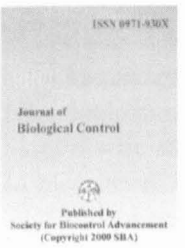


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**Review Article**

# Molecular approaches to improvement of biocontrol agents of plant diseases

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**ABSTRACT**: Biological control holds out the prospect of an attractive proposition against plant diseases. Hitherto, a host of microorganisms have been identified as biocontrol agents while only a few have been able to be successfully commercialized. A major difficulty faced while translating the efficacious biocontrol agents into merchandise was that the efficacy of the developed strains was largely unstable under diverse environmental conditions in which the finished product of organismal origin was to tread before reaching the farmer's field. To add to the problem, desirable properties of a given biocontrol agent are not usually determined by a single attribute; on the contrary desirable biocontrol behavior of the agent is decided by its competitive ability, nature of antibiosis involved, lysis of the target pathogen and induced systemic resistance of the host plant, if any. Yet, a comprehensive understanding of the nature of genes encoding various observable biocontrol mechanisms of action and their putative role at molecular level is still elusive. A detailed characterization of gene(s) encoding biocontrol properties of a given biocontrol agent with respect to individual mechanism of action is a *sine qua non* for further improvement of biocontrol agents with a view to getting the most out of the individual biocontrol agent. With the advent of molecular biology, several approaches have been made towards achieving the following goals: i) Identification and characterization of genes encoding the specific biocontrol property against a given pathogen and ii) Designer biocontrol strains with potential genes responsible for superior biocontrol properties This review tries to focus on the recent developments on the above areas while bringing out a list of promising biocontrol agents so far worked upon with respect to individual target diseases of major crop plants.

**KEY WORDS**: Biocontrol, genetic manipulation, molecular approaches, *Pseudomonas*, *Trichoderma*

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## INTRODUCTION

Agriculture being the largest private enterprise in India, contributes nearly one-fifth of the national GDP, sustains the livelihood of about two-thirds of the population and is considered to be the backbone of agro-based industries. Keeping abreast of modern agricultural technologies, India has moved from an era of food shortages to that of self-sufficiency in foods to feed the entire country which has witnessed a quantum leap in food exports too. While achieving a green revolution during the early 1970s, chemical pesticides undoubtedly played a significant role in enhancing production and productivity largely mitigating losses due to pests and diseases. Continuous use of pesticides since the introduction of high-yielding varieties during the green revolution has raised several concerns including residual pesticides, their phytotoxicity, environmental pollution due to them and human health hazards that followed them. Thus, an effective, sustainable and a non-pesticide-based means of control of plant pests and diseases naturally assumed greater significance in agriculture. With respect to the management of plant diseases, such practice is possible through the use of microorganisms, genes and gene products that can keep the pathogen load under control. Recently, biocontrol science and technology has received an increasing attention across the globe as an alternate means to chemical pesticides.

Microorganisms isolated from the rhizosphere region are considered to provide a superior control of diseases than organisms originally isolated from that of non-rhizosphere (Cook and Baker, 1983). Such plant-associated microorganisms could serve as better biocontrol agents due to their close association and adaptation to the plant and plant parts and the particular environmental conditions in which they are supposed to function. This fact is authenticated with the performance of plant growth

promoting rhizobacteria (PGPR) against various biotic and abiotic stresses (Glick *et al.*, 1995; Ramamoorthy *et al.*, 2002; Mayak *et al.*, 2004a, b; Saravanakumar and Samiyappan, 2007; Saravanakumar *et al.*, 2009). Thus, identification of an effective antagonist strain is the first step towards the development of an effective biological control. So far, several biocontrol agents from fungi (*Trichoderma, Coniothyrium, Gliocladium,* non-pathogenic Fusaria) and bacteria (*Azotobacter, Azospirillum, Arthrobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella* and *Pseudomonas*) have been indicated in the management of plant diseases.

In spite of the successful identification of several biocontrol agents, inefficacy and inconsistency that coexisted with the biocontrol strains against the specific plant diseases under varied field and environmental conditions were indeed a big impediment. This emphasized the need for evolving a superior biocontrol agent that could withstand many adverse conditions and still perform well to reduce pathogen loads. In this context, it became necessary to work with selected biocontrol strains with a view to determining their specific mechanisms while learning about the limitations and their full potential in biological control as well. However, considering the number of biocontrol agents identified so far in the body of literature available as of date, there is very little information available on the potential genes that confer on the biocontrol agent properties related to either antibiosis or lysis and competition. Nevertheless, the advancements in biotechnology have opened up several avenues in biocontrol research and development. Recently, a considerable degree of attention has been devoted towards the identification of useful genes that control biocontrol activity which in turn helps in evolving a superior biocontrol agent which could withstand a reasonable number of biotic and abiotic stresses. In this context, the current review mainly focuses on i) a certain promising molecular approaches to achieving genetic recombination in biocontrol agents and ii) identifying and characterizing potential biocontrol genes useful in bringing about genetic recombination of a particular microorganism with a view to improvingits biocontrol activity.

