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# IMPROVEMENT OF RESISTANCE TO PLANT PATHOGENS AND PESTS BY DNA TECHNOLOGY

## ABSTRACT

To improve crop yield, DNA technology has been used to enhance plant resistance toward pathogens and pests. Genes identified through understanding of host–pathogen interactions in viral, bacterial and fungal diseases, the mechanism of hypersensitive reaction in *Arabidopsis* and insect toxicity of natural peptides are used for their expression in plants. Progress on the use of simple sequence repeats (SSR) markers for resistance gene identification, development of virus–specific antibody gene expression in plants for virus control, construction of genes for multi–pathogen resistance, and use of viral vectors for gene efficiency evaluation are discussed.

## INTRODUCTION

Since the introduction of the cultivation of agricultural crops, selection and breeding have produced crop varieties with many improved agronomical and horticultural properties including high productivity. However, in recent years, plant improvement for higher yields with conventional methods seems to have reached a plateau. Also, crop yield is reduced mainly due to unfavorable environments such as inclement weather, drought, disease and pest infestation. The actual figures for global crop yield loss due to diseases and pests are not available. In a worldwide estimation, plant disease loss is reported at 60 billion dollars per year, and nearly one eighth of agricultural products are damaged by harmful insects (Gatehause *et al.* 1992).

With the exception of virus and viroid diseases, control measures including chemical, biological and other integrated management have been effective against diseases caused by fungi, bacteria and nematodes, as well as infestation by insects. In order to achieve the maximal pro–

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duction of agriculture crops, huge amounts of resources have to be used for chemical application. The long–term application of synthetic chemicals may have detrimental effect on many non–targeted organisms in our ecosystem. Nondegradable chemical residues may also contaminate the environment and threaten food safety. With increasing public awareness, the development of new, effective, environmentally friendly measures for disease and pest control are strongly needed.

DNA technology has made it possible to transfer useful and desirable traits to a number of important agricultural crops. Direct introduction of genes determining specific traits into plants has several advantages over conventional breeding (Cheng *et al.* 1995). It is a fast procedure without disturbing the genomic balance of the targeted plants. Furthermore, there is no restriction on the source of transgenes. Genes from unrelated plant species or even those from outside of the plant kingdom could be used. DNA technology also provides precise manipulation of a gene at the molecular level for its proper regulation and expression. The enhancement of plant resistance toward diseases and insect pests has been the most successful example of plant genetic engineering.

## PLANT BACTERIAL AND FUNGAL DISEASE RESISTANCE

Molecular markers associated with various disease resistance in plants have been identified using simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and repetitive sequence–based polymerase chain reaction (rep–PCR)(Järve *et al.* 2000, Kawchuk *et al.* 1998, Molnar *et al.* 2000*)*. These markers have been used to construct molecular genetic maps of the plant and to select disease resistance lines in plant breeding. SSR markers, which identify high levels of allelic diversity in plant genetics and produce selective PCR products with specific primers, have been developed in soybean and wheat. A total of 606 *SSR* loci have been assigned to 20 linkage groups based on three soybean crossing populations (Cregan *et al.* 1999). Also, in a preliminary study, *(TAA/ATT)*$_n$ microsatellites were found to be the most abundant and the most polymorphic in wheat cultivar ‘Chinese Spring’ (Song *et al.* 2002). With the aid of ditelosomic and nullisomic–tetrasomic lines of the standard wheat cultivar ’Chinese Spring’, the chromosomal location of these (TAA/ATT)$_n$ microsatellite markers has been determined (Song and Cregan, *personal communication*). The availability of these *SSR* markers in wheat can be used for future gene mapping studies and the identification of quantitative trait loci (QTL) of disease resistance and other agronomically important characters.

