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Use of molecular techniques to elucidate the mechanisms of action of fungal biocontrol agents: A review

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Abstract

Biological control of fungal plant pathogens appears as an attractive and realistic approach, and numerous microorganisms have been identified as biocontrol agents. There have been many efforts to understand the mechanisms of action of fungal biocontrol agents. Microbiological, microscopic, and biochemical techniques applied over many years have shed light on these mechanisms without fully demonstrating them. More recently, the development of molecular techniques has yielded innovative alternative tools for understanding and demonstrating the mechanisms underlying biocontrol properties. To date, more than 70 publications describe the use of molecular techniques for this purpose. They describe work exploiting targeted or non-targeted gene isolation, gene expression profiling, gene inactivation and/or overexpression, the study of regulatory factors. This work has shed considerable light on mechanisms underlying biocontrol properties. It has also fully demonstrated a number of targeted action mechanisms of some biocontrol agents. This review describes the techniques used in such studies, with their potential and limitations. It should provide a guide for researchers wanting to study the molecular basis of the biocontrol in diverse biocontrol agents.

Keywords: Biocontrol; Biological control; Fungus; Molecular techniques; *Trichoderma*; Yeast

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1. Introduction

The biocontrol of fungal plant pathogens with microorganisms has been studied for more than 70 years (Howell, 2003) and is becoming a realistic alternative to chemical treatments (Punja and Utkhede, 2003). Although such treatments are still widely used to control diseases caused by plant pathogens, the emergence of fungicide-resistant strains, the deregistration of fungicides, and public concerns regarding the health and environmental impacts of agrochemicals may limit their future application. This should increase the need to develop alternative

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plant protection strategies such as biological control. Already, numerous strains of yeast and mold species have been isolated for their ability to antagonize plant pathogens. Furthermore, several biological control products, based on fungi or yeast, are commercially available (Punja and Utkhede, 2003).

Understanding how biocontrol agents exert their protective effects is a prerequisite to their effective practical application. This knowledge will allow their suitable selection, production, formulation, and use and will facilitate registration procedures. The action mechanisms of numerous biocontrol agents have been studied but not fully elucidated. Various mechanisms, based mainly on competition for space and nutrients, mycoparasitism, antibiosis, or elicitation of plant defenses, are reported to contribute simultaneously or sequentially to the biocontrol properties of microorganisms (Janisiewicz and Korsten, 2002). This mechanistic complexity is mirrored by pathosystem complexity: there exist numerous interactions between plant, microflora, pathogen, and biocontrol agent, all under the influence of the microenvironment. Unraveling biocontrol mechanisms is therefore difficult.

Microbiological, microscopic, and/or biochemical studies have focused on biocontrol mechanisms for many years (Elad, 1996, 2000; Spadaro and Gullino, 2004). More recently, advanced molecular techniques have contributed to the development of innovative alternative tools for improving knowledge on the antagonistic mechanisms of biocontrol agents and for building on insights provided by microbiological, microscopic, and/or biochemical studies. Thanks to the identification of genes involved in biocontrol properties, the genetic basis of action mechanisms can be understood. Gene inactivation and overexpression studies can provide information on the transcription and regulation of these genes. The advantages of molecular techniques have made them very popular in the last decade, as shown by the publication of over 70 papers describing their use in elucidating the biocontrol properties of fungal biocontrol agents.

This review summarizes the molecular techniques that have been used to understand the action mechanisms of fungal biocontrol agents. It discusses their potential and limitations in grasping the complexity of these mechanisms.

2. Gene isolation and sequencing

Gene isolation and sequencing are the first steps in gene characterization (Fig. 1).The literature describes two different gene identification strategies: targeted and open. The targeted strategy focuses on the specific identification of one or a few targeted genes. The open strategy involves identifying a larger number of genes by means of differential expression techniques, large-scale sequencing, or random mutation, without any prior knowledge of the genes that will emerge or their functions.

Table 1 lists the genes identified to date as being potentially involved in biocontrol. The first molecular studies aiming to shed light on biocontrol agent action mechanisms focused on genes coding for enzymes having a direct effect on a plant pathogen (glucanases, proteases, chitinases...). Such genes still represent the majority of identified genes. In a second phase, investigators focused on genes involved in regulating or addressing the above-mentioned enzymes. This work has highlighted genes coding for the G-protein α-subunit, for mitogen activated protein kinases, or for other cellular transporters. A recent paper (Woo et al., 2006) reports the identification in *Trichoderma harzianum* and *Trichoderma atroviride* of avirulence genes (*Avr*) related to the induction of plant resistance.

The abundance of genes presumably involved in mycoparasitism in *T. atroviride* (67 genes) and *T. virens* (21 genes)

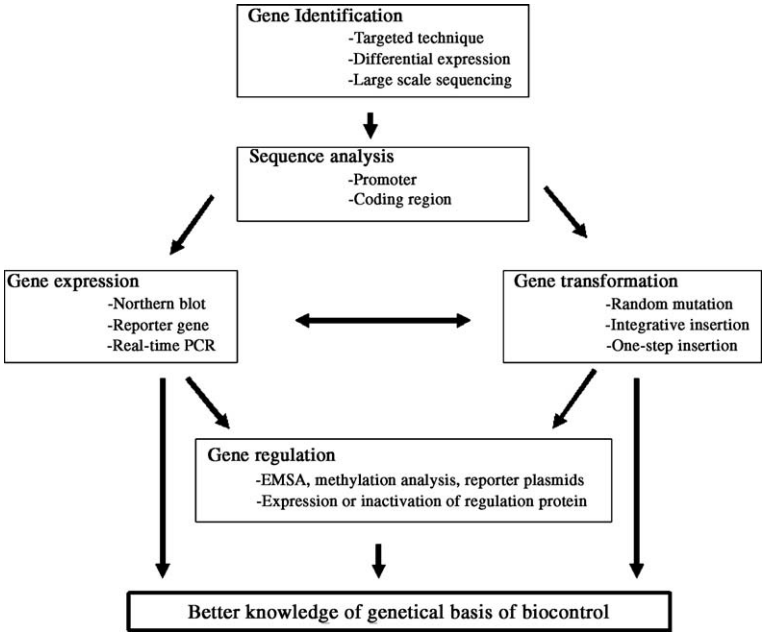

Fig. 1. Step-by-step schema to study the molecular basis of the biocontrol properties of fungal biocontrol agents.

