Genetic transformation strategies to address the major constraints to banana and plantain production in Africa

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PROMUSA
A Global Programme for *Musa* Improvement

INIBAP is a network of the International Plant Genetic Resources Institute (IPGRI), a Future Harvest Centre

FUTURE HARVEST

August 2003

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Engineering resistance to pathogenic fungi

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Introduction

Fungi are far more complex organisms than viruses or bacteria and have developed numerous strategies to survive in nature, which include saprotrophy, necrotrophy, hemibiotrophy and biotrophy. Interactions between plant pathogenic fungi and their hosts are particularly complex and involve different mechanisms, such as the production of fungal toxins and enzymes that degrade the plant cell wall and the formation of occlusions in vascular tissue (Rodriguez and Redman, 1997). It is difficult, therefore, to design a simple defense strategy, such as the use of a single gene or a few genes from the genome of the pathogen, as in the case of viruses. The development of efficient antifungal strategies requires a detailed genetic, cytological and biochemical characterization of the particular host-fungal pathogen interaction. Higher plants and other organisms including fungi use a wide range of defense mechanisms to protect themselves against fungal invasion, some of which might be utilized in gene transfer technologies.

Fungal diseases of bananas

All major organs and tissues of bananas, including the foliage, the root system, the vascular tissues and the fruits are affected by some 50 fungal pathogens. Of these, the most important ones are those causing leaf spot diseases (black leaf streak disease caused by *Mycosphaerella fijiensis* and Sigatoka disease caused by *Mycosphaerella musicola*), Fusarium wilt, also known as Panama disease, (caused by *Fusarium oxysporum* f. sp. *cubense*) and fruit anthracnose (*Colletotrichum musae*) (Table 1). *Mycosphaerella* pathogens have consecutive asexual (conidial) and sexual (ascospores) cycles, whereas only the asexual state is known in Fusarium. Spores of *Mycosphaerella* can spread relatively far by wind and raindrop splashes (Parnell *et al.*, 1998) whereas Fusarium commonly disseminates via infected rhizomes.

The spores of *Mycosphaerella* infect via appressoria over the stomata and produce a mass of hyphae in the intercellular space. Symptoms appear after an incubation time of 2-4 weeks. These features indicate that the pathogen (at least initially) has the characteristics of a biotroph. Black leaf streak disease has been present in East Africa since the end of the 1980s and was first reported in Uganda in 1990 (Tushemereirwe and Waller, 1993). Many East African highland banana cultivars are susceptible and the disease commonly occurs in the region, although data on production losses and the economic impact of the disease are not well known.

Chlamydospores of Fusarium mainly enter root tips, and occasionally wounded surfaces. After germination, microconidia colonize the xylem vessels using the transpiration flux and finally block the vascular system, which results in typical

wilt symptoms. Panama disease (mainly the so-called race 1) has become common in East Africa since the early 1990s (Tushemereirwe and Ploetz, 1993), but the susceptibility of highland banana cultivars is still debated (Ploetz *et al.*, 1994; Kangire *et al.*, 2001) and the distribution and economic impact of the disease has not been studied systematically in the region.

Table 1. Major fungal diseases of banana

Name	Pathogen (teleomorph)	Host range	Occurrence	Damage
Mycosphaerella complex	*Mycosphaerella fijiensis* Morelet and *Mycosphaerella musicola* Leach	All banana types	Worldwide	Chlorotic and necrotic leaves
Fusarium wilt (Panama disease)	*Fusarium oxysporum* Schlect. f. sp. *cubense* Snyder and Hansen	All *Musa* spp. most *Heliconia* spp.	Worldwide	Xylem invasion, vascular colonization and wilt
Anthracnose	*Colletotrichum musae* (Berk. and Curtis) Arx	AAA/AAB bananas	Commercial plantations	Fruit spots and lesions

Based on Jones (1999)

Much can be learned from research on pathogenic fungi related to the ones infecting banana. Though it is a temperate climate pathogen, *Mycosphaerella graminicola* could be an interesting candidate, especially because of recently initiated functional genomic studies (Hamer *et al.*, 2001; Palmer and Skinner, 2002). In addition, an EST (expressed sequence tags) database of *M. graminicola* has been initiated (Keon *et al.*, 2000), which currently contains 5181 ESTs assembled into 2926 unique sequences (http://cogeme.ex.ac.uk). Current research on the improvement of fungal disease resistance by genetic modification of bananas is summarized in Table 2.

Table 2. Current transgenic research on resistance to fungal diseases

Institute	Pathogen	Gene	Cultivar	State of research
BARC	*Fusarium oxysporum* f. sp. *cubense* *Mycosphaerella musicola*	Magainin (MSI-99)	Rasthali (AAB)	Laboratory test (Chakrabarti *et al.*, 2003)
QUT	*Fusarium oxysporum* f. sp. *cubense*	R gene(s) to be cloned	Cavendish	Map-based cloning
KULeuven	*Mycosphaerella fijiensis*	AMPs, chitinases	AAB plantains	Laboratory and greenhouse tests
Syngenta	*Mycosphaerella fijiensis*	AMPs	Grande naine	Field tests

BARC – Bhabha Atomic Research Centre, Bombay (India)
QUT – Queensland University of Technology, Brisbane (Australia)
KULeuven – Catholic University of Leuven (Belgium);
AMP – antimicrobial peptides

Host-derived resistance strategies

Inhibition of fungal penetration

One of the first barriers plant pathogens, in particular necrotrophs, encounter during penetration and subsequent colonization is the plant cell wall. Fungal pathogens secrete a number of enzymes to degrade the major plant cell-wall polymers. The major enzymes used for this purpose are cutinases, endopolygalacturonases and pectate-lyases. In response, higher plants apply a number of strategies to inhibit penetration of the pathogen through the cell wall.

One strategy is cell wall reinforcement. This is a complex process that involves the rapid synthesis of phenolic compounds leading to the accumulation of lignins in the cell wall and the synthesis of hydroxyproline-rich and other glycoproteins to strengthen the extracellular matrix. Another protective measure is initiated by the degradation of cell walls. Cell wall components, such as sugar oligomers, which are released by fungal pectinolytic enzymes, can serve as elicitors to activate plant defense reactions. It is possible, therefore, that the engineered expression of a pectinolytic enzyme may result in the transgenic plant having an activated defense status. Wegener *et al.* (1996) found that when the pectate-lyase gene from the bacterium *Erwinia carotovora* was expressed in potato tubers, the transgenic plants were resistant to infection by this bacterium. However, this strategy has not yet been tested against fungal pathogens.

Plants also synthesize proteins that inhibit fungal enzymes that degrade plant cell wall homogalacturonans to small monomeric uronides. One such inhibitor is the polygalacturonase-inhibiting protein (PGIP), which is specific to fungal endopolygalacturonase and secreted into the extracellular matrix. It is believed that PGIPs only slightly inhibit fungal polygalacturonases and this leads to the production of longer, oligomeric degradation products, which are large enough to act as elicitors of plant defense responses (Cervone *et al.*, 1989). A gene encoding PGIP has been isolated from bean (Toubart *et al.*, 1992) and it became possible to test if PGIP expression in transgenic plants does confer resistance. However, the transgenic tomato expressing the bean PGIP was not resistant to *F. oxysporum*, *Botrytis cinerea* or *Alternaria solani* and the purified recombinant PGIP did not inhibit fungal polygalacturonases (Desiderio *et al.*, 1997). The authors concluded that the expression of more than one PGIP might be required to confer resistance to fungi. In contrast, transgenic expression of a pear PGIP in tomato resulted in inhibition *in vitro* of polygalacturonase activity from *B. cinerea* and delayed symptom developments *in vivo* (Powell *et al.*, 2000). Recently, Ferrari *et al.* (2003) have characterized two differentially regulated PGIP genes in *Arabidopsis* and demonstrated that their overexpression in *Arabidopsis* significantly reduced the disease symptoms caused by *B. cinerea*.

Phytoalexins

Phytoalexins are low molecular weight (<ca. 1000 Da) antimicrobial (primarily antifungal) plant secondary metabolites that are synthesized or accumulated in

response to infection or a stress related to infection. More than 350 phytoalexins have been reported to be present in vegetative and generative parts of higher plants in about 30 botanical families (Kuć, 1995). While a third of them have been isolated from leguminous plants, banana and its relatives also appear to contain at least 25 phytoalexins including resveratrol (Hölscher and Schneider, 1996), musanolones (Luis *et al.*, 1996) and phenalenone-type phytoalexins (Hirai *et al.*, 1994; Luis *et al.*, 1994 and 1995; Binks *et al.*, 1997; Hölscher and Schneider, 1998 and 2000; Kamo *et al.*, 1998, 2000 and 2001). The concentration of some of the latter phytoalexins and intermediate products showed a correlation with resistant or susceptible phenotypes to *M. fijiensis* and *F. oxysporum* f. sp. *cubense* (Otálvaro *et al.*, 2002).

Although the production of phytoalexins is induced by infection, their involvement in disease resistance is by no means certain. However, two lines of evidence strongly suggest that some phytoalexins may play a role in resistance in certain plant-fungus interactions. Firstly, numerous fungal pathogens have active detoxifying systems against phytoalexins (VanEtten *et al.*, 1995). Pisatin, a phytoalexin from pea is enzymatically inactivated by a pisatin demethylase from *Nectria haematococca*. When the corresponding fungal gene was transferred to the maize pathogen *Cochliobolus heterostrophus*, a non-pathogen of pea, the fungus showed a limited virulence on pea (Schäfer *et al.*, 1989). This indicates that pisatin might function as an antifungal agent in pea. Secondly, transgenic suppression of phenylalanine ammonialyase results in decreased levels of chlorogenic acid, a phytoalexin in tobacco, which confers increased susceptibility to *Cercospora nicotianae* (Maher *et al.*, 1994). Another enzyme, stilbene synthase catalyses the conversion of *para*-coumarate to the phytoalexin resveratrol. The expression of stilbene synthase in rice (Stark-Lorenzen *et al.*, 1997; Tian *et al.*, 1998), tobacco (Hain *et al.*, 1993), tomato (Thomzik *et al.*, 1997), barley (Leckband and Lörz, 1998), grape (Coutos-Thévenot *et al.*, 2001), alfalfa (Hipskind and Paiva, 2000) and wheat (Liang *et al.*, 2000) resulted in increased resistance to *Magnaporthe grisea* (*Pyricularia oryzae*), *Phytophthora infestans*, *B. cinerea*, *Phoma medicaginis* and *Erysiphe graminis*, respectively. As to the mode of action of resveratrol in plants, Schouten *et al.* (2002) have recently demonstrated that resveratrol is like a defence reservoir, which is induced upon infection with *B. cinerea*. The accumulated compound induces a phenol oxidase (laccase) from the pathogen, which in turn converts resveratrol to a more toxic dimer, viniferin. This substrate-induced self-intoxication is a good example when plants make use of pathogen-derived enzymes for self-defense, which is activated only in the presence of the pathogen.

Phenylalanine ammonia-lyase (PAL) is one of the key enzymes in the phenylpropanoid pathway, which leads to the synthesis of various defense-related compounds including phytoalexins and salicylic acid. Overexpression of a plant PAL gene in transgenic tobacco revealed decreased lesion sizes after

inoculation with *Phytophthora parasitica* pv. *nicotianae* and *C. nicotianae*, but the plants exhibited a stunted phenotype (Way *et al.*, 2002).

Though resveratrol has been found in banana rhizomes (Hölscher and Schneider, 1996), it may be absent in leaves and fruit. High and constitutive expression of stilbene synthase in banana fruit may confer resistance to preharvest and postharvest diseases.

Antimicrobial peptides

Several reviews have been published recently on the characterization and application of various classes of plant proteins with distinct antimicrobial activities (Broekaert *et al.*, 1997; Shewry and Lucas, 1997; Yun *et al.*, 1997). Antimicrobial peptides (AMPs) have a broad-spectrum antimicrobial activity against fungi as well as bacteria and most are non-toxic to plant and mammalian cells.

Thionins

Besides their antibacterial activity, thionins inhibit the growth *in vitro* of about 20 different fungal plant pathogens including *B. cinerea*, *Fusarium* spp., *P. infestans* and *Rhizoctonia solani* (Cammue *et al.*, 1992; Molina *et al.*, 1993a). Thionins, which have a mass of 5 kDa and are six or eight cysteine-containing basic peptides, are divided into five classes. Class III thionins are viscotoxins from the plant semiparasite *Viscum album* L. including the 46-aa ligatoxins from mistletoe (Li *et al.*, 2002). Holtorf *et al.* (1998) have expressed viscotoxin A3 in *Arabidopsis thaliana* and transgenic plants showed increased resistance to infections of the clubroot pathogen *Plasmodiophora brassicae*. Epple *et al.* (1997) also observed that constitutive overexpression of an endogenous thionin in transgenic *Arabidopsis* resulted in enhanced resistance against *F. oxysporum* f. sp. *matthiolae*, which indicates that thionins are defense proteins. As to their mode of action, thionins are known to form cation-selective ion channels (Hughes *et al.*, 2000) by binding to phosphatidylserine head groups in lipid bilayer membranes (Coulon *et al.*, 2002), which causes permeabilization and oxidative burst followed by cell death in target cells (Bussing *et al.*, 1998) and via hypersensitive reaction in the host tissue as well (Hilpert *et al.*, 2001). More recent molecular modeling data suggest that thionins might be a novel group of DNA-binding proteins (Romagnoli *et al.*, 2000; Li *et al.*, 2002) belonging to helix-turn-helix type proteins, though experimental evidence is currently lacking for binding of thionins to DNA. As thionins are located in the extracellular space as well as intracellularly, it is proposed that thionins have a double function: extracellular thionin exerts the activity via the pore-forming action whereas intracellular thionin may enhance the transcription of proteins that direct programmed cell death.

Plant defensins

The number of known plant defensins, which are structurally related to insect defensins (Broekaert *et al.*, 1995) and contain eight disulphide-linked cysteines, is

steadily increasing. They are constitutively secreted from seeds and flowers or induced mainly in leaves by pathogen infection. At least 20 plant species are now known to contain more than 80 defensin genes (Thomma *et al.*, 2002) including those recently discovered in bell pepper (Meyer *et al.*, 1996), broad bean (Zhang and Lewis, 1997), spinach (Segura *et al.*, 1998), maize (Kushmerick *et al.*, 1998) and *Nicotiana alata* and petunia (Lay *et al.*, 2003). So far, the constitutive expression of three defensins in transgenic plants has delivered strong evidence about their potential for controlling phytopathogenic fungi. The radish defensin *Rs*-AFP2 conferred partial resistance to the tobacco pathogen *Alternaria longipes* (Terras *et al.*, 1995). A defensin from alfalfa provided resistance to *V. dahliae* in potato in the greenhouse as well as in the field (Gao *et al.*, 2000). Finally, overexpression of the WT1 defensin gene from wasabi (Japanese horseradish) in rice resulted in significantly reduced lesion size after inoculation with *M. grisea* (Kanzaki *et al.*, 2002) and extracts purified from *Nicotiana benthamiana* after virus-mediated expression of WT1 were highly active against *M. grisea* and *B. cinerea* (Saitoh *et al.*, 2001). Two members of another defensin family from pea have also been expressed in canola (Wang *et al.*, 1999) and in tobacco (Lai *et al.*, 2002) but only extracts were tested positive *in vitro* to several fungal pathogens including *F. oxysporum* f. sp. *pisi* and *Mycosphaerella pinodes*. As to their mode of action, defensins (at least the one from dahlia) appear to specifically permeabilize fungal membranes via a direct or indirect interaction with membrane sphingolipids rather than with phosphoglycerolipids, as is the case with other AMPs (Thevissen *et al.*, 2000).

In laboratory assays, several defensins, isolated from radish and dahlia have been found toxic to *M. fijiensis* and *F. oxysporum* f. sp. *cubense* (Cammue *et al.*, 1993). The cDNAs encoding three different defensins (from radish, dahlia and horse chestnut) have been transferred to banana (Remy *et al.*, 1998).

Non-specific lipid-transfer proteins

Non-specific lipid-transfer proteins (nsLTPs) in plants are highly basic, 9-10 kDa peptides containing eight disulphide-linked cysteines (Kader, 1996). A few nsLTPs with moderate or high antifungal activity to a broad range of fungi including *B. cinerea*, *Fusarium* spp., *M. grisea* and *Trichoderma viridae* have been isolated from radish (Terras *et al.*, 1992), onion (Cammue *et al.*, 1995) and cereals (Molina *et al.*, 1993b; Segura *et al.*, 1993; García-Olmedo *et al.*, 1995; Velazhahan *et al.*, 2001). The onion nsLTP (*Ace*-AMP2) has been recently expressed in transgenic geranium (Bi *et al.*, 1999) and shown to have increased resistance to leaf infection by *B. cinerea*. The cDNA for *Ace*-AMP2 has also been transferred to several banana varieties, which are currently analysed in greenhouse infection tests.

Non-enzymatic chitin-binding proteins

In addition to class I endochitinases that contain a chitin-binding domain, other chitin-binding proteins such as hevein from rubber tree, a lectin from stinging

nettle (Raikhel *et al.*, 1993) and AMPs from seeds of amaranth (De Bolle *et al.*, 1993) and *Pharbitis nil* L. (Koo *et al.*, 1998), and from leaves of wasabi (Kiba *et al.*, 2003) are also inhibitory *in vitro* to a diverse range of pathogenic fungi.

A cDNA encoding hevein was expressed in Indian mustard (*Brassica juncea*) and conferred resistance in the greenhouse to *Alternaria brassicae* (Kanrar *et al.*, 2002a). Lectins, in general, may be suitable for the control of insects or nematodes (Peumans and Van Damme, 1995; Kanrar *et al.*, 2002b).

The gene encoding the amaranth AMP has been introduced to tobacco, but no increased resistance to *A. longipes* was observed, which might have been caused by the sensitivity of this AMP to the presence of antagonistic cations (De Bolle *et al.*, 1996). In contrast, the seed-specific *Pn*-AMPs from *Pharbitis* showed antifungal activity to the oomycete *P. parasitica* in transgenic tobacco (Koo *et al.*, 2002), and both the chitin-containing fungus *F. oxysporum* and the non-chitinous pathogen *Phytophthora capsici* by *in vitro* extracts as well as in transgenic tomato (Lee *et al.*, 2003). The virus-mediated expression of the wasabi *Wj*AMP-1 in *N. benthamiana* also resulted in growth inhibition of several fungi and bacteria (Kiba *et al.*, 2003). In this case, however, the mature WjAMP-1 lacked the N-terminal hevein domain.

Other plant AMPs

Distinct AMPs containing four disulphide-linked cysteine residues have been isolated from maize seeds (Duvick *et al.*, 1992) and *Impatiens balsamina* (Tailor *et al.*, 1997). Another distinct, highly basic AMP (*Mi*AMP1) with a mass of 8.1 kDa and six cysteine residues has been purified from nut kernels of *Macadamia integrifolia* (Marcus *et al.*, 1997) and expressed in *Escherichia coli* (Harrison *et al.*, 1999). In transgenic canola, *Mi*AMP1 was active in *in vitro* extracts to *Leptosphaeria maculans*, and lesion development after inoculation with the fungus was significantly reduced as well (Kazan *et al.*, 2002). The 63-aa snakins, which contain 12 cysteines, is a new family of AMPs from potato tubers (Segura *et al.*, 1999). Snakins have been inhibitory to a wide range of pathogenic fungi and to Gram-positive bacteria, but have not been expressed yet in transgenic plants.

Wheat puroindolines (PIN-a and PIN-b) are lipid binding, basic (isoelectric point > 10) and cysteine-rich (five disulphide bonds) seed proteins of 13 kDa. They contain a tryptophan-rich domain (cfr. indolicidin in the bacterial chapter), which together with the cysteine skeleton that is similar to (puro)thionin and nsLTPs suggests antimicrobial activity (Blochet *et al.*, 1993; Gautier *et al.*, 1994). Indeed, Dubreil *et al.* (1998) observed activity of PIN-b against five pathogenic fungi, which was abolished by the presence of mono- and divalent cations in the medium. Expression of the puroindoline genes *pinA* and/or *pinB* in transgenic rice resulted in increased tolerance to *M. grisea* and *R. solani* both in inhibition assays with plant extracts and *in planta* infections (Krishnamurthy *et al.*, 2001).

Recently, Charnet et al. (2003) have found that puroindolines cause membrane permeabilization via the formation of voltage-dependent cation channels, which could be prevented by the addition of Ca2+ ions. Though direct evidence still needs to be delivered, ion channel formation could be a mechanism for the antimicrobial activity.of puroindolines.

Whereas 2S albumins, which contain two subunits of about 9 kDa and 4 kDa, function primarily as seed storage proteins, those from seeds of plants in the *Brassica* genus are able to inhibit fungal growth. Although antagonized by inorganic cations, these 2S albumins could be enhanced by a synergistic action with thionins (Terras *et al.*, 1993). 2S albumin proteins have not yet been evaluated for disease resistance in transgenic plants.

A new type of antifungal protein has been isolated from pearl millet seeds (Joshi *et al.*, 1998). This basic, 24 kDa protein was a cysteine protease inhibitor and showed *in vitro* antifungal activity against important plant pathogenic fungi such as *Alternaria* spp., *Fusarium* spp. and *Helminthosporium* spp. In general, protease inhibitors are not strictly antimicrobial in character and are more effective against insect or nematode pests. However, Lorito *et al.* (1994) have also found that the application of a mixture of trypsin and chymotrypsin inhibitors from tobacco inhibited the spore germination of *B. cinerea* and *F. solani*. The significance of this finding awaits further confirmation.

Pathogenesis-related proteins

A large number of diverse plant proteins are synthesized *de novo* after infection with viruses, bacterial and fungal pathogens, or after treatment with biotic or chemical elicitors. These pathogenesis-related proteins (PRPs) differ in their structure, expression, and spectrum of antimicrobial activity and modes of action. Five major types of PRPs can be distinguished (Shewry and Lucas, 1997; Yun *et al.*, 1997; Kitajima and Sato, 1999).

PR-1 proteins are the most abundant PRPs and have a mass of 14-16 kDa. They have been isolated from both monocots (barley and maize) and dicots (tobacco and *Arabidopsis*), but their precise mode of action is still unknown.

PR-2 and PR-3 proteins have endohydrolytic enzyme activity and are present in a wide range of plant species. PR-2 proteins are β-1,3-endoglucanases and may induce defense reaction by releasing elicitors from the fungal cell wall. PR-3 proteins are endochitinases with a similar function, but catalyzing the hydrolysis of the other major component in the fungal cell wall. A combination of PR-2 and PR-3 proteins has a synergistic inhibitory effect on pathogenic fungi both *in vitro* and in transgenic plants (Table 3). PR-2 proteins alone can also be effective against pathogens that belong to the Oomycetes in which the cell wall does not contain chitin.

PR-4 proteins are a heterogenous group of PRPs, many of which have chitin-binding activity. Some of these proteins show homology to products of wound

inducible (*win*) genes that have been isolated from potato, barley and wheat. PR-4 proteins have not yet been expressed in transgenic plants and even their *in vitro* antifungal activity needs to be confirmed.

PR-5 proteins comprise a group of proteins, such as permatin, zeamatin and linusitin that are homologous to salt-induced osmotins and the super sweet protein thaumatin. Nevertheless, they have a clear antifungal activity against a wide range of fungi *in vitro* as well as in transgenic plants. This activity is believed to be caused by permeabilization of the fungal plasma membrane.

Within each of the above PRP groups, except for PR-4 proteins, several classes exist according to structure and expression in the cell. The two major groups, class I and class II, contain basic and acidic proteins, respectively. Acidic PRPs are secreted in the extracellular space, while the much more potent basic homologues are targeted to the vacuole. The class I (vacuolar) PR-3 proteins have a N-terminal, cysteine-rich, chitin-binding domain that is homologous to the chitin-binding region of lectins (see above). Expression of acidic PRP genes is mediated by salicylic acid and actice oxygen species (see below) whereas ethylene and methyl jasmonate are known to mediate the expression of basic PRP genes. Surprisingly, PR-1-like and PR-5-like proteins or genes have also been identified in the animal kingdom, though their functions are not yet known.

