Fungal Biology

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Fungal Biotechnology and Bioengineering

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Fungal Biology

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About the Series

Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

More information about this series at http://www.springer.com/series/11224

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Foreword

For more than a century, fungi have had an enormous impact on industrial biotechnology and bioengineering. They are used in industrial processes such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids, biofuels, and glycolipids. Some of these products are produced commercially, while others are potentially valuable in biotechnology.

Fungal secondary metabolites are important to our health and nutrition and have a tremendous economic impact. In addition to the several reaction sequences involved in fermentation, fungi are extremely useful in carrying out biotransformation processes.

Over the past 20 years, the tools of molecular biology have been successfully adapted for the study of filamentous fungi. These applications have elevated the status of fungal genetics to a fascinating and, at times, truly insightful field of study. Molecular genetics has provided a toolbox of immensely powerful experimental approaches, and it now entails virtually all groups of economically and environmentally important fungi and is having a significant impact on commercial bioprocesses.

Fungi, owing to their metabolic versatility, ecological diversity, complex life cycles, and essential role in nature, have attracted the attention of engineers, biologists, geneticists, ecologists, chemists, and biochemists in myriad ways.

In this sense, *Fungal Biotechnology and Bioengineering* was edited by Abd El-Latif Hesham, R.S. Upadhyay, G. D. Sharma, C. Manoharachary, and V. K. Gupta. The topics were organized so as to provide a very important contribution to science and technological advances to be applied to the fields of biotechnology and bioengineering.

The 20 chapters of this book present, in detail, relevant information that can be useful to students, teachers, researchers, and professionals interested in the area of industrial biotechnology and microbiology.

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He is one of the leading experts in the areas of "Microbial Genetics and Biotechnology," "Biodegradation, Bioremediation and Phytoremediation," "Microbial community structure," "Soil Microbiology and Enzyme activities," "Biological Control," "Anti-microbial activates," "Biofertilizer," "Biofuels," and "Environmental Meta-genome Biotechnology." He has coauthored more than 80 peer-reviewed publications in reputed Thomson Reuters impact factor journals and 4 book chapters in international reputed publishers like Elsevier, Springer-Nature, Taylor & Francis, and John Wiley & Sons. He is key person in many national and international research projects related to field of Microbial Genetics and Applied Biotechnology.

Prof. Hesham has been the scientific and organizing committee member and invited speaker in various international conferences. He is also a recipient of several prestigious national and international awards. Most recently, he has been elected as Member of the Egyptian National Biotechnology Network of Expertise (NBNE), and he has been appointed as the Country Representative for Egypt and the Arab Counties by International Biodeterioration & Biodegradation Society (IBBS) UK, which belongs to the Federation of European Microbiological Societies (FEMS).

He is also Fellow of Microbiology Society, UK; European Federation of Biotechnology, Spain; the Bio-Processing Network, Australia; and the International Biodeterioration & Biodegradation Society.

Prof. Hesham serves as Associate Editor and Editorial Board Member for international reputed journals such as *Scientific Reports*, *Frontiers in Microbiology*, *Frontiers in Plant Science*, *PeerJ*, *Current Bioinformatics*, *International Journal of Agriculture and Biology*, *Journal of Environmental Biology,* and *Biocatalysis and Agricultural Biotechnology*.

Ram Sanmukh Upadhyay is Professor and Head of Botany Department at the most prestigious University of India, Banaras Hindu University (BHU). He is credited with the discovery of the perfect state of *Fusarium udum*, the causal agent of the wilt of pigeon pea. He is also acclaimed for his contributions to the biology of this pathogen. He has published 143 research papers, 60 book chapters, 3 edited books, and 1 monograph of national and international repute. He has produced 27 Ph.D. students ranging on the topics such as biological control of plant pathogens, programmed plant cell death in response to pathogens, bioremediation of toxic effluents, induced resistance in plants and their immunization, plant growth promoting microbes, chitinase production from actinomycetes, mycorrhizal technology for reclamation of wastelands, role of rhizobacteria in detoxifying phytotoxic effect, development of molecular markers in tracking microbes in environment, effect of biotic and abiotic factors on plants, and molecular basis of plant-microbe interaction. Six Ph.D. students are currently working under his supervision. He has supervised more than 60 M.S./M.D. level dissertations. His current research interest focuses mainly on biological control of fungal plant pathogens, induced resistance in plants against pathogens, and microbial technology. He is a member of various scientific societies and is on the editorial board of various journals. Dr. Upadhyay is a recipient of the prestigious Young Scientist Award of the Indian National Science Academy (INSA) and Professor L. S. S. Kumar Memorial Award of INSA. He was conferred by the Prime Minister of India with Prof. Hira Lal Chakravorty Award of the Indian Science Congress Association in 1986. He has carried out collaborative research at Rothamsted Experimental Station (England; 1988–1989) with Dr. David Hornby as Visiting Fellow of the Royal Society of London; Illinois State University (USA; 1990–1991) with Prof. R. K. Jayaswal, Distinguished Professor; and an INSA-JSPS Fellow (Japan; 1994–1995) with Prof. Hajimu Komada at Shimane University. Dr. Upadhyay was the Coordinator of Environmental Science Program of BHU, and currently, he is the Program Coordinator of the Centre of Advanced Study (CAS) in Botany, Banaras Hindu University. He was the Convener of the Mycology and Plant Pathology Group of the CAS in Botany at BHU. His biography has been published by various biographical publishing houses including World Who's Who.

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Chapter 1
Fungal Epigenetic Engineering

Jalal Soltani

1.1 Endosymbiosis in Fungi: An Introduction

Eukaryotic organisms live in a prokaryote-dominated world. Increasing evidence has shown that almost every niche is occupied by prokaryotic microorganisms. These niches include marine, arid, and polar regions, as well as external and internal tissues of organisms' bodies, including fungi. Recent investigations of human, insect, and plant internal tissues have revealed the presence of a diverse community of endosymbiotic bacteria, archaea, and viruses that interact with host organism and modulate its phenotypes (Moran et al. 2008; Soltani 2017). Indeed, the extended phenotype of endosymbiotic entities in eukaryotic world is prevalent. This is becoming a fact in fungal kingdom as well.

Fungi live in diverse habitats and have adapted a vast variety of mechanisms to cope with such diverse niches. Among these mechanisms are endosymbiont exploitation, i.e., making use of endosymbiotic microbiome, such as endofungal bacteria and viruses (Araldi-Brondolo et al. 2017; Arora and Riyaz-Ul-Hassan 2019; Bao and Roossinck 2013; Soltani 2017). As we will see in the next sections, this phenomenon has great implications for fungal epigenetic engineering for potential uses in agroforestry, medicine, pharmacology, and bioindustry.

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1.2 Bacterial Endosymbionts of Fungi: Endofungal or Endohyphal Bacteria (EHB)

Fungi live in close association with bacteria in their shared environments and niches. Fungi-bacteria interactions include parasitism, mutualism, and commensalism (Benoit et al. 2015; Kobayashi and Crouch 2009; Leveau and Preston 2008; Soltani 2017). These interactions occur around the fungal mycelium or mycelosphere, on living fungal hyphae or myceloplane, and inside the living fungal hyphae and spores (Frey-Klett et al. 2011). Of special interest are the bacteria that live within fungal living structures, e.g., hyphae and spores, and are called endofungal or endohyphal bacteria (EHB) (Hoffman and Arnold 2010; Pakvaz and Soltani 2016; Soltani 2017). EHB have adapted a range of lifestyles, from facultative to obligatory, and diverse internal localizations inside fungal cells (Araldi-Brondolo et al. 2017; Arora and Riyaz-Ul-Hassan 2019; Soltani 2017). Recent evidence have highlighted the prevalence of plant-associated EHB. Endohyphal bacteria have the capability of modulating host fungi growth and reproduction, as well as their primary and secondary metabolism, enzyme synthesis, phytohormone synthesis, substrate use, stress tolerance, virulence, and fungal-plant interaction (Deveau et al. 2010; Hoffman and Arnold 2010; Hoffman et al. 2013; Mondo et al. 2017; Pakvaz and Soltani 2016; Partida-Martinez and Hertweck 2005; Soltani 2017). Thus, they can be exploited in various ways in fungal bioengineering and biotechnology.

1.2.1 Classification of Endohyphal Bacteria

According to a recent proposal, EHB can be classified into three functional classes as Class 1, Class 2, and Class 3 EHB (Araldi-Brondolo et al. 2017). So, I discuss, in brief, this classification here.

1.2.1.1 Class 1 EHB

Class 1 EFB refers to Mollicutes-related endohyphal bacteria (MRE) observed in arbuscular mycorrhizal fungi (AMF) (Naito et al. 2015). The genus *Candidatus Moeniiplasma* is proposed to encompass MRE (Naito et al. 2017). Compared to other known EHB, the genomes of Class 1 EFB are small (ca. 0.7–1.2 Mb) with a GC content of ca. 32.0–34.3% (Naito et al. 2015; Torres-Cortés et al. 2015; Kuo 2015). Thus, their metabolic reliance on host fungal cell is high (Kuo 2015). These apparently globally distributed bacteria are observed in the cytoplasm of fungal cells and spores from *Glomeromycotina*, *Mortierellomycotina*, and *Mucoromycotina* (*Mucoromycota*) (Desirò et al. 2013, 2014; Kuo 2015; Naumann et al. 2010; Toomer et al. 2015). They are considered to have an obligatory association with the fungal

cells and are vertically transmitted (Naito et al. 2015, 2017). However, whether MRE are mutualistic or antagonistic to their host cell is under question because they could be antagonistic to their hosts (Desirò et al. 2013; Naito et al. 2015; Toomer et al. 2015; Torres-Cortés et al. 2015).

1.2.1.2 Class 2 EHB

Class 2 EHB include bacteria of *Burkholderiaceae* which associate cytoplasm of fungi belonging to *Glomeromycotina*, *Mortierellomycotina*, and *Mucoromycotina* (*Mucoromycota*) such as ectomycorrhiza and arbuscular mycorrhizal fungi, *Rhizopus microsporus* and *Mortierella elongata* (Bianciotto et al. 2003; Desirò et al. 2014; Oliveira and Garbaye 1989; Garbaye 1994; Partida-Martinez and Hertweck 2005; Partida-Martinez et al. 2007a, b; Ohshima et al. 2016; Sato et al. 2009), as well as bacteria known as *Cytophaga-Flexibacter-Bacteroides* clade associating ectomycorrhizal *Ascomycota* such as *Tuber borchii* (Barbieri et al. 2000, 2002) and *Bacteroidetes* associating soilborne *Ascomycota* such as *Fusarium keratoplasticum* (Barbieri et al. 2002; Shaffer et al. 2017). Compared to other known EHB, the genome sizes of Class 2 EHB are moderate (ca. 1.4–3.8 Mb) with a GC content of ca. 46.1–60.7% (Lumini et al. 2006; Desirò et al. 2014). Their metabolic reliance on host fungal cell is moderate to high (Araldi-Brondolo et al. 2017). These bacteria are observed in vegetative and reproductive structures (hyphae and spores) of fungal hosts (Desirò et al. 2014; Kuo 2015; Naumann et al. 2010; Toomer et al. 2015). They are considered to have an obligatory to facultative association with the fungal cells and are vertically, and potentially horizontally, transmitted (Bianciotto et al. 2004; Araldi-Brondolo et al. 2017). It seems that Class 2 EHB interact in a mutualistic manner with their host cell (Shaffer et al. 2017).

Mycorrhizae/EHB Association

The EHB *Candidatus Glomeribacter gigasporarum*, which is commonly observed in association with AMF of *Gigasporaceae*, proliferates in the cytoplasm of the host fungi's mycelium and transmits vertically by fungal sporulation (Bianciotto et al. 2003). It enhances fungal ATP synthesis, detoxification of reactive oxygen species, and sporulation. Thus, it increases fungal energetic capacity and metabolism, stress tolerance, and reproduction (Salvioli et al. 2016). It is notable that an individual AMF can harbor both Ca. *Moeniiplasma* and Ca. *G. gigasporarum* (Desirò et al. 2014).

Ectomycorrhiza associating EHB enhances fungal growth and the survival and germination of fungal spores, as well as fungal-plant root interaction (Oliveira and Garbaye 1989). Thus they are vital components in the establishment of mycorrhiza in agroforestry, especially under poor soil conditions (Garbaye 1994). Furthermore, it is found in ascomycetous ectomycorrhizal *Tuber borchii* that *Cytophaga-*

Flexibacter-Bacteroides associate internally the fungal mycelia (Barbieri et al. 2000). Further analyses revealed that a number of ca. 10^6 bacteria/fungal cells resided within the mycelia of *T. borchii* (Barbieri et al. 2002).

Rhizopus microsporus/EHB Association

It is observed that some fungi exploit EHB for toxin or metabolite production (Lackner et al. 2009, 2011; Pakvaz and Soltani 2016; Partida-Martinez and Hertweck 2005). An example of such a phenomenon is seen in *Rhizopus-Paraburkholderia* interaction. EHB of the genus *Paraburkholderia* (formerly *Burkholderia*) are found to associate the plant pathogenic fungi *Rhizopus microspores* which cause rice seedling blight (Partida-Martinez and Hertweck 2005; Lackner and Hertweck 2011). Rhizoxin phytotoxin secreted by the fungus induces the disease symptom in host plants. However, rhizoxin is produced by fungus's EHB, i.e., *Paraburkholderia rhizoxinica*, not the fungus itself (Partida-Martinez and Hertweck 2005). It is interesting that fungus provides enzymes for the biosynthesis of 2,3-oxirane ring of the rhizoxin precursor (Scherlach et al. 2012). This indicates a close cooperation between the phytopathogen and its resident bacteria for phytotoxin production. Moreover, *R. microspores*, deprived of EHB, is not capable of vegetative reproduction, i.e., sporangia and spore production. The fungus vegetative reproduction restores only when the *P. rhizoxinica* is reintroduced (Partida-Martinez et al. 2007b). Moreover, it is further found that cyclopeptides such as rhizonin secreted by the phytopathogenic *R. microsporus* are not mycotoxin but are synthesized by the EHB *Paraburkholderia endofungorum* (Partida-Martinez et al. 2007a, b; Lackner et al. 2009). Together, these observations extend the interaction of fungal pathogens with their host cells to the third player, i.e., EHB. This phenomenon has the capability to be used in biotechnological applications of fungi or in combating fungal diseases.

Mortierella elongata/EHB Association

The bacterium *Mycoavidus cysteinexigens* (*Burkholderiaceae*) is found as endobacterium of the nitrous oxide (N_2O)-producing fungus *Mortierella elongata* (Ohshima et al. 2016; Sato et al. 2009). However, a positive correlation between EHB and N_2O production was not seen (Sato et al. 2010). Genome analyses have revealed that the bacterium lacks the genes encoding chitinolytic enzymes (Sharmin et al. 2018). This may indicate a facilitated bacterial entrance into host fungi without provoking defensive mechanisms. EHB also lacked crucial metabolic genes such as the glycolytic pathway and cysteine biosynthesis genes, indicating their reliance on the host fungus for such metabolites (Sato et al. 2010).

Fusarium keratoplasticum/EHB Association

Ascomycetous fungus *F. keratoplasticum*, isolated from a seed of the plant *Cecropia insignis*, has been shown to harbor *Chitinophaga* sp. (*Bacteroidetes*). Phenotypic studies that compare carbon substrate use in *F. keratoplasticum*, with or without EHB *Chitinophaga*, have revealed that EHB increased the growth and use of carbon substrates by its host fungus (Shaffer et al. 2016, 2017). Indeed, *F. keratoplasticum* showed increased growth on 59 of 95 substrates (Shaffer et al. 2017). This is important from an ecological point of view, as well as in biotechnological applications.

Geosiphon pyriformis/EHB Association

It is reported that the cyanobacterium *Nostoc punctiforme* associates the arbuscular mycorrhizal fungus *Geosiphon pyriformis* (Knapp 1933; Kluge et al. 1992; Schüßler et al. 1994; Schüßler 2002). In this symbiosis, *N. punctiforme* fixes CO_2 into organic sugar and under certain conditions fixes N_2 to provide nitrogen for its host fungus (Schüßler and Wolf 2005). In response, the fungus provides mineral nutrients, such as P and CO_2, required for the photosynthesis of the cyanobacterium (Schüßler 2012).

1.2.1.3 Class 3 EHB

Class 3 EHB include a phylogenetically diverse group of bacteria that, in a facultative lifestyle, are affiliated with the hyphae of diverse fungi belonging to plant-associated *Dikarya*, i.e., *Ascomycota* and *Basidiomycota* (Arendt 2015; Arendt et al. 2016; Bertaux et al. 2003; Hoffman and Arnold 2010; Pakvaz and Soltani 2016; Sharma et al. 2008). These bacteria, mostly belonging to *Proteobacteria* and *Firmicutes*, associate diverse plant foliage- and seed-associated fungi from *Pezizomycotina* (Pakvaz and Soltani 2016; Hoffman and Arnold 2010), as well as ectomycorrhizal and endophytic *Basidiomycota* such as *Laccaria* (Bertaux et al. 2005) and *Piriformospora indica* (Sharma et al. 2008). Compared to other known EHB, the genome sizes of Class 2 EFB are larger (ca. 3.9–8.5 Mb) with a GC content of ca. 55.2–70.6% (Araldi-Brondolo et al. 2017). Thus, they do not show significant genome reduction. Their metabolic reliance on host fungal cell is moderate. These bacteria are observed in vegetative and reproductive structures (hyphae and spores) of fungal hosts and can be recovered from the hyphae and be reintroduced to their host or a novel fungal host (Arendt 2015; Hoffman et al. 2013; Partida-Martinez et al. 2007b). Thus, they have a facultative association with the fungal cells and are horizontally transmitted. The mutualistic interaction with the host cell is approved for this Class of EHB. Class 3 EHB are close relatives of free-living or non-EHB taxa. It is observed that individual fungal colonies usually harbor one species of EHB (Pakvaz and Soltani 2016), but presence of several species in different parts of the same mycelium is also reported (Shaffer et al. 2016).

Basidiomycota-Associated EHB

Laccaria bicolor/EHB Association

Ectomycorrhizal fungus *Laccaria bicolor* is used in nurseries to inoculate seedlings of Douglas fir for reforestation (Perry et al. 1987). Fermentations to proliferate the fungus were recurrently polluted by a bacterium identified as *Paenibacillus*. Further research showed the endohyphal association of *Paenibacillus* with *L. bicolor* (Bertaux et al. 2003). However, the outcome of this symbiosis for fungus is not explored yet.

Ustilago maydis/EHB Association

The capability of a pathogenic isolate of *Ustilago maydis* to grow on culture media deprived of free nitrogen resulted in identification of endosymbiotic N_2-fixing *Bacillus pumilus* within the fungal mycelium. This association allows fungus to fix atmospheric nitrogen (Ruiz-Herrera et al. 2015).

Piriformospora indica/EHB Association

Fungi belonging to the order *Sebacinales*, *Basidiomycota*, live in close associations with EHB. It is found that the bacteria *Paenibacillus*, *Acinetobacter*, and *Rhodococcus* associate *Sebacina vermifera* and *Rhizobium radiobacter* (Syn. *Agrobacterium radiobacter*) internally associates *Piriformospora indica* (Guo et al. 2017; Sharma et al. 2008). Findings indicate that *R. radiobacter*/*P. indica* association is highly tight. Antibiotic application studies indicated a decreased fitness of fungi harboring EHB, probably due to the bacterial inhibited growth (Glaeser et al. 2016). Further studies have approved the enhancing effect of *R. radiobacter* on *P. indica*'s fitness (Spraker et al. 2014; Guo et al. 2017).

Ascomycota-Associated EHB

It has become evident over the past decade that EHB are prevalent in endophytic fungi associating healthy plants (Hoffman and Arnold 2010; Pakvaz and Soltani 2016). The recovered endophytic fungi have been mainly from *Ascomycota*, although a minority of *Basidiomycota* has also been observed (our unpublished data). Findings in Arnold's group at Arizona University indicated the association of a diverse number of EHB with endophytic fungi, from *Pezizomycotina*, residing in the foliage of the members of Cupressaceae plant family (Hoffman and Arnold 2010). The bacteria included the members of *Proteobacteria* and *Firmicutes* (Hoffman and Arnold 2010). Their further work on the interaction of endophytic fungus *Pestalotiopsis neglecta* and EHB *Luteibacter* revealed that the EHB enhanced fungal production of the phytohormone indole-3-acetic acid (IAA) (Hoffman et al. 2013); thus EHB indirectly affects the plant health. Research in our lab on

endophyte-associating cupressaceous trees in Iran confirmed the prevalence of endophytic colonization of those trees by *Pezizomycotina* and *Ascomycota* (Hosseyni Moghaddam and Soltani 2013, 2014a, b; Hosseyni Moghaddam et al. 2013; Soltani and Hosseyni Moghaddam 2014a, b, 2015). We further showed that a fraction of fungal endophytes, isolated from *Cupressus sempervirens*, harbored EHB from *Proteobacteria* and *Firmicutes* (Pakvaz and Soltani 2016). Axenic EHB exhibited antifungal activities in vitro, suggesting a protective role for their host fungus against competing fungi (Pakvaz and Soltani 2016). A notable finding in our and Arnold's lab was that *Bacillus subtilis* was isolated both as an endophytic and an endohyphal bacterium (Hoffman and Arnold 2010; Pakvaz and Soltani 2016; Soltani et al. 2016), indicating adapting different lifestyles by some EHB. These findings provide insight into the complicated interrelationship among the EHB, their fungal hosts, and the harboring host plant. This could be of high significance in agroforestry for plant health management and in biotechnology. Thus, future research has to consider EHB as novel epigenetic modulators of fungi phenotypes in association with host plants.

1.2.1.4 Perspective of EHB in Fungal Epigenetic Engineering

Fungi live in close association with various bacteria in their vicinity. The bacteria known as EHB can in an obligatory or a facultative manner colonize the cytoplasm of fungal cells or their reproductive organs. Cumulative evidence suggest that EHB are capable of modulating the host fungus's phenotype and its reproduction. The extended phenotype of EHB in their host fungus includes primary and secondary metabolite production, enzyme and phytohormone synthesis, substrate use, stress tolerance, virulence, as well as fungal-plant interaction. This implicates potential applications of such organisms, as a kind of epigenetic engineering, to modulate the fungal phenotypes for different biotechnological purposes.

1.3 Viral Endosymbionts of Fungi: Endofungal Viruses or Mycoviruses

Viruses that reside within fungal cells are called mycoviruses. Viruses are obligatory residents of the host cells; thus in many cases, they exhibit a parasitic interaction with their host organism. Accordingly, the first case of mycoviruses indicated the pathogenic potential of such entities in fungi such as mushrooms (Hollings 1962). However, experimental studies over the last 50 years have indicated that fungi host a diverse range of cryptic or persistent mycoviruses with benign effects which transmit vertically and in many cases modulate fungal host's morphology and its various phenotypes such as pathogenicity, virulence, stress tolerance, and fungal-plant interaction (Roossinck 2011a, b). These phenomena are mainly studied in

fungal endophytes of plants (Bao and Roossinck 2013), and it is becoming evident that viral mutualistic symbioses in endophytic fungi are common (Feldman et al. 2012; Roossinck 2014). Moreover, it is now clear that multiplexed interactions among mycoviruses, endophytic fungi, and the host plants have great implications for the biology and evolution of all partners (Márquez et al. 2007; Roossinck 2015). Mycoviruses are traditionally used in plant pathology to manage a number of detrimental plant diseases *in planta* (Agrios 2005). However, recent findings on the symbiotic relationship of virus-fungus-plant consortia indicate a huge potential for application of such mycoviruses in fungal epigenetic engineering and biotechnology. This will be discussed further, with a focus on viruses of endophytic fungi, in the next sections.

1.3.1 Prevalence and the Beneficial Role of Viruses in Endophytic Fungi and Virus-Fungus-Plant Consortia

Mycoviruses are ubiquitous in fungi (Buck 1998; Hammond et al. 2008) and play significant roles in biology of their host fungus. Traditionally most viruses investigated in fungal kingdom are viruses infecting fungal plant pathogens (Pearson et al. 2009). These are mainly dsRNA viruses, although a number of +ssRNA viruses and few geminiviruses have also been reported to infect such fungi (Son et al. 2015). A well-known extended phenotype of these viruses is hypovirulence of plant pathogenic fungi, e.g., in *Botrytis cinerea*, *Cryphonectria parasitica*, *Fusarium graminearum*, and *Sclerotinia sclerotiorum* (Bryner et al. 2012; Chu et al. 2002; Hillman and Suzuki 2004; Pearson and Bailey 2013; Xie and Jiang 2014). This phenomenon is already used to infect *C. parasitica* isolates by mycovirus *Cryphonectria hypovirus 1* (CHV-1) and spread it into the natural populations to reduce fungus parasitic growth and sporulation capacity (Rigling and Prospero 2018).

Recently, it has been noted that mycoviruses are prevalent in endophytic fungi populations (Bao and Roossinck 2013). As with plant pathogenic fungi, dsRNA viruses are dominant in endophytic fungi (Bao and Roossinck 2013). Endophytic fungi are classified, according to their life histories, into Class 1, 2, 3, and 4 endophytes (Rodriguez et al. 2009). Since many of these viruses are persistent in their fungal hosts, it could be inferred that they probably play different roles to benefit host fungi.

1.3.1.1 Viral Endosymbionts of Class 1 Endophytes

Class 1 endophytes are fungal members of *Clavicipitaceae*, infecting in an obligatory and host-specific manner the grasses belonging to Poaceae and Cyperaceae (Rodriguez et al. 2009). They are often transmitted vertically through host plant's seeds and play a protective role in their host grasses against

biotic and abiotic stresses (Kuldau and Bacon 2008). Investigations on clavicipitaceous fungi *Beauveria bassiana*, *Epichloë festucae*, and *Torrubiella confragosa* have detected dsRNA viruses (Bao and Roossinck 2013; Herrero and Zabalgogeazcoa 2011; Romo et al. 2007). Studies on *Epichloë festucae virus* 1 (EfV1) have indicated that it is common in different host populations and is transmitted horizontally, most likely by asexual reproduction of the host fungus (Romo et al. 2007). Whether or not such viral associates confer any benefit to the host fungi remains to be clarified.

1.3.1.2 Viral Endosymbionts of Class 2 and 3 Endophytes

Class 2 and Class 3 endophytes are highly diverse fungi, belonging to *Ascomycota* and *Basidiomycota* (Arnold 2007; Arnold et al. 2007; Rodriguez et al. 2009). Class 2 endophytes colonize the whole plant, but Class 3 endophytes are restricted to aboveground plant tissues (Arnold 2007; Rodriguez et al. 2009). Horizontal transmission in both classes is common, but vertical transmission is also seen in the Class 2 endophytes. Indeed, depending on the ecological condition, some fungal species can be classified as Class 2 or 3.

It is found that some Class 2 endophytes confer habitat-adapted benefits to their host plants, including biotic and abiotic stress tolerances such as heat and salinity tolerances (Redman et al. 2002; Rodriguez et al. 2004, 2008; our unpublished data). Indeed, in a pioneering work, the group of Redman and Rodriguez brilliantly showed that the viral symbiont of the endophytic fungus *Curvularia protuberata*, colonizing panic grasses, was necessary to confer heat tolerance to the virus-fungus-plant symbiotic consortium (Márquez et al. 2007; Redman et al. 2002; Rodriguez et al. 2008). Indeed, the fungus was unable to confer heat tolerance to its host plant in the absence of the virus. However, virus-harboring fungus was capable of conferring heat tolerance to not only monocot but also dicot host plants (Márquez et al. 2007). *C. protuberata* had been isolated from panic grasses growing in geothermal lands, tolerating up to 65 °C (Redman et al. 2002). Further analyses revealed that the genome of the mycovirus, named *Curvularia thermal tolerance virus* (CThTV), contained two dsRNA segments. The virus could be transmitted vertically by fungal asexual reproduction with a rate of ca. 100% and horizontally by hyphae anastomosis (Márquez et al. 2007). Furthermore, the same group recovered *Fusarium culmorum* isolates from coastal dune grass, conferring salt tolerance to the host plants (Rodriguez et al. 2008). Putative dsRNA viruses have been detected in various isolates of *F. culmorum* (Herrero et al. 2009), including salt habitat-adapted isolates, but their possible benefits to the symbiotic consortium are not characterized. The group of Roossinck has shown that viruses are common in Class 2 and Class 3 endophytes (Feldman et al. 2012). Thus, future research has to investigate the outcome of viral infection of fungal endophytes for both fungi and plant hosts. This could find immediate application in fungal and plant epigenetic engineering.

1.3.1.3 Viral Endosymbionts of Class 4 Endophytes

Class 4 endophytes are mainly dark septate endophytes colonizing the roots of more than 600 plant species (Jumpponen and Trappe 1998; Sieber and Grünig 2006; Rodriguez et al. 2009). Although virology of dark septate endophytes is poorly investigated, several studies indicate the presence of virus-like particles and dsRNAs in *Phialophora* (Tel. *Gaeumannomyces*) isolates associating different plant hosts (Herrero et al. 2009; Rawlinson and Muthyalu 1974a, b, 1975; Sieber and Grünig 2006). Whether or not mycorrhizal fungi belong to the Class 4 endophytes is controversial. However, existence of dsRNA mycoviruses in mycorrhizal fungi is shown, and interestingly it is reported that viral disinfection of the mycorrhizae improves its plant growth promotion (Ikeda et al. 2012).

1.3.2 Perspective of EFV Application in Fungal Bioengineering

Plants have occupied the planet for more than 400 million years by the aid of their symbionts (Rodriguez and Redman 2008). Research over the last two decades has shown that every individual plant on the planet harbors one or more endophytes. Except for Class 1 endophytes, other endophytic fungi can colonize a diverse range of plant hosts (Higgins et al. 2011). Given the prevalence of endofungal viruses in natural populations of endophytic fungi (Bao and Roossinck 2013), and possible biotic and abiotic stress tolerances conferred by them to both host fungus and host plant in an epigenetic manner (Márquez et al. 2007; Redman et al. 2002; Rodriguez et al. 2004, 2008; Roossinck 2011a, b), it is time to think of their application in fungal epigenetic engineering for agricultural, industrial, and possibly pharmaceutical uses.

1.4 Conclusion and Prospects

Endosymbiosis is one of the key forces of evolution (O'Malley 2015). Fungi are among the eukaryotic organisms that host a diverse range of endosymbiotic bacteria and viruses. Currently, this is most evident in plant symbiotic endophytic fungi which harbor EHB and cryptic or persistent mycoviruses. As discussed, endosymbionts are master modulators of fungal host phenotypes, morphology, and reproduction, in an epigenetic manner. In the documented tripartite plant-fungus-endosymbiont consortia, it has been demonstrated that the presence of endosymbiont is vital for the activity of consortium and for the phenotypes that host plant or fungus express. It seems we are on the onset of a flourishing research agenda on fungal endosymbionts, and this will cover biotechnological aspects of these findings, mainly epigenetic engineering of fungi phenotypes. So, future research should consider this possibility as a novel tool in fungal bioengineering.

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Chapter 2
Yeast Engineering for New Antifungal Compounds: A Contextualized Overview

Alexandre Gomes Rodrigues

2.1 Introduction

The human body hosts trillions of microorganisms, and still, we manage to find equilibrium or paths that keep us healthy and the diseases at bay (Scorzoni et al. 2017). However, immunocompromised hosts, e.g., HIV and diabetes patients or those who underwent transplantation, lack the ability to avoid such threat as their defense lines are not intact (Alangaden 2011). At this point fungi and bacteria can become hostile to us, and their pathogenic behavior leads to infection, opportunistic diseases, and possibly devastating consequences, such as death.

Fungal infections, also known as nosocomial infections (when hospital-acquired), such as cryptococcosis, candidiasis, and aspergillosis, are responsible for many deaths and worsened clinical cases in humans (Kronstad et al. 2011). Despite all and every step forward to treat patients, the future does not look all positive. In the time span of 60 years, we have seen the birth of several antifungal medicines successfully introduced into the clinics, saving many lives. Yet, the threat we have been facing shadows the advances by reminding us that this case is multifaceted and will take more than good work to be handled.

From the years when amphotericin B made its way into the patient's bedside to date, several other drugs have assisted and healed patients with fungal infections (Dismukes 2000). But the persistence and evolution that nature follows have been a constant and implacable challenge that defies the ability of scientists in a battle that put millions of human lives at risk. At the moment, antimicrobial resistance is out of control, alarming patients, healthcare takers, and governmental bodies around the world. Morbidity and mortality have been taking the lives of 1.5–2 million people every year (Denning and Bromley 2015; Robbins et al. 2017). The moment has arrived, when we must look at this problem from another perspective and tackle it

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with well-designed and integrated solutions. This may be possible if we employ what has been developed at the frontiers of science, associating it with technological advances, along with the professional skills of clinicians and scientists.

In this chapter, you will find a brief story about the antifungal agents most efficiently used to treat fungal infections and how pathogens have resisted the treatment. Following that step though, you will see what and how scientists have been resiliently working to solve the puzzle, unfold the genetic secrets of antifungal resistance, and prepare highly targeted strategies that may lead to overcoming this threat.

2.2 Fungal Infections

Fungal infections on the human skin have been reported as early as 1837, whereas the description of aspergillosis came in 1856 by Rudolf Virchow (Wainwright 2008). Sixty-five years have passed since the introduction of nystatin to the market in 1954 (Vicente et al. 2003). It started an era of lifesaving therapy for fungal-infected patients. Since then, several other antifungal agents have come into usage.

However, as paradoxical as it can be, the use of these medicines to save lives has also caused the appearance of antifungal resistance (Ostrosky-Zeichner et al. 2016). *Aspergillus*, *Candida*, and *Cryptococcus* are the leading pathogens when it comes to fungal species involved in as much as 90% of fungal-related infections (Pu et al. 2017; Robbins et al. 2017; Van De Veerdonk et al. 2017). Although the number of deaths caused by severe infections is astonishing, the recognition of such urgency has not been sufficient, with no high investment made by the involved bodies (Brown et al. 2012). The severity and incidence of fungal infections vary according to several factors, such as socioeconomic conditions, geographic region, and cultural habits (Brown et al. 2012; Perlin et al. 2017). Table 2.1 illustrates some of the most common fungal infections observed worldwide.

Apart from that, oral infections are also common in babies and denture wearers, in asthma patients under steroidal therapy, in patients suffering from leukemia and submitted to transplants, and in people who have had radiotherapy for head and

Table 2.1 Common fungal infections and the number of patients affected worldwide (Brown et al. 2012)

Fungal infection	Number of patients affected
Athlete's foot	1 in 5 adults
Ringworm of the scalp	200 million
Infection of the nails	10% of the world population
Infection of the nails	50% of the world population over 70 years and older
Vulvovaginal candidiasis	50–75% of women in childbearing years
Esophageal fungal infections	2 million (in HIV-infected patients)
Oral thrush	10 million (in HIV-infected patients)

neck cancers and transplantation (Havlickova et al. 2009; Brown et al. 2012; Omrani and Almaghrabi 2017; Kabir et al. 2018). In low-birth-weight infants, the mortality due to mycosis may reach 34% of the patients (Puia-Dumitrescu and Smith 2017). Invasive fungal infections affect fewer patients than bacterial infections, but with devastating effects and high mortality rates. Even though the number of reported deaths is between 1.5 and 2 million per year, this count may be underrepresented considering the manner the reporting is made and the fact that misdiagnosing may be occurring due to lack of accurate data (Brown et al. 2012; Revie et al. 2018).

Fungal infections are caused by a series of fungal species and threaten patients who are immunocompromised, such as HIV-infected and postoperative patients. The medicines used to treat such infections are not as efficient as they used to be in the past, as the pathogens become resistant. Besides that, the efficacy of the therapy is complicated by the fact that fungi are close to humans in evolutionary terms (Srinivasan et al. 2014).

2.2.1 *Antifungal Agents*

Fungi are eukaryotic and present ergosterol in their fungal cell wall, as opposed to cholesterol synthesized in the human body, with only slight chemical differences on their structures (see Fig. 2.1) (Carmona and Limper 2017). Most antifungal agents target the cell membrane of the pathogens in different ways (Vicente et al. 2003; Rautenbach et al. 2016). The following sections will briefly describe the main antifungal agents brought to the market and successfully used to treat different fungal infections.

2.2.2 *Polyenes*

Nystatin was the first polyene to treat fungal infections. It was discovered by Elizabeth Lee Hazen and Rachel Fuller Brown in the 1950s while working at the Division of Laboratories and Research of the New York State Department of Health

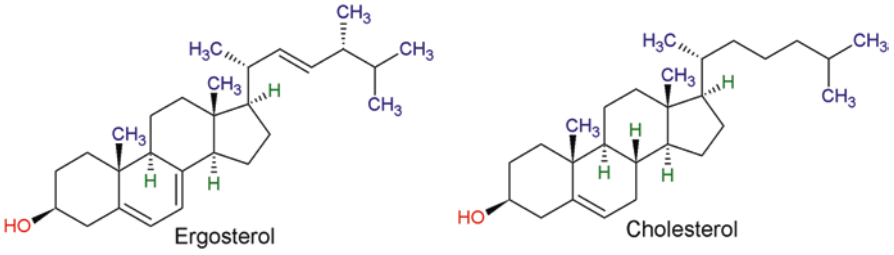

Fig. 2.1 Chemical structures of ergosterol (**a**) and cholesterol (**b**)

(Wainwright 2008). It was isolated from *Streptomyces noursei* and the first antifungal to enter the market in 1954 by E.R. Squibb & Sons (today Bristol-Myers Squibb R&D) under the name of Mycostatin®.

