Field trial of *Xanthomonas* wilt disease-resistant bananas in East Africa

To the Editor:

Banana is a major staple crop in East Africa produced mostly by smallholder subsistence farmers. More bananas are produced and consumed in East Africa than in any region of the world. Uganda is the world's second foremost grower with a total annual production of about 10.5 million tons. The average daily per capita consumption in Uganda ranges from 0.61 to over 1.6 kg, one of the highest in the world. In this Correspondence, we report preliminary results from a confined field trial in Uganda of transgenic bananas resistant to the deadly banana *Xanthomonas* wilt (BXW) disease.

BXW caused by *Xanthomonas campestris* pv. *musacearum* is threatening banana production, the livelihoods of the smallholder growers in East and Central Africa[1], and the stability of food security in the region. The disease has caused estimated economic losses of about $2–8 billion over the past decade and substantial reductions in production have resulted in major price increases[1]. BXW was originally reported[1] in Ethiopia and first identified in Uganda in 2001 and subsequently in the Democratic Republic of Congo, Rwanda, Kenya, Tanzania and Burundi. The disease is very destructive, infecting all banana varieties, including both East African Highland bananas (EAHBs) and exotic dessert and beer bananas. The economic impact of the disease is potentially disastrous because it destroys whole plants leading to complete yield loss. Once these pathogens have become established in smallholder plantations, disease control is very difficult. As diseases continue to spread, demand grows for new improved varieties.

There are currently no commercial pesticides, biocontrol agents or resistant cultivars available to control BXW[1]. Although BXW can be managed by following phytosanitary practices, the adoption of such practices has been inconsistent as they are labor intensive[1]. Given the rapid spread and devastation of BXW across Africa, the lack of known genetic resistance in banana against *X. campestris* pv. *musacearum*, and the difficulties associated with conventional breeding of this highly sterile crop, genetic transformation through the use of modern biotech tools offers an effective and viable way to develop resistant varieties.

Two potential transgenes for controlling BXW are those encoding hypersensitive response-assisting protein (*Hrap*) and plant ferredoxin-like protein (*Pflp*) from sweet pepper (*Capsicum annuum*). Both have been proven effective against related bacterial pathogens, such as *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas* spp., in other crops[2–8]. *Hrap* is one of the important hypersensitive cell death–associated genes that could be used to protect plants from bacterial pathogen attack[2]. HRAP has been reported to intensify the hypersensitive response mediated by harpin$_{PSS}$ (harpin derived from *Pseudomonas syringae* pv. *syringae*), by dissociating multimeric forms of the hairpin into dimers and monomers that trigger stronger hypersensitive cell death necrosis. Constitutive expression of *Hrap* genes in transgenic tobacco[2] and *Arabidopsis*[3] has been shown to confer enhanced resistance against virulent pathogens under laboratory and glasshouse conditions. Resistance resulting from overexpression of the *Pflp* gene in transgenic plants is due to intensified production of active oxygen species and activation of the hypersensitive response when plants are challenged with bacterial pathogens[4]. Overexpression of *Pflp* has been shown to provide resistance against various bacterial pathogens, such as *Erwinia, Pseudomonas*, *Ralstonia* and *Xanthomonas* spp., in transgenic tobacco[4], tomato[5], orchids[6], calla lily[7] and rice[8]. However, none of these transgenic plants was tested for disease resistance under field conditions.

In previous work, transgenic plants of two banana cultivars 'Sukali ndiizi' (apple banana; AAB group) and 'Nakinyika' (EAHBs; AAA group) were generated by constitutively expressing the *Hrap*[9] or *Pflp*[10] gene. Here we evaluated the best 65 resistant lines (40 lines expressing *Hrap* gene and 25 lines with *Pflp* gene) in a confined field trial at National Agriculture Research Laboratories, Kawanda, Uganda, against *X. campestris* pv. *musacearum* for two successive crop cycles (see **Supplementary Methods**) (**Fig. 1**).