Recent progress in the understanding of host–pathogen interactions, systemic acquired resistance (SAR) and host hypersensitive reaction (HR) enables us to use genetic engineering to enhance plant disease resistance (Mourgues *et al.* 1998, Rommens and Kishore 2000, Shirasu and Schulze–Lefert 2000). A family of plant resistance (*R*) genes related to disease resistance has been isolated by map–based cloning techniques (Brommonschenkel *et al.* 2000, Gassmann *et al.* 1999, Milligan *et al.* 1998). These plant *R* genes reportedly encode a group of proteins which contains potential nucleotide–binding site domains (NBS) specific for kinase activity and leucine rich repeats (LRR) at their C–terminal. The LRR regions of different plant *R* genes can recognize and form a complex with specific C–terminal regions of bacterial avirulence gene proteins (Axtell *et al.* 2001, Leister and Katagiri 2000, Shan *et al.* 2000). The recognition of pathogen invasion by plant *R* gene products on the plasma membrane initiates a series of pathological reactions and leads to plant disease resistance. It is evident that usage of a strong promoter for high *R* gene expression and cloning of a modified R gene containing recombinant LRR sequence will raise the level of plant resistance against the particular pathogen and other unrelated pathogens (Ellis *et al.* 1999, Tang *et al.* 1999).

Another important component of plant defense responses is the accumulation of salicylic acid (SA) and subsequent induction of SAR near the infection site (Alvarez 2000, Delaney 2000, Yu *et al.* 1997). Several key gene products which transmit the SA signal and activate pathogenesis–related (PR) gene expression, such as *NPR1* and *Pad4* in *Arabidopsis* and *Prf* in tomato, have been recently identified. Over–expression of these genes would activate the SAR pathway and ward off a broad spectrum of pathogens (Cao *et al.* 1998, Jirage *et al.* 1999, Perlak *et al.* 1991). SA is proposed to be a product of the phenylpropanoid metabolism pathway formed via L–phenylalanine, *trans*–cinnamic acid and benzoic acid in tobacco (Lee *et al.* 1995). In application, cloning and expressing two bacterial alternative SA synthesis genes encoding isochorismate synthase and isochorismate pyruvate lyase enzymes in tobacco were reported to enhance SA accumulation, induce PR gene expression and confer SAR to viral and fungal infection (Verberne *et al.* 2000). Nevertheless, the level of SA induction should be optimally controlled since the highly active SA signaling pathway could lead to severe tissue senescence (Morris *et al.* 2000). Most recently, the possible involvement of nitric oxide (NO) in molecular signaling toward SAR and plant disease resistance has drawn significant attention (Delledonne *et al.* 2000, Durner *et al.* 2000, Klessig *et al.* 2000). It was shown that NO synthase activity was highly increased in resistant tobacco after infection with tobacco mosaic virus. Feeding of NO donor to tobacco and soybean cells triggers the expression of PR protein and phenylalanine ammonia lyase genes, and induces hypersensitive cell death. So far no gene isolation related to NO accumulation and signalings has been reported.

The locally transient massive production of hydrogen peroxide ($H_2O_2$) and reactive oxygen intermediates (ROI) in incompatible plant–pathogen interactions may also play an important role in plant disease resis–

tance (Baker *et al.* 1997, Hilder and Boulter 1999). This oxidative burst which is accompanied by the accumulation of SA and the localized change in peroxidase activity may reinforce the plant cell wall, exert antimicrobial activity, induce localized programmed cell death, and confer plant disease resistance (Bestwick *et al.* 1998, León *et al.* 1995). Barley genes, *Rar 1* and *Rar2*, which are required for the functioning of powdery mildew resistance gene, *Mla12*, were shown to be involved in the accumulation of ROI at the sites of fungal invasion (Hückelhoven *et al.* 2000). Genes encoding bacterial nonheme chloroperoxidase and two $H_2O_2$–generating enzymes, glucose oxidase and oxalate oxidase, have been expressed in various transgenic plants for fungal disease control (Rajasekaran *et al.*2000, Rommens and Kishore 2000, Wu *et al.* 1995).