Following that, amphotericin B (AmB) was developed in 1959 as a natural product isolated from *Streptomyces nodosus* and has been used as a broad-spectrum antifungal to treat patients with *Candida* spp. and *Aspergillus* spp. infections, as well as several other fungemias. It binds to ergosterol and disrupts the fungal cell membrane, leading to fungal death. However, AmB is poorly soluble and has been successfully administered in liposomal formulations (Lanternier and Lortholary 2008). Liposomal formulation presents relevant side effects though. The most concerning is nephrotoxicity (Puia-Dumitrescu and Smith 2017). Yet, it is the preferred medicine for antifungal infection during pregnancy. Because ergosterol has different chemical properties from cholesterol, it has been exploited as a target for polyene-compound agents, such as amphotericin B, which are incorporated into fungal cell membranes containing ergosterol but are less readily incorporated into host cell membranes, which contain cholesterol. Amphotericin B, in association with ergosterol, is thought to form membrane-spanning channels with hydrophilic interiors that allow the leakage of essential components, which ultimately results in fungal cell death (Anderson 2005).

2.2.3 Azoles

Azole antifungals have been tested since the 1960s as an important class of molecules in the treatment of mycoses caused by *Candida* species and a unique option for oral therapy of systemic fungal infection (Maertens 2004; Flowers et al. 2015). Azoles can be divided into three generations. The first generation comprises fluconazole and itraconazole, available for oral and parenteral administration. The second generation includes voriconazole and posaconazole, with ravuconazole belonging to the third azole generation (Fig. 2.2).

Azoles are synthetic agents divided into imidazoles and triazoles (Maertens 2004) and inhibit the enzyme lanosterol 14-α-demethylase (CYP51A1), which plays an important role in the conversion of lanosterol to ergosterol (Waterman and Michael 2008). This class of fungistatic compounds is the most used antifungal agents for invasive and superficial infections (Flowers et al. 2015). This same fact possibly led to the resistance of azole antimicrobials in filamentous fungi and *Candida* yeasts. In yeasts, three main mechanisms have been described as responsible for the resistance to the azole therapy: (1) point mutation of the *ERG11* gene, (2) overexpression of gene *ERG11,* and (3) overexpression of efflux membrane transporters. Evolutionary studies have been important to establish the key factors in the development of antifungal agents (Song et al. 2018).

Ketoconazole, an imidazole developed by Janssen Pharmaceutica, was approved in 1981 against systemic fungal infections and remained the therapeutic choice until the introduction of triazoles a decade later. As a fungistatic drug that presented no

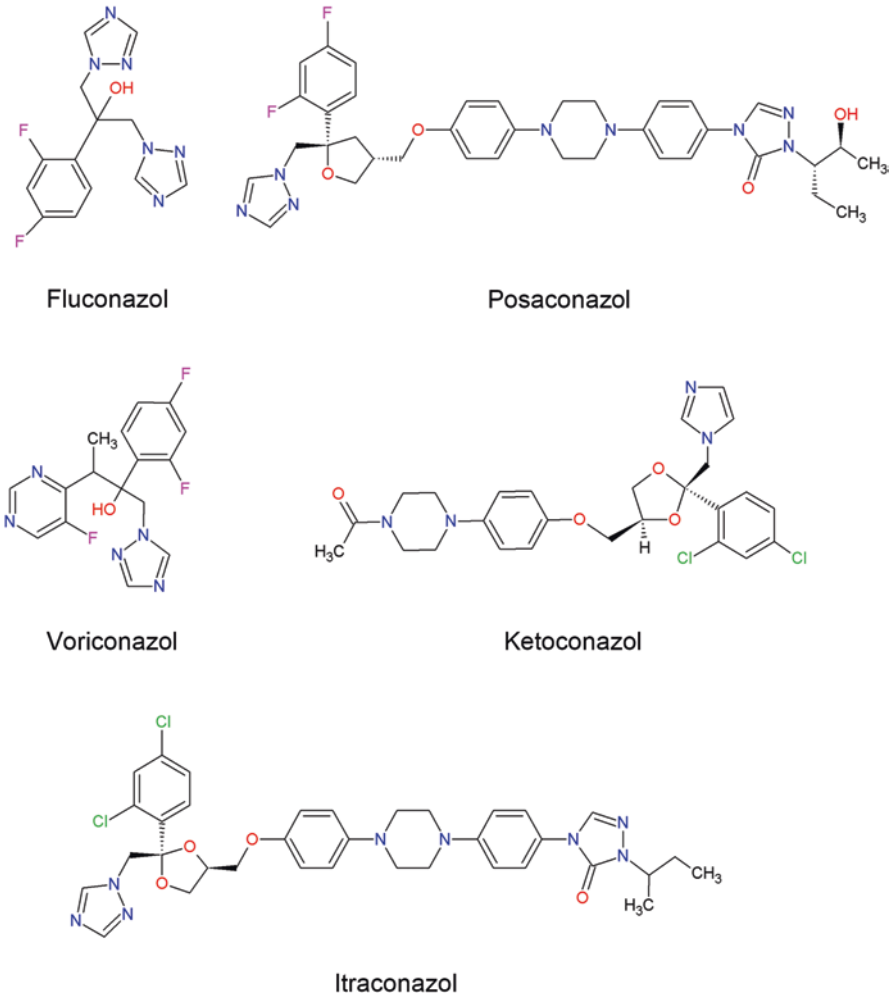

Fig. 2.2 Chemical structures of azoles of different generations

penetration to the blood-brain barrier and was sensitive to gastric pH variations (among other factors), ketoconazole was not an optimal choice for a range of patients (Maertens 2004).

Fluconazole (a triazole) is one of the preferred azoles in the clinic. It is available in oral and i.v. formulations and belongs to the first generation of triazoles. It is a broad-spectrum triazole developed by Pfizer and approved by the FDA (Food and Drug Administration) in the 1990s (Maertens 2004). Due to its good bioavailability, it is one of the most used azoles, and an alternative to ketoconazole for chronic mucocutaneous candidiasis, with fewer drug interactions (Maertens 2004). It is resistant to gastric pH and food. Besides that, only 10% of the protein bound to it. Despite its efficacy, several targets have been identified as antifungal resistance.

More recently, the wide use of fluconazole has contributed to fungal resistance to this compound.

Itraconazole, used for the treatment of oropharyngeal and esophageal candidiasis in adult HIV-infected patients or other immunocompromised patients, was approved by the FDA in 1992 (Maertens 2004). It has been replacing ketoconazole and presents advantages over fluconazole in aspergillosis and sporotrichosis treatments (Maertens 2004). Itraconazole is extensively metabolized in the liver, primarily by cytochrome P450 3A448 and presents a variable oral absorption. The clinical use of itraconazole is primarily limited to the treatment of fungi that do not cause central nervous system (CNS) disease. Routine drug monitoring should be considered, whereas the use of itraconazole in infants is still not recommended due to lack of efficacy and safety data (Puia-Dumitrescu and Smith 2017).

Voriconazole, structurally related to fluconazole, was developed by Pfizer. It displays wide-spectrum in vitro activity against fungi from all clinically relevant pathogenic groups such as *Candida* spp., *Aspergillus* spp., and *C. neoformans*. Compared to reference triazoles, voriconazole is several-fold more active than fluconazole and itraconazole against *Candida* spp. However, *C. albicans* isolates with decreased susceptibility to fluconazole and itraconazole also demonstrate significantly higher MICs for voriconazole. The medicine is orally and parenterally active but exhibits complex pharmacokinetics. Animal studies have revealed good penetration into the cerebrospinal fluid (CSF) and central nervous system (CNS). The promising in vitro activity has been confirmed in a range of infections in immunosuppressed animal models where voriconazole proved to be more effective than amphotericin B, fluconazole, and itraconazole. Data from phase II and III clinical trials indicate that voriconazole is a promising agent for the treatment of oropharyngeal candidiasis in AIDS patients, esophageal candidiasis, and acute and chronic invasive aspergillosis, including cerebral aspergillosis (Maertens 2004).

Posaconazole, a hydroxylated analog of itraconazole developed by Schering-Plough, is a broad-spectrum compound against opportunistic and endemic fungal pathogens, including zygomycetes and some of the dematiaceous molds. In vitro, it is highly active against *Aspergillus* spp. and at least eightfold more potent than fluconazole against *Candida* spp. Posaconazole was more effective than amphotericin B, fluconazole, and itraconazole in animal studies (Maertens 2004). Ravuconazole, another derivative of fluconazole developed by Bristol-Myers Squibb, represents the third second-generation triazole. Ravuconazole has a broader antifungal spectrum than fluconazole and itraconazole, particularly against strains of *C. krusei* and *C. neoformans* (Maertens 2004).

2.3 Echinocandins

They are a class of antifungal that act by disrupting the fungal cell wall by inhibiting the (1,3)-ß-D-synthase complex, which is an important compound in the ß-glucan synthesis (Vicente et al. 2003) (general structure in Fig. 2.3). ß-Glucan varies from

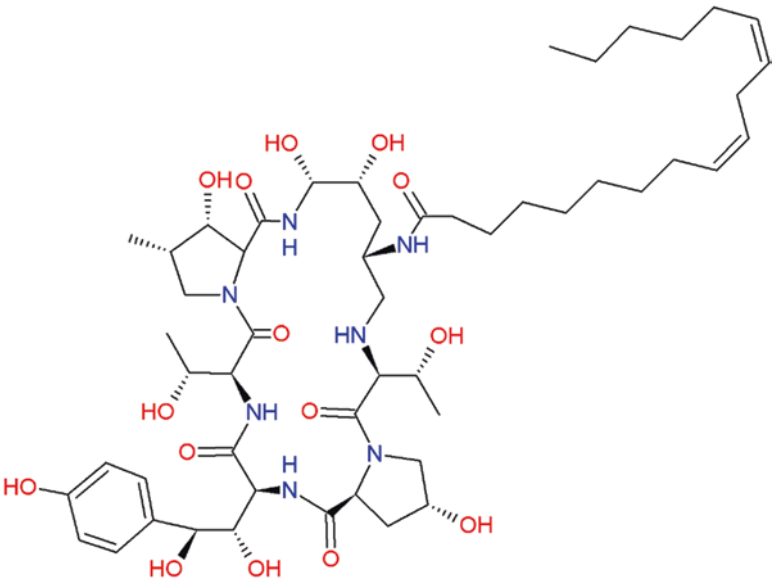

Fig. 2.3 Echinocandin general structure

30% to 60% in the yeast cell wall. By acting on this molecule, echinocandins present a fungicidal effect on yeasts (Nailor and Sobel 2006). In filamentous fungi, ß-glucan is present in the hyphae, and, as they are blocked by the antifungal, the pathogen suffers a fungistatic effect (Aminov 2017). The echinocandins are large, semisynthetic lipopeptide molecules that noncompetitively inhibit the (1,3)-β-D-glucan synthase enzyme encoded by the FKS1 gene. They are fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp. (Denning 2003). No drug target is present in mammalian cells. The first of the class to be licensed was caspofungin, for refractory invasive aspergillosis (Denning 2003).

Echinocandins are the newest class of antifungals, with caspofungin, micafungin, and anidulafungin approved by the FDA for i.v. administration (Kartsonis et al. 2003). The mechanism of action is characterized by inhibition of the b-(1,3)-D-glucan synthase complex, which disrupts cell membrane permeability. Given this unique pathway, they are considered the safest antifungals at the present. Similar to the polyenes, these agents do not penetrate cerebrospinal fluid but can penetrate brain parenchyma. Echinocandins have a broad spectrum of coverage consisting of fungicidal activity against most *Candida* spp., including fluconazole-resistant species, low toxicity, and minimal drug interactions. In the 2016 Infectious Diseases Society of America candidiasis guidelines, echinocandins were the primary medicines of choice for the treatment of invasive candidiasis. Echinocandins However, they are not active against *Cryptococcus*, dimorphic fungi, or *Zygomycetes* (Puia-Dumitrescu and Smith 2017). They share properties with respect to azoles while presenting fewer drug interactions (Nailor and Sobel 2006).

Caspofungin is one classical example of approved echinocandin from Merck & Co., Inc. discovered by James Balkovec, Regina Black, and Frances A. Bouffard (Maertens and Boogaerts 2003; Lacroix et al. 2003). Caspofungin is a lipopeptide and fungicidal agent against *Candida* spp. with a concentration-dependent effect and up to 12 hours' post-antifungal effect. Caspofungin has hepatic metabolism, with a terminal half-life of approximately 10 hours. Its efficacy for invasive candidiasis in infants with less than 3 months of age is limited and has been shown to achieve exposures similar to adults. Caspofungin is well tolerated in this age group, and the most common adverse effects include fever, hypokalemia, and elevated liver enzyme concentration (Puia-Dumitrescu and Smith 2017).

Mutations in the FKS1 gene lead to alterations in the conformation of the encoded enzyme, resulting in lower affinity between FKS1 and echinocandins and consequently resistance to these drugs (Gonçalves et al. 2016). Mutations in two hotspot regions of FKS1 are conserved in clinical isolates of *C. albicans*: there on between 641–648 (comprising a cytoplasmic domain/binding site of echinocandins) and 1345–1365 are hotspot 1 (HS1) and hotspot 2 (HS2), respectively (Scorzoni et al. 2016).

The medicines in Fig. 2.4 are used to treat fungal infections as well. All of them are on the list of the World Health Organization (WHO) for essential medicines. Flucytosine was first produced in 1964 and is commonly administered with amphotericin B to treat cryptococcosis (Vicente et al. 2003). It is used to treat athlete's foot mycosis. Griseofulvin is used for dermatophytosis treatment. It was discovered in 1939 and is used in the therapy of ringworm and the scalp (Petersen et al. 2016). Terbinafine was discovered in 1983 and is employed against pityriasis versicolor and nail infections in oral and topical formulations, being more effective than griseofulvin; it belongs to the allylamine class of antifungals (Abdel-Rahman and Nahata 1997). Despite their successful use, these medicines also face therapeutical challenges and restrictions.

The cell wall of fungi is well equipped and may sense slight changes in the environment (Van De Veerdonk et al. 2017), enabling the microorganism to detect pH variations, reactive oxygen species (ROS), and antifungal agents. With that information the pathogen neutralizes the threat with an arsenal that includes highly specialized enzymes and secreted toxins to circumvent the danger, resisting the

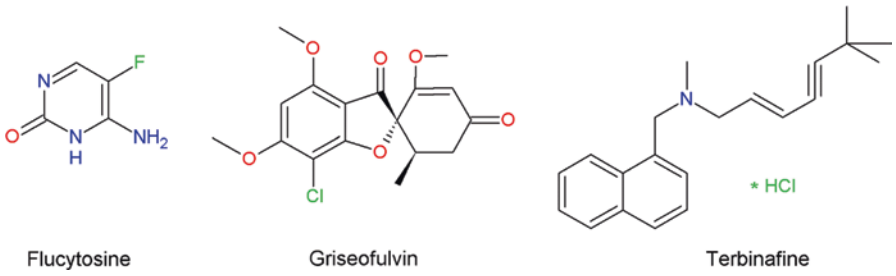

Fig. 2.4 Chemical structures of flucytosine, griseofulvin, and terbinafine

treatment by adapting and developing strategies to escape the host immune system. Azoles have been a successful class of antifungal agents but have faced resistance due to the spread use it has been undergone. Therefore, a detailed understanding of its mechanisms is imperative to design specific and efficient therapeutics (Morio et al. 2010).

2.3.1 Antifungal Resistance

The antifungal drugs currently on the market target the cell wall of the fungi in several ways. The indiscriminate use of antifungal and increase use of implantable devices have prompted the resistance (Reyes-Montes et al. 2017). The incidence of candidemia seems to be variable, with Latin America, the USA, Spain, and Denmark leading the cases, when compared to Australia, Canada, and Europe (Quindós 2014). Researchers have focused their attention on the biofilm formation to discover mechanisms by which antifungal drugs could be developed and contribute to reducing drug resistance (Pierce et al. 2013; Perlin et al. 2017). Such concerns and facts have been shown in different countries as evidenced by epidemiological data.

In Norway, from 1993 to 2003, Sandven et al. (2006) determined that candidemia occurred in 1348 patients, with high incidence in patients aged <1 and > 70 years and *C. albicans* as a predominant species followed by *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. A more comprehensive study was performed from 1991 to 2012 (Hesstvedt et al. 2015). The researchers analyzed 1724 samples from 1653 patients. After a period of 22 years, the incidence of candidemia increased from 2.4 (1991–2003) to 3.9 (2004–2012) per 100,000 inhabitants. After investigating 89 patients with invasive candidiasis at two hospitals in Switzerland, Osthoff et al. (2016) found out that *C. albicans* predominated as the main isolated yeast, as described in many other studies. Yet, despite the attempt to determine the association of mannose-binding lecithin in patients with invasive candidiasis, inconclusive results did not allow to precisely distinguish between the *Candida* colonization and lecithin pathway proteins levels. In Istanbul, Turkey, Sutcu et al. (2016) established species distribution of *Candida* isolates from 134 pediatric patients admitted in teaching hospitals between June 2013 and June 2014, as well as determined the risk factors associated with nosocomial *Candida* infections. The investigation revealed that *C. albicans* and *C. parapsilosis* were the most present yeasts with a prevalence of 13.4% and 8.2%, respectively.

In Spain Nieto et al. (2015) found 705 documented cases of invasive candidiasis during 12 months between 2011 and 2012. *C. albicans* remained the most prevalent but non-*C. albicans* increased. In Mexico, the number of *Candida* infections between 2005 and 2015 reached 8490 as described by Reyes-Montes et al. (2017). France is estimated to have over 968,000 patients suffering from a serious fungal infection during the course of each year according to Gangneux et al. (2016). Salari et al. (2017) reported 1782 cases in the Iranian Province of Kerman from 2004 to 2014 (Mahmoudi et al. 2011). In the Chinese Hospital of Chongqing Medical

University, Sun et al. (2015) detected 297 cases of *Candida* infections from 2011 to 2013, with *C. albicans* being responsible for most of the isolates and *Candida* causing 86% of the fungal invasive infections. In Poland, Małek et al. (2017) investigated 132 *Candida* isolates from a center in the intensive care unit in Krakow. *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* made up a significant amount of the infections. South Korea had 521 cases of invasive fungal infections reported in patients under hematopoietic stem cell transplant, corresponding in a multicenter observational study, highlighting the need for a personalized therapy (Choi et al. 2017). Guimarães et al. (2016) reported an epidemiological study on solid organ transplantation (SOT) and how it was associated with invasive fungal disease (IFD) in a hospital in Rio de Janeiro, Brazil. The study was carried out between 1998 and 2009 with a follow-up until 2015, in 908 patients. In this case, *Cryptococcus* spp. dominated the number of infections being responsible for 36% of the cases.

Odds et al. (2007) conducted a 1-year pilot prospective survey throughout hospitals in Scotland between March 2005 and February 2006. They collected and investigated 300 isolates from 242 patients. Once again, *C. albicans* predominated species among the isolates, followed by *C. glabrata* and *C. parapsilosis*. Later on, an article published in 2015 described another nationwide study in Scotland, with 280 isolates from 217 patients (Rajendran et al. 2016). The study was conducted between 2012 and 2013, with special attention to the impact of biofilm formation. They observed that, from the available data on mortality of 134 patients, 41% passed away. *C. albicans* remains the main pathogen responsible for around 50% of those cases. All in all, the incidence in Scotland dropped from 4.8 (2005–2006) to 4.1 (2012–2013) per 100,000 people each year.

In 2005, Arendrup et al. (2005) documented the number of fungemia episodes in Denmark for 12 months between 2003 and 2004. Data from earlier studies had reported a continuous increase in candidemia cases in that country in the early 1990s. In the report of 2005, after performing a semi-national surveillance study (covering 53% of the Danish population), they found that 97.4% of the 303 episodes from 272 patients were from *Candida* species and that Denmark presented greater number of fungemia than most of the other countries, considering an estimation of 11 cases in every 100,000 against much lower numbers, such as 2.17 in Norway, 3.5 in Sweden, or 6–8 in the USA published during the 1990s. The study also included susceptibility tests. Together with the results, they also detected that there was a 44% increase in the use of fluconazole between 1999 and 2003 and 152% between 2001 and 2006. In the following study, Arendrup et al. (2008) reported data from the period of 36 months from 2004 to 2006 of fungemia in Denmark. This time, 1089 cases from 1040 patients were registered, which points to 10.4 cases in every 100,000 inhabitants and *Candida* species remained as the main pathogen. At that point, no clear reason was suggested as the remarkedly high rates of fungal infection among the Danish population compared to the global numbers. In an attempt to uncover the underlining reasons for the high incidence and mortality, Lausch et al. (2018) conducted a comprehensive study including 912 episodes out of 882 patients for 24 months between 2010 and 2011. The researchers concluded that one possible explanation for the high incidence of fungal infections in Denmark

was the increasing number of severely ill patients with prolonged supportive treatment, with more than 40% of all cases found in the intensive care units (ICU).

Gugnani and Denning (2016) estimate that about 220,000 people suffer from serious fungal infections per year. These cases appear to be related to HIV and tuberculosis-infected patients. These are important parameters taking into account that the Dominican Republic, which is located within the Caribbean region, contributes to the highest prevalence of HIV after the sub-Saharan Africa and the highest rate of TB in the Americas. Besides candidiasis, aspergillosis and cryptococcosis prevalence are also described in the DR population. Taiwan had 2856 cases reported between 1994 and 2005. *C. albicans* predominated there once more as the main *Candida* species (>50%). With a mortality rate between 35 and 60%, candidemia has been reported as a serious healthcare problem in the country. Moghnieh et al. (2017) also described invasive fungal infection (IFI) due to candidiasis and aspergillosis information in Lebanon and Saudi Arabia between 2011 and 2012, covering 207,498 discharges. *C. albicans* was responsible for 56% of the cases, whereas *A. fumigatus* was responsible for 60% of the cases. The investigators concluded that there is a significant burden of such serious infections in the Middle East. It also represents a chance for hospitals to improve the delivery of patient care for serious infections by promoting early diagnosis and appropriate antimicrobial therapy.

Researchers have also driven attention to the fact that fungal infections involve different pathogens according to patient age range and geographical location (Quindós 2014; Antinori et al. 2016). Fungal infection outbreaks have been registered in other places as well, and having detailed surveillance and vigilance is a key measure to help the decision-making in such cases (Benedict et al. 2017).

The rate of antimicrobial-resistant pathogens is increasing at a pace it has been challenging to keep up with (Srinivasan et al. 2014). Under strong global directives, scientists at research institutes, universities, and companies have been working to bring contributions that may help in this theme (Fisher et al. 2018). The World Health Organization has warned about how deep and how dangerous antimicrobial resistance has become. Several leading institutions, such as Stanford University's scientists, started launching initiatives to reach society and penetrate into levels beyond those of science and business. Multidisciplinary teams have been involved in managing the therapy in some studies and evidenced the need for a specialized group to deal with and reduce the inappropriate use of antifungals (Poulat et al. 2017).

Antifungal resistance has become a significant concern to clinicians who are charged with caring for patients at high risk for invasive mycoses (Vanden Bossche et al. 1998). Resistance to currently available antifungal agents can develop secondary to acquired mechanisms following exposure to these drugs. Recent trends in acquired antifungal resistance include increased azole resistance among non-*Candida albicans* isolates, azole resistance in *A. fumigatus*, and echinocandin resistance in *C. glabrata*. In contrast, some fungal species are intrinsically resistant to certain drugs (e.g., *C. krusei* and fluconazole or *C. lusitaniae* and amphotericin B), while others demonstrate microbiologic resistance to all clinically available antifungals (e.g., *Lomentospora* [formerly *Scedosporium*] *prolificans* and *Fusarium solani*). New species are also emerging that may demonstrate resistance to multiple

class of available agents (e.g., *C. auris*). The prevalence of antifungal resistance is not as high as that observed for bacteria against antibiotics, but the treatment available for invasive fungal infections is not efficient enough, and more vulnerable patients often have multiple comorbidities, including immunosuppression, which may limit the effectiveness of therapy even in the absence of drug resistance. Clearly, new treatment strategies are needed to address this issue, in addition to overcoming the toxicities/adverse effects and drug interactions that are associated with currently available antifungals, which themselves can limit the effectiveness of therapy. New antifungals are currently being investigated that may help to address the problem of antifungal resistance (Wiederhold 2017).

The evolution of antimicrobial drug resistance is an almost inevitable process that is ubiquitous in the microbial world. Although the development of antifungal drug resistance has not paralleled their antibacterial antibiotic counterparts, the economic facets associated with fungal infections remain unacceptably high. In addition to this, the arsenal of antifungal agents is extremely limited; therefore, overcoming antifungal resistance can be considered as the mainstay for improving therapeutic strategies to treat mycoses. One of the major factors exacerbating antifungal drug resistance is the inappropriate use of antifungals, forcing the fungi to evolve against selective pressure (Poulat et al. 2017). Numerous studies have elucidated the association of improper antifungal drug exposure and the emergence of resistance. Thus, antifungal therapy should employ effective agents to alleviate the promotion of drug resistance. Furthermore, the dose and spectrum of action of the antimicrobial agent have important ramifications because of their potential effects on the human microbiome (Kronstad et al. 2011). It has been identified that the imbalance in the host microbial diversity imparts deleterious effect and sometimes the development of tolerance in less-susceptible fungi. Considering these factors, the potential strategies to embark in order to overcome antifungal resistance will be discussed further (Pierce et al. 2013).

Despite all efforts, the situation is near to despair, and an outbreak would cause a disaster in many regions with a number as high as 1.2 billion fungal infections worldwide as we strive to understand and fight such pathogens (Enkler et al. 2016; Kontoyiannis and Lewis 2017). To cope with such a threat, innovative strategies and a deep understanding of the mechanisms that lead to resistance are in urgent need (Van De Veerdonk et al. 2017). In that manner, we may have chances of developing a tailored solution to halt the advance of super-resistant pathogens that have been putting humans' lives at risk.

The wide use of antifungals has contributed to the development of resistance and the mechanisms of this resistance have been investigated, as in the case of azoles (Morio et al. 2010; Noël 2012). Not only azoles are susceptible to fungal resistance, and methods to test fungal resistance have also been described (Marichal et al. 1999; Lelièvre et al. 2013; Morace et al. 2014; Matzaraki et al. 2017). To face this challenge and save human lives, antifungal agents have been developed and applied successfully. With a market value of over 9.8 billion dollars a year in 2009, it is supposed to have reached over $ 11 billion in 2017 (https://www.grandviewresearch.com/industry-analysis/antifungal-drugs-market) and over $ 13 billion in 2018, with

azoles and echinocandins clearly dominating the sales (https://www.bccresearch.com/pressroom/phm/global-market-for-human-antifungal-therapeutics-to-reach-nearly-$13.9-billion-in-2018).

2.4 Recent Research: Yeast Engineering

As it has been exposed, the epidemiological data clearly show that a change of direction must be taken and an assertive and accurate approach has to be followed. It has got to a global scale and the World Health Organization has been calling for a triad solution. This solution involves a set of three main steps, first targeting the development of new antimicrobial agents, second directed to the rational use of antimicrobial agents and conscious prescription by physicians, and third focused on public outreaching, informing, and involving the society on the rational and precise use of medicines as illustrated in Fig. 2.5.

Less progress has been made in the development of new antifungal agents, in contrast to antibacterial research, which has been justified by the low occurrence of fungal infections. However, the current increase in the incidence of fungal infec-

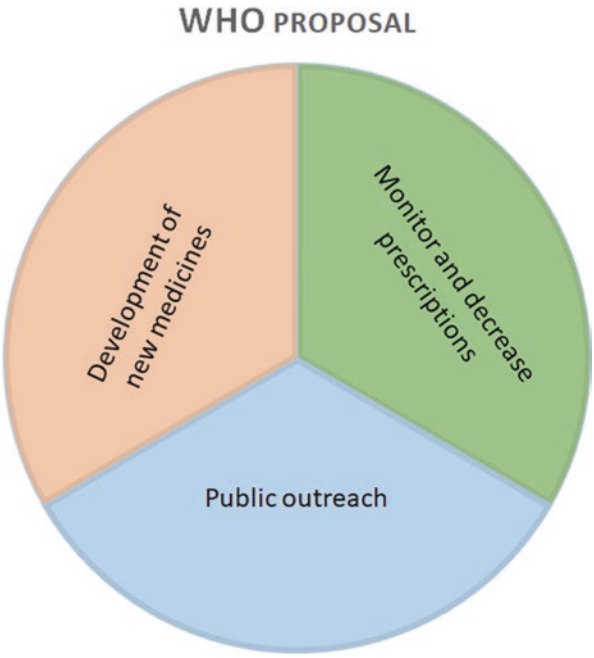

Fig. 2.5 Scheme of the proposed solution by the World Health Organization (WHO) to deal with the antimicrobial resistance

tions has led to aggressive research on new antifungal agents as highlighted by the increasing number of publications since the 1960s (Scorzoni et al. 2016).

Metabolic engineering of microorganisms is developing into a major field in biotechnology. Metabolic analyses of cells to identify the most promising targets for manipulation are followed by genetic engineering of cells. The goals of metabolic engineering are: the optimization of strains for overproducing recombinant proteins and small chemical molecules; extension of substrate range; enhancement of productivity and yield; elimination of by-products; and improvement of process performance of cellular properties (de Backe and van Dijck 2003). Regardless of the desired product, the common aim of metabolic engineering is to transfer product-specific enzymes to microorganisms in order to generate an optimized biosynthetic pathway in terms of minimal cost and maximum productivity (Wriessnegger and Pichler 2013). One of the systems applied to manipulate the yeast genome is CRISPR-Cas9 (*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats) (Raschmanová et al. 2018). Several genes have been investigated as potential targets for antifungal therapy (Sanglard 2002; Nicola et al. 2018).

In *C. albicans*, modulation of the *ERG11* gene in the ergosterol biosynthesis pathway and alteration of the *ERG11* protein targeted by azole antifungals have been shown to contribute to azole resistance (Flowers et al. 2015). Eager to unfold the secrets of antifungal resistance, researchers use genomic techniques to investigate such a phenomenon. Flowers et al. (2015) investigated the overexpression of *ERG11* and overexpression of efflux pump genes *CDR1, CDR2,* and *MDR1* in *C. albicans* resistance to fluconazole. The overexpression of *ERG11* could be explained by a gain-of-function mutation in UPC2 451 in many but not all cases. Gain-of-function mutations led to increased resistance to azole 452 antifungals and terbinafine, increased cellular ergosterol levels, and increased expression of genes 453 and 454 involved in the ergosterol biosynthesis and oxidoreductase activity, as well as the transporter genes *MDR1* and *CDR11*.

Later, they constructed a mutant *ERG11* to understand *C. albicans* resistance to azoles. They found that mutations in the *ERG11*gene have been shown to be significant and 416 prevalent mechanism of resistance in *C. albicans*. Notably, in addition to carrying mutations in 417 *ERG11*, 20 of the clinical isolates also carried activating mutations in *UPC2* which 418 have been shown to have a combinatorial effect on azole susceptibility. Data 419 demonstrate that many *ERG11* mutations result in fluconazole resistance, but most are not as 420,421 significant when tested against voriconazole or itraconazole. Susceptibility to itraconazole, in particular, seems to be less affected by *ERG11* mutations which produce significant resistance to fluconazole. Despite that general observation, they have identified a specific combination of 423 amino acid substitutions that significantly reduce itraconazole and voriconazole susceptibility (Flowers et al. 2015).

A tool to edit genomes was designed by Xie et al. (2014). The novel open-source application named sgRNA-cas9 contains seven Perl (Practical Extraction and Report Language) scripts that can be reliably used to design scored sgRNA expression vectors. Equipped with such tools, researchers increase the ability to understand the mechanisms of biological processes and construct tailored biological systems to

mutate genes and delete and insert DNA, ultimately being used to correct genetic diseases. In this regard, the advantages of such a system, as displayed by the authors, would be the specificity and the short time required to accomplish the task. Another aspect of the tool created by Xie et al. was the range of possible tasks performed, with other online tools (e.g., Cas9 Design, gRNA, ZiFit, E-CRISP) having limitations as only predicting off-target sites.

Vyas et al. (2015) described a *C. albicans* CRISPR system that overcomes many of the obstacles to genetic engineering in this organism. The high frequency with which CRISPR-induced mutations can be directed to target genes enables easy isolation of homozygous gene knockouts, even without selection. Moreover, the system permits the creation of strains with mutations in multiple genes, gene families, and genes that encode essential functions. They were able to produce mutants sensitive to fluconazole by the upregulation of drug pump, which is one of the key mechanisms of azole resistance.

Varshney et al. (2015) investigated the AGC kinase Sch9 regulate filamentation in *C. albicans*. Here, they showed that Sch9 binding is most enriched at the centromeres in *C. albicans*, but not in *Saccharomyces cerevisiae*. Moreover, deletion of CaSch9 leads to a 150- to 750-fold increase in chromosome loss. It was important to confirm the role Ssch9 plays in the chromosome segregation process.

Accoceberry et al. (2018) genetically modified *Candida lusitaniae*, an opportunistic pathogen, and used it as a cellular model to assess the impact of lanosterol C14 alpha-demethylase *ERG11* mutations on azole resistance. The resistance to the action of azoles is caused by allelic variability and missense mutations of *ERG11*, among other molecular mechanisms. They chose *C. lusitaniae* because it is susceptible to azoles and is easily modified genetically and by using molecular modeling. Besides that, *C. lusitaniae* is in an interesting phylogenetic position, close to a node that separates two lineages of pathogenic yeasts. By targeting allelic replacement at the *ERG11* locus to validate the replacement, they expressed the clinically retrieved alleles from *Candida lusitaniae* into *C. albicans* and *Kluyveromyces marxianus*, with and without mutations. In several results, the study provides findings pointing to the differences in systems used in different works and the lack of support to compare the results due to the parameters applied using species. They were able to show that the amino acid was responsible for the fluconazole resistance and that the alleles played an important role in that event. Moreover, the resistance mechanism to fluconazole was characterized by its phenotype in a species-specific case and in several yeasts' species. The researchers still point to the changes the mutations may cause in the biology of the targeted yeasts, with changes in the amino acids possibly leading to changes in the resistance to azoles, as a call for one aspect that should be better investigated. Finally, there is a proposal for *C. lusitaniae* to be used as an expression system in functional characterization of interactions.

The group of Professor Jossinet (Enkler et al. 2016) studied the mutations in *C. glabrata*, a highly relevant pathogen associated with higher mortality and more resistant to antifungal therapy when compared to *C. albicans*. Some of the mutants were tested regarding their virulence in vivo in *Drosophila melanogaster* infection model. The results indicate that *yps11* and a serine/threonine kinase play a role in

this process. As reinforced by the authors, understanding the pathogen enables to draw accurate paths to hold such aggressive pathogen. *C. glabrata* behaves in a stealth way to develop its processes.

In a recent review, Salci et al. (2018) described important characteristics that are relevant to understanding how antifungal resistance happens. They included null mutants, virulence, cytolocalization, co-regulatory genes, and compounds that are related to protein expression, as key factors.

2.5 Conclusions

In the space of nearly one century, our knowledge and skills about antifungal agents, fungal infection, and the paths underlying these phenomena have been uncovered in greater and greater details. In the process to find the answers, we have got to a point where our capacity has been challenged by the evolutionary path nature takes. At the same time that we need microorganisms to survive, we must avoid the pathogenic effects they may exercise on the human organism. The threats that fungal pathogenic species have increasingly imposed on us, have led scientists to the edge of the knowledge and contributed to a better understanding about a deeper layer on the genetic level, to develop tools that will enable us to draw a strategy and prevent the loss of human lives by an antimicrobial resistance that could have devastating effects. With the work being developed, although a challenging path lies ahead, researchers will be focusing on narrowing the ability of pathogens to respond successfully to antifungal therapies and secure the lives of patients.

Apart from developing new strategies to treat fungal infections, other routes can be taken, such as vaccines, more recognition by shifting investments in mycology (Brown et al. 2012), diagnostic tests, and gathering of more accurate epidemiological data. Beyond those possible actions, an integrated solution has to be delineated, so that not only new treatments become available, but also an accurate, quick and inexpensive early diagnosis equips clinicians with tools for better therapeutic decisions, and the use of an antifungal agent be decided based on a thoughtful understanding of the patient condition together with possible drug interactions, genetic characteristics, correct posology, and correct monitoring.

