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Recent Developments in Biotechnological Research on Bananas (*Musa* spp.)

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Introduction

The term banana is meant here to cover dessert, cooking and beer bananas as well as plantains. Banana is probably the most important fruit crop in the world with an annual production of more than 80 million tonnes (Anonymous, 1995). In many of the 120 banana producing countries which are mainly located in the Third World, banana is locally consumed either fresh, prepared by cooking or processed as beer, chips and other food products. In addition to being the major source of carbohydrates, banana fruits contain high levels of potassium, and the vitamins B and C. Plantain is also rich in vitamin A which is often deficient in human diet in the tropics. Banana fruit has been found to exert a cholesterol-lowering effect when freeze-dried pulps were added to a cholesterol-rich diet of rats (Horigome *et al.*, 1992). Banana is a staple food for at least 400 million people. It is (after rice, milk and wheat) the fourth major food source for the developing world and also ranks fourth (after rice, wheat and maize) in terms of gross value of production.

Some 10% of banana production is exported and serves as dessert for many more millions of people. Export banana trade has a gross value of more than three billion US$ (Anonymous, 1994), therefore banana cultivation is of great socio-economic importance for a number of developing countries which rely on banana exports to generate their foreign exchange.

The distribution of production is relatively even among Latin America and the Caribbean (36%), Africa (34%) and Asia with the Pacific (29%). However, a considerable difference exists in the banana types cultivated in the different regions. More than 80% of dessert banana is produced in Latin America and the Caribbean, while cooking banana is cultivated mostly in the Asian-Pacific region. Plantains are

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produced mainly in West and Central Africa and Latin America and highland banana production is limited to East Africa.

MAJOR CONSTRAINTS IN BANANA PRODUCTION

Although banana is one of the world's most important crops, it is the least researched among all those major food sources, perhaps because of the biology of the banana plant. Most cultivated banana varieties are sterile triploids and this prevents elaboration of successful hybridization programmes. In addition, the long life cycle of this crop makes even simple field testing experiments last for 2–3 years. These obstacles hamper banana breeding to deal with the attacks of a number of virulent diseases and pests whose protection costs may account for more than 40% of the total production cost.

The most devastating disease is undoubtly the Sigatoka complex caused by the fungal pathogens *Mycosphaerella fijiensis* Morelet (black sigatoka) and *M. musicola* Leach ex Mulder (yellow sigatoka). Sigatoka disease attacks all types of banana and is common in most banana producing regions where yield losses may reach up to 30–50% (Stover, 1983; Mobambo *et al.*, 1993). Annual costs of fungicide spraying control in plantations range between US $ 600 and 1800 per hectare. However, in addition to their environmental pressure, application of fungicides in backyards or small farms is economically questionable. Recent promising results in resistance breeding are still very limited and, in addition, have required a long time (Vuylsteke *et al.*, 1993a, 1993b; Rowe, 1994).

The second major fungal disease is Panama or banana wilt caused by *Fusarium oxysporum* Schlecht. f.sp.*cubense* (E.F. Smith) Snyd. and Hansen (Smith, 1910). This soil-inhabiting fungus has been the cause of one of the most destructive epidemics in history as by 1960 its race 1 had destroyed approx. 40,000 ha commercial plantation. A new race 4 (Sun *et al.*, 1978) presently threatens export banana industry in the subtropics (Ploetz, 1990). The fungus infects through lateral roots and blocks the host vascular system which results in typical wilt symptoms. Since no fungicide control is available for this disease, production can only be continued by new plantings in noninfested soil.

The most serious viral disease affecting banana is bunchy top disease. The causal agent, banana bunchy top virus (BBTV) is a ssDNA virus which may represent a new group of plant viruses (Harding *et al.*, 1993). BBTV is persistently transmitted by the aphid *Pentalonia nigronervosa* Coquerel or by vegetative propagation (Drew *et al.*, 1989; Thomas *et al.*, 1995) and is widely distributed in the Asian-Pacific region. In Africa, BBTV is present in a few countries, while in Latin America, it is still absent. Growth of heavily infected plants is significantly reduced and yields are entirely lost. In addition, no BBTV-resistant source has yet been identified. Recently, banana streak virus, banana bract mosaic virus and cucumber mosaic virus have been recognized to cause increased damage to banana. Banana streak virus, a badnavirus, first described from Morocco ten years ago (Lockhart, 1986), is becoming another major threat to banana production all over the world (Diekmann and Putter, 1996). It is a pararetrovirus which has recently been found to be able to integrate into the banana genome (Lockhart, 1996). Banana bract mosaic virus has been identified as a potyvirus, and becomes a serious quarantine threat in the Philippines and in India due to the lack of