## 1. Characterization of genes involved in biocontrol

Several microbiological and biochemical studies have directed the efforts at biocontrol mechanisms (Elad, 2000; Ramamoorthy *et al.*, 2001, Spadaro and Gullino, 2004; Saveetha *et al.*, 2009). More recently, advancements in molecular biology-based methods have contributed to the development of innovative alternative means for getting to know the mechanisms of biocontrol agents and for building further on insights provided by microbiological and biochemical studies. From the genes involved in biocontrol properties, the genetic basis of mechanism of action can be understood. Of several strategies available, targeted gene sequence from a known data base, differential display techniques and whole genome sequence of an organism may provide better insights into mechanisms of action (Haggag and Mohamed, 2007). Besides these techniques, gene inactivation and over-expression studies through insertion mutagenesis, PCR Tilling, RNA silencing studies could also be employed to provide information on the transcription and regulation of these genes (Massart and Jijakli, 2007).

### 1.1 Targeted gene sequence

The targeted strategy requires a prior selection of one or few genes. This selection can be based either on pre-existing data or on an extrapolation of the existing model developed in a study of other biocontrol agents. Whatever be the selection process, the first step would be to design degenerate primers amplifying part of the gene sequence. The degenerate primers are selected according to the amino acid sequence of the studied protein, the sequence alignment of similar proteins from other microorganisms, or the primers used previously for other biocontrol agents. After PCR amplification with the degenerate primers, the amplified DNA fragment is isolated, cloned and sequenced. The obtained sequence is further used to identify the whole gene sequence, including cis- and trans-regions, by hybridization of the cloned fragment with a genomic DNA library or by a modified cRACE protocol using single strand extension. Yet, this strategy focuses only on one or a few genes where in biocontrol properties often depend on the regulation and the mutual interaction of numerous genes. The targeted strategy demonstrated to yield only a small percentage of the genes involved in the biocontrol properties. Thus, in some cases, the techniques like differential display is used to gain knowledge on the whole pattern of genes involved in biocontrol.

### 1.2 Differential expression studies

The genes involved in the biocontrol could be identified by assessing the differential gene expression under varied exposure conditions. The molecular techniques, cDNA amplified fragment length polymorphism analysis (cDNA-AFLP), differential display and subtractive hybridization are normally used for the differential expression studies. For example the assessment of gene expression by the biocontrol agents in the presence

and absence of pathogenic microorganisms may provide information on the over expression or repression of novel genes. Those genes can be isolated, cloned and sequenced for further characterization and property studies. Recently in our laboratory, two-dimensional polyacrylamide gel electrophoresis and mass spectrometry analyses were adopted to identify the plant growth promoting rhizobacteria responsive proteins in rice plants. The study revealed the differential expression of p23 co-chaperone,thioredoxin h-rice, ribulose-bisphosphate carboxylase large chain precursor, nucleotide diphosphate kinase, proteosome subunit protein and putative glutathione S-transferase proteins in rice leaves primed with *Pseudomonas fluorescens* strain KH-1. The functional analyses of those proteins reported to be directly or indirectly involved in growth promotion in plants (Saveetha *et al.*, 2009; Saveetha *et al.*, 2010). Nevertheless, the relevance of the genes identified by such methods with regards to their putative involvement in biocontrol properties depends heavily on the selected comparison model.

### 1.3 Genome sequencing

The whole genomic sequencing of several micro-organisms including *Pseudomonas*, *Bacillus* and *Trichoderma* opens up a new avenue to advance the knowledge on beneficial biocontrol agents through genomics approach. The complete genome sequencing of the biocontrol bacterium, *P. fluorescens* Pf-5 revealed that 6% of 7.07 Mb genome is devoted to the biosynthesis of secondary metabolites including antibiotics and siderophores. Of three orphan gene clusters, new group of bioactive cyclic lipopeptides (orfamide A) with a putative role in biological control of plant disease was identified, using a new 'genomisotopic approach', which employs a combination of genomic sequence analysis and isotope guided fractionation (Paulsen *et al.*, 2005). Yet, the various strategies so far developed to identify genes by the research workers have their own advantages and drawbacks. Hence, the selection of technique highly depends on the information to be derived.

## 2. Molecular approaches to improvement of biocontrol agents

As was indicated elsewhere in this review, the construction of superior biocontrol agents after isolation and characte ization of genes involved in biocontrol properties is necessary for the successful management of plant diseases. Moreover, molecular techniques allow modification of wild type strains to improve their ability to control diseases. The selected biocontrol agents could be improved by genetic modification and recombination techniques. In general, Protoplast fusion (Harman *et al.*, 1989; Minucci *et al.*, 1991), transposon mutagenesis (Brown and Holden, 1998) and transformation techniques are used for the genetic manipulation of biocontrol agents. Protoplasts are commonly used for insertion of exogenous DNA (Penttila *et al.*, 1987) while the whole fungal cells can be transformed using the lithium acetate method (Dickman, 1988) through electroporation (Goldman *et al.*, 1990), particle bombardment (Lorito *et al.*, 1993) and through *Agrobacterium*-mediated transformation (Zeilinger, 2004). Bacteria transformation can also be performed by using electroporation, osmotic shock or *Escherichia coli* mediated conjugation. Similarly, the genes encoding to the undesirable characters that reduce the efficacy of biocontrol agents could also be deleted or suppressed using molecular techniques like transposon mutagenesis.