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Chapter 3
G-protein-coupled Receptors in Fungi

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3.1 Introduction

A wide range of bioactivemolecules, biotic or abiotic stimuli as diverse as light, visual, protons, taste stimuli, biogenic amines, Ca^{2+}, odorants, amino acids, nucleotides, proteins, peptides, steroids, fatty acids, hormones, yeast mating factors, and even photons, transduce their extracellular signals to the intracellular environment by specific interaction with a class of G proteins coupled to receptors (GPCRs) (Maller 2003). Alfred Gilman and Martin Rodbell received the Nobel prize in 1994 for discovering GPCRs. The DNA and deduced amino acid sequences of more than 700 GPCRs are known, and all of these have stretches of 20–28 hydrophobic amino acids capable of forming transmembrane α-helices. Structural homology in the putative transmembrane regions between different members of the receptor superfamily have facilitated the molecular cloning of cDNAs encoding novel receptor sequences, and studies have highlighted the significance of discontinuous structural determinants in the definition of functional domains of these receptors (for more details, see Lismaa and Shine 1992). Josefsson (1999) suggested that the GPCRs can be classified into three superfamilies. The generation of multigene families by ectopic gene conversion (EGC) was first recognized as important for maintaining sequence identity between repeat copies of genes within the large rRNA gene cluster. The many multigene families include, for example, histones, GPCRs,

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ubiquitins, immunoglobulins, and major histocompatibility complex (MHC) genes. Functional analyses have demonstrated that the diversity of function elicited by individual neurotransmitters, hormones, etc. is at least partly derived from the existence of distinct structural receptor subtypes. Moreover, studies of the expression of cloned receptors in different cell lines have demonstrated that the functional response to receptor activation depends not only on which receptor subtype is involved, but also on the available repertoire of G proteins and intracellular effector systems. GPCRs consist of a single polypeptide that is folded into a globular shape and embedded in the plasma membrane of the cell. Seven segments of this molecule span the entire width of the membrane, which is the reason why GPCRs are sometimes called seven-transmembrane hydrophobic domain receptors (7TMs). The intervening portions loop both inside as well as outside the cell and have an extracellular N-terminus and a cytosolic C-terminus. GPCRs consist of a heterotrimer, possessing a predominantly hydrophilic guanine nucleotide-binding α-subunit (38–52 kDa), a β-subunit (35 kDa), and a γ-subunit (8–10 kDa) (Vauquelin and Von Mentzer 2007). The β- and γ-subunits are always closely associated, and the β–γ--heterodimers are presumed to be interchangeable from one G protein to another. These heterodimers are associated with the membrane via isoprenyl modifications of the γ-subunit and promote the predominantly hydrophilic α-subunit association with membranes and receptors. Generally, the α-subunits constitute the receptor-recognizing part of the G proteins and are largely involved in the recognition of effector components, which explains why the identity of a G protein is determined by the identity of its α-subunit. Based on the sequence of the α-subunits, G proteins have been grouped into four families: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$, encoded by 16 genes (Vauquelin and Von Mentzer 2007). Repeated stimulation of a GPCR with its agonist over minutes results in a response that is decreased compared to the initial response. These effects that limit repeated GPCR activation are referred to as desensitization (Rajagopal and Shenoy 2018).

In higher plant genomes, only a single gene (or at most, a few genes) for the putative Gαs are found in contrast with the existence of a large number of genes for Gαs in mammalian genomes (23 Gα, 5 Gβ, 12 Gγ in humans). Members of each of the four families regulate key effectors (e.g., adenylate cyclase, phospholipase C, or directly regulate ion channel or kinase function) and generate secondary messengers (e.g., cAMP, Ca^{2+}, IP3) that in turn trigger distinct signaling cascades. Therefore, GPCRs are likely to represent the most diverse signal transduction systems in eukaryotic cells. Signal perception occurs at the GPCRs, which act as guanine nucleotide exchange factors (GEFs) and facilitate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on Gα (Fig. 3.1). The replacement of GDP bound to the α-subunit of the G protein by GTP after the activation of the receptor causes the dissociation of the GTP–Gα complex from the β–γ-dimer. In turn, GTP bound to the α-subunits of G protein initiates intracellular signaling responses by acting on effector molecules such as adenylate cyclases or phospholipases or by directly regulating ion channel or kinase function. The signal is turned

off when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Gα, resulting in the reformation of the inactive heterotrimer GDP–Gαβγ. During the resting phase the Gα is guanosine diphosphate (GDP) bound and the three subunits form an inactive trimeric complex, GDP–Gαβγ. The rates of GTP hydrolysis of the Gα-subunit determine the intensity of the signal. Among many regulatory mechanisms, regulators of G-protein signaling (RGS proteins, COLD1, or phospholipases, Dα1) have a key function in the tight control of GPCR-G-protein-mediated signaling by accelerating the inherent GTPase activity of Gα, causing a faster turnover of the cycle. RGS proteins are pivotal in upstream regulation of fundamental biological processes in filamentous fungi, including vegetative growth, sporulation, mycotoxin/pigment production, pathogenicity, and mating. As an example, five distinct RGS proteins are found in the *Aspergillus nidulans* genome. Some genetic studies on yeast and studies on mammalian cells suggest that β–γ-subunits of G proteins may also regulate effector pathways. GPCRs are also expressed in proliferating cells, not only in fully differentiated cell functions. GPCRs have been implicated in embryogenesis, tissue regeneration, and growth stimulation. Many ligands acting via GPCRs are known to elicit a mitogenic response in a variety of cell types. Accumulated evidence indicates that GPCRs and their signaling molecules can harbor oncogenic potential. Plants possess hundreds of membrane-localized receptor-like kinases (RLKs). Interestingly, there is a surplus of receptor-like kinases (RLKs) that provide signal recognition at the plant cell surface. RLKs have conserved domain architecture, an N-terminal extracellular domain that is involved in signal perception, one to three transmembrane regions, and an intracellular protein kinase domain that transduces the signal downstream, typically by phosphorylating the effectors. There are multiple examples of interactions between plant G-protein components and RLKs (Choudhury and Pandey 2016).

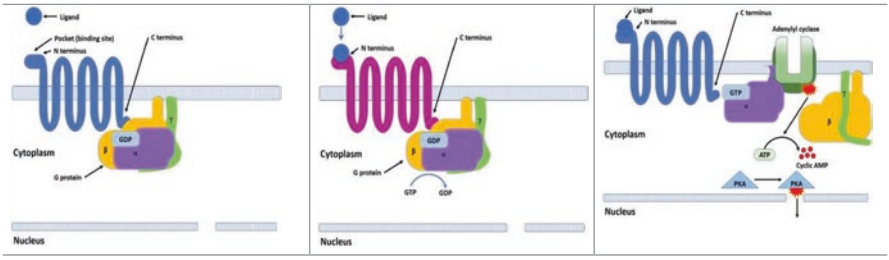

Fig. 3.1 (**a**) GPCRs consist of a single polypeptide folded into a globular shape and embedded in the plasma membrane of the cell. Seven segments of this molecule span the entire width of the membrane. (**b**) Signal perception act as guanine nucleotide exchange factors (GEFs) and facilitate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on Gα. (**c**) α-GTP carries the signal to the effector adenylate cyclase to produce cAMP

3.2 Fungal GPCRs

In fungi, G proteins are integral for cell growth and division, mating, cell–cell fusion, morphogenesis, chemotaxis, virulence establishment, pathogenic development, and secondary metabolite production. Most filamentous fungi have three conserved Gα-subunits (I, II, III), one Gβ protein, and one Gγ protein. Several studies have identified bioinformatically the GPCRs encoded by various fungi: these include *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Magnaporthe grisea*, *Cryptococcus neoformans*, *Neurospora crassa*, *Verticillium* spp., and *Trichoderma* spp. (Lafon et al. 2006). GPCRs have been divided into six families: A, B, C, D, E, and F. Among these families the following are related to fungi: family D is unique to fungi and comprises fungal pheromone receptors: fungal pheromone P-, α-factor receptors, and yeast GPR1 glucose receptors; and family E contains fungal pheromone A- and M-factor and cAMP receptors (Harmar 2001; Kulkarni et al. 2005). Han et al. (2004) identified nine GPCRs (GprA-I) in the *A. nidulans* genome, which are categorized into classes. Classes I and II include GprA (PreB) and GprB (PreA), which are similar to the yeast pheromone receptors Ste2 and Ste3, and function in self-fertilized sexual development (Seo et al. 2004). Class III includes GprC, GprD, and GprE receptors that might be involved in carbon source sensing on the basis of their high similarity to the *Saccharomyces cerevisiae* Gpr1 receptor (Xue et al. 1998; Kraakman et al. 1999). Class IV includes GprF and GprG, which are similar to the *Schizosaccharomyces pombe* Stm1 receptor, and the nutrient sensor Stm1-like proteins (Chung et al. 2001). The Stm1 receptor senses the cell nutritional state, thereby driving the cells to enter meiosis when encountering nutritionally deficient conditions. Class V includes GprH and GprI, which are similar to the *Dictyostelium discoideum* cAMP receptor cAR1 and thus have been proposed to be involved in cAMP sensing (Galagan et al. 2003). Later, Lafon et al. (2006) carried out an exhaustive comparative analysis of the genomes of three aspergilli: *Aspergillus nidulans*, *A. fumigatus*, and *A. oryzae*, and identified 7 additional GPCRs in *A. nidulans*: GprJ (class IV), GprK (class VI), GprM and GprN (class VII), GprO and GprP (class VIII), and NopA (class IX), totaling 16 potential GPCRs classified into nine classes (Lafon et al. 2006). Recently, a total of 10 GPCRs in *N. crassa* were divided into five classes: pheromone receptors (Pre-1 and Pre-2), cAMP receptor-like proteins (Gpr-1, Gpr-2, Gpr-3), carbon sensors (Gpr-4), putative nitrogen sensors (Gpr-5 and Gpr-6), and microbial opsins (Nop-1 and Orp-1) (Borkovich et al. 2004; Li et al. 2007). In the basidiomycete*Cryptococcus neoformans*, Xue et al. (2006) identified a large gene family of 7-TM proteins. Krishnan et al. (2012) provided the first evidence that four of the five main mammalian families of GPCRs, namely rhodopsin, adhesion, glutamate, and frizzled, are present in fungi. In the *N. crassa* genome a total of 10 receptors were predicted (Galagan et al. 2003). A recent report for *A. nidulans* identified GPCRs similar to the yeast pheromone receptors, the glucose-sensing receptor GPR1, the

nitrogen-starvation sensing STM1, and the *D. discoideum* cAMP receptors (Han et al. 2004). In *A. nidulans*, the G-alpha subunit GanB and the G-protein-coupled receptor (GPCR) GprH have been shown to be involved in glucose sensing. GanB is involved in mediating activation of cAMP synthesis and subsequent PKA activation in the presence of glucose during early conidial germination events. The near-complete identification and characterization of both positive (GPCRs, G proteins, PhLPs, and effectors) and negative (RGS proteins) controllers of G-protein signaling in *A. nidulans* will provide us with insights into understanding the mechanisms underlying morphogenesis, pathogenicity, and toxigenesis in less genetically tractable but otherwise medically and agriculturally important fungi. Moreover, as many human diseases are associated with deleterious G-protein-mediated signals, understanding the molecular events resulting from dysfunctional regulation of G-protein signaling in *A. nidulans* may illuminate the nature of certain human diseases (Yu 2006). It is established that G proteins are involved in plant defense and suggested that they relay signals from defense-related receptor-like proteins (RLKS).

3.3 GPCR and Yeast-Secreted Pheromones

Yeast, which was the first eukaryotic genome to be sequenced, provides an exemplary model system and tools for improving our knowledge of GPCRs and their signaling in multicellular eukaryotes (Xue et al. 2008). In *Saccharomyces cerevisiae*, two different haploid cells exist: mating type a (*MAT*a, a-cell) and mating type α (*MAT*α, α-cell) as a result of meiosis, defining what is termed a bipolar system. The two types of haploid (ascospores) are often called mating types because they describe the mating behavior: mating occurs only between an a-cell and an α-cell. The mating type of a haploid cell is determined by its genotype at the mating-type (MAT) locus on chromosome III. The two variants of the MAT locus, MATα and MATa, are referred to as idiomorphs rather than alleles because they differ in sequence, size, and gene content (Seraj Uddin et al. 2016; Singh et al. 1983). The *MAT*α idiomorph contains two genes, *MAT*α1 and *MAT*α2, whereas the *MAT*a idiomorph contains a single gene, *MAT*a1, and those three genes code for transcription regulators (Hanson and Wolfe 2017). They determine the cell type of the haploid by activating or repressing the expression of a-specific genes (asg) and α-specific (αsg) genes (Haber 2012). In *Saccharomyces cerevisiae*, the pheromone genes MFa1 (a-mating factor, MFa, a-pheromone, which is a post-translationally modified peptide, its precursor protein does not enter the secretory pathway but is processed and matured in the cytoplasm where the generated peptide exits the cell via a transporter) and MFα1 (α-mating factor, MFα, α-pheromone produced from a long precursor that enters the secretory pathway and is processed by KEX1, KEX2, and STE13 proteases to finally release repeated peptides through exocytosis) are asg and αsg genes, respectively. The α-pheromone is produced from prepro-proteins, which

display a signal peptide and are not post-translationally modified, a proregion, and four (MFα1) or two (MFα2) repetitions of nearly identical motifs, each of which is preceded by an easily recognizable protease cleavage site. This cleavage site is composed of the "KR" dipeptide followed by "EA" or "DA" dipeptides (e.g., KREA or KRDA). The KEX1 and KEX2 proteases, respectively, cut before the K and after the R of the KR dipeptide. STE13 then cleaves after the A of the EA or DA residues, thus releasing the repeated peptides. The motif recognized by STE13 is, however, more variable in sequence and in length and is often a repetition of XA or XP dipeptides (X, any amino acid). This processing occurs in the Golgi apparatus while the protein passes through the secretory pathway. The pheromone signaling pathway G-protein-subunit genes GPA1, STE4, and STE18 are haploid-specific genes (hsg) hsgs, and the mitogen-activated protein kinase "MAP kinase" FUS3 is a general pheromone-activated gene (Sorrells et al. 2015). In the haploid α-cells, the MATα1 gene codes for the HMG-domain transcription activator α1 (previously referred to as an "α-domain" protein but now recognized as a divergent HMG domain) (Martin et al. 2010) and the MATα2 gene code for the homeodomain-transcription repressor α2. The α1 and α2 proteins can both individually form complexes with the constitutively expressed Mcm1 (MADS domain) protein, which binds upstream of asgs and αsgs. In α-cells, the transcription of αsgs is activated because the α1–Mcm1 complex recruits the transcription factor Ste12 to their promoters, whereas the transcription of asgs is repressed because the α2–Mcm1 complex recruits the Tup1-Ssn6 corepressor. The MAT locus in a-haploid cells contains only the MATa1 gene coding for the homeodomain protein a1, but this protein is not required for a cell-type identity, which is instead defined by the absence of both α1 (the activator of αsgs) and α2 (the repressor of asgs). Instead of requiring an a-specific activator, asgs are activated by Mcm1 and Ste12, which are constitutively expressed in all cell types. Thus, in *S. cerevisiae*, the a-cell type is the default type, and yeast cells lacking a MAT locus will mate with α-haploid cells. In a/α diploid cells, αsgs, asgs, and hsgs are all repressed. These cells have MATα1 and MATα2 genes at the MAT locus on one chromosome, and MATa1 on the other, which results in the formation of a heterodimer a1–α2 of the two homeodomain proteins. There are approximately 5 to 12 asgs and αsgs, depending on the species (Sorrells et al. 2015). In addition to these, a shared set of haploid-specific genes (hsgs) (~12–16 in number) that facilitate mating is constitutively expressed in both a and α cells but not in a/α diploids (Booth et al. 2010), and a larger group of about 100 general pheromone-activated genes is induced in haploids of both types once a pheromone signal from the opposite type of haploid is detected (Sorrells et al. 2015). Because *S. cerevisiae* uses the formation of a heterodimer to sense heterozygosity of its MAT locus, and because this heterodimer is a repressor, there are no "diploid-specific" genes in *S. cerevisiae* (Galgoczy et al. 2004). Indeed, diploid-specific processes such as meiosis and sporulation are repressed in haploids. This repression is achieved via the hsg RME1, a haploid-specific activator that transcribes IRT1, a noncoding RNA which in turn represses IME1, the master inducer of meiosis. Thus, the combined action of *RME1*

and *IRT1* inverts the output of the hsg regulatory logic to restrict *IME1* expression to diploids. *IME1* expression also requires the environmental signals of nitrogen and glucose depletion that initiate meiosis. No genes have constitutive diploid (a/α) specific expression in the same way that hsgs, αsgs, and asgs have constitutive cell type-specific expression in haploids. Mating of *MAT*a and *MAT*α cells produces diploid zygotes (*MAT*a/*MAT*α), which will not exhibit any mating type and therefore cease to secrete pheromones. Mating is initiated in response to the pheromone secreted by haploid cells. The lipopeptide a-pheromone released by a-cells is a C-terminally methylated and farnesylated 12-residue peptide that makes it very hydrophobic and acts on α-cells whereas the simple α-pheromone is an ordinary 13-residue peptide that acts on a-cells (Aksam et al. 2013). Their presence is communicated to the response machinery within the cell by means of a heterotrimeric G-protein complex that, when activated by the pheromone-bound receptor, serves in turn to activate a downstream mitogen-activated protein (MAP) kinase cascade module. The two loci MFAL1 and MFAL2 (MFαl and MFα2) are coding for the α-pheromone that arrests the a-cells at the G_1 stage of the cell cycle as a method of synchronization of the two haploid cells before mating, thus ensuring that only unbudded and mononucleate haploids fuse to form diploid zygotes. Similarly, a-cells produce the peptide a-factor, which arrests the division cycle of α-cells at the G_1 phase. In addition to regulating cell division, these factors induce formation of cell-surface agglutinins encoded by the alpha-agglutinin structural gene, AG alpha1. Alpha-agglutinin is a cell adhesion glycoprotein that promotes the aggregation of opposite cell types, and α-factor as well as a-factor elicit localized elongations "shmoos" of the target a-cell and α-cell, respectively, which may form the site for nuclear migration and fusion. Although α-cell mating requires only one of its two genes to be functional, typically both loci are transcribed, albeit at different rates (MFAL1 ⋙ MFAL2) (Kurjan 1985). The ability to respond to pheromones is controlled by at least eight additional genes: STE2, STE4, STE5, STE7, STE8, STE9, STE11, and STE12. Among these STE genes, STE2 and STE3 are receptors responsible for pheromone sensing. The a-cells express a unique GPCR, Ste2, which is the receptor for α-factor; α-cells express a different GPCR, Ste3, which is the receptor for a-factor. Mutations in any one of these genes prevent a-cells from arresting cell division, producing agglutinins, and altering cell morphology in response to α-factor. Similarly, pheromone sensitivity of α-cells requires the same genes with the exception of STE2. Cell-type specificity of the ste2 mutation raises the possibility that the STE2 gene encodes a cell-surface receptor that recognizes α-factor (Hartwell 1980; Jenness et al. 1983; MacKay and Manney 1974). The STE2 gene, which is necessary for stability of the binding activity, is likely to encode a structural component of the α-factor receptor, and it is likely to encode an integral part of the α-factor receptor because mutations in this gene affect the physical properties of the binding activity (Jenness et al. 1983). Their results were consistent with the view that a single type of receptor elicits different responses at different α-factor concentrations. Because of the large number of α-factor binding sites detected, they

concluded that a-cells can potentially sense a wide range of α-factor concentrations. Physiological responses may be controlled by an intracellular "signal" generated by the binding of α-factor to its receptor. Di Segni et al. (2008, 2011) showed that a cryptic polyadenylation site is present inside the coding region of the a-specific STE2 gene, encoding the receptor for the α-factor. The two cell types (a- and α-cells) produce an incomplete STE2 transcript, but only a-cells generate full-length STE2 mRNA. The tRNA splicing endonuclease is able to produce trans-spliced mRNAs. During their work, Di Segni et al. discovered a previously unnoticed cryptic polyadenylation site early in the STE2 coding region. Cleavage and polyadenylation of pre-mRNAs are essential to ensure transcription termination. For this kind of regulation to be effective, the repression should be very tight. If α-cells produced even a small amount of the Ste2 receptor, they would undergo autocrine activation of the mating pathway by the α-factor that these cells secrete, leading to growth arrest. Therefore, the genes encoding the pheromone receptors should be very strictly regulated. The internal poly(A) site would eliminate rare transcripts of STE2 escaping repression. Conversely, the other mating-type receptor gene, STE3, is induced only in a-cells and not expressed at all in α-cells. The regulation of yeast mating genes is achieved through a concerted mechanism that involves transcriptional and posttranscriptional events. In a-cells, the STE2 gene is actively transcribed, the upstream poly(A) site is skipped as a result of the high transcription rate, and the canonical poly(A) site in the 3′-UTR is prevalently used. In contrast, in α-cells, STE2 is repressed and rare transcripts escaping the repression will abort as a result of polyadenylation at the cryptic site inside the coding region. The early cryptic polyadenylation site in STE2 contributes to its shutoff in α-cells, thus avoiding autocrine activation of the pheromone response pathway that could occur as a result of a leaky repression of transcription. Conversely, the STE3 gene, being always turned off except for where it should be expressed, does not need this further level of control, and indeed, no cryptic polyadenylation site is found in its coding region. The α-factor secreted by α-cells is bound by the Ste2 receptor and ectopically expressed; it induces pheromone response. Mating is elicited by the binding of α-factor and a-factor, respectively, to G-protein-coupled receptors Ste2 and Ste3, specifically expressed in a- or α-cells (Burkholder and Hartwell 1985; Hartig et al. 1986). Ste2 and Ste3 both activate the same Gα-subunit Gpa1 to facilitate the replacement of GDP by GTP, which dissociates the G-protein subunits Gα from the Gβγ (Ste4/Ste18) complex. Expression of all these genes (SCGI, STE4, STE78, STE2, STE3) is haploid specific (they are transcribed in both haploids a- and α-cells, but not in a/α diploids), as is the response to mating pheromones. Unusually, in the *Saccharomyces cerevisiae* pheromone-signaling pathway, it is the Gβγ complex that functions as the main driving force, not Gα, to induce the downstream pheromone-signaling responses, and cells lacking either subunit of the Gβγ complex are blocked for all mating responses (Whiteway et al. 1989). Free Gβγ then activates the signaling branch responsible for regulating cell division and the cell polarity branch responsible for polarized growth (Johnson et al. 2011). The Gβγ-subunit binds to a Ste5–Ste11 complex and to the Ste20 kinase (Leeuw et al. 1998). The scaffold protein Ste5, the PAK kinase Ste20, and the Cdc24/Far1 complex are three main

downstream targets of the Gβγ complex. When bound to Ste5, the Ste4–Ste18 complex facilitates its membrane recruitment and places the scaffold protein, the entire mitogen-activated protein kinase (MAPK) module and Ste20, into close proximity to enable signaling circuit activation (Leeuw et al. 1998; Pryciak and Huntress 1998). The MAPK module is a three-tiered phospho-relay system composed of Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3 (MAPK). Upon signal activation, the phosphor-activated Fus3 releases the downstream transcription factor Ste12 from inhibition by Dig1/Dig2, which induces the expression of several mating-specific genes (Bardwell et al. 1994). Haploid yeast cells use a prototypic cell signaling system to transmit information about the extracellular concentration of mating pheromone secreted by potential mating partners (Yu et al. 2008). Recent studies on the yeast pheromone response have shown how positive feedback generates switches, negative feedback enables gradient detection, and coherent feedforward regulation underlies cellular memory (Atay and Skotheim 2017). The ability of cells to respond distinguishably to different pheromone concentrations depends on how much information about pheromone concentration the system can transmit. They showed that the MAPK Fus3 mediates fast-acting negative feedback that adjusts the dose response of the downstream system response to match that of receptor–ligand binding. This "dose–response alignment," defined by a linear relationship between receptor occupancy and downstream response, can improve the fidelity of information transmission by making downstream responses corresponding to different receptor occupancies more distinguishable and reducing amplification of stochastic noise during signal transmission. They also showed that one target of the feedback is a novel signal-promoting function of the RGS protein Sst2. Negative feedback is a general mechanism used in signaling systems to align dose responses and thereby increase the fidelity of information transmission (Yu et al. 2008). When cells sense pheromones, the interaction between a pheromone and its receptor in either haploid cell type triggers a MAP-kinase signaling cascade resulting in G_1-phase arrest of mitotic proliferation. Then, the mating pathway is activated, the transcriptional profile changes, and they exhibit a chemotactic response by the formation of a mating projection (shmooing) toward the mating partner polarized toward the pheromone source, and finally mating by cell and nuclear fusion to generate a diploid zygote (Fig. 3.2). Components of the pheromone-signaling pathway, from the upstream receptor–G-protein complex to the downstream transcription factor, are all required for these mating responses (Herskowitz 1995).

For haploid cells (both a and α), cell type-specific processes include the induction of competence to mate and the repression of sporulation, whereas diploid cells require repression of mating and the ability to initiate meiosis and sporulation. The mating process of budding yeast (*S. cerevisiae*) is, to date, the best studied example of chemotropism. In mating mixtures, haploid yeast cells can interpret a shallow pheromone gradient, the chemotrope, toward the closest mating partner, and fuse to form a diploid zygote. How yeast cells accurately position the polarity machinery toward the source of pheromone is unclear. It is well known that the pheromone receptor and its cognate G protein are uniformly distributed on the plasma membrane of vegetative cells and that they polarize in response to pheromone (Wang and

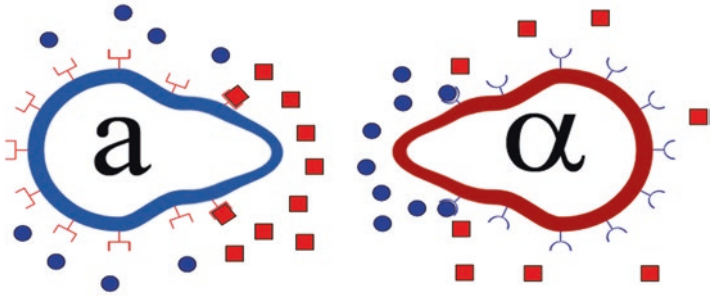

Fig. 3.2 The mating pathway is activated, the transcriptional profile changes, and they exhibit a chemotactic response by the formation of a mating projection (shmooing) toward the mating partner polarized toward the pheromone source. Blue balls refer to a pheromone while red square refer to α pheromone and each of which are bound to their respective receptors

Stone 2017). Gradient sensing, inhibition of receptor phosphorylation by Gβγ, results in differential phosphorylation of the receptor across the cell surface, and, consequently, lesser internalization of the receptor and G protein on the up-gradient side of the cell. A key question is how the uniformly distributed surface receptor competes for a limited amount of G protein. Wang and Stone (2017) showed that in mating cells the initially uniform receptor and G protein first localize as polarized crescents at the default polarity site. The receptor and G-protein crescents then track along the plasma membrane until they reach the region of highest pheromone concentration, centered around the position at which the cell ultimately shmoos toward its partner. They also showed that polarization of Gβ to the default polarity site is independent of receptor phosphorylation and polarization, whereas Gβ tracking from the default site to the eventual chemotropic site does not occur if receptor phosphorylation and redistribution are blocked. These observations suggest a new mechanism that localizes the receptor with its much less abundant G protein. In their revised model, they proposed that mating cells that are arrested in G_1 cell-cycle phase concentrate Gβg at the default polarity site, likely through its interaction with Far1-Bem1-Cdc24-Cdc42. The polarized Gβg then protects the receptor from being phosphorylated and internalized, thereby triggering local accumulation of the receptor and G protein. Because the pheromone gradient is mirrored by a gradient of signaling activation within the receptor/G-protein crescent, there are higher proportions of active unphosphorylated receptors and active G protein closer to the pheromone source. The peak of signaling activity incrementally moves up the pheromone gradient, as unprotected receptors are phosphorylated and co-internalized with G proteins at the back, while vesicles containing nascent receptors and G proteins preferentially dock where the receptor is most abundant.

The phenomenon that the two haploid cell types of yeast (a and α) are able to interconvert in a reversible manner by means of a programmed DNA rearrangement process is called mating-type switching. Mating-type switching is the process by which a haploid a-cell can become a haploid α-cell, by changing its

genotype at the mating-type (MAT) locus from MATa to MATα, or vice versa. Unicellular organisms that do not contain separate germline and somatic DNA cannot make permanent changes to their genomes during development, as these will be transmitted to offspring. Instead, programmed DNA rearrangements underlying cell-type specification in these organisms must be reversible (Nieuwenhuis and Immler 2016). In unicellular organisms every cell must retain the capacity to produce every other type of cell. Mating-type switching was the subject of early studies in *S. cerevisiae* genetics and molecular biology (Oshima 1993; Barnett 2007; Klar 2010). Its mechanism of switching is complex and involves multiple components as well as multiple levels of regulation. The dissection of how cell-type specification and mating-type switching is controlled in *S. cerevisiae* led to breakthroughs in our understanding of many other fundamental cellular processes including homologous recombination, cell signaling pathways, gene silencing, and mechanisms of transcriptional regulation (Haber 2012). In fact, the idea of using arrows and T-bar symbols in network diagrams to symbolize gene activation and repression, respectively, is attributable to Ira Herskowitz (Botstein 2004), whose laboratory discovered the cassette mechanism of switching in *S. cerevisiae* (Fig. 3.3a and b). Switching seemed to appear abruptly within the family Saccharomycetaceae (Butler et al. 2004). The characterization of homothallic and heterothallic strains of *S. cerevisiae* led to the discovery of genetic loci controlling homothallism and ultimately to the cassette model of mating-type switching. Switching mating types in *S. cerevisiae* involves a unidirectional DNA replacement. The cassette model states that a haploid cell can switch its genotype at the MAT locus (from MATa to MATα idiomorph or vice versa) by a gene conversion process. Although mating-type switching in *S. cerevisiae* is often called gene conversion, it is more accurately described as a synthesis-dependent strand annealing (SDSA) process because of the nonhomology of the Y-regions (Fig. 3.3a) between the outgoing and incoming alleles (Ira et al. 2006). The current active gene content at the MAT locus of a haploid cell is replaced by copying a reserve version of the MAT gene of the opposite allele, stored at a transcriptionally silent location (Lee and Haber 2015). This process requires the genome to have three copies of mating-type sequence information, all of which are on chromosome III in *S. cerevisiae*: the active MAT locus (either MATa or MATα), two silent loci termed HML (containing the reserve copy of MATα sequence information) and HMR (containing the reserve copy of MATa sequence information) (Fig. 3.3b). All three loci are flanked by identical sequence regions called X and Z (Fig. 3.3a). The Y-region in the center comes in two forms, Ya and Yα, that are allelic but completely different in sequence. During switching, the actively expressed MAT locus is cleaved by the endonuclease HO encoded by the HO gene on chromosome IV, at a site that marks the boundary between the Y-sequences unique to the MATa or MATα idiomorphs and the shared Z-sequence flanking them, and eliminates the allele at the active MAT locus. Switching is initiated when the HO endonuclease makes a dsDNA break at the Y–Z junction of MAT. The 3′-end of a DNA strand from the Z-region beside MAT then invades the donor (HMR or HML) locus and is extended by a DNA

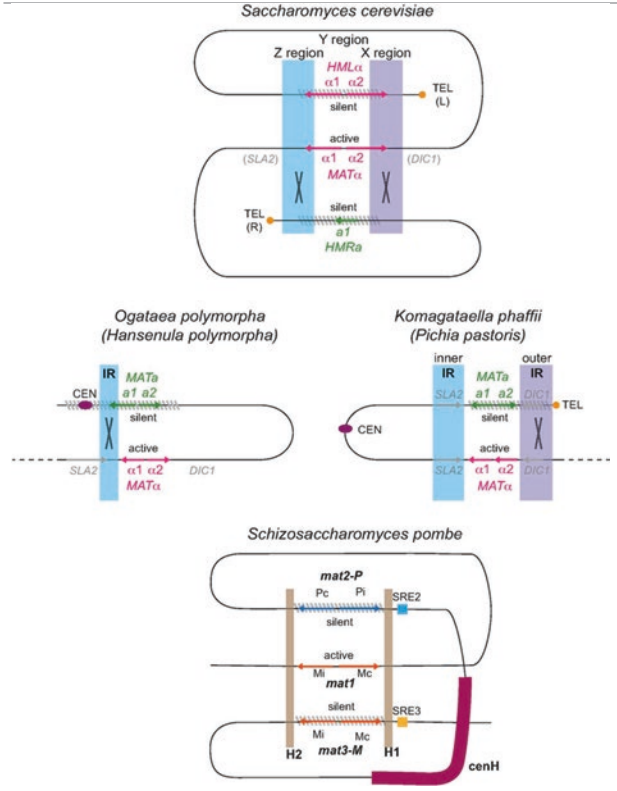

Fig. 3.3 (**a**) Organization of repeat sequences flanking the *MAT* loci in four species (Klar 2007; Hanson et al. 2014). In *Komagataella phaffii*, the region that becomes inverted during mating-type switching is 138 kb long and was recently discovered to contain a centromere at its approximate center (Coughlan et al. 2016). *CEN*, centromere; *TEL*, telomere. (Source: By courtesy and permission of Kenneth H. Wolfe and Genetics Society of America) (https://doi.org/10.1534/genetics.117.202036)

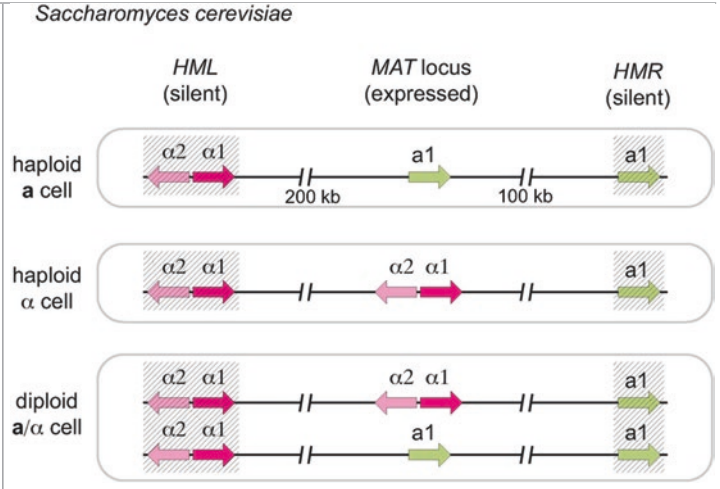

Fig. 3.3 (**b**) Gene organization in the *MAT*, *HML*, and *HMR* loci on *Sacchormyces cerevisiae* chromosome *III*. *Shading* indicates genes whose transcription is repressed. (Source: By courtesy and permission of Kenneth H. Wolfe and Genetics Society of America) (https://doi.org/10.1534/genetics.117.202036)

polymerase through the donor Y-region and into the X-region, after which it reinvades the MAT locus. Finally, the second strand of the new Y-region at MAT is synthesized in the direction from X to Z. Switching is slow, taking approximately 70 min, and is more than 100 times more error prone than normal DNA replication (Hicks et al. 2011; Hanson and Wolfe 2017). In other words, the gap is subsequently filled in through gene conversion guided by homology at the X- and Z-regions; the cleaved MAT locus uses HML or HMR as a template for DNA repair with a strong preference for the silent locus containing the mating-type information opposite to the current MAT genotype (Haber 2012). After MATa is cleaved by HO endonuclease, it is normally repaired by copying HML-alpha, creating a new MAT-alpha cell; this occurs in about 95% of the cells. In the other 5% of cells, the cleaved MATa is repaired by copying HMRa, creating MATa again. This MATa to MATa switching is called "futile switching" because it does not make any difference to the cell. Similarly, when a MAT-alpha cell tries to switch, about 5% are futile MAT-alpha to MAT-alpha switches, and 95% are productive MAT-alpha to MATa switches. The futile switches occur because the recombination enhancer (RE) element is not perfect; that is, the RE makes the direction switching strongly biased but not completely biased. It is possible that during meiosis DNA double-strand breaks are induced similarly at high frequency in the MAT flanking recombination hot spots and the intra-MAT gene conversion hot spot. In *S. cerevisiae*, cells typically switch their mating types every other generation and only mother cells that have divided twice can switch (Sun and Heitman 2016). HO is expressed only in cells that have budded once, which means that only mother cells switch mating type and can mate their second daughter neighboring cells. Hence, most natural isolates of *S. cerevisiae* are diploid and phenotypically homothallic. The question of whether a mating-type system similar to that of *S. cerevisiae* is found in other hemiascomycetes has become pertinent recently because of the discovery of mating-type-like (MTL) loci in *Candida* species that had been regarded as asexual. The *Candida albicans* genome sequence includes an MTL locus but neither silent cassettes nor a HO endonuclease gene (Butler et al. 2004), although *C. albicans* seems to have homologues of all the elements of a functional pheromone response pathway involved in mating in *S. cerevisiae* (*STE2*, *STE3*, Gα(Gpa1), Gβ(Ste4), Gγ(Ste18), (MAP) *STE20*, *STE11*, *STE7*, *FUS3*, and Ste12p) but lacks many homologues of *S. cerevisiae* genes for meiosis (Tzung et al. 2001).