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Table 1
Genes identified in biocontrol agents for their putative involvement in the biocontrol properties

Species	Genes	Authors
Acremonium ophalosporium	1 chitinase	Giczey et al. (NCBI, http://www.ncbi.nlm.nih.gov/)
	1 α-*N*-acetylgalactosaminidase	Ashida et al., 2000
Ampelomyces quisqualis	1 chitinase class 1	Weiss et al., 1996
	1 exo-β-1,3-glucanase	Rotem et al. (NCBI, http://www.ncbi.nlm.nih.gov/)
Candida oleophila	1 exo-β-1,3-glucanase	Segal et al., 2002
Coniothyrium minitans	1 chitinase	Lu et al. (NCBI, http://www.ncbi.nlm.nih.gov/)
	1 exo-β-1,3-glucanase	Giczey et al., 2001
Pichia anomala	2 exo-β-1,3-glucanases	Grevesse et al., 2003
	11 cDNA[a]	Massart and Jijakli, 2006
Physisporinus sanguinolentus	1 exo-β-1,3-glucanase	Rotem and Yarden, 1999
	5 cDNA[b]	Iakovlev et al., 2004
Stachybotrys elegans	1 endochitinase	Morissette et al., 2003
Talaromyces flavus	1 glucose oxidase	Murray et al., 1997
Trichoderma asperellum	2 aspartyl proteases	Viterbo et al., 2004
T. virens	8 endochitinases	Baek et al., 1999 Carsolio et al., 1994 Garcia et al., 1994 Kim et al., 2002 Steyaert et al., 2003
	1 serine protease	Pozo et al., 2004
	2 exochitinases	Kim et al., 2002
	2 *N*-acetylglucosaminidases	Kim et al., 2002
	2 exo-β-1,3-glucanases	Kim et al., 2002
	1 exo-β-1,6-glucanase	Kim et al., 2002
	1 peptaibol synthase	Wiest et al., 2002
	1 peptide synthase	Wilhite et al., 2001
	2 G-protein alpha subunits	Mukherjee et al., 2004
	1 MAPK kinase	Mukherjee et al., 2003 Mendoza-Mendoza et al., 2003
T. hamatum	1 exo-β-1,3-glucanase	Steyaert et al., 2003
	1 endochitinase	Steyaert et al., 2003
	19 cDNA[d]	Carpenter et al., 2005
T. harzianum/ *T. atroviride*	*N*-acetylglucosaminidases	Peterbauer et al., 1996
	4 exo-β-1,3-glucanases	De la Cruz et al., 1995 Donzelli et al., 2001 Cohen-Kupiec et al., 1999 Liu and Yang, 2005
	3 proteases	Geremia et al., 1993 Delgado-Jarana et al., 2002 Liu and Yang, 2005
	1 endochitinase	Dragborg et al., 1996
	2 exochitinases	Dragborg et al., 1995
	1 exo-α-1,3-glucanases	Ait-Lahsen et al., 2001
	1 décarboxylase	Heidenreich and Kubicek, 1994
	2 G-protein alpha subunits	Zeilinger et al., 2005 Rocha-Ramirez et al., 2002
	53 cDNA[c]	Liu and Yang, 2005
	1 di/tri-peptide transporter	Vizcaino et al., 2006
	1 permease	Vasseur et al., 1995
	1 oxydo-reductase	Vasseur et al., 1995
	1 endo-β-1,6-glucanases	Steyaert et al., 2003
	1 glucose transporter	Delgado-Jarana et al., 2003
Verticillium lecanii	1 chitinase	Lu et al. (NCBI, http://www.ncbi.nlm.nih.gov/)

illustrates the complexity of the genetic basis of biocontrol properties. Table 1 also shows that several distinct genes can code for enzymes having the same function. As an example of this redundancy, Kim et al. (2002) have identified three distinct 42-kD endochitinase genes in *T. virens*. So far, few genes have been identified in biocontrol agents belonging to genera other than *Trichoderma*. For example, only 14 whole or partial genes potentially involved in biocontrol has been isolated and sequenced from fungal biocontrol agents of postharvest fruit diseases (Grevesse, 2003; Grevesse et al., 2003; Massart and Jijakli, 2006; Yehuda et al., 2003).

2.1. Targeted strategy

The targeted strategy requires prior selection of one or a few genes. This selection can be based either on pre-existing data or on extrapolation of an existing model developed in a study of (an)other biocontrol agent(s). Whatever the selection process, the first step is 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 micro-organisms, 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 (Grevesse et al., 2003) or by a modified cRACE protocol using single-strand extension (Donzelli et al., 2001).

In the literature several authors report having designed primers according to the amino acid sequence of a protein potentially involved in the biocontrol properties of a biocontrol agent. This strategy was used to isolate the genes coding for various *T. harzianum* endo-β-1,3-glucanases (Cohen-Kupiec et al., 1999; De la Cruz et al., 1995; Donzelli et al., 2001), a *T. harzianum* protease (Geremia et al., 1993), a *T. harzianum* exo-α-1,3-glucanase (Ait-Lahsen et al., 2001), and a *T. atroviride* laminarinase (Nobe et al., 2003, 2004).

Having detected exoglucanase activity in culture filtrates, Grevesse et al. (2003) and Segal et al., (2002) selected degenerate primers in conserved regions identified by multiple amino acid alignment of a number of known exoglucanase genes. They successfully used these primers to amplify exo-β-1,3-glucanase genes of post-harvest biocontrol agents: a *Pichia anomala* gene (Grevesse et al., 2003) and a *Candida oleophila* gene (Segal et al., 2002).

Extrapolation of a model studied in other species or strains was used to isolate a *T. harzianum* decarboxylase gene

[a] 11 cDNA fragments potentially involved in the mycoparasitic response against *B. cinerea*.
[b] 5 genes involved in stress response or in the initiation of secondary metabolism.
[c] Results obtained from the sequencing of 3298 cloned cDNA.
[d] 19 genes overexpressed by *T. hamatum* during *in vitro* confrontation assay with *Sclerotinia sclerotiorum*.
(NCBI): sequences published on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

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(Heidenreich and Kubicek, 1994), a *T. virens* mitogen activated protein kinase gene, and two *T. virens* genes encoding G-protein α-subunits (Mukherjee et al., 2003, 2004), an *Ampelomyces quisqualis* class I chitin synthase gene (Weiss et al., 1996), a *T. harzianum* endochitinase gene (Carsolio et al., 1994), a *T. virens* serine protease gene (Pozo et al., 2004), and a *T. hamatum* chitinase gene (Steyaert et al., 2003).

Peterbauer et al. (1996) used an original but technically challenging approach to isolate a gene coding for an *N*-acetyl-β-D-glucosaminidase (*nag*1). The enzyme purified to homogeneity was used to raise antibodies in mice. A cDNA expression library was constructed in the λgt11 vector. After plating and cell lysis, the membrane was transferred to filters and screened with anti-Nag1 serum. A bacterial colony was selected and the corresponding cloned cDNA fragment sequenced.

