

Sustainable disease management in a European context

David B. Collinge · Lisa Munk · B.M. Cooke

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Cover photos:

From top to bottom: A simplified model of defence against pathogens illustrating successful transgenic strategies for making disease resistant plants (Collinge et al., 2008).

Control of leaf blight of tomato by spraying tomato plants with garlic juice 2 h before inoculation. Inoculation was done by spraying whole plants with a sporangial suspension of *P. infestans* ($4-5 \times 10^{-4}$ sporangia ml⁻¹). Top panel, inoculated plants; lower panel, inoculated and sprayed with diluted garlic juice containing 110 µg ml⁻¹ allicin (Slusarenko et al., 2008).

Accumulation of H₂O₂ as seen by DAB-staining in (a) & (b) the barley –*B. graminis* f.sp. *hordei* interaction and (c) & (d) the wheat –*Septoria tritici* interaction. In (a), a cell is undergoing HR as a response to penetration and is completely stained with DAB, in (b) only papillae are stained. (c) and (d) show wheat cvs Stakado and Sevin exhibiting resistance and susceptibility to the same isolate of *S. tritici* (Shetty et al., 2008).

Rice cultivar mixtures in China. Single rows of tall traditional varieties are interspersed within fields of high yielding hybrid cultivars (Finckh, 2008).

Schematic representation of domains found in plant LRR Resistance proteins. TIR Toll/interleukin-1 receptor, CC coiled coil, NB nucleotide binding, ARC1/2 APAF1, R protein and CED4, LRR leucine-rich repeat, SD solanaceous domain, BED BEAF/DREAF zinc finger domain, TM transmembrane, Kin kinase, WRKY WRKY transcription factor (Tameling and Takken, 2008).

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Foreword

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Sustainable disease management in a European context

The main theme of the book is sustainable disease management in a European context. Of course the issues are global and the papers reflect this. Some of the questions addressed are: How does society benefit from plant pathology research? How can new molecular approaches solve relevant problems in disease management? What other fields can we exploit in plant pathology research? What challenges are associated with free trade across the new borders? How can we contribute to solving problems of developing countries? How does plant pathology contribute to food quality and safety? How does globalization/internationalization affect teaching and extension in plant pathology?

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The authors of papers in this special issue of European Journal of Plant Pathology were selected among the invited speakers at the 8th Conference of the European Foundation for Plant Pathology & British Society of Plant Pathology Presidential Meeting 2006¹ that was held at The Royal Veterinary and Agricultural University², Copenhagen from 13th–17th of August 2006. This was an intimate conference attended by some 200 largely European delegates from more than 30 countries. The result of the conference and of this volume is an insight into the diversity of problems facing pathologists and the remarkable progress made in recent years. This book is intended to be more than a proceedings volume, and clearly, given the breadth of the subject, it represents a series of readings and not a comprehensive account of the state of research in the field in the middle of 2007 (the deadline for submission of these articles) or even of the excellent research presented at the conference that has not resulted in a paper in this special issue. There are many interesting relevant topics that were not presented at the conference — an obvious example is toxins in our food. Notwithstanding this, we believe the authors have provided a useful series of review articles and case studies of many key areas that we hope can

¹ <http://www.efpp06.kvl.dk/index.html>

² Now the Faculty of Life Sciences, the University of Copenhagen.

inspire future research. We have asked the authors to prepare the papers so that they can be used as teaching material for advanced courses and are well satisfied with the result.

We have organised the papers in four sections. As in all classifications of biological material, there is ambiguity as to the correct order and precise classification: alternative models would be entirely appropriate.

The first and largest section **How can biotechnology contribute to sustainable development?** presents an overview of the biological knowledge obtained using molecular biological techniques of the nature of plant-microbe interactions. We start with a review by Collinge et al. of the success stories, progress and challenges associated with developing transgenic disease resistant plants. Weed resistant or rather herbicide and/or insect resistant crops have been grown extensively in various parts of the world, though largely not in Europe, for over a decade, and the area increases annually. Cultivated transgenic disease resistance crops are currently restricted to virus-resistant papaya and courgettes (zucchini) in the USA. The biological — and political — reasons for this are presented and discussed. The techniques of molecular biology and molecular genetics continue to provide an ever deeper understanding of the nature, recognition and regulation of the active and passive defence mechanisms protecting plants from pathogens. Two papers delve into different aspects of the regulation of disease defence mechanisms. Goellner and Conrath review the priming of defences and innate immunity, and in the process cover both the molecular basis of different forms of induced resistance to pathogens and pests and practical experience with the application of the concept. Tameling and Takken consider the downstream signalling associated with race-specificity. Recent knowledge shows how these processes respond to targeting by pathogen effector proteins — of which avirulence gene products represent a special case. The traditional models for and our understanding of Flor's gene-for-gene hypothesis are brought into context. The use of tools of functional genomics to study defence responses are illustrated by Collinge et al. and exemplified by a case study with the NAC transcription protein family of barley in the next paper. Two papers by Shetty et al. and Pruvsky highlight the importance of reactive oxygen species (ROS) in defence and signalling processes. The former reviews the current knowledge of ROS — as directly antimicro-

bial defences, as signals inducing defence responses and in oxidative cross-linking. The latter looks at the role of ROS and other factors in the switch from quiescent to necrotrophic interactions. The implications for control of post-harvest diseases are discussed. Both these papers challenge the simple classification of pathogens into biotrophs, hemibiotrophs and necrotrophs. The final paper in the section, by Ludwig-Müller and Schuller, concerns the study of *Plasmodiophora brassicae*, a fascinating organism quite unlike most of the pathogens we meet, and the use of the model plant *Arabidopsis thaliana* in its study. The major output described in the papers of this section is still fundamental biological knowledge where comparative genomics is an emerging theme. The real and projected impact of this knowledge in combating plant pathogen interactions is discussed.

The second section concerns **Strategies for disease control**. Jørgensen et al. present an analysis of the actual needs and habits of different types of farmer in order to optimise disease control in cereals. The advisory service integrates disease resistance information with fungicide recommendations. Organic agricultural systems are not concerned with the use of fungicides. Two papers consider alternative strategies appropriate for organic growers and provide interesting case studies. Slusarenko reviews the control of plant diseases by natural products and exemplifies this with Allicin from garlic. Finally, Whipps et al. present and review the mycoparasite *Coniothyrium minitans* as a biocontrol agent.