The ambiguity of the meiotic occurrence in *Candida albicans* to date does not let us know much about its switching mating-type mechanism. Chlamydospore formation is a characteristic of many fungal species, among them the closely related human-pathogenic dimorphic yeasts *C. albicans* and *Candida dubliniensis*. Although function and regulation of filamentation are well studied in these species, the basis of chlamydospore formation (although chlamydospores are non-meiotic, but are asexual spores) is mostly unknown (Böttcher et al. 2016; Navarathna et al. 2016). Ho-mediated switching between an active *MAT* locus and silent cassettes exists only in the *Saccharomyces* sensu stricto group and their closest relatives: *Candida glabrata*, *Kluyveromyces delphensis*, and *Saccharomyces castellii*. Butler

et al. (2004) identified, in *C. glabrata*, an *MTL1* as the ortholog of the *MAT* locus of *K. delphensis* and showed that switching between *C. glabrata* MTL1a and MTL1α genotypes occurs in vivo. The more distantly related species *Kluyveromyces lactis* has silent cassettes but switches mating type without the aid of Ho endonuclease. Very distantly related species such as *C. albicans* and *Yarrowia lipolytica* do not have silent cassettes. The *Naumovozyma castellii* mating-type system is organized as in *S. cerevisiae*, and in many other Saccharomycetaceae members, such as *C. glabrata* and *Nakaseomyces delphensis* (syn. *Kluyveromyces delphensis*) (Butler et al. 2004). The system includes a MAT locus, an HO endonuclease gene, silent HMR and HML cassettes, and the MATα1, MATα2, and MATa1 genes that express and regulate mating-type specific features (Butler et al. 2004). In *N. castellii* the MAT locus and HMRa and HMLα silent cassette are located on chromosome II. The sexual state of the cell is defined by expressing the genetic information present at the MAT locus. Thus, a mating-type switch involves replacement of this information with a copy of the opposite genotype that resides in the silent locus, that is, by a gene conversion process. The mating-type switch in *N. castellii* initiates with the introduction of a double-stranded break at the MAT locus, performed by Ho endonuclease encoded by the HO gene. The mating-type information is then copied from the silent HMRa and HMLα cassettes that store a- and α-specific sequence information, respectively. Overall, *N. castellii* therefore conforms to the conventional mating-type system as known for the very well studied pathway in *S. cerevisiae*, although with some deviations in the details (Andersson and Cohn 2017). The work by Le Marquer et al. (2019) presents the largest analysis of fungal secreted proteins predicted to release repeated peptides through KEX2 activity. The best characterized representatives of these proteins are the α sexual pheromones of *Ascomycota*. Cyclic peptides produced in Ascomycota from KEX2-processed precursor proteins belong to the family of ribosomally synthesized and post-translationally modified peptides (RiPPs), and they act as mycotoxins. Le Marquer et al. hypothesized that many proteins with characteristics similar to α sexual pheromones would be present in many if not all fungal species. A few species, scattered all along the fungal kingdom, were shown to possess such proteins. They reported that sexual pheromone genes are present in the following presumed asexual fungi: *Cladosporium fulvum*, *Aspergillus niger*, *Verticillium dahliae*, *Fusarium oxysporum*, and *Trichoderma reesei*. It is also important to consider that sexual pheromones have other important sex-independent roles such as biofilm production or conglutination of hyphae. These KEX2-processed repeat proteins (KEPs) present a clear STE13 signature, identical to that of α-type pheromone protein precursors of Ascomycota. GPCRs are also present in these fungi to perceive these peptides. The investigators speculated that KEPs with clear STE13 signatures may act as hormones or pheromones regulating distinct cellular programs. KEPs may be precursors of peptides, regulating some of the hyphal polar growth, septation, branching, fusion or healing, hyphal network coordination, and production of sexual and/or vegetative reproduction bodies and spores. KEPs displaying peptides with a STE13 signature are therefore good candidates for the release of peptidic hormones that may have distinct roles from sexuality and may regulate other aspects of fungal biology.

3.4 Mating in the *Basidiomycota Ustilago maydis and Sporisorium scitamineum*

Although Basidiomycota do not use α-type pheromones for their sexual reproduction, Le Marquer et al. (2019) identified several proteins with α-type pheromone features in their secretomes. Basidiomycota have conserved proteins with striking similarities to α-sexual pheromones so far only described in Ascomycota. The close relationship between *Ustilago maydis* and *Sporisorium scitamineum* (subphylum Ustilaginomycotina) can be evidenced by the misclassification of *S. scitamineum* as *U. scitaminea*. Generally, *Ustilago* infects all aerial parts of the corn plant and rapidly forms galls or tumors filled with spores. In contrast, *Sporisorium* infects young sugarcane seedlings, remains asymptomatic, and grows systemically until the emergence of a mass of sooty spores. The whole genome character of *S. scitamineum* is most similar to *U. maydis*, which belongs to a separate genus. In the phylum Basidiomycota, a wide variety of lifestyles are represented, ranging from well-known and conspicuous wood-decaying mushrooms, plant growth-promoting and mutualistic mycorrhizae, and crop-destroying smut and rust fungi, to yeast-like human pathogens. Lifestyle differences have consequences for the mating and breeding systems of these fungi. Basidiomycetes have been recognized as having diverse breeding systems, from homothallism to heterothallism. The study of breeding systems, for example, led to the discovery of the astounding variability in mating-type alleles among mushrooms, with thousands of different mating types in some species, and to the realization that in many fungal pathogens the process of sexual reproduction is closely linked to infection and pathogenicity. In basidiomycetes, the sexual cycle typically involves the fusion of genetically distinct homokaryotic hyphae or haploid yeast cells to produce a dikaryon in which the two haploid parental nuclei are replicated in a coordinated fashion without fusion during hyphal elongation. Nuclear fusion (karyogamy) then takes place in the basidia or in other specialized structures (e.g., teliospores), after which the diploid nucleus undergoes meiosis to generate haploid basidiospores (meiospores) and complete the life cycle. One common underlying feature shared by most fungi is the lack of genetically determined anisogamy [i.e., a situation in which a species has two classes of gamete sizes: one class of large gametes (female function) and one class of small gametes (male function) and a single genotype can produce both types of gametes in most anisogamous fungi]. When the two MAT loci are unlinked, four mating types can be generated by meiosis among the haploid progeny, defining this as a tetrapolar breeding system. Other basidiomycetes have instead a bipolar system controlled by a single MAT locus, either because the pheromones and pheromone receptors P/R and the heterodimeric or homeodomain-type HD loci are linked or because one has lost its function in mating-type determinism (Coelho et al. 2017). In mushroom-forming species (Agaricomycetes), there has been a generalized diversification of alleles at both MAT loci, in some cases yielding species with hundreds or thousands of possible mating types (Coelho et al. 2017). Mating is regulated by two genetic mating-type MAT loci: one locus (a) encodes two, a1 and a2,

tightly linked genes encoding P/R (Rowell and DeVay 1954; Holliday 1961; Puhalla 1968), whereas the b locus encodes two subunits that harbor conserved genes of a HD transcription factor determining the viability following syngamy (i.e., haploid cell fusion during mating) (Bakkeren et al. 2008). Different a-alleles are necessary, together with different b-alleles, for the development of the filamentous form (Banuett and Herskowitz 1989). Schulz et al. (1990) reported that the fungal pathogen of corn, *U. maydis*, has two forms: one is yeast like and nonpathogenic, and the other is a dikaryotic filamentous and pathogenic form. The b mating-type genes encode two subunits consisting of an HD1 class and an HD2-class protein that are not related to each other in primary sequence; however, they share a common domain organization. The N-terminal regions of the HD proteins contain the highest degree of variation when different alleles are compared and are thus designated as the variable regions, whereas the C-terminal regions of the proteins, including the homeodomains, are highly conserved (Gillissen et al. 1992; Kronstad and Leong 1990; Schulz et al. 1990). The b locus, with 25 different alleles and most probably more than that, regulates this dimorphism: any combination of 2 different alleles triggers pathogenic development, whereas the presence of identical alleles results in the yeast-like form. Syngamy is governed by, in its simplest form, small 10- to 15-amino acid–lipopeptide pheromones derived from 35 to 40 amino acid precursors through post-translation modifications at both the N- and C-termini. These diffusible pheromones are received by seven transmembrane-domain pheromone receptors, coupled to a G protein for downstream signal transduction. This molecular determination of mating fusion is similar in part to the a- and α-factor P/R system in the Ascomycetes. Therefore, instead of the a-factor/Ste3 and α-factor/Ste2 coupled sensing system characteristic of *Saccharomyces cerevisiae*, "chemosensing" specificity in basidiomycetes is mediated by allelic variants of the same type of genes. Mating is often initiated by a reciprocal exchange of pheromones recognized by matching pheromone receptor variants in both mating types, and thus two strains are needed to carry different alleles of the pheromone and receptor genes at the P/R locus. Gillissen et al. (1992), Kronstad and Leong (1990), and Schulz et al. (1990) have cloned four b alleles (bi, b2, b3, b4) and showed that the b locus contains a single open reading frame (ORF) of 410 amino acids with a variable N-terminal region and a highly conserved C-terminal region (60% and 93% identity, respectively). Mutational analysis confirms that this ORF is responsible for b activity. The b polypeptides appear to be DNA-binding proteins because they contain a motif related to the homeodomain in their constant region. The investigators proposed that combinatorial interactions between b polypeptides generate regulatory proteins that determine the developmental program of the fungus. They discussed also the association of b polypeptides. Snetselaar and McCann (2017) suggested revising the *U. maydis* life cycle based upon their recent findings. Their microscopic examination of both living and fixed tumor material showed that nuclei fuse long before sporulation begins and that tumors are filled with uninucleate cells undergoing mitosis. Quantification of DNA in the nuclei confirmed these observations. Additionally, fungal cells from tumor material placed on nutrient agar produced colonies of diploid budding cells. Time-lapse observations showed that at

least some of these colonies arose from thin-walled fungal cells rather than from immature spores. Ultrastructural examination of developing teliospores from tumors confirmed that these were uninucleate. Condensed chromatin and other structures characteristic of nuclei in prophase I of meiosis were observed. The bipolar species *Sporisorium scitamineum* and the tetrapolar species *Ustilago maydis* possess one divergently transcribed gene pair that encodes the homeodomain proteins bE (HD1) and bW (HD2). The MAT-1 locus, gene order, and orientation, as well as the genomic context, are conserved in the b mating-type genes. Interestingly, both bE and bW mating-type genes are present in the genomes of Ustilaginaceae, including the two genera of *Ustilago* and *Sporisorium*. The a and b loci are linked, and the mating-type locus (MAT) segregates independently in the tetrapolar *Sporisorium reilianum* and *U. maydis. S. scitamineum* is a bipolar mating fungus; the *a* and *b* loci are linked, and the mating-type locus segregates as a single locus. Only the dikaryotic hyphae formed by fusion of compatible sporidia can infect the host (Feldbrügge et al. 2004). Que et al. (2014) explored the genomes of 12 fungi, *S. scitamineum* plus 11 other fungi, and identified members of the G-protein-coupled receptor family from the entire deduced proteomes. They demonstrated that *S. scitamineum* possesses only 6 GPCRs, which are grouped into five classes that are responsible for transducing extracellular signals into intracellular responses; however, the genome is without any PTH11-like GPCR. This total set of analyses also resulted in the identification of 7 and 5 putative GPCRs in *S. reilianum* and *U. maydis*, respectively; this was the first high-quality genome sequence of *S. scitamineum* and was also the first reported genome of sugarcane fungi. There are 192 virulence-associated genes in the genome of *S. scitamineum*, among which 31 are expressed in all the stages, which mainly encode for energy metabolism and redox of short-chain compound-related enzymes. Sixty-eight candidates for secreted effector proteins (CSEPs) were found in the genome of *S. scitamineum*, and 32 of them expressed in the different stages of sugarcane infection, which are probably involved in infection or triggering defense responses (Que et al. 2014).

The basidiomycete *U. maydis* is a member of the smut fungi, a large group of biotrophic parasites that infect mostly grasses. Genome-wide analysis demonstrated that *U. maydis* is more closely related to humans than to budding yeasts (Steinberg and Perez-Martin 2008). With the advent of molecular genetics, a wide range of tools was developed allowing precise genome modifications. Research in *U. maydis* initially followed three main directions: (i) the characterization of genes involved in DNA recombination, (ii) the study of mating-type loci, and (iii) the so-called killer phenomenon, related to the presence of virus-like particles. Subsequently, researchers became interested in different aspects of the biology ranging from gene regulation, signaling, and virulence to cell biology. Community efforts led to a high-quality genome sequence that now serves as a blueprint for comparative studies (Kämper et al. 2006). Under appropriate conditions, such as ambient temperature and humidity, the diploid spores germinate, undergo meiosis, and form a promycelium into which the four haploid nuclei migrate. Septation then produces compartments that contain one haploid nucleus. Following mitosis, haploid cells bud off from the promycelium and enter the vegetative life cycle, during

which they proliferate by budding. On the leaf surface, haploid cells of different mating types fuse and form a filamentous cell cycle-arrested dikaryon, which is the pathogenic form (Snetselaar and Mims 1992; García-Muse et al. 2003). These filaments differentiate into infection structures known as appressoria (for more details, see Matei and Doehlemann 2016; Lanver et al. 2017). Cells differing in the a mating-type locus recognize each other via lipopeptide pheromones, the cell cycle arrests in the G_2 phase, budding is stopped, and conjugation tubes are formed. These structures develop at one cell tip and grow toward each other guided through the pheromone gradient until they merge (Brefort et al. 2009). The resulting dikaryon switches to polar growth if the two mating partners carry different alleles of the two unlinked mating type loci, a and b. The a locus, which exists in two alleles, a1 and a2, controls the fusion of haploid cells and is, together with the b locus, responsible for maintenance of the filamentous form (Banuett and Herskowitz 1989). The a locus has been shown to encode an a-pheromone-based recognition system (Bölker et al. 1992). The b locus is multiallelic; each allele codes for a pair of homeodomain proteins termed bE and bW, which are assumed to act as transcriptional regulators when appropriately combined. This assertion is based on data which show that development cannot be initiated in the absence of b gene products (Gillissen et al. 1992). The gene products exist in inactive combinations in haploid strains and in active combinations in the dikaryon. Recent experimental evidence has indicated that inactive bE-bW combinations differ from active combinations in their ability to form the heterodimer. The b locus codes for a pair of homeodomain transcription factors that dimerize when derived from different alleles and control subsequent sexual and pathogenic development. In response to both chemical and physical signals of the plant surface, the dikaryotic filament forms poorly differentiated appressoria that penetrate the cuticle, probably via the action of lytic enzymes (Brefort et al. 2009). After penetration, the cell cycle is reactivated concomitantly with the development of clamp-like structures that allow the correct sorting of nuclei to maintain the dikaryotic status (see Brefort et al. 2009). In this way, the fungus proliferates, forms a massive network of hyphae, and induces plant tumors. Hyphal growth inside the tumors is followed by sporogenesis, a poorly understood process that includes karyogamy, hyphal fragmentation, and differentiation into melanized diploid teliospores. Eventually the tumors dry up, rupture, and release the diploid spores, which are dispersed by air, and this closes the life cycle. Here the author covered two members of the Basidiomycetes. For more details about mating-type genes in *Pucciniomycotina*, *Ustilaginomycotina*, and *Agaricomycotina*, see Kües et al. (2011), Coelho et al. (2017), and Snetselaar and McCann (2017).

Fungus-growing termites (Macrotermitinae, *Isoptera*) have practiced farming with their fungal symbionts of the basidiomycete genus *Termitomyces*. Surprisingly, unlike other basidiomycetes in which where sex is largely restricted to basidia, *Termitomyces* maximizes sexuality at the somatic stage, resulting in an ever-changing genotype composed of a myriad of coexisting heterogeneous nuclei in a heterokaryon. Somatic meiotic-like recombination may endow *Termitomyces* with the agility to cope with termite consumption by maximized genetic variability (Hsieh et al. 2017).

3.5 GPCR and Fungal Response to Stress

When used in traditional technologies such as baking, brewing, and distillers' fermentations, yeasts are exposed to numerous environmental stresses that can be encountered in concert and sequentially. Yeasts exhibit a complex array of stress responses when under conditions that are less than physiologically ideal. These responses involve aspects of cell sensing, signal transduction, transcriptional and post-translational control, protein targeting to organelles, accumulation of protectants, and activity of repair functions. Attfield (1997) reviewed the aspects of stress and stress response in the context of baker's yeast manufacturing and applications, and discussed the potential of improving the general robustness of the industrial baker's yeast strains in relationship to physiological and genetic manipulations. Yun et al. (1997) suggested that Gpr1p is a G-protein-coupled receptor localized at the plasma membrane of *Saccharomyces cerevisiae* that is likely to monitor an extracellular signal such as nutrition and transduce it via Gpa2p, a possible positive regulator of cAMP level. The dual regulation of gene expression by two different stress conditions is observed in the regulation of the CUP1 gene, encoding a yeast metallothionein. If an essential nutrient is missing from the medium, cells are arrested, enter the stationary phase, and display phenotypes associated with low pKa activity, including high expression of stress-related genes and the production of stored carbohydrates. The protein phosphatase calcineurin is activated by heat shock. CUP1 expression is activated in response to heat shock and glucose starvation through the action of heat-shock factor (Hsf1) and heat-shock element (HSE) located within the CUP1 promoter. It has been observed that in sucrose nonfermenting 1 (snf1# or snf4#) mutants, induction of CUP1 by carbon starvation is abolished, without affecting heat-shock-induced regulation. Glucose starvation induces the activation of CUP1 by a process dependent on the phosphorylation of Hsf1 by Snf1 kinase. This phosphorylation causes trimerization and activation of Hsf1 (Hahn et al. 2001); this is probably the reason why snf1# mutants are more sensitive to heat stress because Hsf1 activity may not be appropriately regulated (Thompson-Jaeger et al. 1991). Snf1 protein kinase participates in the regulation of different cellular responses to different forms of stress, which could be an indication of the key role of the kinase as a sensor of the fuel and stress status of the cell. Sanz (2003) concluded that sucrose nonfermenting 1 (Snf1) protein kinase has a main role in transcriptional activation, repression of gene expression, the regulation of different cellular responses to stress status of yeast cell:nutrient limitation stress, glycogen synthesis stimulated by nutrient limitation, glucose depletion, salt stress, and heat shock. In contrast to the pheromone GPCRs, which are expressed in only haploid cells and in a mating-type-specific fashion, the Gpr1 receptor is expressed in both diploid and haploid cells of either mating type. Broach (2012) showed that we have a detailed understanding of some of the circuitry underlying nutritional sensing in yeast, but we are still somewhat vague on others. The availability of key nutrients, such as sugars, amino acids, and nitrogen compounds, dictates the developmental programs and the growth rates of yeast cells. A number of overlapping signaling

networks, those centered on Ras/protein kinase A, AMP-activated kinase, and target of rapamycin complex I, for example, inform cells on nutrient availability and influence the cell transcriptional, translational, post-translational, and metabolic profiles as well as their developmental decisions. For instance, the interplay of positive and negative regulators and the various feedforward and feedback loops in regulating expression of glucose transporters is so well described that modeling efforts have yielded highly predictive dynamic descriptions of its behavior. In *S. cerevisiae*, Rgt2p and Snf3p are two sensors that upon glucose detection increase the expression of transporters, therefore enhancing glucose uptake. In the absence of glucose, Rgt1p forms a repressor complex with Mth1p, Std1p, and Ssn6p-Tup1p, inhibiting the expression of *HXT* transporter-encoding genes. When glucose is available, Mth1 and Std1 are phosphorylated by casein kinase I and ubiquitinated by the SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase Grr1, which leads to their degradation by the 26S proteasome. Depletion of the corepressors dissociates Rgt1 and relieves repression of hexose transporter gene transcription. On the other hand, we still have no clear understanding of the upstream components of glucose signaling that regulate protein kinase A or the interplay between the various small G proteins in that process. Even more poorly described are the pathways sensing and responding to nitrogen levels, especially that nitrogen regulates pathogenicity and filamentation. In addition, cell growth and cell-cycle progression are dependent on the availability of nitrogen and other nutrients and subsequent signaling via the target of rapamycin complex1. Although many of the components of the target of rapamycin complex TORC1, which controls G_2/M progression in yeast, and the signaling network have been identified and their interactions defined, we have less understanding of the pathways emanating from TORC1, particularly through protein phosphatases. Moreover, we can infer the existence of a second nitrogen-sensing pathway from the limits of TORC1 effects, but this pathway is poorly defined. Finally, we appreciate that significant crosstalk exists between and among the various nutrient signaling pathways, for instance, glucose sparing in nitrogen- or phosphate-starved cells, but the nature of that interplay is undefined. Thus, we have a number of important details to fill in regarding the structure of the nutrient-sensing networks. In addition, several fundamental questions regarding the interplay of nutrient availability and growth have yet to be solved. Roelants et al. (2017) reported that for eukaryotic cell growth, they must expand by inserting glycerolipids, sphingolipids, sterols, and proteins into their plasma membrane and maintain the proper levels and bilayer distribution. When it was found that the antibiotic rapamycin was a potent inhibitor of the proliferation of virtually every eukaryotic cell type examined (e.g., fungi, T cells, and tumor cells), it became clear that the molecular target of rapamycin must be highly conserved and its function is critical for cell survival. Indeed, ever since the authentic target of rapamycin (TOR) was first discovered using an elegant genetic approach in the budding yeast *Saccharomyces cerevisiae*, TOR has emerged as a universal, centrally important sensor, integrator, and controller of eukaryotic cell growth. TOR is a serine/threonine kinase of the phosphatidylinositol kinase-related kinase family, which shares conserved motifs (such as HEAT repeats, FAT, and FATC domains), and is structurally and functionally conserved in eukaryotes.

The TOR pathway orchestrates the growth of cells in response to nutrients. Tor proteins sense nutrient signals, including amino acids, and regulate a broad range of cell developmental and signaling processes, including ribosome biosynthesis, protein translation, starvation-related transcriptional regulation, and autophagy. A fungal cell must coordinate growth with enlargement of its cell wall. In *S. cerevisiae*, a plasma membrane-localized protein kinase complex, TORC2, serves as a sensor and master regulator of these plasma membrane- and cell wall-associated events by directly phosphorylating and thereby stimulating the activity of two types of effector protein kinases: Ypk1, along with a paralog (Ypk2), and Pkc1. Ypk1 is a central regulator of pathways and processes required for plasma membrane lipid as well as protein homeostasis requires phosphorylation on its T-loop by eisosome-associated protein kinase Pkh1 and a paralog (Pkh2). For cell survival under various stresses, Ypk1 function requires TORC2-mediated phosphorylation at multiple sites near its C-terminus. Pkc1 controls diverse processes, especially cell wall synthesis and integrity. Pkc1 is also regulated by Pkh1- and TORC2-dependent phosphorylation, but in addition, by interaction with Rho1-GTP and lipids phosphatidylserine (PtdSer) and diacylglycerol (DAG).

There are an increasing number of studies on 14-3-3 proteins and GTPase families that are defined as functionally conserved eukaryotic proteins which participate in many important cellular processes in fungi. These studies reveal that 14-3-3 proteins are related to fungal growth, which is a complex phenomenon related to nutrient assimilation and development, such as dimorphic transition and mycelial growth, cell program regulation, development, autophagy, apoptosis, that might be a universal stress response signaling cascades and virulence. Fungi have genetic interactions with TOR signaling transduction pathways, which are also important during nutrient stress and could regulate metabolic processes, regulation of carbon and nitrogen metabolism, by directly interacting with and modifying the functions of target enzymes, with numerous roles in the regulation of biological processes as adapters, activators, or repressors in the regulation of signal transduction pathways. The BMH1 gene from *Candida albicans* is essential, and both wild-type alleles are necessary for the optimal growth and morphogenesis of this organism. They could control filamentation and the dimorphic transition in some dimorphic fungi by responding to different environmental factors, especially in *C. albicans*. In basidiomycetous fungus, it was reported that Pdc1 (a homolog of 14-3-3 in *Ustilago maydis*), had an important role in the regulation of the dimorphic switch in *U. maydis*. In *S. cerevisiae*, 14-3-3 proteins are required for the G_1/S transition; in contrast, in *U. maydis* these are essential during the G_2/M transition and have an important function in its sexual reproduction. To date, some 14-3-3 interacting proteins that regulate growth have been discovered in fungi; however, numerous biological processes (especially in mycelial development and secondary metabolism in filamentous fungi) and the regulatory mechanisms of 14-3-3 proteins are still unclear (Shi et al. 2019). The reprogramming of gene expression during stress typically involves initial global repression of protein synthesis, accompanied by the activation of stress-responsive mRNAs through both translational and transcriptional responses. Crawford and Pavitt (2018) summarized the major repressive mechanisms and

discussed the mechanisms of translational activation in response to different stresses in *S. cerevisiae*. Taken together, a wide range of studies indicated that multiple elements act in concert to bring about appropriate translational responses: these include regulatory elements within mRNAs, altered mRNA interactions with RNA-binding proteins, and the specialization of ribosomes that each contribute toward regulating protein expression to suit the changing environmental conditions.

In budding yeast, an important part of extracellular glucose sensing and signaling is mediated by the cAMP-PKA pathway. A dual glucose-sensing system is involved in the activation of the cAMP-PKA pathway: on the one hand, extracellular glucose sensing occurs through the GPCR system composed of Gpr1 and its associated Gα protein, Gpa2, and on the other hand, an intracellular system dependent on glucose uptake and hexokinase-mediated phosphorylation that activates in some unknown way the Ras proteins (Rutherford et al. 2019). All Ras proteins are members of a eukaryotic subfamily of small GTPases involved mainly in cellular signal transduction. The structurally and functionally conserved TOR pathway has for a long time been suggested to contribute to the regulation of cell growth and many related properties by nutrient availability. However, no clear mechanisms have been identified by which the TOR pathway would detect extracellular nutrients, and more recent work suggests that the TOR proteins rather sense intracellular nitrogen availability, in particular mobilization of nitrogen reserves from the vacuole/lysosome. *S. cerevisiae* possesses two *TOR* genes: Tor1 and Tor2. The discovery of several types of plasma membrane nutrient sensors, including a GPCR, several transporter-like sensors, and multiple transceptors, has firmly established yeast as the leading model organism in the field of cellular nutrient sensing. A favored hypothesis is that the PKA-regulating transceptors act in a way that is analogous to GPCRs. A major challenge for the future is the elucidation of the molecular mechanisms involved in nutrient responses that at least partially depend on the metabolism of the nutrient. These mechanisms are much more difficult to identify because of the complex nature of metabolism and the many side effects caused by genetic modification of metabolic pathways. It can be predicted easily that there must be many more allosteric interactions between metabolic intermediates and components of signaling pathways than are currently known. A major mechanism likely to be identified soon is that involved in activation of the Ras proteins by one or more intermediates of glucose catabolism. Because of the importance of Ras in cancer induction in mammalian cells and the well-known overactive glycolysis in cancer cells, that is, the Warburg effect, elucidation of this mechanism may have major consequences for our general understanding of the connection between glycolysis and control of cellular proliferation. The TOR pathway has long been considered as the main integrator of multiple nutrient signals for cellular growth control. However, more and more evidence indicates that the TOR pathway is primarily a specific nitrogen-sensing pathway, with a main role in coordinating the availability of extracellular nitrogen with that of intracellular nitrogen reserves and with its effect on cellular growth being one of the multiple outcomes of this function. Hence, a major challenge for the future remains the identification of the nutrient sensors that regulate cellular growth. In this respect, it is important to realize that all essential nutrients,

the macronutrients providing carbon, nitrogen, phosphorus, and sulfur, as well as the micronutrients such as metal ions and vitamins, have a decisive effect on cellular growth control and hence should all be sensed in some way to exert this function. A specific mechanism may exist for the regulation of cellular growth by each nutrient, but alternatively, a common principle may be involved in sensing all essential nutrients for cellular growth control, and these nutrient sensors may interact much more directly with the protein synthesis machinery than previously anticipated. At present, nutrient control of bulk protein synthesis remains vague and the true relevance of the few controls identified remains poorly defined. Another gap in our understanding is the link between initial nutrient responses and long-term adaptation to the same nutrient. At present, we know that in the nutrient responses for which it has been investigated, the two processes have different requirements, but how the rapid response proceeds to the long-term response at the molecular level is unknown. The powerful genomic and proteomic technologies currently available have led to rapid progress in identifying the scope of signal transduction pathway targets. Most of this information, however, has been obtained with gene deletion or overexpression strains, or using small-molecule inhibitors that completely inactivate the target protein. This point raises the question to what extent the very many changes in target genes or proteins usually detected are physiologically relevant. Another outcome of these studies has been that the signaling pathways investigated affect targets in other parts of metabolism than previously considered. Here too, the true physiological relevance of the widened scope remains to be determined (Conrad et al. 2014). It is well established that the protein kinases PKA and Sch9, and the tor complex 1 (TORC1), have a central role in the nutrient-induced signaling network that controls growth, survival, and longevity by maintaining a tight balance between proliferation and stress defense. PKA activity is regulated by the Ras–cAMP pathway and activation of adenylate cyclase. The latter requires extracellular sensing of glucose via the Gpr1–Gpa2 GPCR system as well as intracellular glucose sensing via the hexokinases Hxk1/2 and glucokinase Glk1, which in turn stimulate the small GTPases Ras1 and Ras2. Nitrogen sources activate TORC1 at the vacuolar membrane. Depending on their quality as a nitrogen source, amino acids act through an evolutionarily conserved mechanism comprising EGOC, a complex containing the Rag-like GTPases Gtr1 and Gtr2. Sch9 is a well-known TORC1 effector and shuttles between the cytoplasm and the vacuole in a glucose-dependent manner. Besides TORC1, Sch9 activity is also regulated by the sphingolipid-dependent PDK1 orthologs Pkh1-3 and the protein kinase Snf1, a key factor in glucose repression. The pathways controlled by PKA, TORC1, and Sch9 converge on the protein kinase Rim15, which ensures proper entry into the stationary phase by activating the expression of the so-called STRE- and PDS-controlled genes during the diauxic shift. When the stress is halted or when nutrients are again plentiful, trehalose is rapidly degraded and, here, the availability of Pi dictates the PKA-dependent activation of Nth1, the neutral trehalase that hydrolyzes the disaccharide in the cytoplasm.

To sense the changes in nutrient availability, the yeast *S. cerevisiae* has three different classes of nutrient-sensing proteins acting at the plasma membrane: GPCRs, which detect the presence of certain nutrients and activate signal transduction in

association with a G protein; non-transporting transceptors, that is, nutrient carrier homologs with only a receptor function, previously called nutrient sensors; and transporting transceptors (*trans*port and rece*ptor*), active nutrient carriers that combine the functions of a nutrient transporter and receptor. Rubio-Texeira et al. (2010) provided an updated overview of the proteins involved in sensing nutrients for rapid activation of the protein kinase A pathway, which belong to the first and the third category, and they also provided a comparison with the best-known examples of the second category, the non-transporting transceptors, which control the expression of the regular transporters for the nutrient sensed by these proteins. Three well-known types of molecular machines employed for the sensing of the environment (receptors, transporters, and channels) are polytopic transmembrane proteins, monomeric or oligomeric, located in the plasma membrane of all types of cells. Strikingly, the transmembrane receptors involved in nutrient sensing (e.g., Ssy1, Mep2, Snf3, and Rgt2) are structurally homologous to nutrient transporters. In contrast to bona fide receptors, transporters and channels mediate the uptake of solutes, metabolites, drugs, or ions, which themselves can act as molecular signals once intracellularly accumulated (Diallinas 2017). Snf3 is used by *S. cerevisiae* to sense glucose, fructose, and mannose.

Microorganisms have multiple sensing systems to sense extracellular and intracellular nutrient signals to adapt to the environment and their own metabolic state. Fungal pathogens prefer certain carbon sources for rapid uptake and metabolism to provide energy for growth and host colonization. The GPCR gene family represents one important sensor system that has been found to have important roles in nutrient sensing in many fungal species (Xue et al. 1998, 2006; Lorenz et al. 2000; Bardwell 2004; Han et al. 2004; Lemaire et al. 2004; Miwa et al. 2004; Maidan et al. 2005a, b). In the yeast *S. cerevisiae*, Ca^{2+} signaling mediated by the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, is required for survival during environmental stress. The phosphatase activates gene expression through its regulation of the Crz1p ("crazy") transcription factor. Calcineurin dephosphorylates Crz1p and causes its rapid translocation from the cytosol to the nucleus, then Crz1p activates the transcription of genes whose products promote cell survival (Cyert 2003). Permeases and their homologs function as sensors for nutrients, including homologs of permeases for sugars, amino acids, ammonia, and phosphate (Bahn et al. 2007). Starved yeast cells anticipate exposure to glucose by activating the Hxt5p (hexose transporter 5) glucose transporter, providing an advantage during early phases after glucose resupply. When cAMP and glucose fluorescence resonance energy transfer (FRET) sensors were used to identify three signaling pathways that cooperate in the anticipatory Hxt5p activity in glucose-starved cells, it was found as expected that Snf1 and the AMP kinase pathway do cooperate, but surprisingly also are dependent on an extracellular G-protein-coupled Gpr1 (G-protein-coupled receptor1)/cAMP/PKA (protein kinase A) pathway and the Pho85 (phosphate metabolism85)/Plc (phospholipase C) 6/7 pathway. Gpr1/cAMP/PKA are key elements of a G-protein-coupled sugar response pathway that produces a transient cAMP peak to induce growth-related genes. A novel function of the Gpr1/cAMP/PKA pathway was identified in glucose-starved cells: during starvation, the Gpr1/

cAMP/PKA pathway is required to maintain Hxt5p activity in the absence of glucose-induced cAMP spiking. During starvation, cAMP levels remain low, triggering expression of HXT5, whereas cAMP spiking leads to a shift to the high-capacity Hxt isoforms (Bermejo et al. 2013). Further work is needed specifically whether the pathway is constitutively active in the absence of glucose, whether PKA exists in different phosphorylation states, and whether the two states regulate different sets of genes. An interesting question to address will also be how the two pathways cooperate to trigger the ability to accumulate glucose and whether they measure distinct signals, such as lack of extracellular glucose or reduced energy status. A putative seven-transmembrane protein gene, *stm1*+, which is required for proper recognition of nitrogen starvation signals, was isolated as a multicopy suppressor of a *ras1* synthetic lethal mutant in *Schizosaccharomyces pombe* (Chung et al. 2001). Under nitrogen-deficient conditions, transcription of the *stm1* gene was induced. Under nutritionally sufficient conditions, overexpression of Stm1 inhibited vegetative cell growth, resulting in decreased intracellular cAMP levels, increased the expression of the meiosis-specific genes *ste11*, *mei2*, and *mam2*, and facilitated sexual development in homothallic cells. Induction of *ste11*, a meiosis-specific gene transcription factor, by Stm1 overexpression was enhanced in *gpa2*-deleted cells but was absent in a deletion mutant of *sty1*, a key protein kinase that links mitotic control with environmental signals and induces stress-responsive genes. Moreover, deletion of both *stm1* and *ras1* caused delayed entry into G_1 arrest in *S. pombe* when the cells were grown on a nitrogen-deficient medium. Thus, it is considered that the *stm1* gene can function through Gpa2-dependent and/or -independent pathways and may have a role in providing the prerequisite state for entering the pheromone-dependent differentiation cycle in which heterotrimeric Gα1 protein, Gpa1, and Ras1 are major factors. Stm1 could function as a sentinel molecule sensing the nutritional state of the cells, stopping the proliferative cell cycle, and preparing the cell to enter meiosis under nutritionally deficient conditions.

In several fungi, responses to contact with a surface are promoted by GPCR family members. As some GPCRs have been shown to be mechanosensitive, the mechanical forces that result from contact might activate fungal GPCRs and contribute to contact-dependent differentiation. The details of how mechanical forces influence GPCR function await clarification (Kumamoto 2008). A modified form of the human B2 bradykinin GPCR, which was fused to two fluorescent proteins to allow fluorescence resonance energy transfer (FRET) between the two fluorescent proteins, was shown to undergo conformational changes that reduced FRET in response to shear stress (Chachisvilis et al. 2006). Thus, GPCRs are mechanosensitive and undergo conformational changes in response to mechanical forces, such as stretching. The fungal cell wall is essential for its viability and an important target of antifungal drugs. In fungi, a conserved MAPK signaling module is responsible for maintaining cellular integrity, shape, and resilience to environmental stresses (Lesage and Bussey 2006). The plant pathogen *Magnaporthe grisea*, the causative agent of rice blast, undergoes appressorium formation following contact with a surface. Contact with a hard surface is required for appressorium formation, and neither soft surfaces nor liquids are inductive for appressorium development. Members

of the GPCR family also regulate morphogenesis in *Candida albicans* and in the model organism *S. cerevisiae*. In *C. albicans*, the formation of filamentous hyphae is promoted by numerous cues from the environment, including contact. Contact sensing results in the formation of hyphae that penetrate into the substratum below the *C. albicans* cells. Filamentation in response to contact is defective in *C. albicans* mutants that lack the GPCR Gpr1p, whereas filamentation of this mutant in response to other environmental cues is normal. *S. cerevisiae* forms pseudohyphae when grown in contact with agar medium of the appropriate composition (for example, low nitrogen content), and *S. cerevisiae* GPR1 is important for pseudohyphal development. *S. cerevisiae* Gpr1p binds sugars such as glucose and sucrose directly and is thought to be a nutrient sensor. In the yeast *S. cerevisiae*, rapid activation of the cAMP pathway by glucose and sucrose requires the GPCR Gpr1. Lemaire et al. (2004) obtained results by cysteine scanning mutagenesis and the substituted cysteine accessibility method (SCAM) of residues in TMD VI that provided strong evidence that glucose and sucrose directly interact as ligands with Gpr1, with the affinity for sucrose being much higher (Rutherford et al. 2019). Structurally, mannose acts as an antagonist for both sucrose and glucose. Although the mechanosensitivity of these fungal GPCRs has not been directly investigated, the mechanosensitivity of other members of this protein family suggests that fungal GPCRs might respond to physical forces which act on the membrane. In summary, GPCR signaling is a conserved feature of contact-dependent morphogenesis in several fungal species. The observation that activation of some GPCRs is influenced by mechanical forces that act on the membrane suggests that contact-dependent perturbation of the membrane could potentiate or activate GPCRs in fungal contact sensing (Kumamoto 2008).

Although much is known about the mechanisms of stress-dependent signaling, less is known about coordination between the stress response and other cell signaling processes. Cells routinely experience changing and often unfavorable conditions in their environment. All cells respond to osmotic stress by implementing molecular signaling events to protect the organism. Many are transmitted by GPCRs or the high osmolarity glycerol (HOG) pathway. Salt and hyperosmotic stress trigger a complex adaptive response in yeast cells, which is coordinated by the HOG MAP kinase pathway. MAPKs are activated in response to osmotic stress, as well as by signals acting through GPCRs. For proper adaptation, the action of these kinases must be coordinated. To identify second messengers of stress adaptation. Shellhammer et al. (2017) conducted a mass spectrometry-based global metabolomics profiling analysis, quantifying nearly 300 metabolites in the yeast *S. cerevisiae*. They showed that three branched-chain amino acid (BCAA) metabolites increase in response to osmotic stress and require the MAPK Hog1. Ectopic addition of these BCAA derivatives promotes phosphorylation of the G protein α-subunit and dampens G-protein-dependent transcription, similar to that seen in response to osmotic stress. Conversely, genetic ablation of Hog1 activity or the BCAA regulatory enzymes leads to diminished phosphorylation of Gα and increased transcription. Taken together, their results define a new class of candidate second messengers that mediate crosstalk between osmotic stress and GPCR signaling pathways.