The targeted strategy serves to isolate one or a few genes. It frequently relies on knowledge derived from biochemical or microbiological studies. Such knowledge makes it possible to design an experiment rationally in order to target relevant genes. Yet this strategy focuses on only one or a few genes, whereas biocontrol properties often depend on the regulation and the mutual interaction of numerous genes. Hence, the targeted strategy yields only a small percentage of the genes involved in the biocontrol properties.

2.2. Open strategy

A first important 'open' approach is the use of differential gene expression techniques. Such techniques have been used in studies designed mainly to identify genes involved in biocontrol. They notably include cDNA Amplified Fragment Length Polymorphism Analysis (cDNA-AFLP), differential display, and subtractive hybridization. Some of these techniques are illustrated in Fig. 2.

Massart and Jijakli (2006) used cDNA-AFLP to identify genes potentially involved in the biological control of *Botrytis cinerea* by *Pichia anomala* strain Kh5. They grew strain Kh5 in a medium containing either glucose or *B. cinerea* cell walls. They were thus able to identify eleven cDNA fragments corresponding to genes overexpressed in the presence of *B. cinerea* cell walls and putatively involved in enzyme secretion, the stress response, sensing or transmission of environmental signals, or energy production. Vasseur et al. (1995) used subtractive hybridization to compare gene expression in *T. harzianum* growing in a medium containing either glucose or *R. solani* cell wall as the sole carbon source. They isolated a permease gene (*inda*1) and a gene (*indc*11) showing similarity to an oxidoreductase. Viterbo et al. (2004) studied the modulation of *T. asperellum* gene expression during plant root colonization. Using the differential display technique to compare the gene expression profiles of *T. asperellum* grown in hydroponic solution with or without cucumber seedlings, they identified an aspartyl protease that may play a role in mycoparasitism and plant symbiosis. Iakovlev et al. (2004) also used differential display to identify 5 genes differentially expressed by the biocontrol agent *Physisporinus sanguinolentus* according to whether it was cultured alone or cocultured with the plant pathogen *Heterobasidion annosum*.

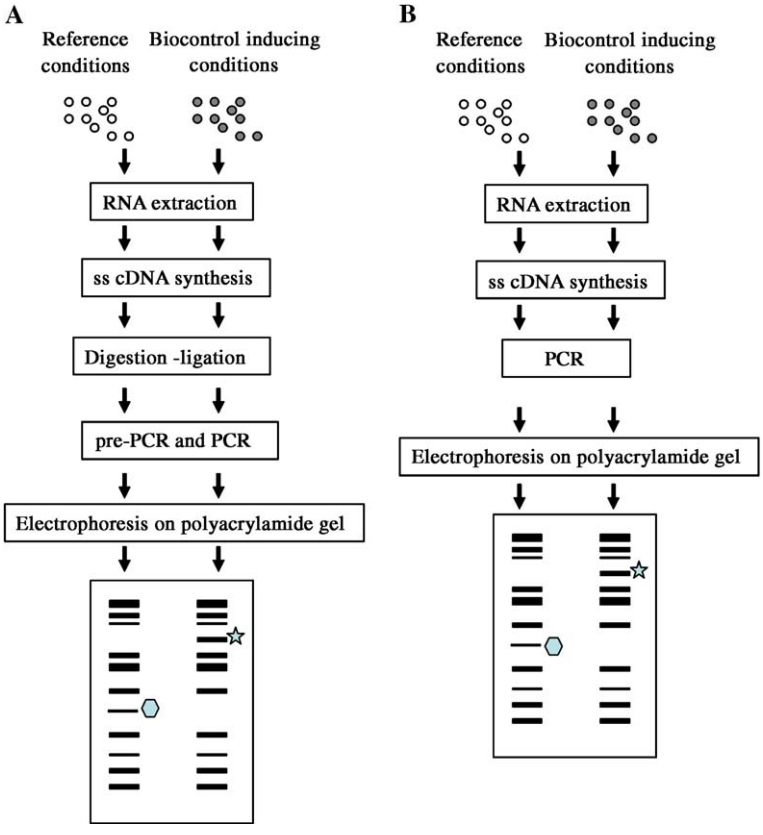

Fig. 2. Differential gene expression techniques to isolate biocontrol-related genes. cDNA-AFLP (A) and differential display (B) protocols. ○ and ●: fungal cells. ☆: mRNA overexpressed in biocontrol inducing conditions. ⬡: mRNA repressed under biocontrol inducing conditions.

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They isolated five genes involved in the stress response, secondary metabolism, or cytoskeleton construction. More recently, Carpenter et al. (2005) identified 19 genes over-expressed by *T. hamatum* in *in vitro* confrontation assays with *Sclerotinia sclerotiorum*. These genes show homology to three monooxygenases, a metalloendopeptidase, a gluconate dehydrogenase, an endonuclease, or a proton ATPase or share no similarity with known proteins.

Numerous other differential techniques have been developed. Their respective advantages and drawbacks are reviewed elsewhere (Donson et al., 2002; Lorkowski and Cullen, 2004). It should be stressed that the relevance of the genes identified by such methods with regards to their putative involvement in biocontrol depends heavily on the selected comparison model. The comparison model can be designed on the basis of previous knowledge on the strain's action mechanism or without any *a priori*. In the latter case the risk of isolating genes that are not involved in biocontrol is greater.

Large-scale sequencing techniques, such as Expressed Sequence Tag sequencing, constitute a second group of open' techniques. They focus on generating abundant genetic information on a strain by identifying thousands of genes, most of them unrelated to the strain's biocontrol properties. This approach does not specifically target genes involved in biocontrol, so a drastic selection has to be made among the identified genes, as the downstream characterization process can only deal with a few genes. For example, Liu and Yang (2005) sequenced 3298 *T. harzianum* cDNAs by the Expressed Sequence Tag technique. They identified 55 cDNAs displaying similarity to proteins involved in biocontrol and classified them according to their potential action mechanisms: antifungal action (23 cDNAs), mycoparasitism (22 cDNAs), competition for space and nutrients (5 cDNAs), and pathogen protease inactivation (5 cDNA).