Under the title **Quarantine and diagnostics**, the third section addresses the issues of global pathogen spread. Despite our level of knowledge, new threats from pathogens continue to emerge, resulting in the spread of disease to agricultural systems and natural ecosystems around the world. Both increased free trade and climatic change contribute to these developments. Petter et al. review the progress made to harmonise methods for diagnostics and provide access to the materials developed. Thrane describes the implementation of these tools and provides a case study: potato testing in Denmark. Two papers in this section present two modern methods for diagnostics and identification of pathogens that allow rapid diagnosis of problems without the need for the taxonomic and identification skills developed through a life-time of study of diverse pathogens. Thornton describes the use of monoclonal antibodies for

detecting fungi (*Trichoderma* spp.) in soil and compares their use to other techniques, e.g., nucleic acid-based methods. The use of these methods for following population dynamics and quantities of fungi are discussed. Boonham et al. look at the development and prospects of generic platform technologies, specifically Real-Time PCR, for diagnostics. The final paper of the section by Smith et al. shows how information on new outbreaks can be used globally but, using Africa as a case study, also discusses the global challenges facing agro-industry and quarantine systems.

The final section concerns **Population diversity and dynamics**. Disease resistance is the most effective form of controlling disease, when available. Plant breeding strategies for disease resistance are dependent on an understanding of the diversity of the pathogen population with respect to the frequency of avirulence genes to which the crop is exposed. Hovmøller describes the impact of virulence surveys — mapping virulence specificity (i.e. avirulence genes) in *Puccinia striiformis* populations in Denmark and the use of the data in dissemination to breeders and farmers. Kaur et al. describe how molecular biological methods have developed as essential tools in efficient plant breeding for disease resistance, and cover both the methods now available for identifying allelic variation (eco-TILLING) and the use of molecular markers in the breeding process. Wheat resistance used against powdery mildew is the case study described. Finckh advocates breeding for resistance diversity to provide the rationale for exploiting and implementing resistance.

The future for plant pathology

So what are the most significant advances made in the last few years and what challenges remain?

An increased understanding of the nature of race specificity is currently emerging. It is increasingly clear that resistance genes can function in two ways. They either recognise pathogen molecules directly, or recognise the effect of the pathogen molecule on the host cell. These pathogen molecules are now called effector proteins. A subset of these can be recognised by the host and they are then called specific elicitors, which are coded by the pathogen's avirulence genes. Race specificity is now considered to represent a

second level of defence which functions to guard the primary mechanisms of resistance, now commonly known as innate immunity, from being disarmed by the effector molecules produced by the pathogen. At the same time, molecular genetic analyses, especially but not exclusively, in *Arabidopsis*, have vastly expanded our knowledge of the nature and regulation of distinct forms of induced resistance. In other words, several distinct, but interacting (i.e. so-called 'crosstalk') signal transduction pathways have been identified which regulate the defence responses activated by plant pathogens. The challenge now is to put this fascinating knowledge to use.

The increased knowledge of microbial genomes has not only led to major revisions in the taxonomy of plant pathogens but also to the development of molecular diagnostic tools, for instance gene chips — i.e. microarrays which allow specific identifications and PCR-based tools. These tools are useful in some systems but there are still many challenges for other pathogens, especially for asexual organisms where closely related organisms that cause diseases on different crops can be difficult to distinguish. We are getting closer to the idea of being able to put diseased material into one end of a machine and obtain a printout of the probable diagnosis from the other end. This would provide a cost-effective solution and bring diagnostics to technicians rather than letting it remain in the hands of a few specialists. These developments are welcome as increasing free trade can facilitate more rapid spread of pathogens between continents and the more variable climates we seem to be experiencing that are attributed to global warming create new habitats for pathogens in regions where particular pests and pathogens can find new niches.

Major progress has been made in the development of models for forecasting the development of diseases both between and within seasons. This can have considerable impact in the development of decision systems for farmers in terms of which varieties to plant in the next season, and how and when to spray. A major challenge is to devise the means for providing appropriate input data for the models. A challenge for the industry is the increasing costs of the development of new pesticides versus the potential for profit.

Plant pathology can both benefit from and contribute to biotechnology. In industrial countries, plant

pathogens are emerging as a resource for new enzyme products for preparing biomass for biofuels. The study of the biochemistry of the complexity of cell walls and the tools that pathogens use to degrade them are one of many opportunities for plant pathologists. We hope you are inspired.

A challenge for the subject of plant pathology lies in the way the universities and research institutions worldwide are funded. In an increasing number of countries, assessment systems and the division of resources is based on impact factors and the like, rather than societal benefit. The field of plant pathology covers both fundamental research and finding solutions for problems facing growers.

The impact of our work is not always measured in citations in the scientific literature but in increased yields of healthy crops. Universities often appoint new lecturers on the former criterion. This is a worrying trend. In addition, although the numbers of university students continue to increase worldwide, the interest in biological sciences and agriculture is waning among the young. As a profession it is vital that we continue to demonstrate that the research and teaching that we perform reaches our targets — society, the industry and students, and that the benefit of our efforts is visible to society. We hope this volume contributes to these aims and needs.

What are the prospects for genetically engineered, disease resistant plants?

David B. Collinge · Ole Søgaard Lund ·
Hans Thordal-Christensen

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Abstract Insect and herbicide-resistant plants are the most widely grown transgenics in agricultural production. No strategy using genetically engineered plants for disease resistance has had a comparable impact. Why is this? What are the prospects for introducing transgenic disease resistant plants to agriculture? We review the biological background for strategies used to make disease resistant GM crops, illustrate examples of these different strategies and discuss future prospects.

Keywords Genetically engineering ·
Disease resistant plants · Plant virus ·
Fungal disease · Bacterial disease

