

Genetic transformation for the improvement of bananas - a critical assessment

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Introduction

Genetic transformation has been the subject of much discussion in recent years. On one side it has been described as a “bandwagon” and a useless bunch of “molecular tricks”, while on the other side, it has been suggested that genetic transformation will result in a second “green revolution” of such magnitude that all problems of food insecurity will become a thing of the past. The reality of course is likely to be somewhere in between these two extremes. While it is true that genetic engineering alone will not provide the solution to feeding an ever expanding human population in years to come, it is also clear that genetic engineering techniques already play an important role in crop improvement and, in combination with conventional breeding, will provide the tools with which new improved varieties will be developed at a faster pace in the future.

It is the intention of this paper to describe briefly genetic transformation and identify some of the constraints which remain to be overcome. The paper will then discuss the potential impact that genetic transformation may have on the future improvement of banana and plantain production, and identify those areas where it is believed genetic transformation has an important role to play.

Plant genetic transformation

More than 120 plant species have already been successfully transformed and these include representatives from the major vegetables, fruits, trees, pastures and ornamentals. Over 60 species are involved in field tests in 45, mainly industrialized, countries. In the USA, insect-resistant cotton and maize, virus resistant squash, several herbicide-resistant transgenic crops

including soybean are commercially produced and different genetically engineered tomatoes with extended shelf life are sold in supermarkets. In total, these transgenic crops were grown on more than 12.5 million hectares in 1997 and this area is expected to reach some 29 million hectares (equivalent to about 40% of arable land in Europe) in 1998. These figures demonstrate that plant transformation has now become a routine methodology whereby the key issue is no longer the production of individual transgenic plants but the generation of transgenic populations with a uniform and predictable pattern of transgene expression. In other words, plant transformation itself has followed a natural progression, developing from early promises of potential during the experimental phase (from the end of 80's to the mid 90's) to an industrial scale (second half of the 90's) with real examples of practical applications.

It should be clear that a major driving force behind investment in transformation research was the desire to have better intellectual property protection on the end-products than was possible with varieties bred through conventional means. To this end, breeding companies have formed alliances with biotechnology companies, in order to ensure access to the technologies and end-products. As a result, many companies that are actively involved in plant breeding have become leaders in one or more fields of biotechnology and their inventions in the quickly evolving, but costly field of plant transformation are frequently protected by patents.

Technical aspects

Plant transformation can be defined as the introduction and stable integration of genes into a plant nuclear genome and their expression in the transgenic plant. Plant transformation involves the introduction of



at least two genes (a selectable marker gene and a gene of interest), but the number of simultaneously introduced genes may reach many more.

During the last decade, wide (and sometimes wild) ranges of transformation techniques have been tested. This has been particularly the case for the transformation of graminaceous monocotyledons, which were previously considered to be recalcitrant. It is now however clear that probably all the major regenerable cell types can be transformed through one of just two techniques, either by particle bombardment or through co-cultivation with *Agrobacterium tumefaciens*.

Particle bombardment (or biolistic transformation) uses accelerated metal (gold or tungsten) microprojectiles coated with DNA to penetrate and deliver foreign genes into plant cells, which are then selected and regenerated into plants. On the other hand, *Agrobacterium tumefaciens*, a soil bacterium, transforms its hosts by integrating a segment (called T-DNA) of its tumor-inducing plasmid into the nuclear genome. The transfer of this T-DNA is regulated by a complex process with the involvement of numerous bacterial genes (the majority called virulence genes) that are located outside the T-DNA. This interesting feature allows for the use of T-DNA as a vehicle to introduce virtually any gene into plant cells.

Whichever technique is used, the precise transformation conditions for a specific regenerable cell type require optimization for each species, and possibly even, each variety.

A critical factor in transformation is the selection of transformed cells, since for each transformation event, the introduced gene(s) will be incorporated into only a fraction of the cells subjected to transformation. This selection is performed with the help of a selectable marker gene which confers resistance to chemical agents, such as antibiotics, herbicides, or amino acid/sugar analogues, which are otherwise toxic to plant cells.

The transformed nature of the transgenic plants must be verified. This is done at the molecular level by DNA hybridization analysis to demonstrate incorporation of the transgenes in primary transformants as well as in their progeny. If large numbers of plants are to be analysed a PCR pre-screening may be carried out. Verification of transformation must also be done by enzymatic or immunological assay or phenotypical observation for the gene product to show the expression (or on the contrary, the silencing) of the integrated genes in parallel with correct controls.