reliable diagnostic tests (Bateson and Dale, 1995). Cucumber mosaic virus (causing infectious chlorosis) is present in all banana producing areas and its broad host spectrum makes it difficult to control (Singh *et al.*, 1995; Diekmann and Putter, 1996).

Among the migratory endoparasitic nematodes which are the most damaging and widespread (e.g. the root-lesion nematodes, *Pratylenchus* spp., and the spiral nematode, *Helicotylenchus multicinctus* (Cobb) Golden), the burrowing nematode, *Radopholus similis* (Cobb) Thorne is the most dangerous (Gowen and Quénéhervé, 1990). This nematode causes steadily increasing problems in commercial plantations in Latin America.

The bacterial wilt or Moko disease which is caused by the bacterium *Pseudomonas solanacearum* (recently proposed to move to the new genus Burkholderia by Yabuuchi *et al.*, 1992) is more destructive in very humid areas. In some restricted regions, the bacterial head-rot disease caused by *Erwinia carotovora* presents significant problems. Similarly, the banana borer weevil, *Cosmopolites sordidus* (Germar) (Ostmark, 1974), by laying its eggs close to the base of the pseudostem after which the larvae bore into the corm, causes serious damage in certain regions, especially in Africa.

Biotechnological approaches in banana research

Since in the past several reviews were published on various aspects of banana biotechnology (Cronauer and Krikorian, 1986; Dale, 1990; Novak, 1992; Cote *et al.*, 1993), this review will focus mainly on the recent developments in this field.

IN VITRO CELL AND PROTOPLAST CULTURE

Banana *in vitro* clonal propagation started in the early 1970s (Ma and Shii, 1972) and became a routine technique for a wide range of cultivars by the mid 1980s (Vuylsteke, 1989; Smith and Drew, 1990). However, only a few reports exist on more sophisticated culture techniques including somatic embryogenesis and embryogenic cell suspension cultures (ECSs) (reviewed by Panis *et al.*, 1994). At present, three main procedures have been described for somatic embryogenesis in banana and all of them are based on vegetative tissues such as rhizome fragments or leaf bases (Novak *et al.*, 1989), *in vitro* proliferating meristems (Dhed'a *et al.*, 1991) and immature male inflorescences (Escalant *et al.*, 1994). Since most edible bananas rarely set seeds, these techniques represent a significant advance when compared to the previous ones which rely on immature zygotic embryos (Cronauer and Krikorian, 1988; Escalant and Teisson, 1989; Marroquin *et al.*, 1993). The three above-mentioned procedures have been reported to result in plant regeneration through somatic embryogenesis, though at a different rate. Histologically, the process of somatic embryogenesis appears to be closely related in the three different explants (Schoofs, 1997). It is therefore not surprising that it was also possible to establish long-term ECSs first from proliferating meristems (Dhed'a, 1992) and very recently from immature male flowers (Cote *et al.*, 1996). In parallel, a temporary immersion culture system which was originally developed by Alvard *et al.* (1993) for meristem propagation of banana, has been applied to multiply banana somatic embryos (J.V. Escalant, pers. comm.) as well as to maintain banana cell suspensions (Schoofs, 1997).

The method of Dhed'a *et al.* (1991) has been successfully combined with

cryopreservation. Several ECSs including those of the cultivar Bluggoe or *Musa balbisiana* Colla were subjected to cryopreservation and showed high rates of survival (90–94%) after being stored in liquid nitrogen (Panis *et al.*, 1992). Major parameters of successful cryostorage were found to be (i) dimethylsulfoxide at a concentration of 7.5% as cryoprotectant, (ii) slow freezing at a rate of 1°C min^{-1} to –40°C, and (iii) initiation of ice crystallization by seeding at –10°C during the slow freezing process. Fluorescein diacetate staining revealed that only highly embryogenic cells survived freezing and were able to regenerate plants through somatic embryogenesis (Panis *et al.*, 1990). Until now, ECSs have been the only material available for cryopreservation. However, very recently *in vitro* meristems (Panis *et al.*, 1996) and somatic embryos (Abdelnour-Esquivel and Escalant, 1994) have been reported to survive cryopreservation, though at a lower rate (40 to 50%) than ECSs.