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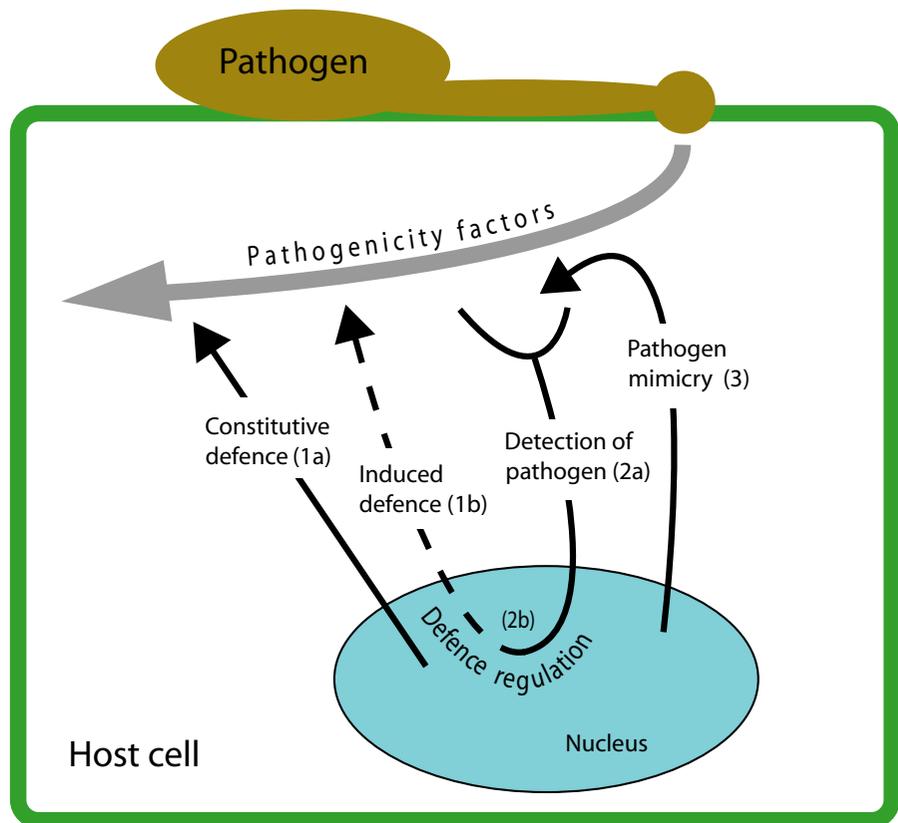
Introduction

Disease resistance is the most effective means of controlling disease. However, there are many pathogens for which no effective sources of disease resistance have been identified. Genetic engineering has been promoted for two decades as a solution for this problem, but to date only very few GM disease resistant cultivars have been introduced to commercial agriculture. This is in stark contrast to the situation for two other key disciplines of plant protection, namely insect pest and weed control where Bt¹ and herbicide-tolerant crops represent well over 90% of all GM crops (James 2006), and have been on the market for more than ten years. The answer to this lies primarily in the complexity of the biology of the traits concerned. Economics has undoubtedly also played a role in that investment in transgenic insect and herbicide resistance was considered safe since the key technologies concerned were well established in agricultural practice prior to their biotechnological application. Furthermore, the implementation of new products is delayed as a result of moratoria resulting from negative public opinion and expense of commercialisation.

Enhanced disease resistance has been achieved using several strategies. These are depicted in Fig. 1 and are described briefly here. The most straightfor-

¹Abbreviations: Bt *Bacillus thuringiensis* toxins; GM genetically modified.

Fig. 1 A simplified model of defence illustrating successful transgenic strategies. See Table 1 for examples. Strategy 1 concerns direct interference with pathogenicity or inhibition of pathogen physiology. Thus 1a involves constitutive expression of antimicrobial factors and 1b involves pathogen-induced expression of one or more genes in the transgenic plant. Strategy 2 concerns the regulation of the natural induced host defences. 2a concerns altering recognition of the pathogen (e.g., R-genes) and 2b concerns downstream regulatory pathways (e.g., SAR), and includes transcription factors. Strategy 3 is pathogen mimicry: the manipulation of the plant to prime recognition of a specific pathogen through pathogen derived gene sequences (genetic vaccination). See Table 1 for examples



ward approach is to add genes encoding antimicrobial proteins or peptides originating from plants or other organisms either alone or in combination (1a). The addition of new antimicrobial secondary metabolites to a species, which can be achieved by adding genes encoding the appropriate biosynthetic enzymes, lies under this strategy. Other strategies involving detoxification, quenching pathogen signals etc also belong in this category. A variant concerns the use of pathogen-inducible promoters to regulate these antimicrobial factors (1b). Plants have their own effective defences – induced resistance, basal resistance and race-specific resistance, which activate the plant's own antimicrobial defences. Therefore, a second strategy concerns the manipulation of the regulation of these processes (2) and can exploit the recognition processes as well as the regulatory signal transduction pathways. A third strategy (3) is pathogen mimicry by which we mean the manipulation of the plant to prime the plant to recognise a specific pathogen. Mechanistically, this is also termed pathogen derived resistance or genetic vaccination. The unique nature of viruses

has made it possible to combat them effectively through gene silencing, which can be considered part of strategy 3.

In this review, we do not attempt to generate a comprehensive review of the many studies which have demonstrated improved disease resistance by transgenic approaches, but illustrate the strategies used with pertinent examples (see Table 1). Indeed, one of the major challenges in writing this review lies in the discrepancy in the quality of documentation at different stages in the process. There are many examples where enhanced resistance has been documented in refereed journals for transgenic plants in the laboratory, but few where documentation extends to field conditions or adoption by practical agriculture.

Herbicide tolerance

Weed control using GM crops has been possible because of the biology of herbicide tolerance. Synthetic herbicides have been developed to be effective

killers of many plants and target different vital processes common to vascular plants. Plants have not been exposed to these substances during evolutionary timescales and natural resistance is therefore not present in target plants. Transgenic herbicide-resistant plants are in commercial use with tolerance against Glyphosate (*N*-(phosphonomethyl) glycine) and Glufosinate, best known under the trade names Roundup® and Basta® (among others), respectively. High durability of the transgenic herbicide tolerance approach has been anticipated, since the main herbicide involved, glyphosate, has proven efficient for more than two decades before being incorporated into the GMO strategy (see Cerdeira and Duke 2006; Senior and Bavage 2003; Senior and Dale 2002 for further discussion of herbicide tolerance).

Insect resistance

In our view, the success with GM insect resistance is attributable both to good fortune, in that appropriate genes are known, and fundamental biological differences between biting insects and pathogens. In contrast to the synthetic herbicides, Bt toxins are natural products of the common soil bacteria *Bacillus thuringiensis* which were originally isolated from moribund insects. This topic has been the subject of several recent reviews (Babu et al. 2003; Christou et al. 2006; Ferry et al. 2004, 2006). As for herbicide tolerance, robust durability of the transgenic approach has been anticipated since Bt toxins have been used for decades as durable insecticides, though some resistance has been observed (e.g., Perez and Shelton 1997). Furthermore, Bt resistance has been demonstrated to be associated with a fitness cost for the insects (Bird and Akhurst 2004; Carrière et al. 2001). There are, nevertheless, several documented examples of Bt resistant insect pests, e.g., (Gahan et al. 2001; Huang et al. 1999) and, if this strategy is to continue to be successful, the use of Bt transgenes must be managed carefully.

