In principle, genetic engineering could also be used to engineer the secretion of weed-suppressing allelochemicals from crop roots or to enhance the vitality, virulence and persistence of biocontrol agents ('bioherbicides') such that they might become commercially viable. Such efforts to use the tools of modern biotech to attack weeds would clearly reduce the inappropriate drudgery that most women are relegated to enduring in the developing world.

Confucius is quoted as saying: "If language is incorrect, then what is said is not meant; if what is said is not what is meant, then what should be done remains undone." If it is not clearly stated with meaning that weeds are a major constraint on the quality of life of most women in the developing world, then what should be done remains undone, and gender issues have not been adequately addressed with biotechnology.

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1. http://www.worldfoodprize.org/Symposium/2009/transcripts.htm

First transgenic geminivirus-resistant plant in the field

To the Editor:

The article by Lucioli *et al.*[1] in last year's June issue states not only the drawbacks to the use of pathogen-derived resistance (PDR) for geminiviruses, but also that RNA interference (RNAi) thus far has not proved to be a robust technology for obtaining plants resistant to these viral pathogens. We present here data on the generation of two transgenic geminivirus-resistant common bean lines. On the basis of these results, we feel that the conclusions of Lucioli *et al.*[1] are premature.

The family Geminiviridae infects a wide range of economically important crop species (e.g., common bean, *Phaseolus vulgaris*; tomato, *Solanum lycopersicum*; cassava, *Manihot esculenta*; maize, *Zea mays* and cotton, *Gossypium hirsutum*) in tropical and subtropical regions and has become a major threat to agriculture worldwide. Bean golden mosaic virus (BGMV) belongs to the genus *Begomovirus*, whose genome is composed of two single-stranded DNA molecules, designated DNA-A and DNA-B, both of which are essential for infectivity. BGMV is transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a persistent, circulative manner, causing golden mosaic in common bean. This disease is characterized by yellow-green mosaic of leaves, stunted growth and distorted pods, which may vary among genotypes. Similar diseases have been described in Puerto Rico, Guatemala, the Dominican Republic, Mexico and the United States[2,3]. Phylogenetic studies and differences in biological properties, such as sap transmissibility, support the taxonomic separation of bean-infecting geminivirus isolates from Brazil (designated BGMV) and isolates from Central America, the Caribbean basin and Mexico (designated Bean golden yellow mosaic virus, BGYMV)[3].

Control practices have focused primarily on controlling the vector by contact or systemic high-toxicity insecticides, with the concomitant problems of development of pesticide-resistant forms, low cost-benefit ratio and environmental concerns. This disease is the heaviest constraint on bean production in Latin America, causing significant yield losses ranging from 40% to 100%[2]. Since 1972, Brazilian bean production has been severely reduced by the disease. The increasing seriousness of the disease has been attributed to growing whitefly populations associated with expansion of soybean production in bean-growing areas. Extensive screening of common bean germ plasm for resistance to BGMV has not yielded genotypes with high resistance to the virus. Indeed, of more than 20,000 accessions of *P. vulgaris* and some accessions of *P. lunatus*, *P. acutifolius* and *P. coccineus* evaluated under field and laboratory conditions, not a single accession has proven immune. Resistance is often unsatisfactory, and commercial cultivars are susceptible to early, moderate or severe infection[2,4].

After the first demonstration of pathogen-derived resistance (PDR) in the pioneering work from Roger Beachy´s lab describing coat protein–mediated resistance to tobacco mosaic virus[5], several strategies have been used to genetically engineer tolerance or immunity to viruses in transgenic plants. These strategies are based on two broad classes: protein-mediated resistance and RNA silencing–mediated resistance. Now that we better understand the mechanisms of RNA interference (RNAi) and its biological functions, it is possible to look back on initial experiments from a new perspective.

It is now known that plants naturally process viral RNAs to generate small sequences of a pathogen's genetic material that can be specifically used against that pathogen through the RNA-induced silencing complex. An RNA-silencing (post-transcriptional gene silencing) mechanism was recognized as being responsible for resistance to RNA viruses. This mechanism depends on the formation of double-stranded RNA (dsRNA) whose antisense strand is complementary to the transcript of a targeted gene.