There are a wide variety of antimicrobial peptides present in plants. Several small cysteine–rich peptides, such as cecropin and thionin, have shown to be active *in vitro* against bacteria (Broekaert *et al.* 1997, García–Olmedo *et al* 1996, Segura *et al.* 1999, Shewry and Lucas 1997). Expression of genes encoding these peptides in transgenic plants results in enhanced tolerance to bacterial and fungal pathogens (Arce *et al.* 1999, Epple *et al.* 1997, Molina *et al.* 1997, Terras *et al.* 1995). Recently, plant viruses with broad host plant ranges, such as cucumber mosaic virus, potato virus X and several potyviruses, have been manipulated as transient vectors to deliver antimicrobial protein genes into plants (Arazi *et al.* 2001, Choi *et al.* 2000, Rommens and Kishore 2000, Toth *et al.* 2001, Zhao *et al.* 2001). The advantage of virus–based vectors is to have a simple, quick delivery of target genes and rapid evaluation of gene expression, antimicrobial property and host toxicity in the whole plants. Once the expression and function of the gene in plants meets the expectation, the gene would be stably incorporated into plants by plant transformation.

## PLANT VIRAL DISEASE RESISTANCE

Introducing resistance to viruses and their virus–transmitting insect vectors into plant cultivars by gene transfer technology has been successful in combating plant virus diseases (Dempsey *et al.* 1998). Several approaches for producing transgenic virus–resistant plants have been explored (Table 1)(Gutierrez–Campos *et al.* 1999, Hadidi *et al.* 1998). Among these, plants expressing viral coat protein (CP) genes, non–structural protein (NS) genes, or virus satellite ribonucleic acids have been shown to offer the best control (Beachy 1999, Maiti *et al.* 1999, Prins and Goldbach 1996). Plants expressing antisense viral RNAs, ribozymes, pathogenesis–related proteins, or virus–specific antibody genes may also confer resistance to viral infection (Hadidi *et al.* 1998).

Viral CP genes are most commonly cloned into transgenic plants to elevate virus disease resistance (Miller and Hemenway 1998). The concept of CP protection in engineered plants is based on cross protection

Table 1

**Genes which were evaluated for their ability to control viral diseases in plants**

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| Virus derived gene sequences |
| Coat proteins |
| Replicase |
| Movement proteins |
| Polyprotein proteases |
| Satellite RNAs |
| RNAs (Sense and antisense) |
| Plant derived transgenes |
| Pathogenesis–related proteins |
| Anti–viral proteins |
| Proteinase inhibitors |
| Natural resistance (R) genes |
| Lectins |
| Other transgenes and sequences |
| Virus–specific antibodies |
| Interferon–induced mammalian oligoadenylate synthetase |
| Antiviral ribozymes |
| Insect toxins |

that infection of plants by a mild strain of one virus may prevent or inhibit the development of symptoms caused by a second more severe strain of the same virus. The mechanisms of CP–mediated resistance were discussed in a recent review (Reimann–Philipp 1998). One of possible roles for CP is to act as an avirulence gene to induce early oxidative burst and elicit the resistance response within the host plants (Allan *et al.* 2001, Knorr and Dawson 1988, Malcuit 2000, Saito *et al.* 2000, Takahashi *et al.* 2001). The CP–mediated resistant plants against positive sense RNA viruses, a tospovirus, and a DNA geminivirus have been developed (Beachy 1993, de Haan *et al.* 1996, Kunik *et al.* 1994).

Other viral genes encoding NS proteins, such as replicase and proteases, are required for virus replication (Matthews 1991). Cloning of these two NS protein genes in transgenic plants reported to provide high degrees of resistance to many virus infections (Anderson *et al.* 1992, Gatehouse and Gatehouse 2000, Longstaff *et al.* 1993, Maiti *et al.* 1993). Steady expression of cucumber mosaic virus replicase gene in tobacco is necessary for CMV resistance (Wintermantel and Zaitlin 2000). And tobacco mosaic virus replicase protein has been implicated as the virus avirulence factor that triggers tobacco *N* gene–mediated resistance (Erickson *et al.* 1999, Erickson *et al.* 1999).