In conclusion, the various strategies developed to identify genes have their advantages and drawbacks. Which technique is most

Table 2
Regulatory motifs identified in the promoter sequence of genes potentially involved in biocontrol properties of [(1)]: *Trichoderma atroviride*, [(2)]: *T. hamatum*, [(3)]: *T. virens* [(4)]: *Candida oleophila*

Motifs	Potential role	Binding protein	Gene	Authors
5′-TATA-3′	Transcription initiation	–	*ech*42[(1)]	Lorito et al., 1996a,b
			*chit*33[(1)]	Kubicek et al., 2001
			*nag*1[(1)]	Kubicek et al., 2001
5′-CSYGGRG-3′	Carbon repression	Cre1	*ech*42[(1)]	Lorito et al., 1996a,b
			*chit*33[(1)]	Kubicek et al., 2001
			*chit*42[(2)]	Steyaert et al., 2004
			*prb*1[(2)]	Steyaert et al., 2004
5′-AGGGG-3′	Stress response by zinc finger protein	Msn2/Msn4	*nag*1[(1)]	Kubicek et al., 2001
			*ech*42[(1)]	Kubicek et al., 2001
			*chit*33[(1)]	Kubicek et al., 2001
			*gluc*78[(1)]	Kubicek et al., 2001
			papa[(1)]	Peterbauer et al., 2002a,b
			*seb*1[(1)]	Peterbauer et al., 2002a,b
			*tvsp*1[(3)]	Pozo et al., 2004
			*chit*42[(2)]	Steyaert et al., 2004
			*prb*1[(2)]	Steyaert et al., 2004
5′-MRAGGGR-3′	Response to carbon depletion	brlA	*ech*42[(1)]	Brunner et al., 2003
5′-CCAAT-3′	Establishment of an open chromatin structure	Hap2, Hap3, Hap5	*nag*1[(1)]	Kubicek et al., 2001
			*ech*42[(1)]	Carsolio et al., 1994
5′-GCCARG-3′	pH regulation	PacC	*ech*42[(1)]	Steyaert et al., 2004
			papA[(1)]	Delgado-Jarana et al., 2002
			*tvsp*1[(3)]	Pozo et al., 2004
			CoExg1[(4)]	Segal et al., 2002
5′-AGGCA-3′	Induction of cellulobiohydrolase genes in response of cellulose	AceI	*ech*42[(1)]	Steyaert et al., 2004
			*prb*1[(1)]	Steyaert et al., 2004
5′-HGATAR-3′	Nitrogen repression	AreA/Nit2	*ech*42[(1)]	Brunner et al., 2003
			*chit*33[(1)]	Brunner et al., 2003
			*prb*1[(1)]	Brunner et al., 2003
			*gluc*78[(1)]	Brunner et al., 2003
			papa[(1)]	Brunner et al., 2003
			*chit*42[(2)]	Steyaert et al., 2004
	Induction of mycoparasitic-related genes	MYRE1-MYRE4	*ech*42[(1)]	Cortes et al., 1998
			*prb*1[(1)]	Cortes et al., 1998
			*chit*42[(2)]	Steyaert et al., 2003
			*prb*1[(2)]	Steyaert et al., 2003
5′-GGCAWTCGGCAT-3′	Mycoparasitic response	MYC motifs	*ech*42[(1)]	Cortes et al., 1998
5′-GGGCAC-3′			*prb*1[(1)]	Cortes et al., 1998
5′-TTGGCAA-3′			*tvsp*1[(3)]	Pozo et al., 2004
5′-GCTTCA-3′			*chit*42[(2)]	Steyaert et al., 2004
			*prb*1[(2)]	Steyaert et al., 2004

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adequate for any given study depends on the objective. In all cases, gene identification and sequencing constitute the first steps essential to gene characterization, although they do not prove that a gene is involved in biocontrol. Proof must come from additional work including sequence analysis, expression and regulation studies, and inactivation and/or overexpression of the gene.

3. Sequence analysis

Once a gene is identified and its sequence known, the next step is to analyze its sequence. This analysis, performed with specially developed software, provides a first theoretical characterization of gene function and regulation.

After identifying the open reading frame, the potential introns, the cis-region including the promoter sequence, and the trans-region including the terminator sequence, it is possible to translate the coding region of the gene into the corresponding amino acid sequence. With the Blastx software (Altschul et al., 1997), for instance, the translated sequence can be further compared to sequences in protein databases in order to find similarities to published sequences.

The cis-region can be analyzed to find motifs, i.e. small nucleotide sequences, potentially involved in gene regulation through their interaction with proteins. This analysis can be carried out with MDscan (Liu and Saint, 2002) or Matinspector algorithm (Catharius et al., 2005) software. Numerous motifs, presented in Table 2, have been identified in the cis-regions of genes potentially involved in biocontrol. For example, the presence of the motif 5′-CSYGGRG-3′ or 5′-HGATAR-3′ in the promoter sequence of a gene may means that its expression is repressed, respectively, in the presence of a carbon or nitrogen source. Stress elements can trigger the expression of genes having a 5′-AGGGG-3′ motif. Carbon or nitrogen availability and stress seem to be important factors regulating the expression of numerous genes involved in biocontrol through mycoparasitism. One explanation for this observation may be that in the absence of directly available carbon or nitrogen, the cell activates enzymes that can metabolize new carbon or nitrogen sources.

Although sequence analysis provides useful information, the actual function of a gene and the actual involvement of identified motifs in its regulation have to be confirmed by additional studies (see the following sections).

4. Gene transformation

Integrating foreign DNA into the genome of a biocontrol agent is a powerful way to study the possible involvement of genes in biological control. Its purpose is to inactivate or increase transcription of the recipient gene. After integration, the biocontrol properties of the transformed strain can be compared with those of the wild strain.

Alternatively, genes involved in biocontrol can be transferred to other organisms. In one case, an endochitinase gene from *T. atroviride* was transferred to apple plants in order to confer resistance to apple scab (Bolar et al., 2000). Heterologous genes can also be transferred to a biocontrol agent. A transgenic *T. atroviride* strain expressing the glucose oxidase gene of *Aspergillus niger* under a homologous chitinase promoter was developed by Mach et al. (1999). Brunner et al. (2005) showed by means of in vitro and in vivo assays that this strain is more able than the wild strain to induce systemic resistance in a plant and to develop mycoparasitic activity against two plant pathogens.

Transformation with a foreign gene can be either specific or random. Random gene mutations can be generated by random oligonucleotide insertion. This technique was used to identify the role of a lipidic cellobiose in the mycoparasitic action of *Pseudozyma flocculosa* (Cheng et al., 2003). In another study, insertion of T-DNA from *Agrobacterium tumefaciens* into the genome of the biocontrol agent *Coniothyrium minitans* made it possible to identify mutants displaying diminished antagonistic activity against *S. sclerotiorum* or a deficiency in the production of antifungal compounds (Li et al., 2005). Alternatively, chemical mutagenesis was used by Rey et al. (2001) to isolate a mutant of *T. harzianum* showing increased antifungal activity against *B. cinerea* and *R. solani*. Random gene mutation, however, has a major drawback: numerous mutated strains are produced and their biological control properties have to be tested. The screening process is often time-consuming, so random mutation is feasible only if a quick and reliable test of biocontrol properties is available. Moreover, as the mutations are random and uncontrolled, it may be difficult to identify all the mutated genes and to select the inactivated genes really involved in biocontrol.

