Ecologically acceptable genetic transformation of banana and plantain? Proposal for a theoretical experiment not restricted to *Musa* crops alone

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Abstract — Introduction. Improvement of plant species involves the selection of plants expressing important characteristics. Breeders find novel gene sources to bypass the normal barriers to sexual crosses in transgenetics. **Ecological concerns**. A major concern about genetically transformed crops is the resilience of "marker" genes (a laboratory device designed to select genetically transformed organisms) in the final transformed crop where they are not needed anymore. **Technical answers**. The use of different types of selection genes and the containment of transgenes are reviewed and discussed. Inactivation of selection marker genes after transformation and selection-less production of transgenic plants are further alternatives. **Proposals**. We propose different approaches to transgenic bananas, in view of their specific biology, without any extra-species/genus DNA. One proposal is based on restoration of the original phenotype by genetic complementation of an artificially auxotrophic mutant created for this purpose by gene surgery or gene silencing. Complementation is performed using native genes or "alleles" to be found in the natural biodiversity and relatives concerned. **Conclusion**. The ability to adapt rapidly and in a cost-effective way is essential for the survival of banana growers, so it is vital to consider the requirements for environmentally safer transgenics. The centerpiece of "ecologically acceptable genetically modified bananas" will be the methodology which emerges as the winner out of two competing approaches: (1) direct biolistic transformation (biolistics of nonvector transgenetic DNA fragments) and (2) complementation assays of a natural or synthetic auxotroph. © Éditions scientifiques et médicales Elsevier SAS

***Musa* / plant biotechnology / genetic transformation / genetic marker / complementation**

Une transformation génétique des bananes et plantains écologiquement acceptable ? Proposition pour une expérimentation théorique, non limitée au seul genre *Musa*.

Résumé — Introduction. L'amélioration des plantes est basée sur la sélection d'individus exprimant des caractères agronomiquement plus performants. La transgénèse fournit aux améliorateurs la possibilité de s'affranchir des barrières naturelles de fertilité pour utiliser de nouveaux gènes d'intérêt. **Considérations écologiques**. La préoccupation majeure concernant les plantes transgéniques est la rémanence de gènes marqueurs (un outil de laboratoire servant à la sélection des transformants) dans la plante transformée. **Réponse technique**. L'utilisation de différents types de gènes marqueurs et leur « confinement » fait l'objet d'une revue bibliographique et d'une discussion. L'inactivation du gène de sélection après transformation ainsi que la production de plantes transgéniques sans aucun gène de sélection sont d'autres possibilités. **Propositions**. Nous proposons différentes approches pour obtenir des plantes transgéniques ne contenant pas d'ADN provenant d'autres espèces ou genres, et cela en considérant les caractéristiques biologiques des bananiers. La première proposition est basée sur la complémentation d'un mutant auxotrophe, artificiellement construit dans cette optique par chirurgie génique ou extinction génique. La complémentation est effectuée en apportant un gène ou un « allèle » natif qu'il conviendra de rechercher dans les ressources naturelles des plantes apparentées. **Conclusion**. La capacité d'adaptation et de réaction aux changements du marché, et cela de façon économiquement étudiée, est essentielle pour la survie des planteurs de banane. Il est donc vital de prendre en compte certains éléments incontournables pour la construction d'un bananier transgénique « écologiquement plus acceptable ». La pièce maîtresse de cette « banane modifiée génétiquement et acceptable écologiquement » sera la méthodologie la plus performante résultant de l'évaluation de deux approches différentes : (1) transformation par biolistique sans utiliser ni vecteur ni gène de sélection et (2) complémentation d'un mutant naturel ou non, par un gène naturel. © Éditions scientifiques et médicales Elsevier SAS© Éditions scientifiques et médicales Elsevier SAS

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1. introduction

Since man stopped being a nomad hunter-gatherer and became a sedentary farmer, good husbandry has implied "selecting" appropriate robust and productive individuals for plant and animal stocks. Henceforth, breeding was empirical and based on morphological aspects of the individuals chosen to carry the crop into the next generation. Plants have thus been cultivated by humans for thousands of years, during which time crops have been continually selected for improved agronomic, horticultural and production traits. The systematic application of empirical knowledge became a selective pressure, the results of which are our present-day cultivated crops. The food requirements of today's population of nearly 6 billion people could not have been met by the conventional technologies used until the 1 940s (*table I*), and so developed the so-called "green revolution".

The improvement of a plant species by conventional, albeit improved, techniques involves the selection for breeding purposes of certain plants expressing important characteristics. As a result of human intervention to select which plants to use for production of offspring, it is possible to produce new varieties at a much faster rate than would occur in the wild. Crop species have therefore been selected for a large number of different characteristics, resulting in a great number of varieties being produced to help feed an expanding world population. The pressure on modern breeders, due to the urgent demand for more and better, disease-resistant and high-yielding food crops, increases the need for continuous improvement of plant varieties. In fact, the basis of genetic improvement is the optimization of gene interactions, based on genetic diversity, which can be obtained from several sources, including: natural populations, products of sexual crosses (via recombination, segregation and selection), spontaneous mutations (aneuploidy, polyploidy and other mutations), mutations induced by physical or chemical agents, and insertional mutagenesis (transposable elements, T-DNA, retroposons). Thus, plant breeders are pressed to seek novel sources of breeding material (in fine: genes) to bypass the normal barriers to sexual crosses. Modern genetics provides an important additional source of such genes: the characteristics of an organism are determined by its DNA (deoxyribonucleic acid) which is the information-containing component of the chromosome. DNA provides the genetic code to determine how individual cells, and, consequently, the whole organism will be constructed. This code is divided up into functional units, or genes, in the same way as a paragraph can be divided into individual words. The total characteristics of a plant will depend on which genes it has received from the parent plants, whether or not they are "switched on" (expressed) and also on interactions between the genes and environmental factors. The advent of modern genetic modification techniques has enabled researchers to remove individual genes from one species and insert them into another, without the need for sexual compatibility.

