Chapter 2

Phytosanitation and the development of transgenic biocontrol agents

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By the year 2050, there will be at least 9 billion people on Earth to feed using the same amount or less land and water than is available today. Currently, about one-third of all potential agricultural commodities grown worldwide are lost to diseases, weeds, insects and other pests. Farmers will be challenged to produce more, but to do so using sustainable cropping practices and less fertilizer and pesticides. Biological control is an integral part of sustainable agriculture. This chapter provides an overview of the topics of the construction, activity and use of transgenic biocontrol agents (BCAs) and their future potential in 21st century agriculture.

Introduction

It is expected that by the year 2050 there will be more than 9 billion people on Earth to feed using the same amount or even less land and water than is now available for agricultural production. Currently, about one-third of all potential agricultural commodities grown worldwide are lost to diseases, weeds, insects and other pests, either before or after harvest. Farmers are being challenged to grow more, but with less fertilizer, pesticides and fumigants, and to use more sustainable practices such as direct seeding (no-till), precision farming and biological control. In the United States and elsewhere, farmers also are being asked to produce the biomass for 21st century biofuels. To meet these challenges to reduce losses from pests and to increase production, all types of traditional and new pest management technologies are needed. Genetically engineered biocontrol agents (BCAs) will need to be a part of these agricultural technologies. This chapter provides an overview of the topics of the construction, activity and use of transgenic BCAs and their potential in 21st century agriculture.

Mechanisms of plant defense

Plants defend themselves against pathogens and insects by several well-described mechanisms: *i)* innate (non-host) immunity; *ii)* localised race-specific resistance; *iii)* systemic resistance; *iv)* microbial-based mechanisms of defense (biological control). Microbial-based defense is especially important because plants lack genetic resistance to some of the most common pathogens and insects, especially organisms that are soilborne. For example in wheat production, the diseases Pythium root rot, Rhizoctonia root rot and Take-all cause billions of dollars in losses annually, yet no commercial variety has resistance. Thus, microbial-based mechanisms serve as the first line of defense against these and other diseases and insects. These mechanisms are modulated by the plant through processes of leaf exudation and rhizodeposition, which stimulate and support specific groups of antagonist microbes (Weller et al., 2007). Pathogen or insect suppression by antagonistic micro-organisms occurs through the mechanisms of competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance, and/or antibiosis/toxin production. Multiple mechanisms of antagonism can operate simultaneously, and in addition, a micro-organism may both suppress pathogens and/or insects and directly stimulate plant growth by enhancing the uptake of nutrients, producing phytohormones and/or degrading ethylene.

Biological control by indigenous and introduced micro-organisms

Disease-suppressive soils provide some of the best examples of indigenous micro-organisms protecting plants' roots against plant pathogens (Weller et al., 2002). "Suppressive soils are soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for awhile but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker and Cook, 1974). In contrast, conducive (non-suppressive) soils are soils in which disease readily occurs. Suppressive soils occur globally and are known for many different pathogens (Weller et al., 2002).

Instances of natural pathogen and insect suppression have been rich sources of micro-organisms for development into BCAs. For example, crown gall caused by *Agrobacterium tumefaciens* is a disease of a wide variety of plant species, but it is especially serious in deciduous fruit nurseries. The observation four decades ago by

Allen Kerr (New and Kerr, 1972) that the incidence of crown gall on almond correlated with the ratio of pathogenic to nonpathogenic agrobacteria suggested the potential for biocontrol by bacterization with nonpathogenic strains. *Agrobacterium radiobacter* strain K84 (isolated from soil around a peach gall) applied to seeds or roots resulted in dramatic control of crown gall (Kerr, 1980). K84 and its transfer-deficient mutant (K1026, see below) (Jones et al., 1988) are used worldwide for crown gall control.

During the last four decades, thousands of putative BCAs have been isolated and then tested on hundreds of diseases, insects, weeds and other pests. Although the use of biocontrol technology remains only a small fraction of that of chemical pesticides, the number of new BCAs, their performance and acceptance by growers continues to increase steadily. *Bacillus* and *Trichoderma* spp. have been the micro-organisms of choice for development into commercial BCAs of plant diseases (Harman et al., 2010; Kloepper et al., 2004; McSpadden Gardener and Driks, 2004), and *Bacillus*, *Beauveria* and *Metarhizium* spp. have been the microbes of choice for development as insect BCAs.[1] These micro-organisms are appealing because they are easily mass produced and formulated. Interestingly, *Pseudomonas* spp. have been the microbes of choice for fundamental studies of biocontrol mechanisms because they are easily genetically modified and engineered. Although they are easily mass produced, they are harder to formulate because they do not produce a dormant spore like *Bacillus* spp. do.