Similarly to other monocotyledonous species, the development of ECSs have provided the only opportunity for isolation of banana protoplasts with high embryogenic potential. Consequently, earlier work on protoplast culture in banana with various organized tissues resulted in very limited success (reviewed by Panis *et al.*, 1994). Megia *et al.* (1992) were the first to describe protoplast isolation from banana ECS. However, only sustained division and callus formation was observed. High-frequency plant regeneration through direct somatic embryogenesis was reported by Panis *et al.* (1993) for the cooking banana cultivar Bluggoe. In addition, high plating efficiencies (between 20 and 40%) were observed when protoplasts were cultured on a feeder layer of embryogenic banana cells or plated alone at a high (10^6 mL^{-1}) density. Cell suspensions initiated from the same cultivar (named by its synonyme) also proved to be a reliable source for regenerable protoplasts for another research group (Megia *et al.*, 1993).

GENETIC TRANSFORMATION AND TRANSGENIC BANANA

The development of ECSs and of regenerable protoplast cultures in banana have made it possible to introduce foreign genes into this crop. Large number of transgenic banana plants have been produced by particle bombardment of ECSs (Sági *et al.*, 1995a, 1995b). Recently, *Agrobacterium*-mediated transformation of meristematic tissues has also been reported to result in regeneration of transgenic banana plants (May *et al.*, 1995).

At present, three systems appear to be feasible for banana transformation: (i) introduction of DNA into regenerable, cell suspension-derived protoplasts by electroporation (Sági *et al.*, 1994), (ii) particle bombardment using an in-house developed particle gun device which has been optimized for transformation of ECSs (Sági *et al.*, 1995a, 1995b), and (iii) *Agrobacterium*-mediated transformation of *in vitro* meristems (May *et al.*, 1995). In the following sections each of these systems is briefly described.

Transient gene expression in banana protoplasts by electroporation

Electroporation conditions were established for transient expression of introduced DNA in banana protoplasts isolated from regenerable embryogenic cell suspensions of the cooking banana cultivar Bluggoe. When using a 960 μF capacitor, the estab-

lished parameters are: (i) electric field strength, 800 V cm^{-1}; (ii) ASP-electroporation buffer, containing 70 mM potassium-aspartate, 5 mM calcium-gluconate, 5 mM MES, and 0.55 M mannitol (pH 5.8) (Tada *et al.*, 1990); (iii) polyethylene glycol (PEG) concentration, 5%; (iv) heat shock, 45°C for 5 min before addition of PEG; (v) protoplast parameters, highly viable protoplasts isolated from 1-week-old embryogenic cell suspensions, and (vi) chimaeric gene constructs for optimized expression. The maximum frequency of DNA introduction as detected by an *in situ* assay for transient expression of the *gusA* gene amounted to approx. 2% of total protoplasts (Sági *et al.*, 1994) (*Figure 1A*).

The present method directly provides a basis for analysis of promoters in banana (Sági *et al.*, 1995c) and may in the future allow the production of transgenic banana using the plant regeneration protocol previously described (Panis *et al.*, 1993).

Particle bombardment of embryogenic cell suspensions

In order to search for promoters which can drive high expression of foreign genes in banana cells, transient expression was tested of the *gusA* gene under the control of different heterologous promoters which had been known to be active in monocotyledonous species. When an enhanced CaMV 35S promoter in combination with the untranslated leader sequence of the alfalfa mosaic virus (35S-35S-AMV, Datla *et al.*, 1993) was bombarded into banana cells using a modified particle gun (Sági *et al.*, 1995a), on average close to 1000 blue foci per shot (i.e. 25 mg cells) were observed (*Figure 1B*). This high transient transformation frequency was comparable with that obtained in ECSs of other monocotyledonous species (*Table 1*). Increased transformation frequency was coupled with a higher expression rate: blue foci appeared much faster and were more intense than with other constructs. Fluorometric GUS assay also confirmed that 35S-35S-AMV drives almost two times higher GUS expression than the recombinant Emu promoter (Last *et al.*, 1991). Other promoters isolated from monocots including the promoter of the rice *Act1* gene (Zhang *et al.*, 1991) and the maize ubiquitin promoter (Christensen and Quail, 1996) have been found to be even more active in ECSs of bananas (Sági *et al.*, 1995a).