In resistant transgenic plants cloned with potyvirus CP or replicase genes, there are no high levels of transgenic RNA that can be detected in plant tissues. The mechanism of virus resistance is hypothesized as a post–transcriptional gene silencing (Jan *et al.* 1999, Jones *et al.* 1998). In a recent important application, cloning of a chimeric gene construct which contains a full–length CP gene of turnip mosaic virus and a partial nucleocapsid protein gene of tomato spotted wilt virus confers the plants with multi–virus resistance (Jan *et al.* 2000).

Expression of virus–specific antibody genes in transgenic plants could potentially interfere with the functions of virus encoded structural and nonstructural proteins that are essential to the completion of the viral replication cycle (de Jaeger *et al.* 2000). Antibodies that bind CPs can affect virus uncoating, thus neutralizing initial establishment of the virus infection. They may also interfere with virus assembly or insect transmission. Antibodies that bind replicase may prevent virus replication. The development of hybridoma monoclonal antibodies and gene cloning techniques has made this strategy very appealing. Genes encoding antibodies or antibody fragments against tobacco mosaic virus and tospoviruses have been expressed in transgenic plants for virus protection (Franconi *et al.* 1999, Tavladoraki *et al.* 1993, Voss *et al.* 1995).

Control of virus-transmitting vectors by introducing insect toxins such as trypsin inhibitor, lectin, and α-endotoxin (*Bt*) toxin genes into plants would undoubtedly contribute toward achieving the goal of controlling plant viral diseases. Recently, the potato leafroll virus replicase gene and the *cry3A Bt* gene were recombined and expressed in potato plants to confer high levels of resistance to virus infection and virus transmission by the aphid vector, *Myzus persicae* (Thomas *et al.* 2000).

## PEST MANAGEMENT BY HOST RESISTANCE

Expression of bacterial δ-endotoxin (*Bt*) genes in commercial crops to confer insect resistance is the most successful example of applying DNA technology for pest control (de Maagd *et al.* 1999, Gatehouse and Gatehouse 2000, Jouanin *et al.* 1998, Navon 2000, Schuler *et al.* 1998). The gram positive bacterium, *Bacillus thuringiensis*, was first found to produce the insecticidal crystalline inclusion, δ-endotoxin, during its sporulation. For the past forty years, *Bt* toxin has been the major bio–pesticide to control lepidopteran pests (Hilder and Boulter 1999, Knowles 1994). The gene encoding *CrylA Bt* toxin was cloned and subsequently transferred to tobacco and tomato for tobacco hornworm (*Manduca sexta*) and cotton bollworm (*Heliothus zea*) resistance evaluation in the 1980’s (Barton *et al.* 1988, Fischhoff *et al.* 1987, Schnepf and Whiteley 1981, Vaeck *et al.* 1987). Since then, *Cry1A*–cotton for cotton bollworm (*H. zea*) and pink bollworm (*Pectinophora gossypiella*), *Cry3A*–potato for Colorado potato beetle (*Leptinotarsa decemlineata*), and *Cry1A*–elite maize for European corn borer (*Ostrinia nubilalis*) control has been developed (Armstrong *et al.* 1995, Perlak *et al.* 1993, Wilson *et al.* 1992). However, these transgenic crops did not receive satisfactory results in field tests mainly due to inconsistent, low *Bt* gene expression (Hilder and Boulter 1999). Several approaches have been developed to elevate *Bt* gene ex–

pression in plants and enhance insect resistance. These include use of specific promoters, such as CaMV34S, reconstructing the *Bt* protein coding sequence following the typical plant genetic code, and targeting the unmodified *Bt* sequence to plant chloroplasts (Jansens *et al.* 1995, Kota *et al.* 1999, Koziel *et al.* 1993, Perlak *et al.* 1991). Based on the evolutionary view, the plant chloroplast genome is evolutionally closely related to bacterial chromosome. An unmodified bacterial *Bt* toxin gene has been stably integrated and highly expressed in tobacco chloroplasts (McBride *et al.* 1995).