Barriers to the wider use of biocontrol technology

There are several historic and chronic problems that need to be overcome before the use of biocontrol technology can reach its full potential as an integral component of sustainable agriculture in the 21st century. The first problem is inconsistent performance. Why a BCA suppresses a disease or kills an insect pest in one field or year but not the next is a fundamental unanswered question. In contrast, there is a perception that chemical pesticides are always effective, but chemicals also can perform inconsistently. A second problem is the narrow spectrum of activity of most BCAs. An agent may be highly effective against a single pest, but often a complex of pathogens or insects must be controlled. Most chemical pesticides have broader activity than BCAs, and thus one chemical often can be used for multiple pests. Thirdly, BCAs are thought to operate over a narrower range of environmental conditions and are much more sensitive to environmental extremes than chemical pesticides. For this reason, BCAs have been shown to be especially successful in the production of glasshouse-grown crops where the environment can be controlled.

The problem of inconsistent performance stems in part from lack of a fundamental understanding of the complex *in situ* interactions among the BCA, host plant, pathogen/insect, indigenous organisms and the environment. For example, what are the *in situ* biotic and abiotic factors that promote and constrain the expression of traits (e.g. root colonisation; ecological fitness; and production of antibiotics/toxins, siderophores, biosurfactants, chitinases, lipases and proteases) that are often important to successful biocontrol? How rapidly are biocontrol metabolites like antibiotics and toxins produced and degraded in the rhizosphere, bulk soil and phyllosphere? In addition, some biocontrol traits are subject to phase variation, "a process of reversible high-frequency phenotypic switching that is mediated by mutation, reorgani[s]ation, or modification of DNA" (Lugtenberg and Kamilova, 2009). This process is well-described *in vitro* but the dynamics and frequency of its occurrence in the rhizosphere, bulk soil and phyllosphere is poorly described.

Without such fundamental information about biocontrol mechanisms *in situ*, it is difficult to predict where and under what conditions a BCA can be expected to perform. "Omics" research (e.g. genomics, proteomics, metabolomics, etc.) will be on the forefront in generating fundamental new information about the biocontrol process. It is notable that in the last several years, at least ten genomes of well-described *Pseudomonas* BCAs have been sequenced and each month more sequences of BCAs and related strains appear in the literature. Knowledge gained from analysis of these genomes is already helping to unravel the fundamental *in situ* interactions leading to biocontrol and also revealing new biocontrol genes.

One example of the benefit of genomics to biological control is seen in the analysis of the genome of *Pseudomonas protegens* Pf-5 (formerly *Pseudomonas fluorescens*) (Loper et al., 2012), the first BCA to be sequenced (Paulsen et al., 2005). In strain Pf-5 and the closely related strain *Pseudomonas protegens* CHA0 (formerly *Pseudomonas fluorescens*), surprisingly, a novel genomic locus encoding a large protein insect toxin termed Fit (for *Pseudomonas fluorescens* insecticidal toxin) was discovered. This toxin is related to the insect toxin Mcf (Makes caterpillars floppy) produced by the entomopathogen *Photorhabdus luminescens*, a mutualist of insect-invading nematodes. When injected into the haemocoel, strain Pf-5 or CHA0 killed larvae of the tobacco hornworm (*Manduca sexta*) and the wax moth (*Galleria mellonella*), whereas mutants of these two strains with deletions in the Fit toxin gene were significantly less virulent to these larvae (Péchy-Tarr et al., 2008).

Why transgenic biocontrol agents?

Genetic engineering offers an approach to enhance the consistency of performance, spectrum of activity and colonising ability of BCAs. All mechanisms of biocontrol (competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance and antibiosis/toxin production) have been targeted for improvement during the last 25 years. Selected examples of proof of concept studies are given below.

Competition/pre-emptive exclusion

Expression of the *Pseudomonas putida* WCS358 ferric siderophore receptor *pupA* in strain WCS374 increased the competitiveness of WCS374 against WCS358 when both strains were co-inoculated (Raaijmakers et al., 1995). Increasing the copy number of the *Pseudomonas fluorescens* WCS365 site-specific recombinase gene *sss* in F113 and WCS307 increased the competitive colonisation ability of the recombinant strains on tomato root tips (Dekkers et al., 2000). This gene plays a role in DNA rearrangements and is thought to help keep bacterial cells from becoming "locked in" a state unfavourable for competitive colonisation.