### 2.1 Protoplast fusion

Protoplast fusion is a quick and easy method in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi (Lalithakumari and Mathivanan, 2003) and antagonistic bacteria (Abdel-Salam *et al.*, 2007). This technique is mainly used to combine the advantageous properties of distinct promising strains. Most frequently, the introduction of exogenous DNA through protoplasts fusion is done in presence of polyethylene glycol and calcium chloride (Penttila *et al.*, 1987). Isolation, fusion and regeneration of protoplasts have been carried out in *Trichoderma* strains mainly for improving the biocontrol potential (Mrinalini and Lalithakumari, 1998). Ogawa *et al.* (1989) reported the enhanced cellulase production in *T. reesei* by inter-specific protoplast fusion. Similarly, self-fusion of protoplasts from *T. harzianum* strain PTh18 showed two-fold increase in chitinase and biocontrol activity as compared to the parent strain against *Rhizoctonia solani* (Prabavathy *et al.*, 2006). Recent studies indicated the protoplast fusion between antagonistic bacterial strains. The protoplast fusion between two antibiotic producing *P. aeruginosa* and *P. fluorescens* strains demonstrated its improved efficacy against *F. oxysporum*. The superiority of the fusant in reducing the pathogen was attributed to the more toxic system that derived from both parental strains and more gene copies of an antagonistic organism (Abdel-Salam *et al.*, 2007).

### 2.2 Genetic recombination

Integrating foreign DNA into the genome of a biocontrol agent is a powerful way to improve the biocontrol activity. To a certain extent, this involves the

construction of strains that produce increased levels of lytic enzymes and antiobiotics (Glick and Bashan, 1997). On the other hand, the suppression or deletion of gene from the biocontrol strains could also enhance the sustained biocontrol activity in some of the strains. This was demonstrated in a modified strain of *Agrobacterium radiobacter* strain K84 that controlled crown gall disease caused by *Agrobacterium tumefaciens*. The antibiotic agrocin 84 that is produced by *A. radiobacter* is normally toxic to agrobacteria carrying a nopaline-agrocinopine A type Ti plasmid (McClure *et al.*, 1994). However, agrocin 84 resistant strains of the pathogen *A. tumefaciens* can develop if the plasmid carrying the genes for the biosynthesis of agrocin 84 is accidentally transferred from *A. radiobacter*. To avoid this possibility, the region of DNA responsible for plasmid transfer was removed from the agrocin 84 plasmid. Thus, a mutant of the biocontrol *A. radiobacter* strain was constructed which no longer can transfer the modified agrocin plasmid to pathogenic agrobacteria, thereby retaining the capacity to act as a biocontrol agent (Jones *et al.*, 1988). Similarly, Smith and Saddler (2001) reported that biocontrol activity of avirulent mutants of *Ralstonia solanacearum* against bacterial wilt in potato. These strains containing transposon induced insertions in the *hrp* gene were able to invade the plant, survive and multiply within the plant excluding pathogenic strains.

## 3. Tapping potential biocontrol genes

Since biocontrol properties are attributed to more than one character, it is imperative to study the genes involved in different mechanisms of action. An extensive work on biocontrol properties elucidated that i. competition, colonization and growth promotion ii. antibiosis, iii. lysis (mycoparasitism) and iv. induced systemic resistance (ISR) either individually or in combination determine the success of biological control of plant diseases. Thus an in depth study on mechanism of action may offer a pedestal for tapping potential genes for further improvement of biocontrol strains.

### 3.1 Colonization and competition

A biocontrol agent that can stimulate plant growth and inhibit the pathogens in the laboratory may not have significant impact on plants in the field conditions unless it is able to colonize, persist and grow in the natural environment. Thus, an effective colonization and enhanced competition by the biocontrol agents will have the greater role in reducing the disease incidence. This was successfully demonstrated with the introduction of *sss* colonization gene from *P. fluorescens* strain WCS365 into poor colonizers of *P. fluorescens*. This has increased the colonization ability of *P. fluorescens* strain against *Fusarium oxysporum* f.sp. *radicis-lycopersici* several times, showing that improvement of the colonization ability by genetic engineering is a realistic goal (Dekkers *et al.*, 2000).

Another strategy of genetic transformation with respect to the survival of biocontrol strains under field condition is an insertion of genes that improves resistance of strains to cold, heat, drought, high salinity, heavy metal rich soils or acid soils. Rainey (1999) has identified more than 20 *rhi* genes specifically expressed in the rhizosphere strains of *P. fluorescens* that are involved in nutrient acquisition and stress response. Similarly, the transfer of NAH7 plasmid, which carries the genes encoding the enzymes of the naphthalene and salicylate biodegradative pathway into an established biocontrol strain showed an enhanced survival capacity of the biocontrol bacterium (Colbert *et al.*, 1993) over host bacterium when salicylate was in the soil. So the presence of herbicide, pesticide or other organic pollutant in soil may facilitate the proliferation of bacteria engineered to degrade these compounds. Moreover, these chemicals may also suppress the proliferation of the other microorganisms in the same soil and possibly offers a significant competitive advantage. This strategy may the advantage that in addition to increasing the competitiveness of a biocontrol strain, it could be a useful strategy for the biodegradation of some recalcitrant organic molecules in the soil (Mohamed and Haggag Wafaa, 2006).