Plants do not suffer from producing these proteins, which are toxic to insects. This is an obvious advantage of the approach. Results suggesting toxic effects to mammals are controversial and inconclusive (Séralini et al. 2007), and it should be borne in mind that this technology has been applied in crops used for both human and stock consumption for over a decade without any prior indications of problems.

Many different strains of *B. thuringiensis* have been described which are toxic to different families of insects, where the proteinaceous toxins act in the midgut of the digestive system (Babu et al. 2003). In particular, the *Cry1a* toxin, affecting Lepidoptera, has proven an effective means of controlling several stem-boring insect larvae, especially in maize (*Zea mays*), where the Corn Borers *Sesamia nonagrioides* and *Ostrinia nubilalis* are among the most serious. The *Cry3a* gene, from another *B. thuringiensis* strain, encodes a Bt toxin effective against Coleoptera, and is used extensively against the Cotton Bollworm *Helicoverpa armigera* in cotton (*Gossypium hirsutum*).

It should be noted that, while the known Bt toxins used in plants work against biting insect pests, there are large groups of insect pests, including sucking insects (Homoptera, such as leaf hoppers and aphids) against which no natural Bt toxins are known. Synthetic, chimeric toxins have been developed which extend the range of these toxins to other insect groups (Mehlo et al. 2005). Furthermore, as for fungi, there are examples of transgenic strategies using genes encoding other insecticidal proteins, for example lectins, which have led to resistance against these types of insects in the laboratory (Saha et al. 2006; Yao et al. 2003).

What issues affect plant disease resistance?

The differing biology of the various types of plant pathogens presents substantial problems in developing GM resistant plants. Firstly, the kinds of organisms causing disease are taxonomically highly diverse; the major groups include cellular pathogens (e.g., bacteria, fungi and the algal Oomycetes) and molecular pathogens (i.e. viruses). These are physiologically very different from each other, and therefore no single gene product can be expected to have a direct toxic effect on all types of pathogens. Secondly, pathogens use two major life strategies, namely biotrophy and necrotrophy. Biotrophic pathogens essentially act as a sink for the host's anabolic assimilates, and therefore keep it alive. Meanwhile, necrotrophic pathogens consume the host tissues as invaded. Hemibiotrophs combine both strategies in their life cycle. Consequently, plants have developed quite different ways for dealing with these two strategies (see below).

Table 1 Examples of transgenic strategies resulting in enhanced disease resistance, named in the text

Strategy ^a	Gene	Donor	Recipient	Pathogen	Reference
Constitutive (a) and inducible (b) defences					
1a	<i>AitaA</i>	Bacillus	Potato (<i>Solanum tuberosum</i>)	<i>Pectobacterium (Erwinia) carotovora</i>	(Dong et al. 2001)
1a	<i>Chi11</i> (chitinase)	Rice (<i>Oryza sativa</i>)	Rice (<i>Oryza sativa</i>)	<i>Rhizoctonia solani</i>	(Kalpana et al. 2006)
1a	Cry1Ab (Bt toxin)	<i>Bacillus thuringiensis</i>	Maize (<i>Zea mays</i>)	<i>Fusarium</i> spp	(Clements et al. 2003; Hammond et al. 2004)
1a	gf-2.8 (oxalate oxidase)	Wheat (<i>Triticum aestivum</i>)	Soybean (<i>Glycine max</i>)	<i>Sclerotinia sclerotiorum</i>	(Cober et al. 2003; Donaldson et al. 2001)
1a	gf-2.8 (oxalate oxidase)	Wheat (<i>Triticum aestivum</i>)	Sunflower (<i>Helianthus annuus</i>)	<i>Sclerotinia sclerotiorum</i>	(Hu et al. 2003)
1a	gf-2.8 (oxalate oxidase)	Wheat (<i>Triticum aestivum</i>)	Poplar (<i>Populus × euramericana</i>)	<i>Septoria musiva</i>	(Liang et al. 2004)
1a	<i>Gfhd101</i>	<i>Clonostachys rosea</i>	Maize (<i>Zea mays</i>)	<i>Fusarium graminearum</i>	(Igawa et al. 2007)
1a	KP4	Virus infecting <i>Ustilago maydis</i>	Wheat (<i>Triticum aestivum</i>)	<i>Tilletia caries</i>	(Clausen et al. 2000; Schlaich et al. 2006; Schlaich et al. 2007)
1a	Suppressor 2b (CMV)	Cucumber mosaic virus (CMV)	Tobacco (<i>Nicotiana tabacum</i>)	CMV	(Qu et al. 2007)
1a	Synthetic sequence generating miRNA based on P69 (TYMV) & HC-Pro (TuMV)	Turnip yellow mosaic virus (TYMV) & turnip mosaic virus (TuMV)	<i>Arabidopsis thaliana</i>	TYMV & TuMV	(Niu et al. 2006)
1a	RCH10 (PR-3) & AGLU1 (PR-2)	Rice (<i>Oryza sativa</i>) & alfalfa (<i>Medicago sativa</i>)	Tobacco (<i>Nicotiana tabacum</i>)	<i>Cercospora nicotianae</i>	(Zhu et al. 1994)
1a	Synthetic D4E1	Cecropia (insect)	Tobacco (<i>Nicotiana tabacum</i>)	<i>Colletotrichum destructivum</i>	(Cary et al. 2000)
1a	Synthetic D4E1	Cecropia (insect)	Poplar (<i>Populus tremula</i>) <i>Populus alba</i>	<i>Agrobacterium tumefaciens</i> , <i>Xanthomonas populi</i> pv. <i>populi</i> & <i>Hyphoxylon manniiatum</i>	(Mentag et al. 2003)
1a	Synthetic D4E1	Cecropia (insect)	Cotton (<i>Gossypium hirsutum</i>)	<i>Thielaviopsis basicola</i>	(Rajasekaran et al. 2007)
1a & 1b	<i>Vst1</i> (Stilbene (resveratrol) synthase)	Grapevine (<i>Vitis vinifera</i>)	Tobacco (<i>Nicotiana tabacum</i>)	<i>Botrytis cinerea</i>	(Hain et al. 1993)
1a & 1b	<i>Vst1</i> (Stilbene (resveratrol) synthase) pss (pinosylvin synthase)	Grapevine (<i>Vitis vinifera</i>); Scots Pine (<i>Pinus sylvestris</i>)	Barley (<i>Hordeum vulgare</i>) & Wheat (<i>Triticum aestivum</i>)	<i>Botrytis cinerea</i> ; <i>Puccinia recondita</i> f.sp. <i>tritici</i> & <i>Stagonospora (Septoria) nodorum</i>	(Leckband and Lorz 1998; Serebriakova et al. 2005)
1b	9f-2.8 (oxalate oxidase) & TaPERO (peroxidase)	Wheat (<i>Triticum aestivum</i>)	Wheat (<i>Triticum aestivum</i>)	<i>Blumeria graminis</i> f.sp. <i>tritici</i>	(Altpeter et al. 2005)

