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Banana and Plantain

Leena Tripathi[1], Jaindra Nath Tripathi[1], Abdou Tenkouano[2] and Paula Bramel[3]

[1]*International Institute of Tropical Agriculture, Kampala, Uganda*
[2]*International Institute of Tropical Agriculture, Messa, Yaoundé, Cameroon*
[3]*International Institute of Tropical Agriculture, Ibadan, Nigeria*

1. INTRODUCTION

Bananas and plantains (*Musa* spp.) are the world's fourth most important food crop after rice, wheat, and maize in terms of gross value of production, with the vast majority of the crop grown and consumed in the tropical and subtropical zones. They provide a staple food for millions of people, particularly in Africa, an area where the green revolution has had little influence.

The performance of bananas and plantains can be severely affected by diseases and pests, including black Sigatoka (*Mycosphaerella fijiensis*), *Fusarium* wilt (*Fusarium oxysporum* f. sp. *cubense*), bacterial wilt (*Xanthomonas campestris* pv. *musacearum*), viruses (banana bunchy top virus, banana streak virus), nematodes, and weevils. These pest and diseases can cause economically significant food and income losses. Consequently, the largest single component in the cost of production of dessert banana and plantain in the tropics is disease control. Current control measures, which vary from cultural and biological to chemical control, comprise approximately 40% of total production cost (Ganry, 1993). Bananas and plantains are predominantly smallholders' crops; most growers cannot afford costly chemicals to control pests and diseases. This leaves host plant resistance as the most sustainable approach to counteracting pest and disease pressure, both under small-scale and large-scale banana and plantain production schemes. Banana and plantain present many challenges for genetic improvement, notably associated with the reproductive biology, the complexity of trait expression, and the long generation time of the crop. Transgenic technology, together with conventional methods can assist in overcoming these problems in developing improved cultivars of banana and plantain.

1.1 History, Origin, and Distribution

Bananas and plantains are giant herbs that belong to the Musaceae, one of the six families of the order Zingiberales. The Musaceae have been associated with human history for a long time (Rowe and Rosales, 1996). The banana plant has been described as the "Tree of Paradise" in the Koran and as the "Tree of Knowledge" in the Book of Genesis in the Old Testament, hence the earlier taxonomic identification of bananas as *Musa paradisiaca* L. or *M. sapientum* L., which some authors still use nowadays.

The cultivation of bananas was first documented in ancient scriptures of India dating back to 500–600 BC (Reynolds, 1927). However, there is mounting archaeological evidence in support of earlier cultivation in Papua New Guinea, at least 7000 years ago and possibly as far back as 10 000 years ago (Denham *et al*., 2003). Thus,

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the New Guinean highlands would be the place where bananas were first domesticated. Organized plantation of bananas existed in China in 200 AD, four centuries before Islamic conquerors brought the banana to Palestine, and Arab merchants eventually spread the plant over much of Africa. The word *banana* is of West African origin, but it is derived from the Arabic banaan (finger), and passed into English via Spanish or Portuguese. However, the long debated when and by what routes *Musa* (bananas) were introduced to tropical Africa and South America remain unresolved.

Cultivated bananas and plantains are predominantly triploid ($2n = 3x = 33$) female-sterile varieties that evolved from two diploid ($2n = 2x = 22$) seminiferous species, *Musa acuminata* Colla (genome AA) and *Musa balbisiana* Colla (genome BB), originating from the tropical Malay region and the Northern Indian region, respectively (Simmonds 1962).

It is hypothesized that the evolutionary pathway leading to the emergence of cultivated varieties from the seminiferous ancestors comprised two critical events, both of which occurred in Southeast Asia, the center of origin and primary diversity of bananas. Firstly, vegetative parthenocarpy and female sterility appeared in *M. acuminata*, allowing for production of pulp without seeds as evidenced by the occurrence of parthenocarpic and seedless diploid *M. acuminata*. Secondly, crosses within *M. acuminata* or between *M. acuminata* and *M. balbisiana* occurred, facilitated by female restitution and haploid fertilization, and produced two groups of natural hybrids. One group comprises autoploid and homogenomic hybrids that are essentially AAA dessert and East African Highland bananas. The other group contains alloploid and heterogenomic types: the AAB plantains and starchy bananas and the ABB cooking bananas.

The natural hybrids do not produce seeds and can only reproduce by vegetative propagation, implying that survival in nature and geographical dispersal could not occur without human intervention. Therefore, it is widely accepted that secondary diversification in areas devoid of wild *Musa* plants, e.g., Western and Central Africa, must be due to somatic mutations of a small number of introduced materials, rather than through multiple introductions (De Langhe *et al*., 2005).

In Africa, the cultivars are usually known under a multitude of vernacular names, superimposing linguistic diversity to genetic and ecological diversity of the varieties in any given area (Rossel, 1998). Similar interactions between linguistics and genecology occur throughout Asia, the geographical origin and primary diversification center of the *Musaceae*. Thus, a good understanding of demographic history and dispersal mechanisms may help explain the structure of genetic diversity among populations of bananas and plantains across geographical regions.

1.2 Botanical Description

The taxonomy of bananas and plantains has attracted many excellent treatises that also describe the distinguishing features among cultivated forms (Simmonds, 1962; Stover and Simmonds, 1987; Robinson, 1996; De Langhe *et al*., 2005). Baker (1893) was first to establish a formal classification of the genus *Musa*. He recognized three subgenera: *Physocaulis* (wild and inedible seeded bananas), *Eumusa* (species with edible fruits), and *Rhodochlamys* (inedible fruits and brightly colored bracts). Bakers's classification was revised by Cheesman (1947) whose detailed taxonomic treatment was based primarily on chromosome number, pseudostem stature, inflorescence characters, and seed morphology. Cheesman adopted the concept of sections or series at the subgeneric level, dividing the genus *Musa* into *Eumusa* ($x = 11$), *Rhodochlamys* ($x = 11$), *Australimusa* ($x = 10$), and *Callimusa* ($x = 10$). The majority of the cultivated bananas belong to the section *Eumusa*. This section is the biggest in the genus and the most geographically widespread with species found throughout Southeast Asia from India to the Pacific Islands.

Cheesman's classification is still widely accepted, despite subsequent reports of basic chromosome numbers of 7 for *Musa ingens* and 9 for *Musa beccarrii* (Shepherd, 1959) or the discovery of *M. ingens* that could not be fitted to any of the sections (Argent, 1976). In fact, several unresolved issues remain, including the evolutionary relationships among the four sections of the genus *Musa*, the separation of sections *Eumusa* and *Rhodochlamys* with apparently little taxonomic support, and the relationship between

the wild progenitors and the cultivated clones (Shepherd, 1999).

It is now accepted that the diversity of bananas in the wild has not been exhaustively described and classified, with new information prompting taxonomic revision from time to time at the species level, and more rarely, at the genus level (Valmayor and Danh, 2002; Häkkinen, 2006).

Ploidy and genome configurations have played a determinant role in the classification of bananas, with major groups including diploids (AA, BB, AB), triploids (AAA, AAB, ABB), and tetraploids (AAAA, AAAB, AABB, ABBB). There is wide consensus about the attributes conferred by A or B genomes in interspecific natural or artificial hybrids of *M. acuminata* and *M. balbisiana*. Hence, it is accepted that edibility of mature fruits arose from mutations causing parthenocarpy and female sterility in diploid *M. acuminata* (Simmonds, 1962). It is equally accepted that hardiness is contributed by the B genome since *M. balbisiana* clones strive abundantly in areas experiencing pronounced dry seasons alternating with monsoons. Also attributed to the B genome are fruit characteristics, such as starchiness and acid taste, causing AAB plantain to be starchier but less sweet and less palatable when raw than the AAA dessert bananas (Simmonds, 1962).

The African plantains (AAB) display greater variation than any other subgroup of triploid bananas in the world, with morphological variation occurring for inflorescence type, plant size, fruit orientation, fruit shape, pseudostem, and fruit color. Based on inflorescence morphology, four types have been distinguished, namely, French plantain, French Horn plantain, Horn, and False Horn plantain, which are further divided into giant, medium, and small types.

While various combinations of the A and B genomes predominate in most cultivars, two other genomes, denoted as S (from *M. schizocarpa*) and T (from *M. textilis*), occur albeit in a few accessions. Thus, genomic groups with the S genome include AS, AAS, and ABBS while those with the T genome are AAT, AAAT, and ABBT (Sharrock, 1990).

Pillay *et al*. (2004) provided a succinct review of studies on genome size and cytological features of bananas. Relatively few studies have been conducted in *Musa* to determine genome size, with large differences in the DNA content estimates obtained by different authors (Lysak *et al*., 1999; D'Hont *et al*., 2000; Kamaté *et al*., 2001). Thus, DNA content ranging from 1.11 $pg2C^{-1}$ to 1.33 $pg2C^{-1}$ was reported for diploid *M. acuminata* accessions. Likewise, nuclear DNA content ranging from 1.03 $pg2C^{-1}$ to 1.16 $pg2C^{-1}$ were found for diploid *M. balbisiana*, while S and T genomes were reported to contain 1.18 $pg2C^{-1}$ and 1.27 $pg2C^{-1}$ DNA, respectively. Clearly, additional research may be needed before robust comparisons of DNA content in the A, B, S, and T genomes can be made, prompting Pillay *et al*. (2004) to advise that caution be exercised in interpreting DNA content data in *Musa*.

1.3 Economic Importance

Banana and plantain are important cash and subsistence crops in most tropical and subtropical regions of the world, growing on production cycles of 12–18 months, essentially as perennial crops that can be harvested all year round (Robinson, 1996). Almost all banana and plantain cultivation falls within 30° latitude north and south of the equator (Stover and Simmonds, 1987). They require an average temperature of about 30 °C and a minimal rainfall of 100 mm per month (Swennen and Rosales, 1994). These crops are cultivated on approximately 10 million ha worldwide with an annual production exceeding 100 million metric tons, which are distributed among Africa (40%), Asia (30%), Latin America, and the Caribbean (30%).

As mentioned above, the existing cultivars are assigned to three genome groups, AAA, AAB, and ABB but varieties grouped in the same genomic category can be very dissimilar (Simmonds, 1962; Stover and Simmonds, 1987; Robinson, 1996). Thus, the AAA group contains sweet dessert bananas that are eaten raw when ripe and bananas of the East African highlands that require cooking before they can be eaten or brewing before drinking. Likewise, the West African and Asia Pacific (Maia maoli/Popoulou) plantains that are cooked before eating dominate the AAB group, which also contains dessert bananas of the Pome subgroup. Varieties in the ABB group are essentially used for cooking, but some may be eaten raw as dessert bananas usually when overripe.

Economically, bananas and plantains constitute major export crops in some countries

predominantly in Latin America and the Caribbean and a few countries in Western and Central Africa. However, only about 10% of world production, predominantly AAA dessert bananas of the Cavendish type, enters international trade. The bulk of the production provides an important staple food for rural and urban consumers in the production areas where bananas and plantains also constitute an important source of income for rural populations.