Parasitism/predation

Expression in *Pseudomonas putida* of *chiA* from *Serratia marcescens* gave improved protection of beans against *Sclerotium rolfsii* (Chet et al., 1993). Dunne et al. (2000) showed that overproduction of an extracellular serine protease by *Stenotrophomonas maltophilia* W81M3 or W81M4 resulted in improved control of Pythium damping-off of sugar beet by the recombinant strains as compared to the wild-type strain W81.

Induced resistance

Introduction of *pchCBA* from *Pseudomonas protegens* CHA0 (formerly *Pseudomonas fluorescens*) into strain P3 enabled salicylic acid production and improved the ability of P3 to induce systemic resistance in tobacco against tobacco necrosis virus (Maurhofer et al., 1998).

Antibiosis/toxin production

Transfer and expression of the HCN biosynthesis operon *hcnABC* from *Pseudomonas protegens* CHA0 into *Pseudomonas fluorescens* P3 resulted in improved control of black root rot of tobacco by the transgenic strain (Voisard et al., 1989).

Transfer of a recombinant plasmid *pCU203*, containing genes for the biosynthesis of 2,4-diacetylphloroglucinol (DAPG) cloned from *Pseudomonas* sp. F113, into *Pseudomonas* sp. strain M114 yielded M114(pCU203), which gained the ability to synthesise DAPG and control *Pythium ultimum* damping-off of sugar beet better than did M114 (Fenton et al., 1992).

Molecular genetic modifications to biocontrol agents

A very wide variety of genetic approaches have been used to genetically engineer BCAs with improved biocontrol or plant colonising ability, and these approaches can be grouped in three categories: *i)* deletion or mutation of existing genes; *ii)* alteration of gene regulation; *iii)* introduction of heterologous genes. Selected examples of these approaches are given below.

Deletion or mutation of existing genes

Agrobacterium radiobacter K84 is a well-described BCA of crown gall that is sold worldwide (Kerr, 1980). A transfer (Tra⁻) mutant of A*grobacterium* K84 (designated K1026) was constructed to prevent the possible transfer of pAgK84 encoding agrocin 84 to *Agrobacterium tumefaciens*, which could result in the pathogen becoming resistant to the BCA (Jones et al., 1988). The recombinant strain K1026 is as effective as the wild type and is used commercially (Jones and Kerr, 1989).

Another excellent example of this type of genetic modification involves biocontrol of ice nucleating bacteria by an ice nucleating deficient *Pseudomonas syringae* (Hirano and Upper, 2000). An Ice strain of *Pseudomonas syringae* was constructed by deleting a fragment of the ice gene, followed by marker exchange of the mutated gene into the wild type. This engineered derivative was the first recombinant microbe deliberately released into the environment. Application of Ice⁻ mutants reduced populations of Ice⁺ *Pseudomonas syringae* on potato and strawberry 50-fold by pre-emptive exclusion and reduced frost damage in the field (Lindow, 1995; Lindow and Panopoulos, 1988). The Ice⁻ strain faced a difficult path through regulatory, social and political obstacles prior to field release, which contrasted strikingly with the release of A*grobacterium* K1026, which faced little resistance.

Finally, Barahona et al. (2011) constructed a triple mutant of *Pseudomonas fluorescens* F113 in the genes *sadB*, *wspR* and *kinB*, resulting in hypermotility and better root colonisation. In addition, the mutant strain had improved biocontrol activity against *Fusarium oxysporum* f. sp. *Radicis-lycopersici* on tomato and *Phytophthora cactorum* on strawberry as compared to F113.

Alteration of gene regulation

Bacillus subtilis strain ATCC 6633 produces the lipopeptide mycosubtilin. Replacing the native promotor of the mycosubtilin operon in ATCC 6633 with a constitutive promotor yielded the recombinant strain BBG100. This recombinant produced up to 15-fold more mycosubtilin and suppressed *Pythium aphanidermatum* on tomato significantly better than the wild type did (Leclère et al., 2005).

The two-component regulatory system consisting of GacS (sensor kinase) and GacA (response regulator) is involved in the regulation of secondary metabolism. In a second example of altered gene regulation, Ligon et al. (2000) enhanced expression of the biosynthesis genes (*prnABCD)* for the antibiotic pyrrolnitrin in *P. fluorescens* BL915 by adding additional plasmid-borne copies of *gacA*, by changing the first base in the coding sequence of the *gacA* gene to a more efficient codon, or by replacing the native promoter of *gacA* with the stronger P$_{tac}$ promoter. Each of these alterations resulted in a marked increase in both the amount of pyrrolnitrin produced by the various genetically modified strains and their level of control of *Rhizoctonia solani* on cucumber and impatiens. The level of antibiotic production was directly related to the level of control of *Rhizoctonia solani*.