Detection of pathogen attack					
2a	Rpi- <i>blb2</i> (NB-LRR)	<i>Solanum bulbocastanum</i>	Potato (<i>Solanum tuberosum</i>)	<i>Phytophthora infestans</i>	(van der Vossen et al. 2003, 2005)
2a	<i>Rxo1</i> (NBS-LRR)	Maize (<i>Zea mays</i>)	Rice (<i>Oryza sativa</i>)	<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>	(Zhao et al. 2005)
2a	<i>Vf</i> (CF)	<i>Malus floribunda</i>	<i>Malus domestica</i> (apple)	<i>Yeniuria inaequalis</i>	(Belfanti et al. 2004)
2a	<i>Xa21</i> (NBS-LRR)	Rice (<i>Oryza sativa</i>)	Rice (<i>Oryza sativa</i>)	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	(Wang et al. 2007; Zhai et al. 2002)
2a/2b	<i>Pto</i>	<i>Lycopersicon pimpinellifolium</i>	Tomato (<i>Lycopersicon esculentum</i>)	<i>Pseudomonas syringae</i> pv. <i>tomato</i>	(Tang et al. 1999)
Regulation of inducible defences					
2b	NHI (NPR1)	Rice (<i>Oryza sativa</i>)	Rice (<i>Oryza sativa</i>)	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	(Chern et al. 2005)
2b	NPR1	<i>Arabidopsis thaliana</i>	Wheat (<i>Triticum aestivum</i>)	<i>Fusarium graminearum</i>	(Makandar et al. 2006)
2b	NPR1	<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i> and <i>Peronospora parasitica</i>	(Cao et al. 1998)
Pathogen mimicry					
3	<i>C1</i> (TYLCV)	Tomato Yellow Leaf Curl Virus (TYLCV)	Tomato (<i>Lycopersicon esculentum</i>)	TYLCV	(Fuentes et al. 2006)
3	ORF2b (PLRV)	Potato leaf-roll virus (PLRV)	Potato (<i>Solanum tuberosum</i>)	PLRV	(Vazquez Rovere et al. 2007)
3	PSRV coat protein gene	Papaya ringspot virus (PRSV)	Papaya (<i>Carica papaya</i>)	PRSV	(Fuchs and Gonsalves 2007)
3	REP (TYLCV)	Tomato yellow leaf curl virus (TYLCV)	Tomato (<i>Lycopersicon esculentum</i>)	TYLCV	(Yang et al. 2004)

^a See Fig. 1 for explanation.

R-genes

The so-called resistance (R) genes have been widely employed for many years, through conventional breeding programmes, with great success. These genes control many plant diseases caused by biotrophic pathogens such as rusts and powdery mildew fungi. These genes have the advantage of conferring complete resistance against specific races of the pathogen. Many specific resistance genes are available in the major crops (Hovmøller et al. 1997; Hovmøller 2007; McDonald and Linde 2003). Typically, 40–70 specific R-genes and alleles have been described for the rust and powdery mildew diseases of major crops. Most R-genes encode the so-called nucleotide binding-site, leucine-rich repeat (NB-LRR) proteins, which activate down-stream defence to combat the disease, when the pathogen has a specific avirulence-gene (*Avr*) that corresponds to the specific R-gene (see the recent review by (McHale et al. 2006). However, resistance obtained by introgression of these types of gene generally has the drawback that pathogen populations eventually adapt to their presence and overcome them (Hovmøller et al. 1997; McDonald and Linde 2003). In other words, when the *Avr*-gene in the pathogen is inactivated by a mutation, the resistance is no longer functional. As the *Avr*-genes often encode effector proteins which have evolved to function in pathogenicity, there is strong balancing selection in natural plant and pathogen populations for polymorphism at the genetic loci in host and pathogen. This means that many *Avr*-alleles are present in natural pathogen populations. However, genotypes carrying a virulent allele of any *Avr*-gene locus will eventually migrate to and invade the resistant plant population, leading to reduced efficiency of the specific resistance gene. These types of resistance genes operate at the recognition stage of an interaction and generally against biotrophic pathogens, where the expression of resistance is often associated with a form of programmed cell death (PCD), known as the hypersensitive response (HR).

In some cases, R-genes can provide effective protection against pathogens when transformed into new species and even into new genera, and this protection can be broad spectrum, i.e., independent of pathogen race and even species (Oldroyd and Staskawicz 1998; Rommens et al. 1995; Tai et al. 1999). This represents strategy 2a in Fig. 1. Recently, *Rxo1*, an

R-gene derived from maize (*Zea mays*), a non-host of the rice bacterial pathogen, *Xanthomonas oryzae* pv. *oryzicola* was successfully transformed into rice (*Oryza sativa*) and shown to confer resistance against *X.o. oryzicola* (Zhao et al. 2005). Thus, the potential of using R-genes as transgenes across natural breeding barriers exists. However, inter-species differences may radically influence R-gene function (Ayliffe et al. 2004) and therefore it is preferable to use R-genes from closely related species. The transgenic approach circumvents tedious backcrossing and has successfully been accomplished in rice for the R-gene, *Xa21*, conferring broad, but nevertheless race-specific resistance to the bacterial leaf blight disease (Wang et al. 2007). *Xa21* has subsequently been transformed into a restorer line for hybrid rice and shown to provide resistance without compromising elite traits (Zhai et al. 2002). Field tests of *Xa21* transgenic rice in The Philippines, China and India have shown satisfactory results (Datta 2004). However, deregulation of transgenic *Xa21* rice for large scale cultivation is still pending. It should be noted that conventional breeding assisted by the use of molecular marker techniques has already provided hybrids containing *Xa21*, pyramided with other resistance genes (Joseph et al. 2004; Zhang et al. 2006), thereby creating a competitive alternative to the transgenic approach.