Recently, six new groups of PRPs have been classified, which are proteinase inhibitors (PR-6), proteinases (PR-7), chitinases with lysozyme activity (PR-8), extracellular peroxidases (PR-9), and other PRPs (PR-10 and 11). The expression of a peroxidase from the tropical legume *Stylosanthes humilis* in transgenic tobacco has resulted in reduced symptom development after infection with *P. parasitica* var. *nicotianae.* In canola, the same peroxidase reduced symptoms caused by *Leptosphaeria maculans* (Kazan *et al.*, 1998a)

Table 3 lists several PRPs that have been expressed in transgenic plants and conferred increased resistance to various plant pathogenic fungi. A possible deleterious side effect of PRPs in transgenic plants may be their action against mycorrhizal fungi, which are important for stimulating the growth of many plants including banana (Declerck *et al.*, 1995; Jaizme-Vega and Azcón, 1995). When class I (vacuolar) PR-2 or PR-3 proteins, the most commonly used PRPs for transformation, have been expressed in transgenic plants, no harmful effect has been observed on the symbiotic bacterium *Rhizobium meliloti* (Masoud *et al.*, 1996) or on the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Vierheilig *et al.*, 1995). However, a delay of colonization by *G. mosseae* was observed in tobacco plants expressing a class II (extracellular) PR-2 protein (Vierheilig *et al.*, 1995) indicating that transgenic expression of antifungal proteins in the apoplast, which is preferred for efficient protection, may have adverse effects on endophytic symbionts.

Table 3. Examples of transgenic resistance to fungal diseases conferred by genes encoding for pathogenesis-related proteins (PRP) (Someone retyped this table from the original landscape version and made a few mistakes)

PRP gene (origin)	Fungal pathogen	Transgenic plant	Observed effect	Reference
Class II PR-1a (tobacco)	*Peronospora tabacina* *Phytophthora parasitica*	Tobacco	Disease symptoms reduced	Alexander *et al.* (1993)
Class I PR-2 (soybean)	*Phytophthora parasitica* *Alternaria longipes*	Tobacco	Symptoms significantly reduced	Yoshikawa *et al.* (1993)
	Phytophthora infestans	Potato	Disease symptoms reduced	Borkowska *et al.* (1998)
	Botrytis cinerea	Kiwifruit	Disease symptoms reduced	Nakamura *et al.* (1999)
Class II PR-2 (alfalfa)	*Phytophthora megasperma*	Alfalfa	Disease symptoms reduced	Masoud *et al.* (1996)
	Cercospora nicotianae	Tobacco	Disease symptoms reduced	Zhu *et al.* (1994)
Class I PR-3 (bean)	*Rhizoctonia solani*	Tobacco, canola	Disease symptoms reduced	Broglie *et al.* (1991)
Class I PR-3 (tobacco)	*Rhizoctonia solani*	*Nicotiana sylvestris*	Disease symptoms reduced	Vierheilig *et al.* (1993)
	Botrytis cinerea, *R. solani*	Carrot	Disease symptoms reduced	Punja and Raharjo (1996)
	Sclerotinium rolfsii			
Chimaeric class I PR-3 (tomato-tobacco)	*Sclerotinia sclerotiorum* *Phoma lingam*	Oilseed rape	Disease symptoms reduced	Grison *et al.* (1996)
Class I PR-3 (rice)	*Cercospora nicotianae*	Tobacco	Disease symptoms reduced	Zhu *et al.* (1994)
	Rhizoctonia solani	Rice (indica)	Disease symptoms reduced	Lin *et al.* (1995)
	Botrytis cinerea	Cucumber	Symptoms significantly reduced	Tabei *et al.* (1998)
	Diplocarpon rosae	Rose	Disease symptoms reduced	Marchant *et al.* (1998)
Class II PR-2 (alfalfa)/ class I PR-3 (rice)	*Cercospora nicotianae*	Tobacco	Symptoms significantly reduced	Zhu *et al.* (1994)
Class II PR-2/PR-3 (barley)	*Rhizoctonia solani*	Tobacco	Symptoms significantly reduced	Jach *et al.* (1995)
Basic PR-5 (tobacco)	*Phytophthora infestans*	Potato	Disease symptoms reduced	Liu *et al*. (1994)

Ribosome-inactivating proteins

The RNA *N*-glycosidase activity of ribosome-inactivating proteins (RIPs) has been successfully applied to generate virus-resistant crops, but RIPs isolated from cereals are also active on fungal ribosomes (Stirpe and Hughes, 1989). The inhibitory effect of RIPs on the growth of fungi *in vitro* has been demonstrated (Leah *et al.*, 1991) in combination with chitinase or glucanase. A barley RIP has been expressed in transgenic tobacco either alone under the control of a wound-inducible promoter from a potato gene (Logemann *et al.*, 1992) or in combination with a barley class II chitinase (Jach *et al.*, 1995). In both cases, increased resistance to *R. solani* was observed with enhanced protection when the combined expression strategy was used. Since then, a maize RIP (Maddaloni *et al.*, 1997) and a mutant of the pokeweed RIP that is non-toxic to plants have been used in transgenic tobacco to confer resistance to *R. solani* (Zoubenko *et al*., 1997).

Antibodies

The concept of plant antibodies for pathogen control is developed in the section on bacterial pathogens. A few potential applications for plant-fungus control are mentioned here. Antibodies for immunodiagnosis of pathogenic fungi (Bossi and Dewey, 1992) are not automatically useful for control because they should not only be able to simply bind a specific target but by interfering with it they should stably inhibit the pathogen. One possible direction could be the inhibition of spore germination by a surface-binding antibody, as demonstrated *in vitro* with zoospores of *Peronospora capsici* (Bishop-Hurley *et al.*, 2002). Mycelial growth has also been inhibited by monoclonal antibodies (Hiatt *et al.*, 2001). Another direction could be the immunological inactivation of fungal toxins either for crop protection or for protection of animal and human health (Yuan *et al.*, 2000). This requires still long-term research for application in banana.

Induction of an oxidative burst and hypersensitive response

As described for defense mechanisms activated by bacterial infections, fungal attack also results in an oxidative burst in plants, *i.e.* the release of reactive oxygen species (Lamb and Dixon, 1997). Generation of hydrogen peroxide by the expression of a fungal glucose oxidase in potato has also resulted in enhanced resistance to late blight disease caused by *P. infestans* (Wu *et al.*, 1995). This resistance has been linked to an increase in lignin content and the accumulation of peroxidases plus a class II PR-3 protein (Wu *et al.*, 1997). Increased resistance to *C. nicotianae* has also been correlated with the induction of the PR-1a gene (Kazan *et al.*, 1998b). It is possible that plant genes, such as germin that encodes an oxalate oxidase (Berna and Bernier, 1997), can also be used for this purpose.

Deák *et al.* (1999) have described the transgenic use of ferritin, an iron-binding protein, to reduce cell damage caused by a variety of environmental stress conditions. It is proposed that overexpression of ferritin in transgenic plants would give protection to oxidative stress, which is caused amongst other things by pathogen infection. Overexpression of alfalfa ferritin in transgenic tobacco has resulted in increased resistance to infections of *Alternaria alata* and *B. cinerea.*

Mittler *et al.* (1995) have introduced into tobacco a gene from *Halobacterium halobium*, which encodes a proton pump called bacterio-opsin (bO), and observed a disease lesion mimic phenotype (a hypersensitive response in the absence of a pathogen) in various organs of the transgenic plants. These plants turned out to be resistant to infections with TMV or *Pseudomonas syringae* pv. *tabaci*, but fungal pathogens were not tested. More recently, the same bO gene was transferred to potato and the same lesion mimic phenotype was again observed (Abad *et al.*, 1997). The transgenic plants were resistant to an isolate of *P. infestans,* but not to another isolate of the same pathogen, and were susceptible to infections with potato virus X and the bacterial pathogen *E. carotovora.* This result indicates that such induced defence reactions may be specific to certain pathogens or distinct plant-pathogen interactions.

Plant resistance genes and fungal avirulence genes

At least 26 fungal disease-resistance genes have been cloned so far by either transposon tagging or map-based cloning (Table 4). Many of these genes show structural similarity indicating a similar function, which is related to signalling a pathogen attack. Of particular interest for banana is the *I2* gene from tomato that confers resistance to *F. oxysporum* (Ori *et al.*, 1997). In tomato, three loci have been mapped which confer resistance to *F. oxysporum* f. sp. *lycopersici* race 1, race 2, and to races 1, 2 and 3, respectively. The *I2* gene that confers resistance to race 2 contains a cluster of at least four homologous genes. Transgenic expression of antisense genes in plants containing the *I2* gene conferred a high susceptibility to race 2, but the plants remained completely resistant to race 1. This result indicates that the *I2* gene is indeed specific to race 2 and may not be a promising candidate for transformation into banana in an attempt to create resistance to *F. oxysporum* f. sp. *cubense*.

At the time of writing, eight fungal avirulence genes had been cloned. Five of these genes direct race-specific interactions with specific plant resistance genes as predicted by the gene-for-gene hypothesis (Laugé and de Wit, 1998). However, the other three genes direct a broader, species-specific, plant-pathogen interaction. Interestingly, the low-molecular-weight protein products of these avirulence genes appear to be elicitins, a proteinaceous class of elicitors that are able to trigger a broad-spectrum defense reaction. This indicates that at least some of the elicitins may have functions related to avirulence and perhaps to virulence. One of the most active elicitins is cryptogein, which is produced by *Phytophthora cryptogea*. Recently, Keller *et al.* (1999) have generated transgenic tobacco plants containing a fusion between a pathogen-inducible tobacco promoter and the cryptogein gene. Infection with *Phytophthora parasitica* turned on cryptogein production in the transgenic plants that induced a localized hypersensitive response and activation of defense-related genes. As a result, these plants displayed a broad-spectrum resistance to several unrelated fungal pathogens, such as *B. cinerea* and *Erysiphe cichoracearum*.

Table 4. Cloned fungal disease-resistance genes (until 1997)

Resistance gene	Plant	Pathogen	Avirulence gene	Structure	Cloning method	Reference
Hm	Maize	*Cochliobolus carbonum*	none	enzyme	Transposon tagging	Johal and Briggs, 1992
L6	Flax	*Melampsora lini*	*AL6*	NBS-LRR	Transposon tagging	Lawrence *et al.*, 1995
M	Flax	*Melampsora lini*	*AM*	NBS-LRR	Transposon tagging	Anderson *et al.*, 1997
RPP5	*Arabidopsis*	*Peronospora parasitica*	*avrPp5*	NBS-LRR	Map-based cloning	Parker *et al.*, 1997
I2	Tomato	*Fusarium oxysporum*		NBS-LRR	Map-based cloning	Ori *et al.*, 1997
Cf-9	Tomato	*Cladosporium fulvum*	*Avr9*	LRR-TM	Transposon tagging	Jones *et al.*, 1994
Cf-4	Tomato	*Cladosporium fulvum*	*Avr4*	LRR-TM	Map-based cloning	Thomas *et al.*, 1997
Cf-2	Tomato	*Cladosporium fulvum*	*Avr2*	LRR-TM	Map-based cloning	Dixon *et al.*, 1996
Mlo	Barley	*Erysiphe graminis*		new	Map-based cloning	Büschges *et al.*, 1997

NBS - nucleotide binding site
LRR - leucine-rich repeat
TM - transmembrane region

Non-host-derived strategies

Non-plant antimicrobial peptides

The antibacterial properties of cecropin are well known, but its potential for fungus control in plants has been discovered only recently. Both cecropin (De Lucca *et al.*, 1997; Alan and Earle, 2002) and its derivatives (D5-C: Qui *et al.*, 1995; D4E1: De Lucca *et al.*, 1998; Rajasekaran *et al.*, 2001) as well as its hybrid peptides with melittin (Cavallarin *et al.*, 1998) have been found to inhibit the *in vitro* growth of several important fungal pathogens including *A. solani*, *B. cinerea*, *Leptosphaeria maculens*, *P. infestans* and *P. parasitica*, *Verticillium albo-atrum* and *V. dahliae*, and numerous f. sp. of *F. oxysporum*. The same proteins, cecropin and its derivatives (D4E1 and D2A21) as well as a derivative of magainin interacted *in vitro* with the plasma membrane of several ascomycete tree pathogens (Rioux *et al.*, 2000) or inhibited their growth *in vitro* (Jacobi *et al.*, 2000). The cecropine analog D4E1 was also more resistant to protease degradation than the natural cecropine (De Lucca *et al.*, 1998) but the *in vitro* activity of D2A21 was abolished in the presence of divalent cations (Jacobi *et al.*, 2000). When recombinant D4E1 was purified from transgenic tobacco it significantly inhibited the growth of *Aspergillus flavus* and *V. dahliae* and showed increased *in planta* resistance to *Colletotrichum destructivum* (Cary *et al.*, 2000). Besides bacterial pathogens, the cecropin-melittin chimaeric peptide MsrA1 was also active in transgenic potato against *Fusarium solani* and *Phytophthora cactorum* (Osusky *et al.*, 2000).

Similarly, Kristyanne *et al.* (1997) have demonstrated that magainin, another bacterial membrane-interactive peptide, interfered with membrane integrity and inhibited the *in vitro* growth of the plant pathogenic fungi *F. oxysporum*, *R. solani* and *V. dahliae*. Alan and Earle (2002) also found that MSI-99, a synthetic magainin, was active *in vitro* against *A. solani* and *P. infestans*. Transgenic poinsettia plants expressing a magainin analogue displayed increased resistance to powdery mildew (*Oidium* sp.) apparently caused by reduced infection and conidial production of the pathogen (Smith *et al.*, 1998). More recently, Chakrabarti *et al.* (2003) reported that MSI-99 inhibited the *in vitro* growth and spore germination of *F. oxysporum* f. sp. *cubense* at 16 mg/L concentration though efficient inhibition could be observed only at significantly higher concentrations. Two constructs were then made to target MSI-99 to the cytoplasm or the extracellular space under the control of an *Arabidopsis* ubiquitin promoter and introduced to tobacco and banana via *Agrobacterium*-mediated transformation. Transgenic plants were confirmed by PCR-Southern analysis and by RT-PCR and tested with inoculations of detached leaves or the corm/root system. Six out of seven transgenic tobacco plants showed reduced lesion sizes after infection with *Alternaria alternata*, *B. cinerea* and *S. sclerotiorum*, though one transgenic plant proved to be even more susceptible than controls. About half of the transgenic banana plants showed less discoloration in the corm after inoculation with spores of *F. oxysporum* f. sp. *cubense* and no striking difference was found between the two constructs used, whereas no major effect was observed after inoculations with *M. musicola*. A major limitation of this work is that expression and cellular localization of MSI-99 was not demonstrated at protein level and thus quantitative correlation between the resistant (or susceptible) phenotype and MSI-99 expression was not established.

Besides resistance to *Pseudomonas* spp., the transgenic tobacco plants containing a mutant esculentin of 46-aa in the intercellular fluid (Ponti *et al.*, 2003) also showed increased resistance to *Phytophthora nicotianae*.

Drosomycin, the first defensin type antimicrobial peptide from *Drosophila melanogaster* (Fehlbaum *et al.*, 1994), was found to show a structural similarity to the *Rs*-AFP2 antifungal defensin from radish (see above, p. 7) (Landon *et al.*, 2000). Recombinant forms of drosomycin and a related but more salt-insensitive defensin heliomicin (Lamberty *et al.*, 1999) both possessed a potent inhibitory activity to germinating spores and/or growing mycelia of a wide range of pathogenic fungi, including *F. oxysporum* in the case of drosomycin (Banzet *et al.*, 2002). When expressed in tobacco plants, both defensins were processed and targeted to the apoplast correctly and retained antifungal activity *in vitro* as well as *in planta* after inoculation with spores of *C. nicotianae* (Banzet *et al.*, 2002). A more potent mutant of heliomicin has been generated and characterized for antimicrobial spectrum (Lamberty *et al.*, 2001).

On the basis of their broad-spectrum activity against fungal as well as bacterial pathogens, individual or combined expression of cecropin, magainin and their derivatives in banana may result in increased resistance to several pathogens. However, problems related to *in planta* expression and stability still need to be solved. Synthetic peptides from combinatorial libraries can also be effective in controlling fungal plant pathogens (*F. oxysporum* and *R. solani*) as demonstrated by Reed *et al.* (1997).

Non-plant hydrolytic enzymes

Chitin is commonly found in cell walls of most fungi (except for the class Oomycetes) or in the exoskeletons of insects. These organisms need to synthesize chitinases in order to outcompete other species and to regulate their own growth and development. Other organisms, in particular bacteria or entomopathogenic nematodes, also contain chitinases in order to degrade the chitin polymer for food. Though a number of fungal chitinase genes, especially from mycoparasitic fungi (Blaiseau and Lafay, 1992; Hayes *et al.*, 1994), have been available for several years, the first gene (from *Rhizopus oligosporus)* was only recently introduced into plants (Terakawa *et al.*, 1997). The transgenic tobacco plants produced were resistant to *Sclerotinia sclerotiorum* and *B. cinerea*. The activity of chitinolytic endochitinases from the mycoparasite *Trichoderma harzianum* has been compared to that of plant chitinases. These enzymes exerted a much stronger activity to almost all pathogenic fungi tested than the plant chitinases, which showed only weak activity against a few fungal species (Lorito *et al.*, 1993). This result indicated that a single chitinase might produce a broad-spectrum resistance to plant pathogenic fungi. This was confirmed by transgenic expression of the corresponding gene whose *in planta* activity conferred resistance not only to the foliar pathogens *B. cinerea, Venturia inaequalis* and two *Alternaria* species, but also to the soil-borne pathogen *R. solani* (Bolar *et al.*, 1997; Lorito *et al.*, 1998; Mora and Earle, 2001). The antifungal effect against *V. inaequalis* was further enhanced by the synergistic action of a co-expressed exochitinase (*N*-acetylhexosaminidase) from *T. harzianum* in transgenic apple (Bolar *et al.*, 2001). No effect of the fungal endochitinase was observed on the life cycle of the symbotic mycorrhiza *Gigantus margaritus* (Lorito *et al.*, 1997a).

Chitinase genes of bacterial origin have not been used extensively. This may be because they are exochitanases and presumed to be less active against fungi (Roberts and Selitrennikoff, 1988). However, when Jones (1988) and Suslow *et al.* (1988) transferred the chitinase gene of *Serratia marcescens* into tobacco, the transgenic plants exhibited elevated chitinase activity and increased resistance to *A. longipes*. Later, Howie *et al.* (1994) found that the same gene provided tolerance to *R. solani* in the field. The protective role of bacterial chitinases was also confirmed by Toyoda *et al.* (1991a) who injected chitinase from *Streptomyces griseus* into barley epidermis cells, and found that the enzyme digested the

haustoria of *E. graminis*, the powdery mildew pathogen. Other sources such as chitinases from nematode-associated bacteria (Chen *et al.*, 1996) or insects (Ding *et al.*, 1998) may also be effective against fungal infections.

Glucan is another component of the fungal cell wall. Glucanases have also been purified from numerous bacteria as well as from fungi including mycoparasites (de la Cruz *et al.*, 1995; Lorito *et al.*, 1997b) and may be as effective as chitinases. However, bacterial glucanase genes transferred to plants (Darbinyan *et al.*, 1996; Jensen *et al.*, 1996; Monzavi-Karbassi *et al.*, 1998) have not yet been tested for resistance to plant pathogenic fungi.

Chitosan, which is a deacetylated derivative of chitin, is another important polymer component of the fungal cell wall, especially of species in the class Zygomycetes. Chitosan and its oligomers have been shown to have antifungal activity (El Ghaouth *et al.*, 1992) and to elicit defense reactions (Kendra *et al.*, 1989). Therefore, genes encoding chitosanase, a chitosan-degrading enzyme, may be useful in transgenic plants. El Quakfaoui *et al.* (1995) found that the recombinant chitosanase of *Streptomyces* sp. inhibited the growth of *Rhizopus nigricans* (a Zygomycete) and also *F. oxysporum* and *V. albo-atrum*. The enzyme was also active in transgenic tobacco.

Human lysozyme, unlike some animal and bacteriophage lysozymes, is able to degrade both bacterial peptidoglycan and chitin. This raises the possibility that it may be an efficient tool for simultaneous control of both fungal and bacterial pathogens. A synthetic human lysozyme gene has been transferred to plants and exhibited enhanced resistance to *E. cichoracearum* as well as the bacterium *P. syringae* in transgenic tobacco (Nakajima *et al.*, 1997), and to *Erysiphe heraclei* as well as *Alternaria dauci* in transgenic carrot (Takaichi and Oeda, 2000).

Host-selective toxins

Host-selective toxins (HSTs) are produced by approximately 20 species of pathogenic fungi and are believed to be primary determinants of host range and pathogenicity (Walton and Panaccione, 1993). HSTs are toxic only to hosts susceptible to the fungus that produce them. Both HST production by the pathogen and toxin sensitivity by the host are required for disease to occur. Thus, in theory, HST-resistant plants should be less susceptible to HST-producing pathogens. Most HSTs, such as victorin, which is produced by *Cochliobolus victoriae* and is one of the most phytotoxic and most selective compounds known, are highly specific. Victorin is active against sensitive oat cultivars at 10 ng/L while resistant oats are not affected even at 10g/L. Due to this specificity some HSTs may turn out to function as elicitors of plant defense reactions (Walton, 1996). Victorin has recently been found to induce a specific proteolytic cleavage of the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme that is encoded in the chloroplasts (Navarre and Wolpert, 1999). In susceptible plants, victorin results in chlorophyll loss, which is also characteristic

of banana leaves treated with a crude toxic extract from *in vitro* cultures of *Mycosphaerella fijiensis* (Harelimana *et al*., 1997).

The banana pathogen *M. fijiensis* is reported to synthesize 2,4,8-trihydroxytetralone, a pentaketide compound that is believed to be a HST (Stierle *et al*., 1991; Okole and Schulz, 1997). However, its role in pathogenesis and any plant resistance mechanism to the toxin remains to be determined. Hoss *et al.* (2000) proposed that in the highly resistant 'Yangambi Km 5' early activation of 2,4,8-trihydroxytetralone production induces cell death and synthesis of a phytoalexin, which leads to an incompatible reaction, whereas in susceptible cultivars the toxin is produced later when compatible interaction has already been established. This is consistent with the suggested similarities between HSTs and avirulence determinants that both of them can directly elicit an array of host defense responses (Wolpert *et al.*, 2002). This phenomenon is similar to two sides of the same coin, in which timing apparently plays a crucial role. When the toxin is produced early (as in resistant genotypes) it appears to be an avirulence factor to activate host defense, and when it is released in a later phase (as in susceptible cultivars) it becomes a virulence factor causing necrosis of the host. That also raises the question that quorum sensing (see the bacterial chapter) or host factors might be responsible for triggering toxin production or both? In support of the first, the first eukaryotic quorum sensing molecule farnesol has recently been identified in the fungus *Candida albicans* (Hornby *et al.*, 2001).

As several HSTs appear to be immunogenic and anti-HST antibodies can be produced (Akimitsu *et al.*, 1992), it is possible to express antibodies that bind to a toxin in a transgenic plant to confer resistance to the toxin and the pathogen. There are so far only a few cases that antibodies (though not against HSTs) have been considered for possible fungus control (Yuan *et al.*, 2000; Hiatt *et al.*, 2001).

Non-host specific toxins

Non-host specific toxins are active on both host and non-host species, but they may nevertheless have a significant role during pathogenesis in particular plant-pathogen interactions. A large number of plant pathogenic fungi synthesize non-host specific toxins; *Fusarium* o*xysporum* f. sp. *cubense* produces fusaric acid (5-n-butyl-2-pyridine-carboxylic acid) and *Cercospora* spp. produce the polyketide toxin cercosporin. After light-activation, cercosporin generates reactive oxygen which is toxic to plants, many bacteria and fungi, but not to the fungus synthesizing the toxin. A cercosporin resistance gene has been cloned from *Cercospora nicotianae* (Chung *et al.*, 1999), and previously attempted to transfer into tobacco (Upchurch *et al.*, 1997). It is not known whether *Paracercospora fijiensis*, the imperfect (anamorph) stage of *M. fijiensis*, produces cercosporin or a related toxin and has resistance genes that could be exploited in a similar manner.