The molecular mechanisms that enable yeast cells to detect and transmit cold signals and their physiological significance in the adaptive response to low temperatures are unknown. To overcome environmental stresses, fungi, as do other eukaryotes, rely on the rapid transduction of signals through mitogen-activated protein kinase (MAPK) pathways. Panadero et al. (2006) demonstrated that the MAPK Hog1p is specifically activated in response to cold. Phosphorylation of Hog1p was dependent on Pbs2p, the MAPK kinase (MAPKK) of the high osmolarity glycerol (HOG) pathway, and Ssk1p, the response regulator of the two-component system Sln1p-Ypd1p. Although *C. albicans* Sln1 can functionally replace *S. cerevisiae* Sln1 and is indeed involved in osmosensing, Sln1 is not essential for viability of *C. albicans.* However, the HOG pathway is activated by heat and cold stress in *S. cerevisiae*, but these stressors do not activate the HOG pathway in *C. albicans*, in which mitochondrial function appears to be required for activation of CaHog1 in response to oxidative stress. Interestingly, phosphorylation of Hog1p was stimulated at 30°C in cells exposed to the membrane rigidifier agent dimethyl sulfoxide. Moreover, Hog1p activation occurred specifically through the Sln1 branch, which suggests that Sln1p monitors changes in membrane fluidity caused by cold. Quite remarkably, the activation of Hog1p at low temperatures affected the transcriptional response to cold shock. Indeed, the absence of Hog1p impaired the cold-instigated expression of genes for trehalose- and glycerol-synthesizing enzymes and small chaperones. Moreover, a downward transfer to 12° or 4°C stimulated the overproduction of glycerol in a Hog1p-dependent manner. However, hog1Δ mutant cells showed no growth defects at 12°C as compared with the wild type. On the contrary, deletion of HOG1 or GPD1 decreased tolerance to freezing of wild-type cells preincubated at a low temperature, whereas no differences could be detected in cells shifted directly from 30° to −20°C. Thus, exposure to low temperatures triggered a Hog1p-dependent accumulation of glycerol, which is essential for freeze protection. Steroids are perceived as stress by yeast cells that triggers general stress response leading to activation of heat-shock proteins, cell-cycle regulators, MDR transporters, etc. (Prasad et al. 2012).

Flammulina velutipes (Curt. ex Fr.) Sing, a white-rot fungus, is known as golden-needle mushroom, winter mushroom, or enokitake. Because of its high nutritional and medicinal value, *F. velutipes* is cultivated on a significant scale worldwide, which has both ecological and commercial importance. The fruit-body quality of *F. velutipes* is determined by both genotype and environment. Both yield and quality of *F. velutipes* fruit-bodies differ largely depending on their primordium number, and the initiation of primordium is prone to environmental factors. Knowledge of chronological protein expression patterns in *F. velutipes* mycelia in response to cold stress is an important metabolic engineering strategy that considers the impact of novel genes and pathways on cold adaptation in fungi. Proteomics is a broad instrument-intensive research area that has advanced rapidly since its inception. Liu et al. (2017) undertook to investigate chronological changes of protein expression during *F. velutipes* mycelia in response to cold stress. The proteomics data revealed that some interesting proteins upregulated in some central metabolic pathways might be important during mycelia response to cold stress in *F. velutipes.* More than

four functional categories selected to evaluate the data sets were mainly involved in energy metabolic processes, amino acid biosynthesis and metabolism, signaling pathway, transport, and translation. They found that the global expressed and differentially expressed proteins participated in energy metabolism pathways including the citrate cycle, pentose phosphate pathway, glyoxylate and dicarboxylate metabolism, and sucrose metabolism. They showed complex protein abundance change patterns in acute normal culture to cold stress transfer in mycelia of *F. velutipes* at the molecular level. The four proteins that had higher expression levels after cold stress in mycelia and involved in energy metabolism were catalase, glucose-6-phosphate isomerase, trehalase, and beta-glucosidase. Catalase, universal in many fungi, a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), was significantly upregulated during exposure of mycelia to short-term cold stress, but its expression levels returned to normal after long-term cold stress. Worth noting, *S. cerevisiae* bZip transcription factor ScYap1 and its *C. albicans* homolog CaCap1 are also involved in oxidative stress response. To prevent damage to cells and tissues, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide, a harmful by-product of many normal metabolic processes, into less reactive gaseous oxygen and water molecules. Glucose-6-phosphate isomerase (GPI), a dimeric enzyme universally distributed in eukaryotes, catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate, trehalase, and beta-glucosidase expression, and dramatically increased after long-term cold stress. In the cytoplasm, it is involved in both glycolysis and gluconeogenesis, as well as the pentose phosphate pathway. Outside the cell, GPI is also known to function as a maturation factor. Cloning experiments revealed that GPI is identical to the protein known as autocrine motility factor (AMF) produced and secreted by cancer cells that is thought to have a key role in regulating endoplasmic reticulum (ER) calcium release to protect against apoptosis in response to ER stress, and stimulates cell growth and motility as a growth factor by activating the MAPK/ERK or PI3K/AKT pathways. Such pathways are signal transduction pathways that promote survival and growth in response to extracellular signals. Beta-glucosidase, a heterogeneous group of exo-type glycosyl hydrolases, catalyzes the hydrolysis of β-glucosidic linkages in β-D-glucosides and oligosaccharides, with release of glucose monomers by breaking the β-1,4 glucosidic bonds of cellobiose. Beta-glucosidases are important in fundamental biological processes, such as nutrient uptake and developmental regulation or chemical defense against pathogen attack. Beta-glucosidase catalyzes the hydrolysis of β-glucosidic linkages in β-D-glucosides and oligosaccharides, with release of glucose monomers by breaking the β-1,4 glucosidic bonds of cellobiose. Trehalase, one of the important carbohydrate storages in fungi, is an important physiological process for fungal spore germination and the resumption of mycelium growth, and catalyzes the conversion of trehalose to glucose: this suggests that trehalase may be important in energy metabolism during fungal mycelia to fruit-bodies transfer and regulating fruit-body formation. The sugar is thought to form a gel phase as cells dehydrate, which prevents disruption of internal cell organelles, by effectively splinting them in position. Knowing the proteins associated with energy metabolic pathways

responsible for the development of the mycelia is helpful to better understanding of the molecular mechanisms of mycelium resistance to cold stress and fruiting-body formation in *F. velutipes.* Amino acids serve as major nitrogen carriers in the long-distance transport systems, precursors to important metabolites, stress response molecules, and signaling molecules. Eleven DPSs were shown to be involved in the biosynthesis and metabolism of nine amino acids. Argininosuccinate synthase and class V chitinase ChiB1 were significantly upregulated in mycelia under short-term cold stress, but its expression levels were dramatically decreased during long-term cold stress. The metabolic pathways in which argininosuccinate synthase participates are linked to the varied uses of the amino acid arginine, such as arginine synthesis, urea synthesis, nitric oxide synthesis, polyamine synthesis, and creatine synthesis. Arginine is the main precursor for urea formation whereas accumulation of large quantities in fruit-bodies is a known feature of larger basidiomycetes. Arginine is a significant nutrient molecule for the transport from the mycelium during early primordium development. The class V chitinase expression level increased in mycelia under short-term cold stress, and it may increase energy supply to promote resistance to cold stress. An increased abundance of signaling pathways and other process-related proteins was shown after mycelia were exposed to cold stress. A total of 1198 proteins were identified and quantified in three different mycelium growth stages of *F. velutipes*, demonstrating the applicability of the iTRAQ approach to multiplexed proteomic profiling was successful. A broader comparison of the proteomes of the three mycelium developments revealed that changes in some central metabolic pathways may be involved in the cold stress responses of *F. velutipes* mycelia.

Recent advances on the control of pH homeostasis revealed interesting new aspects underpinning the crosstalk within the nutrient signaling network (Eskes et al. 2017). In the model ascomycete and occasional pathogen *Aspergillus nidulans*, the PacC transcription factor governs gene expression in response to extracellular pH and is vital for mammalian pathogenicity (Díez et al. 2002). Under alkaline conditions, a signaling cascade involving seven proteins is involved in activation of PacC. A putative pH sensor, PalH, has 7TMDs and a cytoplasmic C-terminus that interacts with a cognate arrestin encoded by palF. Unlike canonical GPCR receptors, PalH is not thought to act via interaction with G-protein subunits. When an alkaline response is triggered, PalF is phosphorylated and subsequently ubiquitinated in a PalH-dependent manner, leading to PalB-mediated, signal-dependent, proteolytic cleavage of the pH-responsive transcription factor PacC. Subsequent translocation of the truncated PacC protein, from cytoplasm to nucleus, permits alkaline adaptation via differential expression of genes required to enable growth under alkaline extracellular conditions (Espeso et al. 2000). In *Aspergillus fumigatus* the amino acid residues crucial for PalH and PalF interaction are conserved, and in split-ubiquitin analyses the proteins enter into close proximity.

Peroxisomes are ubiquitous, DNA-free organelles present in most eukaryotic cells. Yeast peroxisomes readily adapt their number and physiological function in response to changes in the metabolic state of the cell. Manzanares-Estreder et al. (2017) quantified the growth efficiency of yeast cultures upon NaCl stress while

continuously lowering the glucose content. Growth on high-salinity media was dramatically affected by low sugar availability. They suggested that a possible explanation for this dramatic effect was that salt stress caused glucose starvation, which in turn would make necessary the use of alternative energy sources on stress. They suggest that during the defense to salt stress, yeast cells switch from a fermentative metabolism to fatty acid oxidation to cover the energetic needs during the environmental challenge. One important energy resource are intracellular lipid stores, which can be mobilized during sugar starvation by peroxisomal β-oxidation in yeast cells. Their results indicated that peroxisomal function was growth limiting upon salt stress especially during low sugar availability. They found that the HOG MAP kinase pathway was the master regulator of gene expression upon osmostress in yeast cells and confirmed that the Hog1 MAP kinase was indispensable for the increase of mRNA levels of genes involved in the mobilization of fatty acids. They confirmed that the Hog1 kinase function was indispensable for the NaCl induction of genes involved in fatty acid metabolism. Peroxisomal function was responsible for reinforced yeast mitochondrial respiration upon salt stress. For more details about fungal sensing of the host environment and stress, the reader is referred to Braunsdorf et al. (2016).

3.6 GPCRs and Fungal Pathogenicity Determinants

Only a handful of GPCRs have been identified in fungal genomes. However, in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* only three and four receptors, respectively, are well characterized. In the *Neurospora crassa* genome, a total of ten receptors are predicted. A recent report for *Aspergillus nidulans* identified GPCRs similar to the yeast pheromone receptors, the glucose-sensing receptor GPR1, the nitrogen-starvation sensing STM1, and the *Dictyostelium discoideum* cAMP receptors (Kulkarni et al. 2005). Fungal pathogens sense nitrogen levels to control their rate of growth and changes in their morphology, processes that are important for host infection.

Magnaporthe grisea causes rice blast disease, the most destructive disease of rice worldwide. *M. grisea* is amenable to molecular genetic manipulation and the subject of large-scale genome-wide functional studies following the recent completion of its draft genome sequence. It worth noting that *M. grisea* and *Magnaporthe oryzae* are not interfertile and are considered different species (Couch and Kohn 2002). Cell-surface receptors, as previously mentioned, that perceive signals at critical times in the life cycle of *M. grisea* and other pathogenic fungi are strongly implicated as pathogenicity determinants. Signaling has a key role in appressorium formation and infection in *M. grisea*. The cAMP-dependent and pheromone response, as well as other mitogen-activated protein kinase (MAPK)-, phospholipase-, and calmodulin-dependent pathways, are essential for pathogenicity and are likely to involve perception of signals through GPCRs (Beckerman et al. 1997; Mitchell and Dean 1995). The three identified G-protein subunits required for

different aspects of development and pathogenicity possibly transduce perceived signals to the aforementioned pathways (Liu and Dean 1997). The *M. grisea* G proteins probably receive signals from receptors such as PTH11, an integral membrane protein required for development of the appressorium and pathogenicity. It was proposed to act upstream of the cAMP pathway that restores both appressorium formation and pathogenicity (DeZwaan et al. 1999). A BLASTP search against the genome of the closely related filamentous fungus *Neurospora crassa*, using all the *M. grisea* GPCR-like proteins as query, revealed the presence of similar proteins in *N. crassa*, including PTH11 homologs (Galagan et al. 2003; Kulkarni et al. 2005). Analyses revealed putative homologs of the mPR-1 class in both yeasts in which they had not previously been identified (Kulkarni et al. 2005). They found three members of the mPR class and one (MG0532.4) with weak similarity to animal GPCRs. No members of these classes have been reported previously in fungi. Homologs of known fungal GPCRs were found in the *M. grisea* proteome, including the pheromone receptors STE2 and STE3 and the glucose-sensing receptor GPR1. In total, 76 GPCR-like proteins were identified in their study, of which 61 represent a large novel class related to PTH11, a receptor implicated in fungal development and pathogenicity that they proposed acts upstream of the cAMP-dependent pathway. Many of these novel receptors will have roles in known pathways or may define new pathways involved in fungal development. The cAMP, STM1, and mPR receptors are shared between fungi and other eukaryotic species. However, the fungal pheromone receptors (class D) and GPR1-like receptors appear to be fungus specific. For fungus-specific receptors, the conserved domain spanned almost the entire length of the seven transmembrane regions. Members of the large class of PTH11-related receptors were restricted to a fungal subphylum. In the rice blast fungus *M. oryzae*, a non-canonical G-protein-coupled receptor, Pth11, and membrane sensors MoMsb2 and MoSho1 are thought to function upstream of G-protein/cAMP signaling and the Pmk1 MAPK pathway to regulate appressorium formation and pathogenesis (Li et al. 2017). BLASTP of all the PTH11 class members, and PSI-BLAST using conserved regions, against the GenBank (nonredundant) and Swiss-Prot databases and publicly available fungal genomes, retrieved matches in members of the subphylum Pezizomycotina within the Ascomycota, including *Podospora anserina*, *Blumeria graminis*, *Fusarium graminearum*, and *Aspergillus* species. Other fungi belonging to the Ascomycota but not to this subphylum, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, and *Pneumocystis carinii* lacked PTH11-related sequences. Also, no PTH11-related sequences were found in the genomes of the Basidiomycetes *Cryptococcus neoformans*, *Ustilago maydis*, and *Phanerochaete chrysosporium*. For fungus-specific receptors, the conserved domain spanned almost the entire length of the seven transmembrane regions (Kulkarni et al. 2005; Zhao et al. 2007), containing a conserved fungus-specific extracellular membrane-spanning domain (CFEM) at the amino terminus that is unique to filamentous fungi and contains eight conserved cysteine residues. It is similar to epidermal growth factor (EGF)-like domains that function as extracellular receptor or signal transducers or as adhesion molecules in host–pathogen interactions. PTH11 GPCRs containing CFEM domains

have been identified in *Trichoderma atroviride* and *Trichoderma reesei*, and four in *Trichoderma virens* (Gruber et al. 2013). Sabnam and Barman (2017) selected and characterized a novel and unique conserved fungal-specific extracellular membrane-spanning (CFEM) domain containing a PTH11-like G-protein-coupled receptor (GPCR) in *M. oryzae* and called it WISH. The CFEM domain has been reported to function as a cell-surface transmembrane receptor or adhesion molecule during host–pathogen interactions. Their data suggest that WISH protein mediates appressorium differentiation and has a pleiotropic effect as well as acting as a negative regulator of conidium and conidiophore development in *M. oryzae*. They suggested that PTH11 and WISH might be functionally related to recognizing different chemical and physical signals present on the rice leaf surface; the WISH gene appears much more critical and vital than PTH11 in regulating commitment of the pathogen in the process of infection-related morphogenesis. In general, when microorganisms attempt to invade plants they are recognized by the plant immune system through pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs), which are highly conserved molecules such as flagellin in bacteria, glucans in oomycetes, and chitin in fungi. Microbial invasions often trigger the production of host-derived endogenous signals referred to as danger- or damage-associated molecular patterns (DAMPs), which are also perceived by pattern recognition receptors (PRRs) to modulate PAMP-triggered immunity PTI responses (reviewed recently by Yu et al. 2017). The recognition of such molecules by PRRs that reside in the plant plasma membrane triggers an innate immune response known as PTI or MAMP-triggered immunity (MTI) (Maffei et al. 2012). Fungi change their global gene expression upon recognition of the host environment, leading to secretion of effectors, enzymes, and secondary metabolites; changes in metabolism; and defense against toxic compounds. During infection, pathogens secrete an arsenal of several hundred effectors, to target multiple host cell compartments and enable parasitic infection, which seem to be produced in consecutive expression waves during the course of an infection and/or are associated with pathogenic transitions (Toruno et al. 2016). Such effectors are the key elements of pathogenesis that modulate innate immunity of the plant and facilitate infection. Successful invaders are able to dampen or overcome PTI (or MTI) through the secretion of pathogen effectors that either remain at the plant–pathogen interface (apoplastic effectors) or are taken up by host cells (translocated or cytoplasmic effectors). Doehlemann et al. (2011) identified the PIT (proteins important for tumors) cluster, a novel gene cluster composed of four genes, two of which are predicted to encode secreted effectors. The apoplastic effectors Pep1 and Pit2 are functionally characterized (Doehlemann et al. 2009, 2011; Hemetsberger et al. 2012; Mueller et al. 2013) as well as the three cytoplasmic effectors Cmu1, Tin2, and See1 (Djamei et al. 2011; Tanaka et al. 2014; Redkar et al. 2015). Cmu1 is a protein rechannelling chorismate present in the phenylpropanoid pathway and thereby outcompeting the biosynthesis pathway of the plant defense salicylic acid. Pep1 is known to inhibit plant peroxidases and thereby interferes with the oxidative burst, whereas Pit2 inhibits cysteine proteases in the biotrophic interface. See1 is required for the reactivation of plant DNA synthesis, which is crucial for tumor progression

in leaf cells and does not affect tumor formation in immature tassel floral tissues, where maize cell proliferation occurs independently of fungal infection. It interferes with the MAPK-triggered phosphorylation of maize SGT1 at a monocot-specific phosphorylation site, which identifies See1 as a fungal effector that directly and specifically contributes to the formation of leaf tumors in maize. Stirnberg and Djamei (2016) characterized ApB73, a protein transcriptionally upregulated during the biotrophic stage and essential for the successful colonization of *Zea mays* accession B73 by *U. maydis*. ApB73 is secreted by the fungus, but then localizes to the fungal surface during biotrophy. It seems to remain associated with fungal cell wall components or the fungal plasma membrane. Previous genomics studies revealed that the interaction with the respective host is largely determined by approximately 300 genes predicted to encode novel secreted protein effectors (Kämper et al. 2006). Further, genome analysis of several smut fungi including *Ustilago maydis* revealed a singular clustered organization of genes encoding secreted effectors. In *U. maydis*, many of these clusters have a role in virulence (Dutheil et al. 2016). They showed that the genome of *Sporisorium scitamineum* contains more and larger gene clusters encoding secreted effectors than any previously described species in this group [*U. maydis*, *Sporisorium scitamineum*, and *Sphacelotheca* (*Sporisorium*) *reilianum*]. Djamei et al. (2011) showed that *U. maydis* actively suppresses plant defense responses by secreted protein effectors. Its effector repertoire comprises at least 386 genes, mostly encoding proteins of unknown function and expressed exclusively during the biotrophic stage. The *U. maydis* secretome also contains about 150 proteins with probable functions in fungal nutrition, fungal cell wall modification, and host penetration as well as proteins unlikely to act in the fungal–host interface. Secreted proteins are critical virulence determinants in *U. maydis*. Schuster et al. (2017) compared the secretomes of 12 basidiomycete species with very different lifestyles to gain information about their composition and conservation. Of the secreted proteins with domains, they detected a small core secretome in all 12 species, whereas only one cluster with novel secreted proteins without domain was conserved. This finding does not necessarily mean that related novel secreted proteins with similar functions do not exist; rather, they may have diverged to an extent that their homology cannot be detected by the methods used. One way to approach the search for homologs with a very low sequence identity may lie in obtaining structural information. Their study revealed that a significant percentage of core effectors predicted to be secreted resided in gene families in one or several of the species they compared. The analyses of gene families have been lagging behind in most pathogen–host systems, mostly because of technical difficulties. Avirulence (avr) genes encoding effectors from a certain race of pathogens and their corresponding resistance (R) genes in specific host cultivars have been identified as major contributing factors for gene-for-gene resistance whereby the absence of products produced by any of these genes can cause disease. The recognition of a pathogen AVR protein triggers a set of immune responses grouped under the term effector-triggered immunity (ETI), frequently leading to a rapid localized cell death termed the hypersensitive response (HR). The interactions between five blast Avr effectors, namely AvrPita, Avr-Pik, AvrPiz-t, Avr-Pia, and AVr1-Co39, and their cognate rice

R proteins have been reviewed by Liu et al. (2013a, b). *AVR* genes recognized by two distinct *R* genes both necessary for recognition were reported in the *M. oryzae*–rice pathosystem. *M. oryzae*, the causal agent of rice blast, is mostly controlled by using resistant rice cultivars harboring major *R* genes. Seven *M. oryzae AVR* genes have been cloned (Liu et al. 2013b; Petit-Houdenot and Fudal 2017). Some effectors can be recognized directly or indirectly by resistance (R) proteins from the plant and are then called avirulence (AVR) proteins. One class of R proteins corresponds to cell-surface (leucine-rich repeat LRR domain) LRR-containing R proteins that are anchored to the plasma membrane via a transmembrane (TM) domain and sometimes included an intracellular kinase domain: receptor-like proteins, RLP/receptor-like kinases (RLK) (Yang et al. 2012). *R–AVR*gene interactions are frequently exploited in the field to control diseases. Recently, the availability of fungal genomes has accelerated the identification of *AVR* genes in plant pathogenic fungi, including in those infecting agronomically important crops. Although single *AVR* genes recognized by their corresponding *R* gene were identified, more and more complex interactions between *AVR* and *R* genes are reported (e.g., *AVR* genes recognized by several *R* genes, *R* genes recognized several *AVR* genes in distinct organisms, one *AVR* gene suppressing recognition of another *AVR* gene by its corresponding *R* gene, and two cooperating *R* genes were both necessary for the recognition of *AVR* gene). These complex interactions were particularly reported in pathosystems showing a long co-evolution with their host plant but could also result from the way agronomic crops were obtained and improved (e.g., through interspecific hybridization or introgression of resistance genes from wild related species into cultivated crops). Petit-Houdenot and Fudal (2017) described some complex *R—AVR* interactions between plants and fungi that were recently reported and discussed their implications for *AVR* gene evolution and *R* gene management. Resistance genes recognizing several avirulence genes in distinct organisms were also covered. Under the selection pressure exerted by R genes, pathogens can become virulent through evolution of their AVR gene repertoire. The characterization of plant–fungal interactions that are emerging show some similarities (cooperating *R* genes, *R* genes recognizing distinct pathogens, *AVR* gene suppressing recognition of another *AVR* gene) but also specificities (bipartite *AVR* gene). Among the *R* genes displaying complex interaction with *AVR* genes, some of the most promising are those conferring broad-spectrum resistance because they guard key components of plant immunity and, as such, target essential effectors. Even if they exert a strong selection pressure on pathogen populations, they may remain effective through pyramiding with other specific or quantitative *R* genes. Another promising strategy to manage durable resistances would be to target antagonistic interactions between *AVR* genes and to combine the corresponding *R* genes in the same cultivars through pyramiding or to sequentially use the *R* genes in rotation. Established techniques for cataloguing effectors and identifying interactors have matured, and novel approaches to understand function have been applied or developed. Although much has been learned in recent years, we have only been chipping at the tip of the iceberg. Hundreds of putative effectors of unknown function have been identified, particularly in filamentous plant pathogens, and a challenge for the

future is to develop high-throughput methods to validate and characterize these putative effectors to keep up with the pace of identification. It seems certain that novel activities of these proteins will be found, and there will be new insights into how pathogens remodel host cells for their own benefit and how plants recognize effectors and mount an immune response. Studies of effectors also continue to offer opportunities for the development of tools to probe host cell biology in the absence of disease. As our knowledge of fundamental effector biology develops, it will be important to consider how this information can be translated into policies and products to positively impact agriculture. Note for the future: everyone should look out for the sequels (Varden et al. 2017). As fungal pathogens invading mammalian hosts are exposed to systemic pH values above neutrality, it is unsurprising that the *pal/RIM* pathway has been identified as an important factor determining pathogenicity (Davis 2009; Cornet and Gaillardin 2014).

Despite their fungal-like morphology, oomycetes are related to photosynthetic red algae rather than to fungi. Multinucleated sporangia and uninucleate zoospores represent their most common dispersal forms. On the plant surface, sporangia germinate directly and enter host tissue via either stomata, wounds, hyphopodia, or appressoria; fungi can grow within the inner cell layers as extracellular hyphae, intracellular invasive hyphae, or extracellular hyphae that project feeding structures (haustoria) into host cells, in contrast to *U. maydis*, which does not develop haustoria. Both intracellular hyphae and haustoria are surrounded by the plant plasma membrane, termed EHM in haustoriated cells. Alternatively, sporangia may release zoospores that encyst, germinate, and develop appressoria which penetrate the epidermal cell layer (Tyler 2002). Despite significant advances in the field of effector biology, our understanding of the events leading to effector translocation is lagging behind. Oomycete effectors seem to share conserved motifs, whose exact role in effector uptake needs to be unequivocally demonstrated by in vivo and biochemical experiments; fungal effectors are highly divergent at the level of the primary amino acid sequence. Additionally, the lack of conserved motifs in candidate cytoplasmic effectors within a given fungal species could either suggest that the uptake mechanism is rather nonspecific or that a certain degree of conservation between effectors exists at the protein structure level that cannot be recognized at the primary amino acid sequence level. The data available for a subset of oomycete and fungal effectors suggest that effector entry into host cells may occur via endocytosis and depend on a membrane-binding motif, be it a conserved charged motif, a degenerate charged motif, or a solvent-exposed hydrophobic patch. Elucidating the composition of host–microbe interfaces, at both the molecular and ultrastructural level, promises to reveal important insights in the mechanism of effector uptake. The presence of exosomes in biotrophic interfaces, the identification of unconventionally secreted effectors in both oomycetes, and the possible involvement of exosomes in oomycete effector delivery all indicate that extracellular vesicles (EVs) might have a direct or indirect role in effector translocation and warrant further investigation. Lo Presti and Kahmann (2017) summarized current knowledge in the field of oomycete and fungal effector uptake and highlighted emerging themes that may unite rather than set apart these unrelated filamentous pathogens. How the endocytosed effectors

escape host endosomes remains to be determined. Effector genes are not randomly distributed across the genome, but often reside in polymorphic regions of the genome, clustering with repetitive DNA. Despite the ubiquity and importance of fungal effectors, our mechanistic understanding of their transcriptional regulation and genome organization remains inadequate (Cook et al. 2017). As such, they are addressing two key questions: (1) How are in planta effectors transcriptionally regulated? (2) How does repetitive DNA contribute to the highly variable genomic regions that contribute to fungal virulence? They characterized how DNA modifications and chromatin structure (the organization of DNA in a cell) contribute to the evolution of virulence using the soil-borne fungal pathogen *Verticillium dahliae*. The genome of *V. dahliae* is predicted to express numerous homologs of known DNA and chromatin-modifying proteins, including three putative DNA methyltransferases. A single DNA methyltransferase was shown to control a significant portion of the observed DNA methylation at repetitive DNA. Interestingly, repetitive DNA arising from segmental genome duplications are devoid of DNA methylation and are more transcriptionally active relative to repetitive DNA at other loci. Additionally, the genome was assayed for open chromatin to develop a comprehensive view of how gene regulation and chromatin architecture impact the evolution of fungal virulence. Chitin-binding lysin motif (LysM) effectors contribute to the virulence of various plant-pathogenic fungi that are causal agents of foliar diseases (Kombrink et al. 2017). They reported that the LysM effectors of the soil-borne fungal vascular wilt pathogen *V. dahliae* have three core LysM effectors that are conserved in a collection of *V. dahliae* strains. Remarkably, and in contrast with the previously studied LysM effectors of other plant pathogens, no expression of core LysM effectors was monitored in planta in a taxonomically diverse panel of host plants. Moreover, targeted deletion of the individual LysM effector genes in *V. dahliae* strain JR2 did not compromise virulence in infections on *Arabidopsis*, tomato, or *Nicotiana benthamiana*. Interestingly, an additional LysM effector is encoded in the genome of *V. dahliae* strain VdLs17, but not in any other *V. dahliae* strain sequenced to date. Remarkably, this effector is expressed in planta and contributes to the virulence of *V. dahliae* strain VdLs17 on tomato, but on neither *Arabidopsis* nor *N. benthamiana*. Functional analysis revealed that this LysM effector binds chitin, is able to suppress chitin-induced immune responses, and protects fungal hyphae against hydrolysis by plant hydrolytic enzymes. Thus, in contrast with the core LysM effectors of *V. dahliae*, this LysM effector of strain VdLs17 contributes to virulence in planta. Pathogen effectors are considered to be secreted proteins that modify host cell structure and function to promote the success of a pathogen. In *U. maydis*, about 40% of all candidate secreted proteins are completely novel, as they lack known structural or functional domains (Schuster et al. 2017). Effector proteins can be grouped into functional groups, facilitating distinct steps of disease progression. The first group consists of general (core) effector proteins that facilitate breaking of the first defense barrier of the plants. After successful suppression of the epidermal defense barrier, the biotrophic interaction needs to be maintained while the fungus is progressing through the different tissues it encounters (Matei and Doehlemann 2016). The FLP-mediated selectable marker-removal technique

was successfully applied to delete a family of 11 effector genes (eff1) using five sequential rounds of recombination. They showed that expression of all 11 genes is upregulated during the biotrophic phase. Strains carrying deletions of 9 or all 11 genes showed a significant reduction in virulence, and this phenotype could be partially complemented by the introduction of different members from the gene family, demonstrating redundancy (Khrunyk et al. 2010). Lanver et al. (2017) used the term effector for all *U. maydis* proteins that are predicted to be secreted through the classic endoplasmic reticulum (ER)–Golgi route and lack known domains (novel effectors), as well as for secreted proteins with a confirmed virulence function that contain a known domain, are transcriptionally induced following plant colonization, and are not required for growth or morphology when *U. maydis* is propagated in axenic culture. In their review, Lanver et al. summarized their current knowledge of the effector repertoire that has been gained from comparative genomics in smut fungi. They discussed the molecular mechanisms by which the effectors of *U. maydis* change host cell processes to dampen plant defenses, how the respective genes are regulated. Of the 6784 proteins expressed by *U. maydis*, 467 are potentially secreted (that is, they contain a signal peptide and lack transmembrane domains), and of these 203 are completely novel, which means that they lack functional or structural domains (Schuster et al. 2017). Many of these effectors were shown to contribute to virulence.

Aspergillus flavus, a soil-borne pathogen, represents a danger for humans and animals since it produces the carcinogenic mycotoxin Aflatoxin B1 (AFB1) in oil-rich crops such as maize (*Zea mays* L. ssp. *mays*) (Majumdar et al. 2017). The first full-genome assessment of fungal GPCRs through characterization of null mutants of all revealed 15 GPCRs encoded by the aflatoxin-producing fungus *Aspergillus flavus*. Most GPCR mutants were aberrant in one or more response processes, possibly indicative of crosstalk in downstream signaling pathways. Interestingly, the biological defects of the mutants did not correspond with assignment to established GPCR classes, likely because of the paucity of data for characterized fungal GPCRs. Many of the GPCR transcripts were differentially regulated under various conditions as well. Pathogenesis-related (PR) proteins serve as an important defense mechanism against invading pathogens by conferring systemic acquired resistance in plants (Affeldt et al. 2014). Among these, the production of the PR maize seed protein ZmPRms (AC205274.3_FG001) has been speculated to be involved in resistance to infection by *A. flavus* and other pathogens. Involvement of both elicitor-response element (ERE) and enhancing sequences in the *PRms* promoter were implicated in induction of the promoter by fungal elicitors. The presence of a specific motif in the ZmPRms gene promoter region showed strong association between promoter induction and biotic stressors. Involvement of both elicitor-response element (ERE) and enhancing sequences in the PRms promoter were implicated in induction of the promoter by fungal elicitors. In another study, transgenic expression of the *ZmPRms* gene in rice resulted in broad-spectrum resistance against fungal (*Magnaporthe oryzae*, *Fusarium verticillioides*, and *Helminthosporium oryzae*) pathogens suggesting involvement of this gene in the central defense mechanism in plants (Gómez-Ariza et al. 2007). In their study,

Majumdar et al. (2017) have demonstrated a significant role for ZmPRms in resistance to *A. flavus* infection of maize kernels through global regulation of genes associated with biotic and abiotic stress responses in plants. This category includes genes associated with disease resistance, carbohydrate metabolism, and transcription factors that are known to be upregulated in plants under stress conditions. The observed increase in *A. flavus* growth and aflatoxin production in the ZmPRms-RNAi lines supports the RNA-seq interactome analysis that indicated ZmPRms might serve as a major network hub for regulation of downstream resistance-associated gene expression. ZmPRms–RNAi lines and their progenies were morphologically normal, suggesting that it will be a good candidate host-resistance gene for overexpression in maize for increased resistance to *A. flavus* and possibly against other pathogens. Their results are promising and it might be possible to fine-tune ZmPRms expression in a tissue-specific manner (using modern functional genomic tools) in the future, or use ZmPRms expression as a marker to screen for *A. flavus*-resistant maize genotypes to reduce aflatoxin contamination in maize and potentially in other economically important crop plants. Interestingly, El Khoury et al. (2017) demonstrated that an aqueous extract of the medicinal plant *Micromeria graeca*, known as hyssop, completely inhibits aflatoxin production by *Aspergillus flavus*.

The process of cell death is, itself, a way in which organisms control intracellular infections, removing the infected cell before the pathogen can replicate (Green 2017). Franco-Orozco et al. (2017) characterized a novel fungal PAMP, cell death inducing 1 (RcCDI1), identified in the *Rhynchosporium commune* transcriptome sampled at an early stage of barley (*Hordeum vulgare*) infection. *R. commune* is the causal agent of scald, one of the most destructive and economically important diseases of barley: it is a hemibiotroph with an extended asymptomatic phase. Following conidial germination and cuticle penetration, *R. commune* hyphae spread between the epidermal cells. As do several other important fungal pathogens of cereals, including *M. oryzae*, *Zymoseptoria tritici*, and *Parastagonospora nodorum*, *R. commune* belongs to the Ascomycota. This phylum also contains major pathogens of dicots, such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*, as well as the model fungus *Neurospora crassa.* They identified a *R. commune* transcript, highly abundant at an early stage of barley colonization, and coding for a small secreted protein with four cysteine residues. Expression of the full-length coding sequence in *Nicotania benthamiana* triggered cell death, which led to a hypothesis that this protein, named cell death inducing 1 (RcCDI1), is a PAMP. They showed this protein to be conserved across different Ascomycetes, with RcCDI1 homologs from *Neurospora crassa*, *Z. tritici*, *M. oryzae*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* also capable of inducing cell death in Solanaceae. They showed that RcCDI1-triggered cell death is dependent on NbBAK1, NbSOBIR1, and NbSGT1. However, cell death was not suppressed by the effector PiAVR3a or PexRD2, suggesting that it does not require NbCMPG1 or NbMAPKKKe.

3.7 Fungal Mycotoxins

Among the 300 reported mycotoxins, AFB1 is the most dangerous one. In fact, AFB1 is a potent carcinogenic agent in humans, inducing liver cancer. For instance, piperine, a major active component of black and long peppers (*Piper nigrum* L. and *Piper longum* L.), has been previously demonstrated to be an effective inhibitor of aflatoxin production. The impact of piperine was analyzed by targeting a regulatory network of 20 genes including atfA, atfB, ap-1, msnA, srrA, sskA, and sakA coding for stress response transcription factors; genes involved in fungal antioxidant defense (catA, cat2, sod1, mnSOD); genes coding for oxylipins (ppoA, ppoB, ppoC); genes belonging to GPCRs (gprK, gprH, gprG, gprA, gprP); and the global regulator *veA*. Several of the GPCRs studied have been linked to aflatoxin production and fungal stress (Caceres et al. 2017). Overexpressed gprK levels were associated with AFB1 inhibition by piperine. As mentioned, GPCRs are responsible for oxylipin modulation. Within the analyzed oxylipins, ppoB have been shown to be the most impacted gene upon treatment. In *A. nidulans*, ppoB deletion resulted in a precocious production of sterigmatocystin, a precursor of the AFB1 metabolite. This result suggests that in *A. flavus* the overexpression of ppoB could also have a negative impact on aflatoxin production. Piperine significantly induced the overexpression of 11 genes (ppoA, ppoB, atfA, atfB, ap-1, catA, cat2, sod1, sskA, gprK, gprH) and 5 others (ppoC, veA, msnA, srrA and gprP) were downregulated. Results demonstrated that piperine inhibits almost all genes of the aflatoxin biosynthetic pathway, leading to inhibition of mycotoxin biosynthesis. Gene stress response was accompanied by an enhancement of catalase enzymatic activity. In conclusion, these findings strongly suggest that piperine inhibits AFB1 production by *Aspergillus flavus* via the perturbation of the oxidative stress balance (Caceres et al. 2017).