Plant proteinase inhibitors (PIs) which interfere with the insect digestive system by disrupting protein and amino acid metabolism have been used as a source of transgenes for insect resistance study. Plant serine PIs which have two active sites inhibiting trypsin and chymotrypsin activity are reported to affect larval growth and development, and cause insect death (Gatehouse *et al.* 1992, Hilder *et al.* 1987). Genes encoding PIs from various plant and insect sources have been cloned and expressed in alfalfa, cotton, tobacco and sweetpotato to provide protection against various insects (Ishimoto *et al.* 1999, Thomas *et al.* 1995a, Thomas *et al.* 1995b, Thomas *et al.* 1994, Voss *et al.* 1995, Wasmann *et al.* 1994, Yeh *et al.* 1997). Most of the transgenic plants harboring PI genes showed increased levels of pest resistance. However, control of some insect species is unsuccessful since they have the ability to overcome the plant PI's activity by switching protein and amino acid metabolism to an alternative pathway (Hilder and Boulter 1999).

Plant lectins are a group of sugar–binding proteins which have chronic effects on the survival and development of certain insect species (Czapla and Lang 1990, Powell *et al.* 1995, Shukle and Murdock 1983). The lectins reportedly have low insect toxicity to many insects, except those sap–sucking species in the Order Hemiptera (Hilder *et al.* 1995). A lectin gene from pea (*Pisum sativum*) was transferred and expressed in tobacco for control of *Heliothus virescens* (Boulter *et al.* 1990 ).

## PEST CONTROL BY NATURAL ENEMIES

Usage of recombinant DNA to produce genetically improved strains of natural insect enemies and biocontrol agents also receives certain attention (Harrison and Bonning 2000, Hoy 1994, Hoy 2000, Hoy *et al.* 1997, Hughes *et al.* 2000, Pfeifer and Grigliatti 1996). Parasitoid wasps are the major natural enemy of many insect pests but are sensitive to chemical insecticide sprayings (Schuler *et al.* 1999). Recently, the braconid wasp (*Cardiochiles diaphaniae*) was genetically modified by maternal microinjection with a plasmid carrying organophosphorus dehydrogenase (*opd*) gene to enhance their insecticide (paraoxon) resistance (Presnail and Hoy 1992, Presnail and Hoy 1996). Transposable elements, microbial symbionts and plasmid vectors have been used commonly for gene transformation of non–drosophilids and some medi–

cally important insects (Ashburner *et al.* 2000, Durvasula *et al.* 1997, Heilmann *et al.* 1994, Jasinskiene *et al.* 1998, Loukeris *et al.* 1995, O'Brochta *et al.* 1996, Robertson *et al.* 1992).

Research on genetic engineering of insect pathogens in order to enhance their pesticidal properties has also been carried out (Bonning and Hammock 1996, de Vault *et al.* 1996, Harrison and Bonning 2000). Extensive work has been conducted in bacteria and viruses, but the study of nematodes and fungi is still in the early stages.

## PERSPECTIVE

Usage of resistant plant cultivars for disease and pest control is by far one of the modern approaches to raise world crop production. DNA technology undoubtedly will play a significant role in new crop development and economic growth of many parts of the world. However, the release of agricultural biotechnology products in the United States markets and other countries has recently been closely scrutinized and criticized due to increasing public concerns on human health, and possible environmental and ecological impacts. Recently, a "U.S. Risk Assessment Protocols" act was implemented by the U.S. government legislature. Proper application of DNA technology and thorough analyses of transgenic agricultural products will allow an effective management of disease and pest control while maintaining the long–term interests of agricultural productivity and the environment.

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