Introduction of heterologous genes

Most research on engineered strains has focused on adding new biocontrol genes into known BCAs of pathogens, insects and weeds. For example, *Trichoderma atroviride* P1 suppresses a wide range of foliar and soilborne pathogens. Insertion of the *Aspergillus niger* glucose oxidase-encoding gene (*goxA*) under the control of the homologous chitinase (*nag1*) promotor into strain P1 yielded the transgenic strain SJ3-4 (containing 12-14 *goxA* copies) that induced systemic resistance against *Botrytis cinerea* and controlled *Pythium ultimum* and *Rhizoctonea solani* on bean better than did P1 (Brunner et al., 2005).

Bacillus thuringiensis cry genes have been introduced into a wide variety of bacteria (e.g. *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Ancylobacter aquaticus*, *Clivibacter xyli* and *Herbaspirillum seropedicae*). These transgenic strains inhibited a variety of pests, including tobacco hornworm (*Manduca sexta*), malaria mosquito (*Anopheles stephensi*), leatherjacket (*Tipula oleraceae*) and European corn borer (*Ostrinia nubilalis*) (Downing et al., 2000; Obukowicz et al., 1986a, 1986b; Yap et al., 1994). *Bacillus* transformed with the mosquitocidal Cry and Cyt proteins of *Bacillus thuringiensis* and the binary toxin of *Bacillus sphaericus* showed 10-fold better efficacy against Culex spp. (Federici et al., 2003).

In another line of research, *Metarhizium anisopliae* ARSEF 549 was engineered to express the insect-specific neurotoxin AaIT from the scorpion (*Androctonus australis*). Toxicity of the transgenic strain increased 22-fold against tobacco hornworm (*Manduca sexta*) catepillars and nine-fold against adult yellow fever mosquitoes (*Aedes aegypti*) (Wang and St. Leger, 2007).

Most interesting was the report by Fang et al. (2011) who engineered *Metarhizium anisopliae* to produce and deliver molecules that selectively block the development of the causal agent of malaria (*Plasmodium falciparum*) in the mosquito.

A final example relates to biocontrol of weeds. Introduction of *NEP1* (encodes a phytotoxic protein from *Fusarium)* into *Colletotrichum coccodes* increased nine-fold the virulence of the fungus on the herbicide-resistant weed velvetleaf (*Abutilon theophrasti*).

The transgenic strain killed more rapidly and at a lower dose that the wild-type strain (Amsellem et al., 2002).

Case study: Introduction of phenazine genes into *Pseudomonas* spp.

Phenazines are colourful, redox-active antibiotics produced by members of some fluorescent *Pseudomonas* spp. and a few other bacterial genera (Mavrodi et al., 2006). Phenazines are produced in the rhizosphere (Mavrodi et al., 2012), where they are involved in the suppression of plant pathogens (Chin-A-Woeng et al., 2003; Mavrodi et al., 2006; Thomashow et al., 1990), can act as electron shuttles (Hernandez et al., 2004; Rabaey et al., 2005) and contribute to the ecology (Maddula et al., 2008; Mazzola et al., 1992), physiology and morphology (Dietrich et al., 2008; Price-Whelan, 2006) of the strains that produce them. Expression of the core seven-gene phenazine (*phz*) biosynthesis operon (*phzABCDEFG*) is controlled in pseudomonads by homoserine lactone (HSL)-mediated quorum sensing (Mavrodi et al., 2006). Phenazines and quorum sensing are required for the establishment and development of biofilms on surfaces, seeds and roots (Maddula et al., 2008; Mavrodi et al., 2006). In the rhizosphere, expression of *phz* genes can be induced by homoserine lactones produced by heterologous isolates (Pierson et al., 1998; Pierson and Pierson, 2007) or quenched by HSL-degrading rhizosphere inhabitants (Morello et al., 2004).

A disarmed Tn*5* vector (pUT: Ptac-*phz*ABCDEFG), originally constructed by L.S. Thomashow and colleagues, has been used extensively to stably introduce a single copy of the phenazine-1-carboxylic acid biosynthesis genes (isolated from *Pseudomonas fluorescens* 2-79) under the control of a P*tac* promotor into *Pseudomonas* spp. from sources worldwide to improve biocontrol activity. Strains transformed with the *phz* locus also serve as model organisms to determine the impact of transgenes on the ecological fitness and the impact of recombinant strains and on the indigenous rhizosphere microbial community (Ryan et al., 2009). For example, the *phz* operon was introduced into *Pseudomonas brassicacearum* (formerly *Pseudomonas fluorescens*) Q8r1-96 (Loper et al., 2012), a strain that naturally produces the antibiotic DAPG and suppresses Take-all disease of wheat. Several recombinants of Q8r1-96 were selected (Z30-97, Z32-97, Z33-97 and Z34-97) and all produced greater amounts of PCA than strain 2-79, the source of the *phz* operon, because the genes were under the control of a constitutive promotor. Surprisingly however, addition of the *phz* genes also caused elevated production of DAPG in all of the transgenic strains as compared to the wild type Q8r1-96. Although the transgenic strains were no more suppressive of Take-all and Pythium root rot than Q8r1-96, they showed remarkable suppression of Rhizoctonia root rot at a dose of only 100 CFU seed^{-1}, which was 100 to 1 000 times less than the dose required for similar disease control by the wild type Q8r1-96 (Huang et al., 2004).