Both mycotoxins zearalenone (an estrogenic mycotoxin produced mainly by *Fusarium* fungi) and resveratrol (RES, 3,5,49-trihydroxystilbene, or RSV, a phytoalexin that participates in plant defense) are antagonistic on both estrogen receptors (ERα and ERβ) at high doses (Mueller et al. 2004). Resveratrol has been characterized as a phytoestrogen based on its ability to bind to and activate estrogen receptor (ER). ER is a nuclear steroid receptor that binds estrogens and regulates the transcription of estrogen-responsive genes by either binding directly to DNA, at particular sequences called estrogen response elements (EREs), or by interacting with other transcription factors, such as Sp1, bound to their cognate sites on DNA. When activated by an agonist ligand, ERα interacts with coactivators, such as SRC-1 and CBP, that either uses acetylate lysine residues in histones to alter chromatin conformation or interacts with components of the RNA polymerase II initiation complex to enhance target gene transcription. Ligands that bind ER can act as agonists, antagonists, or mixed agonist/antagonists. The archetype mixed agonist/antagonist is tamoxifen (TAM), used clinically to prevent breast cancer promotion and recurrence (Bowers et al. 2000). Although resveratrol was reported to interact with ERs, its agonist or antagonistic effects remain controversial (Le Corre et al. 2005). Gehm et al. (1997) showed that RES is a superagonist when combined with E2, and Lu and

Serrero (1999) reported ER antagonism of RES (5 l M) in the presence of E2 and partial agonism in its absence. Bowers et al. (2000) observed partial to full agonism in CHO-K1 cells transfected with ERα or ERβ and reporter genes based on various estrogen receptor elements (EREs). They showed that RES acts as a mixed agonist/antagonist in cells transiently transfected with ER and mediates higher transcriptional activity when bound to ERb than to ERa. Moreover, RES showed antagonist activity with ERa, but not with ERb (Bowers et al. 2000).

Zearalenone (ZEA) is mainly produced by *Fusarium graminearum* Schwabe that causes ear rot of corn in many corn-producing areas of the world. The ascomycete *F. graminearum* (FG) is a filamentous fungus dwelling on and in a wide range of plant species, on crop debris and within soil, and is responsible for various corn and rice diseases. Zearalenone (ZEA), a nonsteroidal estrogenic mycotoxin, is widely present in cereals and agricultural products. This chemical and its metabolites, zearalanone, α-zearalanol (α-ZOL), and β-zearalanol (β-ZOL), particularly α-zearalenol (α-ZEL), which is used as a growth promoter in cattle, have been shown to competitively bind to estrogen receptors (ERα and Erβ), thereby interfering with the endogenous estrogenic response leading to activated transcription of estrogen-responsive genes and acting as strong ERα agonists. Their endocrine-disrupting behavior is tightly related to their capability to competitively bind the ligand-binding pocket (LBP) and to stabilize at least one of the functionally active conformational assets of the ligand-binding domain (LBD). ZEA, which could be used as an estrogenic effector, is implicated in reproductive disorders and hyperestrogenic syndromes in animals and humans exposed to contaminated food (Videmann et al. 2008). Zearalenone, the endocrine disruptor, has adverse effects such as genotoxicity, oxidative stress, alterations of immunological parameters, pituitary adenoma, and renal toxicity (Le Guevel and Pakdel 2001) and (EFSA Panel on Contaminants in the Food Chain Scientific 2011). ZEN as well as its metabolites exert harmful health effects via their strong estrogenic activities, resulting in decreased fertility, increased fetal resorption, and changes in the weight of endocrine glands and serum hormone levels (Lin et al. 2015; Ji et al. 2017). It has been reported that ZEA is associated with many mycotoxicosis diseases in farm animals; of course, the small intestine absorbs ZEA first, so it is exposed to high concentrations of the toxin, which will certainly influence intestinal tract health. Evidence indicates that a disruption of the epithelial cell integrity and functions induced by ZEA are well established. The review by Robert et al. (2017) aimed at providing a summary of DON, ZEN, OTA, FB1, AFB1, and PAT effects on intestinal barrier function, with special focus on mucus and microbiota. Their data showed that DON, ZEN, OTA, FB1, AFB1, and PAT were known to markedly affect epithelial cell integrity and functions. Previously, Fan et al. (2017) proved that ZEA could induce an accumulation of reactive oxygen species (ROS) in mitochondria and that elevated ROS levels can act as signaling molecules in pathological conditions. ZEA is also a genotoxic agent with DNA-adduct formation, DNA fragmentation, micronuclei production, and chromosome aberrations. Moreover, ZEA inhibits protein and DNA synthesis and triggers lipid peroxidation and apoptotic cell death (Sang et al. 2016). They reported also that the activity of the major antioxidant enzyme of MnSOD in mitochondria was

decreased after ZEA treatment. The oxidative damage to cells as well as the lost mitochondrial membrane potential induced by ZEA are intimately associated with mitochondria-mediated apoptotic cell death. Fan et al. (2018) investigated inflammatory cytokine release and the activation of the NLRP3 inflammasome after ZEA treatment both in vitro and in vivo. First, intestinal porcine enterocyte cell line (IPEC-J2) cells and mouse peritoneal macrophages were treated with ZEA to detect NLRP3 inflammasome activation, and the role of ROS in ZEA-induced inflammation was investigated. Then, Balb/c mice were fed a gavage of ZEA, and the disease activity indices (DAIs) and histological analysis were used to assess intestinal inflammation. Their study showed that the mRNA expression of NLRP3 inflammasome, pro-interleukin-1b (pro-IL-1b), and pro-interleukin-18 (pro-IL-18) was upregulated 0.5- to 1 fold and that the release of IL-1b and IL-18 increased. However, the ROS inhibitor *N*-acetyl-L-cysteine (NAC) reduced IL-1b and IL-18 release. Moreover, the same phenomenon was observed in intestinal tissues of ZEA-treated mice. In addition, clinical parameters of treated mice showed the stools became loose and contained mucus. The presence of gross stool blood was observed in the last 2 days. Histological analysis showed obvious inflammatory cell infiltration and tissue damage in the colon. These findings uncovered a possible mechanism of intestinal mucosal innate immunity in response to mycotoxin ZEA, that it could activate the ROS-mediated NLRP3 inflammasome and, in turn, contribute to the caspase-1-dependent activation of the inflammatory cytokines IL-1b and IL-18.

The phenolic compound resveratrol (RSV) is one type of phytoalexin synthesized by plants in response to fungal attack or ultraviolet light exposure. Sang et al. (2016) treated the human embryo kidney (HEK) 293 cell line with RSV and found it caused a potential protective effect against ZEA cytotoxicity. It has been suggested that resveratrol displays several beneficial human health properties, including anti-high-fat diet-induced senescence and protective mycotoxin-induced cytotoxicity, reduced carbon monoxide-induced cardiotoxicity, and suppressed xenobiotic-induced toxicity through the regulation of xenobiotic-induced AhR activation. Exposure of plants to abiotic stresses or pathogenic attack induces RSV synthesis. Recent studies demonstrated that resveratrol acts a chemoprotective agent against a wide range of PAH-induced toxicities, which is mediated through reduced production of reactive metabolites and DNA adduct formation by specifically inhibiting CYP1A1 and -1B1 activation. Additionally, resveratrol opposed PAH-induced oxidative stress through activation of the nuclear factor E2-related factor 2 (Nrf2) signaling pathway to produce antioxidation molecules, such as superoxide dismutase, glutathione, glutathione reductase, and catalase (Wu et al. 2017). Pangeni et al. (2014) reported that it has been found to potentially exhibit anticancer, antiangiogenic, immunomodulatory, and cardioprotective activities as well as being an antioxidant, and that this is in addition to its usefulness in the treatment of neurodegenerative disease, diabetes, and cardiac ailments. RSV in combination with DON significantly stimulated the progesterone release by GCs at the highest doses (Kolesarova et al. 2012; Wu et al. 2017). The literature reports suggest that oxidative damage seems to be a key determinant of ZEA-induced toxicity, and the protective effect of resveratrol (RSV), an antioxidant phenolic compound, on

ZEA-induced cytotoxicity to HEK293 cells was investigated. The experimental results showed that ZEA decreased cell viability in a dose-dependent manner and induced an increase in intracellular ROS in HEK293 cells. A remarkable elevation of MDA and decreased activity of manganese superoxide dismutase (MnSOD) were also observed. A decrease in mitochondrial membrane potential (MMP), cell-cycle arrest at the G_0/G_1 phase, and increased cell apoptosis indicate a mitochondria-mediated apoptosis. RSV pretreatment not only recovered the activity of MnSOD but also improved ZEA-induced cytotoxicity, evidenced by increased MMP and cell viability and decreased ROS. Furthermore, RSV pretreatment substantially upregulated the expression of the *SIRT1* gene by 6.8 fold, reduced the acetylation level of the forkhead transcription factor FOXO3a, and decreased the expression ratio of *Bax*/*Bcl-2*. All these results demonstrated that RSV exhibited significant protective effects on ZEA-induced cell damage, and this effect may be attributed to the upregulation of SIRT1 and activation of FOXO3a-mediated pathways to enhance the resistance of cells to oxidative stress induced by ZEA exposure (Sang et al. 2016).

3.8 Fungal Effectors and Elicitors

Candidates for secreted effector proteins (CSEPs) in *Sporisorium scitamineum* are clustered into ten families (21 CSEPs). Most of the CSEPs in these ten families have more than 2.0% cysteine content and longer amino acids (Que et al. 2014). Many of the functions of CSEPs are unknown in biotrophic phytopathogenic fungi, such as *Ustilago maydis* (Kämper et al. 2006). Que et al. (2014) showed that 47% of CSEPs were expressed in the infection process. Among them, 7 expressed at all stages, whereas 12, 9, 5, 2, 2, and 1 CSEPs started to express at 0, 12, 24, 48, 96, and 120 hpi, respectively. Four of the aforementioned 7 CSEPs belong to conserved hypothetical proteins with unknown function and did not receive hits from any protein database. The remaining 3 CSEPs have a clan GH-D domain (IPR000111), a catalytic domain (IPR008258), and a Barwin-related endoglucanase domain (IPR009009), as well as sections encoding glycoside hydrolase and lytic transglycosylase-like protein. These CSEPs are mainly involved in carbohydrate degradation. It is unclear how these CSEPs facilitate infection or trigger defense responses, how they are secreted, and whether there is a specific invasion structure to help the secretion. These conundrums require additional endeavors and deeper research. During infection, *U. maydis* secretes hundreds of effector proteins to suppress plant defense responses and to reprogram plant signaling and metabolism (Djamei and Kahmann 2012). However, the function of most of these novel effectors is unknown (Djamei et al. 2011; Djamei and Kahmann 2012; Mueller et al. 2013). Endocytosis is a vesicular transport pathway in eukaryotic cells that internalizes extracellular fluid and particles, as well as plasma membrane molecules. A role of endocytosis in hyphal growth and development, and in fungal pathogenicity in particular, is still subject to debate (Read and Kalkman 2003). However, recent studies showed that a t-SNARE protein, Yup1, is required for endocytosis and

pathogenicity in *U. maydis*. At later stages of host colonization, large plant tumors develop that provide the environment for massive fungal proliferation. During biotrophic growth, a high-affinity fungal sucrose H+ symporter, Srt1, is upregulated and is crucial for fungal nutrition (Wahl et al. 2010). Tanaka et al. (2014) revealed that an effector called Tin2, which is secreted by the corn smut fungus, causes the production of this anthocyanin pigment. Tin2 moves inside plant cells, where it blocks the breakdown of a protein-modifying enzyme that is necessary to 'switch on' the production of anthocyanin. As the building blocks for making lignin are also required for making anthocyanins, Tanaka et al. (2014) suggested a model whereby Tin2 compromises the ability of plants to protect themselves by diverting resources away from making lignin. The link between Tin2 and anthocyanin biosynthesis appears direct and occurs through the stabilization of the kinase ZmTTK1 via Tin2. They have shown that after *U. maydis* infection, when Tin2 is present, the stabilization of ZmTTK1 increases the biosynthesis of anthocyanin. Tin2 affected all steps of the anthocyanin pathway, but did not affect transcription of all genes coding for lignin pathway components. Conversely, when *U. maydis* is lacking tin2, more of the common precursor for anthocyanin and lignin is likely to be available for lignin biosynthesis, resulting in cell wall fortification in vascular tissue. The deposition of lignin in the plant cell wall is considered to provide an undegradable physical barrier to infection. At present, there is only a limited understanding of how the lignin biosynthetic pathway in maize is regulated. Several Myb and NAC transcriptional activators involved in secondary cell wall formation have been identified, but the mechanisms behind lignin tissue-specific and developmentally regulated gene expression, and the role of posttranscriptional regulation of gene expression, are yet to be fully elucidated. The increased susceptibility of maize bm mutants to *U. maydis* and the increased leaf areas displaying disease symptoms provide strong evidence for a role of lignification in disease resistance. A less lignified cell wall will be more easily penetrated by the fungus. The plant may also be less effective at producing defense-related lignin that could slow down the migration of the fungus. Last, because the fungus spreads via the vascular tissue and may rely on nutrients obtained from this tissue for massive proliferation, the altered physicochemical characteristics of the vascular tissue in the bm mutants, combined with altered dimensions of the xylem vessels, may influence entry and the rate of migration of the fungus. The bm2 mutant appears to be an exception in that it does not show increased susceptibility to *U. maydis*, and even appears to attenuate the effect of infection in bm1-bm2 and bm2-bm3 double mutants relative to the bm1 and bm3 single mutants. This effect may be the result of the accumulation of a fungitoxic (phenolic) compound in this mutant. Whether Tin2 is actively diverting the flux from lignin to anthocyanin remains speculative. Recently, a competition between anthocyanin and lignin pathways for their common precursor has also been detected in strawberry (Ring et al. 2013). In that system, it was demonstrated that plant class III peroxidase (FaPRX27), a gene connected with lignin biosynthesis, is linked to a region implied in decrease in fruit color. In addition, it was demonstrated that the downregulation of chalcone synthase led to an induction of FaPRX27 and that this diverted the flux from anthocyanins to lignin (Ring et al. 2013). Thus, there appears

to be a metabolic connection between anthocyanin and lignin biosynthesis pathways in monocot as well as dicot systems, and *U. maydis* may be altering this with the help of Tin2. Future work is needed to address whether this connection is direct with lignin functioning as barrier or indirect with anthocyanin induction negatively affecting the accumulation of other defense compounds. Anthocyanin accumulation is frequently observed under abiotic and biotic stress conditions, provides UV-B stress protection, and allows scavenging of reactive oxygen species and enhanced resistance to microbial pathogens (Zhang et al. 2013). Tanaka et al. (2014) have uncovered a new positive connection between anthocyanin induction and the development of a biotrophic pathogen. They suggested that examples where anthocyanin induction is observed in response to biotic stress should be revised to see whether metabolic rewiring of the phenylpropanoid pathway is adopted as a common strategy by biotrophic microbes colonizing plants.

Elicitors are fast becoming the key instruments in plant disease control because of their competence in activating or inducing resistance in plants. In general, plants utilize systemic acquired resistance (SAR) or induced systemic resistance (ISR) to initiate systemic immunity during their interactions with elicitors (Chen et al. 2015; Hamid and Wong 2017). The term is now often used for any signal-inducing compounds recognized by the innate immune system involved in priming and/or induction of defense responses regardless of its origin. The fungal secretome consists of various functional groups of proteins, many of which participate in nutrient acquisition, self-protection, or manipulation of the environment and neighboring organisms. The least characterized component of the secretome is small secreted proteins (SSPs). Some SSPs have been reported to function as effectors, but most remain to be characterized. The composition of major secretome components, such as carbohydrate-active enzymes, proteases, lipases, and oxidoreductases, appears to reflect the lifestyle and ecological niche of individual species. Kim et al. (2016) hypothesized that many SSPs participate in manipulating plants as effectors. Obligate biotrophs likely encode more and diverse effector-like SSPs to suppress host defense compared to necrotrophs, which generally use cell wall-degrading enzymes and phytotoxins to kill hosts. Because different secretome prediction workflows have been used in different studies, available secretome data are difficult to integrate for comprehensive comparative studies to test this hypothesis. In their study, SSPs encoded by 136 fungal species were identified from data archived in the Fungal Secretome Database (FSD) via a refined secretome workflow. Subsequently, compositions of SSPs and other secretome components were compared in light of taxa and lifestyles. Those species that are intimately associated with host cells, such as biotrophs and symbionts, usually have higher proportion of species-specific SSPs (SSSPs) than hemibiotrophs and necrotrophs, but the latter groups displayed higher proportions of secreted enzymes. Their results established a foundation for functional studies on SSPs and will also help in understanding genomic changes potentially underpinning different fungal lifestyles (Kim et al. 2016). In most cases, adapted pathogens counteract the basal defense responses of the plants by secreting effector proteins into the plant cells (O'Leary et al. 2016). Successful suppression of PTI often results in effector-triggered susceptibility (ETS), as illustrated in the

"zigzag" model by Jones and Dangl (2006) where the plants become susceptible to diseases. Nevertheless, effector-triggered immunity (ETI) is activated when a particular effector protein is recognized either directly or indirectly by its cognate resistance (R) protein in plants (Jones and Dangl 2006; Liu et al. 2013a, b). ETI is an accelerated and augmented response (Coll et al. 2011) that typically results in HR, a localized cell death at the site of infection that kills both the invading pathogen and the infected plant cells (Newman et al. 2013). Consequently, SAR in the host is activated (Jones and Dangl 2006) wherein the host plant becomes resistant to subsequent attacks from obligate biotrophic or hemibiotrophic pathogens. ETI is ineffective against necrotrophic pathogens (Glazebrook 2005). Several studies have shown that necrotrophic pathogens secrete a specialized group of necrotrophic effectors that are known as host-specific toxins (HSTs) which confer susceptibility to the pathogen upon recognition by host PRRs and lead to HST-induced programmed cell death (Friesen et al. 2008a, b; Oliver and Solomon 2010). It was reported that different responses (e.g., in rice: transient membrane depolarization, reactive oxygen generation, expression of typical pathogenesis-related PR genes as well as novel 'early genes,' and biosynthesis of jasmonic acid and phytoalexins) seemed to be a part of a complicated signal transduction cascade mediated by a single class of receptor molecule for chitin fragments elicitor (Tsukada et al. 2002). They conclude that the rice receptor for chitin fragments (*N*-acetyl chitooligosaccharide) elicitor did not couple to Gα, and thus not to the heterotrimeric G protein. For more details about elicitors and effectors in Basidiomycetes, refer to Hamid and Wong (2017).

Elicitins are structurally conserved extracellular proteins in *Phytophthora* and *Pythium* oomycete pathogen species (e.g., PiINF1) (reviewed by Derevnina et al. 2016). Abundant proteins in *Phytophthora* culture filtrates have the capacity to elicit hypersensitive (HR) cell death and disease resistance in tobacco. Later, they became well established as having features of MAMPs, a protein produced by oomycete pathogens known as elicitin that elicits defense in a variety of plant species. Research on elicitins culminated in the recent cloning of the elicitin response (ELR) cell-surface receptor-like protein, from the wild potato *Solanum microdontum*, which mediates response to a broad range of elicitins. Elicitins belong to complex multigene families and form diverse subclasses, defined by Jiang et al. (2006) as elicitin (ELI, class Ia, Ib, II) and elicitin-like (ELL, class III) genes. The number of ELI and ELL genes varies from one species to another, with each showing differential expression patterns and HR-inducing activities. With the exception of the canonical clade ELI-1, all other clades possess, in addition to a signal peptide and elicitin domain, C-terminal domains of variable length (17–291) that tend to be rich in threonine, serine, and proline residues (Jiang et al. 2006). Elicitins bind sterols and other lipids with varying affinities, and sterols are important for oomycete growth and sporulation. However, *Phytophthora* and *Pythium* species are sterol auxotrophs; this may be associated with the parasitic lifestyle of these organisms. Independent studies revealed that elicitins can act as sterol carriers by scavenging sterols from synthetic liposomes and plant plasma membranes. Further work demonstrated that elicitins form a diverse family of oomycete proteins, variously known as cryptogein

(a.k.a. CRY-B elicitin), capsicein (CAP-A), parasiticein (PAR-A), and INF1 from *Phytophthora cryptogea*, *Phytophthora capsici*, *Phytophthora parasitica*, and *Phytophthora infestans*, respectively. These proteins induce potent defense responses in several plant species, particularly in the Solanaceae and Brassicaceae. Elicitin response (ELR), the first cell-surface receptor to mediate specific response to elicitins, was cloned from the wild potato species *Solanum microdontum*. ELR mediates HR cell death to classic elicitins, including INF1 and CRY-B, and also enhances resistance to the Irish potato famine pathogen *Phytophthora infestans*. Successful pathogens overcome MAMP-triggered immunity (MTI) by secreting effector molecules that suppress host immune responses. Elicitins carry many characteristic MAMP attributes: (1) they are structurally conserved; (2) they show no sequence similarity to plant proteins (Jiang et al. 2006), and therefore are viewed as nonself molecules by the host; (3) as sterol transporters, they fulfil an important biological function in oomycetes; (4) they are expressed during host interaction; and (5) they are recognized by cell-surface localized PRRs that trigger an immune response. In addition to tobacco, other reported elicitin-responsive species include tomato, potato, and pepper (Solanaceae), pigeon pea (Fabaceae), grapevine (Vitaceae), citrus (Rutaceae), oak (Fagaceae), and some radish and turnip cultivars (Brassicaceae). However, differential responses to elicitins can occur within a given plant taxon. For example, within the genus *Solanum*, some species respond to INF1 and variation in the response occurs between genotypes of the same species. This finding indicates that the genetic basis of the response to elicitins is likely to be highly variable across plant taxa. Derevnina et al. (2016) in review suggested that oomycete plant pathogens have evolved an effector toolbox to modulate host responses triggered by their elicitins. Many questions remain to be answered. What is the molecular basis of the response to INF1 in other species besides *S. microdontum*? Has elicitin recognition evolved independently in different plant taxa? How does the response of plants such as tomato differ from the typical cell death response observed in *Nicotiana* spp. and wild potato? How do oomycete effectors suppress elicitin-triggered responses? The degree to which these elicitin-like proteins can be detected by plant receptors, and the molecular basis of any potential plant response, remain to be determined. Ultimately, a better understanding of elicitin perception and plant response could help engineer crops with broad-spectrum resistance against oomycete pathogens.

Cerato-platanin (CP) is a noncatalytic, cysteine-rich protein; the first member of the cerato-platanin family is an elicitor of the primary defense response and it has been classified as MAMP/PAMP (Luti et al. 2017). It is a single-domain protein with a double Ψ/β barrel domain resembling the D1 domain of plant and bacterial expansins. Similar to expansins, CP shows a cell wall-loosening activity on cellulose and can be defined as an expanisin-like protein, in spite of the missing D2 domain normally present in plant expansins. The weakening activity shown on cellulose may facilitate the CP–host interaction, corroborating the role of CP in eliciting the plant defense response. Indeed, CP is an elicitor of primary defenses acting as a PAMP. So far, structure–function relationship studies have been mainly performed on the bacterial BsEXLX1 expansin, probably because of difficulties in

expressing plant expansins in heterologous systems. Baccelli et al. (2014) reported a subcloning and purification method of CP in the engineered *Escherichia coli* Shuffle cells, which proved to be suitable to obtain the properly folded and biologically active protein. The method also enabled the production of the mutant D77A, rationally designed to be inactive. The wild-type and the mutated CP were characterized for cellulose-weakening activity and for PAMP activity (i.e., induction of ROS synthesis and phytoalexins production). Their analysis revealed that the carboxyl group of D77 is crucial for expansin-like and PAMP activities, thus permitting establishing a correlation between the ability to weaken cellulose and the capacity to induce defense responses in plants. Their results enable the structural and functional characterization of a mono-domain eukaryotic expansin and identified the essential role of a specific aspartic residue in cellulose weakening. Because CP lacks the D2 domain it can be, therefore, defined as a mono-domain expansin. The ease of producing the protein in high yield in *Pichea pastoris*, as well as the extraordinary heat stability, make this protein suitable for future potential applications in increasing the cellulose loosening rate as, for example, during biofuel production.

Secondary metabolite synthesis and accumulation in plant cell cultures can be stimulated by elicitors, signaling molecules that can boost the formation of secondary metabolites in cell cultures by initiating plant defense, hypersensitive responses, or pathogenesis-related proteins (Zhao et al. 2007). Among biotic elicitors, fungal elicitors have resulted in substantial augmentation in the production of a number of phytochemicals in plant tissue cultures of *Withania somnifera*, also known as Indian ginseng. *W. somnifera* is known to contain valuable bioactive compounds, called anolides, that structurally resemble the ginsenosides of *Panax ginseng*. The extracts as well as different isolated bioactive constituents of *W. somnifera* have been shown to possess adaptogenic, anticancer, anticonvulsant, antioxidative, immunomodulator, and neurological effects. *Piriformospora indica*, a root endophytic fungus that belongs to the family Sebacinaceae, was isolated from rhizosphere soil of xerophytic shrubs in the Thar desert in India; it is an axenically cultivable phytopromotional and biotrophic root endosymbiont. This fungus has multifaceted roles such as nutrient uptake, disease resistance, stress tolerance, and growth encouragement involving value addition. It has been reported to increase the growth of *W. somnifera* in vitro and in cell suspension and hairy root cultures (Ahlawat et al. 2014; Saxena et al. 2017). In the study by Ahlawat et al. (2014), a cell suspension culture of *W. somnifera* was elicited with cell homogenates of the following fungi (*Alternaria alternata*, *Fusarium solani*, *Verticillium dahliae*, and *Piriformospora indica*) in a shake flask; then, the major withanolides such as withanolideA, withaferin A, and withanone were analyzed. Among the biotic elicitor preparations from *P. indica*, the cell homogenate at 3% (v/v) affected the maximum enhancement on the production of withanolides in the cell cultures of *W. somnifera* in a 5.0-l bioreactor. In their study on the positive effect of *P. indica* on withanolides biosynthesis in *W. somnifera*, it appeared that the fungus has the potential of acting as a good source of elicitor in increasing the production of useful secondary metabolites in other plant cell cultures also, which might be worth investigating. Saxena et al. (2017)

examined elicitation by cell homogenate of *P. indica*(CHP) and methyl jasmonate on withanolide accumulation in hairy root cultures as well as on the expression pattern of ten genes encoding enzymes in the withanolide biosynthetic pathway. The expression of all the genes encoding enzymes of withanolide biosynthesis and its related pathways supplying carbon, viz. *HMGR*, *FPPS*, *DXS*, *DXR*, *SQS*, *SE*, *CAS*, *ODM*, *SDS*, and *SMT-1*, was upregulated in CHP treatments compared with the control, although it was less than that observed with 15 μM MeJ during a 4-h treatment. Maximal expressions were found in 3% CHP for 48 h, which also had more biomass and withanolide content.

A wide range of external stress stimuli trigger plant cells to undergo complex network of reactions that ultimately lead to the synthesis and accumulation of secondary metabolites. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Endophytic fungi, an important constituent in the environment of medicinal plants, have been known to form long-term stable and mutually beneficial symbiosis with these plants. The endophytic fungal elicitor can rapidly and specifically induce the expression of specific genes in medicinal plants, which can result in the activation of a series of specific secondary metabolic pathways resulting in the significant accumulation of active ingredients (Zhai et al. 2017). The plant–microbe interactions and plant defense responses, as well as the signal transduction pathways involved, have been studied extensively and continue to be topics of active research and discussion. The use of fungal elicitors has been reported to be one of the most effective strategies for improving the productivity of useful secondary metabolites in plant cell culture (Takeuchi et al. 2013). Recent studies of fungal elicitors focus on fungal elicitor recognition, G-protein, Ca^{2+}, and hydrogen peroxide (H_2O_2) signal transduction, signal amplification of jasmonic acid (JA), nitric oxide (NO), salicylic acid (SA), abscisic acid (ABA), ethylene (ETH), signal crosstalk, gene expression, activation of the key enzymes, and the application of fungal elicitor. The results were summed up as follows: fungal elicitor, receptor to fungal elicitor, and the identification of fungal elicitor (Zhai et al. 2017). Although *Fusarium*, yeast, and *Pythium*species can be used to elicit specific secondary metabolites in various medical plants, they may not be the best elicitor for the induction of a specific secondary metabolite. It is a remarkable fact that the combination of different elicitors may achieve a synergistic effect from the different action of different elicitors. There are some large-scale industrial productions of secondary metabolites in some medical plants.

3.9 Aspergillus GPCRs

The fungal genus *Aspergillus* is of critical importance to humankind because of the medical (*A. fumigatus*, *A. terreus*), food spoilage (*A. flavus*, *A. parasiticus*), and industrial (*A. niger*, *A. aculeatus*, *A. oryzae*) relevance of some of its species. Many *Aspergillus* species are included in important pathogens of humans, animals, and crops, a source of potent carcinogenic contaminants of food, and represent an

important genetic model, especially that of *Aspergillus nidulans*, that have contributed broadly to our understanding of eukaryotic cell biology and molecular processes. The genome sequences of eight aspergilli have already been explored to investigate the aspects of fungal biology. Most filamentous fungi have three conserved Gα subunits (I, II, III), one Gβ protein, and one Gγ protein (de Vries et al. 2017). The number of predicted GPCRs varies widely, with a larger number identified in Ascomycetes than in Basidiomycetes (Han and Prade 2002; Li et al. 2007). Results of the domain-based identification of G proteins and GPCR orthologs in the Eurotiomycetes show that Gα proteins were found to be highly conserved. The Gα group I (fadA) regulates multiple pathways, and most filamentous fungi have a single copy of this gene (de Vries et al. 2017). In contrast, group II Gα proteins (ganA) are less conserved than the other Gα proteins, and as many as three copies were found in one species (*Talaromyces stipitatus*). The group II Gα proteins have a positive role in germination of conidia, possibly through cAMP signaling in carbon sensing (Chang et al. 2004; Lafon et al. 2005). The last Gα belongs to group III (ganB) and was found as a single copy in all Eurotiomycetes, where it is thought to act as a negative regulator (de Vries et al. 2017). Additionally, they found three different Gα groups of orthologs (Clusters FungiJGICTBE21135 only present in *A. niger* ATCC 1015, FungiJGICTBE20122 present in *A. flavus* NRRL 3557 and in *A. oryzae* RIB40, and FungiJGICTBE14195 present in *A. flavus* NRRL 3557) that appeared to encode genes which contain the Gα domain. There was also one copy of the Gβ-negative regulator of asexual reproduction (sfaD) in each genome (Rosen et al. 1999). Finally, all Eurotiomycetes genomes contained a single highly conserved Gγ gene (Downes and Gautam 1999). The pheromone/pheromone receptor genes, differentially regulated in ascomycetes and basidiomycetes, are discussed in detail in the review article by Xue et al. (2008). *Aspergillus* species are characterized by the unifying feature of the "aspergillum," an asexual reproductive structure. A central regulatory pathway (*brlA*, *abaA*, *wetA*) controls conidiation-specific gene expression and asexual developmental processes. Proper activation of *brlA* requires upstream developmental activators (encoded by *fluG*, *flbA-E*) and removal of repression by several negative regulators including SfgA, VosA, NsdD, and two G-protein signaling pathways (de Vries et al. 2017). WetA is also required for normal vegetative growth, hyphal branching, production of aflatoxins, contribution to spore integrity and maturity by properly regulating the metabolic pathways of trehalose, chitin, α-(1,3)-glucan, β-(1,3)-glucan, melanin, hydrophobins, and secondary metabolism more generally. Light also regulates asexual developmental genes, including Eurotiomycetes, with several photoreceptors identified such as one red-light (FphA), which represses fruit-body formation and induces asexual spore formation, and three blue-light receptors represented by the white-collar complex (WCC) LreA (WC-1) and LreB (WC-2) and the photolyase/cryptochrome CryA. Full stimulation of conidia production was only achieved with a combination of red and blue light. In *Neurospora crassa*, WC-1 and WC-2 interact and form the white-collar complex (WCC). This complex, upon light exposure, binds transiently to the promoters of light-inducible genes, presumably to activate their transcription. LreA and LreB act as positive factors for the sexual cycle. LreB interacted not only with LreA, but also

with the phytochrome FphA, which itself also interacted with the VeA regulatory protein. Light receptors transmit their signal to a number of other regulatory proteins including a bridging protein, VeA, as part of a trimeric complex. VeA has a central role in the balance of asexual and sexual development and in the coordination of morphogenesis and secondary metabolism. After light absorption, the phytochrome shuttles from the cytoplasm to the nucleus upon illumination, then interacts with transcription factors, for example, which in turn regulate the transcription of several genes in plants. In *A. nidulans* the phytochrome was found in the cytoplasm and in the nucleus, where it forms a light-sensing protein complex, but there is yet no evidence for a shuttling mechanism. All the presumed asexual species were found to contain either *MAT1-1* alpha idiomorphs or *MAT1-2* idiomorphs, *ppgA* homologs encoding a pheromone precursor, and *preA* and *preB* homologs encoding pheromone receptors; all of these are expressed in all the asexual species in the same way as known sexual species. Many regulatory genes for asexual sporulation in *A. nidulans* have been identified. *A. nidulans* forms asexual spores in light, but preferentially undergoes sexual reproduction and produces resistant and durable ascospores in the dark. However, it has to be considered that at least 75 other genes are required for completion of the sexual cycle in the aspergilli.

Aspergillus fumigatus is the most pathogenic species among the aspergilli, and is the major fungal agent of human pulmonary infection (Grice et al. 2013). In healthy individuals, mucociliary clearance and pulmonary immune defenses clear the hundreds of conidia inhaled daily. Beyond residual host immune responses, there are additional obstacles to successful colonization of the mammalian lung, including tolerance of host-facilitated stresses, such as iron starvation and alkaline pH. A screen of the predicted proteome using all GPCR sequences at the time available in the GPCR Database (GPCRDB) was applied to *A. fumigatus* and identified 15 putative GPCRs (Lafon et al. 2006). In aspergilli, putative GPCRs are classified by homology, and according to a convention established by Lafon et al. (2006) in *A. nidulans*, into nine groupings. In *A. fumigatus*, classes 1 and 2 are composed, respectively, of two putative pheromone receptors GprA (AFUA_3G14330) and GprB (AFUA_5G07880); class 3 is composed of two putative carbon sensors GprC (AFUA_7G04800), GprD (AFUA_2G12640); class 4 contains three putative nitrogen sensors: GprF (AFUA_5G04100), GprG (AFUA_1G11900), and GprJ (AFUA_1G06840); class 5 has three putative cAMP receptors: GprH (AFUA_5G04140), GprI (AFUA_3G00780), and GprL (AFUA_ 3G01750), the latter being unique to *A. fumigatus*; class 6 is composed of a single putative GPCR, GprK (AFUA_4G01350) having a regulator of G-protein signaling (RGS) domain, unique to filamentous fungi. Kim et al. (2019) characterized the functions of RgsD, one of the six RGS domain proteins present in the human pathogenic fungus *A. fumigatus*. Yeast two-hybrid assays reveal that RgsD can interact with the three Gα proteins GpaB, GanA, and GpaA, showing the highest interaction potential with GpaB. They concluded that RgsD attenuated the cAMP-PKA signaling pathway and negatively regulated asexual development, toxigenesis, melanin production, and virulence in *A. fumigatus*. In *A. fumigatus*, the *rax1* gene encodes for a putative positively controlling growth and development, and modulates intracellular

trehalose amount, cell wall melanin levels in conidia, and spore resistance to H_2O_2; class 7 includes two putative GPCRs with homology to rat growth hormone-releasing factor receptors, only one of which is found in *A. fumigatus*, GprM (AFUA_7G05300); class 8 contains three putative GPCRs with identity to yeast Izh zinc regulators, two of which are found in *A. fumigatus* GprO (AFUA_ 3G10570) and GprP (AFUA_6G07160); and Class 9 is composed of a single putative GPCR, NopA (AFUA_7g01430), having identity to bacterial opsins. The roles of some of these receptors have been identified in other species, although in *A. fumigatus* little is known. Among the 15 predicted GPCR-like proteins in *A. fumigatus*, only 2, GprC (AFUA_7G04800) and GprD (AFUA_2G12640), have been characterized. GprC and GprD have been noted as having homology to Gpr1p of *Saccharomyces cerevisiae*, which activates the cAMP pathway in response to glucose. As well, the *A. nidulans*GprD homolog mediates increase of intracellular cAMP in response to oxygenated polyunsaturated fatty acids (oxylipins), which act as autocrine and paracrine mediators in eukaryotic organisms (Affeldt et al. 2012). In contrast to most *Aspergillus* spp., wherein four predicted Gα-subunits occur, only three (GpaA, AFUA_1G13140, GpaB, AFUA_1G12930, and GpaC, AFUA_3G12400) have been identified for *A. fumigatus*, which presumably act via interaction with the Gβ- and Gγ-subunits (SfaD, AFUA_5G12210 and GpgA, AFUA_1G05210), which are undoubtedly significantly relevant to *A. fumigatus* viability and vegetative growth. Kulkarni et al. (2005) noted, based upon membrane topology, that the number of putative GPCR-like proteins encoded by the *Magnaporthe grisea* genome rose to 76 when the criteria were relaxed to include homologs of the Pth11 receptor (DeZwaan et al. 1999). The CFEM domain of seven-transmembrane protein Pth11 is necessary for proper development of appressoria, appressoria-like structures, and pathogenicity. Applying a more universal approach to *A. fumigatus*, Grice et al. (2013) identified 6496 proteins having putative transition-minimized differential signaling (TMDs). Among them, 161 proteins were found to encode seven predicted TMDs. Histidine kinases (HK) are phospho-relay protein sensors that transduce extracellular signals. HKs are common in the fungal kingdom and apparently absent in humans. Among Archaea, Bacteria, and Fungi, two classes of HK (two-component and hybrid) are found. HK activities have been associated with both the osmo- and peroxide-regulatory pathways in many fungi and have been most extensively characterized in *Saccharomyces cerevisiae*. However, RR proteins are not abundantly encoded by fungal genomes; Skn7 and Ssk1 are two examples of such proteins, which in *S. cerevisiae* and *Candida albicans*, account for the entire RR cohort of these species (Kaserer et al. 2009). The fungal phospho-transfer relay can involve three proteins, as exemplified by the *S. cerevisiae* HOG1 MAPK phospho-relay, where an HK (Sln1), a histidine phospho-intermediate (Ypd1) and an RR (Ssk1) collectively mediate a multistep phospho-transfer (Kaserer et al. 2009). In a study addressing the role of oxidative stress in *A. fumigatus* pathogenicity, Du et al. (2006) characterized the *A. fumigatus* TcsB protein, a putative homolog of Sln1 in *S. cerevisiae*.