Bananas and plantains are the major staple food in the equatorial belt of Africa, where more than 70 million people derive in excess of 25% of their daily calorie intake from plantains in West and Central Africa (Robinson, 1996). Indeed bananas are rich in carbohydrates (about 35%) and fiber (6–7%), and have a relatively low protein and fat content (1–2%), and constitute a good source of major elements, such as potassium, magnesium, phosphorus, calcium, iron, and vitamins A, B6, and C (Marriott and Lancaster, 1983). Bananas play a vital role in the nutrition and well-being, and cultural life of millions of people in Central and Western Africa and South and Central America, often being the first solid food for infants (Price, 1995).

Bananas and plantains can be processed into a variety of secondary products from fibers to wrappings and are fermented to produce alcohol. However, these options are essentially practiced at rudimentary scales, despite their potential for industrial production.

1.4 Traditional Breeding

Banana and plantain were introduced in Africa only about 3000 years ago (De Langhe, 1995) and a remarkable diversity now exists for both groups, but all traditional varieties have become susceptible to a range of pests and diseases, the most serious of which are black Sigatoka (Mycosphaerella fijiensis Morelet), Fusarium wilt (Fusarium oxysporum f. sp. cubense), banana weevil (Cosmopolites sordidus Germar), and a complex of plant parasitic nematodes (Pratylenchus goodeyi, Helicotylenchus multicinctus, and Radopholus similes). Therefore, breeding efforts focus on developing new disease and pest resistant varieties that also retain the organoleptic properties of the traditional varieties.

There are two basic steps in plant breeding: (a) accessing natural variation or artificially creating genetic diversity, and (b) selecting individuals with the desirable gene combinations from existing or artificially created populations. These principles have been applied for seed-propagated crop species using techniques that are often referred to as conventional. However, a commonly held view is that the most widely used so-called conventional or classical plant breeding techniques from sexual, seed-propagated crops are not appropriate for banana and plantain improvement. The distinction between "conventional" and "nonconventional" breeding lies in the methods of gene shuffling and in the origin of the genes being manipulated. Nevertheless, the methodological principles applied for sexually propagated crops can be, and have been, applied to banana and plantain breeding, with the aid of nonfield methods of recovering viable progeny and identifying those progenies with putatively desirable gene combinations. In this regard, there is nothing conventional about banana and plantain breeding (Tenkouano, 2005).

Banana and plantain breeders aim to produce seedless varieties, preferably in the triploid ($3x$) background common to the majority of existing cultivars. This usually involves crossing $3x$ cultivars to diploid ($2x$) accessions that are donors of resistance genes, selecting $4x$ and $2x$ primary hybrids from the $3x - 2x$ progenies, and crossing $4x - 2x$ hybrids to produce secondary $3x$ hybrids. Alternatively, secondary $3x$ hybrids may be produced via $2x - 2x$ crosses where one of the $2x$ parents produces $2n$ pollen. However, this requires screening of diploid lines for $2n$ pollen production, and setting crossing schemes that would allow pollination to coincide with periods that are favorable for $2n$ pollen production.

Other methods of producing triploid hybrids have been explored, such as tetraploidization of diploid accessions using colchicine prior to crossing with another diploid (Tézenas du Montcel *et al*., 1996). Colchicine prevents the formation of mitotic spindles, resulting in mitotic restitution in treated cells. This technique may preserve advantageous linkages selected at diploid level, particularly in the tetraploid derived from the diploid, but recombination can occur when crossing the tetraploid with a diploid to produce a triploid, with the potential of disrupting linkages. Also, colchicine treatment may not affect uniformly all cells in multicellular meristems, causing

cytochimeras that may not be easy to dissociate (Roux *et al*., 2001). Thus, efficient methods for *in vitro* dissociation of chimeras and selection of the desired cells are required, before such cells can be cultured to regenerate a plant that will now be used for crossbreeding. Furthermore, the use of colchicine can cause increased inbreeding, reduced vigor, and reduced genetic variability (Ortiz *et al*., 1992). Thus, colchicine-mediated polyploidization is impractical for routine breeding operations in plantain and banana.

Hence, tetraploid × diploid crosses remain the predominant triploid breeding scheme, although the multiploidy and heterogenomic structure of breeding populations results in unpredictable variation in genome size and structure across and within generations. This causes complex inheritance patterns and complicates phenotypic selection for most yield and growth traits (Ortiz and Vuylsteke, 1996).

Despite these limitations, many improved hybrids combining genetic resistance to black Sigatoka with appropriate agronomic characteristics have been developed (Swennen and Vuylsteke, 1993; Ortiz and vuylsteke, 1998). The improved hybrids are about two to five times more productive than the traditional plantain landraces, under natural conditions with no chemical control of black Sigatoka. These hybrids are now being introduced into farmers' fields in several countries, across Africa (Gallez *et al*., 2004; Tenkouano and Swennen, 2004) with relatively high adoption prospects (Tenkouano *et al*., 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

A good understanding of the structure of diversity in the existing materials is required to access natural variation or to artificially create genetic variation. Early breeders relied essentially on morphological and geographical information, which may not be adequate. Recent advances in the development of molecular or cytological tools stand to benefit modern breeders, notably by clarifying phylogenetic relationships, thus allowing for inferences about gene pools. For example, amplified fragment length polymorphism analysis of total DNA revealed that the *M. acuminata* complex of about 50 morphological subspecies can be ascribed to only three genetic subspecies (*burmannica*, *malaccensis*, and *microcarpa*) while *M. balbisiana* was shown to contains two forms (Singapuri, I-63), suggesting that there might be at least three A genomes and two B genomes (Ude *et al*., 2002a, b). Furthermore, it seems that the A genomes in the plantains come from the subspecies *microcarpa* while the B genome comes from the I-63 form of *M. balbisiana*. More recently, Ge *et al*. (2005) also traced variation in *M. balbisiana* to two main clades, based on simple sequence repeat length polymorphisms and CpDNA analysis. Such information becomes important when attempts are made to diversify and broaden the genetic foundation of breeding populations, particularly in the search for novel genes.

In fact, as new genomic resources become available, or are applied in conjunction with cytogenetic tools (Bartos *et al*., 2005) or numerical tools (De Langhe *et al*., 2005), the taxonomic description of the *Musaceae* will likely evolve. These findings have major implications for evolutionary or reconstructive breeding, aiming to re-enact existing landraces without some of their shortcomings such as disease susceptibility.

Despite these advances, many problems specific to the biology of plantains and bananas impede rapid breeding progress, including low reproductive fertility, triploidy, and slow propagation (Vuylsteke *et al*., 1997). Thus, breeders may not be able to access all available variation, largely due to the high sterility of many cultivated varieties; the conundrum of banana breeding is that seed set is required to produce seedless varieties. Improving female or male sterile accessions can only be achieved by circumventing reproductive barriers, e.g., by deliberate mutagenesis or by direct gene transfer through genetic engineering.

The crossbreeding approach is time consuming, given the long generation time of the crop and it is also technically complex, since it requires both ploidy and genome selection. With the advent of flow cytometric analysis of nuclear DNA content (Dolezel, 1997) and molecular markers for the A and B genomes (Pillay *et al*., 2000), ploidy and genome selection have become easier. However, predicting progeny performance remains a major challenge when ploidy and genome variation occurs within and across generations (Tenkouano *et al*., 1999a, b). Ideally, genetic engineering would provide a more deterministic approach

for substituting undesirable genetic materials with those that would enhance the performance of the recipient line or cultivar with respect to a particular constraint.

Additionally, the available germplasm may not harbor the desired genes with respect to an emerging threat, which is clearly the case for the bacterial wilt caused by *X. campestris* pv. *musacearum* that is threatening livelihoods in the Great Lakes region of central and eastern Africa. There is little that crossbreeding can do to address such a threat in the near future, leaving genetic engineering as the best alternative to control the bacterial wilt epidemics through host resistance.

Genetic engineering could target the development of improved versions of existing cultivars for deployment or, alternatively, insert alien resistance genes into breeding lines for later use in crossbreeding schemes.

2. DEVELOPMENT OF TRANSGENIC BANANAS AND PLANTAINS

In view of the constraints of conventional breeding in banana and plantain already mentioned, the case for using transgenic approaches to improve these crops is particularly compelling. Because of the lack of cross-fertile wild relatives in many banana producing areas, as well as the male and female sterility of most edible bananas and plantains and the clonal mode of propagation, gene flow is not an issue for this crop, making a transgenic approach even more attractive.

Genetic transformation has become an important tool for crop improvement. Genetic engineering, i.e., the introduction and stable integration of genes into the nuclear genome and their expression in a transgenic plant offers a better alternative for the genetic improvement of cultivars not amenable to conventional crossbreeding, such as Cavendish bananas and False Horn plantains (Jones, 2000). The successful genetic transformation in plants requires the production of normal, fertile plants expressing the newly inserted gene(s). The process of genetic transformation involves several distinct steps, namely, identification of useful gene, the cloning of the gene into a suitable plasmid vector, delivery of the vector into plant cell (insertion and integration) followed by expression, and inheritance of the foreign DNA encoding a polypeptide. With the advent of plant biotechnology and the rapid development of gene transfer techniques, the potential to introduce desirable traits is no longer restricted to those occurring in close relatives. In theory, genes, once identified, isolated, and cloned may be introduced to a plant from any organism.

Development of stable and reproducible transformation and regeneration technologies opened new horizons in banana and plantain breeding. Despite technical difficulties of transforming a monocot species, transformation protocols are now available for most *Musa* cultivars. Several transformation strategies have been published in the last 10 years by different banana biotechnologists (May *et al*., 1995; Sagi *et al*., 1995; Becker *et al*., 2000; Ganapathi *et al*., 2001; Khanna *et al*., 2004; Tripathi *et al*., 2005a).

2.1 Donor Gene, Promoter, Termination Sequence, and Selection Marker

The identification of resistance genes for the diseases facing banana and plantain is critical in order to create transgenic varieties resistant to these diseases. Upon identification of resistance genes, a method of introduction of these transgenes into banana and plantain will be necessary. Genetic transformation could be a useful tool for creating plants with improved characteristics. The status of research on genetic engineering of banana and plantain for disease resistance and future possibilities has also been reviewed (Sagi, 2000; Tripathi, 2003; Tripathi *et al*., 2005b). Currently, the transgenes used for banana improvement have been exclusively isolated from heterologous sources like other plant species, insects, microbes, and animals (Tripathi, 2003; Tripathi *et al*., 2004, 2005b).

2.1.1 Isolation and characterization of novel genes: targeted traits

2.1.1.1 Pest resistance

There are several possible approaches for developing transgenic plants with improved weevil and nematode resistance. A variety of genes

are available for genetic engineering for pest resistance (Sharma *et al*., 2000). Among these are proteinase inhibitors (PIs), *Bacillus thuringiensis* (*Bt*) toxins, plant lectins, vegetative insecticidal proteins (VIPs), and ά-amylase inhibitors (AI). PIs contribute to host plant resistance against pests and pathogens (Green and Ryan, 1972). They operate by disrupting protein digestion in the insect mid-gut via inhibition of proteinases. The two major proteinase classes in the digestive systems of phytophagus insects are the serine and cysteine proteinases. Coleopteran insects, including the banana weevil, mainly use cysteine proteinases (Murdock *et al*., 1987) and recent studies indicate a combination of both serine and cysteine proteinase is useful (Gerald *et al*., 1997). These inhibitors have already been used for insect control in genetically modified (GM) plants (Leple *et al*., 1995). Presently, cysteine proteinase activity has been identified in the mid-gut of the banana weevil and *in vitro* studies have shown that cysteine proteinases are strongly inhibited by both a purified recombinant rice (*oryzacystatin*-I [OC-I]) and papaya cystatin (Abe *et al*., 1987; Kiggundu *et al*., 2003).