In this way, since its emergence in the late 1970s and early 1980s, biotechnology has had a dramatic impact on agriculture since it can provide an effective arsenal to meet the food needs of a rapidly expanding world population. Genetic sequencing

Table I.
Historical development of plant breeding.

Year	Plant breeding event
4000 BC	**Neolithic revolution**
1 694 **AD**	Discovery of sexual reproduction in plants
1719	First recorded plant hybrid (intraspecific hybridization)
1799	First report of a cereal hybrid
1866	**Mendel publishes his work on pea crosses**
1876	Interspecific and intergeneric crossing
1900	**Start of hybrid maize breeding in USA**
1909	Protoplast fusion reported
1927	Mutation via X-rays
1937	Polyploidization
1940s	Single-seed descent technique developed (SSD)
1960s	Embryo rescue refined
1970	Recombinant DNA technology (start of modern biotechnology)
1970s	Double haploid techniques
	First improved banana hybrids
1983	**First genetically modified transformed plants (tobacco)**
1990	**First genetically modified cereals**

and transgenic technology, tools developed largely in response to the Human Genome Project, have greatly facilitated efforts to understand the genetic basis of plant traits and investigate the genomes of plant pathogens. The ability to insert genes for defense against adverse conditions or pests, though controversial, will play a role in increased productivity. Of potentially greater benefit may be genetic modifications to enhance the quality or composition of a given crop.

Once the new gene has been inserted into a plant, offspring containing copies of it can be produced in the traditional manner. The advent of genetic modification over the last two decades, in addition to the continued improvement of existing methods of crop (and animal) husbandry and food processing, has enabled plant breeders to develop new varieties of crop at a faster rate than was possible using traditional methods and with huge potential for further beneficial developments. Tobacco was the first plant to be genetically transformed in 1983 [1,2], with cereals beginning in 1990, but it is only recently that products such as genetically modified soybean have reached the market place in Europe and given rise to concern and controversy:

– risk of transfer of genes from genetically modified crop plants to wild living organisms and other crops (which are not genetically modified or are not the target of genetic modifications),

– uptake of genes from genetically modified food by the digestive system.

In contrast to the United States where the biotechnology revolution is sweeping farms and transgenic crops have taken a firm root in the economy, concerns in Europe about the need, safety and overall desirability of transgenic plants, and any produce derived from them, are on the increase. The same concerns about bio-hazards and economic reasons explain that this research has made few inroads into many developing countries where per capita food consumption is low and where the promise of this technology to enhance nutritional security is most enticing. Some genetically modified foods have been produced using antibiotic resistance and/or herbicide tolerance "marker" genes, which are a laboratory device designed to select genetically modified organisms (GMO). When researchers wish to insert a new gene into a plant, for example to express a protein that will make the plant resistant to a specific insect pest, it is often linked to another gene known as a "marker gene" (selection gene). A major concern about genetically transformed crops is the resilience of selection genes in the final product (i.e., the transformed crop) where these transformation markers are no longer needed (i.e., in the field and in the processed food). During the late 1980s, genes for resistance to a range of antibiotics were introduced as markers for selection [3].

Selection markers are necessary to screen for transformed cells because:

1) transformation yield is low (the problem of how to select one transformed cell versus millions of untransformed ones),

2) transformed cells may have lower reactivity due to the stress of transformation as compared to untransformed ones (not only are transformed cells scarce in the bulk of untransformed ones, but intuitively one might expect that "fitness" may have decreased, hence a lower growth rate).

Thus, marker genes are used to facilitate laboratory selection of cells and subsequently plants where the genes have been successfully inserted. Plants containing a gene for resistance to an antibiotic will grow on material which contains that antibiotic, whereas if the genes have not been successfully inserted the plants will not grow. Because the "marker gene" is linked to the "improvement gene", plants that have grown on the antibiotic will also contain the other gene of interest. On the other hand, selection media are stressful for the host organism. In fact, regeneration efficiency of cells in selection media should be lower than that of transformed cells from non-toxic media. Selection pressure may cause the emergence of spontaneous resistant or tolerant mutants depending on the selection marker (e.g., kanamycin resistance is due to a loss of function mutation in sensitive bacteria). But positive selection (killing all the non-transformed cells) more than compensates for the decrease of overall transformation efficiency and fitness.

The most commonly used antibiotic resistance marker genes in genetically modified plants confer resistance for such purposes to kanamycin or hygromycin. In genetically modified bacteria, ampicillin resistance marker genes are more often used.

The question as to whether the selection gene product in processed foodstuffs and/or the presence of the gene itself in the genome of the crop represent an ecological hazard has become the subject of an impassioned debate. For instance, toxicity of the gene product (proteins related to antibiotic resistance and/or herbicide tolerance) is being assessed. There is a possibility that horizontal transfer of antibiotic resistance (or herbicide tolerance) genes from the food to the intestinal flora of humans and cattle and giving rise to pathogenic bacteria (or soil bacteria via the feces, giving rise to weeds) upon infection is bound to spread resistance to antibiotics in the pathogen population, and this adds to the race to discover new anti-bacterial agents. In the field, open trans-pollination and infectious soil bacteria and/or other vectors (e.g., virus/pest systems) are potential intermediates likely to spread herbicide tolerance from crops to weeds - it has been hinted that gene transmission is possible from transgenic lines of potato to a bacterial pathogen [4]. The "transmission" of herbicide tolerance to weeds could become a very serious problem because of the paucity of weed-specific herbicides which are in any case difficult to validate for obvious ecological reasons.