When embryogenic cell suspensions of cultivar Bluggoe were bombarded with a plant expression vector carrying chimaeric *gusA* and *hph* (encoding the hygromycin detoxifying enzyme hygromycin phosphotransferase) genes and subjected to hygromycin selection, GUS-positive cell aggregates appeared after two months of culture on selective medium. Hygromycin resistant plants were regenerated and transferred to the greenhouse (*Figure 1C*) and found to be GUS-positive both in their leaves

Table 1. Comparison of transient transformation frequencies of particle bombardment in embryogenic cell suspensions of monocot species

Species	Number of blue foci/mg fresh weight	References
Barley	7000 /1,000	Ritala *et al.* (1993)
Maize	8700 /100	Vain *et al.* (1993)
Rice	422 /300	Wang *et al.* (1988)
Wheat	880 /100	Vain *et al.* (1993)
Banana	1000 /25	Sági *et al.* (1995a)

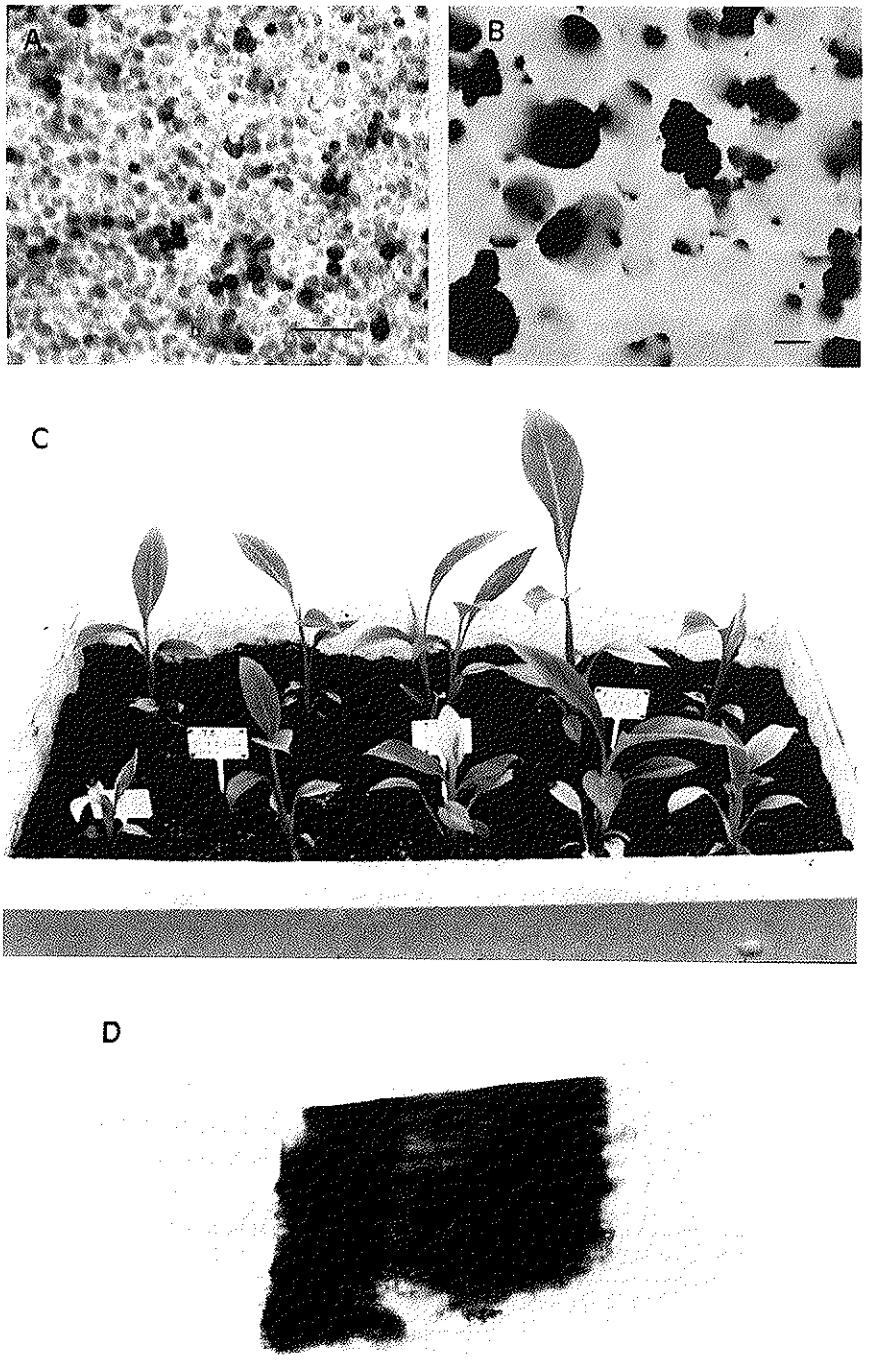

Figure 1. Transient GUS expression in (A) electroporated protoplasts (bar = 100 μm) and (B) bombarded embryogenic cell suspension of the cooking banana cultivar Bluggoe (bar = 100 μm). (C) Hygromycin resistant transgenic plants of the cooking banana cultivar Bluggoe transplanted into soil. (D) Stable expression of the *gusA* gene in a leaf sample of a 6-month-old transgenic Bluggoe plant.

(*Figure 1D*) and roots. Untransformed control plants were always GUS-negative in the same tissues. No evidence of chimaerism has been found so far which is probably due to single-cell origin of regenerated plants. PCR-analysis of DNA isolated from hygromycin resistant plants showed the presence of the correct amplification product corresponding to the coding region of the *hph* gene. In untransformed control plants no PCR products could be amplified while the plasmid DNA used for transformation showed again the same PCR product as in the transfomed plants. Furthermore, Southern hybridization analysis of PCR products, digested and undigested genomic DNA confirmed the identity and integration of the introduced genes (Sági *et al.*, 1995a).

These three lines of evidences, i.e. (i) the hygromycin-resistant phenotype, (ii) stable expression of the *gusA* gene in various plant tissues, and (iii) the presence and integration of the introduced genes in transformed plants, demonstrate that the regenerated plants are indeed transgenic.