The use of PIs, as nematode antifeedants is an important element of natural plant defense strategies (Ryan, 1990). This approach offers prospects for novel plant resistance against nematodes and reduces use of nematicides. The potential of PIs for transgenic crop protection is enhanced by lack of harmful effects when humans consume them in seeds, such as rice and cowpea. Cysteine PIs (cystatins) are inhibitors of cysteine proteinases and have been isolated from seeds of a wide range of crop plants consumed by man including those of sunflower, cowpea, soybean, maize, and rice (Atkinson *et al*., 1995). Transgenic expression of PIs provides effective control of both cyst and root–knot nematodes. The cystatins Oc-I and an engineered variant Oc-IΔD86 were shown to mediate nematode resistance when expressed in tomato hairy root (Urwin *et al*., 1995), *Arabidopsis* plants (Urwin *et al*., 1997), rice (Vain *et al*., 1998a), and pineapple (Urwin *et al*., 2000). The partial resistance was conferred in a small-scale potato field trial on a susceptible cultivar by expressing cystatins under control of the cauliflower mosaic virus (CaMV) 35S promoter (Urwin *et al*., 2001). The enhanced transgenic plant resistance to nematodes has been demonstrated by using dual PI transgenes (Urwin *et al*., 1998). Since cystatins have been shown to function in rice, which like *Musa* is a monocotyledon, and also have clear efficacy against a wide range of nematode species, their usefulness as transgenes for development of transgenic *Musa* for resistance to nematodes can be evaluated as having a high probability of success. Recently, Cavendish banana (Grand Nain) has been transformed using *Agrobacterium tumefaciens* to express a protein-engineered rice cystatin (Atkinson *et al*., 2004) and tested in screen house for nematode resistance.

The expression and biological activity of the *Bt* toxins has been investigated in GM plants for insect control. *Bt* gene technology is currently the most widely used system for lepidopteran control in commercial GM crops (Krettiger, 1997). The expression of a selected *Bt* gene for weevil resistance may be a rather long-term strategy since no potential *Bt* gene with high toxic effects against the banana weevil has been identified as yet (Kiggundu *et al*., 2003). Some *Bt* proteins are also effective against saprophagous nematodes (Borgonie *et al*., 1996). The Cry5B protein is toxic to wild type *Caenorhabditis elegans*, other *C. elegans* mutants are resistant to Cry5B but susceptible to the Cry6A toxin (Marroquin *et al*., 2000). The approach using *cry* genes has potential for plant nematode control (Wei *et al*., 2003).

The other strategies for nematode resistance include the use of natural plant resistance genes (R genes) and lectins genes. Several R genes are targeted against nematodes. The *Hs1pro-1* from a wild species of sugar beet confers resistance to the cyst nematode *Heterodera schachlii* (Cai *et al*., 1997). Plant lectins confer a protective role against a range of organisms (Sharma *et al*., 2000). Lectins have been isolated from a wide range of plants including snowdrop, pea, wheat, rice, and soybean. Their carbohydrate binding capability renders them toxic to insects. Some lectins such as the snowdrop lectin (*Galanthus nivalis* agglutinin: GNA) have biological activity against nematodes (Burrows *et al*., 1998). However, many lectins have toxic effects on insects and mammals, which raise concerns regarding toxicological safety and this, may prove a substantial limitation to the future commercial development of lectins.

AI and chitinase enzymes might also have a future potential for weevil control. AI operate by

inhibiting the enzyme ά-amylase, which breaks down starch to glucose in the insect gut (Morton *et al*., 2000). Transgenic adzuki beans are produced with enhanced resistance to bean bruchids, which are Coleopteran insects like weevils (Ishimoto *et al*., 1996). Therefore, AI may be of interest for banana weevil control in GM banana. Chitinase enzymes are produced as a result of invasion either by fungal pathogens or insects. Transgenic expression of chitinase has shown improved resistance to Lepidopteran insect pests in tobacco (Ding *et al*., 1998).

2.1.1.2 Bacterial and fungal resistance

Currently, no source of any banana germplasm exhibiting resistance to the disease has been identified. As an alternative, genetic transformation technology with fungicidal or bactericidal transgenes may offer an alternative solution to these problems.

The most attractive strategy for serious fungal disease like Black Sigatoka control in *Musa* is probably the production of disease resistant plants through the transgenic approach including the expression of genes encoding plant, fungal or bacterial hydrolytic enzymes, genes encoding elicitors of defense response, and antimicrobial peptides (AMPs) (Tripathi, 2005, 2006). AMPs have a broad-spectrum antimicrobial activity against fungi as well as bacteria and most are nontoxic to plant and mammalian cells. Examples of AMPs are magainin from the African clawed frog (Zasloff, 1987; Bevins and Zasloff, 1990), cecropins from the giant silk moth (Boman and Hultmark, 1987), and plant defensins (Broekaert *et al*., 1995). The cecropin (Alan and Earle, 2002) and its derivatives (D4E1; Rajasekaran *et al*., 2001) have been found to inhibit the *in vitro* growth of several important bacterial and fungal pathogens. Transgenic tobacco plants expressing cecropins have increased resistance to *Pseudomonas syringae* pv. *tabaci*, the cause of tobacco wildfire (Huang *et al*., 1997).

Similarly, magainin is effective against the plant pathogenic fungi (Zasloff, 1987; Kristyanne *et al*., 1997). Li *et al*. (2001) reported enhanced disease resistance in transgenic tobacco expressing Myp30, a magainin analog. Chakrabarti *et al*. (2003) reported successful expression of other synthetic peptide, MSI-99 and enhanced disease resistance in transgenic tobacco and banana. A synthetic substitution analog of magainin, MSI-99, is employed in this study to impart disease resistance in transgenic tobacco (*Nicotiana tabacum* L.) and banana (*Musa* sp. cv. Rasthali). This peptide inhibited the growth and spore germination of *F. oxysporum f.* sp. *cubense* at 16 lg ml^{-1}. MSI-99 has been subcloned into plant expression vectors pMSI164 and pMSI168, targeting the peptide into the cytoplasm and extracellular spaces, respectively. Tobacco plants transformed with pMSI168 showed enhanced resistance against *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Botrytis cinerea*. Transgenic banana pants were obtained for both pMSI164 and pMSI168 transformations and showed resistance to *F. oxysporum f.* sp. *cubense* and *Mycosphaerella musicola*. On the basis of the broad-spectrum activity against fungal pathogens, individual or combined expression of cecropin, magainin, and their derivatives in banana may result in increased resistance to several pathogens.

Another source of antimicrobial proteins has been lysozyme, either from bacteriophage, hen eggs, or bovine. The lysozyme attacks the murein layer of bacterial peptidoglycan resulting in cell wall weakening and eventually leading to lysis of both gram-negative and gram-positive bacteria. The lysozyme genes have been used to confer disease resistance against plant pathogenic bacteria in transgenic tobacco (Trudel *et al*., 1995), potato (Düring *et al*., 1993), and apple (Ko, 1999).

There are many reports on the application of plant proteins with distinct antimicrobial activities (Broekaert *et al*., 1997). Thionins are cystein-rich low molecular weight proteins (about 5 kDa) and have been identified in various organs of a number of plant species. They show antimicrobial activity when tested *in vitro* against various bacteria and fungi (Florack and Stiekema, 1994). The antimicrobial action is based on the ability of thionins to form pores in cell membrane resulting in membrane disruption and cell death. Expression of thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens (Carmona *et al*., 1993). Epple *et al*. (1997) observed that constitutive over expression of thionin in transgenic *Arabidopsis* resulted in enhanced resistance against *F. oxysporum f.* sp.

matthiolae. The expression of thionin, viscotoxin A3, in transgenic *Arabidopsis* showed increased resistance to infection of clubroot pathogen *Plasmodiophora brassicae* (Holtorf *et al*., 1998). Unfortunately, most thionins can be toxic to animal and plant cells and thus may not be ideal for developing transgenic plants (Reimann-Philipp *et al*., 1989).

There are a number of known plant defensins, which are known to protect against plant pathogens. The radish defensin Rs-AFP1 and Rs-AFP2, have been shown to inhibit the growth of several pathogenic fungi *in vitro* (Terras *et al*., 1992). The expression of Rs-AFP2 in transgenic tobacco confers partial resistance to attack by *Alternaria longipes* (Terras *et al*., 1995). Two homologous peptides, Rs-AFP3 and Rs-AFP4, are also induced in radish leaves upon infection by *A. longipes*, thus further substantiating the role of defensins in plant defense. Two sugar beet leaf defensins, AX1 and AX2, homologs of the radish AFP2, have been isolated after infection with the fungal pathogen *Cercospora beticola* (Kragh *et al*., 1995). The preliminary results indicate that the expression of these peptides in transgenic corn plants imparts significant resistance to Northern corn leaf blight caused by the fungal pathogen *Exserohilium turcicum*. A defensin from alfalfa seeds alfAFP (alfalfa antifungal peptide) when expressed under the control of 35S promoter in transgenic potato imparted resistance to *Verticillium dahliae*, *Alternaria solani*, and *Fusarium culmorum* but not to *Phytophthora infestans* (Gao *et al*., 2000). It has shown resistance to fungal diseases in potato in the greenhouse as well as in the field. Kanzaki *et al*. (2002) reported the overexpression of the WTI defensin from wasabi (*Wasabia japonica*) conferring enhanced resistance to blast fungus in transgenic rice.

Several defensins isolated from radish and dahlia have been found toxic to *M. fijiensis* and *F. oxysporum f.* sp. *cubense,* two major fungal pathogens of *Musa* (Cammue *et al*., 1993). They are nontoxic to human or banana cells. Large number of transgenic lines of *Musa* especially plantains expressing defensins have been developed at KULeuven (Remy, 2000). Many hundreds of transformed lines have been generated and screened under greenhouse conditions in Belgium for disease resistance and the most promising lines of transgenic bananas and plantains are currently being evaluated in the greenhouse and field in Cuba and Costa Rica.

Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Pathosystem-specific plant resistance (R) genes have been cloned from several plant species against many different pathogens (Bent, 1996). These include R genes that mediate resistance to bacterial, fungal, viral, and nematode pathogens. Many of these R gene products share structural motifs, which indicate that disease resistance to diverse pathogens may operate through similar pathways. In tomato (*Lycopersicon esculentum*), the R gene *Pto* confers resistance against strains of *P. syringae* pv. *tomato* (Martin *et al*., 1993; Kim *et al*., 2002). *Pto*-overexpressing plants show resistance not only to *P. syringae* pv. *tomato* but also to *X. campestris* pv. *vesicatoria* and to the fungal pathogen *Cladosporium fulvum* (Mysore *et al*., 2003). Similarly, the *Arabidopsis RPS4* gene specifies disease resistance to *P. syringae* pv. *tomato* expressing *avrRps4* (Gassmann *et al*., 1999). The *Bs2* resistance gene of pepper specifically recognizes and confers resistance to strains of *X. campestris* pv. v*esicatoria* (Tai *et al*., 1999). Transgenic tomato plants expressing the pepper *Bs2* gene suppress the growth of *Xcv.*

The *Xa*21 gene isolated from rice has been shown to confer resistance against many isolates of *Xanthomonas oryzae* pv. *oryzae* (Song *et al*., 1995; Wang *et al*., 1996). Transgenic plants expressing *Xa*21 under the control of the native promoter of the genomic fragment of the *Xa*21 gene showed enhanced resistance to bacterial leaf blight caused by most *Xoo* races. The *Xa*1 gene also isolated from rice confers resistance to Japanese race 1 of *X. oryzae* pv. *oryzae*, the causal pathogen of bacterial blight (Yoshimura *et al*., 1998).