Like many other *Fusarium* spp., *Fusarium oxysporum* f. sp. *cubense*, the causative agent of *Fusarium* wilt of banana, also synthesizes fusaric acid (Thangavelu *et al.*, 2001) or its derivatives. Therefore, the isolation of toxin resistance genes from these fungi and their expression in transgenic plants may contribute to a better understanding of the role of the toxin in pathogenesis. In fact, several candidate genes have been isolated earlier (Utsumi *et al.*, 1988; Toyoda *et al.*, 1991b; Utsumi *et al.*, 1991) but never transferred to plants.

Antifungal killer toxins

Several isolates of *Ustilago maydis*, a fungal pathogen of maize, contain resident dsRNA viruses that code for antifungal killer toxins; these are polypeptides with a mass of 7-14 kDa. These fungal isolates, which are resistant to the secreted toxin, are able to gain selective advantage by killing other susceptible isolates. At least some of these toxins appear to act by the formation of ion channels in cellular membranes, and two of the three known killer toxins have been expressed in transgenic tobacco with no adverse effect on the plants (Kinal *et al.*, 1995; Park *et al.*, 1996). Recently, Clausen *et al.* (2000) demonstrated that overexpression of one killer toxin in transgenic wheat correlated with antifungal activity against *U. maydis* and with increased resistance to the wheat pathogen *Tilletia tritici*. In a greenhouse test, the transgenic wheat plants did not exerted any measurable effect on soil microarthropods after evaluation of different parameters in a feeding bioassay (Romeis *et al.*, 2003). If these killer toxins were found to be active against a broader range of plant pathogenic fungi, they could be a novel means for control.

Applications to bananas

Fungi are the cause of some of the most serious diseases of banana and thus are of the greatest economic concern to the commercial banana industry. Until genes conferring resistance to fungal pathogens are identified in banana (Wiame *et al.*, 2000), heterologous genes encoding proteins with antifungal activity are the primary targets for transgenic expression. Perhaps the most promising candidates are AMPs of plant origin (Broekaert *et al.*, 1997) as they display high *in vitro* activity against *Mycosphaerella fijiensis* and *Fusarium oxysporum* f.sp. *cubense* and are not toxic to humans or banana cells.

Particle bombardment technology (Sági *et al.*, 1995) has enabled the production of several hundreds of transgenic lines, which express different genes encoding defensin-type AMPs and nsLTPs alone or in combinations, mainly in plantains (AAB, Three hand planty, Cemsa and Navolean) and Williams (AAA, Cavendish subgroup) at KULeuven. A large-scale molecular analysis by PCR, RT-PCR, Southern and Northern hybridization and biochemical characterization (ELISA, Western analysis and antifungal assay with leaf extracts) has confirmed that a vast majority of these lines contain and express the introduced genes (Remy, 2000). Approximately 100 transgenic lines have been micropropagated for testing

the stability and effect of the transgenes in different field environments. In addition, about 200 individual plants have been transferred to the glasshouse and a simple and reproducible leaf disc bioassay has been developed for the evaluation of resistance to fungal pathogens. Computer image capturing and software based area calculation allowed for the precise measurement of the infected leaf area and for the classification of the level of resistance of each transformant (Remy *et al*., 1999). A differential disease response has been observed among transgenic plants, which ranges from no resistance to a high degree of resistance compared to control plants. However, no correlation has been found between AMP expression and resistant phenotypes. Apparently, the health status of donor plants for the leaf discs highly affects the outcome of the bioassay (Carlier, personal communication). Therefore, repeated bioassays have been performed with about 30 selected lines in collaboration with scientists at CIRAD, Montpellier (France). Highly resistant lines have been identified which show a good correlation between symptom development and AMP expression levels (Carlier, Remy, Abadie, Sági and Swennen, unpublished). In addition, in collaboration with CORBANA in Costa Rica the same set of transgenic lines is currently being tested in a greenhouse experiment.

Up to now, none of these transgenic plants has entered field trials. This is mainly due to the lack of biosafety guidelines and / or regulatory bodies in many tropical countries where these plantain plants need to be evaluated in the field under natural disease pressure. It is hoped that this obstacle to progress can soon be overcome.

More recently, gene combinations of plant AMPs with rice chitinase have been introduced into plantain and the molecular analysis of transgenic plants is in progress (Arinaitwe *et al*., unpublished).

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Engineering resistance to pathogenic bacteria

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Introduction

The development of crops resistant to bacterial pathogens through genetic engineering has not been progressing as rapidly as other areas, which is surprising given the information that is available on interactions between plants and bacteria. The first avirulence genes were cloned from bacteria (Staskawicz *et al.,* 1984) and the very first plant resistance genes defined and cloned in tomato (Martin *et al.,* 1993; Salmeron *et al.,* 1996) and Arabidopsis (Bent *et al.,* 1994; Mindrinos *et al.,* 1994; Grant *et al.,* 1995) conferred resistance specifically to strains of *Pseudomonas syringae*. Plant-bacterial interactions have thus provided one of the best systems for studying plant-pathogen relationships and the molecular basis of disease resistance in plants.

Bacterial diseases of bananas

Banana is affected by several bacterial diseases (Table 1). Most of the classical research on bacterial pathogens of banana was done in the 1950s and 1960s, with little support thereafter. As a result, host-pathogen interactions have not been characterized at the cellular and molecular levels, rapid diagnostic methods are scarce, and the diversity and epidemiology of most pathogens is not well understood. Consequently, sources of resistance are not well known and the mechanisms of infection and resistance have not been studied and, not surprisingly, the economical significance of these diseases has not been assessed thoroughly. To my knowledge, there is no ongoing research on generating bananas resistant to bacterial pathogens via genetic modification. However, some of the transgenic lines produced for fungus tolerance (e. g. those expressing antimicrobial peptides) might be worthwhile for testing against bacteria after the development of sensitive bioassays.

In principle, many of the engineering strategies outlined below may be applied to control these diseases in banana. However, before transformation experiments can proceed, detailed biochemical and genetic studies of well-characterized strains will be necessary. Initial investigations might focus, for example, on the mode of action and structure of bacterial toxins and of extracellular polysaccharides.

The complete genome sequences of *Ralstonia solanacearum* (Salanoubat *et al.,* 2002) and two pathovars of *Xanthomonas campestris* (da Silva *et al.,* 2002) have recently been determined. Comparative and functional genome analysis of these pathogens is now in progress (Van Sluys *et al.,* 2002; da Silva *et al.,* 2002).

Table 1. The main bacterial diseases of banana (based on Thwaites *et al.,* 2000)

Name	Pathogen	Host range	Occurrence	Damage
Moko	*Ralstonia* (syn. *Pseudomonas*) *solanacearum* race 2, biovar 1 (8 strains known)	AAA/AAB/ABB bananas and *Heliconia* spp.	Central and South America Caribbean and Philippines	vascular wilt
Blood disease	*Ralstonia solanacearum* (Smith 1896) Comb. Nov. (race and biovar unidentified)	ABB bananas and *Heliconia* spp.	Indonesia	vascular wilt
Bacterial wilt	*Xanthomonas campestris* pv. *musacearum*	*Ensete* spp. AAA East Africa highland bananas	Ethiopia, East Africa	vascular wilt
Rhizome rot (head rot)	*Erwinia carotovora* ssp. *carotovora* (Jones) Holland *E. chrysanthemi* Buckholder, McFadden & Dimock	AA and AAA bananas	Central America, Jamaica Israel, Papua New Guinea	pseudostem and rhizome
Bugtok (tapurok)	*Ralstonia solanacearum* race and biovar unidentified	ABB bananas	Southern Philippines	male bud, fruit
Finger-tip rot (mokillo)	*Ralstonia* (*Pseudomonas*) sp.	AAA bananas	Central America, Australia Taiwan	fruit

Antibacterial peptides and proteins

Antimicrobial cationic peptides are thought to be an important component in host defense responses against pathogenic agents. There are four structural classes of cationic antimicrobial peptides; the disulfide-linked b-sheet peptides, including the defensins; the amphipathic a-helical peptides such as the cecropins and melittins; the extended peptides, which often have a single amino acid predominating (e.g. indolicidin); and the loop-structured peptides like bactenecin. Most of these peptides interact with the bacterial membrane by forming transient ion channels and pores or blocking the membrane's own ion channels or by inhibiting the synthesis of membrane proteins. Some of these peptides are active against a wide range of organisms, which include viruses and various eukaryotes, while others have specific activity against certain groups of bacteria, e.g. Gram-negative bacteria.

Antibacterial agents from insects

Probably the best-known antibacterial peptides of insect origin are cecropins, which accumulate in the haemolymph of the giant silkmoth (*Hyalophora cecropia*), the silkworm (*Bombyx mori*) and Drosophila as a response to infection. These short, linear peptides (31-39 amino acids, aa) interact with the outer phospholipid membranes of both Gram-negative and Gram-positive bacteria and modify them by forming a large number of transient ion channels (Durell *et al.,* 1992). Native (cecropin B), mutant (SB37=38 aa, MB39=39 aa) and synthetic (Shiva-1 peptide=38 aa, D4E1=17 aa) cecropins are active *in vitro* against a wide range of plant pathogenic bacteria including *Erwinia amylovora*, E*rwinia carotovora* sspp. *carotovora* and *atroseptica*, *Erwinia chrysanthemi*, *Pseudomonas syringae* several pathovars, *R. solanacearum*, and *X. campestris* several pathovars (Nordeen *et al.,* 1992; Mills and Hammerschlag, 1993; Kaduno-Okuda *et al.,* 1995; Mourgues *et al.,* 1998; Rajasekaran *et al.,* 2001; Alan and Earle, 2002) whereas they exert no toxicity at bactericidal concentrations to cultured cells or protoplasts of several plant species (Nordeen *et al.,* 1992; Mills and Hammerschlag, 1993; Mills *et al.,* 1994; Mourgues *et al.,* 1998). Therefore, cecropins have been considered as potential candidates to protect plants against bacterial pathogens.

Initially, apart from a few early positive reports (SB37: Hassan *et al.,* 1993; Shiva-1: Jaynes *et al.,* 1993), accumulating evidence has shown that the expression of cecropins does not result in resistance to bacterial pathogens. This has been attributed to low levels of the peptide being produced *in situ* (Hightower *et al.,* 1994) and to early proteolysis by endogenous proteases in plants (Mills *et al.,* 1994; Florack *et al.,* 1995; Mourgues *et al.,* 1998). Low expression in plants of this and other foreign proteins from insects may also be caused by a difference in codon usage (Perlak *et al.,* 1991) and in potential regulatory sequences that determine intracellular localization or intercellular secretion of the foreign protein. However, Owens and Heutte (1997) found that a synthetic mutant form of cecropin B (MB39) with a single amino acid substitution became, on average,

three times more resistant to degradation by various endogenous peptidases and proteases from the intercellular fluid of plant leaves. When this mutant cecropin gene was fused to a barley α-amylase secretion signal and expressed under the control of a potato proteinase inhibitor gene promoter in transgenic tobacco plants (Huang *et al.,* 1997), there was either no symptom development or a delay in symptom expression after *P. syringae* pv. *tabaci* had been infiltrated into leaves. Huang *et al.* (1996) also introduced a different synthetic cecropin B gene into two japonica rice varieties and found it to be transcriptionally active in the transgenic plants with some lines showing improved resistance to bacterial blight and streak diseases. By the use of improved gene variants and more efficient expression vectors promising results are accumulating now. The Shiva gene in tobacco has been reported to confer resistance to *Pseudomonas solanaceraum* pv. *tabaci* (Xu *et al.,* 1999) whereas the SB37 gene has shown activity in potato to *E. carotovora* ssp. *atroseptica* (Arce *et al.,* 1999). A high degree of resistance to *E. carotovora* was also observed in potato tubers expressing the 34-aa chimaeric peptide MsrA1, which contains a C-terminal eight-aa segment from cecropin A and an N-terminal 16-aa segment of melittin, a 26-aa antibacterial peptide from bee venom (Osusky *et al.,* 2000). More recently, the expression of the D4E1 variant in poplar has resulted in resistance to *Agrobacterium tumefaciens* and *Xanthomonas populi* but not to *Hypoxylon mammatum* (Mentag *et al.,* 2003).

A novel aspect of research on transgenic plants modified to express antimicrobial agents is their effect on non-target organisms in the environment. Sessitsch *et al.* (2003) monitored in the greenhouse the composition of *Bacillus* populations in the rhizosphere of cecropin B expressing transgenic potato plants and found that apart from transient and minor changes no major effects could be observed. However, they failed to show a direct connection between the transient changes in *Bacillus* population structure and cecropin expression. Puterka *et al.* (2002) even found an unintentional beneficial effect against non-target pests in transgenic pear. When D5C1, a cecropin-analog, was expressed for fireblight control together with the neo antibiotic resistance gene a four-fold reduction in population levels of pear psylla (*Cacopsylla pyricola*) was observed in a long-term study.

A group of cecropin-like insect defensins has been purified from flesh fly (*Sarcophaga peregrina*), which includes three families of sarcotoxins and the family of sapecins (Natori, 1994). Sarcotoxins are active against a wide range of Gram-negative bacteria at submicromolar concentrations but not toxic to tobacco and rice suspension cells at up to 25 mM (Ohshima *et al.,* 1999). The gene (sarco) coding for sarcotoxin IA has been introduced into potato by *Agrobacterium*-mediated transformation (Galun *et al.,* 1996) and yeast (Aly *et al.,* 1999). The recombinant sarcotoxin from yeast showed toxic effects to *E. carotovora*, *P. syringae* pv. *lachrymans* and *R. solanacearum*, which was similar to the effects of the native sarcotoxin. Okamoto *et al*. (1998) have found that the low expression of

sarcotoxin IA in transgenic tobacco can be significantly increased when expressed as a fusion protein or under the control of a strong constitutive promoter, which resulted in enhanced resistance to *P. syringae* pv. *tabaci* and *E. carotovora* ssp. *carotovora* (Ohshima *et al.,* 1999). Similar results were obtained when the pathogen- and salicylate-inducible promoter of the pathogenesis-related 1 (PR1a) gene was used instead (Mitsuhara *et al.,* 2000). As to to the effect of sarcotoxin to potential non-target organisms, sarcotoxin IA has exerted a selective effect on bacteria commonly living in the human intestine (Mitsuhara *et al.,* 2001). The growth of harmful and food poisoning bacteria such as strains of *Escherichia coli* and *Clostridium* spp. was inhibited *in vitro* whereas beneficial bacteria were not affected including *Bifidobacterium* spp., *Bacteroides* spp., and *Lactobacillus acidophilus*. This observation supports the conclusion that if sarcotoxin from transgenic plants were to enter the food chain it could even improve human health rather than impairing it.

Sapecins are 40-residue peptides with six half-cystine residues, which are essential for the antibacterial activity against mainly Gram-positive bacteria. They show a significant structural and functional similarity to charybdotoxin from scorpion venom by being able to bind to calcium-activated potassium ion channels (Yamada and Natori, 1991). Other toxins with homology to the sapecin family, such as royalisin from royal jelly of honeybee (Fujiwara *et al.,* 1990), or phormicin from *Phormia terranovae*, have also been isolated. However, these antimicrobial peptides have not yet been tested against plant pathogenic bacteria.

The same insects that synthesize sarcotoxins also produce attacins, which belong to another family of six 20 kDa antibacterial proteins, in response to bacterial infection (Hultmark *et al.,* 1983). Attacins alter the structure and permeability of prokaryotic membranes by binding to lipopolysaccharide in the bacterial envelope (Carlsson *et al.,* 1998) and inhibiting the synthesis of the outer-membrane proteins (Carlsson *et al.,* 1991). Increased *in vitro* and greenhouse resistance to fire blight caused by *E. amylovora* by the expression of the attacin E gene (attE) in transgenic apple plants was reported by Norelli *et al.* (1994). This work is continuing (Borejsza-Wysocka *et al.,* 1997), but the attE gene in combination with the lysozyme gene from the T4 bacteriophage did not result in synergistic effect (Ko *et al.,* 2002). The same research group used the attE gene fused to either to the secretion signal peptide of the PR1b gene or to the potato proteinase inhibitor II promoter. The data obtained with these two constructs indicate that attacin can enhance resistance to *E. amylovora* (Ko *et al.,* 2000). As with cecropin, Arce *et al.* (1999) have shown attacin activity in transgenic potato to *E. carotovora* ssp. *atroseptica*. Chen and Kuehnle (1996) have also demonstrated the expression of attacin in calli induced from transformed *Anthurium*. A number of distinct, antibacterial peptides or proteins have been described in other insects

including apidaecins from honeybees (Casteels *et al.,* 1989) and moricin from silkworm (Hara and Yamakawa, 1995), but have not yet been used in plants.

Antibacterial agents from other invertebrates

Tachyplesins are a family of antimicrobial peptides first isolated from acid extracts of hemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*). These strongly basic 2.3 kDa peptides (17-18 residues) with two disulphide bridges primarily inhibit the growth of both Gram-negative and Gram-positive bacteria by forming a complex with bacterial lipopolysaccharides (Nakamura *et al.,* 1988) or with phospholipid membranes (Oishi *et al.,* 1997). Allefs *et al.* (1996) fused the sequence encoding a synthetic tachyplesin I gene with that of the barley hordothionin signal peptide. A low expression of this chimaeric gene in three potato cultivars revealed slight inhibitory effects to *E. carotovora* ssp. *atroseptica*. Tachyplesin was also found to be effective in controlling the growth of bacteria that are typically found in vase water like *Bacillus*, *Enterobacter* and *Pseudomonas* spp. (Florack *et al.,* 1996).

Antibacterial agents from higher animals and mammals

The expression in plants of antibacterial proteins from higher animals has scarcely been investigated. The best-known peptides are the pore-forming magainins (Bevins and Zasloff, 1990), bombinins (Simmaco *et al.,* 1991), brevinins and esculentin (Simmaco *et al.,* 1993 and 1994), rugosins (Suzuki *et al.,* 1995) and temporins (Simmaco *et al.,* 1996) isolated from frog skin (Barra and Simmaco, 1995). Of these, only magainin analogs (MSI-99 and Myp30) and esculentin have recently been transferred successfully into plants for use against bacteria. Based on the *in vitro* activity of the magainin analogs to different bacteria including *E. carotovora* ssp. *atroseptica* (Li *et al.,* 2001; Alan and Earle, 2002), expression of both MSI-99 and Myp30 conferred resistance to *P. syringae* pv. *tabaci* in transplastomic tobacco (DeGray *et al.,* 2001) and to *E. carotovora* ssp. *atroseptica* in transgenic tobacco (Li *et al.,* 2001), respectively. The 46-aa esculentin and its analogues, which displayed potent *in vitro* activity to *P. syringae* pv. *tabaci* (Ponti *et al.,* 1999), shared N-terminal sequence homology to a defensin family (Segura *et al.,* 1998), which is likely to protect the peptide from plant peptidases. On the basis of these findings, Ponti *et al.* (2003) introduced into tobacco a mutated (M28L) esculentin gene under the control of the leader sequence from a bean endopolygalacturonase-inhibiting protein to provide extracellular localization of the mature product. When transgenic leaves were infiltrated with either *Pseudomonas aeruginosa* or *P. syringae* pv. *tabaci* no bacterial growth and no symptoms were detected.

Indolicidin has been isolated from cytoplasmic granules of bovine blood neutrophils, and it is one of the smallest of the naturally occurring linear antimicrobial peptides (Selsted *et al.,* 1992). This 13-aa peptide contains the highest percentage (39%) of tryptophan of any known protein, and consists of

only six different amino acids. In addition, the tryptophan residues are interspersed with 23% prolines. Although it is presumed to act by disrupting membranes (Falla *et al.,* 1996), its mechanism of action remains to be established unequivocally. Recently, a synthetic reverse indolicidin has been found to be stable in tobacco leaf extracts while retaining a high antimicrobial activity, which correlated with a broad protease inhibitory activity (Li *et al.,* 2002). Indolicidin has been expressed in transgenic tobacco and is currently tested in the field for resistance to *P. syringae* pv. *tabaci*.

Lactoferrin, a member of a family of iron-binding glycoproteins found in human milk, has been reported to have antibacterial properties in transgenic plants. Mitra and Zhang (1994) observed that the introduction of a human lactoferrin cDNA in to tobacco resulted in the expression of a truncated lactoferrin protein which exhibited enhanced activity against several pathovars of *P. syringae*, *X. campestris* pv. *phaseolii* and *Clavibacter flaccumfaciens* pv. *flaccumfaciens*, and more recently to *R. solanacearum* in tobacco (Zhang *et al.,* 1998) and in tomato (Lee, T.J. *et al.,* 2002). These observations may not be surprising since Bellamy *et al.* (1992a, 1992b) have found that lactoferricin, an acid-pepsin cleavage product of lactoferrin, had activity against a broad range of bacteria.

Lysozymes

Another source of antibacterial proteins are lysozymes, either from bacteriophages or from hen eggs. These muramidase type enzymes attack the murein layer of bacterial peptidoglycan resulting in cell wall weakening and eventually leading to the lysis of both Gram-negative and Gram-positive bacteria. Hippe *et al.* (1989) reported that the expression of a bacteriophage T4 lysozyme with a plant signal peptide in transgenic tobacco plants was localized in the intercellular space. The application of this strategy in potato led to increased resistance to infection by *E. carotovora* ssp. *atroseptica* in the greenhouse (Düring *et al.,* 1993). A lysozyme gene from bacteriophage T7 has also been used to construct expression vectors for plant transformation (Huang *et al.,* 1994). Initial experiments with the hen egg white lysozyme (HEWL) for bacterial resistance met with little success (Trudel *et al.,* 1992) due to a low level secretion of the lysozyme with its own signal peptide to the intercellular space. However, high extracellular secretion of HEWL in transgenic tobacco resulted in growth inhibition of *Clavibacter michiganense* and *Micrococcus luteus* (Trudel *et al.,* 1995) in laboratory assays. Likewise, a synthetic human lysozyme gene has been expressed in transgenic tobacco plants and found to inhibit the growth of *P. syringae* pv. *tabaci* (Nakajima *et al.,* 1997). It was only in this stage that *in vitro* activity of lysozyme was confirmed on *E. amylovora* and (in contrast to the combination with attacin) appeared to be synergistic with cecropin B against this pathogen (Mourgues *et al.,* 1998). Surprisingly, Düring *et al.* (1999) found that the enzymatic activity of lysozyme was not responsible for the antibacterial activity but that smaller peptides, which disrupt the bacterial membrane and are

generated during degradation or denaturation of lysozymes, were. However, this might be in contradiction with the observation that addition of HEWL at a concentration of 18 mg/ml to a liquid culture of different prune shoot cultures eliminated *Bacillus circulans* without compromising the proliferation of plant shoots (Marino *et al.,* 2003). Recently, potato plants transformed with the HEWL gene showed increased resistance to *E. carotovora* ssp. *atroseptica*, and the level of resistance correlated with the level of transgene expression in the nine lines tested (Serrano *et al.,* 2000).

Interestingly, the effect of T4 lysozyme expressing transgenic potato plants on non-target bacteria in the rhizosphere has been intensively studied. According to one standpoint, the T4 lysozyme was secreted from plant roots and was toxic *in vitro* on both Gram-negative and Gram-positive bacteria (de Vries *et al.,* 1999; Ahrenholtz *et al.,* 2000). In contrast, in a field trial no negative influence was found on the genetic make-up of antagonistic (Lottmann *et al.,* 1999; Lottmann *et al.,* 2000; Lottmann and Berg, 2001) and plant-associated soil bacteria (Heuer and Smalla, 1999; Lottmann *et al.,* 1999; Heuer *et al.,* 2002). It can be concluded that in plants a lysozyme might confer resistance to leaf pathogens without a major influence on soil bacteria.