These discoveries led to the introduction of constructs to produce intracellular generation of small interfering RNA (siRNA)-like species in transgenic plants, inducing targeted gene silencing and virus resistance. This is an important tool in generating plants resistant to a broad range of viruses[6]. However, not all viral genes used in transgenic constructs render plants resistant to infection. The use of inverted repeat constructs, resulting in dsRNA transcripts, is the most efficient means of generating transformed lines showing effective gene knockdown or virus resistance[7]. The most likely reason for this is that dsRNAs are fed directly into the silencing pathway at the level of the RNaseIII-like enzyme Dicer, and therefore they are not reliant on the action of plant-encoded RNA-dependent RNA polymerase proteins. Nevertheless, most examples of RNAi-mediated virus resistance pertain to RNA plant viruses. Indeed, attempts to obtain robust PDR to geminiviruses have not been as successful as those against RNA viruses, and development of geminivirus-resistant plants is considered a major challenge[8].

Because of the social and economic importance of common bean as a source of protein in the diet of over a billion people worldwide, we have been attempting since

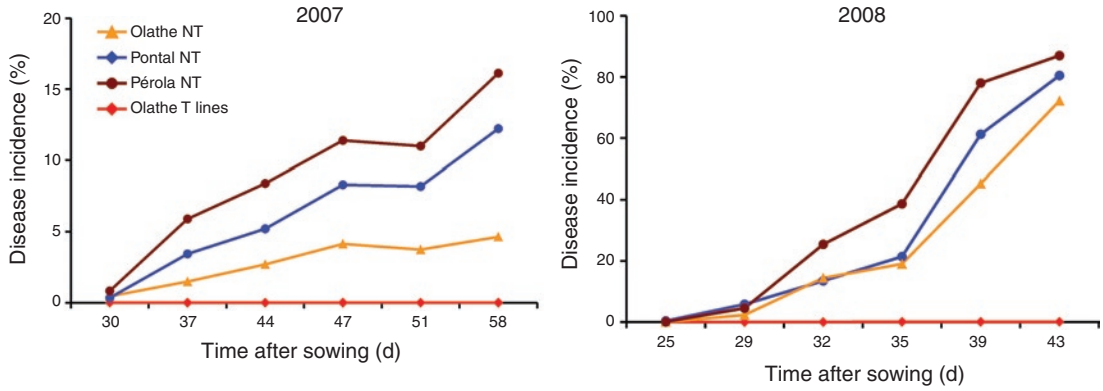

Figure 1 Development of golden mosaic disease in the transgenic (T) common bean lines engineered for resistance to BGMV-mediated RNAi compared with non-transgenic varieties (NT). Two transgenic lines showed similar resistance curves. Plants were cultivated in the field in 2007 and 2008. Disease evaluation was done visually by recording the first date of vein clearing.

the early 1990s to obtain BGMV-resistant engineered lines of the common bean. We have studied the molecular biology and diversity of BGMV and have developed a bean transformation system that allows us to obtain transgenic plants with high efficiency. We first created transgenic lines that express BGMV coat protein gene—the primary choice for PDR in begomoviruses—but these failed to show resistance to the virus (D. Maxwell, unpublished data).

We also explored a second strategy involving expression of the construct comprising the viral genes encoding replication initiator protein (*rep*; *AC1*), transactivator protein (*TrAP*; *AC2*), replication enhancer protein (*REn*; *AC3*) and movement protein (*BC1*) in an antisense orientation[9]. The resulting transgenic lines showed delayed and attenuated golden mosaic symptoms upon whitefly-mediated inoculation. Using the strategy of transdominance, we then obtained bean lines with a vector containing the mutated *rep* (*AC1*) gene. This mutated *rep* gene encodes a mutated AC1 (REP) protein with an amino acid change (D262R) in the putative NTP-binding motif ($EGX_4GKTX_{32}DD$). One of these lines showed resistance to the virus. After studying resistance for several generations, however, we determined that immunity to infection depended on the number of viral particles inoculated[10].