Specific transformation with a gene has been more widely used than random mutation. Gene inactivation can be achieved by two different methods, called integrative disruption and one-step gene disruption (Fig. 3). Alternatively, *Agrobacterium*-mediated transformation has been used for *T. atroviride* (Zeilinger et al., 2005).

Integrative disruption relies on the use of a non-replicative plasmid bearing an internal sequence of the targeted chromosomal gene. The plasmid is introduced into the chromosome to inactivate the targeted gene following one recombination event. Alternatively, transformation causes gene duplication, but neither copy of the gene is an entire copy. This technique can

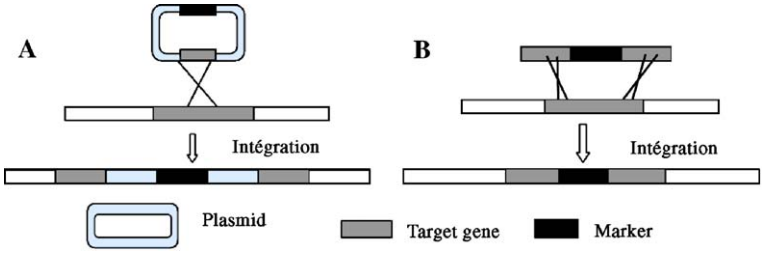

Fig. 3. Gene transformation by integrative disruption (A) or one-step disruption (B).

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Table 3
Disruption or overexpression of genes potentially involved in biocontrol properties

Species	Gene	Plant pathogen	Biocontrol activity[a]		Authors
			Gene disruption	Gene overexpression	
C. oleophila	Exo-β-1,3-glucanase (CoExg1)	*Penicillium digitatum*	Identical	Identical	Yehuda et al., 2003
P. anomala	Exo-β-1,3-glucanase (*PaExg1*)	*Botrytis cinerea*	Identical	–	Grevesse, 2003
	Exo-β-1,3-glucanase (*PaExg2*)	*B. cinerea*	Identical	–	Grevesse et al., 2003
	PaExg1 and *PaExg2*	*B. cinerea*	Reduce (up to 46%)[b]	–	Friel et al., in press
T. harzianum/*T. atroviride*	*N*-acetylglucosaminidase (*nag*1)	*B. cinerea*	Identical	–	Brunner et al., 2003
	*chit*33	*Rhizoctonia solani*	–	Improved[c]	Limon et al., 1999
	Endochitinase (*ech*42)	*R. solani*	Improved (16%)	–	Woo et al., 1999
		B. cinerea	Reduced (33%)	–	
		Pyhtium ultimum	Identical		
	Endochitinase (*ech*42)	*R. solani*	Identical	Identical	Carsolio et al., 1999
		Sclerotium rolfsii	Identical	Identical	
	G-protein α subunit (*tga*3)	*B. cinerea*	Reduced (from 25 to 75%)[d]	–	Zeilinger et al., 2005
	Protease (*prb*1)	*R. solani*		Improved (from 200 to 500%)[e]	Flores et al., 1997
T. longibrachiatum	β-1,4-endoglucanase	*Pythium ultimum*	–	Improved	Migheli et al., 1998
T. virens	Endochitinase (*chit*42)	*R. solani*	Reduced (10%)	Improved (15%)	Baek et al., 1999
	Serine-protease (*tvsp*1)	*R. solani*	Identical	Improved (15%)[f]	Pozo et al., 2004
	Peptide synthase (*Psy*1)	*R. solani*	Identical	–	Wilhite et al., 2001
		P. ultimum			
	MAPK kinase	*R. solani*	Improved (400%)[e]	–	Mendoza-Mendoza et al., 2003
	MAPK kinase	*R. solani*	Reduced[c]	–	Mukherjee et al., 2003
		S. rofsii	Reduced[c]	–	Mukherjee et al., 2003
	G-protein α subunit (*TgaA*)	*R. solani*	Identicals	–	Mukherjee et al., 2004
		S. rolfsii	Lost	–	Mukherjee et al., 2004
	G-protein α subunit (*TgaB*)	*R. solani*	Identical	–	Mukherjee et al., 2004
		S. rolfsii	Identical	–	Mukherjee et al., 2004

Effect of the genetic transformation on the biocontrol activity of the transformed strain.
[a] Improvement or reduction of the biocontrol activity comparing to those of the wild strain.
[b] Reduction depending on experimental conditions (yeasts population and apple maturity).
[c] Reduction non-quantified by the authors.
[d] Reduction of the antifungal activity of a culture filtrate from the strain.
[e] Improvement of the percentage of healthy plants.
[f] Improvement of the percentage of germinated cotton seeds.

be used to integrate into a genome a plasmid overexpressing a gene of interest. One-step gene disruption relies on the use of an altered DNA fragment with a selectable marker. Cells are transfected with this fragment in the absence of a vector sequence. The selectable marker can either be introduced into the coding sequence of the gene or replace the whole ORF. One-step disruption requires two homologous recombination events, whereas a single event suffices for integrative disruption. Thus, depending on the homologous recombination capacity of the strain, one-step disruption may be more challenging. In both approaches, the marker is flanked by sequences that are homologous to the targeted genomic gene.

Table 3 lists the genes that have been disrupted and/or overexpressed in biocontrol agents. Effects of these transformations on the recipient's biocontrol properties are also mentioned. Nearly all of these experiments focused on disruption of a single gene in the studied strain. Recently, Friel et al. (in press) used the URA3-blaster technique to inactivate simultaneously two exo-β-1,3-glucanases potentially involved in the biocontrol properties of *P. anomala* strain K. The biocontrol efficiency against *B. cinerea* of the disrupted strains was found to be significantly affected upon application to wounded apples.

Most of these studies were carried out with species of the genus *Trichoderma*. An analysis of the papers summarized in Table 3 shows that three factors influence the effect of transformation on biocontrol: the biocontrol agent, the experimental conditions, and the plant pathogen combination.

The influence of the biocontrol agent itself and of its action mechanisms is exemplified by two studies in which a gene coding for a 42-kD endochitinase was inactivated in either *T. harzianum* (Woo et al., 1999) or *T. virens* (Baek et al., 1999). By comparison with the untransformed strain, the *T. harzianum* loss-of-function mutant showed increased biocontrol action against *R. solani*, whilst its *T. virens* counterpart showed diminished biocontrol action.