An NB-LRR R-gene, *Rpi-blb2*, derived from the wild potato relative, *Solanum bulbocastanum*, confers broad-spectrum race-nonspecific resistance in potato (*Solanum tuberosum*) against the Oomycete pathogen *Phytophthora infestans* (van der Vossen et al. 2003, 2005) and patent EP20020075565. A representative of the Cf family of R-genes, *Vf* was cloned from the wild apple species *Malus floribunda*, and transferred to cultivated apples (*Malus domestica*) where resistance to a presumably mixed population of *Venturia inaequalis* isolates was demonstrated (Belfanti et al. 2004). Compared to conventional breeding, the transgene approach will facilitate introduction of more R-genes into a crop at the same time (pyramiding). This will extend the durability of the resistance concerned. The transgenic strategy using R-genes can, however, have negative side effects. For example, over-expression of the *Pto* gene from tomato (*Lycopersicon esculentum*) resulted in a lesion mimic phenotype in the mesophyll tissue (Tang et al. 1999).

Some necrotrophic pathogens are adapted to deliberately use the R-gene-type of recognition in

order to activate PCD through the use of specific toxins that provoke the R-gene signalling (reviewed by Glazebrook 2005; Mayer et al. 2001). R-genes effective against these types of necrotrophic pathogens are simply unknown. This statement needs to read in the light of the fact that many apparently necrotrophic pathogens are really hemibiotrophic and exhibit an albeit brief biotrophic or endophytic phase during early stages of infection, where R-genes can be effective, e.g., *Bipolaris sorokiniana*, *Magnaporthe oryzae*, *Rhynchosporium secalis*, *Phytophthora infestans* (see Parlevliet 2003).

Induced resistance

Plants have effective defences against pathogens. These defences are invariably activated following pathogen attack, though they are not always sufficiently effective to lead to resistance (see the articles by Collinge et al. 2007; Conrath, 2007; Shetty et al., 2007). Briefly, it is often observed that pathogen attack or treatment with various inducer molecules can result in effective local induction of resistance, or less effective systemic resistance which, nevertheless, has a broad effect on many pathogens (Durrant and Dong 2005). Such studies of induced resistance have led to two different strategies aiming for the development of transgenic disease resistant plants. One of these can be considered a first generation strategy, analogous to the strategies used in GM crops to control insect pests, which concerns the use of single gene products that have a direct inhibitory effect on the pathogen (strategy 1 in Fig. 1). Second generation strategies (i.e. more recent studies) are based on an understanding of the mechanisms regulating disease resistance in plants, for example the R-genes as described above (strategy 2). Neither of these strategies has yet led to GM disease-resistant crops in production, though the latter strategy is promising.

First generation strategies for transgenic disease-resistant plants

The study of plant defence mechanisms in the 1970s and 1980s led rapidly to the discovery that various defence proteins (i.e. the PR or pathogenesis-related proteins), certain small peptides and a wealth of secondary metabolites possess direct antimicrobial

activities (Broekaert et al. 2000; Castro and Fontes 2007; Field et al. 2006; Hammerschmidt 1999; van Loon et al. 2006). In contrast to the case with Bt toxins and insects, however, no single protein or metabolite has been identified with a major effect on a range of pathogens. For example, early studies based on *in vitro* data suggested that the plant defence enzyme, chitinase (Collinge et al. 1993), was a promising candidate to provide resistance against any fungal pathogens since the substrate, namely chitin, is a major constituent of fungal cell walls. Many studies used single genes encoding antimicrobial proteins, such as chitinase to make transgenic plants (see Broekaert et al. 2000; van Loon et al. 2006). There are a number of examples where it was demonstrated that constitutive expression of single genes gave a significantly improved disease resistance (see Broekaert et al. 2000 for an early comprehensive review), but in no case was the effect more than partial, even when several genes encoding antimicrobial proteins were combined in the same plant (e.g., Kalpana et al. 2006; Zhu et al. 1994). Some examples are discussed below and listed as 1a/1b in Table 1. Antimicrobial proteins can act through a wealth of physiological mechanisms, few of which are really understood. Some act directly to interfere with pathogen physiology or indirectly by interfering with pathogenicity processes necessary for infection.

A concern associated with the production of antimicrobial proteins is that some might be allergenic or toxic to vertebrates, and there are well-established analyses for allergenic or toxicological risks [see e.g., Schlaich et al. (2007) for examples applied in a relevant case]. Another concern is related to the risk of selecting new microflora resistant to future antibiotics of relevance to humans. From a plant science perspective, it would be interesting to use plant antimicrobial proteins, such as defensins (Broekaert et al. 2000), as alternative medical antibiotics, similar to plectasin (Mygind et al. 2005). A means for reducing toxicological and allergenic risks, whilst simultaneously reducing the risk that pathogens will develop tolerance or resistance to specific proteins, is to use promoters which confer tissue-specific or defence-response-specific expression in the transgene. An example of this strategy is the use of epidermis-specific expression of defence genes in wheat (*Triticum aestivum*) where constitutive expression of a wheat peroxidase gene specifically in the epidermis

provided some protection against the powdery mildew fungus (Altpeter et al. 2005).

Analogous to Bt insect resistance, antimicrobial proteins are found in many organisms other than plants, and have been exploited in transgenic strategies. It can be predicted that increased knowledge of the biology of plant–microbe, and microbe–microbe interactions will provide further examples with potential for GM-strategies. Thus transgenic wheat has been prepared with a gene encoding the protein KP4 from a virus, which infects the smut fungus *Ustilago maydis*. These plants exhibited variously 10–30% protection against the smut (*Tilletia caries*) in field and greenhouse tests (Clausen et al. 2000; Schlaich et al. 2006, 2007). Antimicrobial peptides do not need to be natural. For example, transgenic cotton, prepared using a synthetic peptide, D4E1 (derived from an insect antimicrobial peptide), exhibited enhanced resistance against the fungus *Thielaviopsis basicola* (Rajasekaran et al. 2007). Interestingly, the same peptide provided resistance to bacterial pathogens in transgenic poplar (Mentag et al. 2003; Montesinos 2007).

A new approach to protect plants against bacterial diseases is based on interference with the communication system, quorum-sensing, used by several phytopathogenic bacteria to regulate expression of virulence genes according to population density (reviewed by Cui and Harling 2005). The enzyme, AiiA, isolated from bacterial strain, *Bacillus* sp.240B1, was found to degrade the quorum-sensing signalling molecule of the soft rot pathogen, *Erwinia carotovora*, and thereby rendering the bacteria incapable of infecting the host (Dong et al. 2000). Transgenic expression of AiiA *in planta* was subsequently demonstrated to provide significant enhancement of resistance against soft rot in potato (Dong et al. 2001; US patent 7205452). The strategy looks technically very promising since the microbial target is likely to be strongly conserved. However, since similar quorum-sensing is also known for bacterial pathogens of humans (for example, *Pseudomonas aeruginosa*), this strategy also raises the concern that there is a risk that control of bacterial infection in humans will be impaired.