Antibacterial agents from plants

Higher plants are also a rich source of various proteins with lysozyme activity like a chitinase from cucumber (Métraux *et al.,* 1989) or hevamine from *Hevea brasiliensis* (Jekel *et al.,* 1991). However, these proteins have not been expressed so far in transgenic plants in order to control bacterial infections. The best-characterized plant antimicrobial proteins are thionins (Florack and Stiekema, 1994), which are able to inhibit a broad range of pathogenic bacteria *in vitro* (Molina *et al.,* 1993; Florack *et al.,* 1993). Of these, the expression of a barley µ-thionin gene in transgenic tobacco enhanced resistance to two pathovars of *P. syringae* in laboratory assays (Carmona *et al.,* 1993) while synthetic hordothionin genes were not secreted into the intercellular space in transgenic tobacco plants (Florack *et al.,* 1994). Unfortunately, most thionins can be toxic to animal (Carrasco *et al.,* 1981) and plant cells (Reimann-Philipp *et al.,* 1989), and thus, may not be ideal for expression in transgenic plants. The recently discovered antimicrobial peptides called fabatins that are active against both Gram-negative and Gram-positive bacteria (Zhang and Lewis, 1997), a defensin-type pseudothionin from potato (Moreno *et al.,* 1994) or plant defensins from various seed plants (Broekaert *et al.,* 1995; see section on fungal pathogens) may be more suitable for this purpose. Based on the first positive results obtained in transgenic tobacco against *P. syringae* pv. *tabaci* (Molina and García-Olmedo, 1997), lipid transfer proteins and snakins (see the chapter on fungi) may be good candidates for use against some plant pathogenic bacteria.

Many of the above toxic peptides may be useful for the control of bacterial pathogens in plants and they should be screened for activity in laboratory assays to determine if they have potential for use in transgenic plants. In addition, more efficient synthetic compounds designed by combining different protein domains responsible for toxicity to bacteria (Powell *et al.,* 1995; Desnottes, 1996) could also be tested. Ideally, ecological risks and human health hazards could also be evaluated in preliminary experiments.

Antibodies

An interesting strategy for control of bacteria may be the expression in transgenic plants of antibodies that target specific bacterial pathogenicity factors (Düring, 1996). Specific binding of antibodies to one or more bacterial factors, such as secreted lytic enzymes or extracellular proteins, is expected to compromise bacterial pathogenicity. The feasibility of this concept has already been demonstrated for virus control (Tavladoraki *et al.,* 1993; Voss *et al.,* 1995; Fecker *et al.,* 1997; Zimmermann *et al.,* 1998). The classical hybridoma technology for production of antibodies is laborious and involves the maintenance and care of immunized animals. However, the recently developed phage display technique (Scott and Smith, 1990; Clackson *et al.,* 1991) offers a more convenient and faster means to produce single-chain variable antibody fragments (scFv's). This method is based on bacterial recombination and gene expression techniques and also allows the ready isolation of the gene encoding for the desired antibody. For the antibody strategy to be successful, antibodies must be correctly expressed and secreted in plants. Although full-size antibodies as well as scFv's have been successfully expressed in transgenic plants for various applications (Conrad and Fiedler, 1994; Whitelam and Cockburn, 1996), the obvious advantage of recombinant scFv's over full-size antibodies is that with the former, only correct folding is required, and not the assembly of the light and heavy chains, as is necessary with the latter. It should be stressed, however, that transgenes encoding antibodies, just like many other foreign genes, can be inactivated by various mechanisms in the plant, such as gene silencing (De Neve *et al.,* 1999). To my knowledge, this strategy has not been applied for transgenic control of plant pathogenic bacteria though scFv's against *R. solanacearum* has been produced by phage display (Griep *et al.,* 1998).

Bacterial toxins

Pathogenic organisms produce various toxins that can be classified as either host specific toxins or non-host specific toxins. The former account for the specificity of the host-pathogen interaction at the molecular level while the latter are able to affect a wider range of host and non-host organisms (Mitchell, 1984; Gross, 1991). Only non-host specific toxins can be considered as targets to control plant pathogenic bacteria because host-selective toxins have, so far, only been identified in fungal pathogens.

Bacterial toxins target certain enzymes that are present in both the host plant and bacterial pathogen producing the toxin. Two major strategies have been adopted by bacteria to protect themselves from their own toxin. One is resistance based on the production of target enzymes that are insensitive to the toxin (Durbin and Langston-Unkefer, 1988) and the other is resistance via the production of detoxifying enzymes (De la Fuente-Martínez and Herrera-Estrella, 1993). The best-characterized bacterial toxins originate from various pathovars of *P. syringae* (Table 2). Consequently, transgenic research has so far mainly been undertaken with this family of toxins.

Table 2. Characterized bacterial toxins from pathovars of *Pseudomonas syringae*

Toxin	Pathovar	Biochemical target
Coronatine	Glycinea, tomato	Salicylic acid signaling
Phaseolotoxin	Phaseolicola	Ornithine carbamoyltransferase (OCTase)
Syringomycin	Syringae	Plasma membrane H+-ATPase
Tabtoxin	Tabaci, coronafaciens	Glutamine synthetase
Tagetitoxin	Tagetis	Eukaryotic (plastid) RNA polymerase III

Ornithine carbamoyltransferase (OCTase) is a key enzyme in the arginine and polyamine biosynthetic pathway in plastids. It is inhibited by phaseolotoxin, a tripeptide toxin produced by *P. syringae* pv. *phaseolicola.* Phaseolotoxin-producing bacterial strains, however, synthesize a different OCTase that is resistant to the toxin and its derivatives (Mosqueda *et al.,* 1990). When this toxin-insensitive OCTase was expressed in chloroplasts in transgenic tobacco plants, *in vitro* and biological assays demonstrated its effect in complementing the action of toxin-sensitive OCTase which led to the hypersensitive defense reaction of cells to *P. syringae* pv. *phaseolicola* (De la Fuente-Martínez *et al.,* 1992; Hatziloukas and Panopoulos, 1992).

Tabtoxin, another phytotoxic dipeptide from *P. syringae* pv. *tabaci*, inhibits the target enzyme glutamine synthetase and causes chlorotic symptoms (wildfire disease) in tobacco. The toxin-producing strains are insensitive to the toxin due to inactivation by an acetyltransferase enzyme encoded by the ttr (tabtoxin-resistance) gene. Transgenic tobacco plants expressing this tabtoxin acetylase showed high levels of resistance to the purified toxin or to infection by *P. syringae* pv. *tabaci* (Anzai *et al.,* 1989; Batchvarova *et al.,* 1998).

Albicidins are a family of phytotoxins and antibiotics produced by the xylem invading bacterium *Xanthomonas albilineans* and specifically block prokaryotic (plastid) DNA replication (Birch *et al.,* 1990). They are involved in leaf scald

disease of sugarcane by causing chlorosis in invaded host plants. Several genes that confer resistance to albicidins have been cloned from heterologous, biocontrolling bacteria, such as *Klebsiella oxytoca* (albA: Walker *et al.,* 1988), *Alcaligenes denitrificans* (albB: Basnayake and Birch, 1995), *Pantoea dispersa* (syn. *Erwinia herbicola*) (albD: Zhang and Birch, 1997), and from *X. albilineans* itself (Wall and Birch, 1997). A certain level of homology between some of these genes at the protein level suggests a common functional domain. Therefore, these genes may be useful candidates for transfer into the sugarcane genome. Indeed, expression of albD in transgenic sugarcane resulted in reduced chlorotic disease symptoms and conferred resistance to systemic multiplication of the pathogen (Zhang *et al.,* 1999).

Bacterial polysaccharides

Many plant pathogenic or symbiotic bacteria, including several species of *Clavibacter, Erwinia, Pseudomonas, Ralstonia, Rhizobium*, and *Xanthomonas*, produce large amounts of extracellular polysaccharide (EPS) during growth and pathogenesis (Denny, 1995). EPSs have multiple functions and appear to provide a selective advantage for bacteria during their epiphytic or saprophytic life period in that they protect bacteria from desiccation, concentrate nutrients, and enhance attachment to surfaces. During pathogenesis, EPSs regulate and minimize interaction with plant cells thereby reducing the effect of host defense responses (Király *et al.,* 1997) and contact with toxic substances while promoting colonization. In addition, EPSs may play a primary role in the development of disease symptoms, e.g. wilting caused by plugging of xylem vessels.

At least three gene clusters, which are organized more or less similarly in various phytopathogenic bacteria, are important for EPS biosynthesis. In *R. solanacearum*, an 18-kb operon (eps) with at least nine genes is responsible for the acidic component of EPSs (Huang and Schell, 1995). Another gene cluster (ops) that contains at least seven structural genes seems to be necessary for nucleotide sugar components of both EPSs and lipopolysaccharides (Kao and Sequeira, 1991). Kao *et al.* (1994) have identified a regulator gene whose overexpression resulted in decreased EPS production and reduced virulence in *R. solanacearum*. Though expression of the eps operon in *R. solanacearum* appears to be controlled by a complex regulatory network (Huang *et al.,* 1995) that is environmentally regulated, it may be interesting to express either the regulator gene or another mutated regulatory system in the apoplast of transgenic plants to test whether they affect EPS production and virulence of *R. solanacearum*.

An attractive way to understand the role of EPS in more depth and eventually increase disease resistance by reduced EPS production in plant pathogenic bacteria could be through the use of polysaccharide depolymerase enzymes. Hartung *et al.* (1986) described the isolation of a polysaccharide depolymerase gene from a bacteriophage of *E. amylovora*. The purified recombinant enzyme degraded amylovoran, the acidic component of the EPS (Nimtz *et al.,* 1996),

abolished the virulence of *E. amylovora* in bioassays, and inhibited bacterial cell growth (Kim and Geider, 2000). These observations indicate that correct expression of the gene in plants may be useful for testing this approach to control bacterial diseases. In addition, these bacteriophages apparently use additional ways to facilitate the infection of their host, which includes a lysozyme and a holin that may form a pore to support cell lysis by the lysozyme (Kim and Geider, 2000).

It should be mentioned that numerous bacteriophages of bacterial plant pathogens have been described in the past (Okabe and Goto, 1963) including phages of *R. solanacearum* (Hayward, 1964) and its banana-attacking strain (Buddenhagen, 1960). At least one of these phages has been found to produce a bacteriolytic protein against *R. solanacearum* (Ozawa *et al.,* 2001).

Quorum sensing

A novel strategy for controlling pathogenesis of bacteria can be the engineered interference with their cell-to-cell communication. Besides multicellular organisms, cell-to-cell communication is of vital importance for single-celled organisms, too, bacteria included. Quorum sensing (QS) is a mechanism to control gene expression in response to cell density (Fuqua *et al.,* 1994; Bassler, 1999), i.e. when a population has reached a minimal population size, the 'quorum'. QS is mediated by a group of diffusible signal molecules, also called 'quormones' because they function similarly to insect pheromones. The most common quormone group in Gram-negative bacteria consists of acyl homoserine lactones (AHLs), though for example in *R. solanacearum* another type of quormone (3-hydroxypalmitic acid methyl ester) is also known (Flavier *et al.,* 1997). At low cell density, a small amount of AHL is produced at a basic level by the AHL synthase, which catalyzes the reaction of homoserine lactone from S-adenosylmethionine with the acyl chain synthesized via the common fatty acid biosynthesis pathway. AHL is able to pass through the bacterial membrane by passive diffusion or via active transportation mechanisms depending on the length of the acyl chain. When cell density increases, AHL concentration is elevated via autoinduction of the AHL synthase gene. Above a threshold level AHL binds to a transcription activator (or a repressor in *Erwinia stewartii*) to trigger (or derepress) expression of target genes by a QS-dependent manner. Originally discovered in marine bacteria to play a role in bioluminescence (Nealson *et al.,* 1970; Eberhard *et al.,* 1981), QS is now known to regulate a wide range of biological functions including the production of EPS, degradative enzymes, antibiotics and pigments; swarming motility, conjugative transfer of Ti plasmid and biofilm formation. Since many of these functions are directly or indirectly related to pathogenesis or to controlling competitors, most phytopathogenic bacteria also possess a QS system, which probably functions as a preventive means to avoid early activation of local and systemic plant defense responses. QS may thus ensure that virulence factors are produced only at high population density so that bacteria will be able to overcome plant defense.

All the bacterial species that are pathogenic on banana (Table 1) are known to use QS (Chun *et al.,* 1997; Cha *et al.,* 1998; Elasri *et al.,* 2001; Whitehead *et al.,* 2002), which indicates the potential for control in banana and the general importance of this mechanism in plant pathogenesis. It is therefore not surprising that plants have developed mechanisms that specifically affect their interaction with bacteria via the manipulation of QS. Such a native anti-QS mechanism (called 'quorum-quenching') can basically be of two types; inhibition or degradation of AHLs (or the synthesis thereof) or, on the contrary, releasing AHL-like substances to 'fool' invading bacteria (Teplitski *et al.,* 2000; Mathesius *et al.,* 2003). These and similar strategies can also be applied to engineering bacterial resistance in plants, such as the inactivation of AHLs or overexpression of quenchers including AHLs as well. In the first direction, the AHL lactonase (AiiA from *Bacillus* spp.; Dong *et al.,* 2000) and the AHL acylase (AiiD from *Ralstonia* sp.; Lin *et al.,* 2003) enzymes are known to degrade AHLs. So far, the expression of AiiA in *E. carotovora* resulted in reduced AHL content, decreased extracellular pectolytic enzyme activities and attenuated virulence on a range of plants (Dong *et al.,* 2000). Similarly, transgenic potato and tobacco plants expressing AiiA showed enhanced resistance to *E. carotovora* with a strong correlation between AHL lactonase activity and disease symptoms (Dong *et al.,* 2001). In the second direction, overexpression of AHLs in transgenic plants is expected to trigger a premature attack of the pathogen, which would result in a successful defense response of the host. To this end, two genes have recently been tested, the yenI (Throup *et al.,* 1995) and expI genes (Pirhonen *et al.,* 1993) from *Yersinia enterocolitica* and *E. carotovora* ssp. carotovora, respectively, both of which encode an AHL synthase. Whereas the first gene did not enhance resistance to *E. carotovora* ssp. *atroseptica* in transgenic potato (Fray, 2002), in expI transgenic tobacco plants the same pathogen was efficiently controlled (Mäe *et al.,* 2001). This apparently contradictory result indicates that a fine tuning is required for each particular plant-pathogen combination.

Bacterial avirulence genes and plant disease resistance genes

Gene–for-gene interactions

The gene-for-gene hypothesis (Flor, 1971; Keen, 1990) explains host-pathogen recognition events on the assumption that single genes at particular loci in the plant and single genes at particular loci in the pathogen together determine whether the plant-pathogen interaction will be compatible (resulting in infection and disease development) or incompatible resulting in the initiation of hypersensitive defense response (HR) and disease resistance. Biological races of pathogens that contain a set of genes with alleles for virulence and avirulence will therefore induce a specific pattern of compatible and incompatible reactions when inoculated on a collection of host plant cultivars that contain (or lack) a set of genes with alleles for resistance and susceptibility. Incompatibility results only when the resistance allele at a particular gene locus in the plant complements the corresponding avirulence allele in the pathogen. All other combinations result in

compatibility. This concept presupposes that plants with a particular resistance gene (R gene) recognize a pathogen-produced elicitor, a direct or indirect product of the corresponding avirulence (effector) gene (Gabriel and Rolfe, 1990). It is likely that when the interaction is compatible, the direct or indirect product of most avirulence genes may contribute to the pathogen's virulence (Dangl, 1994).

It has been found that a gene cluster called hrp (hypersensitive response and pathogenicity) determines the expression of avirulence genes (Huynh *et al.,* 1989) in a wide range of plant pathogenic bacteria. The gene cluster controls secretion and transport of effector proteins outside of bacterial cells since at least eight of the known 20 hrp genes show remarkable homology to proteins involved either in protein secretion pathways (Van Gijsegem *et al.,* 1993; Bogdanove *et al.,* 1996) or in flagellum biogenesis (Rosqvist *et al.,* 1995) of animal pathogenic bacteria. In addition, Pirhonen *et al.* (1996) demonstrated that non-pathogenic *E. coli* cells transformed with a functional hrp gene cluster were able to trigger a genotype-specific HR. The main role of the hrp genes is that their products constitute the Hrp pilus (He and Jin, 2003) of the type III secretion (TTS) apparatus, which mediates the delivery of bacterial effector proteins into the host cell (Cornelis and Van Gijsegem, 2000). The hrp gene cluster itself encodes and controls the secretion of a glycine-rich protein called harpin (HrpZ from *P. syringae*, HrpN from *E. amylovora*, and PopA from *R. solanacearum*), which is also able to elicit the HR when infiltrated into plant leaves (He *et al.,* 1993). Though the precise role of harpin is not well understood, some results indicate that it may have potential for some pathogen control in transgenic plants. When PopA was expressed in tobacco under the control of a pathogen-inducible promoter, it rapidly accumulated in the infection sites, which resulted in a localized HR (Belbahri *et al.,* 2001). This result has now been confirmed by constitutive expression of PopA in *R. solanacearum*, which rendered the recombinant bacteria avirulent on tobacco leaves and roots but not in xylems (Kanda *et al.,* 2003). Taken together these observations indicate that an early recognition of PopA by tobacco plants may result in effective protection in the apoplast. However, potential negative effects of the induced HR on plant health should be first addressed by extensive field testings.

Bacterial effector (avirulence) genes

During the past two decades, about 50 bacterial effector genes, mainly from species of *Pseudomonas* and *Xanthomonas*, have been cloned. More recently, several plant disease-resistance genes corresponding to effector genes have also been cloned and characterized at a molecular level. However, little is known about the gene products, their function and the mechanism by which they interact during incompatible plant-bacterial interactions. So far only one effector gene product of *P. syringae* has been shown to function as an enzyme that is involved in the synthesis of syringolides, which are able to elicit the

hypersensitive response (Keen *et al.,* 1990). Further results indicate that the AvrBs2 effector of *X. campestris* may also be an enzyme (Swords *et al.,* 1996) and act within plant cells (Yang and Gabriel, 1995; Gopalan *et al.,* 1996). The predicted sequence of this effector protein shows similarity to enzymes involved in the synthesis or hydrolysis of phosphodiester linkages like agrocinopine synthase from *A. tumefaciens*. The indirect evidence that effector proteins may be delivered into plant cells via the Hrp system indicates that they could be involved in bacterial pathogenesis rather than merely controlling genotype-specific elicitation of the HR (Collmer, 1996; Gabriel, 1999). Indeed, two effectors, AvrBs3 from *X. campestris* pv. *vesicatoria* (Szurek *et al.,* 2002) and PopP2 from *R. solanacearum* (Deslandes *et al.,* 2003) have recently been shown to be localized in plant nuclei by immunolocalization and monitoring GFP fusions, respectively. In the case of PopP2, the corresponding RRS1 resistance gene was colocalized in the nucleus only in the presence of PopP2, which is consistent with the model that R genes might act as a guard to interact with the targets of effector proteins rather than directly interacting with the effectors (van der Biezen and Jones, 1998). Light is also shedding on the potential function and possible targets of bacterial effectors as AvrPto from *P. syringae* has recently been shown to repress a whole set of host genes encoding secreted cell wall and defense proteins (Hauck *et al.,* 2003) whereas AvrPtoB has been found to induce plant susceptibility by inhibiting programmed cell death (Abramovitch *et al.,* 2003).

Although it would be tempting to express an effector gene in transgenic plants in order to create bacterial disease resistance by the elicitation of the HR, there are a number of reasons why this should not be considered for immediate practical use. Firstly, as seen before, effectors determine race-specific plant recognition events, thus, a whole set of genes would be necessary to provide protection against a wide range of pathogenic races. Secondly, as has already been reported for a fungal avirulence gene (Joosten *et al.,* 1994), the introduced gene may mutate which would prevent the induction of the HR. Thirdly, since the function of most effectors is not clear yet, expression of a single transgene might not induce the HR as has already been observed when bacteria harbouring various effector genes were infiltrated into plant leaves (Dangl, 1994; Gopalan *et al.,* 1996). Finally, expression of effectors in plants would require very tightly regulated (preferably pathogen-inducible) promoters, otherwise, background or leaky expression of the avirulence transgene may lead to an uncontrolled HR (Michelmore, 1995). Nevertheless, the feasibility of this approach has been demonstrated by McNellis *et al.* (1998) who expressed the avrRpt2 avirulence gene of *P. syringae* under the control of a chemical-inducible promoter in transgenic Arabidopsis plants that contained the complementary RPS2 resistance gene. After chemical induction of the transgenic plants, transcription of the introduced avirulence gene was detected in 30 min, its protein product was detected within 2 h and a localized HR was observed within 6 h after induction.

Plant resistance genes

At least 11 plant resistance genes conferring resistance to bacterial diseases have been cloned to date (Martin *et al.,* 1993; Bent *et al.,* 1994; Mindrinos *et al.,* 1994; Grant *et al.,* 1995; Song *et al.,* 1995; Salmeron *et al.,* 1996; Yoshimura *et al.,* 1998; Warren *et al.,* 1998; Gassmann *et al.,* 1999; Tai *et al.,* 1999; Swiderski and Innes, 2001; Deslandes *et al.,* 2002). They have been isolated by map-based cloning, which includes genetic localization, marker saturation in the region localized, isolation of large genomic (BAC or YAC) clones, identification of cDNAs and complementation. Though different classes of resistance genes exist (Michelmore, 1995), the genes cloned have structures related not only to each other, but also to other plant resistance genes (Dangl, 1995). A variable number of leucine-rich repeats (LRR), which indicate a function for protein-protein interactions (Kobe and Deisenhofer, 1994), is a common feature. Another is a P-loop motif with a common nucleotide-binding site (NBS) in diverse proteins with ATP / GTP binding activity (Saraste *et al.,* 1990), which suggests that binding of these nucleotide triphosphates to the proteins is essential for their functioning (Traut, 1994).

Basically, all these genes seem to encode components of receptor systems and form part of a signal transduction pathway, which triggers general defense reactions such as reinforcement of the cell wall, synthesis of phytoalexins and oxidation of phenolic compounds, activation of defense-related genes and the HR. That the majority of the plant disease resistance genes isolated so far belongs to the receptor-related class may suggest that many of the classical resistance genes will fall into this category (Michelmore, 1995). Furthermore, while significant sequence similarities were found among the different disease resistance genes this has not been the case for avirulence genes. These observations indicate that pathogen recognition and response mechanisms in plants may be similar, but are activated by a wide range of specific elicitors (Dangl, 1994). This hypothesis was given credence when the first cloned disease-resistance gene Pto (a tomato resistance gene against *P. syringae* pv. *tomato*) was found to function both in *Nicotiana tabacum* (Thilmony *et al.,* 1995) and *N. benthamiana* (Rommens *et al.,* 1995) suggesting that disease resistance functions are conserved in a wide range of plant species.

The most successful example of engineering R genes in crop plants is definitely the Xa21 gene from rice (Song *et al.,* 1995), which confers resistance to all races of *Xanthomonas oryzae* pv. *oryzae*. The gene has been introduced into a wide range of susceptible varieties and was repeatedly found to provide resistance under laboratory conditions as well as in the field (Tu *et al.,* 1998; Zhang, S.P. *et al.,* 1998; Tu *et al.,* 2000; Zhai *et al.,* 2000; Zhai *et al.,* 2002).

The Bs2 gene of pepper confers resistance to strains of *X. campestris* pv. *vesicatoria* and other *X. campestris* pathovars that contain the corresponding bacterial avirulence gene, avrBs2. Thus, the Bs2 gene may be durable in the field and

provide resistance when introduced into other plant species. The gene was cloned and found to belong to the NBS-LRR class of R genes (Tai *et al.,* 1999). Functional expression of Bs2 in transgenic tomatoes has demonstrated that it can be used as a source of resistance in other Solanaceous plant species and perhaps in banana, too.

The RRS1 gene has recently been identified in Arabidopsis at a recessive locus for resistance to *R. solanacearum* (Deslandes *et al.,* 1998), a pathogen of banana. This gene that confers resistance to several races of *R. solanacearum* has recently been cloned and found to fall into a specific class of the above NBS-LRR type of R genes (Deslandes *et al.,* 2002). The gene can be a good candidate for transfer to banana, and testing for resistance to Moko disease.

Nothing was known about how plant R genes trigger defense reactions until Zhou et al. (1997) identified several classes of cDNAs encoding proteins that physically and specifically interact with the Pto protein. The sequence of these Pti proteins shared significant homology to a wide range of transcription factors, some of them with the ability to bind to a GCC core sequence (PR box) present in the promoters of a large number of plant defense-related genes. Zhou *et al.* (1997) also showed that the interaction between the Pto protein and the corresponding avirulence gene product correlated with the early induction and increased expression of defense-related genes that contained the PR box. This provided indirect evidence for a connection between a R gene and the specific activation of plant defense-related genes. Zhou *et al.* (1995) had previously showed that another Pto interacting protein was also involved in the induction of the HR. Thus, at least two pathways of plant defense reactions can be linked to a particular plant R gene. More recently, proteolytic degradation pathways have also been connected to R gene-mediated resistance in plants (Tör *et al.,* 2003).