Similarly, it was reported that *P. putida* GR12-2 secretes antifreeze protein(s) into the surrounding medium when the bacterium is grown at low temperatures (Sun *et al.*, 1995). This protein may regulate the formation of ice crystals outside of the bacterium, thereby protecting it from damage that might otherwise occur at freezing temperatures. The addition of antifreeze protein synthesizing capability to biocontrol bacteria by genetic engineering may make a bacterium more effective by permitting it to thrive under adverse conditions. Further, Bacterial growth on the root surface would expose the cells to plant enzymes that produce superoxide anion ($O_2$) and hydrogen peroxide ($H_2O_2$). These active oxygen species have antimicrobial properties and biocontrol strains need to have mechanisms to counter their toxicity with suitable enzymes. In this context, to increase the levels of one or more of the enzymes that reduce the amount of active oxygen species by genetic manipulation of biocontrol strain could be a very good strategy to increase the root colonizing ability and thereby enhancing effectiveness of biocontrol against plant pathogens.

### 3.1.1 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

In general, ethylene accumulation is greater in plant tissues from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC) during biotic and abiotic stress conditions, which in turn retard root growth and cause senescence in crop plants (Sheehy *et al.*, 1991; Ma *et al.*, 2003). Conspicuously, the presence of biocontrol strains possessing ACC deaminase activity can cleave the plant ethylene precursor ACC and thereby lower the level of ethylene in a developing seedling or stressed plant (Glick *et al.*, 1997; Mayak *et al.* 2004a, b; Saravanakumar and Samiyappan, 2007). Thus, it is quite necessary to genetically engineer the bacterium with ACC deaminase gene *acdS* and their regulatory regions into the biocontrol bacterium that lacks this activity. This could enhance the beneficial effect of biocontrol bacterium on plant growth under different environment conditions. *Pseudomonas* strains that lack ACC deaminase but have been transformed to express a *Pseudomonas acdS* gene are able to promote the elongation of canola roots in growth pouches (Shah *et al.*, 1998). Similarly, the efficacy of biocontrol pseudomonads was also significantly enhanced following the introduction of a *Pseudomonas acdS* gene (Wang *et al.*, 2000). However, it is important that the complex transcriptional regulatory system that controls the expression of many *acdS* genes should work in all bacteria. When *Azospirillum* strains lacking ACC deaminase were transformed with a *Pseudomonas acdS* gene under the control of the regulatory *acdR* gene, ACC deaminase was not expressed (Holguin and Glick 2001). But, when the native regulatory region of the *Pseudomonas acdS* gene was replaced by either the *E. coli lac* promoter or the *tet* promoter, ACC deaminase was expressed at a high level and the growth promoting activity of the transformed *Azospirillum* strain was significantly improved (Holguin and Glick 2001). Similarly, *Sinorhizobium meliloti* strain transformed with *acdS* gene from *Rhizobium leguminosarum* enables the transformed bacterium to nodulate alfalfa plants and stimulates their growth by 35–40% more than the non-transformed strain (Ma *et al.*, 2004). These studies clearly indicated the possibility of improving the efficacy different biocontrol strains with the genetic engineering of ACC deaminase gene.

### 3.1.2 Siderophore

Siderophores are low molecular weight molecules that are secreted by microorganisms to take up iron from the environment and their modes of action in suppression of disease were thought to be solely based on competition for iron with the pathogen (Duijff *et al.*, 1999). The role of siderophore production by biocontrol agents in the rhizosphere has been studied with molecular methods for many years (Moenne Loccoz *et al.*, 1996). Since each siderophore is encoded by a number of different genes, genetically engineering bacteria to produce modified siderophores is not a simple matter. On the other hand, it is possible to improve biocontrol strains by extending the range of iron-siderophore complexes, so that a genetically altered biocontrol strain could take up and use siderophores synthesized by other soil microorganisms, thereby giving it a competitive advantage. Similarly, *P. fluorescens* strain was genetically modified to utilize additional ferric siderophores (Haas, 2003). This gives the scope for genetic modification of biocontrol agents utilizing siderophore related genes and receptors.

## 3.2 Antibiosis

Antibiosis plays an active role in the biocontrol of plant disease and it often acts in concert with competition and parasitism. Fluorescent pseudomonad bacterial strains are known to suppress fungal growth by the production of wide array of antibiotics which includes 2,4-diacetyl phloroglucinol, hydrogen cyanide, kanosamine, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, pyocyanin and viscosinamide as well as several other uncharacterized moieties. Antagonistic *Bacillus* strains produce kanosamine, zwittermycin A, iturin A, bacillomycin, plipastatins A and B compounds (Fernando *et al.*, 2005). In case of *Trichoderma*, the production of secondary metabolites is strain dependent and it includes pyrones, butenolides, volatile terpenes, gliotoxin and gliovirin (Reino *et al.*, 2008). The identification of these extracellular secondary metabolites involved in biocontrol offers the potential for cloning genes that encode their biosynthesis and using these genes to improve a given biocontrol agent. With respect to antibiosis, the biocontrol may be improved either by providing it with genes that encode the biosynthesis of antibiotics normally produced in other bacteria and fungi or by increasing the amount of antibiotic that the bacterium synthesizes through genetic recombination.