Successful transfer of resistance genes to heterogous plant species gives another new option to develop disease resistant plants. R gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently halt pathogen growth with minimal collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, R genes are often quickly

defeated by co-evolving pathogens (Pink, 2002). Many R genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. Also efforts to transfer R genes from model species to crops, or between distantly related crops, could be hampered due to restricted taxonomic functionality.

Plants also employ a wide array of defense mechanisms against pathogen attack. Among those, hypersensitive response (HR) is an induced resistance mechanism, characterized by rapid, localized cell death upon their encounter with a microbial pathogen. Several defense genes have been shown to delay the HR induced by bacterial pathogen in nonhost plants through the release of the proteinaceous elicitor. Elicitor-induced resistance is not specific against particular pathogens. Hence, manipulation of such defense genes may be more ideal.

The ferredoxinlike amphipathic protein (*pflp*, formerly called AP1) and HR-assisting protein (*hrap*), isolated from the sweet pepper, *Capsicum annuum,* are novel plant proteins that can intensify the harpinPSS-mediated HR (Chen *et al*., 2000). These proteins have dual function: iron depletion antibiotic action and harpin triggered HR enhancing. The transgenes have been shown to delay the HR induced by various pathogens like *Erwinia, Pseudomonas, Ralstonia*, and *Xanthomonas* sp. in nonhost plants through the release of the proteinaceous elicitor, harpinPss in various crops including dicots like tobacco, potato, tomato, broccoli, orchids, and monocots like rice (Tang *et al*., 2001; Lu *et al*., 2003; Huang *et al*., 2004). Also elicitor-induced resistance is not specific against particular pathogens, so it could be very useful strategy (Wei and Beer, 1996). The research is in progress at IITA for developing bacterial wilt (caused by *X. campestris* pv. *musacearum*) resistant banana varieties using *pflp* or *hrap* genes.

A series of resistance gene analogs have been isolated from banana, using degenerate polymerase chain reaction (PCR) primers targeting highly conserved regions in proven plant resistance genes (e.g., kinase or transmembrane-encoding domains, or leucine-rich repeat sequences). Plant disease resistance genes involved in signal transduction contain domains that are conserved throughout monocotyledons and dicotyledons. Primers have been designed to those domains in the *RPS2* gene of *Arabidopsis* and the *N* gene of tobacco. Using these primers for PCR, candidate resistance genes have already been cloned from soybean, potato, rice, barley, and *Arabidopsis*. A similar strategy has been applied to clone candidate resistance genes from banana (Wiame *et al*., 2000). A series of disease resistant genes were isolated from the somaclonal mutant CIEN-BTA-03 (resistant to both *M. fijiensis* and *M. musicola*) and the parent "Williams" that fall into two classes: nucleotide-binding site-leucine-rich repeat-containing kinases, and serine-threonine protein kinases of the *pto* type (Kahl, 2004). All the resistance genes were fully sequenced, and eight of them are also transcribed in the mutant, its parental genotype, "Pisang Mas" and a tetraploid *M. acuminata*. The researchers at Queensland University of Technology (QUT)have isolated the complete gene sequence of R gene candidate (*RGC-2*) from *M. acuminata* ssp. *malaccensis,* a wild diploid banana segregating for resistance to *F. oxysporum* f. sp. *cubense* (FOC) race 4. The development of *Fusarium* wilt resistant transgenic banana using this gene is in progress (Dale *et al*., 2004).

2.1.1.3 Virus resistance

The most promising transgenic strategies for the ssDNA viruses like banana bunchy top virus (BBTV) is expressing a defective gene that encodes an essential virus life cycle activity, for instance, the replication of the virus is encoded in the replication gene or genes (*Rep*). Resultant Rep protein may retain the ability to bind to its target viral DNA but lack the functions of the *Rep* (Brunetti *et al*., 2001). The defective Rep protein binds to the invading viral DNA and is thought to outcompete the native viral Rep protein, thus reducing or eliminating virus DNA replication. Lucioli *et al*. (2003) expressed the first 630 nucleotides of the *Rep* gene of tomato yellow leaf curl Sardinia virus (Acronymn) to generate resistance. The duration of the resistance was related to the ability of the invading virus to switch off transgene expression through post-transcriptional gene silencing (PTGS). Many researchers are trying to develop transgenic plants of *Musa* resistant to BBTV targeting the PTGS mechanism using mutated or antisense *Rep* genes.

Unfortunately, there appear to be no strategies that have been developed that generate high-level

resistance to the plant dsDNA or pararetroviruses, including the badnaviruses. Researchers at IITA, Nigeria in collaboration with the John Innes Centre (JIC), UK, are attempting to generate transgenic resistance to banana streak virus (BSV) based upon PTGS. The construct used for transformation is pC-BB with a BSV sequence from the viral reverse transcriptase-RNaseH domain under the regulation of CaMV 35S promoter. This approach involves the specific silencing of a viral gene or genes known to be involved with replication or pathogenesis.

2.1.1.4 Edible vaccine

Researchers are working to develop a banana that is an "edible vaccine" to fend off hepatitis, one of the world's most widespread and devastating diseases. Charles Arntzen's group at Boyce Thompson Institute for Plant Research, USA had successfully developed tobacco plants producing a vaccine against hepatitis B. It was found that the vaccine produced in plants is similar in form and function to that from human serum or recombinant yeast and provoked a strong immune response when injected into mice, while B and T-cell epitopes were preserved. Successful expression of antigens in plants was also achieved for Rabies virus G-protein in tomato (McGarvey *et al*., 1995), Norwalk virus capsid protein in tobacco and potato (Mason *et al*., 1996), Hepatitis B virus surface antigen in tobacco and potato (Thanavala *et al*., 1995), *Escherichia coli* heat-labile enterotoxin B subunit (LT-B) in tobacco and potato (Hirst and Holmgren, 1987), Cholera toxin B subunit (CT-B) in potato (Arakawa, 1997). Foods under study include potatoes, bananas, lettuce, rice, wheat, soybean, corn, and legumes. Banana is a good candidate for edible vaccines since they were eaten raw, appealing to children, inexpensive to produce, native to many developing countries (Hassler, 1995). But the only limitation is the time from transformation to evaluation of fruit is 2 years or more.

Embryogenic cells of banana cv. Rasthali (AAB) have been transformed with the "s" gene of hepatitis B surface antigen (HBsAg, Kumar *et al*., 2005). Higher monoclonal antibody binding of 67.87% of the antigen was observed when it was expressed with a C-terminal ER retention signal. HBsAg obtained from transgenic banana plants is similar to human serum derived one in buoyant density properties. The transgenic plants were grown up to maturity in the green house and the expression of HBsAg in the fruits was confirmed by RT-PCR. These transgenic plants were multiplied under *in vitro* using floral apex cultures. Attempts were also made to enhance the expression of HBsAg in the leaves of transgenic banana plants by wounding and/or treatment with plant growth regulators. This is the only report on the expression of HBsAg in transgenic banana fruits.

2.1.2 Promoters

Plant genetic transformation is a potential tool in different areas such as manipulation and understanding of biochemical processes, knowledge of genome regulation, and integration of genes, which cannot be manipulated by classical breeding. In order to apply genetic engineering to both crop improvement and basic research, the availability of a variety of promoters that confer constitutive, i.e., expression in all cell types, tissue-specific, or developmental-specific transgene expression is required. Often a strong constitutive promoter is required to ensure sufficient expression of the transgene throughout the plant. Several such promoters have been isolated that direct strong constitutive expression in monocot plants. These include the maize ubiquitin promoter, the rice actin1 promoter, various enhanced CaMV 35S, and the synthetic pEmu promoter (Kay *et al*., 1987; McElroy *et al*., 1990; Christensen and Quail, 1996; Mitsuhara *et al*., 1996). However, the promoter activity can vary in different plant species and not all of these promoters can be used for high-level expression in all monocot species. Variation in transgene expression levels between different species and promoters may be due to different abundance of transcription factors, recognition of promoter sequences or intron splicing sites, or other factors (Wilmink *et al*., 1995).

Most commonly used constitutive promoter is the CaMV 35S, a promoter active in most stages of development and in most plant tissues. However, it has been widely used for high-level constitutive expression in dicots, but confers lower levels of expression in monocots. Promoters isolated from monocots generally show higher activity

in monocots, and adding an intron between the promoter and the reporter gene can increase transcription levels (Rathus *et al*., 1993). The rice actin promoter *Act1* (McElroy *et al*., 1990) and the maize ubiquitin promoter *Ubi* (Christensen and Quail, 1996) achieved far better expression than the CaMV 35S in most monocots tested.

Effective applications of genetic engineering, however, are frequently likely to be dependent on the use of promoters specific to particular tissues or developmental events. It is desirable, for example, for nematode resistance to be expressed only after nematodes begin to feed and in the roots requiring the splicing to the resistance gene of a combination of suitable promoters. The approach for finding such promoters is to identify any gene expression with the required tissue or developmental specificity, and then to isolate the regulatory elements and test for specificity.

Currently, only a few promoters are widely used for plant transformation. Promoters can have different expression patterns even in closely related species; therefore information gathered from other transformation experiments may not be completely representative of the expression pattern of that specific promoter in banana. Therefore, the different promoters will have to be tested in banana in order to identify promoters that will be suitable for use in different transformation projects.

The production of transgenic bananas harboring genes encoding an improved genotype requires strong promoters for both constitutive and regulated high-level expression of the transgenes. Generally speaking, not enough promoters are available, though several groups are actively working to isolate useful promoter sequences, or at least fragments with promoter activity. Commonly used promoters in banana transformation are rice actin, CaMV 35S, and maize ubiquitin (Table 1). A number of monocot active promoters have been isolated from viruses infecting both dicot and monocot plants and are potential promoters for banana transformation. A promoter was isolated from sugarcane bacilliform badnavirus and showed expression in constitutive manner in banana and tobacco (Schenk *et al*., 1999). This promoter represents a useful tool for the high-level expression of foreign genes in both monocot and dicot transgenic plants that could be used similarly to the CaMV 35S or maize ubiquitin promoter. Promoters from BSV were proved to drive high expression of *gus*A (β-glucuronidase) reporter genes in transgenic monocot (banana, barley, maize, millet, sorghum) and dicot plants (canola, sunflower, tobacco), and also in *Pinus radiata* and *Nephrolepis cordifolia* (Schenk *et al*., 2001). In greenhouse experiments with banana transgenics, the BSV promoter was found to be superior to the widely used maize ubiquitin and CaMV 35S promoters. Promoter regions of BBTV have been characterized. DNA segments incorporating the intergenic regions of BBTV DNA components S1 and S2 were isolated and fused to the *gus*A reporter gene to assess promoter activity in tobacco and banana (Hermann *et al*., 2001). BBTV S1 and S2 promoters supported vascular tissue-associated reporter gene expression, and can certainly be exploited as tissue-specific promoters.