Another important event associated with plant defense mechanisms is a rapid and transient release of different reactive oxygen species (ROS), such as the superoxide anion radical (O_2-), hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2). Of these ROS, production of hydrogen peroxide appears to occur early and is involved in a direct oxidative reaction with the pathogen, in biosynthesis of phytoalexins, and in activating defense-related genes as well as in the induction of acquired disease resistance. Evidence for a direct role of hydrogen peroxide in plant defense was provided by Wu *et al.* (1995) when they expressed in transgenic potato plants the *Aspergillus niger* gene for glucose oxidase, which catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. Increased level of hydrogen peroxide was observed which resulted in a simultaneously increased resistance to *E. carotovora* ssp. *carotovora.* Similarly, the same gene in transgenic cabbage conferred resistance to *X. campestris* pv. *campestris*, which correlated with glucose oxidase activity in leaves (Lee, Y.H. *et al.,* 2002). Increased hydrogen peroxide may also account for resistance to the soft rot-causing bacterial pathogen *E. carotovora* ssp. *atroseptica* in tubers of antisense

potato plants with decreased activity of the plastidic ATP/ADP transporter protein AATP1 (Linke *et al.,* 2002). It is likely that downregulation of AATP1 results in increased ATP to ADP ratio, which favours protein kinase activation. Decreased AATP1 activity is associated with low starch and high glucose-6-phosphate content, which serves as oxidizable carbohydrate supply for the burst of hydrogen peroxide (Linke *et al.,* 2002).

Concluding remarks

The major focus of research on pathogenic bacteria in banana should likely be on *Ralstonia solanacearum*. First, the widespread damage and economical significance of this and other pathogens (*Erwinia* and *Xanthomonas* spp.) in banana in East Africa need to be firmly established. Since *R. solanacearum* strains from tomato and radish were able to infect *Arabidopsis thaliana* (Yang and Ho, 1998), much of the transgenic research (e.g. testing candidate genes) could be done on *Arabidopsis,* with final tests in banana. For the latter, elaboration of sensitive bioassays would be necessary.

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Strategies for resistance to nematodes in *Musa* spp

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Introduction

Ideally, new technology should not require either additional knowledge or resources from the grower before implementation can be successful. The small size of nematodes ensures that many growers are unaware of the crop loss they cause. This is exacerbated in many developing world countries by the lack of extension advice to subsistence farmers. At best, transgenic plants should involve the simple act of planting without other changes to traditional farming practices. A technology that is effective against many different nematodes eliminates the need to identify them and determine if damaging levels are present in a field. The aim should be a banana crop that is fully and durably resistant to nematodes. Its deployment should be biosafe for both humans and the environment. Its uptake should not require change from the cultivar of choice of the subsistence farmer while assuring freedom of choice to grow or avoid transgenic plants. The planting material should be available to subsistence growers without a royalty premium. Such technology also has potential to reduce the environmental harm caused by nematode control in the banana plantations of agribusiness. The aim of this review is to consider the scientific and technological progress that has been made towards these aims and then to consider the prospects for further progress.

Principal nematode pests of banana and plantain

Plant parasitic nematodes can be subdivided into a matrix with three broad ecological categories. They are 1) root or aerial parasites, 2) ectoparasites or endoparasites and 3) either migratory throughout the life cycle or sedentary for at least part of female development. All the important species that feed on banana are root parasites. *Pratylenchus goodeyi*, *Radopholus similis* and *Helicotylenchus multicinctus* are migratory endoparasites. Both *Meloidogyne* spp and *Rotylenchulus* are sedentary endoparasites. These differences have implications for both the type of damage caused and appropriate biotechnology for their control.

Sedentary parasitism is associated with modification of plant cells into a form that can sustain prolonged feeding at one site. The form of such cells varies. *Meloidogyne* induces a few multinucleate giant cells from which the female feeds in turn during a sedentary phase that typically extends for several weeks. The swollen female also induces characteristic root galls with a lesion to the root surface through which up to 1000 eggs/female are laid. Adult females of the small nematode *Rotylenchulus reniformis* also induce feeding cells in host plants. This nematode is associated with narrow secondary roots and its importance on

banana may be inadequately defined given it is a major pathogen of some other crops such as pineapple and cotton (Caswell *et al.,* 1990; Starr and Page, 1990).

Dropkin (1969) made a distinction between destructive and adaptive plant cell changes that is particularly useful for banana. *Radopholus*, *Pratylenchus* and *Helicotylenchus* all destroy the plant cells on which they feed before advancing to attack other cells. Such nematodes provide a resource for necrotrophic microorganisms. Consequently, they leave a "wake" of necrosis that helps diagnose their attack and provide the burrows from which *Radopholus similis* and *Pratylenchus* spp. gain their common names of burrowing and lesion nematodes respectively. This mode of feeding promotes root rots and hence these nematodes are associated with banana toppling which is a major cause of yield loss where high winds occur. Neither *Meloidogyne* nor *Rotylenchulus* cause extensive root rots and their damage to hosts is principally due to the resources they remove from the plant roots.

Occurrence on banana

All the above genera of nematodes are widespread and important pathogens of banana and plantain. Many country or locality specific surveys of banana nematodes establish that the relative prevalence of these genera and their species varies. For instance *Helicotylenchus multicinctus*, *Meloidogyne* spp., *Hoplolaimus pararobustus*, *Pratylenchus coffeae* and *Radopholus similis* were found at 100%, 68% 64%, 50% and 46% respectively of the 68 plantain sites sampled in Southern Nigeria. Other nematode species occurred at less than 5% of the sites. *H. multicinctus* and *P. coffeae* occurred at high densities and there was a geographical basis to the relative predominance of these two species. It is concluded that *P. coffeae* followed by *R. similis* are the major biotic constraints of plantain production in southern Nigeria (Speijer *et al.,* 2001). Results from several African countries suggest overall prevalence of 69% for both *R. similis* and *Helicotylenchus* and 31% for species of *Meloidogyne* and *Pratylenchus* (Gowen and Quénéhervé, 1990). Experimental application of nematicides in a range of African countries suggest yield responses of 71 ±16% over three years after nematicide application (geometric mean based on data given by Gowen and Quénéhervé, 1990). The geographical range and importance of *Radopholus similis* seems to have been extended by distribution of the 'Cavendish' varieties as replacements for Gros Michel (Gowen, 1995).

The main conclusion to be drawn is that any approach for nematode control should control all the damaging species present at that locality. Subsistence growers cannot distinguish among species or estimate their densities relative to damage thresholds. They are unlikely to have either the resources or the opportunity to refer to extension advisers. There is little value in plant breeding or biotechnological approaches that result in prevalence changes of damaging nematodes rather than a reduction in crop loss.

Current control

Nematicides are widely used for nematode control in commercial banana plantations. Application frequencies are up to 3 times per year (Gowen 1995). Such pesticides cannot be afforded by resource poor farmers and they are inappropriate for the majority of this group that lack both training in proper chemical use and essential protective clothing.

The intense application of nematicides to banana has been a matter of considerable concern for decades. It has both environmental consequences and implications for the health of agricultural workers. Concerns have been male sterility, damage to liver and kidneys and both teratological and carcinogenic properties. There have been out-of court settlements within USA and successful prosecutions in Nicaragua of the manufacturers of one now withdrawn compound (Lanchen, 2001). FAO considers one of the most compelling reasons for adopting genetic transformation of banana is to reduce current pesticide use. It identifies proteinase inhibitors in roots for nematode control as one of the key approaches (Anon, 2001). Current nematicides have a high mammalian and invertebrate toxicity and cause considerable environmental damage for instance by contaminating water sources (Gustafson, 1993). As a consequence WWF is seeking an eco-friendly banana production in Latin America with a target of 50% reduction in nematicide use. (WWF, 2000). Cultural control practices have value. They can involve flooding or fallowing which cause a reduction in cropping opportunities. Rotational control is problematic in that several of the nematodes of banana have wide host ranges and so knowledge of the species and their abundance is required on a field-by-field basis. The subsistence farmer cannot afford a loss of production and so crop rotation or other practices and must maintain the productivity per hectare that banana achieves.

Host crop resistance is another option for banana nematode management and sources of resistance have been identified (see later). There is a considerable challenge to breed a banana that is resistant to several different nematodes. Transgenic approaches can support such effort by providing crop protection when natural resistance cannot be achieved. Such transgenic resistance could be incorporated in to breeders lines so allowing effort to be concentrated on fewer traits.

Establishing an initial bioassay screen for transgenic resistance

There is a distinction between the needs for screening for resistance to sedentary rather than migratory endoparasites. The former do not induce root rots and transgenic resistance can be evaluated over one or more generations the first of which is completed in approximate synchrony after introducing nematodes to uninfected plants. The procedures can be modified for *Meloidogyne* from those of trials on transgenic *Arabidopsis thaliana*, potato and rice (Urwin *et al.,* 1997; 2001, Vain *et al.,* 1998). Such trials have been also defined for *Rotylenchulus* on *A. thaliana* (Urwin *et al.,* 2000a). The migratory endoparasites differ because of the

root rotting they cause. Under conditions that favour nematodes such as *Radopholus*, their density increases not only per root system but also per unit mass of fresh roots. When this occurs the proportion of necrotic root resulting from feeding increases. This can add to the intraspecific competition between the nematodes for resources because they are biotrophic pathogens. The density of nematodes introduced, the size of the root system at that time and the subsequent rate of both root and nematode population growth should be considered. For instance, Barekeye *et al.* (2001) found little increase between 2 and either 4 or 6 months in the density of *R. similis* after introducing 1,000 nematodes to plantlets (AAA-EA genotype). In contrast the proportion of roots with a necrotic cortex did increase from c20% to 45% between 2 and 6 months. Others have restricted challenge to eight weeks for *Radopholus* (Stoffelen *et al.,* 2000; Marin *et al.,* 2000) or ten weeks for *Pratylenchus* (Stoffelen *et al.,* 2000). Both 200 and 1,000 *R. similis* per pot have been introduced (Stoffelen *et al.,* 2000; Marin *et al.,* 2000). The key issues are to ensure experiments provide for a reproducible infection and completion of the trial before the population of nematodes begins to plateau at an upper level under severe intraspecific competition. Lines from transformation have been screened to identify those expressing sufficient cysteine proteinase inhibitor transgenically to provide some nematode resistance before western blots and ELISA screens to identify expression levels. The transgenic lines were grown in a containment glasshouse at 24-32 °C with a RH >80% and a 12h light: 12h dark cycle provided by automatic blinds. After an initial period of about 4 weeks in 10 cm diameter pots of sandy loam, the banana plants had root masses of about 10g fresh weight. They were then transplanted to 18 cm diameter pots in the same soil type with 5ml of dry granules per pot of a water retaining gel (Raingel). Two hundred *R. similis* were watered onto a 1 cm diameter filter paper (GFA, Whatman) underneath each root system at this re-planting. The pots were then stood in a 1 cm depth of water to maintain a soil moisture regime that favored nematode invasion. The plants were harvested at 8 weeks post infection, the fresh weight of green tissue and root tissue measured and *R. similis* recovered over 3 days from the chopped root system using a misting chamber with water at 25°C (Southey, 1986). The inoculum of 200 *R. similis* typically increased about 12 fold over 8 weeks and the root fresh biomass increased from 10 to 40g and showed only limited root necrosis. The coefficient of variation suggests the assay can detect > 30% resistance with eight replicate plants. This assay is therefore adequate for initial screening of transgenic lines.

Screening banana lines: field evaluation

The complexity of the interactions between root size and a migratory endoparasitic nematode has been established for *Pratylenchus zeae* on rice (Prot and Savary 1993). Care has to be taken to standardize plant size relative to initial intensity of nematode challenge. The variation detected in one field trial in St Lucia suggest that 10-20 plants per line is sufficient to estimate resistance in initial field trials (Atkinson unpublished experiments).

No transgenic field trial for nematode resistance on banana has been reported. A key issue is likely to be biosafety and in particular, isolation to ensure no loss of plants as this would constitute a breech of national biosafety regulations. A mock trial would help establish safe protocols. They should be done in a country possessing national biosafety regulations plus an authority able and willing to scrutinize deliberate release effectively for biosafety. The approving authority should then be able to sanction true transgenic trials it considers safe without excessive bureaucratic delay.

Promoters for transgenic control of nematodes

The characteristics of a promoter required to drive an effective nematode resistance depends upon the type of defense being constructed. The requirement is exacting if the aim is to prevent or attenuate plant cell modification by females of *Rotylenchulus* or *Meloidogyne* spp on banana. It is less exacting if the aim is to deliver biosafe proteins that have a direct effect on nematodes but not plants.

Anti-nematode effectors and constitutive promoters

CaMV35S has been widely deployed in plant biotechnology. It has been used to control expression of a cystatin providing partial resistance to *G. pallida* in the field (Urwin *et al.,* 2001) but it is less than ideal as a promoter for delivering anti-nematode effectors. It is progressively down-regulated in the syncytial feeding cells of cyst nematodes (Goddijn *et al.,* 1993) so its use may underestimate the level of efficacy that might be achieved with a more specific promoter. It is also more active in younger than older roots and may only provide for a patchy protection of the root system. It did provide sufficient cystatin expression for partial resistance against *M. incognita* challenging rice (Vain *et al.,* 1998) although in general it is less active in monocots than dicots. GUS reporter studies established that the UBI-1 promoter showed a gradual decline in activity in older roots but it was active in most of a rice root system after 10 weeks (Green *et al.,* 2002). This promoter provided higher expression levels in banana than either of two root-preferential promoters.

The major nematode pests of banana are restricted to roots and constitutive expression is unnecessary. Promoters that direct expression preferentially in root tissue play an important role in addressing public concerns over transgenic nematode-resistant crops. Nematode protection without expression of transgenic protein in the fruit offers a clear food safety benefit. It also reduces exposure of non-target organisms feeding on aerial tissues to transgene products (see section later).

Promoters active during root invasion

The wun-1 promoter responds to invading cyst-nematodes (Hansen *et al.,* 1996). The invading juveniles of *G. pallida* induce expression that is not restricted to damaged cells in potato roots but also occurs near the invading parasite. Promoter activity is lost once the animal enters a phase of syncytial induction as wun-1 seems to be related to wounding of plant cells by the invading nematode.

Therefore the promoter may be of value for use in banana for control of *Radopholus*, *Pratylenchus* and *Helicotylenchus* all of which wound root cells continually as they feed. The ideal promoter would be responsive in a locally systemic way so ensuring the biopesticide is synthesized before the nematode feeds from the responding plant cell.

Promoters with preferential activity in roots

Several promoters of this type have been identified and the potential of some for delivery of anti-nematode effectors has been established (Lilley and Atkinson, 1997; Lilley *et al.,* 2003). Some of these promoters respond to nematode feeding cell establishment. They have potential for control of *Meloidogyne* and *Rotylenchulus* on banana but the additional need is for promoters of value against migratory endoparasites. Promoters of root preferentially expressed genes have been identified from pineapple (Neuteboom, *et al.,* 2002) and similar approaches could be applied to banana. Ideally, such promoters should be active throughout all the roots that such nematodes attack and they should not be down-regulated by the parasites.

Microarray analysis for further promoters of interest

Microrarrays of the full genome are available for both *A. thaliana* and rice. Several nematodes infect *A. thaliana* (Sijmons *et al.,* 1991). Those that parasite banana include *Meloidogyne* (Urwin *et al.,* 1997), *Rotylenchulus* (Urwin *et al.,* 2000a) and *Radopholus similis* (Carlens *et al.,* 2002). Microarray techniques have been applied to determine changes in plant gene expression on challenge with both a microbial pathogen (Schenk *et al.,* 2000), and *Heterodera schachtii* both during feeding site induction (Puthoff *et al.* 2003) and for tissue enriched for the feeding cells of established females (Haeger, *et al.,* personal communication). In the latter work, signals were detected from c12, 000 genes with many examples of both increased and decreased expression sometimes with >10 fold change between the root lengths with feeding cells and similar regions of control roots. Therefore genes of *A. thaliana* that are responsive to nematodes that attack banana could be identified as well as abundant, non-responsive transcripts limited to roots. When installed in reporter constructs, promoters of such genes could be tested for their activity in banana in a similar way to work on the activity of the TUB-1 promoter of *A. thaliana* in rice (Green *et al.,* 2002). In addition, homologues might be identified from cDNA libraries made from banana root transcripts and the promoter regions defined by genomic library screens. Both approaches would provide additional promoters to restrict transgenic expression to roots if the promoters already available prove less than ideal.

Modifying Promoter Activity

Promoters have elements that provide spatial, temporal or environmental responses to the expression pattern of a gene (Keller and Baumgartner, 1991). Rarely can these elements be recognized from sequence information but deletion

studies may sometimes separate promoter elements providing a useful modification to promoter activity. Deletion of the 5'-flanking region of a root-preferential promoter TobRB7 resulted in a 300 bp promoter fragment just upstream of the coding region that remained active within the giant cells induced by *M. incognita* and silenced in root meristems (Opperman *et al.* 1994). There has been a lack of further progress with this promoter and there are unpublished reports that the promoter shows unwanted activity. A promoter from pyk20 (Puzio *et al,* 1998 and 1999) active in the feeding cells of *H. schachtii* induced in *A. thaliana* was also active in leaf hydathodes and stipules. Promoter deletion studies established that activity could be eliminated at all sites. Unfortunately, activity was restored sequentially to hydathodes, stipules and then the nematode feeding cells as the promoter length was restored (Puzio *et al.,* 1998). More work is required to define how frequently promoter modification can enhance the value of promoters for use in nematode defenses.

Bionematicides

Pesticides that control nematodes are often subdivided into those that kill the animals and those such as aldicarb and oxamyl that disrupt chemoreception and hence host and mating finding behaviour. EPA terms anti-pest proteins expressed transgenically in plants as biopesticides. For simplicity, this review conforms to that view whether or not the protein is lethal to nematodes.

Serine Proteinase inhibitors

Proteinase inhibitors (PI) of all four classes of proteinase occur in plants and they often accumulate in aerial and certain other plant tissues in response to wounding or herbivory (Ryan, 1990). They also accumulate in many seeds such as those of rice (Abe *et al.,* 1987), maize (Arai *et al.,* 2002), cowpea (Fernandes *et al.,* 1993) and potato tubers (Rodis and Hoff, 1984). Cowpea trypsin inhibitor expressed in transgenic potato influences the sexual fate of newly established *G. pallida* (Hepher and Atkinson, 1992; Atkinson, 1993). As a result, the population is biased towards predominance of the much smaller and less damaging males. No reduction in the fecundity of females that establish occurs. CpTI reduces the fecundity of females of *M. incognita* without influencing their sexual fate (Hepher and Atkinson, 1992; Urwin, *et al.,* 1998). Inhibition of cyst nematode development correlated with trypsin inhibitor activity but not with the amount of sporamin. The latter is a Kunitz-type trypsin inhibitor from sweet potato tuber that was expressed experimentally in hairy roots (Cai *et al.,* 2003). Histochemical assays demonstrated serine proteinase activity in the intestine of *H. glycines* that could be inhibited by cowpea trypsin inhibitor, CpTI (Lilley *et al.,* 1996) and this nematode expresses three serine proteinases, HGSP-I, II and III (Lilley *et al.,* 1997).

Cysteine proteinase inhibitors (cystatins)

This class of proteinases was first detected in homogenates of *G. pallida* feeding females (Koritsas and Atkinson, 1994). It has also been localized histochemically to the intestine of *H. glycines* in both fusiform animals and saccate females prior to egg deposition. It seems to be active throughout parasitic establishment. Two cysteine proteinases have been isolated from cDNA library of feeding female *H. glycines*. *Meloidogyne* hapla females possess predominantly cysteine proteinase activity but other proteinase classes are also present in *M. incognita* and *M. javanica* (Michaud *et al.,* 1996).

The rice genes Oc-I and Oc-II were the first plant cystatins to be cloned (Abe *et al.,* 1987; Kondo *et al.,* 1990, 1991) and at least 50 plant cystatins are now known. Oc-I has a lower affinity for the cysteine proteinase papain than chicken egg white cystatin but its affinity for this proteinase was enhanced through protein engineering (see later; Urwin *et al.,* 1995).

Nematode resistance achieved with cystatins in other crops

OcIΔD86 is a protein-engineered version of Oc-I. It inhibited the growth of *G. pallida* more effectively than native Oc-I when expressed in transgenic hairy roots of tomato (Urwin *et al.,* 1995). Under the control of the constitutive CaMV35S promoter, Oc-ID86 at 0.4 % total soluble protein conferred similar resistance levels on *A. thaliana* to both a cyst and a root-knot nematode (Urwin *et al.,* 1997). Fewer female nematodes reached egg-laying size than for controls and those laying eggs were smaller and less fecund. The same line of transgenic *A. thaliana* provided 76 ± 8% resistance for *Rotylenchulus reniformis* and other lines expressing lower levels allowed a correlation to be achieved between expression levels of the cystatin and resistance (Urwin *et al.,* 2000a). Chicken egg white cystatin provided partial resistance to *M. incognita* on rice in containment (Vain *et al.,* 1998) but a resistance level of 83 ± 5% have since been achieved in rice using a maize cystatin and a root preferential promoter (Jayne Green unpublished experiments). Partial resistance was conferred to *Globodera* in a small-scale field trial on a susceptible potato cultivar (Desiree) by expressing chicken egg-white cystatin under control of CaMV35S. The level of resistance was up to 70 ± 9% (Urwin *et al.,* 2001). A somewhat lower level of resistance was obtained in a subsequent trial with the same cultivars with the CaMV35S promoter driving expression of either OcID86 or sunflower cystatin. A third trial was completed expressing OcID86 under control of root-preferential promoters with similar efficacy to the first. There is no evidence that expression of cystatins impairs plant growth or yield in the trial (Urwin *et al.,* 2001). These results establish that cystatins can provide useful partial resistance to plants from a wide range of nematodes.

Nematode resistance achieved with cystatins in banana

'Cavendish' banana has been transformed using *A. tumefaciens* to express a protein engineered rice cystatin (OcID86). Expression was under control of the constitutive promoters maize ubiquitin gene (UBI-1), a chimeric promoter from the octopine and mannopine synthase genes of *A. tumefaciens* (oc3 mas; Ni *et al.,* 1995) or a promoter from a root-preferentially expressed tubulin gene of *A. thaliana* (TUB-1). Western blots confirmed that eight selected lines expressed cystatin with a mean of 0.08 ± 0.04 % tsp. ELISA established the positive lines under control of the UBI promoter provided significantly higher expression levels of OcID86 than recorded for the other two promoters. Eight plants of one UBI promoter line expressing only 0.1 ± 0.004% tsp as cystatin were re-challenged with *R. similis* and achieved a resistance of 70 ± 10% for a line expressing c 0.1% tsp as cystatin.

More recently an African highland genotype has also been transformed with a cystatin (chicken-egg white cystatin) and green fluorescent protein (GFP) under control of the ubiquitin promoter to confirm that transformation occurs. The GFP is a reporter gene to confirm transformation without a chimeric character. Both this protein and the expression of the cystatin were confirmed by western blotting. A large-scale transformation effort is now in progress to determine if adequate expression levels of cystatin can be achieved for nematode resistance (Vain personal communication).

Stacking of R-genes and a bionematicide

Two cultivars with partial natural resistance to *G. pallida* have been transformed with OcID86 under control of CaMV35S. In both cases, the natural partial resistance was enhanced to full resistance (less eggs in the soil post harvest than pre-planting). A similar effect has been achieved in enhancing resistance of tomato to tospovirus infection by crossing transgenic with naturally resistant plants (Gubba *et al.,* 2002). In contrast, adding a transgene did not enhance partial natural resistance in potato to the Colorado Potato Beetle (Douches *et al.,* 2001). This approach may have value in enhancing partial resistance or tolerance detected in screening programs. It may also help suppress nematode species for which plant breeders can offer only partial resistance.