The influence of experimental conditions is illustrated by conflicting results obtained by Mendoza-Mendoza et al. (2003) and Mukherjee et al. (2003) in *in vitro* experiments. Both authors disrupted the same *T. virens* gene, coding for a Mitogen Activated Protein Kinase, but worked with different media, i.e. Vogel's medium or minimal medium and potato dextrose agar respectively. Mendoza-Mendoza et al. (2003) found the null mutant to have a greater capacity to control and reduce damage from *R. solani*, but Mukherjee et al. (2003) found it to be less effective against this pathogen. Using different experimental

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conditions, Woo et al. (1999) and Carsolio et al. (1999) studied *in situ* the effect of disruption of an endochitinase gene (*ech*42) on the biocontrol of *R. solani* by *T. atroviride*. Woo et al. (1999) observed increased protection by the disrupted strain, whilst Carsolio et al. (1999) observed no effect of gene disruption on the biocontrol properties.

The influence of the plant pathogen species on the biocontrol capacity of a transformed strain is illustrated by work in which the biocontrol activity of an *ech*42 knockout mutant strain of *T. atroviride* was compared with that of the wild strain. The mutant was found to exert greater biocontrol activity against *R. solani*, lesser activity against *B. cinerea*, and the same activity as the wild strain against *Pythium ultimum* (Woo et al., 1999). In soil plate assays, a *T. virens tgA* mutant was found to parasitize *R. solani* sclerotia but not *S. rolfsii* sclerotia (Mukherjee et al., 2004). On *R. solani* hyphae, a *tmkA* loss-of-function mutant of *T. virens* exhibited the same mycoparasitic coiling and lysis of host as the wild strain, but was unable to mycoparasitize the hyphae of *Sclerotium rolfsii* (Mukherjee et al., 2003). These results suggest the involvement of several metabolic pathways in the biocontrol agent properties. Activation of these pathways may depend on the plant pathogen.

In several studies and for reasons unknown, transformation did not modify the biocontrol ability of the studied strain. This might be due to the experimental conditions used (Mukherjee et al., 2003), to the existence of a compensatory effect exerted by other enzyme(s) having similar substrate specificity (Baek et al., 1999), or to non-involvement of the gene in biocontrol.

Integration of a foreign DNA fragment to inactivate or overexpress a gene is a useful tool for refining the analysis of the involvement of a gene in biocontrol. As the function of a gene can be inactivated and then restored, it is possible to demonstrate the involvement of this gene in biocontrol by evaluating the biocontrol properties of the studied strain. Yet this method has some drawbacks, such as the difficulty of transforming non-conventional species or the necessity of studying various growth conditions when evaluating the impact of integration on biocontrol properties. The conclusions of such studies must take into account the experimental conditions used. Furthermore, studies tend to focus on one or a few genes, mostly genes coding for secreted proteins, whereas biocontrol properties often depend on numerous genes, with their respective regulatory pathways and mutual interactions. Hence, compensation of effects stemming from gene disruption cannot be ruled out.

5. Gene expression

Gene expression can be studied at the RNA or the protein level. Both approaches have been widely used and are well documented in the literature.

So far Northern blotting has been the most popular technique for studying gene transcription in biocontrol agents. A closely related technique, called dot blotting, was applied by Steyaert et al. (2003). Foreman et al. (2003) used microarrays, a miniaturization of the RNA dot blot system, to study transcription of 35 *T. reesei*, genes coding for enzymes involved in biomass degradation, notably including endoglucanases and β-glucosidases.

Other techniques, such as the use of reporter genes, RT-PCR, or real-time RT-PCR, have also been successfully used. The reporter gene approach involves fusing the reporter gene with the promoter sequence of the studied gene. The whole construct is ligated into a vector and introduced into the genome of the studied strain. This type of genetic engineering provides a useful tool for monitoring gene expression. Its advantages include easy use and sensitive detection, but it is necessary to prove that reporter gene expression does not affect biocontrol properties or gene expression. Moreover, it must be checked that expression of the reporter gene mirrors that of the studied gene. The *A. nidulans* glucose oxidase gene has been used to study the expression of a *T. harzianum* chitinase gene (*chit*33) (De la Mercedes et al., 2001) and of two *T. atroviride* chitinase genes (*ech*42 and *nag*1) (Mach et al., 1999). The gene encoding the green fluorescent protein of *Aquora victoria* has been fused with the promoter sequences of the *T. harzianum chit*33 (De la Mercedes et al., 2001), *ech*42, and *nag*1 (Lu et al., 2004) genes. Other reporter genes, such as a glucuronidase gene (Bae and Knudsen, 2000) or the red fluorescent protein gene (Mikkelsen et al., 2003), have been used successfully in some strains of *T. harzianum* in order to monitor them in the environment. These genes could be fused with genes potentially involved in biocontrol.

Standard RT-PCR has scarcely been used to study gene expression, mainly because of the difficulty of quantifying gene expression. This limitation has been overcome by the development of real-time RT-PCR. Real-time RT-PCR combines the specificity, sensitivity, and ease-of-use of standard PCR with the possibility of quantifying reproducibly the targeted PCR products without using radioactivity. The fluorescence emitted during the reaction is monitored as an indicator of amplicon production during each PCR cycle. This signal increases in direct proportion to the amount of PCR product in a reaction. Various chemistries, based on binding dyes, probes, or beacons, have been developed so far (for a review see Mackay, 2004). Real-time PCR may be used to quantify the population of biocontrol agents (Massart et al., 2005; Schena et al., 2002) or the level of transcription of genes from the biocontrol agents (Iakovlev et al., 2004; Massart and Jijakli, 2006; Morissette et al., 2003). Quantification of gene expression can be absolute or relative. Absolute quantification involves calculating the exact number of gene transcripts by means of a standard curve based on various dilutions of known amounts of the RNA or DNA sequence of the gene. Relative quantification uses a housekeeping gene, whose expression level remains constant under the studied conditions, to quantify the relative expression of a gene under various conditions. Massart and Jijakli (2006) applied real-time PCR to the biocontrol agent *P. anomala* to quantify the relative expression of 11 genes previously identified by cDNA- AFLP. Morissette et al. (2003) used the same technique with the biocontrol agent *Stachybotrys elegans* to quantify the relative expression of the *sechi*44 gene.