In a similar study, *Pseudomonas fluorescens* SBW25 was transformed with the mini-Tn*5* vector carrying the *phz* genes and the transgenic strains gained enhanced ability to suppress *Pythium ultimum* damping-off disease of pea when compared to the wild-type strains SBW25 and 2-79 (source of the *phz* operon) (Timms-Wilson et al., 2000).

Some of the best studies of the population dynamics and non-target effects of transgenic BCAs in the field have been conducted with *Pseudomonas putida* strain WCS358r engineered to produce either PCA or DAPG by using the mini-Tn*5* vector system described above (Glandorf et al. 2001; Leeflang et al. 2002; Viebahn et al. 2003). PCA was shown to be produced in the rhizosphere by the transgenic strain, and both

cultivation-dependent and independent methods indicated that the wild-type and transgenic strains had transient effects on the composition of the rhizosphere fungal and bacterial microflora of wheat. The effects of the transgenic strains sometimes were longer lasting than those of WCS358r, and differed from year to year and study to study. These results were similar to those of others conducted under controlled or field conditions and were not surprising given that strain WCS358r and other BCAs often establish high population sizes soon after inoculation, and then the densities decline over time and distance from the inoculum source. In addition, introduced BCAs do not become uniformly dispersed throughout the rhizosphere or among roots of the same or different plants. Collectively, these and other studies of the non-target effects of wild-type and recombinant BCAs indicate that even though the introduced bacteria have definite impacts on non-target microbial communities, the effects vary from study to study and are transient (Ryan et al., 2009).

Conclusion

Microbial-based mechanisms of defense are especially important because plants lack genetic resistance to many common pathogens and insects, especially soilborne organisms. Suppressive soils are the best examples of indigenous micro-organisms protecting plants against pests. Natural instances of pathogen and insect suppression have been rich sources of micro-organisms for development into BCAs. Although the use of biocontrol technology remains only a small fraction of that of chemical pesticides, the number of new biocontrol agents and their performance continues to increase. However, inconsistent performance and narrow spectra of activity are issues that must be resolved before the use of biocontrol technology can reach its full potential as an integral part of sustainable agriculture in the 21st century. BCAs have been engineered to colonise better, tolerate stress better, perform more consistently and effectively, and have a broader spectrum of activity than their wild-type progenitors. All biocontrol mechanisms have been targeted for improvement: competition/pre-emptive exclusion, parasitism/predation, induction of systemic resistance and antibiosis/toxin production. A very wide variety of genetic approaches have been used to engineer BCAs and they can be grouped into three categories: deletion or mutation of existing genes, alteration of gene regulation and introduction of heterologous genes. When new genes are introduced into a BCA, they can influence the expression of biocontrol traits already present. Competitiveness of the transgenic BCA as compared to the parental strain can depend on the host crop. Current micro-organisms of choice for development as commercial BCAs (*Bacillus*, *Trichoderma*, *Beauveria* and *Metarhizium* spp.) will probably be the microbes of choice for future development as transgenic BCAs and *Pseudomonas* will continue to be an important research tool.

Understanding the biogeography of potential transgenes (i.e. those encoding antibiotics and toxins) and their role in nature should lessen concerns about the commercial use of recombinant BCAs. Future research should continue to focus on the development of novel engineered BCAs but broader field testing is needed for engineered agents that have been constructed during the last 25 years and are known to have enhanced activity. During the last 15 years, there has been much greater research emphasis on transgenic plants than transgenic microbes for pest control.
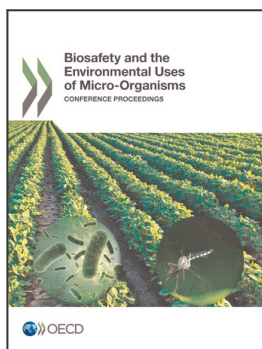
Note

1.   www.epa.gov/pesticides/biopesticides

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