Additive Resistance

The success of stacking an R-gene and a biopesticide suggests that combinations of transgenes also have the potential to provide high levels of transgenic resistance. Co-delivery of OcID86 and CpTI as a fusion protein linked by a short peptide did established an additive effect (Urwin *et al.,* 1997). The linker used was refractory to cleavage *in planta* and proved stable after ingestion by nematodes (Urwin *et al.,* 1998). An appropriate, cleavable peptide linker has the potential to deliver multiple rather than dual inhibitors and potato multicystatins (Waldron *et al.,* 1993) provide a natural precedent for the approach. A second distinct way of delivering multiple biopesticides is by use of Internal Ribosome

Entry Segment (IRES) of the encephalomyocarditis virus. It functions in a plant and can co-deliver multiple, separate polypeptides via a single transgene (Urwin *et al.,* 2000b). Tobacco lines expressing both PIs in a CaMV35S/Oc-ID86/IRES/CpTI construct were challenged with *Globodera tabacum*. The best level of resistance was only 51 ± 3 %. (Urwin *et al.,* 2002). It seems that IRES allowed less CpTI expression in roots than required for effective additive resistance. Engineering of IRES may enhance its value for delivering multiple biopesticides but such effort may not be justifiable as a transgenic DNA sequence from an animal virus is unlikely to be suitable for uptake by banana growers.

Other intestinal targets

cDNA libraries have been constructed and major digestive enzymes defined for *Globodera* and *Longidorus*. Such work has identified additional targets for which some plant genes are known that provide appropriate inhibitors (Catherine Lilley, personal communication). Each inhibitor of interest should be evaluated against *Meloidogyne* and *Radopholus* as representative of banana nematodes. This can be done in *A. thaliana* or in banana if a high throughput transformation program is established for this plant.

Lectins

Snowdrop lectin (GNA) binds to the non-reducing end of a-D-mannosyl residues of glyconjugates and has bioinsecticide activity (Hilder *et al.,* 1995). Some lectins do have biological activity against nematodes. Dis-orientation of nematodes can be achieved by lectins that bind to glyco-conjugates associated with chemoreceptors (Zuckerman and Jansson, 1984). Concanavalin A suppresses *M. incognita* when used as a soil amendment in a tomato crop (Marban-Mendoza *et al.,* 1987). The lectin-binding patterns on the surface of nematodes are complex with different glyco-conjugates varying both in position and species (Dürschner-Pelz and Atkinson, 1988). The choice of lectin is therefore important. Pea lectin that has affinity for a-D-mannosyl residues is an ineffective anti-nematode effector in potato against both invasion and growth of *G. pallida* (Hepher and Atkinson, 1992). Transgenically expressed GNA has a variable effect on *Pratylenchus neglectus* and no effect on *H. schachtii* (Burrows and de Waele, 1997). An effect on the number of females of *G. pallida* was detected when GNA was expressed in potato (Burrows *et al.,* 1998), however, the data show that some GNA-expressing lines also enhanced parasitism. A similar effect has also been reported after soaking *Radopholus citrophilis* in Concanavalin A prior to root invasion (Kaplan and Davis, 1991).

Lectins may have potential as external anti-nematode effectors by causing behavioral disruption but their potential against internal targets may be limited if they are excluded by the feeding tube of at least some parasitic nematodes. Many lectins have toxic effects on insects (see later) and mammals. Concerns regarding toxicological safety may prove a substantial additional limitation to the future commercial development of lectins. GNA from raw, transgenic potato

tubers was reported as having an adverse effect on rats including the mucosal thickness of some parts of the rat gastrointestinal tract (Ewen and Pusztai, 1999). The combination of actual and alleged risk may have left a legacy with consumers and biosafety regulators that compromise the future acceptability of any lectin as a safe transgene.

Endotoxins of Bacillus thuringiensis

The spore-forming bacterium *Bacillus thuringiensis* produces proteinaceous δ-endotoxins (commonly termed Bt) that are encoded by the cry group of genes. This protein binds specifically to receptor sites in the brush border of the midgut epithelial cells. As a result of binding, a conformational change occurs in the endotoxin and a pore forms in the membrane of the epithelial cell that causes a lethal, osmotic lysis. This interaction occurs for a narrow range of host insects but protein engineering raises the possibility of increasing or altering binding specificity. Some Bt proteins have effects against saprophagous nematodes with a pathology that involves disruption of the intestine. The effect takes up to a few days to occur (Borgonie *et al*., 1996). This work has been extended using the Cry5B toxin expressed in *E. coli*. It is toxic to wildtype *C. elegans* and the nematode intestine is damaged (Marroquin *et al.,* 2000). An aspect of the pathology is that a few juveniles hatch within hermaphrodites of *C. elegans*. A similar effect occurs naturally during starvation. Cry5B also has a lethal effect on males, juveniles and a sterile mutant suggesting mortality in the hermaphrodite is not merely due to endothekial matricide. Mutants of *C. elegans* have been identified that are resistant to Cry5B toxin but they remain susceptible to the Cry6A toxin suggesting the binding domain does show receptor specificity (Marroquin *et al.,* 2000). These authors suggest expression in roots may provide an effective basis for plant parasitic nematode control. Borgonie *et al.* (1996) who first identified that Cry5B had activity against nematodes considered that these endotoxins do not hold promise for plant nematode control. It has also been suggested that some of the effects reported by Borgonie *et al*. (1996) may be due to additional uncharacterized crystal and noncrystal proteins exotoxins also made by the isolate used (Wei *et al.* 2003). Cry5B, Cry6A, Cry14A and Cry21A were toxic to several bacterial feeding nematodes with a pathology that seems to act via the intestine. The authors of this work do consider the approach has potential for plant nematode control (Wei *et al.,* 2003).

Protein engineering

Bt proteins characteristically target a narrow range of insects. Therefore, there has been interest in protein engineering of Bt proteins. The aims include countering the prospects of insect resistance to the proteins, altering the range of targeted insects and protecting non-target insects. For instance, the Cry2Aa protein is unusual in having activity against Diptera and Lepidoptera rather than one insect order. It has 87% sequence identity with CryaAb, which lacks activity against Diptera but not Lepidoptera. Correlating the structure of these two

proteins indicates the putative receptor epitope binding site of Cry2Aa and this provides a target for future protein engineering studies towards the above aims (Morse *et al.,* 2001). Marroquin *et al.,* 2000) obtained 50% mortality of *C. elegans* using Cry5B at 12.6µg / ml. This represents 90 nM for this 140,000 kDa protein. More recently Wei *et al.* (2003) have obtained 50% reduction in brood size of *C. elegans* with Bt endotoxins. Values were 16 ng / µl for Cry14A, 47 ng / µl Cry21A, 66 ng / µl Cry5B and 230 ng / ml for Cry6A. They also suggest that structure-function analysis indicates that Cry6A has an unusually small active toxic core with preliminary predicted molecular mass of 43 kDa. The polypeptides of 65-75 kDa that have been used as transgenes for insect control are likely to be excluded from oral uptake by the feeding tube during feeding by cyst and root knot nematodes (see earlier). *Rotylenchulus reniformis* does ingest sufficient GFP to be detected by western blot but not direct visualization (Urwin *et al.,* 2000a). This may indicate Mr 28k is close to the exclusion limit for this nematode. There is a need to resolve the exclusion limit for *Radopholus similis*, *Helicotylenchus* and *Pratylenchus* spp before designing biopesticides that can be effective against them. There may be a need to address two protein-engineering steps before control of plant parasitic nematodes with Cry proteins becomes feasible. An approach must be found to achieve ingestion if the current proteins are too large for ingestion. This may require a further reduction in the size of the molecule. Secondly, it is not yet certain that plant nematodes share receptor specificity with *C. elegans*. Such binding is essential before the conformational change occurs that causes the δ-endotoxin to cause pores in the intestinal cell membrane that result in death. Possibly protein engineering could provide or improve this binding. Further development of the approach is necessary before its potential can be evaluated.

Protein engineering may have aims other than reducing a biopesticide to a size that can be ingested by all the nematode pests of banana. For example, it was carried out with a cystatin to alter its Ki for papain. This work was informed by the crystallographic structure of chicken egg-white cystatin and the complex of human stefin with papain, which allowed a model of Oc-I to be docked into the actives site of papain. As a result, it was found that deleting the Asp-86 from Oc-I to form OcIΔD86 improved the Ki by a factor of 13 fold (Urwin *et al.,* 1995). A similar approach is being adopted to modify a proteinase inhibitor for control of banana weevils (Kiggundu *et al.,* 2002). The digestive proteinase profile of an Andean potato weevil has been defined (Cowgill unpublished experiments) and this study could be extended to banana weevils for target selection. There are common needs for work on nematode and weevil pests of banana.

Directed evolution

The success with protein engineering OcID86 by mutagenesis led to use of directed evolution to explore further changes in Ki of plant cystatins. DNA shuffling was carried out on 11 plant cystatin genes. As a result a large number

of shuffled genes were made with sequences derived from at least two but usually more native cystatin genes. Some of these products had improved Ki relative to many native cystatins (McPherson and Harrison, 2002). This work establishes the potential of gene shuffling to generate new proteins of value for transgenic control of nematodes that may have potential for a wide range of bionematicides.

Peptides

A new technology is emerging that extends anti-nematode effector targets beyond those requiring oral uptake. Aldicarb is a potent inhibitor of acetylcholinesterase and very low levels of this nematicide disrupt chemoreceptive behaviour. Recently Winter *et al.,* (2002) have shown that 50% loss of chemoreceptive orientation of *H. glycines* to Ca2+ occurred for 1.1 ± 3.06pM aldicarb with 90% inhibition at 21pM aldicarb. Aldicarb is frequently used to control nematodes on banana. Much higher levels of 1 µM aldicarb were required to induce paralysis. A possibly related observation is that the chemoreceptive neurons of *H. glycines* fill with FITC beyond their cell bodies to commissures and the nerve ring. Similar uptake of bisbenzimide, a nucleic acid vital stain, results in staining of 8 nuclei in neuronal cells. These seem homologous to the cholinergic amphidial neurons of *C. elegans* which are the only ones to take up FITC in this nematode. Similarly, conjugates of Mr 12 k Dextran / FITC but not Mr 19 k pass along these neurons of *C. elegans*. Winter *et al.* (2002) therefore advanced the hypothesis that aldicarb is effective against nematodes at low concentrations because it also undergoes retrograde transport along cholinergic neurons. A phage display library was biopanned against acetylcholinesterase to obtain a peptide mimetic of aldicarb. A peptide mimetic of levamisole was similarly obtained by biopanning against a *C. elegans* preparation that contained nicotinic receptors. Work with animal parasites suggests that these short peptides are unlikely to enter nematodes via the cuticle (Sheehy *et al.,* 2000). However both disrupted chemoreception of *H. glycines* and *G. pallida* at c 1 nM. J2 cyst-nematodes pre-treated with these peptides showed a reduced success rate at invading plant roots (Winter *et al.,* 2002). Potato can be transformed to express these peptides in roots and suppress nematode numbers in roots (Liu *et al.*, 2005). The key issue now is whether or not plant roots can secrete sufficient peptide to reduce invasion. Biosafety is clearly an issue for the aldicarb mimetic. However, mammals are dosed with levamisole as an anthelmintic (Martin, 1997) and it is used as drug in colorectal cancer treatment (Kraus *et al.,* 1994). Its peptide mimetic is as effective against nematodes as that for aldicarb but it lacks biosafety concerns. Other candidate peptides have also been identified (Bing Liu, personal communication). The possibility that this technology can provide a range of biosafe anti-nematode effectors for control of nematode parasites of banana requires further investigation.

Plant defences against nematodes

There is a wide range of plant defences against pests and pathogens. It is beyond the scope of this work to consider each in detail. However there is relevance in considering both natural genes for resistance (R-genes) and a wide range of molecules that are either pre-formed or occur after nematodes invade roots. The latter may help underpin biotechnology aimed at disrupting root migratory endoparasites. This latter area has received much less attention at the molecular level than characterisation of R-genes that are effective against sedentary endoparasites such as *Meloidogyne* spp.

Natural Resistance Genes (R-genes)

R-genes display a high degree of specificity for a particular pathogen. This specificity stems from a so-called "gene-for-gene" interaction whereby a component of the pathogen, encoded by an avirulence gene, interacts either directly or indirectly with a corresponding R-gene in the plant. The presence of both the avirulence (avr) and the R-gene product results in an incompatible interaction. The plant recognises the pathogen and elicits a suitable defensive response. If the pathogen does not possess an avr product, or it possesses one that is non-functional due to mutational or other change, there will be no interaction with the R-gene product. Such a pathogen will be able to infect a plant.

Several R-genes are targeted against nematodes. The first described was Hs1pro-1 from a wild species of beet that confers resistance to the cyst nematode *Heterodera schachtii* (Cai *et al.,* 1997). Subsequently two genes for resistance to *Globodera* spp have been described (van der Vossen *et al.,* 2000). The resistance gene in tomato to *Meloidogyne* is of greatest interest as this nematode attacks banana. The Mi-1.2 gene of tomato confers resistance against *Meloidogyne incognita* and some other *Meloidogyne* species such as *M. javanica* (Milligan *et al.,* 1998). It also confers resistance to the peach potato aphid *Macrosiphum euphorbiae* (Rossi *et al.,* 1998). This is the first example of a single R-gene conferring resistance to pathogens that differ so remarkably in the nature of their feeding from the host plant. The Mi-1.2 gene encodes a putative protein of 1257 amino acids that belongs to the family of nucleotide binding domain-LRR proteins and has an N-terminal region that encodes a potential leucine zipper domain, perhaps involved in protein dimerization. Some other R-genes are similar to Mi but so far they have only been described from Solanaceous plants (Hwang and Williamson, 2003). Chimeric constructs of the functional gene, Mi-1.2, were produced with a homologue, Mi-1.1 that does not confer nematode resistance. Their phenotypes were used to determine the role of the N-terminus and leucine-rich repeat regions in regulation of localized cell death (Hwang *et al.,* 2000). A model proposed in that work has been studied further. As a result, residues 984-986 have been identified as being involved in nematode recognition. It seems that the LRR region of this gene is involving the regulation of the transmission of the

resistance response and recognition of *Meloidogyne* (Hwang and Williamson, 2003).

There is clearly scope for the transfer of natural R-genes to related crops that may be expected to harbour suitable intracellular signalling pathways and therefore be competent at mounting a resistance response. To-date there has been no reports of Mi-1.2 being functional after transfer to a plant other than tomato. R-genes that are Mi-like may be restricted to solanaceous plants (Hwang and Williamson, 2003). It may be premature to expect Mi-1.2 to be functional in very different plants such as a banana. An attempt has been made to transform banana with Mi-1.2 but to-date its presence and stable integration into banana has not been proven (Møller-Nielsen, 2002) hence its ability to function in banana is not yet tested. Probably more characterization of Mi-1.2 in tomato is required before a portable and functional form can be made that expresses in banana. Molecular evolution may be one basis for allowing new genes to be identified quickly. Possibly a library of R-genes could be based on a common intracellular signalling mechanism but with different recognition characteristics that would trigger this response. Rapid screening of the library against a new pathogen target molecule would provide a basis for creating new transgenic lines that may recognise different avr gene products.

No source of resistance to *Meloidogyne* spp. was found among twenty-six Vietnamese banana accessions, from the AA, AAA, AAB, ABB, AB genome groups and some wild accessions (Van den Bergh *et al.,* 2002). However resistance has been reported to *Meloidogyne megadora* in six banana genomes (Almeida and Santos, 2002). If this response or that of other banana genomes is due to an R-gene against a *Meloidogyne* species, it could be located by marker-assisted breeding which is becoming established for Musa (Crouch *et al.,* 1998) and has been applied to screening genotypes for nematode resistance in soybean plants (Meksem *et al.,* 2001). An alternative way forward is to assume that the gene will have some similar domains to Mi-1.2. In that case, a PCR-based approach could be attempted as in the molecular analysis of the Gpa2 locus in potato. This approach enabled an R-gene cluster to be identified of which at least two genes were active. One corresponded to the previously isolated Rx1 gene that confers resistance to potato virus X, while the other corresponds to the Gpa2 gene that confers resistance to the potato cyst nematode *Globodera pallida.* The proteins encoded by the Gpa2 (912aa) and the Rx1 genes share an overall amino-acid identity of greater than 88% and belong to the leucine-zipper, nucleotide-binding site, leucine-rich repeat containing class of plant resistance genes (van der Vossen *et al.,* 2000).

If a single dominant R-gene conferring resistance against *Meloidogyne* spp does occur in *Musa* spp., it could be cloned and studied as so powerfully achieved for Mi-1.2 (Hwang *et al.,* 2000; Hwang and Williamson, 2003). Possibly its characterization and modification at the molecular level could at least result in its

recognition and control of all *Meloidogyne* spp. It is difficult to judge whether or not this approach or modification of Mi-1.2 to function in banana is likely to be the more rapid way forward. Possibly all R-genes against *Meloidogyne* achieve their effect by targeted cell death of giant cells. If so, it is uncertain that it could be adapted to recognize and control migratory endoparasites. Even if recognition could be engineered, it may be that the defence may induce excessive plant cell death in roots given the mobility and population density of these parasites.

Other basis for resistance in roots

Other protein-based defences occur against pathogens. Pathogenesis related (PR) proteins have received much attention for the potential for their role in providing systemic resistance to certain pathogens. PR-proteins are induced systemically in potato leaves over 14 days following root invasion by *G. rostochiensis* or *G. pallida* (Hammond-Kosack *et al.* 1989, Rahimi, *et al.* 1998) but there is no evidence that this protects plant roots from nematodes. Roots also constitutively express basic PR proteins but their role in reducing invasion success of nematodes is not established. There has been much interest in manipulating PR and other proteins involved in systemic acquired resistance to maintain high levels of the protection throughout plant growth (Uknes *et al.,* 1996). This approach seems unlikely to provide a basis for controlling the nematode parasites of banana roots.

Some secondary metabolites accumulate following nematode invasion of roots. The phytoalexin glyceollin I increases in soybean roots from 2-4 days after their invasion by the soybean cyst-nematode (*H. glycines*). The effect occurs at the onset of an incompatible interaction between race 1 of this nematode and cv Centennial (Huang and Barker, 1991). Nematode invasion of roots can result in responses that induce hypersensitivity leading to localized plant cell death. For some other pathogens this is due to the generation of reactive oxygen intermediates, O_2- and H_2O_2. Nitric oxide also acts in the hypersensitive response to help prevent pathogen spread (Delledonne *et al.,* 1998). It seems to have a complementary activity with the reactive oxygen intermediates. The production of NO and oxygen intermediates seems to induce progressive secondary oxidative bursts that amplify the initial response and potentiate reiteration of the signal (Alvarez *et al.,* 1998). This involves systemic induction of hypersensitive cell death and activation of other defence-related genes occurs. This may provide more than one means of inducing specific defences. Complex control of cell death may limit the unwanted consequences of too widespread a hypersensitive response. However some locally systemic response is necessary to isolate a nematode from living plant cells on which the biotroph must feed.

Increased peroxidase activity is also reported after nematode invasion (Andres *et al.,* 2001). It too may produce bursts of H_2O_2 that have local effects on both nematodes and plant cells. The latter may include: (i) involvement in constructing intermolecular linkages in the wall matrix in wounding repair; (ii) catalysis of phenoxy radical formation in the final stages of lignification; (iii)

polymerisation of phenolic molecules in the production of suberin (this involves highly anionic forms of the peroxidase); (iv) cross-linking proteins with phenolic groups (e.g. in hydroxyproline rich glycoproteins (HRGPs); extensins); (v) polysaccharide gelling, for instance by increasing differulyol bridges in pectins (van Huystee, 1987).

Some of the other bases of root defence to nematodes are of interest. Pre-formed plant defensive compounds occur and by their nature must lack anti-plant cell effects. Many such secondary metabolites have anti-nematode effects (Huang, 1985) and some do occur in roots. Resistance in banana to migratory endoparasites has been reported in some genotypes including both male (AA, AABB genotypes) and female parents (AA, AAB genotypes) and one hybrid (AAAB). This work also confirmed that the resistance previously demonstrated in one genotype Pisang Jari Buaya (AA) could be transferred to other *Musa* genotypes (Vaiene *et al.,* 2003). Possible sources of resistance / tolerance to *P. coffeae* have also been reported in some genotypes of banana. The resistance of dwarf *Musa* cv 'Kunnan' to *R. similis* seems related to its high levels of condensed tannins with levels being unresponsive to infection. The tannins of cv 'Kunnan' had a mostly procyanidin character, but also contained propelargonidins (Collingborn, *et al.,* 2000).

Possibly, plant defence involving pre or post-invasion synthesis of secondary metabolites can be manipulated to provide at least partial protection of banana from migratory endoparasites. This may be a long-term objective but would be important if R-gene mediated resistance is not identified for all nematode parasites of banana. A possible long-term aim is to manipulate both R-genes and secondary metabolite defences to provide protection from a wide range of nematodes and achieve durability by the functioning of different defence responses in tandem. These approaches would be important if all biopesticide-based approaches failed to gain acceptance outside of the scientific community.

Mimicking resistance responses

There have been attempts to destroy the plant cells from which nematodes feed using approaches that are analogous to R-gene function. Emphasis to date has been placed on the modified plant cells that cyst and root-knot nematodes specifically induce. The paradigm for such work is emasculation of maize using barnase, a small RNAse, and a tapetum-specific promoter (Mariani *et al.,* 1990). Unfortunately, nematodes are unlikely to induce a gene to express in just those plant cells from which they feed. However, some unwanted expression in other cells could be countered using barstar, an inhibitor protein of barnase (Hartley, 1988). Fertility was restored to maize when a line emasculated by barnase was crossed with a line expressing barstar (Mariani *et al.,* 1992).

Transgenic nematode resistance has been explored using a promoter expressed predominantly in the feeding cell to control expression of barnase plus barstar expressed constitutively to counter unwanted leaky expression of the RNAse

elsewhere in the plant (Ohl *et al.,* 1997). The risk that the restorer is not always effective can be overcome using a bi-partite effector and using two promoters to provide an expression pattern that overlaps only in nematode feeding cells. The potential of this approach has been explored using a plant ribosome inactivating protein (RIP) from maize delivered in two parts by different promoters. (A. Neelam, C.Thomas & McPherson, personal communication). Possibly such systems could be adapted to mimic a hypersensitive response where migratory endoparasites feed. Appropriate promoters might be identified after microarray-based screening of whole plant genomes. One principal problem might be to ensure that the localised cell death isolates such nematodes from a food source effectively, without causing excessive unwanted cell death. This may be a particular risk given the high density of such nematodes that may challenge a banana root system and their probable ability to re-invade roots when feeding fails. This approach may be even less simple to optimise for a migratory than a sedentary endoparasitic nematode.

Biosafety

There is a wide range of biosafety issues that could be considered. Some are generic to all transgenic crops and fall outside the scope of this review. Therefore, only aspects particularly relevant to development of transgenic resistance to nematodes on banana are considered.

Food safety

There can be no compromise on the safety of food from proteins expressed transgenically. The added problem in the developing world is that labelling may not be practical. A proportion of consumers of the crop may be illiterate and this hampers dissemination of information relating to both human health and pesticide safety. Secondly much of the production is within subsistence farming and so consumption may not involve even informal markets. A practical way forward may be to ensure the transgenically derived protein poses no risk to the consumer. The main issues are proteins that are new to the diet and safe levels of consumption for any protein by the most vulnerable in society such as young children and the elderly. The situation is helped when toxicology and allergenicity information has been determined for first world markets. The reasonable assumption is that humans in different countries are likely to be equally at risk when consuming similar levels of the new food. When a crop like banana provides a staple diet then the exposure of the individual will be greater than when the banana is a dessert crop and is only a minor component of the diet. Therefore, dietary exposure must take into account the extreme consumption rates of the novel protein by those highly dependent on eating bananas or plantains.

There have been thirteen toxicological studies of proteins that may be used in transgenic crops (Kuiper *et al.*, 2001). Three were conducted on humans to examine reactivity of sera and the remainder have relied on rodents. This second

approach allows a wide range of measurements to be made. These have included food intake, body weight, organ weights, histopathology of organs, blood chemistry, and haematological measurements. The protein can be delivered *in planta* but it may be better at first to use the isolated protein in initial studies. This is to avoid the issues of equivalence of diet and over complexity of experimental design.