3.10 *Candida albicans* GPCR

Although *Candida albicans* is commensal in humans, it often causes mucosal or systemic infections that contribute to substantial morbidity and life-threatening bloodstream infections in immunocompromised patients. Four classic *C. albicans*-cell types are known, which switch among at least six distinct forms: yeast-like morphotypes, hyphae, pseudohyphae, and chlamydospores in response to environmental cues, and this adaptability is thought to contribute to its virulence. The chlamydospores are large, spherical, thick-walled cells that are observed in vitro under certain harsh conditions, such as starvation and hypoxia. Macrophages are important innate immune cells that limit the niches in the human body in which *C. albicans* can persist through phagocytic removal. However, following phagocytosis *C. albicans* readily escapes from the immune cell by differentiating into filamentous hyphae. In *C. albicans*, Tor1 has been implicated in the negative regulation of filamentous growth. Inhibition of TORC1 results in the activation of the GATA transcription factor Brg1, which is involved in the regulation of hypha-specific genes and blocking the recruitment of the Nrg1-Tip1 transcriptional repressor complex (Lu et al. 2012; Su et al. 2013). Hyphae express numerous cell type-specific virulence factors such as adhesins [for example, hyphal wall protein 1 (Hwp1), agglutinin-like protein 3 (Als3), Als10, factor activated 2 (Fav2), and Pga55], tissue-degrading enzymes [for example, secreted aspartyl protease 4 (Sap4), Sap5, Sap6], antioxidant defense proteins [for example, superoxide dismutase 5 (Sod5)], and even a recently described cytolytic peptide toxin (extent of cell elongation protein 1, Ece1. The CFEM domain-containing proteins in *C. albicans* are involved in binding and maintenance of iron, adherence, and virulence. Hyphal form has an important function in disease pathogenesis by invading epithelial cells and causing tissue damage by inducing their endocytic uptake by cultured human oral epithelial cells through a specific interaction between the hyphal adhesin, Als3, and host epithelial cadherin (E-cadherin); internalized hyphae then proceed to damage the host cells. They can also actively penetrate into oral epithelial cells, possibly through physical pressure and secreted enzymes. Thus, in a reconstituted model of human oral epithelial tissue, invading hyphae trigger several proinflammatory signaling pathways in the host, whereas yeasts, which merely colonize the surface of the tissue without causing damage, trigger a more muted inflammatory response. Yeasts, hyphae, and pseudohyphae were all present in infected tissues that were recovered from human patients and animals with disseminated candidiasis. Moreover, *C. albicans* mutants that are trapped as either yeasts or filaments are both defective in bloodstream infection models, which suggests that the ability to interconvert between different cell types is required for virulence. Although hyphae and pseudohyphae predominate in most virulence models, with white (a/α) yeasts also being essential in disseminated (bloodstream) infections, the newly described elongated yeasts may be more specialized for commensalism. For example, GUT a/α cells were identified based on their superior fitness in an intestinal commensalism model. Further, opaque (a/α) and opaque (a or α) cells have been reported to outperform

other cell types during skin colonization (Noble et al. 2017). Therefore, inhibition of hyphal formation has significance in the prevention of candidiasis (Kurakado et al. 2017). During the survey of low molecular weight compounds, it was found that various steroids, including 17β-estradiol, inhibit hyphal formation without inhibiting growth. Previous reports have shown that several low molecular weight compounds inhibit the budded to hyphal form transition (Toenjes et al. 2005, 2009). The yeast to hyphae transition is central to *C. albicans* virulence through functions including tissue invasion, cell adhesion, evasion of macrophages, and development of clinically relevant biofilm communities. In response to stimuli such as temperature, nutrients, or serum, signal transduction pathways and transcription factors induce the hyphal gene expression program and filamentous growth (Sudbery 2011). The limited studies in which steroid response in yeasts has already been examined demonstrate that yeast cells are extremely responsive to steroids in affecting the morphology, growth and expression profile of several genes and proteins (Prasad et al. 2012). Interestingly, *C. albicans* has proteins capable of binding to steroids, including estrogen-binding protein (Ebp1). It also possesses corticosterone-binding protein (CBP) and progesterone-binding protein (PBP); however, the steroid signaling cascade does not appear to exist in yeast. Estrogen and progesterone are known to stimulate filamentation and biofilm formation, leading to vulvovaginal candidiasis. To determine whether EBP1 regulates a virulence factor, Kurakado et al. (2017) investigated the effect of 17β-estradiol on the morphological transition of *C. albicans* using an ebp1 deletion mutant. Treatment with 17β-estradiol inhibited hypha formation, whereas its effect on the ebp1 deletion mutant was decreased compared to that on the wild-type and revertant strains. These data suggest a new pathway for the yeast to hypha transition via EBP1 in *C. albicans*. To date it has been difficult to translate these basic science discoveries into new therapies to combat *C. albicans* infections (Vila et al. 2016, 2017).

The plasma membrane has key roles in virulence because it not only functions as a protective barrier but also mediates dynamic functions including secretion of virulence factors, cell wall synthesis, invasive hyphal morphogenesis, endocytosis, and nutrient uptake (Douglas and Konopka 2016). *C. albicans*biofilms forming on implanted medical devices (abiotic surfaces) such as catheters serve as a source of infectious cells that can cause deep-seated and bloodstream infections and are associated with high levels of antifungal resistance (Nobile and Johnson 2015). Drug transporters are not only upregulated during planktonic growth but also remain upregulated during biofilm development, enabling *C. albicans* to persist in the presence of antifungals (Ramage et al. 2002). A typical biofilm architecture consists of layers of yeast and hyphal cells interlaced with each other and stabilized by adhesive interactions between these cell types (Nobile and Johnson 2015). The transcriptional network that controls biofilm formation contains six transcription factors (Efg1, Tec1, Bcr1, Ndt80, Brg1, Rob1), wherein all these excepting Bcr1 regulate hyphal morphogenesis (Schweizer et al. 2000). The downstream targets of these transcription factors constitute genes involved in adhesion, hyphal morphogenesis, matrix production, and drug resistance. Bcr1 regulates the expression of adhesins, including the agglutinin-like Als proteins Als1 and Als3, and the hyphal cell wall

protein Hwp1 that promote adherence during biofilm formation (Nobile et al. 2008). Bcr1 thus serves as a positive regulator for cell–cell and cell–substrate adhesion (Finkel et al. 2012).

Although the diploid fungus*Candida albicans*, a human pathogen, has been thought to have no sexual cycle, it normally possesses mating-type-like orthologs (MTL) of both the *Saccharomyces cerevisiae* mating-type genes (MAT) a and alpha on chromosome 5 (Magee and Magee 2000). Evidence for mating included formation of stable prototrophs from strains with complementing auxotrophic markers; these contained both MTL alleles and molecular markers from both parents and were tetraploid in DNA content and mononucleate. However, mating-competent forms of the organism were recently described that produced tetraploid mating products. Bennett and Johnson (2003) described the conditions in which growth of a tetraploid strain of *C. albicans* on *S. cerevisiae* 'pre-sporulation' medium induced efficient, random chromosome loss in the tetraploid. They proved evidence for a chromosome loss pathway that, combined with mating, completes the parasexual cycle for *C. albicans* that can be readily carried out in the laboratory. Their study leaves open the possibility that *C. albicans* is able to undergo meiosis. They suggested that if meiosis does occur in *C. albicans*, it may occur by a different signaling pathway, perhaps requiring a stimulus from its mammalian host. The *C. albicans* genome encodes CaSte2p, a homolog of the *S. cerevisiae* alpha-mating pheromone receptor Ste2p, and two potential pheromones, alpha-F13 (GFRLTNFGYFEPG) and alpha-F14 (GFRLTNFGYFEPGK). Janiak et al. (2005) indicated that CaSte2p is effectively coupled to the *S. cerevisiae* signal transduction pathway. Functional expression of CaSte2p in *S. cerevisiae* provides a well-defined system for studying the biochemistry and molecular biology of the *C. albicans* pheromone and its receptor. Analyzing more than 500 genes important for sexual differentiation in *S. cerevisiae*, Janiak et al. found many homologs of genes that are implicated in the initiation of meiosis (*SPO11*, *DMC1*, *NDT80*), chromosome recombination, and the formation of synaptonemal complexes. However, others (*ME1*, *ZIP2*, and *SPO13*, which are strictly required for meiosis in *S. cerevisiae*) are striking by their absence. *C. albicans* seems to have homologs of all the elements of a functional pheromone response pathway involved in mating in *S. cerevisiae* (*STE2*, *STE3*, Gα(Gpa1), Gβ(Ste4), Gγ(Ste18), (MAP)*STE20*, *STE11*, *STE7*, *FUS3*, Ste12p) but lacks many homologs of *S. cerevisiae* genes for meiosis (Tzung et al. 2001). However, the identification of a mating-type-like (*MTL*) locus and genes such as *CPH1*, *CAG1*, *DLH1*, *NDT80*, and *HST6* in *C. albicans* (*C. albicans* genome project information http://alces.med.umn.edu/Candida.html and http://www-sequence.stanford.edu/group/candida), which participate in meiotic differentiation in *S. cerevisiae*, suggests that the classification of this diploid fungus belies the existence of a sexual cycle. The investigation of pathogenesis-related underlying processes, which are mediated by protein–protein interactions (PPI), is restricted to a limited number of genetic tools available in *C. albicans* (Subotić et al. 2017). Unique features of *C. albicans*, such as its alternative codon usage and incomplete meiosis, have enforced the optimization of standard genetic methods as well as development of novel approaches. Hence, Subotić et al. (2017) have successfully established an additional tool for PPI

studies in *C. albicans* by creating an optimized set of vectors allowing the use of the BiFC assay to detect PPI, and confirmed and visualized the interaction of the membrane-bound receptors, Gpr1 and Gpa2. Full-length Gpr1 interacted with Gpa2 in the membrane, whereas the C-tail, devoid of the membrane-anchoring part, formed a complex with Gpa2 in the cytosol. The BiFC assay proved to be useful in visualizing in vivo, for the first time, the interaction of PKA subunits Bcy1-Tpk1 and Bcy1-Tpk2 in *C. albicans*. Rta3, a member of the Rta1-like family of lipid-translocating exporters, has a 7-transmembrane domain topology, similar to the G-protein-coupled receptors, and is unique to the fungal kingdom. Transcriptome analysis revealed that Rta3 regulates the expression of Bcr1 target genes involved in cell-surface properties, adhesion, and hyphal growth. The identification of this novel Rta3-dependent regulatory network that governs biofilm formation and PC asymmetry across the plasma membrane will provide important insights into *C. albicans* pathogenesis (Srivastava et al. 2017). Their findings point toward a role for the plasma membrane localized Rta3 in providing tolerance to miltefosine, an analog of alkylphosphocholine, by maintaining mitochondrial energetics. There is a complex relationship among the Cek1 and Cek2 MAP kinases (redundant for mating in *C. albicans*) and the MAP kinase phosphatase Cpp1 functioning in the *C. albicans* mating response (Rastghalam et al. 2019). Evidence provided in their study suggests that the Cek1 kinase is the major MAP kinase required for the pheromone response. Loss of this kinase greatly compromises all aspects of the mating response, reducing processes such as induction of pheromone-responsive genes, projection formation, and mating, far below the wild-type level. However, the loss of Cek1 function does not create complete sterility, as a residual level of morphological response and mating can be detected in cek1Δ/Δ mutant strains that is dependent on the related MAP kinase Cek2. The deletion of both kinases completely blocked pheromone response and mating. This pattern of two kinases, with distinct roles but having overlapping functions that require both to be deleted to generate sterility, is similar to that seen for the MAP kinases Fus3 and Kss1 of the post-whole-genome duplication (WGD) yeast *S. cerevisiae* (Marcet-Houben and Gabaldón 2015). The fact that Cek1/Cek2 paralogs are present in the pre-WGD yeast *C. albicans* establishes that the MAPK duplication preceded the WGD, occurring coincidentally with the appearance of an Ste5 equivalent (Cote et al. 2011). Therefore, during the WGD, duplication of the Cek1/Cek2 paralogs should have created four paralogous genes, a pair of CEK1 orthologs, and a pair of Cek2 orthologs. To get to the two-paralog pattern that exists in *S. cerevisiae*, two of these duplicated genes must be lost. Synteny, sequence similarity, and functional relatedness all are consistent with FUS3 and CEK1 being orthologs. The function of the Cek1 MAP kinase in the pheromone response appears regulated by the Cpp1 phosphatase. In response to pheromone treatment, the activation loop of Cek1 shows enhanced phosphorylation, and the level of phosphorylation in both pheromone-treated and -untreated cells was enhanced by the loss of the Cpp1 phosphatase. Phenotypic analysis of the cpp1Δ/Δ strain showed that all aspects of pheromone response were modified in such strains: the cells showed enhanced projection formation and pheromone-induced gene expression in response to pheromone

application, they showed greatly increased pheromone-mediated cell-cycle arrest measured by halo assays, and they showed significantly reduced mating compared to nonmutant cells. As well, pheromone-treated cells or cells lacking Cpp1 showed a greater concentration of Cek1 in the nucleus, consistent with activation loop phosphorylation being involved, directly or indirectly, in nuclear localization. Rastghalam et al. (2019) provided a speculative model for the roles of the MAP kinases and MAP kinase phosphatase in the *C. albicans* pheromone response. It appears that the major signaling pathway involved the receptor–G-protein module activating the upstream elements leading to Hst7 phosphorylating Cek1 on the activation loop, and this in turn enhanced nuclear localization of the kinase and activation of downstream events. This activation is opposed by the action of the Cpp1 phosphatase; in the absence of Cpp1, Cek1 activation is enhanced and sustained, leading to increases in pheromone-mediated gene induction, projection production, and halo formation. In the absence of Cek1, a low level of mating still occurs, and this mating is dependent on Cek2. However, overall Cek2 appears to have a minor role in the mating process: its most significant role in this process in wild-type cells appears to be regulating reentry into the cell cycle after pheromone-mediated arrest. Overall, loss of Cek2 causes only a minor decrease in mating and in morphological changes in response to pheromone and has essentially no effect on pheromone-mediated gene induction. However, loss of Cek2 leads to a significant increase in pheromone-mediated cell-cycle arrest as measured by halo assays. Because the frequency of projections in the mutant strain is not dramatically enhanced relative to WT cells, it is likely the larger halos result from an inability to adapt to pheromone treatment and to reenter the cell cycle. They concluded that further work will be necessary to determine the details of MAP kinase phosphorylation/dephosphorylation and cellular function, with the ultimate goal of understanding how a single MAP kinase with roles in different pathways is properly directed and regulated and how closely related kinases are connected to unique regulatory events.

Under laboratory conditions, the human fungal pathogen*Candida albicans* can undergo both heterothallic and homothallic (opposite- and same-sex) mating. However, both mating modes require the presence of cells with two opposite mating types (MTLa/a and α/α) in close proximity. Given the predominant clonal feature of this yeast in the human host, both opposite- and same-sex mating would be rare in nature. Glucose starvation and oxidative stress, common environmental stresses encountered by the pathogen, induce the development of mating projections and efficiently permit same-sex mating in *C. albicans* with an “a” mating type (MTLa/a). This induction bypasses the requirement for the presence of cells with an opposite mating type and allows efficient sexual mating between cells derived from a single progenitor. Glucose starvation causes an increase in intracellular oxidative species, overwhelming the heat-shock transcription factor-1 (Hsf1)- and heat-shock protein (Hsp) 90-mediated stress-response pathway (Guan et al. 2019). Candida transactivating protein 4 (Cta4) and cell wall transcription factor 1 (Cwt1), downstream effectors of the Hsf1–Hsp90 pathway, regulate same-sex mating in *C. albicans* through the transcriptional control of the master regulator of a-type mating, MTLa2,

and the pheromone precursor-encoding gene mating α-factor precursor (MFα). Their results suggest that mating could occur much more frequently in nature than was originally appreciated and that same-sex mating could be an important mode of sexual reproduction in *C. albicans.*

C. albicans exhibits several survival mechanisms to evade attack by antifungals and colonize host tissues; therefore, their importance derives not only from the severity of their infections but also from their ability to develop resistance against antifungals, such as azoles, in patients undergoing long-term or prophylactic treatment (Saini et al. 2005). In *C. albicans*, upregulation of genes encoding the Tac1-regulated ABC drug transporters (*CDR1* and *CDR2*) is a predominant cause for the development of antifungal resistance. With the increasing number of *Candida* infections in the recent years, multidrug resistance (MDR) has become a growing health concern. Three efflux pumps are responsible for decreasing the intracellular concentration of azoles in *C. albicans*: these pumps are encoded by genes for *Candida* drug resistance (CDR1 and CDR2) and multidrug resistance (MDR1). Notably, human steroid hormones are also shown to be substrates of yeast and mammal ABC multidrug transporters. The fact that *C. albicans* often come in direct contact with these hormones suggests that these could indeed be the physiological substrates of this transporter protein. Notably, their competition data revealed that progesterone was unable to compete with the steroid transport and extrapolated that progesterone may not be the substrate of Cdr1p. However, it is very likely that progesterone might interact with a different set of residues of Cdr1p and has different binding sites. Mdr1p, a MFS H+/drug antiporter that is also a multidrug transporter and relevant in clinical drug resistance of *Candida*, cannot export steroid hormones. It should be noted that although both are promiscuous multidrug transporters having many common substrates, yet they remain selective toward steroid transport.

Invading hyphae trigger several proinflammatory signaling pathways; during inflammation host- and microbial-derived proteases trigger the activation of protease-activated receptors (PARs), a family of G-protein-coupled receptors, that are encoded in the mammalian genome. The activation of PARs occurs through proteolytic cleavage of the extracellular N-terminal domain, which generates a new N-terminus that functions as a tethered ligand and binds to the receptor through an intramolecular interaction to trigger transmembrane signaling. Once cleaved, activated PARs undergo conformational changes within transmembrane helices that facilitate interaction with heterotrimeric G proteins. PARs display biased agonism or functional selectivity, which refers to the capacity of different ligands to stabilize unique active conformations of a GPCR that facilitates activation of distinct signaling responses. PAR1 is promiscuous and interacts with multiple distinct types of these G proteins, including Gαi, Gαq, and Gα12/13. PARs are expressed differentially in distinct cell types in a species-specific manner. Moretti et al. (2008) reported that activation of Toll-like receptors (TLRs) by fungi unmasks an essential and divergent role for PAR1 and PAR2 in downstream signaling and inflammation. TLRs activated PARs and triggered distinct signal transduction pathways involved in inflammation and immunity to *C. albicans* and *Aspergillus fumigatus.*

Inflammation is promoted by PAR1 and PAR2 activation in response to *Candida* and by PAR2 inhibition in response to *Aspergillus*, which occurs by TLR regulation of PAR signaling, with TLR2 promoting PAR1 activity and TLR4 suppressing PAR2 activity. Thus, tissue injury and pathogens induce signals that are integrated at the level of distinct TLR/PAR-dependent pathways, the exploitation or subversion of which contributes to divergence in microbial promotion of the inflammatory response. The only current clinical agent that targets PARs is the PAR1 antagonist vorapaxar for the prevention of thrombotic cardiovascular events (Hamilton and Trejo 2017).

3.11 GPCRs Used in the Pharmaceutical Industry

Endogenous natural ligands Fig. 3.4 (a) are those that are produced in the body, not those introduced into the body, such as certain drugs.

Antagonist ligands include these:

- *Competitive antagonists* Fig. 3.4 (b), which are drugs that bind to the same site as the natural ligand, agonists, or partial agonist, and inhibit their effects: these would be analogous to competitive inhibitors of an enzyme. One could also imagine a scenario in which an "allosteric" antagonist binds to an allosteric site on the receptor, inducing a conformational change in the receptor so the ligand, agonist, or partial agonist could not bind.
- *Noncompetitive antagonists* (or perhaps more generally mixed antagonists) Fig. 3.4 (c) are drugs that bind to a different site on the receptor than the natural ligand, agonist, or partial agonist, and inhibit the biological function of the receptor. In analogy to noncompetitive and mixed enzyme inhibitors, the noncompetitive antagonist may change the apparent dissociation constant (K_d) for the ligand, agonist, or partial agonist (the ligand concentration required to achieve half-maximal biological effects), but will change the maximal response to the ligand (as mixed inhibitors change the apparent V_{max}). The following figure shows the action of a competitive and noncompetitive antagonist.
- *Irreversible agonists*, or 'negative antagonists,' arise from covalent modification of the receptor.

As pockets or binding sites of the receptors are of great importance in the pharmaceutical point of view for designing the proper ligand, it is valuable to look at a relevant review. Gao and Skolnick (2012) reported a comprehensive study of the distribution of protein–ligand interaction sites, namely, ligand-binding pockets, around protein–protein interfaces where protein–protein interactions occur. They inspected a representative set of 1611 representative protein–protein complexes and identified pockets with a potential for binding small molecule ligands. The majority of these pockets are within a 6 Å distance from protein interfaces. Accordingly, in about half of ligand-bound protein–protein complexes, amino acids from both sides of a protein interface are involved in direct contact with at least one ligand.

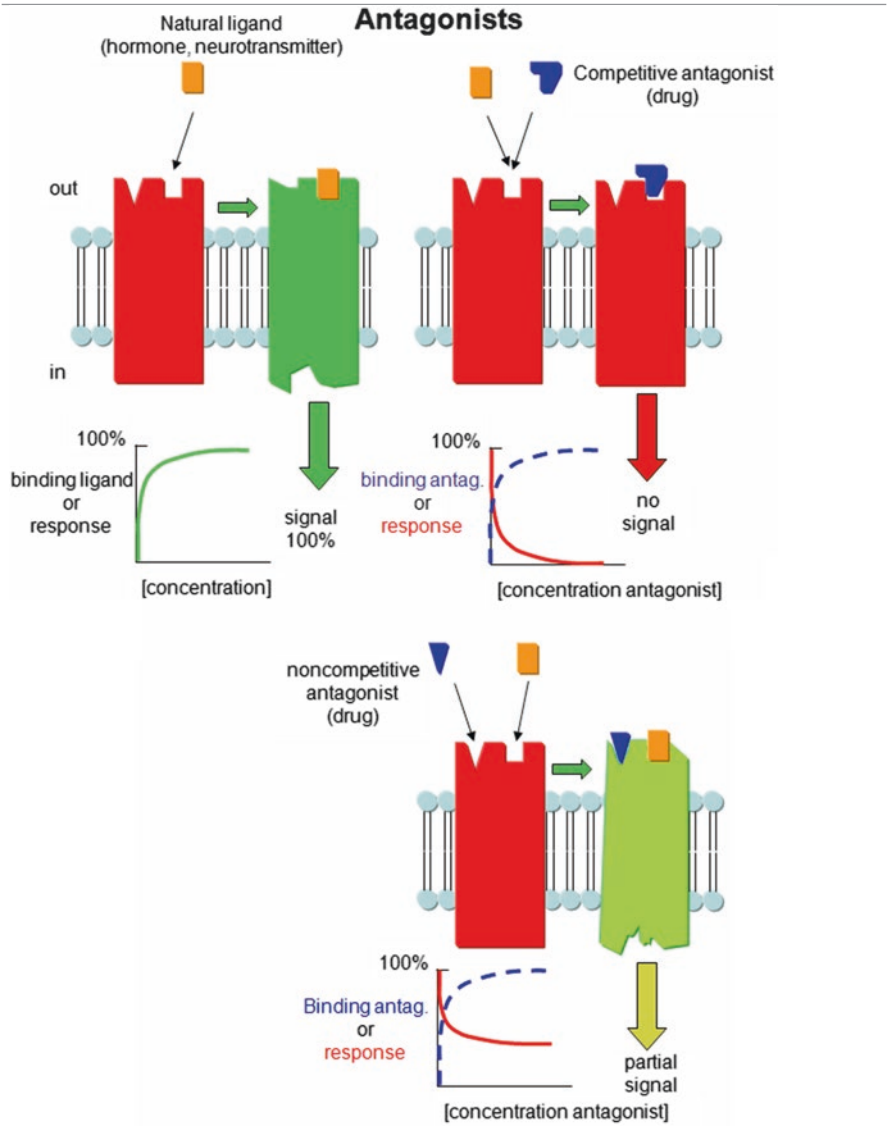

Fig. 3.4 (**a**) Natural ligand at the upper left hand side. (**b**) competitive antagonist at the upper right hand side. (**c**) noncompetitive antagonist at the bottom of the figure. (Source: By courtesy and permission of Jakubowski, Henry)

Statistically, ligands are closer to a protein–protein interface than a random surface patch of the same solvent-accessible surface area. Similar results were obtained in an analysis of the ligand distribution around domain–domain interfaces of 1416 nonredundant, two-domain protein structures. Furthermore, comparably sized pockets as observed in experimental structures are present in artificially generated

protein complexes, suggesting that the prominent appearance of pockets around protein interfaces is mainly a structural consequence of protein packing and, thus, is an intrinsic geometric feature of protein structure. Humankind may take advantage of such a structural feature by selecting and further optimizing for biological function. The authors propose that packing nearby protein–protein or domain–domain interfaces is a major route to the formation of ligand-binding pockets. The author refers the reader to Landry and Gies (2007) for more details.

An important role in the study of ligand-binding sites (pockets) is the role of the N-terminus of the GPCR. This topic is elegantly covered by Coleman et al. (2017), who concluded that recent advances in the field of GPCRs show the humble N-terminus to be more than a ligand-binding site, a substrate for glycosylation, or an anchor for a signal peptide in the diverse examples summarized in their work. The protease-activated receptors are exemplars of this, revealing that GPCRs can harbor their own agonists within their N-termini, uncovered following a regulated proteolytic event. Similarly, the MC4 receptor N-terminus contains an agonist for its own receptor, allowing evolution of agouti-related peptide as an endogenous inverse agonist, through its ability to antagonize the N-terminus. In contrast, they reported the orphan receptor GPR37L1 to have constitutive activity maintained by its N-terminus and abolished by metalloprotease-mediated removal of the region. This differs again from the N-terminus of yeast pheromone α-factor receptor Ste2p, which reportedly serves to constrain the receptor response to its agonist. The receptors that have been discussed in their review represent emerging examples of novel modalities of GPCR signaling whereby the N-terminus is shown to be a crucial and dynamic contributor to signal transduction.

G-protein-coupled receptors (GPCRs) are the starting point for the control of several signaling pathways and are therefore considered a potentially rich source of innovation as drug targets and for drug design to alleviate many human diseases of genetic or biotic origin. The most appropriate GPCR candidate target for developing new fungicides needs searching for new compounds blocking this particular target and requires the knowledge of its 3D structure. Second, to identify potential new fungicide target(s), an important step is to verify that the identified target(s) are not present in host organisms and humans. Bresso et al. (2016) provided a detailed example of an integrated process with detailed steps merging genomics, structural bioinformatics, and drug design for *Fusarium graminearum*, leading to propose valuable and innovative solutions to a worldwide threat to wheat grain producers and consumers.

The trehalose biosynthetic pathway is found in a wide variety of organisms, including human-pathogenic fungi, but not in humans. Genes encoding proteins involved in trehalose biosynthesis are mechanistically linked to the metabolism, cell wall homeostasis, stress responses, and virulence of *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus.* By further defining the biology and functions of trehalose and its biosynthetic pathway components in pathogenic fungi, an opportunity exists to leverage this pathway as a potent antifungal drug target (Thammahong et al. 2017). It will be necessary to identify antagonists or inverse agonists for these receptors, which will require good screening systems and large

compound libraries (Van Dijck 2009). Considering the abundant expression of several nuclear receptors, it has also been suggested that one and the same nuclear receptor may have distinct endogenous ligands in distinct tissues or cell types.

Analysis of the human genome in 2002 led to the estimation of 6000–8000 targets of pharmacological interest. Only a small part of these targets relates to approved drugs. Lagerström and Schiöth (2008) overviewed a comprehensive overview of the five main human GPCR families–Rhodopsin, Secretin, Adhesion, Glutamate and Frizzled/Taste2–with a focus on gene repertoire, general ligand preference, common and unique structural features, and the potential for future drug discovery. A consensus number of 324 drug targets for all classes of approved therapeutic drugs was proposed by Overington et al. (2006). Of these, 266 are human genome-derived proteins, and 58 are bacterial, viral, fungal or other pathogenic organism targets. A large number of druggable targets have been recently proposed from preclinical and first clinical data, but a huge reservoir of putative drug targets, possibly several thousands, remains to be explored. The review of Landry and Gies (2008) considered the different types of ligands and their selectivity in the main superfamilies of drug targets, enzymes, membrane transporters, and ion channels, and the various classes of membrane and nuclear receptors with their signaling pathway. Recently approved drugs such as monoclonal antibodies, tyrosine kinase, and proteasome inhibitors, and major drugs under clinical studies, are reviewed with their molecular target and therapeutic interest. The druggability of emerging targets such as multidrug resistance transporters and cystic fibrosis transmembrane conductance regulator (CFTR), hyperpolarization-activated cyclic nucleotides-gated (HCN), cyclic nucleotide-gated (CNG) and transient receptor potential (TRP) ion channels, tumor necrosis factor (TNF) and receptor activator of NFjB (RANK) receptors, integrins, and orphan or recently deorphanized G-protein-coupled and nuclear receptors were discussed. Large advances have been made in the therapeutic use of recombinant cytokines and growth factors [i.e., tasonermin, TNFa-1a; becaplermin, platelet-derived growth factor (PDGF); dibotermin-alpha, bone morphogenetic proteins (BMP)2; anakinra, interleukin-1 receptor antagonist protein (IRAP); and in enzyme replacement therapy, i.e., algasidase (alpha-galactosidase) and laronidase (alpha-L-iduronidase)]. New receptor classes are emerging, such as membrane aminopeptidases, and novel concepts are stimulating drug research, such as epigenetic therapy, but the molecular target of some approved drugs, such as paracetamol and imidazolines, must be identified (Landry and Gies 2008). G-protein-coupled receptors are the largest class of receptors mediating the effects of small neurotransmitters, all known neuropeptides, many peptide hormones and inflammatory mediators, some lipids, and even calcium for the control of its blood concentration. Almost 30% of all marketed drugs act on GPCRs. The most familiar GPCRs as historical drug targets are the muscarinic acetylcholine receptors, the alpha- and beta-adrenergic, dopaminergic, histaminergic, and opioid receptors. Some other GPCR ligands have been developed as drugs during the past three decades: serotonin 5-HT, prostaglandins, leucotrienes, ADP, or calcium receptor ligands. The actual top-selling GPCRs ligands are clopidogrel (ADP-P2Y12 antagonist, platelet antiaggregant), olanzapin (mixed serotonin-5HT2/dopamine-D2

antagonist, neuroleptic), valsartan (angiotensin-AT1 antagonist, antihypertensive), fexofenadine (histamine-H1 antagonist, antiallergic), sumatriptan (serotonin-5HT1D antagonist, anti-migrainous), and leuprorelin (GnRH/LH-RH peptidic agonist, antihormone-dependent cancer). Cellular differentiation, mating, and filamentous growth are regulated in many fungi by environmental and nutritional signaling cascades. The mitogen-activated protein kinase (MAPK) pathway is a network of signaling pathways that allow adaptation to environmental changes and is evolutionarily conserved from yeasts to mammals. The divergent point between fungal and mammalian MAPK pathways is their upstream signaling module. Based on this, several MAPK signaling pathways in fungi have been identified that may have therapeutic implications: the Mkc1 pathway, the Cek1 pathway, the Cek2 pathway, and the high-osmolarity glycerol (HOG) pathway. In *C. albicans*, these pathways are involved in invasive hyphal growth, morphogenesis, biogenesis of the cell wall, dimorphism, and the stress response, all of which likely have an important role in virulence and serve as potential targets for new drugs. Among these potential targets, the HOG pathway appears to be the most promising as it is the main signal transduction system that is responsible for cellular stress responses (Rutherford et al. 2019). The Ssk2-Pbs2-Hog1 MAPK module is mainly regulated by the multicomponent phospho-relay system, which consists of sensor hybrid histidine kinases (HHKs), histidine-containing phospho-transfer (HPt) protein, and response regulator RRs in *C. albicans*. Its transduction network is composed of a two-component-system-like phospho-relay system and the Hog1-type MAPK cascade. Overactivation of the HOG pathway can be devastating to an organism, as witnessed by the action of fludioxonil, which overactivates the HOG pathway and confers lethal effects. This observation suggests that drugs targeting the negative regulators of the HOG pathway could be more efficient at killing a pathogen than those targeting its positive regulators. Other compounds targeting the HOG system have been identified including the ambruticins, a family of cyclopropyl-pyran acids with broad antifungal activity. Ambruticin S, an antifungal cyclopropyl-pyran acid, has long been known to have curative effects against murine pulmonary coccidioidal infection. Recently, two analogs of ambruticin S with greater in vitro potency, KOSN-2079 and KOSN-2089, were tested against lethal murine *Coccidioides* infection; both improved the survival of mice over that of controls, suggesting that larger studies in animals may be warranted (Landry and Gies 2008). In their review article, McCarthy et al. (2017) reported that recent advances in our understanding of the fungal life cycle, functional genomics, proteomics, and gene mapping have enabled the identification of multiple potential new drug targets that could bolster the arsenal of available options to treat resistant invasive fungal infections (IFI). In their review, they examined those targets mechanistically and described how promising new therapies might be developed, with special attention paid to molecules that promote growth inhibition. They explored fungal architecture, the anchoring of proteins to the plasma membrane through covalent attachment to glycosylphosphatidylinositol (GPI), which provides an appealing potential target for the development of antifungal agents. E1210 is a novel isoxazolyl bis-pyridine wall-active antifungal compound that inhibits an early step in the GPI-dependent anchoring of mannosylated

cell wall proteins attached to the polysaccharide wall component. Its target is Gwp1p, the fourth enzyme in the GPI-anchoring pathway, responsible for inositol acylation (the mammalian homolog is not inhibited). A second GPI inhibitor has recently been discovered. Gepinacin, a monocarboxylic amide, selectively inhibits Gwt1, a critical acyltransferase required for the biosynthesis of fungal GPI anchors. In contrast to all three major classes of antifungal agents in current use, its direct antifungal activity results from its ability to induce overwhelming stress to the endoplasmic reticulum (ER) and does not affect the viability of mammalian cells, nor does it inhibit their orthologous acyltransferase. It was noted to increase the immunogenicity of *Candida albicans*, further enhancing its antifungal activity by disrupting the mannoprotein outer layer of the cell wall and unmasking the more immunogenic inner β-glucan layer. Glycosphingolipids (GSLs) are a type of glycolipid found in cell membranes that have emerged as key regulators of pathogenicity in a variety of fungi. When yeast and mold lack the GSL called glucosylceramide (GlcCer), they are unable to replicate. Two newly identified compounds have been found to decrease levels of fungal, but not mammalian, GlcCer. Metabolic pathways are essential for fungal viability and virulence. The glyoxylate cycle, a modified tricarboxylic acid cycle, bypasses the carbon dioxide-generating steps to conserve carbons as substrates for gluconeogenesis, which enables fungi to survive in nutrient-limited host niches. The cycle consists of five enzymes, including isocitrate lyase and malate synthase, which are unique to this cycle, and three others that are essential for the virulence of both yeasts (including *C. albicans*) and molds (including *Aspergillus fumigatus*), making it a promising target for the development of novel antifungal agents. The pyrimidine salvage pathway offers another potential target for antifungal therapies. Discovered as the result of an *Aspergillus nidulans* library screen, it targets the enzyme dihydroorotate dehydrogenase, which catalyzes the fourth enzymatic step of pyrimidine biosynthesis. Cytochrome P450 enzymes (CYPs) are a superfamily of proteins that catalyze a wide range of metabolic reactions and present an attractive site for drug development. The commonly used triazole-class drugs work by selectively inhibiting fungal cytochrome P450 14α-demethylase. The MAPK pathway is a network of signaling pathways is their upstream signaling module. Several MAPK signaling pathways in fungi that have been identified may have therapeutic implications: the Mkc1 pathway, the Cek1 pathway, the Cek2 pathway, and the high-osmolarity glycerol (HOG) pathway. In *C. albicans* these pathways are involved in invasive hyphal growth, morphogenesis, biogenesis of the cell wall, dimorphism, and the stress response, all of which likely have an important role in virulence and serve as potential targets for new drugs. Others have sought to develop novel antifungal agents by interrupting the modification of nucleic acids. Epigenetic therapy is being widely developed for the treatment of human neoplastic disease and has recently found a role in the treatment of invasive candidiasis, using histone deacetylases (HDACs) as a target, which are involved in a wide range of cellular functions, ranging from cell proliferation to cell death, by regulating chromatin structure and transcription through lysine deacetylation of histones. The report ended by anticipating future directions: IFIs continue to be an important cause of morbidity and mortality, particularly in immunocompromised

patients. The emergence of antifungal resistance is a growing global health problem, underscoring the need for new therapeutic options. Significant progress has been made in the identification of potential targets for novel agents that exploit inherent differences between fungi and humans. Although many of these targets remain theoretical, several molecules have recently entered early clinical development. Complementing these therapies are advances in innate host defense: a pioneering clinical study using adoptive transfer of *Aspergillus*-stimulated T cells showed significant efficacy in treating stem cell transplant recipients with invasive aspergillosis. Other investigators have generated T-cytotoxic cells expressing Dectin-1 chimeric antigen receptors (CARs) that recognize surface fungal glucans. New treatment options for IFIs are urgently needed, but it remains a challenge to bring novel agents to the market. The discovery of a new therapy is difficult, chemical genetic-based screens are labor intensive, traditional regulatory requirements are stringent, and the financial reward may be limited if the IFI is uncommon. In the face of these hurdles, patients continue to suffer. We must address this unmet need by bolstering support for research, considering alternative approval pathways, repurposing existing non-antifungal drugs, strengthening ties between academia and industry, and providing financial incentives for the development of new therapeutic options to treat these uncommon yet devastating infections. Beyond small molecules, new approaches for augmenting host responses are critical and include the development of vaccines, immunomodulators, and novel approaches for reducing immunosuppression and exploiting the immunopharmacology of existing antifungal agents.