A superior biocontrol strain was designed by transferring the cloned antibiotic genes (12-kb fragment) from *P. fluorescens* 2-79 into strains of *P. putida* and *P. fluorescens* to produce high amount of phenazine-1-carboxylic acid (Bull *et al.* 1991). Similarly, Haas *et al.* (1990) isolated a cosmid (containing a 22-kb insert) from *P. fluorescens* CHAO which contained genes involved in the biosynthesis of pyoluteorin and phloroglucinol. Increasing the copy number of this fragment in strain CHA0 enhanced the production of pyoluteorin fourfold

and that of phloroglucinol two fold on agar medium and improved the ability of strain CHA0 to protect cucumber from *Pythium ultimum* in a gnotobiotic system. Vincent *et al.* (1991) isolated a cosmid from *P. aureofaciens* Q2-87 which contained genes involved in the biosynthesis of phloroglucinol. Mobilization of this cosmid into two heterologous *Pseudomonas* strains conferred the ability to synthesize Phl and increased their activity against *G. graminis* var. *tritici*, *P. ultimum* and *Rhizoctonia solani in vitro*. Similarly, the isolation and over expression of *tri5* (trichodiene synthase) gene in *T. brevicompactum* Tb41tri5 transformant increased the trichodermin production and antifungal activity against *Aspergillus fumigatus* and *Fusarium* spp. (Tijerino *et al.*, 2011). The greater efficacy of antibiotic engineered biocontrol strains raises the scope for designing effective biocontrol strains against various crop diseases.

### 3.3 Lysis

The production of extracellular cell wall degrading enzymes including chitinases, cellulases, ?-1,3-glucanases and proteases by fungal and bacterial antagonistics play an important role in the biological control of plant pathogenic fungi (Steyaert *et al.*, 2003). Since many of the lytic enzymes secreted by biocontrol agents are encoded by a single gene, it is considered to be a straightforward technique to isolate some of these genes and then transfer them to other biocontrol agents. In terms of antifungal activity, many bacteria including *Aeromonas, Bacillus, Pseudomonas, Serratia marcescens* and *Streptomyces* have been shown to produce multiple chitinases (Lee *et al.*, 2002; Duzhak *et al.*, 2002).

More specifically, de la Cruz *et al.* (1992) cloned *chit33* chitinase gene from *S. marcescens* and Garcia *et al.* (1994) cloned *ech42* chitinase gene from *T. harzianum*. Chitinase gene isolated from the bacterium *S. marcescens* and transferred into *T. harzianum* and *Rhizobium meliloti* cells expressed the chitinase and subsequently displayed increased antifungal activity (Chet and Inbar, 1994). When the *S. marcescens* chitinase gene was introduced into a *P. fluorescens* strain, the transformant stably expressed and secreted active chitinase and showed higher efficacy against the pathogen *R. solani* (Koby *et al.*, 1994). Similarly, *T. virens* containing two *ech42* copies showed an increased antagonistic activity against *R. solani* on cotton (Baek *et al.*, 1999). With constitutive expression of *chit33* in *T. harzianum*, the recombinant strains provided a superior biocontrol activity against *R. solani* on agar plates (Limon *et al.*, 1999). On contrast, no difference was observed between biocontrol activity of over expression and deletion *T. atroviride chit42* mutants and wild type (Carsolio *et al.*, 1999). The ability of *chit42* deletion mutants to offer the same level of protection suggested some redundancy in endochitinase activity. Flores *et al.* (1997) demonstrated a five fold increase in biocontrol activity of *T. atroviride* transformant against *R. solani* over the wild type by integrating multiple copies of the prb1 (proteinase) gene. A review by Steyaert *et al.* (2003) discussed in detail regarding the mycoparasitism related genes from *Trichoderma* species and their involvement in biological control. In addition, the review clearly provides greater information on genes that encodes lytic enzymes for genetic manipulation. The isolation of three endochitinase encoding genes, *cr-ech58, cr-ech42* and *cr-ech37* from biocontrol fungus *Clonostachys rosea* strain IK726 offers potential lytic genes for cloning and genetic transformation of other biocontrol agents (Mamarabadi *et al.*, 2008).

Similarly, an endophytic bacterial strain, *P. fluorescens* transformed with the *chiA* gene encoding the major chitinase of the *S. marcescens* showed an effective control of *R. solani* on bean seedlings under plant growth chamber conditions (Downing and Thomson, 2000). The *P. fluorescens* P5 proved to be an effective biocontrol agent of various soil-borne plant diseases was introduced with a *chiB* chitinase with a size of 6.5 kb and its efficacy was compared with wild-type P5 in pot experiments. The transformant had an increased effect against rice sheath blight and cotton damping-off caused by *R. solani*. It also increased the effect on suppression of wheat take-all caused by *Gaeumannomyces graminis* var. *tritici* (Xiao-Jing *et al.*, 2005).