2. ecological concerns

Crop systems can be divided into three groups with regard to the possibility of natural transfer of genes from genetically modified crop plants to wild relatives:

– no sexually compatible wild relatives in the region where the crop is grown, therefore no gene transfer to other species can occur,

– gene transfer unlikely due to the nature of the crop species (e.g., inbreeding species),

– gene transfer likely (e.g., outbreeding species).

The likelihood of gene transfer to wild relatives depends on the species of crop and the location where the crop is to be grown. There are several steps involved in gene transfer: the pollen must contain a copy of the inserted gene(s); it must then move away from the area where the crop plant is grown and come into contact with the part of a compatible plant which receives pollen; if fertilization occurs successfully, it may not always result in a plant able to grow successfully, or if a plant is produced then it may not compete well with other species in the environment. Furthermore, if the resulting plant produces pollen that goes on to fertilize other plants, then the inserted gene(s) will become increasingly diluted in the overall population if there is no selective advantage for the plants that contain it. As with the transfer of genes to unrelated wild plant species, the likelihood of transfer will depend on the biology of the crop species and the location where it is grown. It should be also noticed that, even in cases of unlikely transmission or absence of wild relatives, transmission to other non transgenic crops has to be monitored.

Many important food and horticultural crops are vegetatively propagated hybrids or cultivars or perennial crops or have long reproductive cycles. The material is mainly vegetatively propagated because of parthenocarpy and sterility or else to maintain the elite genomes: banana, potato, grapevine, strawberry, cassava, yam, hybrids of fruit and forest trees including poplar, citrus, eucalyptus, oil palm, hybrid aspen. Gene transfer from crops that are hybrids may not be a problem because such seed is not propagated in the next generation and the farmer buys new hybrid seed each year, or replants new suckers or shoots from micropropagation.

As it is well known that pests (e.g., insects, nematodes) are able to transmit viruses from plant to plant, the potential horizontal gene transfer via pests has to be taken into account for vegetatively propagated as well as sexually propagated crops.

But one of the major concerns [5] about genetically modified crops, linked to the introduction into the diet of foods and

ingredients derived from or produced with the aid of genetically modified plants, is the possibility that the genes from such plants may be taken up by consumers when eaten, and become part of their own genetic makeup. The use of antibiotic resistance as a marker for selection in genetically modified plants for human or animal consumption has resulted in the fear that these genes might be introgressed into the cells of the gut, or transferred into the bacteria present in the stomach of the consumer. If this were to happen, the genes might be transferred from these bacteria into bacteria causing disease in humans, making them resistant to the antibiotics that are usually prescribed.

The uptake of genes via the food chain is not a new issue because genes (i.e., DNA) are normal constituents of the human diet. Many products from genetically modified plants, such as sugar prepared from transformed sugarbeet, are absolutely identical to conventional products. Others such as tomato paste from genetically modified tomatoes are so similar that they are regarded as "substantially equivalent". Others, such as flour from genetically modified soybean, may contain a new gene or its product, although many of the purification processes involved in food production will degrade any DNA present in the raw material (though not completely destroy it). It is likely that, if transfer of a functional (whole) gene occurs, it would only occur following consumption of the unprocessed genetically modified plant, since processing of food causes DNA present in the food to be degraded.

As it cannot be assumed that current practices will feed the population of eight billion expected by 2020, it is worthwhile to think now about alternative techniques for plant transformation.

3. technical answers

3.1. use of different types of selection gene

As antibiotic resistance or herbicide tolerance genes could lead to potentially harmful products, it has been proposed for the selection step to rely on genes which confer neither resistance nor tolerance to drugs but add a selectable metabolic advantage to the transformed cells. Given the fact that most animal cells require the use of several vitamins or amino acids to grow, it has been proposed to use genes conferring growth capacity to the transformed cell, on media lacking a particular "vitamin" or amino acid.

The use of glutamine, histidine or tryptophan free media coupled with introduction of the glutamine synthase [6] *hisD* and *trpD* genes [7], respectively, has been reported for the selection of transformed mammalian cells.

The example of the *hisD* gene is also interesting because it gives the transformed cell the capacity to grow in the presence of histidinol. Histidinol is usually toxic for mammalian cells, so this gene enables a double selection of transformed cells which are able to produce an essential amino acid from a usually poisonous compound.

This approach is suitable for animal cells which are known to need vitamins and essential amino acids to grow, but for plant cells, which are autotrophs, it is much more difficult to implement.

3.2. containment of the transgene

Inserted genes, or "transgenes", can be introduced into the chromosomes of plant cells or, though less easily, into the additional genetic material contained in other parts of the plant cell such as chloroplasts. The advantage of insertion into the chloroplasts is that the genes cannot be transferred by the pollen, as chloroplasts can be excluded during fertilisation. On the other hand, the transgene is active in all the plant organs harboring a chloroplast, mature or not.

3.3. inactivation of selection/marker genes after transformation

To inactivate expression (i.e., the selective gene is unexpressed or produces no protein in the final transformed plant), the use of anti-sense and ribozyme technolo-

gies has been proposed to inactivate expression of selectable markers in transgenic plants [8]. This approach is based on the fact that:

(i) translation requires single-strand mRNA,

(ii) double-stranded RNA has autocatalytic activities for degradation in particular conditions.

Thus, introduction of anti-sense-engineered RNA into the transformed plants, targeted against selectable genes, will produce selective inactivation of specific mRNA by autocatalytic cleavage. The use of inducible promoters for selection genes could be of interest for modulating their expression in the transformed plant in the field. However, these strategies are a specific answer to the potential "toxicity" of selectable gene products but do not address the risk of transmission of resistance or metabolic advantages to other organisms. One answer to this is the total removal of the selectable marker gene from the genome. Methods for the removal of the potentially harmful selectable marker gene from the transgenic plant genome have been proposed and explored since the early 1990s.