Up to date, the only constitutive heterologous promoters (the maize ubiquitin 1 and the rice actin 1 promoters) or viral promoters (derived from the BSV and BBTV) have been used for the production of transgenic banana plants (Table 1). For a number of future applications, transgenes will have to be expressed differentially or under specific abiotic (e.g., salt, wounding) and biotic stress conditions, which requires the use of a set of specific promoters to drive regulated gene expression. However, relatively few promoters are currently available for a specific or fine regulation of gene expression. It is expected that for these purposes homologous promoters will be more functional than heterologous ones, which should also raise less biosafety concerns. Many promoters have been identified directly from the banana genome by using a T-DNA tagging method with a promoterless codon-optimized luciferase (*luc*) gene (Remy *et al*., 2005). A series of promoters has also been isolated from genes induced after challenge with a fungus. Isolation of promoters for banana transgenics, using modern technique like TAIL-PCR or other walking techniques is routine these days (Terauchi and Kahl, 2000). Commonly used terminators for banana transformation are the nopaline synthesis (NOS) and CaMV 35S.

2.1.3 Selection marker and reporter genes

Plant transformation is based on the delivery, integration, and expression of defined genes into plant cells, which can be grown to generate

Table 1 Genetic transformation of banana

Explant	Method	Constructs	Transgene	Selection marker	Reporter gene	Promoter	References
AM[d]	Agro[b] LBA4404	pBI141		*npt*II[l]	*gus*A[h]	Act1[a]	May *et al.* (1995)
ECS[g]	MPB[j]	pWRG1515		*hpt*[i]	*gus*A[h]	CaMV 35S	Sagi *et al*. (1995)
ECS[g]	MPB[j] Co-	pUbi-BTintORF1[o] pBT6.3-Ubi-NPT[o]	BBTV[e]	*npt*II[l]		Ubi[m] BBTV[e]	Becker *et al*. (2000)
ECS[g]	MPB	pUbi-BTintORF5[o] pBT6.3-Ubi-NPT[o]	BBTV[e]	*npt*II[l]		Ubi[m] BBTV[e]	Becker *et al*. (2000)
ECS[g]	MPB[j]	pUGR73[o] pDHKan[o]		*npt*II[l]	*gus*A[h]	Ubi[m] CaMV 35S	Becker *et al*. (2000)
ECS[g]	Agro[b] EHA105	pGVSUN		*als*[c]	*gus*A[h]	Gelvin	Ganapathi *et al*. (2001)
ECS[g]	Agro[b] LBA4404 & AGL1	pCAMBIA1305.1		*hpt*[i]	*gus*A[h]	CaMV 35S	Khanna *et al*. (2004)
ECS[g]	Agro[b] LBA4404 & AGL1	pART-Test7		*npt*II[l]	m*gfp*	CaMV 35S	Khanna *et al*. (2004)
ECS[g]	Agro[b] EHA105	pSAN164, 168	MSI-99[k]	*npt*II[l]		Ubq3[n]	Chakrabarti *et al*. (2003)
AM[d]	Agro[b] EHA105	pCAMBIA1201		*hpt*[i]	*gus*A[h]	CaMV 35S	Tripathi *et al*. (2005a)
AM[d]	Agro[b] EHA105	pC-BB	BSV[f]	*hpt*[i]	*gus*A[h]	CaMV 35S	Author's lab
AM[d]	Agro[b] EHA105	pSPFLP	*pflp*	*hpt*[i]	*gus*A[h]	CaMV 35S	Author's lab

[a] Rice actin promoter
[b] *Agrobacterium*
[c] Confers resistance to sulfonylurea herbicide
[d] Apical meristem
[e] Banana bunchy top virus
[f] Banana streak virus
[g] Embryogenic cell suspension
[h] β-glucuronidase
[i] Hygromycin phosphotransferase
[j] Microprojectile bombardment
[k] Magainin analog
[l] Neomycin phosphotransferase II
[m] Maize ubiquitin promoter
[n] Promoter from *Arabidopsis*
[o] Plants were co-transformed with either a BBTV-derived construct or *GUS* reporter gene and a selectable marker constructs

transformed plants. Efficiency of stable gene transfer is not high even in the most successful transfer systems and only a fraction of the cells exposed integrate the DNA construct into their genomes. Moreover, a successful gene transfer does not guarantee expression, even by using signals for the regulation of transgene expression. Therefore, systems to select the transformed cells, tissues, or organisms from the nontransformed ones are indispensable to regenerate the truly genetically transformed organisms.

The use of genes encoding for antibiotic or herbicide resistance has assisted the selection of transformants in the background of untransformed ones. The neomycin phosphotransferase II (*npt*II) gene that confers resistance to aminoglycoside antibiotics such as kanamycin and its analogs paromomycin and geneticin, and hygromycin

phosphotransferase (*hpt*) gene, which confers resistance to hygromycin have been successfully used as selectable marker in transformation of a variety of crop plants, including dicots and monocots. Similarly the *bar* (bialaphos resistance) and *pat* (phosphinothricin acetyltransferase) genes that confer resistance to the herbicide BASTA® and phosphinothricin respectively, have also provided an efficient selection of transgenics in a number of crops.

Herbicide or antibiotic assays, which kill or suppress nontransformed cells, while allowing proliferation of transformed tissues, are attractive, but sensitivity of tissues to the selecting agent is critical. A high incidence of untransformed "escapes" has been demonstrated in some studies; therefore, better selection markers are needed.

The *ipt* gene encodes the enzyme isopentenyl transferase and is located on Ti-plasmids of *A. tumefaciens*. This enzyme catalyzes the condensation of isopentenyl pyrophosphate with AMP to produce isopentenyl AMP, a precursor of several cytokinins (Akiyoshi *et al*., 1984). Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants from cultured cells after transformation. When a chimeric *ipt* gene under the control of the CaMV 35S promoter was introduced into cells of potato, cucumber, and several *Nicotiana* species, transgenic cells proliferated and adventitious shoots differentiated in hormone-free medium (Smigocki and Owens, 1988). These transgenic plants exhibited loss of apical dominance. Therefore, it is easy to detect visually transgenic plants that carry a functional *ipt* gene. Chimeric *ipt* genes are not commonly used as selectable markers because the resulting transgenic plants lose apical dominance and are unable to root due to overproduction of cytokinins. Ebinuma *et al*. (1997) developed the multi-auto-transformation (MAT) vector system, in which the selectable marker is composed of a chimeric *ipt* gene inserted into the maize transposable element *Ac* to overcome some of the difficulties of the current transformation methods. It has been demonstrated that marker-free transgenic plants can be visually selected by using the chimeric *ipt* gene as a marker gene in tobacco plants and hybrid aspen. This method could be particularly valuable for fruit like banana and forest trees, for which long generation times are a more significant barrier to breeding and genetic analysis.

There are several ways of determining where and when a particular gene is expressed in a plant. Most commonly used approach is the use of a reporter gene. A reporter gene produces a protein that is easily detectable in transformed organisms. Often, the protein possesses an enzymatic activity that can turn a colorless substrate into a colored product. Thus, one can see the location and amount of gene expression in a transformed organism by looking at the location and intensity of the colored product. Theβ-galactosidase (*lac*Z) and β-glucuronidase (*gus*A) genes are two examples of these reporter genes. When the reporter gene is fused to the promoter of the gene of interest, the reporter gene will be expressed only at the times and locations where the gene is expressed. This provides a method to detect a very limited expression of a gene, such as in small patches of cells (like root tips or pollen) or at certain times (such as after a certain stress or hormone treatment).

The green fluorescent protein (*gfp*) gene is an ideal selectable marker and reporter for gene expression analysis and plant transformation. The *gfp* gene, isolated from jellyfish, *Aequorea victoria*, encodes a small, barrel-shaped protein surrounding a fluorescent chromophore, which immediately emits green fluorescent light in the blue to ultraviolet range. Visual detection is possible at any time in living cells without their destruction and without the addition of any cofactor or external substrate. In addition, *gfp* gene product does not adversely affect cell growth, regeneration, and fertility of transformed plants. The availability of mutant forms of *gfp* differing in solubilities and emission spectra make it possible to simultaneously monitor multiple transformation events within an individual transformant (Stuber *et al*., 1998). Green fluorescent protein (GFP) has been of great use when the organogenesis or conversion segments of transformation are inefficient under antibiotic and herbicide selection. In the beginning, GFP was used in conjunction with antibiotics and herbicides and has been shown to decrease the number of escapes for a number of tree species (Tian *et al*., 1997). Recently, *gfp* has been used as a sole visual selectable marker in genetic transformation

of monocots like barley (Ahlandsberg *et al*., 1999), oat (Kaeppler *et al*., 2000), and rice (Vain *et al*., 1998b). GFP selection system seems to be promising for tissue culture/transformation systems that are inefficient, for recalcitrant genotypes, and for plant species for which no system exists. Most commonly used reporter genes in banana transformation are *gus*A and *gfp* under the regulation of constitutive promoters Act1, CaMV 35S, and Ubi (Table 1).

2.2 Transformation Methods Employed

Genetic transformation offers an attractive means for introduction of agronomically important genes into banana cultivars. Some success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells. Gene insertion in plants can be achieved by direct gene transfer through particle bombardment or through biological vectors like a disarmed Ti (tumor inducing)-plasmid of *A. tumefaciens*. Genetic transformation using microprojectile bombardment of embryogenic cell suspension (ECS) is now routine (Sagi *et al*., 1995; Becker *et al*., 2000). An efficient method for direct gene transfer via particle bombardment of ECS has been reported in cooking banana cultivar Bluggoe and plantain Three Hand Plant (Sagi *et al*., 1995). While Becker *et al*. (2000) reported the genetic transformation of Cavendish banana cv. Grand Nain.

Agrobacterium-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc.), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Shibata and Liu, 2000). Therefore, transformation of plants by *Agrobacterium*-mediated DNA transfer is currently the most commonly used phenomenon in accomplishing plant gene transfer (Lindsey, 1992). For quite some time banana was generally regarded as recalcitrant for *Agrobacterium*-mediated transformation. However, Hernandez *et al*. (1999)has reported that *A. tumefaciens* is compatible with banana indicating the potential for genetic transformation. The recovery of transgenic plants of banana obtained by means of *Agrobacterium*-mediated transformation has been reported. The protocol has been developed for *Agrobacterium*-mediated transformation of ECS of the banana cultivars Rasthali, Cavendish, and Lady finger (Ganapathi *et al*., 2001; Khanna *et al*., 2004). Ganapathi *et al*. (2001) reported *Agrobacterium*-mediated transformation of shoot apex derived ECS of cultivar Rasthali (AAB) recording production of up to 40 plants per 0.5 ml packed cell volume in this cultivar. Banana functional genomics and plant improvement initiatives demand higher transformation frequencies and a standard protocol that can be used to transform all banana genomic groups including the high demand cultivars of AAA group, e.g., Cavendish. Khanna *et al*. (2004) described centrifugation-assisted *Agrobacterium*-mediated transformation protocol developed using banana cultivars from two economically important genomic groups (AAA and AAB) of cultivated *Musa.* This protocol resulted in 25–65 plants per 50 mg of settled cell volume of embryogenic suspension cells, depending upon the *Agrobacterium* strain used, and gave rise to hundreds of morphologically normal, transgenic plants in two banana cultivars from the two genomic groups.