The maximum level of a novel protein that a human might consume from plantains or bananas expressing it transgenically to protect from nematodes can be estimated. The soluble proteins of banana and plantain represent 1% of the fresh weight. The maximum quantity of plantains or banana consumed per person is 272 g / day for Uganda. This provides 2.72 g day of protein to the diet. Assuming transgenic expression is not higher than 1% tsp, the maximum exposure to a transgenic protein is 27.2 mg protein per day or 0.54 mg / kg / day for 40 Kg person. The expectation should be that this is equal or less than the maximum amount needed for a no effect level (NOEL) with rodents. There has to a margin of safety between that level and the actual dietary consumption. A dietary margin of exposure (MOE) of 100 fold or more is unlikely to cause dietary concern (EPA). This suggests a level of 5.4 mg / Kg body weight / day is the maximum level of transgenic protein that an adult dependent on banana should consume to ensure a safe MOE. The MOE has been determined for the δ-endotoxin of *Bacillus thuringiensis* (Bt) used in insect control (Betz *et al.,* 2000). The MOE values for Bt are 2000 to 250,000 with the lower factor pertaining when the maximum dose used with rats was insufficient to define the true NOEL. NOEL for OcIΔD86 is >200 with constitutive expression in potato tubers and >2000 with root specific expression that reduces the level in tubers. The values are underestimates as the NOEL was not defined using a maximum dose of 10mg OcID86 / Kg / day / rat. However MOEs of >100 tend not to cause dietary concern (EPA, 1998). This seems an appropriate point in this case as cystatins are normally consumed by humans. For instance consuming two chicken eggs with a combined weight of 100g provides 3.72 mg cystatin (Awade, 1996) and human saliva also provides 13mg of swallowed cystatin / day (Veerman *et al.,* 1996).

There is a case for carrying out some initial test of a protein under development for nematode control before the expense of expressing it in banana or plantain. A protein is unlikely to be a toxin if it is readily digested. The Bt proteins Cry1Ab, Cry1Ac, Cry2Aa and Cry3a are all no longer detectable after 30-60 seconds in simulated gastric fluid (Betz *et al.,* 2000). One indicator that a protein is unlikely to be an allergen is its rapid partial digestion by pepsin in simulated gastric fluid (Astwood *et al.,* 1996) providing the fragments do not retain allergenicity (Fu, 2002). Proteins do need to be studied individually. Unlike several other Bt proteins, that from the Cry9 gene causes concern over its allergenicity and EPA now restricts its commercial use to fodder crops (EPA, 2001). Snowdrop lectin (GNA) has also been developed for insect control but some concerns about its toxicity have been expressed as it alters some enzyme activity in the intestinal

brush border cells of rats (Pusztai *et al.,* 1996; Ewen and Pusztai, 1999). In contrast, Puzstai *et al.*(1992) suggested that CpTI was not toxic to rats although a 30% increase in pancreas size was reported in toxicological tests. OcIΔD86 is also readily digested within 15 seconds in simulated gastric fluid. It does not form stable fragments that could be allergens. A further check is to complete an *in silico* study to determine if the bionematicide shares a motif with known allergenic determinants of other proteins in the database. Such an approach would enable effort to be invested only in bionematicides that are likely to pass later toxicological and allergenicity testing. Hopefully such extensive work will be part of the deployment of the protein in first world crops for nematode control.

Environmental concerns

Deployment of transgenic nematode resistance is justifiable at several levels but a key need in many commercial banana plantations is a reduction in nematicide use. This has been a matter of considerable concern for decades. It has both environmental consequences and implications for the health of agricultural workers. Therefore FAO considers one of the most compelling reasons for adopting genetic transformation of banana is to reduce pesticide use (Anon, 2001). Another concern is a gradual spread in the use of nematicides among poor farmers who are ill equipped to safeguard themselves from incidents of pesticide poisoning. Possibly, their uptake may occur for growers in a periurban environment that provides opportunity for marketing bananas. Benefits of a transgenic approach are a reduction in nematode-induced losses, a reduction in the frequency at which re-planting is required and a reduced entry rate of some growers to the pesticide market. Future uptake of transgenic plants may occur for the very many subsistence growers that do not currently use nematicides.

The methodologies for determining if the impact of a transgenic nematode resistant plant on non-target organisms is benign or harmful are still in development. Birch *et al.* (1999) suggested that non-target effects could occur with GNA as a bioinsecticide. They showed that aphids feeding on GNA adversely affect the reproduction of the ladybirds that prey upon them. This effect could arise because compromised aphids are inadequate prey rather than the lectin having a direct effect on the predator (Down *et al.,* 2000). GNA fed in an artificial diet to aphids can also have a dose-dependent effect on aphid parasitoid development (Couty *et al.,* 2001). The ecological significance of these findings on predators and parasitoids of aphids requires thorough evaluation. This would also be necessary if GNA was used as a bionematicide unless expression was restricted to roots.

Effort has been made to set up an appropriate basis for assessing any environmental risks that cystatins pose for non-target organisms when used as anti-nematode effectors. Using the potato crop as a specific example, a general, sequential approach to risk assessment has been developed for non-target

organisms (Cowgill and Atkinson, 2003). This could be adapted for banana. First, the non-target, herbivorous insects of the canopy are surveyed. The prevalent groups plus ones that represent different invertebrate groups are selected for screening. In the case of cystatins, the aim would be to determine which non-target invertebrates use cysteine proteases as digestive enzymes. That sub-group was then screened in containment against transgenic plant material. For instance, the leafhopper *Eupteryx aurata* does have a digestive cysteine proteinase but seems not to receive an adequate dose of cystatin when feeding on potato leaves in containment or the field to compromise its growth (Cowgill and Atkinson, 2003). Similarly, the aphid *Myzus persicae* is not harmed by such plants although a cystatin does reduce its growth when added to artificial diet (Cowgill *et al.,* 2002a). It seems that neither a phloem feeding aphid nor *Eupteryx aurata* receives sufficient cystatin for an adverse effect although the same plants provided partial resistance to a nematode. In contrast, the slug *Deroceras reticulatum* is adversely affected by consuming leaves expressing cystatin (Walker *et al.,* 1999). Presumably, it is an example of an invertebrate with digestive cysteine protease that browses on leaves rather than being a specific tissue feeder. In response to concerns about effects on non-target organisms, anti-nematode proteins can be restricted to expression in roots (see earlier) and significant, adventitious exposure for leaf feeders can be avoided.

Since most nematodes attack roots, it follows that root expression may have non-target consequences for other soil organisms. Ester-linked phospholipid fatty acid analysis (PLFA) provides one means of determining if the number of soil organisms is perturbed (Cowgill *et al.,* 2002b). PLFA indicate active microbial biomass as these compounds are turned over rapidly and some provide "signatures" for different types of microbes such as bacteria or fungi. Major temporal changes in PFLAs during the growing season of potatoes were revealed (Cowgill *et al.,* 2002b) probably related to changes in soil moisture. Superimposed on this change was a small effect of cystatin expressing potatoes on the microbial community. The latter effect was insufficient to alter the rate of leaf litter decomposition. There were also no effects on abundance of micro arthropods or free-living nematodes. More work is required to complete the audit of effects on soil organisms. One issue is the persistence of anti-nematode effectors in soil. For instance, a CRY protein adheres to soil particles and this enhances its persistence (Saxena and Stotzky, 2001).

Technology transfer issues

Technology transfer to subsistence growers of banana should be on a royalty-free basis to the grower. Separation of agribusiness and subsistence banana production could be made by distinguishing growth for internal consumption and export to the developed world as for other crops (Herrera-Estrella, 2000). The distinction is readily underpinned by the difference in cultivars required for export commerce such as cv 'Cavendish' and other uses including cooking banana and plantains. Under these terms, it is likely that patent and or license

holders will allow technology transfer to occur. Patents for proteinase inhibitor based control of nematodes are owned by the University of Leeds and have been donated for several developing world applications including banana in Africa. The pro-vitamin A rice (Golden Rice) developed by a Swiss government institute (Beyer *et al.,* 2002) is another well-studied example. ISAAA is one organization brokering IP from first world laboratories to third world donation. Such approaches help avoid the issue of the very many patents that would otherwise impinge upon work such as Golden Rice. Consensus for donation is preferable to relying on a lack of patent coverage in many developing countries. Many judge WTO agreements as less than fair to the developing world (Tansey, 1999) and donation of first world IP is one aspect of improving the situation for poor farmers.

The need for a step change in progress

One weakness of agricultural research in the developing world is the inadequacy of resource levels. This lack of resource defines the type of science that can be attempted by scientists active in such countries. The value of more work on issues such as the prevalence and damage of nematodes on banana is limited. The case for the damage they cause is already well made and the real issue is the need for low-cost, effective and biosafe control. What is required is a step change in approach that results in a large reduction in nematode induced yield loss to banana worldwide. This is a key argument in favor of developing biotechnology for banana. CGIAR institutes such as INIBAP, government agencies such as USAID, DFID and major charities such as The Rockefeller Foundation could concentrate resources and co-ordinate their efforts to achieve this. Transgenic crops have been rapidly taken up by farmers when made available to them in countries as diverse as USA, Argentina, India and China. The potential advantages for first and developing world applications are not the same even with one application such as Bt for insect resistance in cotton. In the USA, the main advantage is reduced cost of control of cotton insect pests. In the developing world, effective insecticides are often neither available nor affordable by poor farmers while the pressure of insect pest damage is higher than in the USA. Consequently, deployment of Bt cotton in India reduces cost to growers but unlike in the USA, it enhances yields (Qaim and Zilberman, 2003). Nematicides for banana nematode control are neither affordable for subsistence growers or biosafe. Therefore a transgenic banana with full and durable resistance to all its nematode problems offers clear advantages that merit development of the approach.

Work to-date suggests that resistance to all nematodes is achievable within 5 years at an appropriate level of investment. Priorities are: a) to define which approach(es) have highest potential, b) to provide appropriate promoters c) to evaluate efficacy against the target nematode species d) to ensure necessary biosafety pre-dates considerable investment in any novel protein e) to cover socio-economic and IP related issues to ensure safe and rapid uptake of

technology, once proof of principle is achieved. The details of the various potential tasks are summarized in table below.

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Annexe 1. Time frame and level of effort required to achieve control of nematodes

Activity	Comment	Milestones	Timeframe	
			Lab work	Field proof
Manipulation of natural resistance	Resistance may not be conferred by single genes Resistance may not involve R-genes similar to structure of Mi etc Resistance that is not based on R-gene mechanisms may not be manipulated effectively with current understanding Resistance may not be achievable against all nematodes	Determine if R-genes do exist in *Musa* spp Evaluate whether or not other approaches can be defined, critical genes cloned and their expression controlled in an effective manner	3 years* 3 years* (Concurrent)	8 years# if progresses
Underpinning studies	Determination of the exclusion limit of proteins by targeted nematodes to ensure biopesticides of appropriate size are prioritised Protein engineering/molecular evolution to modify proteins of interest Development of cleavable linkers to allow a single fusion protein to deliver several bionematicides for durability			
Bionematicides	A wide range is available for evaluation	Select the best 3 approaches based on biosafety plus efficacy and ability to affect all banana nematodes	3 years *(basis for more than 1 project; linkers to C)	2 years# for genes of known efficacy
	Several have already been shown to be effective against plant nematodes	Deliver the three concurrently for effective and durable resistance		5 years# for genes not yet of proven value
	Suitable for stacking to achieve durability and high levels of resistance	Evaluation of expression, activity and efficacy of lines		
Promoters	Promoters with activity appropriate for endoparasites	Screening of banana root cDNA library Microarray screening of *A. thaliana* infected with *Radopholus* Use *A. thaliana* promoter heterologously or obtain a homologue gene from banana	3 years*	5 years to function with bionematicides of known value
	Comparison of promoters effective against all banana nematodes with an approach based on separate promoters for migratory and sedentary species	Spatial and temporal reporter studies of nematode distribution in roots and promoter activity		7 years for deployment of new promoters with currently not evaluated bionematicides
Transformation	Transformation capacity that does not delay evaluation of approaches	Judgement to be made whether high throughput transformation of banana is/ is not a bottleneck to progress. If it is then *A. thaliana* to be used for selecting	2 years*	

Activity	Comment	Milestones	Timeframe	
			Lab work	Field proof
		genes of interest by their efficacy before entry to banana transformation programme *A. thaliana* has uncertain value for promoter-related studies that needs to be resolved If banana used, a genotype to be selected on basis of transformation ease and utility for field evaluations		
Biosafety	Initial toxicological and allergenicity studies on candidate novel proteins	Ideally before investment in transformation and field evaluation of bananas Rapid initial screen of each new protein obtained. Could be accommodated within B or C above		
	Environmental work to evaluate non-target effects	Concurrent with field evaluation of novel resistance		
Supportive	Access to all IP required for use	Also required to underpin other banana biotechnology programmes		
	Social-economic studies to ensure acceptability locally, nationally and internationally the work	Also required to underpin other banana biotechnology programmes		

* 1 year is 1 Post-doctoral worker 100% full time plus technical support at 40% full time.
# 1 year involves field trial effort involving technical staff while effort indicated by * continues.

Strategies for the generation of virus resistant bananas

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Introduction

As a group, viruses have had and continue to have a major negative impact on the production of bananas worldwide. This negative impact is manifested in three distinct areas: (i) yield reduction as a direct consequence of virus infection, (ii) limitation on the multiplication and distribution of vegetative planting material and (iii) impact on conventional breeding programs. However, the impact of the individual viruses differs significantly. For instance, banana bunchy top babuvirus, banana bract mosaic potyvirus and, to a lesser extent, banana streak badnavirus are clearly the most important viruses from a crop damage perspective. Banana streak badnavirus is the only virus impacting conventional breeding programs whereas all banana viruses need to be excluded from vegetative planting material and therefore limit the multiplication and distribution of this material.

There are relatively few viruses reported from bananas and the majority of these are reasonably well characterised, if not controlled. Interestingly, the viruses are from quite diverse backgrounds. The virion and genome properties of the major viruses infecting bananas are summarised in Table 1.

The Viruses

Banana Bunchy Top Babuvirus

Banana bunchy top babuvirus (BBTV) is almost certainly the most important of the banana viruses from a yield reduction perspective. This virus, transmitted persistently by the black banana aphid, has been reported from all the major banana producing areas except for the Americas. It is already present in a number of countries in Africa and reports suggest it is spreading. Infected plants do not usually produce a bunch and plantations can rapidly become unproductive. The virus virtually destroyed a major part of the Australian banana industry in the 1920s but is now well controlled through strictly enforced phytosanitory regulations (Dale, 1987). Despite considerable effort, the virus has not been eradicated. In areas such as Pakistan, India, the Philippines, Vietnam and the South Pacific islands, attempts at similar control measures have been far less successful. The virus exists as two groups, the Asian group with isolates from the Philippines, Vietnam, China, Taiwan and Indonesia and the South Pacific group with isolates from Australia, the Pacific Islands, India, Pakistan and Africa (Egypt, Gabon and Burundi) (Karan *et al.* 1994; Wanitchakorn *et al.* 2000). While there is a reasonable level of genomic sequence variability, it appears that this will probably not be a major obstacle to generating stable transgenic resistance.

Table 1. Virion and genome characteristics of the significant banana viruses

Virus	Virions	Genome	Replication	Classification
Banana bunchy top babuvirus (BBTV)	Isometric	Multi-component circular ssDNA	Rolling circle replication	Babuvirus, Nanoviridae
Banana streak badnavirus (BSV)	Bacilliform	Single component circular dsDNA	Via RNA using reverse transcriptase	Badnavirus, Caulimoviridae
Banana bract mosaic potyvirus (BBrMV)	Filamentous	Single component linear ssRNA	RNA dependent RNA polymerase	Potyvirus, Potyviridae
Banana mild mosaic virus (BanMMV)	Filamentous	Single component linear ssRNA	RNA dependent RNA polymerase	
Cucumber mosaic cucumovirus (CMV)	Isometric	Multi-component linear ssRNA	RNA dependent RNA polymerase	Cucumovirus, Bromoviridae

Banana Streak Badnavirus

Banana streak badnavirus (BSV) certainly causes the most complex of the banana virus diseases as it exists in two forms, a "conventional" virion or episomal form and an integrated form (Ndowora *et al.* 1999; Harper and Hull 1999). The episomal form appears to be geographically widespread and has been reported in most banana producing countries. However, there are only a few examples of significant levels of infection and yield loss. Importantly, one of these examples is from Africa (Harper *et al.* 2002a). As an episomal form, BSV is transmitted by mealybugs and is hypervariable, that is, there is a very high level of genome sequence variation which has complicated efforts to develop reliable diagnostics (Dahal *et al.* 1998). This hypervariability may also provide a major challenge in developing transgenic resistance.

It has now been determined that the BSV genome is integrated in the genome of many accessions of *Musa balbisiana* in such a way that after activation the integrated BSV genome fragments can recombine to produce an episomal virus (Harper *et al.* 2002b). The most common activator is hybridisation; a number of banana breeding programs have used *M. balbisiana* as a parent or have used a parent with a B genome containing activatable integrated BSV and many of the resultant progeny are infected with episomal BSV. As knowledge and understanding of this phenomenon has increased, breeding programs are screening prospective parents to avoid where possible, the use of B genomes with activatable, integrated BSV.

Banana Bract Mosaic Potyvirus

Banana bract mosaic potyvirus is quite geographically restricted but in most regions where it occurs, it causes significant damage. It is widespread in the Philippines, certain parts of India, Sri Lanka and has been recorded in Vietnam and Samoa (Rodoni *et al.* 1999). It is transmitted non-persistently by aphids and appears to spread rapidly in the field. There are reports of yield losses of up to 40% and it has been extremely difficult to control. There is genomic variability reportedly up to 10% but this may be very regional.

Banana Mild Mosaic Virus

Banana mild mosaic virus (BanMMV) has only recently been discovered and characterised (Gambley & Thomas, 2001). It appears to be geographically widespread but there have been no reports of this virus causing yield losses of any consequence. Reliable diagnostics are now available for this virus and therefore this virus cannot be considered as an important target for transgenic resistance.

Cucumber Mosaic Cucumovirus

Cucumber mosaic cucumovirus (CMV) is one of the most common of all plant viruses (Palukaitis *et al.* 1992). It has an incredibly wide host range which includes both dicots and monocots and it has been recorded in virtually every country with extensive agriculture. In many crops, particularly cucurbit crops, it can occur at high incidence and cause major yield loss. This, however, is not the case in bananas. Incidence is normally low and sporadic and it can be considered an opportunistic virus in bananas. The highest incidence seems to occur when bananas are inter-cropped with a cucurbit crop. Further, bananas appear to be a dead-end host as it is very difficult to transmit the virus from bananas.

There is wide variability with the CMV genome which would make generating broad spectrum transgenic resistance a challenge. Transgenic resistance to CMV has been generated in other species using both coat protein mediated resistance and posttranscriptional gene silencing. However, again it would be difficult to justify generating transgenic bananas resistant to CMV.

The Strategies

Strategies for the RNA Viruses

Transgenic virus resistance is the most advanced of the applications of biotechnology to the control of plant pathogens. The first example of transgenic virus resistance was published in 1986 (Abel *et al.* 1986), reporting generation of transgenic tobacco with resistance to tobacco mosaic tobamovirus (TMV) through the constitutive expression of the TMV coat protein gene. While coat protein mediated resistance has not proven to be widely applicable to the control of other viruses, it represented the beginning and the incentive to develop transgenic resistances for many other plant viruses. This led to the discovery of RNA mediated resistance and from this the discovery of posttranscriptional gene

silencing. Posttranscriptional gene silencing (PTGS), also referred to as RNAi, is now recognised as an innate mechanism to control gene expression which appears to be present in virtually all eukaryotes.

At least in plants, PTGS is highly effective in generating immunity to most, if not all, the RNA plant virus genera for which it has been tested rigorously. The mechanism of PTGS in plants is also now reasonably well understood. Essentially, the plant recognises an RNA species as aberrant and targets this RNA for degradation. The plant generates small 20-22 nucleotide molecules which are complementary to the target RNA. These molecules hybridise with the target RNA and these resultant dsRNAs are then degraded by a plant encoded dsRNase, Dicer. Further, there are now very efficient mechanisms for triggering PTGS against a virus sequence in plants. Waterhouse *et al.* (2001) have developed a series of vectors in which the target RNA sequence derived from the target virus is inserted (as DNA) as an inverted repeat with the repeats separated by an intron. Once transformed into the plant and transcribed, a novel dsRNA is formed and this is very rapidly and efficiently recognised by the plant as an aberrant RNA and is silenced. The plant is then "pre-immunised" for invasion by the homologous virus and is effectively immune to infection by that virus. One of the significant advantages of this approach is that the transgenic plant does not express any recombinant proteins (Goldbach *et al.* 2003).

There are two important disadvantages to this strategy. The first is that by definition it is highly sequence specific whereas most plant viruses exists as a continuum of strains which differ from one another to a greater or lesser extent in their genome sequence. Thus, PTGS targeted against one strain of a virus may not be effective against a distant strain. This can be partially overcome by (i) surveying the virus isolates extensively and particularly regionally so that selection of the integrated sequence is the most appropriate to generate broad immunity and (ii) to design the transgene such that it covers the breadth of sequence variation likely to be encountered. The second disadvantage is that virtually all plant viruses encode silencing suppressors, proteins that suppress the plant's silencing mechanism. This does not appear to affect the immunity of the plant to the initial invading homologous virus as presumably the viral RNA is degraded before the virus encoded suppressor can take effect. However, if the transgenic plant is either previously infected with a heterologous virus (a virus from another genus/family or a strain that is too different in sequence to be recognised by the silencing mechanism) or post infected by a heterologous virus, then that virus' silencing suppressor can be and the plant can potentially become susceptible to infection by the heterologous virus. This is obviously of most concern in regions where plants are regularly infected by multiple viruses and/or where there is high sequence variability within the local target virus population. It is highly likely that these disadvantages can be overcome by understanding the virus population structures in the target regions and by careful design of the virus derived transgene.

Transgenic virus resistant plants using this strategy have already been commercialised; transgenic papaya immune to papaya ringspot potyvirus (PRSV) are now commercially grown in Hawaii (Gonsalvez, 2002) and transgenic papaya with PRSV resistance have been developed for and tested in a number of other countries including Australia (Lines *et al.* 2002) and Thailand. The isolates of PRSV-P (the type of PRSV that infects papaya) have quite significant sequence differences which are very much correlated with geographical distribution and therefore it is essential that transgenic papaya are developed for a region only after extensive surveying. Interestingly, this survey should also include PRSV in cucurbits (PRSV-W) which does not infect papaya but almost certainly is the source of mutants that do infect papaya (Bateson *et al.* 2002).

There have been no convincing reports of PTGS as a mechanism to generate transgenic resistance to the dsRNA viruses of plants. However, no dsRNA viruses have been reported in bananas.

Strategies for the ssDNA Viruses

Development of effective strategies for generating transgenic resistance to ssDNA viruses has remained one of the major challenges particularly for virus control in tropical and sub-tropical crops. The ssDNA viruses of plants include both the Geminiviridae and the Nanoviridae. These two families include some of the most devastating of all the plant viruses such as Africa cassava mosaic begomovirus, maize streak mastrevirus, cotton leaf curl begomovirus, tomato (yellow) leaf curl begomovirus, bean golden mosaic begomovirus, wheat dwarf mastrevirus and many other major viruses of other crops. Significantly, BBTV is also a ssDNA virus.

Unfortunately, the strategies, particularly PTGS, that have been so successful for generating resistance to the RNA viruses have not been reported to be effective for the ssDNA viruses; with one very recent and unconfirmed exception published as Correspondence to Nature Biotechnology (Pooggin *et al.* 2003) where the target sequence was the viral intergenic region. This would appear not to be the same mechanism that is operating with ssRNA viruses. There are also very few reports of coat protein mediated resistance against ssDNA viruses (Kunik *et al.* 1994) and it is generally accepted that this strategy is ineffective. Therefore, there have been concerted efforts to develop strategies that target specific characteristics of these viruses and in particular to interfere with the replication of the virus.