3.4. removal of selectable genes from the transformed plant genome

The insertion of "marker genes" is a necessary part of the selection process, but it should also be emphasized that it is possible (though time-consuming) to remove such genes later.

3.4.1. by genetic recombination through breeding

Because the transgene lacks homologous copies in the recipient plant, the likelihood of cross-overs between the selectable gene and the gene of interest is extremely small, even if possible. Thus repeated backcross between the transformed plant and the untransformed genitor to select a recombination event is bound to fail. However, genetic transformation using two separate vectors, one harboring the gene of interest and the other the selectable gene, has been proposed to separate the selectable gene from the other genetic material [9, 10]. It has been reported that, amongst plants selected for resistance to an antibiotic, 30 to 80% integrated the second gene. The frequency of independent segregation exhibited by these plants is between 30 and 100%.

Consequently, co-transformation may be a useful method for removing a selectable gene from the plant genome.

3.4.2. by transposase-mediated excision of the selectable marker

Since the discovery of the maize transposon Activator (Ac) and its non-active derivative Dissociation (Ds) by McClintock [11], transposon systems have been used for cloning genes from plants [12] and have been transferred into plants lacking well-characterized endogenous mobile elements for tagging purposes [13]. In plants where transposons have been characterized, endogenous systems have been used.

Besides "natural" active plant transposons, site-specific recombination systems have been developed in plants starting from the P1 bacteriophage with Cre/*lox* [14, 15].

The basic idea is to use transposons or site-specific recombination systems to "eject" the selectable gene from the plant genome. First, plants are transformed and selected with conventional chemicals, then activation of transposase or recombinase activity excises the targeted gene from the plant DNA. As excision is linked to DNA replication, it occurs in both somatic and germinal cell lines. Excision and eventual reinsertion, early in plant development, lead to chimeric plants. However, there is a potential for selecting non-chimeric plants from progeny as reproduction starts from a single cell.

Two opposing principles can be used as it has been shown that transposition or site-specific recombination is followed either by loss of the targeted DNA sequence or by its re-integration into the genome at a different locus.

Excision of the selectable marker and selection for loss of the targeted sequence

is preferred mainly for plants where crosses are difficult or impossible because this strategy directly leads to plants without selectable genes.

The other strategy consists in targeting the gene of interest and selecting by reinsertion. Physical separation between the selectable gene and the gene of interest allows for segregation through backcrosses and selection of the marker-less progenies (see part 3.4.1). The advantage of this strategy is the complete elimination of residual vector parts (e.g., bacterial origin of replication) linked to the selectable gene. The selected plant retains two short sequences, "scars" resulting from the transposase activity in the genome as well as the gene of interest. On the other hand, this strategy requires at least one backcross.

Other types of selection system implicitly constructed for removal have been assayed according to this methodology. One example is the integration into an Ac element of the *ipt* gene encoding for isopenthyl transferase which is involved in the biosynthesis of several cytokinins [16]. Overproduction of the Ipt protein leads to loss of apical dominance in the transformed plants. The resulting extreme shooty phenotype (ESP) allows for visual selection of the transformants. Removal of the *ipt* gene from the trangenic plants is performed by growing ESP plants and selecting reversion to normal phenotype after excision of the Ac derivative.

The Cre/*lox* recombination system has been successfully used in tobacco [15], *Arabidopsis* [17]. The lox site sequences are artificially integrated into the transfected sequence, flanking the selectable gene. As no endogenous Cre proteins exist in plants, no excision occurs. Trans-recombinase activity is introduced by crossing the transformed plant with a "donor": a transformed plant expressing the phage P1 Cre protein. In the zygotes inheriting the two transformed haplotypes, the Cre protein will recognize the lox sites flanking the selectable gene and excise it. Finally, genetic segregation of self-pollinated marker-deficient plants produces transformed plants exclusively integrating the gene of interest.

It has been proposed to obtain excision of the selectable marker by transient expression of the Cre protein after a second round of transformation, where sexual crosses are difficult or impossible.

3.5. alternative methodologies

3.5.1. reporter genes

Besides the use of selectable genes, founding experiments on plant transformation are built using (bacterial) reporter genes. These genes are bound to produce a protein which leads under certain conditions to visually-detectable (colored) products allowing for easy visual identification (selection) of the transformed plants. As reporter genes do not produce any resistance-related products, their use does not interfere with human health. Gene spread into weeds or bacterial flora may not be of concern because these reporter genes are endogenous to bacteria. But the question of allergenicity, if not toxicity, for humans – and even for the target plants – remains to be posed.

3.5.2. obviation of selectable or reporter marker genes

It has been proposed to transform immature barley embryos by biolistics without any selection [18]. They prove, though this does not establish a routine gene transfer procedure, that production of transgenic plants without selection is possible. According to barley data, stable integration occurs in one plant out of 300 and, beyond that, in 10% of its seedlings.

Even though the stable and functional integration frequency of a transgene without selection is lower for other plant systems, recent developments in automatization of molecular biology techniques (mainly robotics in DNA extraction, PCR and sequencing) now allow for individual checks of increasing numbers of plants.

PCR screenings of thousands of individuals are both conceivable and manageable. Thus it will become possible in the near future to prepare plant transformation assays without any selectable marker genes

by handling large populations and regenerating candidate plants bound to be individually tested.

A complement to this technique to increase the number of transformation events against a background of untransformed plants could be direct DNA microinjection. [19].

4. proposals

Despite the advantages of transformed crops *versus* untransformed ones, no extensive use of the technique has been made, especially in Europe. This is mainly due to public concerns over environmental safety. Thus, host genomes should not, at least for commercial use, contain the selectable marker.