Specific characteristics of genetic transformation

Source of variation

One advantage of genetic transformation over conventional breeding lies in the availability of sources of genetic variability. While conventional breeders are limited to the gene pool that exists in sexually compatible species, this barrier does not apply for genetic transformation. Genes isolated from lower organisms (viroids, viruses, bacteria and fungi) as well as from higher animals (including human genes) can be introduced into plant cells, and such genes have been shown to be correctly expressed in the transformed plants. This allows for the generation of transgenic plants expressing useful traits, which could not be achieved by classical breeding. The list of preliminary yet encouraging examples is wide ranging, and includes the production of vaccines as well as biodegradable plastics in such plants.

Introduction of large DNA segments

It is often assumed that plant transformation involves only single genes, and is therefore of limited usefulness in crop improvement, as most agronomically important traits are multigenic. However, it has been shown that up to 600 kb DNA can be introduced into soybean cells with one shot by particle bombardment and similarly, *Agrobacterium* efficiently delivers and integrates at least 150 kb foreign DNA into the plant genome. This size range is more than sufficient for the introduction of multiple genes. Since it is known that similar sizes of DNA can be incorporated in the genome of prokaryotic as well as eukaryotic organisms such as yeast, this is not altogether surprising.

Constraints remaining

Somaclonal variation

An important scientific concern relates to the tissue culture component of transformation. In many culture systems, the rate of somaclonal variation, i.e. random genetic changes that occur during *in vitro* culture, can be correlated to the time the explants spend in tissue culture. This is particularly the case for banana. Regeneration systems requiring a minimum of time in culture, but still compatible with

transformation, are therefore needed. It should be noted that somaclonal variation is inherent to *in vitro* culture and is not related to the transformation techniques. Therefore, somaclonal variation could be eliminated by a tissue culture-free transformation method, but at present, this is available only for *Arabidopsis thaliana*.

Gene expression

Highly variable levels of transgene expression and complete suppression (also called gene silencing) are also a cause for concern. Variable gene expression is thought to be due to the random integration of the transgene into the plant DNA, thus resulting in different genetic contexts around the transgene in the independent transgenic plants. On the other hand, gene silencing appears to be related to multicopy integration and/or DNA methylation, although the precise mechanism remains to be resolved.

The major consequence of unpredictable transgene expression is the cost of having to screen large numbers of plants for the desired transformants and the continuing evaluation of stability in subsequent progenies.

Transformation efficiency

The constraints outlined above call for a precise definition of transformation efficiency. In the development of transformation technologies, scientists strive to achieve high transformation frequencies, i.e. they try to generate as many transformed plants as possible. However, in practice the issue is not the absolute number of transgenic plants but the number of useful transgenic events that fulfil the required conditions. Therefore, precise gene targeting, which is at present more advanced for the transformation of animal species, is also needed for the improvement of plant transformation efficiencies.

Management and field release of genetically transformed plants

Once transgenic plants have been produced, their further management and field release requires the smooth coordination of a large number of factors. Field release must be authorized by national governments through regulatory bodies. In many developing countries, operational regulatory bodies are not yet in place, and this causes delays in implementing field testing of transgenic plants. Field testing should involve an active breeding programme to ensure that plants

are evaluated correctly and precise observations are taken. Such collaboration with a breeding programme should also form the basis for integrated breeding activities, where plant transformation provides transgenic parental lines that, after field testing, could be incorporated into existing crossing and selection programmes.

Why is genetic transformation important in *Musa*?

Genetically resistant banana and plantain varieties are the basis upon which sustainable improved production can be developed. Since a very wide range of varieties of bananas and plantains are grown worldwide, each adapted to a specific ecoregion and selected for specific eating or cooking qualities, a similarly wide range of improved varieties is required.

Classical breeding of bananas is hampered by long-generation time, triploidy and sterility of most edible cultivars. Sources of resistance to many of the major pests and diseases are known in both landraces and wild species. However, landraces are often sterile and cannot be used in breeding, while crosses involving wild species result in the transfer of many unwanted traits together with the desired resistance genes.

Furthermore, there are certain diseases for which sources of resistance are not known, an important example being banana bunchy top virus (BBTV).

Genetic transformation provides an opportunity for single genes or gene combinations, (such as those associated with disease resistance) to be extracted from the genome of the source organism and transferred directly into the desired variety. This allows the variety in question to retain all its original characteristics, with the simple addition of the desired trait.

Furthermore, since most banana cultivars do not produce seeds under natural conditions, crosses with other varieties or species will not occur. In these cases, the introduced gene remains confined to the variety in which it has been introduced.