Besides genetic recombination, random mutagenesis have also induced higher chitinase, ß-1,3-glucanase, and ß-1,6-glucanase and antibiotic activity in *T. harzianum* mutant than the wild type (Rey *et al.*, 2001). Though the increase in lytic enzyme activity was low in comparison to the transformants, the mutant afforded increased antifungal activity towards *R. solani* and disease protection from *Botrytis cinerea*. These studies have also demonstrated that the small increases in lytic enzymes such as chitinase, ß-1,3-glucanase, and ß-1,6-glucanase may be more conducive than large increases of single enzymes in improving biocontrol potential (Rey *et al.*, 2001). Thus, comparing the colonization and growth promoting genes, the information on genes encoding for lytic enzymes and antibiosis play a significant role in improvement of biocontrol strains.

### 3.4 Induced resistance

Induced resistance is associated with plant's defensive capacity against a broad spectrum of pathogens that is

acquired after appropriate stimulation. Since most of the plant growth promoting biocontrol bacteria, *Pseudomonas* and biocontrol fungi, *Trichoderma* appear to switch on the synthesis of some anti-pathogen metabolites including ?-1,3-glucanases, chitinases, thaumatin-related proteins and some pathogenesis-related proteins (Tuzun, 2001; Harman *et al.*, 2004), a better understanding of the metabolic signals that activate the synthesis of these proteins may allow for the construction of biocontrol strains that act more rapidly and more efficiently in eliciting induced resistance. The construction of *T. atroviride* SJ3-4 strain with glucose oxidase encoding gene (goxA) from *Aspergillus niger* under a homologous chitinase (nag1) promoter has significantly induced systemic resistance in plants (Bruner *et al.*, 2005). The transgenic strain has the advantage that $H_2O_2$ accumulation occurs only in the presence of the pathogen, due to the host-induced regulation of the *nag1* promoter. This could eliminate or at least strongly reduces the accumulation of $H_2O_2$ in the absence of a pathogen attack. Furthermore, the induction of systemic resistance in plants might occur before the pathogen attacks the plant roots, since the *nag1* promoter could trigger glucose oxidase expression as soon as the *Trichoderma* hyphae contact the pathogen. Similar construction of biocontrol strains for the enhanced induction of disease resistance in crop plants may provide better biocontrol strategies. Further, the genetically modified strains possessing different biocontrol properties so far constructed also raise the scope for designing newer biocontrol agents (Table 1).

## 4. Use of regulators for expression of biocontrol genes

One of the most important factors for successful transgenesis of biocontrol strains is the availability of suitable biocontrol related promoters to drive the expression of exogenous or endogenous novel transgenes. Margolles-Clark et al. (1996) used a transformation construct that contained the coding region of the 42 kDa endochitinase gene (*ThEn-42*) of *T. harzianum* under the control of the cellulase promoter, *cbh1* from *T. reesei*. A ten-fold increase of the chitinase activity was measured in transformants. Yet, the behavior of these transformant under natural conditions is difficult to predict, as their increased chitinase production was elicited by cellulose inducing components (owing to the *chb1* promoter) and no specific induction occurred in the presence of chitin, the real substrate of the overproduced enzyme. On the other hand, genes encoding 42 kDa endo-chitinase cloned by screening the genomic library of *T. hamatum* strain Tam-61 with a PCR amplified chitinase sequence from the same fungus (Giczey *et al.*, 1998). A 3.5 kb genomic DNA fragment containing the coding region, as well as the 5' and 3' regulatory sequences was reintroduced into the host strain by PEG-mediated homologous transformation under selection pressure provided by hygromycin B. Duplicating the copy number of the entire endo-chitinase gene under its own regulatory sequences increased five-fold higher chitinase activity. This is suitable for improving the biocontrol capability of *Trichoderma* as the highly conserved 42-kDa endochitinase encoding gene present in all mycophagous species of *Trichoderma* (Fekete *et al.*, 1996) and specifically triggered in mycoparasitic interactions (Carsolio *et al.*, 1994). Triggering occurs when a specific "mycoparasitic" protein complex binds to the promoter sequences of the gene and displaces the binding of a catabolite repressor protein (Lorito *et al.*, 1996).

Similarly, the transgenic strain *T. atroviride* SJ3-4 constructed with glucose oxidase-encoding gene (goxA) from *Aspergillus niger* under a homologous chitinase (nag1) promoter had increased capabilities as a fungal biocontrol agent against *R. solani*, *B. cinerea* and *P. ultimum*. goxA expression occurred immediately after contact with the plant pathogen and the glucose oxidase formed was secreted. The transgenic strain also more quickly overgrew and lysed the plant pathogens, *R. solani* and *P. ultimum*. This clearly demonstrates that heterologous genes driven by pathogen inducible promoters can increase the biocontrol and systemic resistance inducing properties of biocontrol agents. Further, these microbes could be used as vectors to provide plants with useful molecules such as glucose oxidase that can increase their resistance to pathogens (Brunner *et al.*, 2005).

A genetically modified rice-indigenous epiphytic bacterium *Erwinia ananas* NR1 that colonizes rice leaves but does not produce antifungal factors such as lytic enzymes or antibiotics was developed by introducing genes encoding for antifungal factors (58-kDa endochitinase ChiA) isolated and cloned from *Serratia marcescens* strain B2. The genes were put under the control of several types of promoters, which were isolated from the recipient i.e. *E. ananas* NR1. These genetically modified micro-organisms effectively suppressed rice blast disease and are not affected by abiotic or biotic factors. Therefore an introduction of disease inhibitory genes controlled by promoters and derived from the recipient offers a novel technology for the development of new biocontrol agents (Someya and Akutsu, 2007).