4.1. specificity of banana

The specificlty of commercially propagated bananas implies that original methods should be sought to obtain ecologically safe, transformed plants. Banana and plantain belong to the Musaceae family and are among the tallest of the monocotyledons. Dessert bananas are intensively cultivated for export to Europe (West, Central and Eastern), China and North America, while cooking and some dessert bananas are most often cultivated in back gardens for local consumption in tropical countries (although export of these crops is also increasing). The genomes of four different wild Musa species are involved in cultivated bananas: the A, B, S and T genomes related to *M. acuminata*, *M. balbisiana*, *M. schizocarpa* (2n = 2× = 22) and the *Australimusa* species (2n = 2× = 20), respectively. Most cultivated clones are triploid or diploid, highly sterile, parthenocarpic and clonally propagated. They have been classified into genomic groups according to chromosome numbers and morphological traits. The main groups are AA, AAA, AAB, ABB and, to a lesser extent, AS and AAT [20]. They are susceptible to several diseases that seriously threaten plantations.

Biotechnology now allows for precise and early characterization of the genomic nature of the accessions. Molecular markers have led to a better understanding of the origin of cultivated bananas, particularly through the analysis of A genome variability. The maternal and paternal origin of the clones may be inferred respectively from the RFLP analysis of the chloroplast and mitochondria genomes [21]. RFLP analysis of the nuclear genome of cultivated bananas reveals all the wild *acuminata* subspecies at their origin. For instance, it has been shown that fruit quality is related to the nature of the A genome: cooking types with a *M. acuminata banksii / errans* origin, and dessert types with a *M. acuminata malaccensis / zebrina* origin. Germplasm collections worldwide can be screened for naturally resistant gene alleles and *Musa* biodiversity is far from being exhaustively sampled. Whereas banana cultivation has greatly improved during the green revolution, genetic improvement of banana seems to have been left somewhat by the wayside. For example, yield of 1 ha of Cavendish banana has increased from 6 t of banana fruit to 80 or even 120 t in experimental plantations, owing to improvement of agricultural techniques, pest management and an integrated approach to cultivar management, including micropropagation. By contrast, only a small number of genetically improved banana varieties are internationally available and commercially accepted either by farmers or by consumers at the present time. Thus it is conceivable that all the genetic progress documented on cereals still represents an untapped potential in banana. Genetic improvement has been restricted to picking out new clones from amongst the natural biodiversity. Several attempts at genetic improvement using crosses have been started, with more or less success regarding quality traits. An additional problem lies in the fact that production infrastructures are adapted to the Cavendish type cultivar. It may not be easy to reconstruct an elite cultivar, acceptable to local growers and consumers, from genetically improved diploid, fertile donors in a reasonable space of time. Thus the question as to whether genetic transformation of banana is of major concern has been

positively answered and this technique remains a suitable tool.

Is there an "ecological" way?

Commercial varieties of banana are sterile to the extent that meiotic events cannot be observed. It follows that removal of selectable marker genes either by genetic recombination through breeding (3.4.1) or transposase-mediated excision (3.4.2) is impossible in these varieties. Excision of the selectable marker combined with selection for loss of the target sequence may be obtained asexually, but requires a second transformation assay. Further, transposase-driven excision is likely to produce chimerical plants which will have to be cloned. Dechimerizing banana using suckers is labor-intensive and time-consuming. Micropropagation, on the other hand, produces variable amounts of off-types, adding yet another dimension to the complexities of the problem.

We propose to consider different methods for building transgenic plants, obviating the use of antibiotic or herbicide resistance genes and actually without any transfer of extra-species/genus DNA.

4.2. transformation without selection marker genes

Producing transgenic plants without selection marker genes seems possible by screening only, which could be counted as "selection". A huge number of transiently transformed cells may regenerate in a first stage and the eventual proportion of stable transformed cells amongst the total totipotent population can be extremely low. However, this method is only really possible when transformation efficiency is very high (% to ‰ range) and screening for character is possible at the plantlet level. But generation of transgenic plants without some built-in selection might be highly impractical in instances where transformation and regeneration frequencies are lower. Efficacy of selection by screening depends on the manageability of the screening method, for example, high throughput techniques at affordable cost and reduced labor intensity by robotized laboratory infrastructures.

4.3. complementation

Original proposals, based on genetic complementation of a natural or artificial deficient mutant, may bypass some of these difficulties and impossibilities. An artificial auxotroph will first have to be created through some means of directed mutation or screening. Then the original, and natural, phenotype will be restored by transformation using the native gene, or any "allele" to be found in the biodiversity of the crop species (and relatives) concerned. Unfortunately, the Cavendish series of cultivars seems to be very homogeneous. Furthermore, the favorable genomic composition of banana cultivars is triploid. Thus, one additionally important question is how to "switch off" three copies of a functional gene to obtain a homozygous knock-out mutant. However, it might be possible to obtain selectable mutants shutting down one or two alleles of a locus. Different alternatives may be explored:

4.3.1. origin of the auxotroph

4.3.1.1. mutagenesis

Since the beginning of genetics, mutant collections have been built in a variety of organisms, starting with simple ones (*E. coli*), continuing with animals (*Drosophila melanogaster* and finally plants (*Arabidopsis thaliana*). All these model systems have in common that they can be rapidly and efficiently crossed to perform genetic analyses. When considering a polyploid, sterile plant, subjected to mutagenesis, questions of unicity, stability of mutations and the chimeric nature of the organism will be very difficult to answer, since segregation is impossible to observe.

In fact, after identification of the mutant based on its phenotype, building a complementation system suitable for use as a selectable marker for genetic transformation requires at least:

a) characterization of the mutation at molecular level,

b) cloning of the endogenous intact gene for functional complementation.