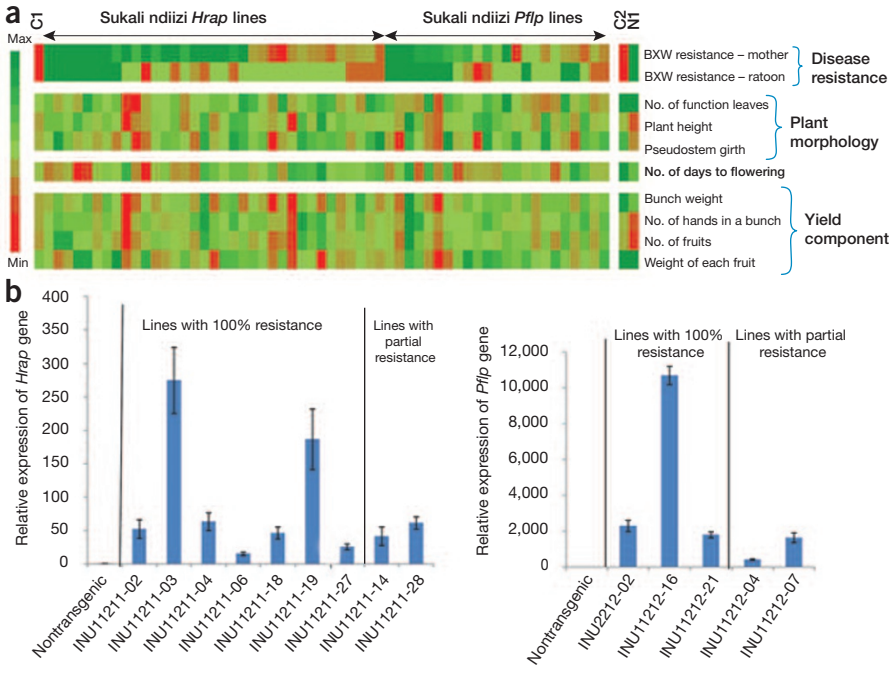

Figure 1 Performance and molecular analysis of transgenic lines under confined field trial. (**a**) Heat map showing disease resistance, plant morphology and yield component of transgenic lines in comparison with nontransgenic plants. C1, 'Sukali ndiizi' nontransgenic control; C2, 'Nakinyika' nontransgenic control; N1, 'Nakinyika' *Pflp* line. (**b**) Relative transcript levels of the *Hrap or Pflp* gene in transgenic lines in comparison with nontransgenic plants. Expression of transgene was normalized with *Musa 25S* ribosomal gene (internal control) and nontransgenic plant served as a calibrator. Relative expression was determined from replicate measurements in two independent biological replicates and three technical replicates. Data are mean ± s.e.m.

The resistant lines were selected from a large number of transgenic lines originally generated on the basis of enhanced resistance to BXW using potted plants in the glasshouse, the presence of low copies of the transgene and detectable expression of expected RNA signals. The majority of lines (64/65) tested in this study were of cultivar 'Sukali ndiizi', an easily transformable cultivar. Genetic transformation protocols using cell suspension are cultivar-dependent and EAHBs are recalcitrant for establishing cell suspension, resulting in only a few transgenic lines for cultivar 'Nakinyika'. Although this proof of concept work used 'Sukali ndiizi', we have successfully established cell suspensions of 'Nakinyika' and other EAHBs cultivars and are developing more lines using these genes.

Transgenic plants did not show substantial changes in morphology compared with nontransgenic plants (**Supplementary Fig. 1**). Most of the transgenic lines (59/65) showed normal growth and fruit development, suggesting that overexpression of the *Pflp* or *Hrap* gene does not induce negative pleiotropic effects and alter plant physiology (**Fig. 1a**).

We artificially inoculated mature, preflowering-stage plants of mother and ratoon (new growth from banana suckers without replanting) crops with *X. campestris* pv. *musacearum*, continuously assessed them for disease symptoms (chlorosis or necrosis of leaves and complete wilting of plants), and scored disease severity at harvest time. All nontransgenic control plants developed symptoms and eventually wilted completely, whereas several transgenic lines (21 lines of mother crop and 19 lines of ratoon crop) remained asymptomatic until harvest (**Fig. 2**). The majority of transgenic lines had significantly higher ($P \leq 0.05$) resistance in comparison to control nontransgenic plants (**Fig. 1a**, **Supplementary Fig. 2** and **Supplementary Table 1**). Eleven of these transgenic lines (7 *Hrap* lines and 4 *Pflp* lines) were highly resistant, similar to the reference, resistant, wild-type, uncultivated, not-edible variety *Musa balbisiana* (BB group), showing 100% disease resistance with both mother and ratoon crops in comparison to control nontransgenic plants (**Supplementary Table 1**). An additional five lines showed 85–93% resistance with mother plants and 100% resistance with ratoon plants. The higher resistance in ratoon plants might be because ratoon plants were expressing the transgene more consistently compared with the mother plants, resulting in a more-resistant phenotype. The mother plants would have been under more stress owing to hardening in the glasshouse and transplantation into the field.