At present most of the transformation protocols use cell suspension, however establishing cell suspension is a lengthy process and cultivar dependent. The protocol has also been established using shoot tips from various cultivars of *Musa* (May *et al*., 1995; Tripathi *et al*., 2005a). This technique is applicable to a wide range of *Musa* cultivars irrespective of ploidy or genotype (Tripathi *et al*., 2003, 2005a). This process does not incorporate steps using disorganized cell cultures but uses micropropagation, which has the important advantage that it allows regeneration of homogeneous populations of plants in a short period of time. This procedure offers several potential advantages over the use of ECS as it allows for rapid transformation of *Musa* species and meristematic tissues have potential to regenerate plants from many different cultivars, unlike somatic embryogenesis, which is restricted to only a few cultivars. The transformation of meristematic cells may result in chimeric plants when only one or a few cells receive T-DNA. To

obtain uniformly transformed plants, two steps of selection and regeneration were performed to avoid regeneration of any nontransformed cells. The transformation of East African Highland bananas using meristematic tissues has also been established (Tripathi *et al*., 2008).

Development of a highly efficient *Agrobacterium*-mediated transformation protocol for a recalcitrant species like banana requires the identification and optimization of the factors affecting T-DNA delivery and subsequent plant regeneration. Preinduction of *Agrobacterium* with acetosyringone (Khanna *et al*., 2004; Tripathi *et al*., 2005a) and use of surfactants like Pluronic F68 (Cheng *et al*., 1997) have been reported to have a positive effect on *Agrobacterium*-mediated transformation. Hernandez *et al*. (1999) reported chemotactic movement and attachment of *A. tumefaciens* to wounded tissues of banana cultivars belonging to different genomic groups. Microprojectile bombardment is reported to be an effective method of wounding tissues to promote *Agrobacterium*-mediated transformation (Bidney *et al*., 1992). We observed a significant increase in transformation efficiency measured as transient expression of reporter gene, in explants microwounded by microprojectile bombardment with naked gold particle prior to co-cultivation with *A. tumefaciens*. This improvement in transformation efficiency can be attributed to the initiation of active cell division upon wounding (Sangwan *et al*., 1992), the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns, 1991), and the production of *vir*-inducing compounds by the metabolically active cells (Stachel *et al*., 1985).

Differences are known to exist between infection efficiencies of different Ti plasmid vectors and virulence of *Agrobacterium* strains. To date, the published reports of banana transformation via *Agrobacterium* described only three different strains, i.e., LBA4404, disarmed C58, and EHA101; and their derivatives (EHA105 from EHA101, AGL0 and AGL1 from EHA101) as mentioned in Table 1. *Agrobacterium* supervirulant strains EHA105 and AGL1 derivative of EHA101 were found to be better in comparison to the normal strain LBA4404 (Khanna *et al*., 2004; Tripathi *et al*., 2005a). Antibiotics such as cefotaxime, carbenicillin, and timentin have been used regularly in *Agrobacterium*-mediated transformation of banana following coculture to suppress or eliminate *Agrobacterium.*

2.3 Selection of Transformed Tissue

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, which are otherwise toxic to plant cells. An effective selection process is needed to increase the efficiency of transformant recovery. For banana transformation, antibiotic resistance genes are widely used to enable this selection.

The most widely used selectable markers in monocot transformation are the genes encoding *hpt*, phosphinothricin acetyltransferase (*pat* or *bar*), and *npt*II. Use of these marker genes under the control of constitutive promoters such as the 35S promoter from CaMV, or the ubiquitin promoter from maize, works as efficiently for selection of *Agrobacterium*-transformed cells as for biolistic-mediated transformation. For banana, the *npt*II gene under the control of the NOS or CaMV 35S promoter has been used to successfully select stable transformants with kanamycin, similar to many standard dicot transformation protocols (Table 1). Kanamycin and hygromycin proved to be equally effective as selection agents (Khanna *et al*., 2004).

Matsumoto *et al*. (2002) reported the use of *ahas* (acetohydroxyacid synthase) gene as a new selectable marker for banana transformation. AHAS is the target enzyme for AHAS-inhibiting herbicides such as imidazolinones and sulfonylureas. Embryogenic cells of "Maçã" banana (*Musa* sp. AAB group, Silk) were bombarded with a plasmid vector containing the *ahas* gene, under control of the *ahas* promoter from *Arabidopsis*. The bombarded cells were regenerated on selected medium containing Imazapyr herbicide.

Dominant genes encoding either antibiotic or herbicide resistance are widely used as selectable markers in plant transformation (Yoder and Goldsbrough, 1994). The antibiotics and herbicides that select rare transgenic cells from nontransgenic cells generally have negative effects

on proliferation and differentiation. These agents may retard differentiation of adventitious shoots during the transformation process. Some plant species are insensitive to or tolerant of the selective agents and, therefore, it is difficult to separate the transformed and untransformed cells or tissues. Therefore, it is difficult to find appropriate selectable markers and to establish optimal conditions for transformation of such difficult species. Selectable marker genes remain in transgenic plants, and their gene products need to be assessed for safety and environmental impact (Flavell *et al*., 1992).

It is difficult to introduce a second gene of interest into a transgenic plant that already contains a resistance gene as a selectable marker. There are a large number of desirable traits and genes worth incorporating into plants, but only a limited number of selectable marker genes are available for practical use. The problem becomes even more difficult if one wants to introduce a number of genes, and it is impossible to introduce them simultaneously (Goldsbrough, 1992; Yoder and Goldsbrough, 1994). It is desirable, therefore, to develop a system for the removal of selectable marker genes to produce environmentally safe transgenic plants and pyramid a number of transgenes by repeated transformation.

The positive selectable marker phosphomannose isomerase was first used for *Agrobacterium*-mediated transformation of dicot plants, i.e., sugarbeet (Joersbo *et al*., 1998), and it has now been shown to be effective in transformation of monocots, such as rice and maize as well (Negrotto *et al*., 2000; Lucca *et al*., 2001). This can be a potential selection marker for banana transformation also.

2.4 Regeneration of Whole Plant

The introduction of transgene into a desired plant species for the development of stable transgenic plants requires an efficient regeneration system amenable to genetic transformation and stability of transgenes under field conditions. *In vitro* regeneration is the technique of growing plant cells, tissues, or organs isolated from a source plant. It has been found that plants can reproduce whole plants from fragments of plant material when given a nutrient media capable of supporting growth and appropriate hormone control under laboratory condition. With the advent of gene insertion, plant cells with gene material inserted can be regenerated using *in vitro* culture to produce a whole plant. Plant regeneration of banana has been reported from various explant sources and from a variety of cultivars. Depending on different explants in combinations with various growth regulators, regeneration occurs via micropropagation or ECS.

2.4.1 Micropropagation

Micropropagation is the process by which explants having the pre-existing meristems lead to the development of multiple shoots. This is by far the most routinely applied technique used in plant tissue culture. Multiplication rate is several orders of magnitude larger than in the field. Indeed, while *in situ* a banana plant will produce about 10 shoots after 1 year, a shoot meristem *in vitro* can produce about 125–144 shoots within 8 weeks. Through repeated subculturing of proliferating shoots, an open-ended system can be maintained (Tripathi *et al*., 2003). Propagation from existing meristems yields plants that are genetically identical to the donor plants. The shoot tip culture technique offers several additional advantages like clean planting material and *in vitro* preservation and exchange of banana germplasm.

The micropropagation technique for cultivated *Musa* is now well established (Cronauer and Krikorian, 1984; Banerjee and De Langhe, 1985). Shoot tip culture is easy and applicable to a wide range of *Musa* genotypes. An efficient regeneration protocol, which seems to be independent of ploidy level and genomic background, was developed for *Musa* species using apical meristems (Tripathi *et al*., 2003). The selected species represent major groups of *Musa* including fertile diploid bananas (AA and BB genomes), the sterile triploid plantains (AAB), Cavendish bananas (AAA), and tetraploid hybrids (AAAA and AAAB).

Micropropagation has played a key role in banana improvement programs worldwide. Planting material derived from micropropagation performs equal to or superior to conventional material. Micropropagated plants establish faster, grow more vigorously, are taller, have a shorter and more uniform production cycle, and yield higher than conventional propagules.

2.4.2 Embryogenic cell suspension

Somatic embryogenesis is a process where a group of somatic cells/tissues leads to the formation of somatic embryos that resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium. ECS appear, as is the case with most monocotyledons, to be the material of choice for nonconventional *Musa* breeding.

Recently, much progress has been made in the establishment of embryogenic cell culture from banana explant sources and from a variety of cultivars. Novak *et al*. (1989) established embryogenic suspensions from somatic tissues such as leaf sheaths and corm sections of Grand Nain cultivar. For the cultivar Bluggoe, Dheda *et al*. (1991) cultured their sections from highly proliferated shoot tip cultures to produce ECS cultures. Embryogenic suspensions have also been established from immature zygotic embryos (Escalant and Teisson, 1989; Marroquin *et al*., 1993). However, male flowers are the most responsive starting material for initiating embryogenic cultures of cv. Grand Nain (Escalant *et al*., 1994; Cote *et al*., 1996; Navarro *et al*., 1997; Sagi *et al*., 1998) and cultivar Rasthali (Ganapathi *et al*., 2001). These suspension cultures can be regenerated into plantlets through somatic embryogenesis at high frequencies and grown in the field (Novak *et al*., 1989; Dheda *et al*., 1991).

Suspension culture promises a tool for faster multiplication rate than shoot tip cultures. Also, it allows for a quick response to demands and offers possibilities for automated multiplication. ECS cultures have successfully been used for cryopreservation (Panis *et al*., 1996). The conservation of banana germplasm under cryopreserved conditions is an attractive alternative to the conservation of actively growing meristem tips. Plant regeneration from cell suspension cultures was investigated for its potential in mass propagation and as a tool in transformation using recombinant DNA technology. However, most of the procedures of developing ECS are still laborious, time consuming, and genotype dependent.

2.5 Testing

The proof of stable genetic transformation is the determination of integration of the foreign DNA into the chromosome of the recipient plants. In early developing stages of a transformation system qualitative expression assays of the β-glucoronidase (GUS) or the GFP, encoded by the *gus*A and *gfp* reporter-genes, respectively, have been successfully used to monitor transgene delivery into banana tissues. The resulting banana transgenic plants are carefully evaluated for transgene integration, expression, and stability of transgene expression. A molecular and genetical screening is necessary to identify individual transformants with desired characteristics, such as single inserts, desired expression level, and single locus integration, etc.

Two molecular techniques, PCR and Southern blot analysis, are widely used to evaluate banana transgenic plants for transgene integration and integration patterns. Further, the expression of a transgene is evaluated at the RNA, protein, and bioassay levels. The presence of transgenic RNA demonstrates that the transgene is actively transcribed. Translation of transgene into the encoded protein can be demonstrated with Western blot or enzyme-linked immunosorbent assay (ELISA). Several bioassays have been developed for transgene expression for disease resistance in banana for example, leaf bioassay for testing gene expression for resistance to banana *Xanthomonas* wilt.

