and differentiation [2]. Moreover, it is thought to play a role in protecting the genome against damage caused by transposons [44]. More recently, these findings have been extended by the observations that siRNA-directed DNA methylation in plants is linked to histone modification [89]. In fission yeast, hetero-chromatin formation at centromere boundaries is associated with siRNAs [72].

Application of RNAi in crop improvement has been derived from targeted degradation of gene products with significant homology to the introduced sequence. Successful utilization of RNAi-based resistance effects rely on; (i) identification of a target gene (ii) dsRNA delivery, which includes in plantaexpression of dsRNA and (iii) delivery of sufficient amounts of intact dsRNA. RNAi can therefore be an important tool for crop improvement given that the RNAi signal can be both local (cell-cell) and systemic (spread through vascular system) [8, 71]. RNAi mediated silencing for agricultural traits offers the advantage of transmission across many cells and application in multigene family silencing.

9. Application of RNA interference in management of biotic challenges in agriculture

RNAi against crop parasites that include insects, nematodes, viruses and parasitic plants has been demonstrated [3, 9, 10, 11, Day et al., 1991, 22, 31, 82, 83, 88]. For example, the cotton bollworm (Helicoverpaarmiger a; Lepidoptera) and western corn rootworm (Diabroticavirgiferavirgiferaleconte) where dsRNA directed against a gene encoding V-type ATPase A, demonstrated rapid knockdown of endogenous mRNA within 24 hours of ingestion. In addition, dsRNAs directed against three target genes (β-tubulin, V-ATPase A and V-ATPase E) in western corn rootworm effectively resulted in high larval mortality [9].

Root-knot nematodes (Meloidogynespp) cause significant crop losses in Africa with the most damaging ones being M. incognita, M. javanica, M. arenariaand M. hapla. Since the discovery that RNAi is active in worms through oral uptake of dsRNA [22], intense studies on the control of parasitic nematodes through targeting essential parasite genes have been carried out [31, 82]. Yadav [82] described almost complete resistance to Meloidogynespp infection in transgenic tobacco. Geminiviruses, a major problem on crops in tropical and subtropical countries have been targeted via RNAi [Asadet al., 2003, 10, 11, 19, 83, 88]. With dsRNA and antisense RNA (as RNAi technologies), several regions of the viral genome can be targeted by plants expressing fused viral siRNA or hairpin dsRNA sequences. Multiple targeting of the viral genome provides stable and durable resistance considering that the viral genome is highly recombinogenic [12].

Currently it is understood that transcripts can be trafficked from host to parasitic plants [59]. Therefore when the RNAi transformed host plant is attacked by the parasite, the gene specific RNAi transcripts can be trafficked into the parasitic plant via the haustorial connection leading to gene silencing. Some studies have proposed the targeting of KNOX genes which are vital in plant development while others have suggested targeting genes that code for

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aquaporins that aid in loosening of the host plant cell wall during parasite infection [63]. This ability to tap into native pathways has yielded crucial breakthrough in parasitic plant management [23].

10. Prospects of applying RNAi in management of Aspergillus species and aflatoxins in grains

Early genetic studies have identifiedaflatoxin biosynthesis to becontrolled by a cluster of aflatoxin and sterigmatocystin gene in an ~70kb region [85, 13, 69, 76]. A critical analysis of the pathway indicates that their exists three enzymes that catalyse the two rate-limiting steps in aflatoxinbiosynthesis. Two enzymes stcJ and stcKcatalyse the first step that involves the conversion of Acetate and Malonyl-CoA into Hexanoyl-CoA. A further critical examination of the pathway identifies another enzyme stcA which catalyses the conversion of Hexanoyl-CoA to Norsolorinic acid. To addstrength to this observation, Brown et al.,[1996] reported that Aspergillus species with mutations in the stcJ and stcK grew normally but could not produce aflatoxin and sterigmatocystin. In the same study addition of Hexanoic acid to growth media restored aflatoxin and sterigmatocystin production. Recent studies have reported the trafficking of molecular cues between hosts and parasites including fungi. Among the molecules are small interfering RNA SiRNA. This targeted downregulation of gene expression bySiRNA has been used to engineer crops against virueses, nematodes and parasitic plants in cross species version. The key steps for this strategy to succeed are;i) identifying a key gene to a process, ii) cloning the target sequence of the gene from the parasite, iii) making an RNAi construct with the target sequence of the parasite in sense and antisense direction separated by an intron so as to allow formation of primary small intereferingRNAs (SiRNA) in host (maize), iv) transforming the host (maize) with the construct tailored for RNAi. In this case the rate-limiting steps in aflatoxin biosynthesis are known to be catalysed bystcJ, stcK and stcA [85,]. The strategy is therefore be to make an RNAi construct containing either combined partial or full sequences of the stcA, stcK and stcJ in sense and antisense orientation and transform it into maize. Upon colonization with aflatoxigenic fungi in the field, the primary SiRNA molecules will then cross from transgenic maize into Aspergillussppfungi through the haustoria connection at infection. The siRNAs will then cleave the stcJ, stcK and stcA mRNAs into 20 to 28bp long double molecules hence downregulating or inhibiting aflatoxin and sterigmatocystin biosynthesis.The transformed aflatoxigenic species in the field will be unable to synthesizeaflatoxins both in field and storage. RNAi will succeed in this case because it can be both local (cell–cell) and systemic (spread through the vascular system), hence all parts of the transgenic plant shall remain armed against aflatoxin biosynthesis. The stcJ, stcK and stcA do not exist in maize hence their silencing will not affect the crop.

11. Conclusion

Mycotoxins especially aflatoxins are believed to have caused harm to mankind since time immemorial. It is now almost 54 years after the discovery of the Turkeys X disease suspected to have been caused by aflatoxin contamination. Several major steps have been made towards the understanding of the aflatoxin biosynthetic pathway and its related genes. This book chapter not only emphasizes such work, but also focuses on Africa where due to the complex social economic dynamics, aflatoxins have greatly impacted negatively on the grain consuming population. This work goes on to describe the biosynthetic control of aflatoxinand further explores how AF/ST pathways could be altered via cross species RNA interference of key steps. If adopted, together with other existing aflatoxin control methods we believe researchers targeting mycotoxinswill realign their efforts in the development of practical methods for preventing not only aflatoxin contamination but alsoall the major mycotoxins in grains and nuts.

Acknowledgements

This work was funded by a grant from the Bill & Melinda Gates Foundation through the Grand Challenges Explorations initiative.

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