Studies of gene transcription in biocontrol agents are usually carried out *in vitro* by growing the biocontrol agent under various conditions. The variable factor in a medium may be the nitrogen source (Ait-Lahsen et al., 2001; Delgado-Jarana et al., 2002) or the carbon source, such as glucose as a control *vs*. a plant pathogen cell wall preparation, autoclaved mycelium, chitin, pustulan, laminarin, cellulose, scleroglucan, or *N*-

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acetylglucosamine (Ait-Lahsen et al., 2001; De la Cruz et al., 1995; Flores et al., 1997; Geremia et al., 1993; Kim et al., 2002; Pozo et al., 2004). Investigators have observed that the transcription of numerous genes potentially involved in mycoparasitism is repressed in the presence of glucose at high concentration (Ait-Lahsen et al., 2001; De la Cruz et al., 1995; Donzelli et al., 2001; Geremia et al., 1993; Nobe et al., 2004; Peterbauer et al., 1996; Pozo et al., 2004) and that it increases as the glucose concentration drops. This phenomenon may reflect the need of a cell to find a new carbon and energy source in the absence of glucose, which is directly assimilable. Upon growing *T. harzianum* strains on various media, Kim et al. (2002) and Limon et al. (1995) respectively observed differences in the regulation of two *N*-acetyl-glucosaminidases and two endochitinases.

The identification of regulatory factors such as carbon or nitrogen availability, pH, or abiotic stress can strengthen the hypothesis that motifs previously identified in a promoter sequence are involved in the regulation of the corresponding gene (Delgado-Jarana et al., 2002). Mach et al. (1999) found transcription of the endochitinase gene *ech*42 to be stimulated by nutrient limitation in the culture medium and by some stress conditions such as low temperature, low water activity, or the presence of ethanol. Studying the regulation of a protease gene (*Prb*) in *T. atroviride*, Olmedo-Monfil et al. (2002) found its transcriptional induction to depend on nitrogen availability in the environment. Furthermore, expression of the *ThPTR*2 gene, coding for a PTR-family di/tri-peptide transporter, can be triggered by nitrogen starvation (Vizcaino et al., 2006).

Investigators have also studied the gene expression patterns of some *T. harzianum* (Geremia et al., 1993; Pozo et al., 2004) and *T. hamatum* (Steyaert et al., 2003) biocontrol genes. Using confrontation assays or incubation on a medium containing a pathogen cell wall preparation, these authors demonstrated time-dependent transcription of genes potentially involved in biocontrol. They found transcription to peak after a period that depended on the gene and on the studied conditions.

The relevance of transcription analysis depends on the chosen growth conditions. So far, most studies have focused on *in vitro* conditions and have required large amounts of cells for Northern blotting. Now that the development of real-time (RT)-PCR offers new prospects, it is possible to study gene expression on small amounts of cells. An additional advantage of real-time (RT)-PCR is that it does not require designing and constructing a reporter plasmid and integrating it into cells. Real-time (RT)-PCR thus opens the way to simpler *in situ* experiments and to studying the gene expression in cells under real biocontrol conditions without the bias inherent in the design of *in vitro* experiments. Moreover, gene transcription should be studied over time, as its induction or suppression may be time-dependent.

Proteomic experiments study directly the molecules influencing biochemical processes. They avoid the stumbling block that may result from discrepancies that sometimes exist between the level of an mRNA and that of the corresponding protein. Yet despite recent progress, the molecular complexity and diversity of proteins still makes this approach technically more challenging than the transcriptomic approach. In the field of biological control, numerous proteomic studies have focused on individual proteins secreted by biocontrol agents, and particularly on protein expression over time (Berto et al., 2001) or under various *in vitro* conditions (Vazquez-Garciduenas et al., 1998). In such experiments the protein of interest is often detected by staining after polyacrylamide gel electrophoresis (Jijakli and Lepoivre, 1998). The recent technical advances achieved in proteomics, e.g. 2-D electrophoresis and protein chips, will probably popularize the proteomic approach in the future. Grinyer et al. (2005) recently published the first proteomic study in which 2-D gel electrophoresis was applied to proteins of a biocontrol agent (*T. atroviride*). These authors identified 24 proteins that are up-regulated in the presence of *R. solani* cell walls. Among these proteins are an *N*-acetyl-beta-D-glucosaminidase, a 42-kDa endochitinase, and 3 novel proteases. Woo et al. (2006) recently published a paper describing the concept and state of the art in proteomic analysis applied to biocontrol strains of *Trichoderma* spp.

As described above, gene expression studies can be designed with two alternative objectives: to study gene regulation *in vitro* under controlled nutrient supply conditions or to study gene expression *in vivo* or *in vitro* during direct interaction of the biocontrol agent with the plant pathogen. Overexpression of a gene when the biocontrol agent interacts directly with the plant pathogen strengthens the hypothesis of an involvement of this gene in biocontrol (Vizcaino et al., 2006).

6. Promoter analysis and regulatory proteins

Promoter analysis can be used to confirm molecular models of gene regulation deriving from studies carried out under various *in vitro* conditions. Published studies of the promoter regions of genes involved in biocontrol have focused on either promoter sequences or regulatory proteins.

Some investigators have studied the promoter sequence of a gene in order to confirm the involvement of previously identified motifs in the regulation of its transcription under biocontrol conditions. Electromobility Shift Assays (EMSAs), *in vivo* footprinting, and/or promoter deletion analysis (Peterbauer et al., 2002a) are the techniques used. EMSAs are powerful tools for detecting DNA-binding transcription factors. These factors bind to specific DNA sequences and play an important role in regulating gene transcription (Fried and Crothers, 1981). Binding of a factor to a particular sequence is detectable on a gel because it modifies the electromobility of the sequence. *In vivo* footprinting is based on methylation of genomic DNA with dimethylsulfate. The areas protected against cleavage, including the promoter region responsible for transcriptional regulation of the gene, can be further visualized by ligation-mediated PCR. Promoter deletion analysis involves the use of reporter plasmids containing a reporter gene fused with a modified 5′ upstream region of the studied gene.

Lorito et al. (1996a,b) used an EMSA to study the promoter sequence of the *T. atroviride ech*42 gene. Their results suggest that *ech*42 expression is repressed by binding of the carbon catabolite repressor Cre1 to a consensus sequence (5′-SYGGR-3′) in the *ech*42 promoter. These authors also showed that during a mycoparasitic interaction, *ech*42 gene transcription is

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stimulated by binding of a mycoparasitic protein–protein complex in the vicinity of the Cre1 binding sites. This complex hampers binding of the Cre1 protein to the *ech*42 promoter. Peterbauer et al. (2002a) used all three above-mentioned methods to study the promoter region and the regulatory proteins involved in regulation of the *T. atroviride nag*1 gene. They found a mutation in the motif AGGGG (−240), $CCAGN_{13}CTGG$ (−284), or CCAAT (included in the N_{13} sequence of the previous motif) to inhibit *nag*1 induction *in vitro*. Brunner et al. (2003) used reporter genes and EMSAs to identify the DNA sequences controlling transcription of the *T. atroviride* endochitinase gene *ech*42. They found binding of the protein *BrlA* to the motif, at positions−524,−326 and−203, to induce transcription of the gene during carbon starvation. Using the EMSAs approach, Peterbauer et al. (2002b) demonstrated that a stress response binding element (*seb*1) binds to the 5′-AGGGG-3′ motifs of the promoter sequences of *nag*1 and *ech*42.