The disease evaluation results confirmed the transfer of the disease resistance trait from mother to progeny in all the lines except two (**Fig. 1a** and **Supplementary Table 1**). Gene stability and expression in all the ratoon plants was confirmed by PCR and reverse transcriptase PCR (RT-PCR) (**Supplementary Fig. 3**). Using quantitative RT-PCR, we further assessed the expression of the transgene in 14 transgenic lines (7 *Hrap* lines with 100% resistance, 2 *Hrap* lines with partial resistance, 3 *Pflp* lines with 100% resistance and 2 *Pflp* lines with partial resistance). The level of *Hrap* or *Pflp* transcripts varied among the 14 transgenic lines tested. No transcripts were found in the nontransgenic plants (**Fig. 1b**). In only three of the transgenic lines (2 *Hrap* lines and 1 *Pflp* line) transcript levels positively correlated with disease resistance (i.e., the lines with high transcript levels were also highly resistant against *X. campestris* pv. *musacearum*). Other lines did not show any correlation between transcript level and disease resistance. These results suggest that the observed variation in disease resistance in transgenic lines is not due to transcript accumulation. It has been known that the timing of gene expression is important because in some cases gene expression may occur faster during an incompatible host-pathogen interaction in comparison to compatible interaction. It might be interesting to analyze the pattern of gene expression at a different time interval after pathogen infection. We will also investigate the influence of *Hrap* or *Pflp* on the transcription of other genes that are linked to plant defense mechanisms.

About 20% of the *Hrap* lines and 16% of the *Pflp* lines tested showed 100% resistance for both mother and ratoon crops under field conditions in comparison to glasshouse results, where all 65 lines showed high resistance. Resistance under field conditions may be considerably different from the resistance observed in the laboratory or glasshouse owing to variance in environmental conditions, genetic control, number of pathogens and pests, and plant age. The BXW field trial was neighboring with other banana fields and had a lot of disease pressure resulting from other pathogens, such as *Mycosphaerella fijiensis*, which causes black sigatoka, *Fusarium oxysporum*, which causes Fusarium wilt, and pests such as weevils and nematodes. In addition, the age of plants tested in the glasshouse was very different in comparison with plants tested in the field. Very young plants (three months old) were artificially inoculated with *X. campestris* pv. *musacearum* in the glasshouse trial, whereas under field conditions, the plants were inoculated at maturity, just before flowering. Therefore, the difference in the disease resistance might be due to differences in spatial and temporal expression of the transgene during development of plants.

Eleven transgenic lines showed 100% resistance under field conditions and retained the resistance even in the ratoon crop, which suggests that our approach could provide a solution to farmers for controlling BXW. However, to get more robust proof of concept for further product development, we have multiplied the promising lines showing complete resistance in this study and are evaluating them in a second field trial with more replicates to assess the durability of high disease resistance and yield performance. This trial will be expanded to multiple locations to capture the different environmental effect on disease resistance for these lines. It is well known that pathogens can evolve and 'break down' disease resistance. To delay or prevent this outcome, we are also stacking these two genes together in the same line to enhance resistance and make it durable.

All of the resistant transgenic lines produced fruits that appeared normal with no external symptoms (**Fig. 2h**), whereas most of the control nontransgenic plants died before flowering. Some of the control plants produced fruits before wilting completely; however, the fruits showed BXW symptoms, such as premature ripening and rotting (**Fig. 2c**,**i**). Transverse sections of the pseudostem, rachis and fruit at harvest of resistant lines did not show internal symptoms (yellow bacterial ooze) (**Fig. 2d**,**e**). However, yellow ooze and brown scars were observed in the pseudostem and fruits of symptomatic transgenic lines and nontransgenic control plants (**Fig. 2f**,**g**).

Apart from full resistance to BXW, the flowering and yield (bunch weight and fruit size) characteristics of transgenic lines were similar to those of nontransgenic plants, indicating that there were no observable unintended impacts of the transgenes to crop performance (**Fig. 1a**, **Supplementary Figs. 4** and **5** and **Supplementary Table 2**).

The resistant transgenic lines showed localized necrosis at the point of inoculation owing to the rapid, localized death of plants cells at the site of inoculation. This is characteristic of the hypersensitive response, which is thought to be an important defense response to prevent further multiplication