In case of biocontrol pseudomonads, the production of various anti-fungal metabolites is regulated by a global regulator (Laville *et al.*, 1992). Hence, it is possible to enhance the antibiotic production of a biocontrol bacterium by modifying this global regulation. In fact, it was reported

**Table 1. Genetically modified strains with biocontrol properties against various plant pathogens**

| Source organism (gene) | Transformant strain | Crop / Pathogenic organism | Remarks | Reference |
|---|---|---|---|---|
| *P. fluorescens WCS378 (sss)* | *P. fluorescens* WCS365 | Tomato/*F. oxysporum* f.sp. *radicis-lycopersici* | Improved colonization | Dekkers *et al.*, 2000 |
| *Enterobacterium cloacae* UW4 (*acdS)* | *P. fluorescens* | Canola seedlings | Enhanced growth promotion | Shah *et al.*, 1998 |
| *E. cloacae* UW4 (*acdS)* | *P. fluorescens* strain CHA0 | Cucumber / *Pythium ultimum* Potato / *Erwinia carotovora* | Improved phytopathgen control | Wang *et al.*, 2000 |
| *E. cloacae* UW4 (*acdS)* | *Azospirillum brasilense* | Pea | Enhanced plant growth promotion | Holguin & Glick 2001 |
| *Rhizobium leguminosarum* (*acdS)* | *Sinorhizobium meliloti* | Alfalfa plants | Stimulates plant growth | Ma *et al.*, 2003 & Ma *et al.*, 2004 |
| *P. fluorescens* 2-79 (Phenazine-1-carboxylic acid ) | *P. putida* & *P. fluorescens* | Wheat / *Gaeumannomyces graminis* var. *tritici* | Improved antibiosis | Bull *et al.*, 1991 |
| *P. fluorescens* CHAO (22 kb cosmid containing pyoluteorin & phl) | *P. fluorescens* CHA0-1 antibiosis | Cucumber / *Pythium ultimum* | Increased synthesis of pyoluteorin and phloroglucinol | Haas *et al.*, 1990 |
| *Pseudomonas* strain (*phl*) | *Pseudomonas aureofaciens* Q2-87 | Wheat/*G. graminis* var. *tritici*, *Pythium ultimum, Rhizoctonia solani* | Increased phloroglucinol activity | Vincent *et al.*, 1991 |
| *Trichoderma brevicompactum* (*tri5* -trichodiene synthase gene) | *Trichoderma brevi-compactum* Tb41tri5 | *Aspergillus fumigatus* *Fusarium* spp. | Overexpression of *tri5* gene Enhanced trichodermin activity | Tijerino *et al.*, 2011 |
| *Serratia marcescens* B2 (endochitinase *ChiA*) | *Erwinia ananas* NR1 | Rice / *Pyricularia oryzae* | Improved lytic activity | Someya & Akutsu, 2007 |
| *S. marcescens* (*chit33* chitinase gene) | *T. harzianum* | Cotton / *R. solani* | Improved lytic activity | Limon *et al.*, 1999 |
| *T. harzianum* (*ech42* chitinase gene) | *T. virens* | *R. solani in vitro* | Improved lytic activity | Baek *et al.*, 1999 |
| *S. marcescens* (*chiA* chitinase gene) | *P. fluorescens* | Bean / *R. solani* | Improved control | Downing & Thomson, 2000 |
| *P. fluorescens* P5 (*chiB* chitinase) | *P. fluorescens* P5-1 | Wheat /*Gaeumannomyces graminis* var. *tritici*Rice / *R. solani*Cotton / *R. solani* | Enhanced biocontrol activity against soil borne pathogens | Xiao-Jing *et al.*, 2005 |
| *T. atroviride* (*prb1* proteinase gene) | *T. atroviride* strain with multiple copies of prb1 | *R. solani* | Enhanced lysis of pathogen | Flores *et al.*, 1997 |
| *Aspergillus niger* (*goxA*) | *T. atroviride* SJ3-4 | Bean / *R. solani, Pythium ultimum, Botrytis cinerea* | Glucose gene insertion enhances the ISR activity in bean plants | Brunner *et al.*, 2005 |

that amplification of the gene from *P. fluorescens* CHAO encoding the housekeeping sigma factor ~70 enhanced the antibiotic production and improved protection against *P. ultimum* induced damping-off of cucumber (Schnider *et al.*, 1995). Similarly, another strain of *P. fluorescens*, antagonist of *R. solani* has been genetically modified for increased production of the antibiotic pyrrolnitrin (Ligon *et al.*, 1996) by modifying or introducing an extra copy of the wild-type global regulator gene gacA. Interestingly, the GacS/GacA two-component system globally exerts a positive effect at a posttranscriptional level on the production of extracellular metabolites required for the control of plant diseases in many pseudomonads (Haas and Keel, 2003).