4.3.1.2. silencing

Given the triploid nature of the banana cultivar's genome, genetic silencing, using

homologues or anti-sense, could be an alternative method to "switching off" the expression of a particular gene. After the first transformation with an additional copy, sense or anti-sense, of the targeted gene, the resulting phenotype might be that of a defective mutant. This mutant could then be restored by the introduction of a heterologous or engineered gene with less than 70% homology, avoiding the silencing of the newly-introduced gene. Scientifically, mastering and directing gene silencing to produce a tool in transformation assays is a thrilling project, but in practice it may be more like a Pandora's box. Use of silencing for generating mutants may well prohibit original phenotype restoration.

4.3.1.3. gene surgery

"Gene surgery" using RNA-DNA hybrid oligos is based on the following observations and discoveries:

– RNA-DNA hybrids are more efficient than duplex DNA in homologous pairing assays in vitro,

– hairpin caps protect the hybrid molecules at both ends from cellular helicase and exonuclease-based destabilization and destruction,

– hairpin caps do not hinder homologous pairing,

– RNA-DNA hybrids are recombinogenic. They may be designed to align perfectly (mutator sequence) with a specific genomic target sequence except for one nucleotide mismatch. The resulting mismatched base pair will be recognized by the endogenous DNA repair systems, and a change in sequence on either the genomic strand or the oligonucleotide strand will occur. Thus the underlying mechanism is bound to be gene conversion rather than homologous recombination in vivo [22].

Self-complementary chimeric oligonucleotides (COs) composed of DNA and modified RNA residues are a novel technology to modify individual genetic loci by catalyzing either a stable, site-specific base substitution or a base addition in order to introduce a frame shift in specific nuclear genes (developed in human gene therapy).

The frequency of these events appears to be several orders of magnitude higher than the rates of gene targeting, a process involving homologous recombination. Point mutations and single-base deletions can be corrected at frequencies of approximately 0.1% and 0.005% respectively, a 5–11% gene conversion rate can be measured using restriction enzyme polymorphism and direct DNA sequence analyses (depending on the mammalian system). Results of genetic and biochemical studies suggest that the process of mismatch repair functions in site-directed gene correction [23, 24]. COs are designed to contain a 25 nucleotide homology domain comprised of a five-deoxyribonucleotide region (harboring a single-base mismatch to the native targeted codon sequence) flanked by regions each composed of ten ribonucleotides (*figure 1*). The CO can be easily transfected into cells, remain stable within them, and migrate to the nucleus. As the first results on tobacco suggest [25], "gene surgery" using CO is a manageable way to produce mutants in a targeted way based on the repair mechanism; this approach should be of great utility in the area of functional plant genomics [25]. Our proposal is to produce loss of function mutants by destroying specific targeted genes in the anabolitic and/or catabolic pathways using CO to produce "deficient" cells needing "specific vitamins" to survive.

Selection of the mutant is performed by duplicating cells on selective media (nega-

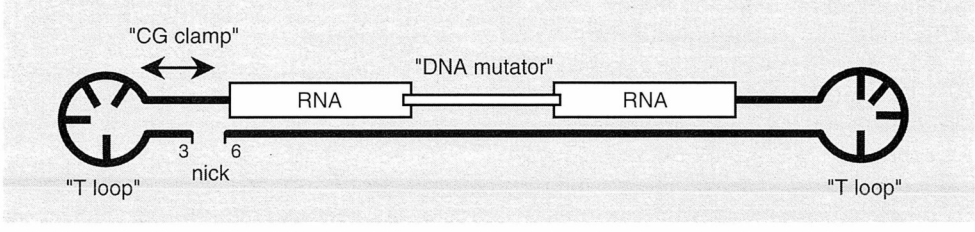

Figure 1.
Chimeric Oligo structure.

tive selection) or by positive selection wherever possible (*hisD*, nitrate reductase).

The endogenous gene becomes a marker gene for transformation assays through complementation using a vector incorporating a native copy of the defective gene which restores the original phenotype.

4.3.2. targeted complementation genes for selection

The proposal to use an inactivated endogenous gene to build a selection system requires prior knowledge of the gene sequence, function and resulting phenotype.

4.3.2.1. well-characterized genes and functions

Due to vast international genome initiatives on model organisms, an increasing number of essential genes is becoming well-characterized and publicly accessible. These data allow for rapid cloning by homology and sequencing of endogenous "alleles".

However, a problem remains in that essential genes are often pleiotropic and targeting them for mutant construction may result in a fatal phenotype. Even a very tormented phenotype might not be successfully restored if the transgene promoter is not "tuned" to the differential expression requirements of the various developmental stages and organs. On the other hand, use of the endogenous promoter might trigger silencing.

4.3.2.2. unknown gene but well-characterized function

In banana, zinc nutrition can be sustained by different mineral sources with a variety of chemical formulations. Inactivation of the capacity to assimilate specific zinc resources should lead to an interesting system: in vitro selection is possible using cultivation media containing Zn only in non-assimilable form, allowing for selection of the restored autotroph. In the field, even if the recently introduced gene is silenced, zinc nutrition can be agronomically complemented by the use of other mineral forms.

However. this approach requires prior characterization of the gene(s) linked to the putative specific function.

5. conclusion

Given the many biological and economic challenges or opportunities growers are faced with in a changing world – sterility, parthenocarpy, nematodes, black sigatoka, weeds, beetles, viruses, organoleptic quality of fruit (sugar, vitamin content), yield, wind resistance, size of the plant, tolerance of frost and drought, banana as a vaccine vector, pharming (pharmaceutical farming) – the genetic transformation of banana and plantain seems to be ineluctable.

What are the requirements for the development of environmentally acceptable transgenic bananas?

Banana biotechnology is sufficiently well-developed to suggest some answers. In fact, cell suspension techniques are well mastered and somatic embryogenesis is developed to industrial production level. Transformation and regeneration techniques are being optimized for various diploid (sub)species and polyploid cultivars.