More recently, we have explored the concept of using RNAi constructs to silence the *AC1* viral gene, which encodes the only protein strictly essential for viral genome replication. We hypothesized that silencing expression of the *AC1* viral gene, by sequence-specific degradation of target mRNA interfering with viral replication, would reduce or prevent viral DNA accumulation and, consequently, appearance of symptoms. We obtained 22 lines with an intron-hairpin construct designed to induce post-transcriptional gene silencing of the *AC1* gene. These lines were first evaluated under greenhouse conditions. Two of these lines (named 2.3 and 5.1) showed high resistance (~93% of the plants were symptom free) upon inoculation at high pressure (>300 viruliferous whiteflies per plant during the plant's entire life cycle) and at a very early stage of plant development[11]. In the field, 2–10 whiteflies per plant led to 100% virus infection of wild-type plants. Homozygous plants were crossed with nontransgenic plants to generate a hemizygous population. Both homozygous and hemizygous plants were inoculated using viruliferous whiteflies. Two weeks after inoculation, 100% of homozygous plants remained symptomless, whereas 28.7% of hemizygous ($n = 164$) plants showed mild symptoms, and all nontransgenic plants ($n = 40$) showed severe symptoms.

It is well known that viral protein, initially identified as a mediator of synergistic viral disease, acts to suppress the establishment of both transgene-induced and virus-induced gene silencing, in which co-infection with two heterologous viruses leads to much more severe symptoms than does infection with either virus alone. Many such synergistic diseases involve a member of the Potyvirus group of plant viruses[12]. Thus, we examined possible resistance suppression by co-inoculating transgenic plants with the bean common mosaic necrotic potyvirus or bean rugose mosaic comovirus and the BGMV. In these experiments, all transgenic plants infected with bean common mosaic potyvirus or bean rugose mosaic comovirus maintained their immunity against the BGMV ($n = 5$).

Table 1 Agronomic traits in bean transgenic line 5.1 cultivated in the field during low-disease-incidence season in three regions of Brazil

Trait	Goiás		Minas Gerais		Paraná	
	Control	Transgenic	Control	Transgenic	Control	Transgenic
Yield (kg/ha)	770.8	628.1	2,460	2,476	2,268	2,344
Seed germination (%)	86.9	91.4	87.9	85.4	75.2	86.2
Initial plant height (cm)	10.4	10.2	13.6	13.5	9.9	9.7
Width of the leaves (cm)	6.8	6.7	7.4	7.3	6.4	6.3
100-seed weight (g)	27.3	29.7	31.0	32.1	31.4	32.7
Flowering time (days after germination)	31	31	32	32	30	30
Seeds per pod	5.8	5.7	5.3	5.4	5.6	5.7

Statistical analyses revealed no significant differences ($P < 0.05$; Tukey studentized range test, $n = 48$) between transgenic and control lines.

We then conducted studies on the behavior of transgenic common bean lines under field conditions. The first two field trials were carried out during the agricultural seasons of 2007 and 2007–2008 in the state of Goiás, Brazil. Results from these trials confirmed earlier greenhouse observations. Experimental plots with five replications (each replication with five 5-m rows and >300 plants), conducted in a random-block design under low and high *B. tabaci* pressure, resulted in no infected transgenic lines. The highest average incidence of infection in nontransgenic control plots was 18% in 2007 and 83% in 2008 (**Fig. 1**). Nontransgenic plants showed characteristic severe symptoms of the golden mosaic disease (yellow-green mosaic in leaves, stunted growth and distorted pods). Off-target effects were not apparent, and transgenic lines showed normal phenotypes (plant height, site of insertion of first pod, number of branches, internode length, foliar area and total number of flowers, flowering time and pods).

We went on to select transgenic line 5.1 for agronomical evaluation in the field during the low-disease-incidence season in three distinct regions of Brazil (Paraná, 23° 11′ S, 51° 10′ W; Goiás, 16° 30′ S, 49° 17′ W; and Minas Gerais, 19° 26′ S, 44° 09′ W). In these trials, no significant differences were observed in the seed germination, initial plant height, width of the leaves, flowering time, yield, number of seeds per pod and 100-seed weight (**Table 1**).