EMSA, *in vivo* methylation, and reporter plasmids containing truncated promoter sequences have scarcely been used to study biocontrol agents, although they are very useful. Not only can they demonstrate the involvement of regulatory motifs in the promoter sequence of a studied gene, but they can also provide an overall view of the regulatory mechanisms affecting a gene involved in biocontrol.

Regulatory proteins can influence gene transcription either directly (by binding to the promoter sequence) or indirectly (via signal transmission). The molecular tools described in the "Gene Transformation" section are used also to inactivate genes coding for regulatory proteins. Peterbauer et al. (2002b) found that inactivation of the *seb*1 gene does not modify transcription of the *nag*1, *chit*33, and *ech*42 genes. They also showed that other proteins can bind to the 5′-AGGGG-3′ promoter motifs of *nag*1 and *ech*42 in the disrupted strain.

In *in vitro* studies, Mukherjee et al. (2003) and Mendoza-Mendoza et al. (2003) examined how inactivating two mitogen activated protein kinases (MAPKs) affects the mycoparasitic properties of *T. virens*. In many fungal species, MAPK proteins participate in cascade signals involved, e.g., in plant parasitism. The obtained results are described hereabove ("Gene transformation" chapter).

The role of two G-protein α-subunits, *TgaA* and *TgaB*, in biocontrol by *T. virens* has been studied by Mukherjee et al. (2004). G-proteins play an important role in intracellular signaling. They amplify receptor responses and influence the amplitude and duration of cellular signals. Using null-*TgaA* and null-*TgaB* strains, these authors showed that *TgaA* is involved in the biocontrol activity against *S. rolfsii*, but that neither *TgaA* nor *TgaB* is required for its activity against *R. solani*. The authors conclude that the involvement of G-proteins in biocontrol by *T. virens* depends on the plant pathogen with which the biocontrol agent is in contact. Zeilinger et al. (2005) have shown that the *tga*3 gene of *T.atroviride*, also coding for a G-protein α-subunit, is involved in this biocontrol agent's vegetative growth and mycoparasitic activity.

Having demonstrated the ability of a MAPK inhibitor to inhibit *prb*1 transcription in *T. atroviridae* whatever the nitrogen availability, Olmedo-Monfil et al. (2002) were led to suggest the involvement of mitogen activated protein kinases in *prb*1 transcription. They also used a reporter gene to identify a new regulatory element in the promoter region of *prb1*.

The ability of a biocontrol agent to respond quickly and adequately to an environmental signal such as the presence of a potential host is a key factor in the development of mycoparasitism or in the metabolization of plant nutrients. The study of regulatory proteins is thus essential to an in-depth understanding of the genetic basis of biocontrol properties. It notably highlights relationships between the environment and biocontrol gene expression. The induction or repression of gene expression in response to environmental signals may occur through various pathways. So far research has focused on G-proteins and MAPK pathways. Other candidate genes, like the Abc transporters or the OPT protein family, should be studied for their possible involvement in biocontrol.

7. Conclusions

The first step in characterizing genes potentially involved in biocontrol is to isolate and sequence them. Which strategy is most appropriate depends on previous results and on the objectives of the study. The targeted strategy should be privileged if the aim is to acquire further genetic knowledge on a well-studied mechanism of action. Differential gene expression techniques are very useful in original research aiming to isolate new genes potentially related to biocontrol properties when there is no a priori reason to suspect their involvement. Large-scale sequencing techniques, finally, support a broader endeavor: to improve genetic knowledge on a strain by sequencing numerous genes. Putative biocontrol genes may emerge from this endeavor.

In the future, numerous genes of various biocontrol agents will be identified by means of open strategies and with the help of advanced gene isolation and sequencing methods. The isolated genes will have to be characterized, and characterization will remain a bottleneck requiring much more time and effort than gene identification and sequencing.

Gene characterization is a multistep process beginning with an *in silico* sequence analysis. Such analyses exploit specially developed softwares. They include a comparison of the translated sequence with sequences stored in databases, identification of particular motifs in the predicted amino-acid sequence (signal peptides, transmembrane signals, consensus sites…), and the identification of regulatory motifs in the promoter sequence. Conclusions drawn from this *in silico* analysis must be verified by means of laboratory experiments such as transcriptional analysis, gene inactivation/overexpression studies, electromobility shift assays, *in vivo* methylation experiments, the use of reporter plasmids, and the study of regulatory proteins.

Most studies using molecular techniques to elucidate the genetic basis of the antagonistic properties of fungal biocontrol agents have focused on species of the genus *Trichoderma* and on a particular action mechanism: mycoparasitism. Genes related to antibiosis have been studied more intensively in the case of prokaryotic biocontrol agents, e.g. *Bacillus subtilis* and *Pseudomonas* spp. Only a few genes involved in the induction of a plant defense response or in competition for space and nutrients have ever been

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identified in biocontrol agents. In the case of the genes involved in competition for space and nutrients, this is probably due at least in part to the complexity of the gene expressions and interactions that contribute to regulate this mechanism.

Biocontrol strains often belong to unconventional species for which few molecular techniques are available. Optimized molecular tools are available only for *Trichoderma* species. Hence, studying the genetic basis of biocontrol requires the transfer and adaptation of molecular biology tools developed with conventional species such as *Saccharomyces cerevisiae*, *Aspergillus nidulans*…This is often a time-consuming process which slows down investigations.

Molecular techniques have been used to study the genetic basis of biological mechanisms and to identify partial or complete molecular pathways regulating gene expression. This is also true in the field of biocontrol. Whatever the technique used, it is paramount to choose an appropriate experimental model, as this will determine the reliability and validity scope of any conclusions drawn from an experiment.

Molecular techniques have shed light on the antagonistic properties of numerous biological control agents, but they have also underlined the complexity of genetic regulation. They are now essential to studying the mechanisms of action of biocontrol agents and must be included in comprehensive studies that should also include microbiological, biochemical, and microscopic approaches.

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