A plethora of different antimicrobial secondary metabolites (known as phytoalexins or phytoanticipins (VanEtten et al. 1994)) are produced in plants. These metabolites can have roles in disease resis-

tance, and in some cases it has been demonstrated that these can indeed limit the host range of specific pathogens (Field et al. 2006; Osbourn 1996). Specific metabolites are often restricted to closely related plant species, and pathogens adapted to a particular plant species need to be able to withstand these antimicrobial metabolites, for example, by detoxifying them. This makes them attractive subjects for exploitation in transgenic strategies. It can be predicted that the pathogens adapted to parasitise one species are not adapted to the phytoalexins of a distantly related species and are therefore incapable of detoxifying them. However, one problem in exploiting secondary antimicrobial metabolites in transgenic disease resistance strategies is that they are usually the products of multi-step biosynthetic pathways, requiring multiple enzymes, each comprised of one or more proteins, which are individually the products of separate genes. Unfortunately, this calls for simultaneous or sequential transformation of many genes into a single plant line. In many cases, the complexities of the biosynthetic pathways remain to be clarified and the necessary genes cloned. The best exploited exception concerns the stilbenes, especially resveratrol. In this case, it has proven possible to make a new phytoalexin following transfer of a single gene, with resulting improved resistance (Hain et al. 1993; Leckband and Lorz 1998; Serebriakova et al. 2005). However, in no case has the desired complete resistance been obtained.

The regulation of disease resistance – the second generation

Whereas cucumber and tobacco, in particular, provided the physiological understanding of induced resistance, mutational studies using *Arabidopsis thaliana* have provided a profound understanding of the nature of regulation of defence mechanisms (Glazebrook 2005). *Arabidopsis* genetics has, for instance, been instrumental in the analysis of the different mechanisms of resistance operating in biotrophs and necrotrophs. This has led to strategies for utilising regulatory genes in developing GM disease-resistant plants (Campbell et al. 2002). The use of R-genes (included in strategy 2a described above) can be considered to fall under the concept of this approach. A general strategy is to activate defence signalling

pathways and thereby simultaneously stimulate a wider collection of the down-stream response genes, which manifest the resistance (strategy 2b). For this purpose, the knowledge obtained from work on mutant plants that constitutively express defence responses can be explored. Such plants are generally resistant to a number of different pathogens, but they often suffer from being lesion mimics and dwarves (see Lorrain et al. 2003). Examples of such mutants are *lsd1* (Torres et al. 2005), *acd2* (Mach et al. 2001), *acd11* (Brodersen et al. 2005), *cpr1*, *cpr5*, *cpr6* (Clarke et al. 2000) and *syp121 syp122* (Zhang et al. 2007). Some mutations causing lesion mimic phenotypes have occurred in NB-LRR-type R-genes. Here, mutations in specific motifs of the NB domain permanently stimulate resistance as they mimic avirulence-activation of the R-protein (Howles et al. 2005; Takken et al. 2006).

An interesting example concerns the *NPR1* (or *NIMI*) gene, a key defence regulator first identified in *Arabidopsis* (Durrant and Dong 2005). Over-expression of this gene confers broad-spectrum resistance against various pathogens (Cao et al. 1998). The effect is not restricted to *Arabidopsis*, thus over-expression of *Arabidopsis* NPR1 in wheat led to resistance against *Fusarium graminearum* (Makandar et al. 2006). Transgenic rice plants over-expressing the rice NPR1 orthologue (NH1) acquire high levels of resistance to *Xanthomonas oryzae* pv. *oryzae* (Chern et al. 2005).

Because of lesion development and dwarfism, the resistance caused by this kind of mutation cannot be used directly. However, if the regulatory gene can be up or down-regulated according to the function of the protein, so that resistance is activated only when a pathogen attacks, then this would provide a useful strategy for developing disease resistance. Here pathogen inducible gene promoters can become useful, although the choice of promoter is not trivial. Such a promoter must not itself be stimulated by the defence response to be regulated; otherwise a runaway lesion response will occur following the first pathogen attack of the plant.

Specific problems – toxins

Necrotrophic pathogens, in contrast to biotrophs, use pathogenicity factors such as toxins and hydrolytic

enzymes to effect successful infection. Indeed, without effective production of the toxin, the pathogen is often incapable of causing infection. Some toxins have the unfortunate side effect of being toxic to mammals and not just the target plant tissue, in which case they fall into the category of mycotoxins. Toxins often accumulate in biologically active concentrations in tissues remote from the site of infection. In some cases, the toxins are therefore a significant factor in crop spoilage disproportionate to actual loss of yield, especially where they are distasteful or poisonous to the consumer of the crop. One strategy for GM disease resistance (falls under strategy 1) is to target the toxin, i.e. cause its degradation, and thereby reduce infection and loss, and simultaneously reduce spoilage where mycotoxins are concerned. Transgenic maize, where the levels of the *Fusarium*-toxin zearalerone were reduced to 10% of the wild-type levels, proves that the approach is feasible (Igawa et al. 2007). A concern for this approach is that it may lead to the accumulation of breakdown products of which little is currently known. This is not likely to be a major problem where removal of the toxin from the system arrests pathogen development.

Oxalic acid has an important role as a toxic pathogenicity factor in several species of necrotroph, of which *Sclerotinia sclerotiorum* is a particular problem in many dicotyledonous species, for example, oil seed rape (*Brassica napus*) and sunflower (*Helianthus annuus*). Several studies have therefore taken the approach of constitutively expressing a heterologous (usually wheat) oxalate oxidase gene in a target crop in order to neutralise the oxalic acid produced by the pathogen. The products of the enzyme include the reactive oxygen species hydrogen peroxide, which itself has an important role in disease resistance (see Shetty et al. 2007 details). Examples where partial resistance has been obtained in the laboratory include sunflower and soybean (*Glycine max*) against *Sclerotinia sclerotiorum* (Cober et al. 2003; Donaldson et al. 2001; Hu et al. 2003) as well as poplar (*Populus* × *euramericana*) against *Septoria musiva* (Liang et al. 2004).

Bt maize and Fusarium toxins – serendipity

Transgenic maize with Bt toxin genes (specifically the Cry1Ab protein) from *Bacillus thuringiensis* are not

just insect-resistant but also consistently less attacked by *Fusarium* spp. and contain consistently reduced levels of toxin (Clements et al. 2003; Hammond et al. 2004). The reduced infection is likely to be a consequence of reduced opportunity for fortuitous fungal infection in tissues less wounded by insects (Duvick 2001).