5.1. exclusion of any exogenous DNA in the transgene construct

As meiotic recombination is impossible in triploid banana cultivars, the only way to obtain a transformed plant without any exogenous DNA is to perform transformation avoiding any kind of vector. This constraint obviates the use of *Agrobacterium*-mediated transformation which is known to transfer variable amounts of unwanted bacterial DNA. Even if progress in *Agrobacterium*-mediated transformation technology allows for reduction or elimination of transfected, but unwanted, DNA, the problem of persistence of *Agrobacterium* bacterial strains themselves within the transformed plant tissues is far from resolved.

Recent biolistics experiments point to the possibility of functional gene constructs integrating into the plant genome without

the need of a bacterial vector. This is of low efficacy but still less than one order of magnitude. Alternative transformation methods (e.g., no vector, transgene DNA protection through protein coating, targeting DNA to the nucleus, protoplast techniques) require further improvement (author's unpublished data).

A perverse effect of public safety concerns is that, new technologies of plant transformation being "cleaner", detection of genetically modified plants is more difficult. Indeed, transgenic plants bearing no exogenous DNA (neither vector nor selectable genes) will be very difficult to detect if information concerning introgressed genes and/or regulating segments (like promoters) is not publicly available. European consumers are asking for products containing GMO to be labeled. This requires the development of "forensic" techniques to detect GMO in manufactured food. The requirement for better ecological management of plant transformation implies the reduction of exogenous DNA introduced into plants and eventually a total lack of any potential GMO marker except the transgene itself. As most transgenic plants are developed by private companies, information concerning the real nature of the transgene will be difficult to access owing to potential patent restrictions.

The state of the art urgently needs improvement and standardization of in vitro protocols: a problem to be mastered is true-to-type multiplication, minimizing or eliminating off-types. Transformed meristems will need to be dechimerized (transformed vs untransformed cells) by in vitro cloning. Transformed varieties need to be multiplied on an industrial scale without somaclonal variation. As it is well known that in vitro micropropagation of banana produces various off-types, the question of whether a transgenic improved banana phenotype will remain stable throughout the multiplication process is of major concern. The stability of transgene(s) needs to be controlled and monitored by high-throughput (molecular) techniques to be developed (based for instance on DNA-chip technology). Current knowledge concerning somaclonal variation in banana points to genetic and/or epigenetic causes. There is a possibility of drastic genomic reorganization occurring during in vitro culture. It has been shown that the aneuploidy level is enhanced in dwarf variant banana cells. On the other hand, epigenetic factors are not to be excluded, by analogy with oil palm, where the global genomic methylation level changes notably between variants and true-to-type individuals [26]. Thus, these uncharted events could have a major impact on transgene expression and/or stability within the genome and should be investigated, at least in the early phases of the development of a transgenics research program.

Finally, techniques and methodologies will have to be "invented": genes of interest will have to be identified in the *Musa* biodiversity as well as endogenous promoters/terminators and enhancers/silencers, through a (structural, functional and expressional) banana genomics initiative (genetic, physical and cytogenetic mapping, reverse genetics, map-based cloning, sequencing, transcriptional analyses). The centerpiece of "ecologically acceptable genetically modified banana and plantain" will be the methodology emerging as the winner out of two competing approaches: (1) direct transformation using biolistics of non-vector transgenetic DNA fragments and (2) the construction of, or the screening for, an auxotroph mutant and gene sources for complementation assays.

The pace is quickening and the ability to adapt rapidly and in a cost-effective way is essential for survival. To manage an improvement program based on modern-biotechnology and genomics, prospecting the growers' opinions about agronomic needs for banana improvement and research for suitable interest genes is of paramount importance.

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references

[1] Barton K.A., Binns A.N., Matzke A.J., Chilton M.D., Regeneration of intact tobacco plants containing full-length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny, Cell 32 (1983) 1033–1043.

[2] Fraley R.T., Rogers S.G., Horsch R.B., Sanders P.R., Flick J.S., Adams S.P., Bittner M.L., Brand L.A., Fink C.L., Fry J.S., Galluppi G.R., Goldberg S.B., Hoffmann N.L., Woo S.C., Expression of bacterial genes in plant cells, Proc. Natl. Acad. Sci. USA 80 (1983) 4803–4807.

[3] Yoder J.I., Goldsbrough P., Transformation systems for generating marker-free transgenic plants, Bio-Technol. 12 (1994) 263–267.

[4] Schlüter K., Fütterer J., Potrykus I., "Horizontal" gene transfer from a transgenic potato line to bacteri pathogen (*Erwinia chrysanthemi*) occurs – if at all – at an extremely low frequency, Bio-Technol. 13 (1995) 1094–1098.

[5] Harding K., Biosafety of selectable marker genes, AgBiotechNet http://agbio.cabweb.org/reviews/misc/sm.htm (1995).

[6] Bebbington C.R., Renner G., Thomson S., King D., Abrams D., Yarranton G.T., High-level expression of a recombinant antibody from myeloma cells using a glutamine synthase gene as anamplifiable selectable marker, Bio-Technol. 10 (1992) 169–175.

[7] Hartman S.C., Mulligan R.C., Two dominant-acting selectable markers for gene transfer studies in mammalian cells, Proc. Natl. Acad. Sci. USA 85 (1988) 8047–8051.

[8] Steinecke P., Herget T., Schreier P.H., Expression of a chimeric ribozyme gene results in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression in vivo, Embo J. 11 (1992) 1525–1530.

[9] De Block M., Debrouwer D., Two T-DNAs co-transformed into *Brassica napus* by a double *Agrobacterium* infection are mainly integrated at the same locus, Theor. Appl. Genet. 82 (1991) 257–263.

[10] McKnight T.D., Lillis M.T., Simpson R.B., Segregation of genes transferred to one plant cell from two separate *Agrobacterium* strains, Plant Mol. Biol. 8 (1987) 439–445.