Using this technology, several hundreds of independent transgenic lines have been produced so far and will be tested for their uniformity in the field. A major question before more widespread utilization of banana ECSs to create transgenic plants is the presence and rate of somaclonal variation. Occurrence of off-types after micropropagation of banana meristems has been reported for a wide range of cultivars (reviewed by Reuveni *et al.*, 1996). Variation in somatic chromosome numbers has also been observed in micropropagated progenies of a dessert banana (Sandoval *et al.*, 1996). On the other hand, though somaclonal variation in plants derived from ECSs is not adequately documented in the generative phase, the frequency of off-types in the vegetative phase of ECS-derived plants of the cultivars Williams and Three Hand Planty has been found to be significantly lower than in that of micropropagated plants (Schoofs, 1997; R. Swennen, pers. comm.).

Agrobacterium-mediated transformation of meristems

The *Agrobacterium*-based banana transformation system utilizes apical or corm meristematic tissues. Meristems of *in vitro* Grande Naine banana plantlets were wounded by microparticle bombardment with uncoated particles. After a brief recovery period, these meristems were subsequently cocultivated with *Agrobacterium tumefaciens* harbouring the plant transformation vector pBI141 in the presence of acetosyringone, a known inducer of the *Agrobacterium* virulence genes. The transformation vector contains a chimaeric *gusA* gene and the *neo* gene conferring resistance to kanamycin. Kanamycin resistant plants were regenerated from meristems at a high frequency. Southern hybridization experiments demonstrated that (i) the regenerated plantlets contained the transgenes in all tissues analysed, (ii) the transgenes were incorporated into high molecular weight genomic DNA, and (iii) no residual *Agrobacterium* persisted in the plants. In addition, plantlets that had undergone multiple rounds of propagation maintained these genotypic and phenotypic traits (May *et al.*, 1995). The main attractiveness of this method is (i) the ease and speed of transgenic plant regeneration, (ii) the lack of requirement for high-tech equipment or protoplast regeneration skills, and (iii) the fact that the desired DNA sequences are specifically transferred to recipient cells. However, prior to wider applications, the genotype specificity of this technique as well as the risk of generating chimaeric transformants needs to be carefully evaluated.