We are now conducting biosafety evaluations, taking into account the demands of the Brazilian Biosafety Committee and other regulatory authorities, with a view to obtaining authorization for commercial release of the first transgenic bean varieties. These studies are evaluating interactions of transgenic plants with microorganisms, insects and other plants from the agricultural and natural environment. In addition, the stability of foreign gene expression, gene flow and factors related to their interaction with the complex physiology of these plants exposed to natural stress in tropical environments are now under study. Furthermore, food biosafety analysis is being carried out by the Biosafety Network of Embrapa to determine differences in nutritional and antinutritional compounds as well as to verify the absence of toxic molecules. All risk assessment data generated so far have suggested no differences between transgenic lines and parental plants. In addition, as RNAi in eukaryotes is a sequence-specific gene silencing mechanism, we are resequencing viruses isolated from several parts of Brazil and have observed no significant variations in the sequence of the *rep* gene. Molecular analysis of the 421-base-pair fragment from the BGMV genome used for the intron-hairpin construct showed isolates with 100% similarity or variants with one point polymorphism.

We believe we are on the road to generating the first commercial transgenic plant produced in Latin America. This work is an example of a public sector effort to develop useful traits, such as resistance to a devastating disease in an 'orphan crop' cultivated by poor farmers throughout Latin America. It has the potential to become a milestone as one of the first 'homemade' biotech crop improvements performed in a non-G8 country as a result of the interaction between bench and field scientists. In addition, in the context of global food deficiency and high prices, where golden mosaic virus causes annual reductions in yields in the range of 90,000 to 280,000 tons, a geminivirus-resistant bean could boost production enough to feed an estimated extra 6 million to 20 million adults. In addition to the economic problems caused by yield reduction, this virus also brings other social consequences as it precludes common bean as a crop in family based agricultural systems. Approximately 180,000 hectares are now unsuitable for common bean cropping in the dry season in Brazil due to the occurrence of BGMV. These areas can be recovered for bean cultivation after development of resistant cultivars. Finally, the success presented here demonstrates the feasibility of genetically engineering geminivirus-resistant plants, a technique that can also be applied to other devastating geminivirus diseases, such as those affecting maize and cassava in Africa and tomatoes worldwide.

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Lucioli & Tavazza reply:

The focus of our letter of correspondence was protein-mediated resistance against geminiviruses (particularly tomato yellow leaf curl Sardinia virus; TYLCSV); in contrast, Aragão and Faria expand on the utility of RNA silencing–mediated resistance to geminiviruses.

In our letter, we described the molecular mechanisms that form the basis for the ability of TYLCSV to overcome protein-mediated PDR. Using an anti-virus-induced transgene silencing strategy, we demonstrated that virus-induced gene silencing (VIGS) is an Achilles' heel for protein-mediated resistance. As we wrote in the original article, "if the transgenic protein does not stop viral expression and/or replication in the cells initially infected, then the virus will shut off transgenic expression, leading to a late-susceptible phenotype." We also proposed a simplified model to represent protein-mediated resistance to geminiviruses that takes into account the impact of VIGS. Our model suggests that strategies to avoid VIGS, while potentiating protein-mediated resistance to geminiviruses, may not generate virus-immune plants.

In the final part of the correspondence, we expressed personal opinions as to the drawbacks of using PDR against geminiviruses. Drawbacks include the lack of robust evidence proving the effectiveness of RNA interference (RNAi) in obtaining geminivirus-resistant plants[1,2] (for reasons that remain unresolved), the possibility that PDR may be compromised by plant viruses encoding proteins that interfere with different steps of the RNA silencing pathway[3] (a not unlikely scenario, given the prevalence of mixed viral infections) and the capacity of geminiviruses to evolve rapidly by mutation, recombination and pseudorecombination[4] (potentially leading to the emergence of resistance within a short time). We think that a deeper investigation of the above aspects is still necessary for thorough exploitation of PDR against geminiviruses.