Further, some of the specific transcriptional activators and repressors could also regulate the transcription of secondary metabolite biosynthetic genes. The PhlF protein, which is expressed from the phloroglucinol (Phl) locus, represses transcription of the PhlA-D operon, which comprises genes encoding proteins that direct the synthesis of phloroglucinol (Delany *et al.*, 2000; Schnider *et al.*, 2000). Mutation of phlF in a *P. fluorescens* strain increased phloroglucinol production *in vitro* during the early logarithmic phase of growth. Similarly, overexpression of phlA-D resulted in phloroglucinol overproduction and concomitantly, enhanced biocontrol efficacy against *P. ultimum* in laboratory microcosm trials (Delany *et al.*, 2001).

## 5. Biocontrol genes for plant transformation

Genetic modification of plant species with genes from beneficial microorganisms encoding for biocontrol properties and disease resistance represents a novel approach to disease control. Incorporation of endochitinase (*ech42*) of *T. harzianum* and *T. atroviride* has been demonstrated to dramatically improve disease resistance of potato and tobacco to *Alternaria alternata*, *A. solani*, *B. cinerea* and *R. solani*. Co-transformation of apple with *nag70* (nag1) and *ech42* resulted in synergistic increase in biocontrol activity against *Venturia inequalis* (Bolar *et al.*, 2001). Further, genes from biocontrol fungi encoding endochitinases or exochitinases were also inserted into broccoli (Mora and Earle, 2001) or *Brassica juncea* (Mondal *et al.*, 2003). Similar to the manipulation of anti-fungal encoding genes in different plants, several transgenic plants including tomato, canola, and tobacco that express ACC deaminase have been engineered. These transgenic plants have been reported to be tolerant of metals (Stearns *et al.*, 2005), high salt (Sergeeva *et al.*, 2006), *Fusarium* wilt pathogen (Robison *et al.*, 2001) and flooding (Grichko and Glick, 2001). Recently, transformation of hsp 70 gene from *T. harzianum* showed thermo tolerance as well cross-tolerance to osmotic, salt and oxidative stresses in *Trichoderma* (Montero-Barrientos *et al.*, 2008) and tolerance to heat in *Arabidopsis* (Montero-Barrientos *et al.*, 2010). It is also opined that the genes isolated from various biocontrol agents could be a potential candidates for the development of transgenic plants with multiple attributes (gene pyramiding). These again depending on regulation of gene expression, components involved in regulation of gene expression, regulation at initiation of transcription, regulation at the level of transcription, post-transcriptional control, factors affecting the expression of a gene.

## 6. Use of microbial consortia for enhanced biocontrol activity

Apart from genetic manipulation of biocontrol strains to improve their activity, the knowledge on mechanism of action by biocontrol strains offers greater insights for combining various modes of action. For plant beneficial pseudomonads, strain mixtures and combinations with other bacteria or fungi often provided more effective disease control than the application of an individual biocontrol pseudomonad alone (de Boer *et al.*, 2003). Another approach to obtain a successful microbial biocontrol consortium is to apply mixtures of biocontrol agents which display different disease-suppressive mechanisms that are complementary to each other. Cocktails of various *Pseudomonas* strains provided enhanced protection than a single organism (Saravana-kumar *et al.*, 2009; Karthiba *et al.*, 2010). Mixtures of plant growth promoting strains significantly reduced the severity of diseases compared to the single strain in tomato, pepper, cucumber and rice (Jetiyanon and Kloepper, 2002; Saravanakumar *et al.*, 2008). de Boer *et al.* (2003) combined *Pseudomonas* strains effective in siderophore mediated competition for iron and induction of systemic resistance to improve the control of *Fusarium* wilt in radish. Dunne *et al.* (1998) applied a mixture of the DAPG producer *P. fluorescens* F113 and a proteolytic rhizobacterium to enhance suppression of *Pythium* damping off in sugar beet. When a biopesticide possessing *P. fluorescens* strain TDK1 (ACC deaminase producer), Pf1 (DAPG secretor) and PY15 (phenazine producer) applied on rice plants, the study demonstrated the greater reduction of sheath rot incidence under varied field conditions through effective colonization, antagonistic activity and ISR (Saravanakumar *et al.*, 2009).

## 7. Conclusion and future directions

In conclusion, there is no skeptic that genetic manipulation using molecular updates could result in new

biocontrol strains with increased production of toxic compounds, lytic enzymes, improved competence and wider host range through enhanced tolerance to biotic and abiotic stresses. Yet, the genetic manipulation of biocontrol agents is in the infancy stage. However, the growing knowledge and understanding of the molecular mechanisms as well as the increased interest by biotechnology companies assure the future development and commercialization of genetically modified biocontrol agents. In this regard, the use of genetically modified microorganisms is likely to play a significant role in near future. At the same time, the monitoring of genetically modified organisms in the environmental conditions assumes greater attention throughout the globe. The growing knowledge on biotechnology and available molecular markers provide gigantic sources of data that can assist researchers in developing tools to monitor the genetic and environmental fate of the genetically modified biocontrol agents. However, development of appropriate molecular technique should be based on the specific characteristics of the organisms and on the desired type of information necessary to evaluate à particular step in the developmental process of a biofungicides. Thus, there is no doubt that the identification and the biological and molecular characterization of microorganisms, useful as biocontrol agents or as producers of bioactive compounds, are of great relevance for the modern and eco-compatible agriculture.

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