The transgenic plants are further tested for the desired agronomic performance in confined field trail. Rahan Meristem Ltd, recently conducted field trials of transgenic bananas growing plants in an area heavily infested with nematodes and the plants showed complete resistance (FreshPlaza, 2006). The nematodes could not reproduce on the transgenic banana plants developed by use of a special technology called RNAi.

During the last 10 years, after the public sector had developed the transformation technology, large-scale commercial producers of banana invested considerable additional resources in the research for transgenic solutions, especially to the problems of black Sigatoka disease and nematodes. Considerable progress has been made but failed to reach the stage of deploying commercially useful varieties. This effort has left a legacy of relevant technologies but the use of many of them, at least beyond the stage of experimentation, is severely limited by intellectual property constraints, many of which

remain unsolved in the absence of a clear policy on the use of transgenic banana by the private sector.

In the absence of commercial transgenic banana varieties, there has not been the level of private sector investment in biosafety testing. However, with the entry of countries such as Brazil and South Africa, which already have substantial areas of transgenic crops, into the field of genetic modification of *Musa*, more rapid progress is now expected. Meanwhile a number of developing countries are putting in place the necessary legal framework for testing and dissemination of GM crops, either specifically in order to facilitate the development of transgenic bananas, as in the case of Uganda, or in order to benefit from a range of transgenic crops that may in due course include banana and plantain.

2.6 Regulatory Measures Adopted

Regulations governing genetically modified organisms (GMOs) can potentially act at a number of key stages. Development of GM variety is a long process. Development of GM banana is in its inititial stages in the laboratory, where the GMOs are produced and presence of the transgene is confirmed etc., and proceeds to field testing of the organisms produced to ensure they have the desired characteristics. Regulations at this stage may cover the conditions under which laboratory experiments take place; exchange of GM material between laboratories and conditions for testing GMOs in greenhouses, other contained facilities, or in the field. After the Research and Development stage, there may be interest in bringing the GM product to the market. Regulations here may cover assessment of the potential human health and environmental risks, to be carried out prior to eventual approval. If approval is granted, the next stage is the commercial release of the GM banana. Till date, there is no commercial release of transgenic banana and plantain.

Banana and plantain are important crops in most tropical and subtropical regions of the world. Many developing countries are beginning to develop regulations related to genetically engineered products (James, 1998). Furthermore, operational field-testing regulations have been implemented in, for example, Argentina, Brazil, Mexico, Chile, Costa Rica, Cuba, India, Philippines, and Thailand. But still the majority of developing countries currently do not have a regulatory system for GMOs in place. Development of a regulatory framework may be a costly, time-consuming process involving extensive consultation and effort.

Many countries in sub-Saharan Africa are now establishing national biosafety committees and biosafety regulations for testing and dissemination of GM crops, specifically in order to facilitate the development of transgenic bananas in many countries like Uganda, Egypt. There are also initiatives to harmonize biosafety regulations at the regional level. The Food and Agricultural Organization (UN) is now focusing its biotechnology programme toward providing technical advice and capacity building regarding biosafety to its member governments. The Program for Biosafety Systems (PBS) is another important initiative that has been established to assist national governments in studying the policies and procedures necessary to evaluate and manage potential harmful effects of modern biotechnology on the environment and human health. The program's unique approach addresses biosafety as part of a sustainable development strategy, anchored by agriculture-led economic growth, trade, and environment objectives. Many countries have signed the Cartagena Protocol on Biosafety to the UN Convention on Biological Diversity agreed to put in place measures to ensure the safe handling of GMOs within countries and their transfer across national boundaries.

A number of existing international agreements have direct relevance to GMOs and they can be of assistance to developing countries in establishing appropriate regulatory structures that deal with potential concerns while, at the same time, promoting harmonization of national regulations at the international level.

3. FUTURE ROAD MAP

Bananas and plantains are seriously threatened by pests and numerous viral, bacterial, and fungal diseases. Thus, resistance to biotic stresses is an important part of regional or national efforts. Since all the cultivated varieties of banana are sterile

and, therefore, do not set seed, traditional breeding is more difficult than genetic transformation using molecular techniques. Although attempts to produce transgenic bananas and plantains are still proceeding too slowly, public acceptance of these novel plants and their products should already be prepared for through sound information and risk assessment, although the chances of transfer of transgenes from transgenic field material to wild species (the major public concern) are expected to be negligible in view of the sterility of many cultivars. The scope for further improvement of *Musa* species is large; along with other methods of crop improvements transgenic technologies should provide fast and effective methods of *Musa* improvement.

The number of transgenic bananas is continuously increasing. Transformation protocols, including tissue culture techniques, suitable transformation constructs with modified promoters driving one or more transgene(s), appropriate transformation techniques such as particle bombardment and *Agrobacterium*-mediated gene transfer, the detection of the transgenes and characterization of their insertion sites (copy number), are well developed. Transgenes have been used exclusively from heterologous sources rather than specifically from bananas.

3.1 Expected Products

The major research institutes/organizations and universities are concentrating on the development of two broad types of proprietary traits: (a) input traits such as pest or disease resistance and (b) output traits that improve the nutritional contents of foods or exhibit unique properties for very specific end uses or markets. Such input and output traits will be incorporated into existing elite varieties to provide material with further added value, which may offer to the farmers at lower costs or higher yield, and increased value of the end product. Initially, it is likely that transgenic banana varieties brought to the market will focus on diseases (bacterial and fungal) and pest (nematode) resistance as the technologies are in advanced stage in many labaroteries. However, the long-term commercial potential of plant biotechnology is considered to be in the development of value-added output traits that will address a wide range of specific needs or market niches.

Genetic modification of banana has also been considered as a means toward increasing the value of this crop to health and nutrition in developing countries. As a crop that is widely consumed as a weaning food by children and as a starchy staple by all sectors of the community in some countries, banana has been advocated as a carrier for vaccines and as a source of carotenoids that can counteract debilitating vitamin A deficiency. However, although much of the necessary technology is now available, these applications have yet to advance to the stage of practical evaluation.

Nutritionally enhanced crops could make a significant contribution to the reduction of micronutrient malnutrition in developing countries. Biofortification (the development of nutritionally enhanced foods) can be advanced through the application of several biotechnologies in combination. Genomic analysis and genetic linkage mapping are needed to identify the genes responsible for natural variation in nutrient levels of common foods. These genes can then be transferred into popular cultivars through breeding or, if sufficient natural variation does not occur within a single species, through genetic engineering. Vitamin and mineral deficiencies, which contribute to the deaths of millions of children each year, can be easily prevented by adding just a few key nutrients to staple food.

Transgenic plants that produce medicinal compounds such as subunit oral vaccines have already been developed, but experts concede that application of this technology is at least a decade away. There are several technical problems, which need to be addressed before plant edible vaccines become a reality in practice. Most inserted genes are expressed in very low levels in plants, which need to be enhanced. Arntzen *et al*. (2005) recently reported that a synthetic cholera vaccine gene that was more "plant" like in its sequence is four times more productive than the original gene. The stability of vaccine proteins when transgenic fruits or leaves are stored at ambient conditions is another concern. There are also concerns about oral tolerance and the dosage of oral vaccine. Only further collaborative research between plant and medical scientists may resolve these and other issues. In the near term,

the edible-vaccine technology might be better targeted at animals. In fact, such an approach may benefit agriculture as billions of dollars are spent presently on vaccinating farm animals and poultry. Transgenic plants supplying feedstock containing edible vaccines may represent the first commercial application of this intriguing technology.

The next future product can be banana varieties with longer shelf life. Banana is the most widely consumed fruit worldwide. Fruits are picked before they are allowed to ripen. They are then transported to their final destination under controlled atmospheric conditions where they are gassed with a plant hormone, ethylene, to induce ripening. Once ripening has been artificially triggered, the fruit has to be eaten or sold immediately before they spoil. First round of banana field trials by Senesco Technologies, Inc. and Rahan Meristem show that using Senesco's delayed ripening technology significantly extends the shelf life of banana. Senesco claims that banana fruit lasted twice as long as the control (nonenhanced) fruit (Crop Biotech Update, 2003). The Senesco bananas ripened normally, but the onset of spoilage and blackening that follows ripening was significantly delayed. The banana field trials indicate that the delayed ripening technology slows the process of cell death once ripening has occurred, without affecting normal growth of the plant and its fruit. This ensures that bananas are the same size, shape, weight, and color as nonenhanced bananas, with the same taste and nutritional characteristics.

Global Agricultural production has been seriously threatened with the continuing deterioration of arable land, scarcity of water, and increasing environmental stress. Over the recent decades, rainfall has seemingly become less reliable, which is putting pressure on the food security and livelihood status of smallholder farmers in sub-Saharan Africa. Drought does not only affect typical dry land but increasingly affects the more semi-humid areas where crops such as cassava and banana dominate. For example, East African highland bananas would need an annual precipitation of at least 1,200 mm, but in some recent years annual precipitation in many banana growing areas dropped to below 800 mm rainfall, resulting in yield losses of more than 50% (P. Van Asten, IITA, per. comm.). Conventional plant breeding and crop physiology have had limited success in building and deploying tolerance to climatic stresses in the developing world. Recent advances in transgenic approaches to provide enhanced drought tolerance hold promise to move forward. The stress-regulated expression of *DREB1A* gene by the *rd29A* promoter has shown to confer tolerance to drought, low temperatures, and salinity in *Arabidopsis*, rice, and wheat (Dubouzet *et al.*, 2003; Pellegrineschi *et al*., 2004). Other gene *XVSAP1* derived from the resurrection plant *Xerophyta viscosa* Baker is implicated in resistance to abiotic stress resistance (Garwe *et al*., 2003). We anticipate that this will be a useful strategy to adopt for banana. Drought resistant banana varieties will extend the geographic spread of banana production in Africa.

3.2 Addressing Risks and Concerns

Before release, any new banana GM varieties will go through an environment and food safety analysis, in addition to variety registration procedures. Several African countries, where GM banana research is in progress, are signatories to the Cartagena Protocol on Biosafety, which deals with the conservation of biological diversity and the equitable sharing of benefits from the use of genetic resources. The Protocol seeks to protect biological diversity from potential environmental risks posed by living modified organisms (LMOs) and GMOs resulting from modern biotechnology, taking into account risks to human health and focusing on trans-boundary movement of LMOs.

There is currently no scientifically accepted evidence to suggest that transgenic crops *per se* are any more or less toxic or allergenic than their conventionally bred counterparts (Ruibal-Mendieta and Lints, 1998). Since banana is an important food and fodder crop in Africa, any effort to genetically modify the crop with eventual goal of producing food and feed, novel genes ought to be made in the context of social, economic, and political considerations of the new technology and proceed in a safe and highly responsible manner. Accordingly, several studies will be conducted on the transgenic banana to guarantee that the product placed on the market as food or feed is safe. In particular, the compositional analysis to ascertain that transformed banana is substantially equivalent

to isogenic nontransformed plants, nutritional trials for digestibility in model animals to confirm nonallergencity using standard protocols, toxicity of transgenic banana to confirm nontoxicity in model animals and indicator species and finally acute gavage mouse studies to confirm nontoxicity will be performed before release of the GM varieties.