[11] McClintock B., Mutable loci in maize, Carnegie Inst. Washington Year Book 47 (1948) 155–169.

[12] Fedoroff N., Furtek D., Nelson O., Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Ac*, Proc. Natl. Acad. Sci. USA 81 (1984) 3825–3829.

[13] Carroll B.J., Klimyuk V.I., Thomas C.M., Bishop G.J., Harrison K., Scofield S.R., Jones J.D.G., Germinal transpositions of the maize element dissociation from T-Dna loci in tomato, Genetics 139 (1995) 407–420.

[14] Dale E.C., Ow D.W., Gene transfer with subsequent removal of the selection gene from the host genome, Proc. Natl. Acad. Sci. USA 88 (1991) 10558–10562.

[15] Odell J., Caimi P., Sauer B., Russell S., Site-directed recombination in the genome of transgenic tobacco, Mol. Gen. Genet. 223 (1990) 369–378.

[16] Ebinuma H., Sugita K., Matsunaga E., Yamakado M., Selection of marker-free transgenic plants using the isopentyl transferase gene, Proc. Natl. Acad. Sci. USA 94 (1997) 2117–2121.

[17] Vergunst A.C., Jansen L.E.T., Hooykaas P.J.J., Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase, Nucleic Acids Res. 26 (1998) 2729–2734.

[18] Ritala A., Aspegren K., Kurtén U., Salmenkallio-Marttila M., Mannonen L., Hannus R., Kauppinen V., Teeri T.H., Enari T.M., Fertile transgenic barley by particle bombardment of immature embryos, Plant Mol. Biol. 24 (1994) 317–325.

[19] Reich T.J., Iyer V.N., Miki B.L., Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti-plasmids, Bio-Technol. 4 (1986) 1001–1004.

[20] Stover R.H., Simmonds N.W., Bananas, Tropical Agriculture Series (3rd Ed), Longman Scientific and Technical, New York, USA, 1987.

[21] Fauré S., Noyer J.L., Carreel F., Horry J.P., Bakry F., Lanaud C., Maternal inheritance of chloroplast genome and paternal inheritance of mitochondrial genome in banana (*Musa acuminata*), Curr. Genet. 25 (1994) 265–269.

[22] Alexeev V., Yoon K., Stable and inheritable changes in genotype and phenotype of albino melanocytes induced by an RNA-DNA oligonucleotide, Nat. Biotechnol. 16 (1998) 1343–1346.

[23] Xiang Y., Cole-Strauss A., Yoon K., Gryn J., Kmiec E.B., Targeted gene conversion in a mammalian CD34$^+$-enriched cell population using a chimeric RNA/DNA oligonucleotide, J. Mol. Med. 75 (1997) 829–835.

[24 Cole-Strauss A., Gamper H., Holloman W.K., Munoz M., Cheng N., Kmiec E.B., Targeted gene repair directed by the chimeric RNA/DNA oligonucleotide in a mammalian cell-free extract, Nucleic Acids Res. 27 (1999) 1323–1330.

[25] Beetham P.R., Kipp P.B., Sawycky X.L., May G.D., A tool for functional plant genomics; chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations, In: Proceedings of the International Symposium on the Molecular and Cellular Biology of Banana, March 22–25, INIBAP/BTI (Eds), Montpellier, France / Ithaca NY, USA, 1999, in press.

[26] Rival A. Somatic embryogenesis in oil palm, In: Somatic embryogenesis in woody plants, vol. 6, Jain S.M. (Ed.), Kluwer Academic Publishers, Dordrecht, Netherlands, 1999, in press.

¿ La transformación genética de la banana y del plátano ecológicamente aceptable, es posible ? Propuesta para experimentos téoricos no restringida al cultivo de *Musa*.

Resumen — Introducción. Le mejoramiento varietal de los plantas tiene como objetivo la selección de individuos con los mejores caracteres agronómicos. La transformación genética ofrece a los selaccionadores la posibilidad de sobrepasar las barreras naturales de fertilidad para utilizar nuevos genes utiles. **Consideraciones ecológicas**. La más importante de las preocupaciones en cuanto a plantas transgénicas es la permanencia de genes marcadores (una herramienta del laboratorio necesaria para la selección de estas). **Repuesta técnica**. La utilización de diferentes tipos de genes marcodores y su confinamiento hace el objeto de una irvestigación bibliográfica y de una discusión. La inactivación del gene de selección (marcador) después de la transformación genética y también de la producción de plantas transgénicas sin este constituyen otras alternativas. **Proposiciones**. Nosotros proponemos diferentes enfoques para obtener plantas transgénicas sin un ADN proveniendo de otras especies o de otros géneros, y porsupuesto considerando las caracteristicas biológicas de los plátanos. La primera proposición esta basada sobre la complementación de un mutante auxotrofo construido artificialmente en esa optica por el método de la quirurgía genética o de la extinción genética. La complementación se efectiva dando un gene o un alelo nativo que convendra de buscar dentro de las reservas naturales de las plantas enperentadas. **Conclusión**. La capasidad de adaptación y de reacción a causa de las fluctuaciones del mercado, y esto de una manera economicamente estudiada, es esencial por la supervivencia delos plantadores de plátanos. Es vital considerar los elementos a tomar en cuenta por la construcción de un platano transgénico ecologimente más aceptado". La pieza primordial de este "platáno modificado geneticamente y aceptado ecologicamente" sera la metodología la más contundante deriva de la evaluación de los enfoques diferentes: (1) la transformación por balistica sin la utilización de un vector, ni de un gene marcador (selección) y (2) la complementación de un mutante natural o no, por un gene natural. © Éditions scientifiques et médicales Elsevier SAS

***Musa* / biotecnología vegetal / transformación genética /marcadores genéticos / enriquecimiento**