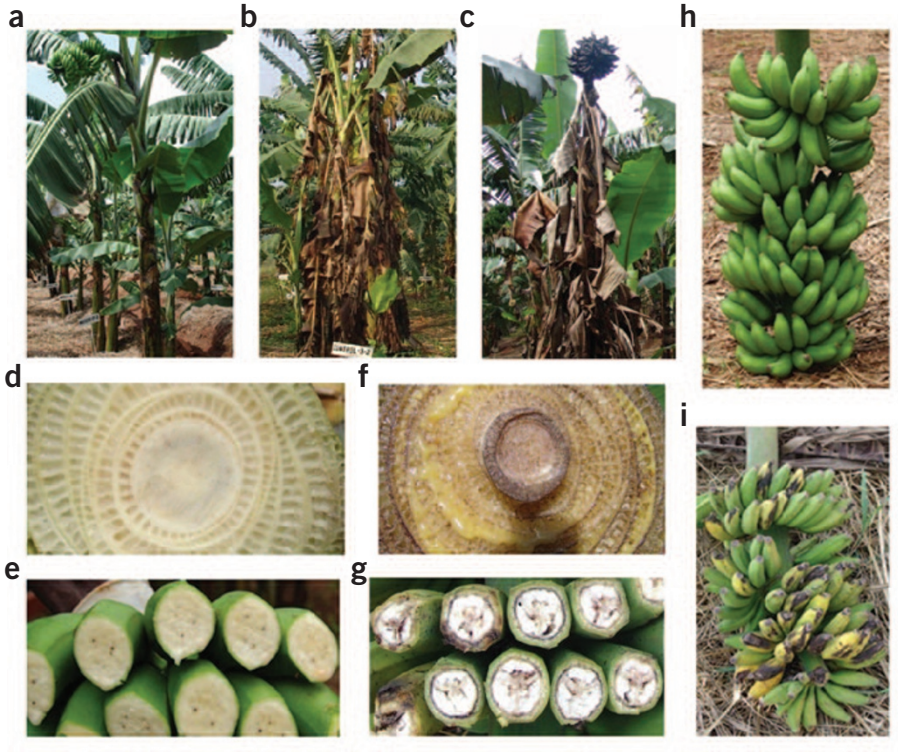

Figure 2 Comparison of transgenic plants with nontransgenic plants after inoculation. (**a**) Asymptomatic transgenic plant showing no symptom after artificial inoculation. (**b**) Nontransgenic plant showing wilting of leaves post inoculation. (**c**) Symptomatic plants showing rotten fruit bunch. (**d**,**e**) Transverse section of pseudostem and fruits of transgenic plants showing no internal symptoms. (**f**,**g**) Transverse section of pseudostem and fruits of nontransgenic plants showing internal symptoms (yellow ooze, brown scars and ooze on the margin). (**h**) Fruit bunch of transgenic plant showing no external symptoms. (**i**) Fruit bunch of nontransgenic plant showing premature ripening.

and restrict the spread of the pathogen to other parts of the plant. To confirm this, we attempted to isolate pathogenic bacteria from the pseudostem and the inoculated leaves of all the asymptomatic transgenic lines. In the transgenic plants we were unable to recover any viable bacteria, even from the site of inoculation, suggesting that the plants mounted a successful resistance response. This type of plant defense mechanism is commonly found in disease-resistant plants and induced by the highly specific recognition of a pathogen-derived elicitor by a plant resistance gene product.

The HRAP and PFLP proteins are not listed as being potential allergens in AllergenOnline (http://www.allergenonline.org/), indicating that these proteins should be safe for human consumption. These proteins are widely distributed throughout a broad range of plant species, including tobacco, *Arabidopsis* and rice, and vegetables, such as pepper, that are eaten raw. Even so, the transgenic lines will be tested for food and environmental safety in compliance with biosafety regulations. Most edible bananas are sterile and the clonal mode of propagation minimizes the possible risk of environmental contamination by gene flow from transgenic bananas to other bananas and another crop species.

Our data provide a proof of concept for control of BXW through *Hrap*- and *Pflp*-mediated resistance and, to our knowledge, are the first field-based evidence for transgenic control of a bacterial disease in banana and progress toward development and release of transgenic bananas resistant to BXW. Such resistant varieties would boost the available arsenal to fight this disease epidemic and save livelihoods in Africa, where the green revolution has had little influence. Banana is an important food and cash crop in the Great Lakes region of East Africa. Food security studies revealed that in Uganda, Rwanda and Burundi, bananas constitute >30% of the daily per capita caloric intake, rising to 60% in some regions[1]. As elicitor-induced resistance is not specific against particular pathogens, this transgenic approach using *Hrap* and *Pflp* may also provide effective control of other bacterial diseases of banana, such as moko or blood disease, in other parts of the world.

AUTHOR CONTRIBUTIONS
L.T. conceived the idea and led the study. L.T., A.K., F.S. and W.K.T. designed the study. L.T. and J.N.T. performed the experiments and S.K. analyzed the data. All authors contributed to the interpretation of data and writing of the paper.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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