Applications and prospects

RESISTANCE TO DISEASES AND PESTS

Since the most significant damage to banana production is caused by fungal pathogens and host resistance genes remain to be identified, heterologous genes encoding proteins with antifungal activity (Broekaert *et al.*, 1996) are the primary targets for expression in banana. Perhaps the most promising candidates are the recently described new types of antifungal proteins (AFPs) which are stable, cysteine-rich small peptides isolated from seeds of diverse plant species (Broekaert *et al.*, 1992; Cammue *et al.*, 1992; Terras *et al.*, 1992; Cammue *et al.*, 1995; Osborn *et al.*, 1995). These AFPs have a broad antifungal spectrum, and show high *in vitro* antifungal activity to *Mycosphaerella fijiensis* and *Fusarium oxysporum*, the main fungal pathogens in banana, while they exert no toxicity to human or banana cells (Cammue *et al.*, 1993). Corresponding cDNA clones to AFPs from amaranth (De Bolle *et al.*, 1993), radish (Terras *et al.*, 1995) and onion (Cammue *et al.*, 1995) seeds were isolated and introduced into plant transformation vectors. One of these AFP constructs has recently been expressed in transgenic tobacco which resulted in an increased resistance to *Alternaria longipes* (Terras *et al.*, 1995). It is expected that expression of these AFPs in transgenic banana plants combined with cytological and ultrastructural analysis of the fungus-banana interaction (Beveraggi *et al.*, 1993) *in planta* will lead to a more detailed understanding of the infection process and symptom development. Furthermore, high and tissue-specific expression of AFPs may result in the production of commercially acceptable fungal disease-resistant bananas.

Engineering resistance to banana bunchy top virus (BBTV) is another obvious objective of banana molecular breeding. Recently, Harding *et al.* (1991) and Thomas and Dietzgen (1991) isolated virus-like particles (18–20 nm in diameter) and reported the association of small ssDNA with the purified virion. The association of this DNA with the bunchy top disease and its transmission was also demonstrated using a cloned DNA probe (Harding *et al.*, 1991) and virion-specific monoclonal antibodies (Thomas and Dietzgen, 1991). The BBTV genome has six different ssDNA components which have now been cloned and sequenced: each of these components but one contains one large open reading frame (ORF) (Burns *et al.*, 1995). One of these ORFs has been identified by Harding *et al.* (1993) as the putative replicase gene and may be utilized for ribozyme technology to prevent BBTV replication in banana (J.L. Dale, pers. comm.). Components of the BBTV genome have also been recently isolated by another research group (Yeh *et al.*, 1994) and confirmed the above findings. It appears, however, that there are two strains of BBTV: an Asian and a South Pacific strain (Karan *et al.*, 1994; Xie and Hu, 1995) and therefore, different constructs may be required to produce resistance to these two strains.

The recent cloning of the C-terminal region of the coat protein and the 3' terminal untranslated region of banana bract mosaic virus (Bateson and Dale, 1995) opens the opportunity for using pathogen-derived resistance to this virus, too. Transformation experiments are now in progress in this direction in the authors' laboratories.

Gene transfer in banana also provides a tool to engineer resistance against nematodes. Basically three strategies can be used: (i) expression of lytic enzymes or nematicidic proteins in transgenic banana for direct killing of nematodes, (ii)

cloning and expression of (heterologous) plant genes conferring resistance to nematodes, and (iii) interfering with the nematode–plant interaction by expressing, e.g. proteins blocking the nematode–plant recognition or plantibodies raised against essential proteins secreted by the nematodes (De Waele *et al.*, 1994). At present, the first strategy appears to have the best perspective against the migratory endoparasitic nematodes of banana, since no genes for resistance to this group of nematodes have been identified and the nematode–plant interaction is not yet well understood.

