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Maize pollen to control mosquitoes

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Introduction

The *Anopheles gambiae* complex of mosquitoes that transmit malaria in Africa develops most abundantly in water bearing the "stamp of human activity" (Gillies & De Meillon, 1968). Larvae of this species complex exploit shallow, vegetation-free puddles that frequently are rendered virtually opaque by suspended clay and other inert colloidal soil particles so ubiquitous throughout the world's tropics (Gillett, 1972). Although microbes (mainly bacteria) may occur abundantly in such water (Kondratieff & Simmons, 1985) or on its surface (Walker & Merritt, 1993; Wotton *et al.*, 1997), any endogenous microbial infusion in this medium would be sparsely dispersed throughout the puddle and would be diluted enormously by indigestible silt. The presence of such large quantities of inert material suspended in the mosquito breeding habitats imposes on them a requirement for some peculiarly adapted feeding strategy, an adaptation that has yet neither been well explored nor exploited.

Rather than concentrating on a poorly-nutritious endogenous microbial infusion, larvae of *A. gambiae* feed on exogenous material that becomes trapped on the water surface, and pollen seems the most likely nutrient source. Maize (*Zea mays*) pollen appears to provide a complete food for certain beetles (Cottrell, 1998) and might also qualify as a food source for *A. gambiae* larvae, because it is wind-borne and is cultivated by small-holders and in gardens maintained by virtually every household in much of rural Ethiopia and many parts of Africa during the wet season. Maize was introduced into Africa in the 16th Century but languished in a few coastal pockets of cultivation until the early 1900s, when large-scale farming began in the South African Transvaal (Spencer, 1910). This crop became well established in East Africa during World War I, when the British encouraged its cultivation in Kenya as a major export product (Byerlee & Heisey, 1997). By the 1930s, maize had become important in much of Africa both as subsistence and cash crops. A recent World Bank Internet newsletter described the increase of maize production in Sub-Saharan Africa since the 1960s as "phenomenal" (www.worldbank.org/html/cgiar/newsletter/april97/8maize.html). Fueling much of this increase has been a "green revolution" in biotechnology. New varieties have been bred that mature far more rapidly and are much more productive than are older varieties.

Maize has become the main cereal crop during the wet season on much of the continent. Its perceived value for ethanol production has further enhanced maize cultivation.

Intensive maize cultivation, development of larval *Anopheles arabiensis* and incidence of malaria in Sub-Saharan Africa occur concurrently, depending on the seasonal rains. Biological relationships linking maize culture with malaria-vector density are evident. Anthropogenic accumulations of water, such as borrow pits, hoof-prints and tire tracks are particularly abundant in villages where maize is usually cultivated (Gillett, 1972), and the intensity of malaria transmission is spatially correlated with the extent of maize cultivation (Kebede *et al.*, 2005).

A medium sized maize plant may produce as many as 50 million soft-walled pollen grains (Paton, 1921), and the density of such pollen in a cornfield may exceed 42 thousand grains/in^2 (Kiesselbach, 1949). Each pollen grain is thin walled, roughly spheroidal, with a diameter of 50-90 μm, well within the size range of particles ingested by mosquito larvae. Maize is an unusually prolific source of pollen. Although a few such grains may be carried over great distances by strong gust of wind, they generally settle to the ground within 60 meters and tend to be trapped in the surface film of any body of water on which they settle (Ye-Ebiyo *et al.*, 2000). Potential anopheline breeding sites that are situated in the immediate vicinity of a maize plant are thus enriched by this important food source.

Taken together, these considerations depict an *intimate relationship between flowering maize and development of the main anopheline vectors of malaria in Africa.* We therefore proceeded to develop a potential powerful *antimalarial* intervention strategy that is based upon an *entomotoxic transgenic variety of maize-pollen* against these larval mosquitoes using the properties of two powerful anti-mosquito molecules *Bacillus thuringiensis israelensis* (Bti) and Trypsin Modulating Oostatic Factor (TMOF). The primary target of the Bti toxin is the larval midgut epithelium in mosquitoes and black flies, where proteases activate the protoxin at alkaline pH. The toxins exert their effects in an impressively specific manner and perforate the gut membrane that selectively channels K^+ and Na^+ cations. Equilibrium of these ions across the insect cell membrane results in an influx of water that leads to a colloid osmotic lysis and death (Margalith & Ben-Dov, 2000).

As with the Bti toxins, mosquito TMOF affects a relatively narrow range of targets. TMOF appears to affect the activity of adult and larval midgut cells that synthesize trypsin and prevents adult from digesting its blood meal and starves mosquito larvae causing anorexia and death (Borovsky *et al.*, 2006). Earlier studies from our laboratories showed that TMOF synergizes with Bti enhancing the potency of Bti several folds when both were expressed in yeast cells (Borovsky *et al.*, 2010).

Material and methods

Combinations of mosquito larvicidal toxin genes were engineered for expression in maize pollen, including *cry* and *cyt* of Bti together with *tmfA* that encodes TMOF which is an unblocked decapeptide hormone (YDPAPPPPPP) of *Aedes aegypti.* TMOF blocks the translation of trypsin transcripts in the midgut and starved larvae are 6-35 fold more sensitive to Bti toxins then fed larvae; the combination of Bti toxins and TMOF is therefore anticipated to suppress larval populations around fields planted with genetically engineered maize expressing these genes in the pollen. Continuous anti-vector coverage of entire village is likely to be achieved with transgenic maize producing such larvicidal pollen.

Results and discussion

The series of constructs designed and prepared included a combination of *cry/cyt* (Ben-Dov *et al.*, 1995) with *tmfA* in tandem flanked by a maize pollen-specific promoter and a terminator sequence used in plants expressing foreign genes. Transformed plantlets with one or several genes were generated by particle bombardment, grown in the greenhouse, and their pollen bioassayed by feeding it to *Ae. aegypti* larvae. Based on the pollen toxicities and qPCR analyses of the transgenic plants, the most toxic lines were selected and selfed to select homozygous lines carrying single and multiple genes combinations. Several of the selected lines killed 85 to 100% of larvae that were fed on the transgenic pollen whereas control pollen did not harm them: larvae that were fed on pollen lacking the transgenes developed normal adult mosquitoes (D. Borovsky, R. G. Shatters Jr., C. Powell, E. Ben-Dov & A. Zaritsky, to be published elsewhere). Extensive tests against many organisms demonstrated that Bti toxins and TMOF do not harm the environment (Borovsky, 2007), and transgenic maize expressing genes derived from various subspecies of *Bacillus thuringiensis* protecting crops against Lepidopterans and Coleopterans is already grown worldwide.

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