Status of transformation in *Musa*

Two systems have resulted in the production of transgenic banana plants: particle bombardment of embryogenic cell suspensions and *Agrobacterium*-mediated transformation of *in vitro* meristems.

Particle bombardment of embryogenic cell suspensions

This technology combines particle bombardment transformation with a regeneration protocol using highly regenerable suspension cultures containing fast-dividing and totipotent somatic cells. It appears that this technology is limited only by the availability of cell cultures with a sufficiently high capacity for plant regeneration. An average of 2-3 transgenic plants per bombardment can be regenerated from a cell suspension with a good morphogenic potential. Transformed plants are ready to be established in the greenhouse six to eight months after bombardment. This method is being used with some considerable success by several research teams worldwide. At KUL alone, close to 900 independent transgenic lines have been produced from the AAB plantain cv. 'Three Hand Planty' and the Cavendish-type dessert (AAA) banana cv. 'Williams'. These plants were transformed with either of two selectable marker genes and a number of chimaeric genes including three different antifungal genes and various genes isolated from banana viruses such as the coat protein gene of banana bract mosaic virus. More than 200 independent lines have been transferred so far to the greenhouse and a significant proportion have been multiplied and prepared for field testing.

Agrobacterium-mediated transformation

The *Agrobacterium*-based banana transformation system as originally reported, used explants containing the apical meristem or corm meristematic tissues which were wounded by microparticle bombardment with uncoated particles. After a brief recovery period, these meristems were subsequently co-cultivated with *Agrobacterium tumefaciens* harbouring the plant transformation vector in the presence of acetosyringone, a known inducer of the *Agrobacterium* virulence genes. Antibiotic resistant plants were regenerated and DNA hybridization demonstrated that the transgenes were incorporated into high molecular weight genomic DNA and no residual *Agrobacterium* persisted in the plants. In addition, plantlets that had undergone multiple rounds of propagation maintained these genotypic and phenotypic traits. The main advantages of this method are (i) the ease and speed of transgenic plant regeneration, (ii) the lack of requirement for high-tech equipment or sophistication in tissue culture and (iii) the assumption that only the desired DNA sequences are

transferred to recipient cells. However, the genotype specificity of this technique in banana needs to be thoroughly evaluated. In addition, several independent laboratories have found that a high incidence of chimaeric transformants is characteristic of this method. This is probably due to the very low chance that all cells contributing to the formation of new organs are accidentally transformed in the organized explants. Recently different research groups have observed that regenerable cell suspensions can also be transformed by *Agrobacterium* at a high frequency. However, the involvement of cell suspensions makes this method slow and more expensive, thus less attractive for routine use.

Introduction of multiple genes

Long-term and multiple disease resistance is likely to be achieved by integrating several genes with different targets or modes of action into the plant genome. Technically, this can be done either in several consecutive steps or simultaneously. Particle bombardment of embryogenic cell cultures, the present method of choice for genetic transformation of banana, relies on the time-consuming development of highly embryogenic cultures from a number of target cultivars, which makes consecutive transformations impractical. It has been shown at KUL that simultaneous gene transfer into banana can be performed by co-precipitation of a mixture of chimaeric gene constructs onto microparticles before bombardment. Transgenic plants regenerated after bombardment with such constructs contained unlinked genes at a frequency of 70 – 80%. As a result of these co-transformation experiments, transgenic banana plants containing up to six different genes have been obtained. These observations indicate that simultaneous bombardment of different plasmid molecules may be a convenient way for the introduction and perhaps co-expression of multiple genes in banana plants.

Current applications of genetic transformation in *Musa*

Fungal disease resistance

Since the most significant damage to banana production is caused by fungal pathogens, the introduction of genes conferring resistance to fungal pathogens is a primary research target. Efforts are presently

focused on the recently described antimicrobial proteins (AMPs) which are stable, cysteine-rich small peptides isolated from seeds of diverse plant species. These AMPs have a broad anti-fungal spectrum and show high *in vitro* activity against field isolates of *Mycosphaerella fijiensis* and *Fusarium oxysporum*, the two main fungal pathogens of *Musa*, while they exert no toxicity to human or plant cells.

To date, five AMP genes have been introduced at KUL into embryogenic cell suspensions of banana and plantain using particle bombardment. Large-scale molecular and biochemical characterization of the transgenic lines confirmed that a vast majority contained the foreign genes. For example, PCR-screening of a total of 776 putative transgenic plants revealed that more than 90% of them contained the selectable marker gene as well as the AMP genes. Similar results were obtained by DNA hybridization of 56 plants, which showed that 89.3% had the foreign gene incorporated in their genome.