There are potential benefits to human health and well-being by using transgenic bananas. The insertion of genes for vitamins into staple crops such as banana can enhance their nutritional value. Genetic engineering could be used to develop pharmaceuticals and vaccines in plants, decreasing the risk of adverse reactions, and enabling faster vaccination of large populations.

A variety of concerns have been expressed regarding the impact of transgenic crops on the environment. The major potential risks of GM crop to the environment and wildlife is the gene flow. Banana is a sterile, vegetatively propagated plant lacking wild relatives in Africa meaning that the potential for outcrossing transgenic banana with other banana cultivars or other crops is negligible.

Commercial banana production requires application of large amounts of pesticides that pollute the environment, and whose residues accumulate in plantation workers. Therefore, it would be ethically justifiable to produce a transgenic banana variety that would allow for a reduction in pesticide application and a subsequent improvement in human health. Genetically engineered pest and disease resistance could reduce the need for pesticides and other chemicals, thereby decreasing the environmental load and farmers' exposure to toxins. The development of bananas resistant to pests, disease, and extreme weather, decreasing the risk of devastating crop failure will be potentially useful contributions to sustainable farming. Although weighing risks and benefits is necessary, it is neither easy nor the sole concern in considering the ethics of agricultural biotechnology.

In Western countries where food supplies are abundant and incomes are high, people can afford to be critical about the introduction of new agricultural technologies and production processes about which they are unsure. In developing economies, by contrast, the benefit/cost ratio is very different. Many food-insecure people in developing countries live in rural areas, earn a significant share of their income from agriculture, and meet a substantial share of their food needs from their own production. For them, increasing agricultural productivity and thereby real income is a high priority. And for the urban poor in those countries, anything that lowers the effective price of basic foods is highly desirable.

Bananas and plantains are staple food crops for over 100 million people in sub-Saharan Africa and over half a billion worldwide. Despite its importance, there are many diseases and pests severely effecting banana production and threatening livelihood of millions of farmers. The production of nematode, fungus, bacterial, and virus-resistant transgenic will be a major benefit for farmers. It may also be possible to incorporate other characteristics such as drought tolerance, thus extending the geographic spread of banana and plantain production, and thus contributing significantly to food security and poverty alleviation in developing countries.

3.3 Expected Technologies

Research in the field of transgenic improvement and functional genomics in banana is constrained by low efficiency and cultivar-specific transformation systems and therefore, an efficient transformation protocol is crucial. Currently, the transformation system based on cell suspension cultures is most commonly used but development of cell suspension is time consuming and cultivar specific. Several labs are trying to develop an efficient and rapid transformation system that can be applied to broad range of cultivars.

Successful genetic transformation requires not only efficient gene delivery, but also an efficient selection system to distinguish transgenic from nontransgenic events. Several different selection strategies have been used in bananas, mainly using antibiotic or herbicide resistance genes. However, there have been concerns over the use of these selective markers. Concerns have been raised that the selectable marker genes could be transferred into microbes and increase the number of resistant pathogens, or that horizontal transmission of the marker gene into wild relatives may result in weedy pests (Nap *et al*., 1992). Several strategies to eliminate marker genes have been employed.

Co-transformation using binary vectors carrying two separate T-DNAs was used for rice transformation and the segregation of the transgenes in the progeny allowed the retrieval of marker-free plants (Komari *et al*., 1996). Becker *et al*. (2000) has demonstrated co-transformation in banana, which should be used further. Transient expression of the *cre*/*lox*P site-specific recombination system (Gleave *et al*., 1999) together with a conditional dominant lethal gene *cod*A (Perera *et al*., 1993) resulted in the elimination of marker genes from transgenic plants without sexual crossing.

Mannose has been reported as a novel selectable agent and the phosphomannose isomerase gene (*pmi*) as its suitable selectable marker (Joersbo and Okkels, 1996). Recently, transgene removal systems denoted as MAT vectors have been developed to generate marker-free transgenic plants through a single step transformation (Ebinuma *et al*., 1997). The introduction of the *pmi* during transformation makes this an ideal system for positive selection whereby plants containing the gene are able to survive on a medium containing mannose. This can be potential selection marker for banana transformation.

Currently, the strong constitutive promoters are routinely used for banana transformation but tissue-specific promoters might be better especially for developing pest resistant varieties. The most useful types of promoters for nematode control strategies are those providing root-specific gene expressions since roots are not consumed in bananas. There is a value in limiting spatial and temporal patterns of expression of an effector to the locale of the parasites and away from food parts of the plant. Root-specific promoters would serve this ideal and drive effective resistance against a number of nematode species. Feeding site-specific promoters would be of more specialized value against particular nematode species. Several root-specific promoters of value for nematode control have been identified at the University of Leeds, UK. Ideally the optimal promoter for nematode control would exhibit the correct spatial and temporal expression pattern only within roots and throughout parasitism.

In order to accelerate efforts in producing improved varieties of banana and plantain, a Global *Musa* Genomics Consortium was established in 2001 with the goal of assuring the sustainability of banana as a staple food crop by developing an integrated genetic and genomic understanding, allowing targeted breeding, transformation, and more efficient use of *Musa* biodiversity. Basically, the consortium aims to apply genomics to the sustainable improvement of banana and plantain. The consortium believes that genomic technologies such as analysis and sequencing of the banana genome, identification of its genes and their expression, recombination and diversity can be applied for the genetic improvement of the crop (Frison *et al*., 2004). Genetic and physical mapping of the *Musa* genome will make it possible to isolate genes that can be used in genetic transformation.

Currently, no transgenic bananas and plantains are commercially available; however there is enormous potential for genetic manipulation of *Musa* species for disease and pest resistance using the existing transformation systems. Using molecular techniques, novel genes encoding agronomically important traits can be identified, isolated, characterized, and introduced into cultivars via genetic transformation. The use of appropriate gene constructs may allow the production of nematode, fungus, bacteria, and virus-resistant plants in a significantly shorter period of time than using conventional breeding, especially if several traits can be introduced at the same time. Long-term and multiple disease resistance can 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. Plant biotechnology has the potential to play a key role in the sustainable production of *Musa*.

3.4 IPR, Public Perceptions, Industrial Perspectives, Political and Economic Consequences

Most innovations in biotechnology are developed using the knowledge or technologies generated from previous innovations. Many plant biotechnology products or techniques are "modular" in that they are assembled from a number of previously developed technologies/transgenes, each of which may be subject to a separate patent. The commercialization of many proprietary biotechnology products is typically contingent on other proprietary biotechnology products or processes and in particular on agreements between

IPR holders regarding the relative contributions of different proprietary technologies to the product in question. Many biotechnology products (e.g. transgenic seeds or transgene constructs) now have a complex IPR pedigree because a large number of proprietary products or processes are involved in developing the product.

The current generation of transgenic bananas and their testing, however, highlights some problems that need to be avoided in future. Some genes of agronomic interest were owned by the industry, and it took much effort by the Catholic University of Leuven before these genes could be used freely for plantain and cooking bananas. Therefore, it is urgent that a mechanism is put in place whereby an authority at the global level will interact with the industry to negotiate access to protected technologies for developing countries. However, in the case of food production by smallholders like banana and plantain, it is absolutely necessary that technologies are royalty free and that the farmers should have privilege to save seed (or planting material) for subsequent replanting and distributed from farmer to farmer without any financial return to the industry. It is clear that delivery of GM products to smallholder farmers in Africa will be expensive. Remington and Bramel (2004) had described handling of GM seeds for small farmers in Africa through partnership with NGOs.

Currently, a number of institutions in Africa are focused on developing and promoting biotechnology for farmers (CBI, 2003). Foundations, international organizations like CGIAR, NGOs, and national governments are promoting the potential benefits of biotechnology in addressing the critical need to increase food production in Africa. Several research networks have been established to promote biotechnology research in crops such as cassava, banana, rice, sweetpotato, and cowpea that can benefit the poorest farmers in Africa.

Donor initiatives are promoting research and development of biotechnology products with public/private partnerships, and donors and international organizations initiatives will strengthen biosafety regulation and technical expertise in developing countries to enhance the application of biotechnology products. Novel partnerships are being formed between the private sector, donors, and nonprofit organizations to find common and acceptable grounds. For example, many donor agencies are providing ways for North–South partnerships to open up African markets in a mutually beneficial and sustainable manner. African Agricultural Technology Foundation (AATF) was launched in 2003, whose mission is to acquire technologies through royalty-free licenses along with associated materials and know-how for use on behalf of SSA's resource-poor farmers, while complying with all laws associated with the use of these technologies. AATF has been instrumental in facilitating technology transfer negotiations whereby proprietary biotechnologies have been made available to Africa. Recently, IITA has negotiated royalty-free license from Academia Sinica, Taiwan through AATF for access of technology for bacterial wilt resistance. AATF has signed the licensing agreement with Academia Sinica, Taiwan for access to the *pflp* gene for use in banana for the commercial production of varieties resistant to banana bacterial wilt in all of sub-Saharan Africa and granted a sublicense to IITA for developing the improved varieties.

In view of the tremendous losses incurred by smallholder *Musa* farmers due to pest and disease attack, and the economic, environmental, and health costs associated with plant protection measures in large-scale commercial plantations of banana, it is very much to be hoped that the remaining technical, intellectual property, and regulatory obstacles to the deployment of transgenic bananas and plantain can soon be overcome. It is not anticipated that transgenic varieties will threaten the diversity of existing varieties grown by banana farmers. Rather, studies in East Africa suggest that varieties modified for pest or disease resistance will be incorporated into the range of varieties already grown as part of a strategy to reduce risk, provide multiple products, and satisfy varying tastes. In the meantime, various biotechnology techniques are already contributing to conventional breeding efforts and are expected to become even more effective in this area as genetic maps and markers are refined. The use of tissue culture plants is already contributing to the development of novel production systems for smallholder farmers and, as part of a balanced program of deploying biotechnology techniques cost effectively in the developing countries, tissue culture is expected to be much more widely used

in increasing the productivity and sustainability of such systems in the future.

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Abstract:

Bananas and plantains (*Musa spp*.) are the world's fourth most important food crop after rice, wheat, and maize in terms of gross value of production. They are major staple food and source of income for millions of people in tropical and subtropical regions; particularly in Africa, an area where the green revolution has had little influence. The performance of bananas and plantains can be severely affected by diseases and pests. These are predominantly small-holders' crops; most growers cannot afford costly chemicals to control pests and diseases. The host plant resistance is the most sustainable approach to counteracting pest and disease pressure. Transgenic technology, together with conventional methods can assist in overcoming these problems in developing improved cultivars of banana and plantain. Some successes in genetic engineering of *Musa* have been achieved, enabling the transfer of foreign genes into the plant cells. The transgenic approach shows potential for the genetic improvement of bananas using a wide set of transgenes currently available that may confer resistance to pests and diseases. The use of appropriate constructs may allow the production of pest- and disease-resistant plants in a significantly shorter period of time than using conventional breeding; especially if several traits can be introduced at the same time.

Keywords: *Musa*, micropropagation, embryogenic cell suspensions, genetic transformation, transgenic bananas IPR