While Geminiviridae and Nanoviridae have different virion morphology and genome organisations, they have the same replication strategy which involves rolling circle replication and requires host cells to be replication competent. The virus encodes a Rep protein which is a replication associated protein involved in nicking and ligating within a highly conserved loop sequence and which both initiates replication and forms the final circular ssDNA molecule. The individual virus Reps recognise specific sequences within the intergenic regions and bind to

these thus providing the specificity between the Rep and its substrate. However, the Reps are not DNA polymerases; this function is provided by the host cell. The virus also encodes a retinoblastoma (Rb) binding-like protein which is involved in switching the host cell from G phase to S phase thus creating an environment for viral DNA replication. The Geminiviridae encode this function with the Rep functions on the same gene whereas the Nanoviridae have separate genes encoding these two functions.

One of the most promising transgenic strategies for the ssDNA viruses is expressing a defective Rep protein which retains the ability to bind to its target viral DNA but lacks the functions of the Rep (Brunetti *et al.* 2001; Hanson & Maxwell, 1999; Sangare *et al.* 1999). The concept is that the defective Rep will bind to the invading viral DNA and out-compete the viral Rep thus reducing or eliminating DNA replication. The two approaches have been to either mutate the Rep gene or to truncate the Rep gene and constitutively express the defective Rep at high level. The mutations are specifically targeted to motifs involved in rolling circle replication. However, merely mutating the Reps of geminiviruses has not been practicable because the resultant gene continues to encode the Rb binding activity and attempts to regenerate plants expressing such proteins is either not possible or results in very abnormal phenotypes. The situation may well be very different for nanoviruses as their Reps do not have Rb binding activity. This is still to be tested. As a consequence, a number of groups have truncated the geminivirus Rep proteins in an attempt to avoid the Rb binding activity. In the most recent report, Lucioli *et al.* (2003) expressed the first 630 nucleotides of the Rep of tomato yellow leaf curl Sardinia virus to generate resistance. They demonstrated that resistance to the homologous virus was conferred by inhibiting transcription of the Rep gene of the invading virus while resistance to a heterologous virus was through the interacting properties of the truncated Rep oligomerisation domain. The duration of the resistance was related to the ability of the invading virus to switch off transgene expression through gene silencing.

Strategies depending on truncated and mutated Reps to inhibit virus replication and or transcription require the transgene Rep to be constitutively expressed at high levels which will always be a disadvantage.

A quite different approach was devised by Hong *et al*. (1997); they utilised the natural activation of the begomovirus coat protein promoter by the Trap gene product. They transformed *Nicotiana benthamiana* with a gene encoding the ribosome inhibiting protein, dianthin under the control of the begomovirus coat protein promoter. The concept was that the invading virus would activate the expression of dianthin by the viral Trap protein and this would inhibit viral replication by inhibiting translation of the viral mRNAs. While there was inhibition of virus replication, ultimately, this strategy was unsuccessful as there was leakage from the coat protein promoter. Further, Padidam *et al.* (1999) have been developing a strategy based on the constitutive expression of a bacterial

ssDNA binding protein in plants. There have been no published reports of the outcomes of this strategy.

At QUT, we have developed a very different and broad resistance strategy that should be applicable to all ssDNA viruses of plants (Dale *et al.* 2001). This strategy, virus activated cell death, involves integrating into the host plant a construct encoding a split suicide gene which is flanked by the target virus intergenic region which in turn are embedded in introns. The suicide gene is only activated upon infection by the target virus and is only expressed in cells that are infected by the target virus. Activation is by viral Rep mediated replicative release and circularisation during which the suicide gene is reconstituted leading to transcription, processing out of the intergenic region embedded in the intron and finally translation of the suicide gene and cell death. We have developed this strategy using tobacco yellow dwarf mastrevirus (TYDV). Regenerated transgenic tobacco carrying the split suicide gene construct were morphologically normal indicating that there was no expression leakage or recombination during transformation. Of the first two transgenic lines challenged by an infectious clone of TYDV, one was completely immune (no symptoms and negative by PCR and Southern analysis) whereas all plants of the other line had symptoms, were positive by PCR but had either undetectable or very low levels of viral DNA as compared with wildtype controls. These results are very promising but will need to be expanded to other virus/host combinations.

Strategies for the dsDNA Viruses

Unfortunately, there appear to be no strategies that have been developed that generate high level resistance to the plant dsDNA or pararetroviruses, including the caulimoviruses and the badnaviruses (R. Hull, personal communication). However, it is important to consider that prior to the characterisation of such badnaviruses as BSV, sugarcane badnavirus and taro bacilliform badnavirus there were very few examples of plant pararetroviruses causing economic damage sufficient to justify large scale or concerted efforts to generate transgenic resistance.

Transgenic Virus Resistance in Bananas: Current Status

There are a number of groups around the world actively involved in the development of transgenic virus resistance in bananas. The three major groups are the Queensland University of Technology (QUT) targeting BBTV and BBrMV, the University of Hawaii (UH) targeting BBTV and the John Innes Institute (JIC) targeting BSV. However, there are other groups including labs in India, Egypt, China and Vietnam who have significant interest in developing transgenic bananas with virus resistance and are primarily focussed on BBTV.

There would appear to be very little interest or justification in targeting transgenic resistance to banana viruses other than BBTV, BSV and BBrMV.

Transgenic resistance to banana bract mosaic potyvirus: current status

BBrMV should be considered a prime target for generating transgenic resistance in bananas for a number of reasons: (i) it is a potyvirus and transgenic resistance to potyviruses has been generated in a number of crops using PTGS, (ii) the virus is quite widespread in Asia and causes a serious disease with significant yield loss, (iii) the virus spreads rapidly in the field and therefore where it occurs it will remain a significant production threat and (iv) while there is reported genome variability up to 10%, rational design of the transgene should facilitate broad resistance to this virus in bananas.

Transgenic 'Cavendish' with potential BBrMV resistance have already been generated at QUT under the World Bank sponsored Banana Improvement Program (BIP). These transgenic lines were transformed with the coat protein coding region of a Philippines isolate under the control of the maize polyubiquitin promoter using microprojectile bombardment. The lines have yet to be challenged primarily because funding for the project finished and subsequent funding applications were unsuccessful. However, if BBrMV again became a target for transgenic resistance, it would be preferable to generate new transgenic lines with new constructs that were designed to cover all known genome variability and were in the form of inverted repeats. From a technical perspective, the chances of generating transgenic bananas immune to BBrMV would be very high.

Transgenic resistance to banana bunchy top babuvirus: current status

At least two groups, QUT and UH are involved in developing transgenic bananas, particularly 'Cavendish', with resistance to BBTV.

At UH, several putative transgenic lines have been generated expressing mutated or anti-sense Rep genes with partial resistance to BBTV. Some of these plants remained symptomless for up to a year. However, the plants were not immune to BBTV and they all eventually developed symptoms. The current strategy is to incorporate the recent findings on gene silencing into bananas for BBTV resistance (John Hu, personal communication).

At QUT, we are investigating two strategies, constitutive expression of mutated Reps and the strategy we have developed, virus activated cell death. For the mutated Rep strategy, we have made mutations in two motifs in the BBTV Rep gene involved in rolling circle replication, with a single mutation in each of the codons. We have demonstrated in transient assays that either mutation renders the Rep incapable of initiating replication. Further, we have demonstrated that over-expression of these mutated Reps will significantly reduce but not eliminate replication of BBTV in transient assays. Consequently, we have transformed both 'Cavendish' and 'Lady finger' with the mutated Reps under the control of high level constitutive promoters. The resultant transgenic lines are currently being regenerated and multiplied in preparation for glasshouse challenge.

We have also developed the virus activated cell death strategy for BBTV. The BBTV intergenic region has been embedded into an intron and inserted into a split barnase gene construct. This construct has been transformed into both 'Cavendish' and 'Lady finger'. The resultant transgenics are currently on selection.

Again, from a technical perspective, the chances of generating transgenic bananas immune to BBTV must be considered good to high as there are now two potentially effective strategies available.

Transgenic resistance to banana streak badnavirus: current status

There is only one reported group, JIC that is either attempting or planning to generate transgenic resistance to BSV (Roger Hull, personal communication). In the absence of further information, this must be considered high risk for a number of reasons: there are no reported successful attempts to generate badnavirus or caulimovirus resistance; banana is a difficult model in which to develop a strategy; and the hypervariability of episomal BSV would seem to make broad resistance difficult.

Conclusion

This is an extremely opportune time to develop transgenic virus resistance in bananas:

- There is an immediate, urgent requirement to develop virus resistant bananas
- The tools to transform and express transgenes in bananas are now well established
- Other strategies, such as conventional breeding, for developing virus resistance in bananas are very unlikely to be successful even in the medium term
- A proven transgenic virus resistance strategy is available for one of the most important viruses, banana bract mosaic potyvirus
- Two very promising strategies are now available to generate resistance to the most important of the banana viruses, banana bunchy top virus.

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Strategies for enhancing resistance to weevils

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Introduction

The banana weevil is a pest of considerable importance in Africa that significantly affects banana and plantain production (Ostmark, 1974; Gold, 1998; Swennen and Vulysteke, 2001; Gold and Messiaen, 2000; Fogein *et al.,* 2002). The weevil has been associated with rapid plantation decline in East Africa (Gold *et al.,* 1999b) and a phenomenon called yield decline syndrome in West Africa. The adult weevils are free living, have a nocturnal habit, and rarely fly. Their eggs are deposited inside the plant tissue at the base of the pseudostem or on an exposed corm. On hatching, the larvae tunnel through the corm for feeding and development. Tunnelling reduces the water and mineral transport, thereby weakening the plant, reducing the bunch weight (yield) and causing plant toppling during windstorms. In severe weevil infestations, crop losses of up to 100% have been reported (Sengooba, 1986). The establishment of new plantings may fail (Price, 1994) and yield loss appears to increase gradually, reaching 44% in the forth ratoon cycle (Rukazambuga *et al.,* 1998).

Weevil control is currently based on the application of cultural practices such as the use of clean planting material, systematic trapping of adult weevils in an effort to control the weevil population, and field sanitation to remove residues that may form breeding grounds for the weevil (Gold, 2000; Gold and Messiaen, 2000). Although cultural control methods contribute to weevil management, both the high labour input and material requirements are often limiting factors for adoption (Gold, 1998). Furthermore, application of effective pesticides is economically unfeasible for subsistence producers and, unfortunately, the banana weevil has developed resistance to a range of these otherwise effective pesticides (Collins *et al.,* 1991; Gold *et al.,* 1999a). Consequently, development of resistant plants has been suggested as a potential long-term intervention for weevil control, especially on small-scale farms, as the inclusion of such plants might be part of an integrated pest management (IPM) framework (Seshu-Reddy and Lubega, 1993).

Classical resistance mechanisms (Painter, 1951) have been investigated in *Musa* germplasm, and so far antibiosis (factors affecting larval performance), rather than antixenosis (attractivity), appear to be the most important weevil resistance

mechanism in banana (Mesquita et al., 1984; Ortiz *et al.,* 1995; Abera *et al.,* 1999; Kiggundu, 2000). Although some differences in attracting adult weevils to different cultivars have been found, there were no direct correlations with plant damage (Budenburg *et al.,* 1993; Pavis and Minost, 1993; Abera *et al.,* 1999; Musabymana et al., 2000; Kiggundu, 2000). Difference in attraction has been rather due to environmental factors such as soil moisture around a cultivar with high sucker number (Ityeipe, 1986).

Several banana plant phenological factors seem to contribute to weevil resistance. Corm hardness was the first biophysical factor associated with resistance. Whereas Pavis and Minost (1993) found a small, negative correlation ($r = -0.47$) between corm hardness and weevil damage, Ortiz *et al.*. (1995) found no relationship between the two factors in segregating plantain progenies. They suggested the existence of other weevil resistance factors such as chemical toxins or anti-feedants. Kiggundu (2000) found corm dry matter content, resin/sap production and suckering ability to negatively correlate with weevil damage. Whereas corm diameter and resin/sap production were important in East African Highland banana (EAHB) accessions, corm dry matter content, corm hardness, resin/sap production and suckering ability (number of suckers) were significant parameters in the resistance response of recently introduced clones. In big corms the weevil larvae can complete their life cycle without burrowing too deep into the corm. This indicates that some form of tolerance exists in cultivars with a large corm (Balachowsky, 1963).

The suggestion that biochemical compounds affected weevil performance led to investigations of resistant selections by using high-performance liquid chromatography (HPLC). HPLC chromatograms from corm extracts of weevil-resistant AB and ABB cultivars (cvs. Kisubi and Kayinja) showed compound peaks that were absent not only in susceptible clones, but also in some resistant clones of the AA and AAA genomes (e.g. Calcutta-4 and Yangambi km-5). This result possibly indicates a type of antibiotic mechanism that may be based on toxic compounds. These compounds are seemingly present in weevil-resistant cultivars with the BB genome whereas a different form of resistance may be present in the genome of weevil-resistant AA cultivars (Kiggundu, 2000). Furthermore, separation of a cultivar 'Pisang Awak' (ABB) methanol extract allowed the isolation of two highly polar sub-fractions with high anti-larval activity (Kiggundu, unpublished). In these experiments, unique HPLC peaks were also found for susceptible cultivars, which indicate the presence of compounds possibly related to weevil susceptibility.

Transgenic weevil resistance in bananas

The application of modern biotechnological tools to produce a genetically modified (GM) banana has been introduced by several research groups. Although remarkable achievements have already been made in banana transformation, the identification and introduction of useful genes into banana to reduce losses caused by the banana weevil is still a major challenge. This is

partially due to the lack of information on expression of endogenous banana genes after weevil infestation. Therefore, the Uganda Banana Biotechnology Project (UBBP) in collaboration with the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria in South Africa has recently initiated a study to identify a greater variety of differentially expressed genes following weevil infestation. In this study, resistant and susceptible Musa varieties are used and the cDNA-AFLP and cDNA Representational Difference Analysis (cDNA-RDA) (Hubank and Schatz, 1994) techniques applied to identify and isolate genes expressed during weevil infestation. These genes might ultimately be useful as (1) specific probes in microarray experiments to study gene regulation, (2) for the development of an Elisa-based protein marker system or (3) as transgenes in the production of future GM banana plants, especially as they will have endogenous promoter sequences.

Proteinase inhibitors

A variety of genes are available for genetic engineering for pest resistance (Carozi and Koziel, 1997; Sharma *et al.,* 2000). Among these are proteinase inhibitors that apparently contribute to host plant resistance against pests and pathogens (Green and Ryan, 1972). There are generally two major proteinase classes in the digestive systems of phytophagus insects ie.the serine and cysteine proteinase class. Serine proteinase activity is present in Lepidoptera, Dictyoptera and Hymenoptera, while the cysteine proteinase activity is characteristic for Odoptera and Hemiptera. Coleopteran insects mainly use cysteine proteinases (Gatehouse *et al.,* 1985; Murdock *et al.,* 1987), but recent studies indicate a combination of both serine and cysteine proteinases in this order (Gerald *et al.,* 1997).

Attention on weevil resistance in GM banana is currently focused on the expression of exogenous cysteine proteinase inhibitors. These inhibitors have been used before for insect control in GM plants (Leple *et al.,* 1995). Proteinase inhibitors are expressed naturally as a plant defence against insect attack (Ryan, 1990; Pernas *et al.,* 2000; Ashouri *et al.,* 2001). They operate by disrupting protein digestion in the insect mid-gut via inhibition of cysteine proteinases. Cysteine proteinases are not part of the human gut system, and therefore relatively safe for human consumption. The UBBP in collaboration with FABI is currently investigating the potential of cysteine proteinase inhibitors from rice and papaya in GM banana. So far, cysteine proteinase activity has been found in the mid-gut of the banana weevil and *in vitro* studies at FABI have shown that these cysteine proteinases are strongly inhibited by both a purified recombinant rice cystatin, oryzacystatin-I (OC-I) (Abe *et al.,* 1987) and papaya cystatin (Kiggundu, unpublished results). By using a newly developed bioassay system based on vacuum infiltration of banana stems, it could be demonstrated that recombinant papaya cystatin significantly reduces the early growth and development of weevil larvae (Kiggundu *et al.,* unpublished results). In addition, the design of optimised inhibitors using site-directed mutagenesis is currently being carried

out at FABI to improve inhibition and stability of the inhibitor for weevil control. Transgenic banana expressing a modified OC-I gene targeting nematode control has been produced by the John Innes Centre (JIC, UK) and the University of Leeds (UL, UK) (Philip Vain, personal communication), field testing of these plants proposed to be done in Uganda.

Genes with potential for banana weevil control

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. A lectin from snowdrop, *Galanthus nivalis* agglutinin (GNA), is toxic to several insect pests in the orders Homotera, Coleoptera and Lepidoptera (Tinjuangjun, 2002). In collaboration with the UBBP, work at Katholieke Universiteit Leuven (KUL) in Belgium is currently being conducted to test the effect of GNA and the *Aegopodium podagraria* lectin (APA) among others on the mortality and reproduction of three nematode species pathogenic to banana (Carlens, 2002). Similar work can be extended to banana weevil using in-vivo assays. A major concern about the use of lectins, however, is that some of them, such as the wheat germ agglutinin (WGA), are toxic to mammals (Jouanin *et al.,* 1998). However, the snowdrop and garlic lectins are toxic only to insects (Boulter, 1993), and these deserve investigation for weevil control.

Expression and biological activity of the Bacillus *thuringiensis* (Bt) toxin has been extensively investigated in GM plants for insect control. Bt plants are currently the most widely used for Lepidopteran control in commercial crops (Krettiger, 1997). Bt genes products are a group of more than fifty insecticidal crystal proteins. When ingested by an insect, they are solubilized in the alkaline environment of the insect's midgut and become toxic by binding to apical border brush membranes of the columnar cells. This causes lysis of the cells and eventual death of the insect. In collaboration with the Centre de Coopération Internationale en Recherché Agronomique pour le Dévelopement (CIRAD) in France, the UBBP screening will be done as a follow-up project at KUL using a range of newly isolated Bt toxins for banana weevil toxicity. Screening however, is hampered by the lack of any artificial diet for the banana weevil, which is a pre-requisite for efficient screening under controlled conditions. The expression of a selected Bt gene for weevil resistance will also be rather a long-term strategy.

Vegetative insecticidal proteins VIPs are similar to that of Bt toxins. VIP1 and VIP2 are contained in supernatants of *Bacillus cereus* (Warren, 1997) while VIP 3 in the supernatants of *B. thuringiensis*. They function by causing gut paralysis followed by lysis of the gut epithelium cells, completely arresting gut function and leading to the death of the insect (Duck and Evola, 1997).

Alpha-amylase inhibitors (AI) and chitinase enzymes might also have a future potential for weevil control. Alpha-amylase inhibitors operate by inhibiting the

enzyme alpha-amylase. They are divided into two types, AI-1 and AI-2, isolated from common and wild beans (*Phaseolus vulgaris*), respectively. They break down starch to glucose in the insect gut (LeBerre-Anton *et al.,* 1997; Morton *et al.,* 2000). Ishimoto *et al.*. (1996) produced transgenic adzuki beans with enhanced resistance to bean bruchids, which are Coleopteran insects. Since they are active against this type of insects, they might 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 in tobacco against Lepidopteran insects (Ding *et al.,* 1998). At KUL, a rice chitinase gene has been transformed into bananas, directed towards the control of fungal pathogens (Arinaitwe, 2002).

The way forward

Plant biotechnology has, without doubt, the potential to play a key role in the sustainable production of bananas and plantains in Africa. This has recently been realised by several African governments and international donors by providing better financial support for research into banana and plantain biotechnology. Such support includes the extensive training of young African scientists in plant biotechnology in both African and overseas research institutions, and the setting up of research networks in plant biotechnology on the African continent. Current efforts at NARO, IITA and CARBAP to develop weevil resistance by conventional means using carefully selected parents and especially improved diploids with resistance should be continued and encouraged. It is, however, also important that work continues towards the better understanding of resistance mechanisms and the development of rapid screening methods as has been attempted by Kéhé *et al.*. (2000).

Certain barriers exist in Africa that limits the opportunity to take advantage of the benefits plant biotechnology might offer. Affordability is a major barrier for the introduction of any costly biotechnology-derived product. What might look exciting in the lab might not necessarily make a good and affordable product when considering the needs of small-scale farmers in Africa with their very limited financial resources. This will especially become true when the potential of traditional, conventional selection is compared to MAS (Morris *et al.,* 2003). Conventional selection has, without doubt, certain advantages where limited financial resources are a concern. Any biotechnology research programme directed towards the development of a genetic marker for weevil resistance has, therefore, to answer the question if genotypic screening is indeed superior to a less costly phenotypic screening as far as the time saved is concerned for releasing an improved line earlier. It has also to be asked if the technique applied will ultimately result in a useful marker. This might require the application of more than one technique to ultimately produce a useful marker (Cullis and Kunert, 2000; Kunert *et al.,* 2003). The techniques widely employed to develop genetic markers include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), AFLP, microsatellites and the

analysis of r-DNA intergenic regions. Besides the fact that identified genome differences detected when using these techniques might not be directly linked to a trait, some of them are also either not robust, difficult to reproduce between different laboratories (e.g. RAPDs), expensive when applied to a large number of plants (e.g. RFLP and AFLP), or have only recently started to be widely used in plants (e.g. microsatellites) (Powell *et al.,* 1996; Jones *et al.,* 1997; Scribner and Pearce, 2000).

Work on the identification of insecticidal proteins against the banana weevil is needed, especially on proteins other than Bt and proteinase inhibitors. Unfortunately, the lack of an artificial diet seriously hampers progress in this direction. Perhaps the most urgent activity at this point is the development of a reliable and efficient bioassay system. An initial working diet should at least allow development to about the 5th instar stage, while work continues refining the diet. This will aid in the rapid discovery of proteins with insecticidal properties to banana weevil. At CIRAD, progress in this direction and preliminary reports indicate a working diet may be available soon (INIBAP, 2004). At FABI a system to rear weevil larvae on banana stems vacuum infiltrated with inhibitors has been developed to test *in vivo* effects on larval development.

A common characteristic of many insecticidal proteins derived from plants is that they are effective against a wide range of organisms (Hilder and Boulter, 1999). Therefore, it would be worthwhile if the laboratories working on proteins such as proteinase inhibitors (JIC and UL), lectins (KUL) and chitinases (KUL), but targeting nematodes and fungal pathogens, could include banana weevil testing. This can be done either concurrently or in later stages to investigate the effect of such proteins or derived transgenic plants on weevils.

The view is often expressed that a sustainable solution to pest problems in Africa involves the development of high yielding, pest resistant cultivars by means of genetic modification. Although this view might ultimately be true, one should consider whether the over-expression of a single selected native plant gene, as is done in many engineering approaches, is not too simplistic and ultimately ineffective in obtaining efficient pest resistance. Natural plant resistance is mostly polygenic and involves more than one gene. Recent evidence shows, however, that these sophisticated defence mechanisms have been lost during selection for domestication (Carlini and Grossi-de-Sa, 2001). Therefore, one approach would be to optimise a “resistance” gene by protein engineering, or a balanced interaction that involves the simultaneous expression of several protective proteins by using gene pyramiding or multiple resistance engineering (Winterer, 2002).

The successful transformation of bananas might depend on many factors. The consistent expression of desired traits, at the required level and in a wide range of natural environments, without significant interference with metabolic processes by the transgene in particular, has to be achieved (Van der Vyver *et al.,*

2003a; Foyer *et al.,* 2003). When a triploid GM banana is produced without any backcrossing, it will have to be propagated vegetatively. This raises questions about the genomic integrity of the off-spring plant, such as the occurrence of mutations and epigenetic changes as a result of the plant transformation process (Karp, 1993; Phillips *et al.,* 1994; Labra *et al.,* 2001; Van der Vyver *et al.,* 2003b). Future research, therefore, has to focus on long-term performance of GM bananas under African field conditions, including the long-term response of weevils and effects to their natural predators. Such research is still at its infancy in Africa and no such data are available for banana.

A key question concerning all GM crops is the commercialisation and consumer acceptance. Without doubt, any GM banana expressing banana weevil resistance would have, despite being of great value to African small-scale farmers, to overcome the current trend for negative public perception and public concerns of bio-safety. Also, concerns about the effect of a GM banana on African agro-systems and biodiversity have ultimately to be addressed. These concerns represent the most important barrier to be crossed even in African countries, where crop losses due to stress are most severe and which would, therefore, benefit most from the successful development of a GM crop. The need to strengthen the whole transgenic approach by developing a wide range of technical expertise through training cannot be over emphasised. The benefits would include the development of integrated biotechnology teams with overlapping expertises required for success.

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