FRUIT QUALITY CONTROL

Modern molecular techniques have been used over the last eight years to create transgenic tomatoes with enhanced fruit ripening characteristics (Klee, 1993). This has led to the 1994 introduction of the FlavrSavr™ tomato by Calgene, Inc. after the company obtained FDA approval for their new food crop which contains a transgene that inhibits a fruit-softening enzyme. A number of technologies developed for tomato are very likely to be directly applicable to other fresh fruits.

An obvious target for genetic modification is ethylene biosynthesis in banana (reviewed by Clendennen *et al.*, in press). Bananas are climacteric fruits that can be induced to ripen by ethylene at any maturity stage. Reduction in ethylene biosynthesis in bananas would result in fruit with an extended green life which will prove beneficial to the producer and consumer alike. Not only will the fruit remain at the customer in preferred stage of ripeness for longer periods of time, but the producer/shipper will reduce losses due to premature or uncontrolled ripening. In addition, this technology will make feasible the shipment of novel banana varieties that are otherwise not commercially viable. Ethylene reduction may also have the added benefit of reducing the detrimental biochemical and physiological responses to fungal pathogens or mechanical wounding.

Other areas of enhancing value-added traits in banana fruit include: (i) modification of carbohydrate biochemistry during fruit filling, to increase yield, or during fruit ripening to modify the starch/sugar interconversion in the proper contexts for both dessert and cooking bananas, (ii) inhibition of discolouration by phenolics in the pulp or peel as a result of mishandling or environmental stresses, and (iii) enhancement of nutritional value by increasing the levels of low-abundance amino acids (such as methionine) through the expression of foreign proteins or synthetic polypeptides high in nutritional value.

The authors and their collaborators have made significant advancements in gaining a fundamental understanding of the changes in gene expression that are associated with the complex biochemical and physiological process which occur during banana fruit ripening. Clendennen and May (submitted), and Medina-Suarez *et al.* (in press) have recently reported their findings on differential screening of banana pulp cDNA libraries before and at the onset of ripening. Both manuscripts report the isolation and identification of transcripts that are differentially expressed during fruit ripening. Current efforts are being directed towards the isolation and characterization of the regulatory elements of these genes.

An abundant 31 kDa (p31) protein from the pulp of banana fruit has also been identified and characterized (Lopez-Gomez *et al.*, in preparation). Amino-terminal

sequence analysis and protein data bank comparisons of purified p31 demonstrates that this protein is homologous with previously characterized chitinases. Western analyses utilizing rabbit anti-p31 antibodies demonstrate that this protein is highly pulp-specific. A partial cDNA for p31 has been isolated from pulp-specific cDNA libraries by differential screening. Northern analyses of RNAs isolated from ripening stage-specific banana pulp tissues indicate that this cDNA is expressed at high levels in the pulp of unripe fruit, and that the abundance of this transcript decreases as the fruit ripens. In addition, a genomic clone containing the 5' proximal and structural element encoding p31 has been isolated and characterized. These promoter elements should prove useful for early fruit-specific expression of foreign proteins in transgenic banana plants.

PHARMACEUTICAL APPLICATIONS

Another application of molecular techniques to banana is based on a recent review of Lyons *et al.* (1996) which describes a number of demonstrations that transgenic plants are capable of producing functional proteins or peptides of pharmaceutical importance.

A number of candidate recombinant vaccines have been produced in transgenic plants. The first of these was the hepatitis B surface antigen (HBsAg) which may be used as a vaccine (Mason *et al.*, 1992). It has been demonstrated that transgenic tobacco plants can produce recombinant HBsAg (rHBsAg), and that plant-derived rHBsAg can assemble into virus-like particles (VLPs) as shown by immunoaffinity purification and by electron microscopy, respectively. It has further been demonstrated that these proteins have retained their immunogenic properties by injecting mice with plant-derived VLPs (Thanavala *et al.*, 1995). These injections resulted in an immune response which was qualitatively similar to that obtained with injections of the commercial hepatitis B vaccine (Recombivax, Merck Sharpe and Dohme). Additional investigations from these laboratories indicate that feeding potato tubers which express other candidate vaccines invokes an immune response in mice (Taq *et al.*, 1995). It is the authors' intention to express oral 'edible' vaccines against diarrheal (Richter *et al.*, 1996) and other diseases in transgenic banana plants (C.J. Arntzen, pers. comm.).

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