To study the expression of antifungal proteins in the transgenic plants, the AMP genes were placed under the control of various regulatory sequences (promoters) which had previously been shown to drive high gene expression in banana as well as in other monocots. Using specific antibodies, the concentration of two antifungal proteins in total leaf proteins was determined in transgenic plant extracts and results ranged from 0.05% to more than 1% depending on the promoter used. Addition of these extracts to germinating spores of a field isolate of *Mycosphaerella fijiensis*, the causal agent of black Sigatoka disease, resulted in a significant inhibition of fungal growth making these plants likely candidates to control this pathogen under field conditions.

Further research will demonstrate whether these transgenic plants express the functional antimicrobial peptides at levels high enough to control fungal leaf or root diseases in the field. It has been shown that one AMP gene is also expressed in the fruit, opening the opportunity to create resistance against pre- and postharvest diseases such as cigar-end rot and crown rot.

Virus resistance

Engineering resistance to banana bunchy top virus (BBTV) is another obvious objective for banana transformation, since no natural resistance to this virus has so far been identified in the *Musa* gene pool. Molecular virologists have gathered more

knowledge about BBTV in the last five years than during the preceding 100 years. The genome organization of BBTV, as well as its movement within the plant, is now starting to be understood in detail.

The further molecular characterization of this virus is ongoing, and this will assist in designing various transformation strategies to achieve protection against the virus by gene transfer. One of the strategies now being tested in several laboratories is the expression of various BBTV genes in transgenic banana plants in order to interfere with the normal replication, encapsidation or movement of the virus. Another approach is the expression in bananas of heterologous antiviral proteins that are known to act by the inhibition of viral replication or translation.

Similar strategies can also be considered against another recently emerged DNA virus, the banana streak virus whose molecular research in laboratories worldwide has already revealed a number of interesting features including its integration into the banana genome.

The future of transgenic research in *Musa*

To date, no genetically transformed banana or plantain plants are being tested in the field. This is essentially due, as mentioned earlier, to the lack of functional regulatory bodies in the countries where testing would be carried out. In view of the fact that more and more transgenic plants are being produced by laboratories worldwide, the lack of regulatory mechanisms is likely to continue to be a major bottleneck to progress. It is therefore recommended that greater efforts, at both national and international level, be made to resolve this situation.

Major gaps in scientific knowledge related to *Musa* pests and diseases still exist. In order for further progress in genetic transformation to be made, these gaps must be filled. For instance it is remarkable that considering the importance of fungal diseases in banana, insufficient knowledge is available at the molecular, or even cellular level on the pathogenesis of fungi in banana. Much more information in this area must be obtained before studies on the molecular interaction and signal transduction between the banana host and the fungal pathogen can be conducted.

Besides the more advanced areas of fungal and virus resistance, there are several areas of banana transformation which are also being investigated and may hold potential for the future. These include resistance to pests such as nematodes and insects, tolerance to cold and water stress, improved fruit quality and the potential use of bananas as bioreactors.

Conclusions

The genetic transformation of cultured cells and tissues has led not only to the production of the first transgenic banana and plantain plants, but also to the generation of a large number of transgenic lines with agronomically useful genes. Several hundreds of independent transgenic plants transformed with genes encoding antifungal proteins are available for field testing. Banana bunchy top, banana streak and bract mosaic viruses have been isolated and their genomes are being characterized. The prospects for improving bananas through the use of molecular techniques therefore are clearly demonstrated. However, it should always be remembered that molecular technologies cannot stand alone, but are complementary to conventional breeding based on hybridization. Indeed molecular biology reveals the specific action and interaction of a particular or a few gene (s) under controlled conditions while breeding deals with a statistical process where large numbers of genes are involved. It is therefore essential that *Musa* breeders and *Musa* biotechnologists work together to accelerate *Musa* improvement and not compete against each other.

In view of the limited resources being devoted to research into *Musa* improvement, and knowing the scale of the problems to be overcome, it is important that donor agencies, and particularly the CGIAR, put emphasis on stimulating collaboration between successful breeding institutions and advanced biotechnology centers. One recent initiative that aims to bring together all the major players in *Musa* improvement research is PROMUSA, the Global Programme for *Musa* Improvement. This programme was launched in 1997 by INIBAP and the World Bank and provides the necessary framework within which collaborative partnerships can be developed and fostered. Further details of PROMUSA are provided in Focus Paper 1.