Virus resistance

Numerous reports concern transgenic resistance to plant viruses (reviewed by Sudarshana et al. 2007; Fuchs and Gonsalves 2007) in which RNA-mediated gene silencing especially is a predominant strategy (viral RNA is degraded and viral DNA is inactivated by methylation). Most of these approaches can be categorised as pathogen mimicry (strategy 3, in Fig. 1). RNA-mediated resistance to both DNA and RNA viruses can be obtained without transgenic expression of a protein and this strategy thereby minimises toxicological and allergenic risks. Transgenes constitutively expressed to provide RNA-mediated virus resistance fall into three major types: (A) Sense or antisense viral sequences, (B) Inverted repeats/hairpin RNA of viral sequences, (C) Sequences of engineered microRNAs targeted against viruses. For examples of the three types: (Fuentes et al. 2006; Niu et al. 2006; Smith et al. 2000; Vazquez Rovere et al. 2007; Yang et al. 2004). The three types of transgenes have been compared for their ability to provide disease protection in short term experiments, and a relative order of efficiency ($A < B$) and ($B < C$) has been reported for RNA viruses (Smith et al. 2000; Qu et al. 2007). However, a more extensive comparison is needed before general conclusions can be drawn.

The best documented examples of transgenic virus resistance applied in farmers' fields have involved transgenes of type (A) above, although the mechanism of RNA-mediated resistance was initially not known. In the 1990s, the Papaya industry on Hawaii suffered a 50% decline in production due to an outbreak of the potyvirus Papaya ringspot virus, PRSV (Gonsalves 1998). Virus resistance was obtained in a high-yielding papaya hybrid using the viral coat protein sequence as the transgene (type A above). Following distribution of transgenic seeds to farmers, a 50% rebound of total papaya production on Hawaii was achieved within 4 years (Gonsalves

2004). A similar approach has been successfully applied in US cucurbit production, although the situation has been more complicated due to the presence of several different viruses (Fuchs et al. 1997; Fuchs and Gonsalves 2007).

For DNA viruses, the geminiviruses constitute a focal area of intense research in a range of crops (tomato, cassava, maize, legumes). For these viruses, strategies for transgenic resistance involves both RNA-mediated resistance and several approaches using mutated viral proteins exerting transdominant negative effects on viral replication (reviewed by Vanderschuren et al. (2007)). Several of the DNA viruses are whitefly-transmitted, and an inherent problem in crops like tomato is that, even though a transgene may protect against the virus, it will not protect the crop against the substantial damage caused by the whiteflies. An approach targeting both viruses and insects might be valuable, and some inspiration for future research in that direction can be obtained from a recent study in rice: under controlled conditions, it was demonstrated that inhibition of phloem-feeding insects, by transgenic expression of a garlic lectin, subsequently reduced the associated viral disease, rice tungro, vectored by the insects (Saha et al. 2006).

Discussion

Given the effort put into biotechnological approaches for introducing disease resistance into crops over the last two decades and the lack of concrete results in terms of transgenic crops in use, it is pertinent to pose the question as to under which circumstances should one attempt to make disease resistant plants by genetic engineering. Would it not be better to use the resources required, especially public funding, to support classic plant breeding initiatives? The answer probably lies in a balance between the two approaches. Most plant breeding in the developed economies is run effectively by private enterprise. The effectiveness of plant breeding has improved dramatically in recent years through the development of molecular marker technologies, which are particularly beneficial for disease resistance breeding where costly (and potentially harmful) phenotypic screening can be minimised. The investment required for making transgenic plants is enormous and the markets

apparently uncertain due to barriers caused by legislation which in themselves represent a political reaction to public opposition to technologies carrying perceived risk. Indeed, it can be argued that the public reaction, especially in Europe, to the success of transgenic herbicide-tolerant crops has set back the opportunities for plant biotechnology by at least a decade.

There is the issue of ineffectiveness. Most strategies tried to date have resulted in at best partial resistance. Partial resistance provides, of course, a clear advantage over susceptibility; the development of a pathogen on a partially resistant plant is slower, which means that the spread through a population (crop) will be slower. This is widely exploited by breeders. However, given the enormous costs associated with developing GM crops, in most cases partial resistance by GM is not considered attractive for commercial development. A related issue is whether a gene product can be expected to confer protection against many different, or a single pathogen species. Some genes offer prospects for general antimicrobial activities, i.e. strategies effective against different taxa; others will prove very narrow in their mode of action. This will not in itself be a disadvantage where a high value crop is threatened by a specific problem with major economic impact (e.g., potato-late blight, wheat-stem rust, coffee-rust, banana-black sigatoka or Panama disease).

Where strategies are based on the introduction of single genes, there is a risk of rapid breakdown, a problem well known from the introduction of race-specific resistance by conventional breeding (Hovmøller et al. 1997; McDonald and Linde 2003; Parlevliet 2003). The potential and need for pyramiding genes must be evaluated carefully in order to avoid the risk of breakdown and prolong the lifespan of the transgenic crop. In addition, it can be an advantage to ensure that the gene products are produced only when needed by using tissue-specific, pathogen-inducible promoters (Altpeter et al. 2005). Another important issue is to ensure that effective resistance is introduced in a sufficiently broad genetic background to avoid exposure to new risks from pests and pathogens associated with monoculture. Finally, the potential health risks – toxicity and allergenicity, have to be borne in mind.

Much has been written about the ethics of making transgenic plants, especially where synthetic genes are

used or where genes are transferred between different Kingdoms or Phyla (from bacteria to plant, from insect to plant). We will not expand on this debate other than to refer to a recent movement – cis-genetics, or “all native” – to emphasize solutions based on gene silencing within species or the alteration of regulation of existing genes (Rommens 2004).

At present, there are no signs that transgenic fungal or bacterial resistance will be introduced in commercial crops in the near future. In contrast, the clear results obtained repeatedly in laboratory and field studies demonstrate that transgenic strategies for virus resistance work effectively. Despite this, virus resistant GM crops have been commercially introduced in only very few cases. Three factors need to be present: the technical solution to a problem which has no other obvious alternative, the economic incentive for implementing the solution, and therefore market and public acceptance. The combination of these factors was present for the Papaya Ringspot Virus in Hawaii. Apparently, continued research into transgenic virus resistance and improved understanding of the mechanisms involved has not led to any significant new introductions of virus resistant GM crops since the late 1990s. Perhaps the expiry of the EU moratorium for the introduction of new transgenic crops in Europe will facilitate this process.

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