There are two main families of membrane proteins involved in drug transport, the major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) proteins. Both types of protein possess multiple membrane spanning α-helices in transmembrane domains (TMDs), and ABC proteins, in addition, contain cytosolic nucleotide-binding domains (NBDs) involved in ATP hydrolysis. ABC proteins, and to a lesser extent MFS proteins, have broad substrate specificities that are determined by the structure and arrangement of the transmembrane α-helices (Lamping et al. 2017b). In an effort to understand the molecular basis of drug recognition, binding, and release, several studies are being pursued, giving an insight into its drug-binding pocket (Baghel et al. 2017). One of the most clinically significant mechanisms of azole resistance in the pathogenic yeast *Candida albicans* is overexpression of the multidrug transporter protein Cdr1p (*Candida* drug resistance), belonging to the ABC superfamily of transporters (MDR), which is a phenotype where cells in parallel develop resistance to multiple chemically unrelated chemotherapeutic agents. Drug efflux pumps belonging to either the ATP-binding cassette (ABC) family or major facilitator superfamily (MFS) transporters have a major role in the development and sustenance of drug tolerance. The induced overexpression of genes encoding these transporter proteins does not allow cells to retain toxic concentrations of the drug, because of their rapid extrusion, thus making it ineffective. Lamping et al. (2010) reported that the overexpression of pleiotropic drug resistance (PDR) efflux pumps of the ATP-binding cassette transporter frequently correlates with multidrug resistance. Fungal, plant, and human ABCG-family Pdrps

possess a nucleotide-binding domain (NBD) and a transmembrane domain (TMD) in a family-defining 'reverse' ABC transporter topology (NBD–TMD) that is duplicated (NBD–TMD2) in full-size fungal and plant Pdrps. The Human Genome Organization (HUGO) classified ABC superfamily members into eight subfamilies in which Cdr1p, as the pleiotropic drug resistance (PDR) family in *Saccharomyces cerevisiae*, belongs to the ABCG subfamily. The subfamily contains a N- and a C-terminal nucleotide-binding domain (NBD), each followed by a trans-membrane domain (TMD) (N- and C-TMDs). Each TMD is composed of six TMHs; although NBDs are highly conserved and participate in ATP catalysis to power drug extrusion, TMDs are more variable in structure and are involved in the formation of overlapping multiple substrate-binding sites. These TMHs are linked to each other via six extracellular loops (ECLs) and four intracellular loops (ICLs). The recent publication of the heterodimer ABCG5/ABCG8 X-ray structure allowed establishing that proteins belonging to the G-subfamily constitute a new type of transporters, called type II, compared to B- and C-subfamilies transporters that belong to the type I. Type II deeply differ from type I by the absence of domain swapping in the TMD between the N- and C-moieties, together with the absence of a large extension of the membrane helices toward the cytoplasm to connect each NBD. These features let us suppose a distinct transport mechanism. Notably, Rawal et al. (2013) in a recent study probed the nature of the drug-binding pocket of Cdr1p by performing a full alanine scanning mutagenesis of its membrane region, wherein each of the residues of 12 TMHs was replaced with an alanine (or a glycine when being an alanine). Their study revealed multiple overlapping mini drug-binding sites within a large centrally located polyspecific drug-binding cavity. Baghel et al. (2017) examined the kinetics of steroids efflux mediated by the *Candida* drug resistance protein 1 (Cdr1p) and evaluated their interaction with the protein. They exploited their in-house mutant library for targeting the 252 residues forming the 12 transmembrane helices (TMHs) of Cdr1p. The selective structural arrangement of the steroid-binding pockets in the core region and at the lipid–TMD interface, which was never reported previously, together with the possible rotation of some TMHs may be the structural basis of the drug-transport mechanism achieved by these type II ABC transporters. Baghel et al. (2017) showed that the central binding pocket in which β-estradiol and R6G competitively bind is made of 14 residues; among them, 10 are identified as critical for the former and 8 for the latter. It mainly engages the N-half of Cdr1p with 7 residues brought by TMHs 2 and 5 while only 3 are recruited on the C-side, brought by TMHs 7 and 8. In this binding pocket F1233 from TMH 8, although highly critical, remains rather distant whereas, in contrast, residues I1237 and N1240, although never found critical, are part of the binding pocket. All this information suggests that the position of TMH 8 in the current model/conformation may probably slightly differ, with F1233 closer to the substrate and I1237 and N1240 further. By contrast, corticosterone engages only 3 critical residues in that pocket, which strongly suggests that it does not interact with Cdr1p as β-estradiol or R6G in that region, and consequently may be transported by the protein with a distinct mechanism. Cdr1p has not only acquired significant clinical importance but is considered an important player in any design of strategies to combat antifungal

resistance. The CDR1 gene encodes an integral plasma membrane (PM) protein. On the basis of its amino acid sequence, Cdr1p is predicted to consist of two homologous halves, each containing one N-terminal hydrophilic domain followed by a C-terminal hydrophobic domain (Shukla et al. 2003). Their data showed for the first time that the drug substrate-binding sites of Cdr1p exhibit striking similarities with those of mammalian drug-transporting P-glycoproteins and, despite differences in topological organization, the transmembrane segment 6 in Cdr1p is also a major contributor to drug substrate-binding site(s). It is clear that much remains to be learned of the signals, fungal signaling pathways, and transcriptional regulatory networks that control morphogenesis in *C. albicans* (Noble et al. 2017). ATP-binding cassette drug transporters are not only considered determinants of multidrug resistance but also have crucial functions in controlling lipid levels. Adenosine triphosphate-binding cassette (ABC) transporters constitute a group of highly conserved cellular transmembrane transporter proteins. ABCA1, ABCA7, and ABCA4 are members of the ABCA subfamily and share extensive sequence and structural similarity. Several studies have shown that ABCA proteins are involved in lipid transport. ABCA7 is thought to have an important role in lipid homeostasis in cells of the immune system. It is a full-size single-subunit ABC transporter consisting of 12 trans-membrane-spanning domains (Nowyhed et al. 2017). Within cells, it is expressed predominantly on the plasma membrane, but it is also detected in intracellular membranes. More recently, ABCA7 was found to be significantly associated with phagocytosis in macrophages both in vivo and in vitro (Jehle et al. 2006; Linsel-Nitschke et al. 2005). Nowyhed et al. (2017) data show that ABCA7 is required for efficient phagocytosis of apoptotic cells. In *C. albicans*, which causes infections associated with venous catheters, urinary catheters, and several other implanted devices, upregulation by genes encoding the Tac1-regulated ABC drug transporters (*CDR1* and *CDR2*) is a predominant cause for the development of antifungal resistance (Coste et al. 2004; Prasad et al. 2015). As a consequence, drug-resistant isolates display enhanced efflux of azole antifungals, leading to reduced inhibition of their target enzyme, lanosterol 14α-demethylase of the ergosterol biosynthesis pathway. The presence of gain-of-function mutations in Tac1 renders it hyperactive, resulting in the simultaneous induction of *CDR1* and *CDR2* along with *RTA3*, *IFU5*, and *HSP12* in azole-resistant isolates (Coste et al. 2004; Liu et al. 2007). The significance of coordinate regulation has been inferred from studies with *S. cerevisiae*, wherein Pdr5, the major ABC drug efflux pump, and genes involved in sphingolipid biosynthesis are induced coordinately via the transcription factors Pdr1/Pdr3 (Devaux et al. 2002). These studies argue for the role of the pleiotropic drug resistance (Pdr) pathway in coordinately controlling lipid levels on the plasma membrane and multidrug resistance in the budding yeast (Johnson et al. 2010; Khakhina et al. 2015). Studies on the noteworthiness of Tac1-coregulated genes have not been established. Thus, among the Tac1-coregulated genes, *RTA3* (named for resistance to 7-aminocholesterol) caught the attention of Srivastava et al. (2017) in particular as it was annotated as a putative lipid translocase in the *Candida* Genome Database (CGD). Lipid translocators can direct inward or outward transbilayer movement of phospholipids and are referred to as flippases and floppases,

respectively. The balanced action of these proteins is crucial for generating plasma membrane asymmetry, such that the aminophospholipids are sequestered in the cytoplasmic leaflet of the membrane, whereas choline lipids are enriched in the outer leaflet (Devaux et al. 2008). Any perturbation in membrane asymmetry serves as a signal for activating multiple cellular events. Other than the identification of Cdr1 and Cdr2 as floppases, molecular entities that can function as flippases or signals which regulate the activity of these lipid translocators to generate membrane asymmetry in *C. albicans* remain unidentified. The problem of drug resistance is not only limited to free-living planktonic forms of *Candida* but also extends to surface attached communities such as biofilms (Nobile and Johnson 2015). Microorganisms naturally exist primarily in association with surfaces in communities called biofilms. Central to the biofilm formation is the ability of microbial cells to adhere to substrates. Adherence of biofilms is clinically significant as the basis for infections associated with implanted medical devices. Adherence mechanisms are diverse, and involve specific cell-surface proteins (adhesins), more complex surface structures such as pili, and secreted extracellular matrix material. Adherence is often found to be highly regulated, reflecting the need for biofilms to release cells to colonize new sites. Biofilms are clinically significant as the basis for infections associated with implanted medical devices. For device-associated biofilms the definition of the mechanisms that regulate cell–substrate adherence provides insight into how these biofilms form (Finkel et al. 2012). Besides the Bcr1 that functions in yeast form cells and is among the best characterized biofilm regulators, its adhesin targets Als1/3 and Hwp1 mediate cell–cell interaction in biofilms as well as genes induced upon hyphal development. The other best characterized biofilm regulator Ace2 is known to affect adherence, biofilm formation, and hyphal morphogenesis. Much of the phenotypic impact of Snf5, a subunit of the eukaryotic SWI/SNF chromatin remodeling complex, stems from its role in ACE2 expression. Their findings also have significant implications on a more global scale: they define a regulatory network through which 12 transcription factors govern expression of more than one quarter of the *C. albicans* cell-surface protein genes. Zinc response regulator Zap1 is among the 12 transcription factors that also govern biofilm matrix accumulation and quorum sensing molecule production. Zap1 is thus positioned to coordinate multiple steps in biofilm formation. Finally, many adherence regulators do not have clear functional targets, based on their analysis. However, their unifying target gene classes will help to direct future studies. In addition to the identification of transcription factors, the role of Ras- and Tor1-mediated signaling pathways in regulating cell–cell adhesion during biofilm formation via Bcr1, Efg1, Nrg1, Tec1, and Tup1 has also been established (Inglis and Sherlock 2013). In aggregate, these studies point toward the complexity of the gene regulatory programs that control biofilm formation in the fungal pathogen *C. albicans*. The predicted topology of Rta3 revealed the presence of 7-transmembrane domains (7TMDs) similar to the family of 7-TM receptor proteins often associated with the G-protein-coupled receptors (GPCRs). The homologs of *RTA3* are referred to as the Rta1-family of proteins or the lipid translocating exporters in *S. cerevisiae* (Manente and Ghislain 2009). In addition to *RTA3*, *C. albicans* has three additional genes, orf19.6224, *RTA2*, and

RTA4, coding for the Rta1-family of proteins. Noteworthy is that these proteins lack an overall sequence conservation with the classical GPCRs. The role of Rta2 in modulating azole tolerance and in endoplasmic reticulum (ER) stress resistance has been established, and the others remain uncharacterized (Thomas et al. 2015). These proteins are unique to the fungal kingdom and can be considered as potential therapeutic targets. In agreement with its 7TMD topology, Srivastava et al. (2017) showed that *C. albicans* Rta3 is localized at the plasma membrane similar to Rsb1 of *S. cerevisiae* and thus is most likely to function as a regulatory protein rather than a transporter or translocator. Moreover, Rta3 and proteins of this family represent targets for new antifungals owing to their exclusivity in the fungal kingdom. RTA3 is a downstream target of the transcription factor Tac1 and is coregulated with CDR1 and CDR2, two well-documented drug efflux pumps in *C. albicans*. Except for a single study where Rta3 was implicated to affect susceptibility to fluconazole in an azole-resistant clinical isolate (Whaley et al. 2016), its functional relevance remains unexplored in *C. albicans*. Srivastava et al. (2017) findings indicate that absence of Rta3 affects a spectrum of biological processes highlighting the role of this 7-TM receptor protein in multiple regulatory pathways. The loss of Rta3 not only affects the tolerance to miltefosine, an alkylphosphocholine analog, but also alters mitochondrial membrane energetics. Miltefosine-induced apoptosis-like cell death in the budding yeast is caused by its direct interaction with COX9, a subunit of the complex IV of the electron transport chain in *S. cerevisiae.* This action results in the disassembly of complex IV, causing disruption of the electron transport chain, which in turn affects the MMP. Consistent with this, they proposed that altered mitochondrial parameters in rta3Δ/Δ cells might sensitize it to a lower dose of miltefosine than is required for the wild-type cells. Furthermore, a reduced flop or export of miltefosine in rta3Δ/Δ as a cause for the increased susceptibility to miltefosine also cannot be ruled out. Additionally, Tac1-regulated RTA3 is essential for curbing miltefosine-induced stress as transcript levels of RTA3 are elevated in wild-type cells treated with miltefosine. Recent studies demonstrate the ability of miltefosine to kill azole-resistant clinical isolates and inhibit biofilm formation by *C. albicans* (Vila et al. 2016, 2017). In this context, identification of Rta3 as one of the determinants of miltefosine tolerance may lead to the identification of its target in *C. albicans*. Srivastava et al. (2017) demonstrated that the loss of Rta3 perturbs the dynamic equilibrium of PC by increasing its inwardly directed (flip) movement across the plasma membrane; this results in the enrichment of PC on the inner leaflet of the plasma membrane and consequently its transfer to intracellular membranes by passive diffusion. They thus proposed that the increased flip of PC may be attributed to the role of Rta3 as a negative regulator of PC-specific flippase activity. In conclusion, given the 7TMD topology of Rta3, their study shows that functional Rta3, instead of functioning as a lipid translocator itself, may have a role in maintaining the asymmetrical distribution of PC across the plasma membrane, possibly by modulating regulatory pathway(s) that signal unidentified PC-specific flippase(s) or floppase(s) in *C. albicans*. The proposal that Rta3 is a positive regulator of biofilm formation and functions upstream to Bcr1 stems from the following observations. First, the catheter inoculated with the rta3Δ/Δ cells was essentially devoid of

any material, similar to bcr1Δ/Δ, suggesting that there was a defect in early events of biofilm formation in vivo. Second, the overexpression of BCR1 in rta3Δ/Δ cells partially rescues the biofilm defect of the mutant. Third, the biofilm defect phenotype of the bcr1Δ/Δ cells was not rescued by the increased expression of RTA3. Their results therefore argue that Rta3 is pivotal for biofilm formation and that Bcr1 is one of the key downstream effector molecules of the Rta3-dependent regulatory pathway that contributes to biofilm formation. The biofilm formation in vivo involves dynamic interactions with host factors. Their transcript analyses are indicative of a positive transcriptional feedback loop between Rta3 and downstream Bcr1 transcription factor. These signaling pathways are modulated by feedback loops where the downstream components of the pathway regulate upstream elements of the same pathway. Positive feedback regulation amplifies the signal, whereas negative feedback regulation helps in attenuation of the response. It is possible that in unique niches of the mammalian host, expression of Rta3 and BCR1 may be interdependent. The mutual regulation of Bcr1 and Rta3 may regulate the magnitude and duration of the signaling event entailing response to host specific cues and thus biofilm formation. Their study also showed a mitochondrial membrane energetics-dependent expression of BCR1. Based on these data, it is evident that Rta3 governs biofilm formation largely by regulating Bcr1. Tac1-regulated drug transporters (Cdr1 and Cdr2) are responsible for the asymmetrical distribution of phosphoglycerides across the plasma membrane, in addition to contributing to the development of azole resistance in *C. albicans.* Considering the role of Rta3 in maintaining the asymmetrical distribution of PC across the plasma membrane, the physiological role of Tac1-coregulated genes under unstressed conditions may be to maintain phospholipid asymmetry in the plasma membrane. Furthermore, the function of RTA3 in conferring tolerance to another broad-spectrum antifungal, miltefosine, highlights the physiological relevance of Tac1-regulated genes in defending *Candida* cells against xenobiotics present in the environment. The fact that Rta3 is unique to the fungal kingdom opens the possibility of interfering with the ability of *C. albicans* to thrive as a biofilm on indwelling devices in the human host. They also propose that the principal purpose of coregulation of Rta3 with drug transporters in *C. albicans* may be to enable this pathogen to efficiently switch to the highly drug-resistant biofilm mode of growth in the human host, upon perceiving a host-specific stimulus. As a consequence, this pathogenic fungus is empowered to resist the onslaught of antifungals in both planktonic as well as the biofilm modes of growth in the host environment. In view of the fact that biofilm-specific drugs do not exist for *C. albicans*, identification of Rta3 as a novel component that primarily regulates Bcr1-dependent adhesion may lead to new strategies to prevent biofilm formation in this human fungal pathogen. *C. albicans* cells with CDR1 deletion showed a significant increase in sensitivity to styrylquinolines in contrast to 8-hydroxyquinoline and 8-hydroxyquinaldine, suggesting that styrylquinolines are Cdr1p substrates. Styrylquinolines consist of two aromatic parts connected by an unsaturated ethylene linker that results in a flat rigid structure with relatively high lipophilicity. Such molecules are generally expected to penetrate cell membranes effectively;

moreover, they may interact with lipid structures such as ergosterol, indicating a possible mode of action as competitive inhibitors of this transporter (Szczepaniak et al. 2017). As Cdr1p has a higher impact on fluconazole resistance than Cdr2p, the strain without Cdr1p transporters is characterized by a lower fluconazole minimum inhibitory concentration (MIC) than strains that express Cdr1p transporters. However, the simultaneous presence of fluconazole and styrylquinolines abolishes the Cdr1p-induced resistance. It was confirmed that the cell membrane of *C. albicans* with treated with styrylquinolines was not disrupted. The treatment of *C. albicans* cells with ABC transporter substrates increases their expression. The Cdr1p-GFP protein level in crude extract was also higher in treated cells. Very similar results to wild-type strain (MICs) were obtained in cytotoxicity testing using normal human fibroblast cells, which suggests excluding the use of higher concentrations of styrylquinolines as antifungal drugs. The structures of styrylquinolines and their lipophilic properties predispose them to accumulate in cellular membranes. One of the mechanisms of action could be reducing the activity of Cdr1p and its relocation by disrupting the construction of the membrane. These findings are promising for designing novel antifungal agents. The identification of the dopamine G-protein-coupled receptor (GPCR) antagonists clozapine and fluspirilene as budded to hyphal transition (BHT) inhibitors was very interesting, especially given that clozapine and a number of its bioactive derivatives are FDA approved for the treatment of atypical schizophrenia (Toenjes et al. 2009). There are only three annotated GPCRs in *C. albicans*, with two being the STE2 and STE3 pheromone receptors. The remaining GPCR is Gpr1p, which has been implicated in a nutrient-regulated BHT signaling pathway upstream of PKA (Maidan et al. 2005a; Miwa et al. 2004). It remains to be determined whether clozapine and fluspirilene function through the Gpr1p GPCR. It is worth noting that the side effects of clozapine increase the disability of patients with schizophrenia and should be monitored regularly (Gürcan et al. 2017). Clozapine and olanzapine were consistently more strongly associated with metabolic adverse events than were other second-generation antipsychotic (SGAs) currently available (Hirsch et al. 2017). Vasudev et al. (2017) showed that about two thirds of the patients on clozapine were overweight or obese and had abnormal triglycerides and HDL cholesterol, with about half the patients having metabolic syndrome. The evidence-based report of the Canadian Agency for Drugs and Technologies in Health (CADTH) clearly indicated that high-dose or combination antipsychotic strategies are not known to be more effective and may be more harmful. Their study confirms that concurrent use of medications known to increase body mass index (BMI) significantly contributed to the increased BMI in patients on clozapine (Hirsch et al. 2017). It is now clear that Gpr1 is the founding member of a novel seventh class of GPCRs. Functional homologues seem to be present in most fungi with the *C. neoformans* Gpr4 receptor as a clear example (Van Dijck 2009). This receptor functions similarly to the *C. albicans* Gpr1 receptor, despite the absence of sequence homology. As this class of GPCRs is fungal specific and GPCRs form the largest groups of therapeutic targets, two important criteria for a good drug target are fulfilled (specificity and possibility to screen for drugs). In humans, histatins are produced by the submandibular and parotid glands and

secreted in saliva. They form part of the innate immune system and have an important role in maintaining oral health. Histatins are small, cationic, histidine-rich peptides of 3–4 kDa with potent antimicrobial activity. The most important members of the histatin family are histatin3 and histatin5 (32 and 24 amino acids, respectively). Histatin5 has potent activity against the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus.* Recent data seem to indicate that it would initiate an osmotic stress response by activation of the Hog1 pathway. Van Dijck (2009) showed that cell defense to histatin 5 is dependent on Gpr1: a homozygous gpr1 mutant is very sensitive to histatin 5. At higher concentrations of histatin 5, this strain no longer grows, whereas a heterozygous or wild-type strain still grows, which would indicate that a combination of histatin 5 and an antagonist of Gpr1 would be an interesting antifungal cocktail. Once the ligand is known, it may be important to identify the exact binding sites. Another *Candida* showing drug efflux pumps and multidrug efflux pump, *C. krusei* is an innately azole-resistant diploid fungal pathogen with a tendency to create aneuploid strains or triploid hybrids when exposed to environmental stress (see Knöppel et al. 2017). The experimental evidence indicates that, although all three genes are under very strong purifying selection, ABC1, ABC11, and ABC12 are not dosage-sensitive genes, and one gene copy appears to be enough for the survival of *C. krusei* (Lamping et al. 2017a). In *C. krusei*, ABC1 and ABC11 are encoding for drug efflux pumps, and a third close homolog, multidrug efflux pump *ABC12* was discovered (Lamping et al. 2017a). *ABC11* and *ABC1* orthologs are found in the closest sequenced relative, *Pichia membranifaciens* in the three *C. krusei* family members. Although *C. krusei*, also known as *Issatchenkia orientalis* or *Pichia kudriavzevii*, is a relatively poorly studied ascomycetous yeast, it is important for natural food fermentation. Overexpression of Abc1p in *S. cerevisiae* ADΔ caused multidrug resistance, is most frequently caused by active transporters, such as P-glycoprotein (ABCB-1), that pump a broad spectrum of chemically distinct molecules out of cells, which could be reversed with known efflux pump inhibitors. Strains overexpressing Abc1p or Abc11p were found to be resistant to a number of xenobiotics: resistant to azole antifungals (FLC, CLT, ITC, MCZ, and VRZ), fluorescent substrates (R6G and R123), large ionophores (NIG), translation inhibitors (CHX and ANI), and anticancer drugs (DOX and DAU). The discovery of a third *C. krusei* multidrug efflux transporter, *ABC12*, which had the same substrate specificities as Abc1p and Abc11p [the seven Abc1/11p: milbemycins α11; α26; β9; and β11; FK506 (FK); ENI; beauvericin (B) or OLI] were also Abc12p substrates. Although all three multidrug efflux pumps transported the same range of substrates, their transport activities for individual compounds differed significantly. Abc1p appeared to pump the selected test compounds best overall. However, Abc12p was the best transporter of the smaller test compounds [i.e., low molecular weight (MW); ANI, CHX, FLC, and CLT], and Abc11p was the best transporter of the larger (i.e., high MW) test compounds (R6G and NIG). Perhaps surprisingly, Abc11p of 89,102 (pump strain A), a chimera with two Abc1p-specific amino acids in TMS6 (patch III) and one in TMS11 (patch V), was an even better multidrug efflux pump (i.e., it was more resistant to most test compounds) than Abc1p. Additional variations in substrate specificities were

observed for the remaining *ABC11-1* and *ABC1-11* chimeras (pump strains B–E and F–G, respectively). Although all chimeras were clearly functional, some effluxed certain compounds even more efficiently than wild-type Abc11p or Abc1p (e.g., FLC efflux by Abc1-11p chimeras; pump strains F and G), whereas some Abc11-1p chimeras showed severely reduced drug efflux, especially of ANI, KTC, or NIG (pump strains B, C, and E). Differences were also observed in relative sensitivities conferred by Abc1p and Abc11p to known efflux pump inhibitors. Milbemycins are acaricides, insecticides, and anthelmintics widely used in agriculture and veterinary medicine. FK506 is a commonly used immunosuppressor, ENI and beauvericin are ionophoric depsipeptides with antibiotic and insecticidal activities, and OLI is an ATPase inhibitor. Abc1p was more sensitive than Abc11p to all eight efflux pump inhibitors tested, especially milbemycin β11, and, as expected, Abc11p of 89102 conferred inhibitor sensitivities that were between those for Abc1p and Abc11p of B2399 because it contains elements of Abc1p. The investigators have previously shown that the constitutive expression of Abc1p may tip the balance in favor of innate azole resistance for *C. krusei* (Lamping et al. 2009). In their report, Lamping et al. (2017a) showed that there may yet be other factors contributing to the innate azole resistance of *C. krusei*, namely, the expression of additional multidrug efflux transporters Abc11p and Abc12p. Lamping et al. (2009) described the isolation and characterization of Abc1p, a constitutively expressed multidrug efflux pump, and investigated *ERG11* and *ABC1* expression in *C. krusei*. The examination of the *ERG11* promoter revealed a conserved azole responsive element that has been shown to be necessary for the transcription factor Upc2p mediated upregulation by azoles in related yeast functional overexpression of *ERG11* and *ABC1* in *S. cerevisiae* and conferred high levels of resistance to azoles and a range of unrelated Abc1p pump substrates, whereas small molecule inhibitors of Abc1p chemosensitized *C. krusei* to azole antifungals. It is worth noting to have a look at Wingard et al. (1991) in the context of drug resistance and prophylaxis precautions. They have previously noted an increasing prevalence of *C. krusei* colonization in their renowned center, although it remained only a sporadic systemic pathogen. The data in their report indicated the emergence of *C. krusei* during 1990 as the chief systemic pathogen in patients with marrow transplants, but not in patients with leukemia. Although they cannot exclude the possibility that there was a common source of introduction of these organisms, they believed that such an explanation is unlikely. The same kitchen did provide food to both the bone marrow transplantation and leukemia services; however, these wards had different rates of colonization and infection. The transplant recipients were cared for by three different teams of nurses and physicians. In contrast, the increased prevalence of *C. krusei* was associated with the use of prophylactic fluconazole. Recent case reports describe a similar inability of fluconazole to suppress *C. krusei* infection. Although susceptibility testing is in the early stages of development, the lack of in vitro activity of fluconazole against *C. krusei* supports these clinical findings. This lack of susceptibility was probably not the only reason for the emergence of *C. krusei*. This species has been noted to be at a disadvantage with respect to adherence, an important determinant of colonization. Unfortunately, the factors that lead to

colonization, tissue invasion, and systemic infection were poorly understood. It was concluded that although fluconazole prophylaxis was associated with a significant decline in the number of disseminated infections caused by *C. albicans* and *C. tropicalis*, and the emergence of a less virulent organism *C. krusei* as a systemic pathogen, they proposed that the suppression of more virulent *Candida* species can contribute to the emergence of other, less virulent *Candida* species that are not susceptible to fluconazole, such as *C. krusei*, as systemic pathogens. Although drug resistance is one of the human major concerns, unexpectedly the findings of Knöppel et al. (2017) showed that antibiotic resistance can evolve in bacteria in response to a novel selection pressure without any antibiotic exposure. The molecular mechanisms that cause drug resistance are naturally occurring in less susceptible species and are acquired in strains of susceptible organisms (Perlin et al. 2017). Another unexplored possibility is that resistance evolves coincidentally in response to other selective pressures. They showed that selection in the absence of antibiotics can coselect for decreased susceptibility to several antibiotics. Thus, genetic adaptation of bacteria to natural environments may drive resistance by generating a pool of resistance mutations on which selection could act to enrich resistant mutants when antibiotic exposure occurs. Prigitano et al. (2015) arrived at a similar conclusion.

Esquivel and White (2017) compared the filamentous fungus *Magnaporthe oryzae* to yeast species such as *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Candida glabrata*, and *Candida krusei*, and found it to contain an unusually high number of genes encoding predicted membrane transporters, including at least 50 ATP-binding cassettes (ABC) and at least 250 major facilitator superfamily (MFS) transporters (Kim et al. 2013). The majority of these transporter genes are still uncharacterized to date. Most filamentous fungal species uncharacterized to date and molds are intrinsically resistant to fluconazole (FLC) because they possess multiple targets of azoles: one is CYP51, an enzyme localized on the endoplasmic reticulum (ER), and required for fungal ergosterol biosynthesis. Most filamentous fungal species and molds are intrinsically resistant to FLC because they possess multiple target CYP51 paralogs: two in *M. oryzae*, *A. fumigatus*, *and A. nidulans*, and three in *A. flavus* and species of *Fusarium.* This CYP51 redundancy allows for slight changes to occur in the active site of one or all Cyp51 copies that affect the binding affinity to azoles. The structure of FLC in particular allows multiple Cyp51 active site-binding conformations that are weak or transitory so that there is incomplete inhibition of the Cyp51 target enzyme compared to other azoles that have stronger Cyp51 binding affinity. Regardless, the E-tests confirmed that even azole drugs used to treat human fungal pathogens are taken up by the plant pathogen *M. oryzae* as evidenced by growth inhibition seen with the medical azoles ITC, KTC, POS, and VRC. Overall, the susceptibility and resistance patterns illustrated by the E-tests suggest a common mechanism of action of azoles on all fungal species, including the requirement for entry into the cell, passing through the cell wall and plasma membrane. This report may be the first of the minimum inhibitory concentration (MICs) of medical antifungals on a distinctly plant-based pathogen. A common mechanism of action of azoles on all fungal species is that it must enter the fungal cell through the cell wall and plasma membrane to inhibit the

intracellular Cyp51 target enzyme. Therefore, reduced or modified drug imports may help to explain why some pathogenic fungi are more resistant to azoles than others. Their assay can be used to compare drug import in agricultural, medical, and other pathogenic fungi. Experiments thus far have demonstrated that azole entry into the fungal cell is not solely by a passive diffusion mechanism. There may be some baseline level of azole passive diffusion into the cell, but their evidence suggests azole import into *M. oryzae* is more substantially through a plasma membrane-localized protein channel or carrier that recognizes a specific moiety found in azole drugs. This finding is in agreement with studies on the human pathogenic fungi *C. albicans*, *C. neoformans*, *and A. fumigatus* as well as the model yeast *S. cerevisiae* (Esquivel et al. 2015). Similarly, there may be a certain amount of azole import from uncharacterized ATP-dependent importers that was masked by the high activity of ATP-dependent efflux transporters. Import of azoles did not require a proton gradient, as no change was observed in uptake over a range of buffered pHs. There was a trend toward alkaline sensitivity for drug uptake as seen by a decrease in labeled FLC (3H-FLC) uptake in samples in pH 7 media. However, a deficiency in cell growth was observed in *M. oryzae* cells at pH 7, so import at this pH may be affected by other cellular factors directly or indirectly related to pH and proton gradients. They did find significant differences in drug accumulation in *M. oryzae* depending on the growth media used. Environmental adaptations that prevent or enhance azole uptake are an important aspect for drug resistance and treatment analysis, and it is difficult to identify a single factor that would cause such a dramatic difference in uptake between the different medias used. Cell adaptation to the different media that affect FLC uptake is probably a complex mixture of differences in protein synthesis, lipid storage, transcriptional activity, and other metabolic activities that may alter the cell membrane composition. The altered azole uptake between the samples argues against passive diffusion entry into the cell, in which case one would expect only minimal reduction or increase in drug accumulation between the samples. The evidence indicates that the efflux of azoles is stimulated by energy, potentially via ABC efflux transporters, which suggests there are distinct transporters for influx and efflux of azoles, as opposed to a single transporter that functions in both directions. Resistance to commonly used antifungals has frequently been shown to develop as a result of overexpression or increased activity of ABC transporters in human fungal pathogens (Sanglard 2016). More recently, ABC transporters are being recognized for their role in pathogenicity, virulence, stress tolerance, and drug resistance in *M. oryzae* and other plant pathogens.

Nutrient receptors could represent a potentially rich source of targets for antifungal drug development. About 40% of all available drugs target GPCRs, making these receptors the most important drug target group. Transceptors are often important for fungal development and virulence, and hence could be an excellent drug target group as well. Indeed, receptors that sense different stress responses have been proposed as drug targets. Current antifungal therapies are limited to drugs such as amphotericins, azoles, and echinocandins that inhibit the growth of *C. albicans* cells rather than specific virulence processes. Unfortunately, these drugs can have inhibitory effects on human cells, leading to serious side effects for the host. The

problem of drug resistance should be manipulated with innovative and unconventional approaches and strategies at the population levels. McCarthy et al. (2017) reviewed the challenges associated with identifying broad-spectrum antifungal drugs and highlighted novel targets that could enhance the armamentarium of agents available to treat drug-resistant invasive fungal infections. Unfortunately, none of the novel targets that they proposed was using GPCR and ligand antagonists in fugal therapy. The emergence of antifungal resistance is a growing global health problem, underscoring the need for new therapeutic options. Significant progress has been made in the identification of potential targets for novel agents that exploit inherent differences between fungi and humans. Many of these targets remain theoretical, but several molecules have recently entered early clinical development. Complementing these therapies are advances in innate host defense: a pioneering clinical study using adoptive transfer of *Aspergillus*-stimulated T cells showed significant efficacy in treating stem cell transplant recipients with invasive aspergillosis. Other investigators have generated T-cytotoxic cells expressing Dectin-1 chimeric antigen receptors (CARs) that recognize surface fungal glucans. Infusion of D-CAR+ T cells reduced both fungal burden and mortality. Beyond small molecules, new approaches for augmenting host responses are critical and include the development of vaccines, immunomodulators, and novel approaches for reducing immunosuppression and exploiting the immunopharmacology of existing antifungal agents. Lundstrom (2017) stated, in his article "G-protein-coupled receptor-based drugs rediscovered," that it seems the drug development process has become more complex, longer in duration, and more expensive. Today, generating a commercially available drug requires synthesis of 10,000 to 30,000 compounds, extensive fine tuning, and thorough testing before being approved. The efficacy requirements and the safety standards have reached new levels.

The author pinpointed that GPCR–ligand antagonists have not yet been adopted as a plan for producing antifungal drugs, at the industrial level, after surveying many scientific publication to date; to his best knowledge, perhaps such work is still under scrutinized scientific research, whether academic or in pharmaceutical factories. The author would like also to take the advantage of writing this chapter about GPCRs to launch a novel and original idea. As population and quantitative geneticists we can statistically partition the phenotypic variance to its components: additive, dominance, epistatic, and environmental. The problem that we face is the genotype × environment interaction. I believe that using marker-assisted selection and the marker in such cases will be the highly responsive GPCR that represent(s) the interface between the cell or organism and its surrounding environment and will leads us to stability in performance, which will be a new approach in breeding. We all know that traits for which breeders have never selected have a great amount of potential encrypted variance, which means that it provides a rich row material for high selection response. Such an idea was handled in another way by Botella (2012), who showed that a recent report identified two important yield quantitative trait loci (QTLs), GS3 and DEP1, cloned in rice (*Oryza sativa*), that were found to be heterotrimeric G-protein γ-subunits. This identification has profound consequences on our current understanding of both QTLs and the plant G-protein signaling network.

Botella discussed how the manipulation of G-protein signaling may lead to yield improvements in rice and other crop species.

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Chapter 4
Prompt and Convenient Preparation of Oral Vaccines Using Yeast Cell Surface Display

Seiji Shibasaki, Miki Karasaki, Wataru Aoki, and Mitsuyoshi Ueda

4.1 Introduction

4.1.1 Molecular Display

Microbial biotechnology has been applied to the production of proteins, lipids, saccharides, or other bioactive compounds for humans owing to advances in genetic engineering technologies (Shibasaki et al. 2008a, b). Recently, there have been drastic developments in genetic engineering techniques focusing on the cell surface as a site of protein production. These technologies, collectively referred to as "cell surface engineering (arming technology)" or "molecular display," have contributed to combinatorial engineering strategies that can produce novel biomolecules or cells with unique properties and broad industrial potential (Shibasaki and Ueda 2010, 2014; Shibasaki et al. 2003).

Among the cell surface engineering strategies reported to date using different kinds of microorganisms, a molecular display system using yeast, especially *Saccharomyces cerevisiae*, has been developed and extensively investigated (Ueda 2016). In brief, a molecular display system using *S. cerevisiae* is based on the construction of genetic fusions to connect a foreign protein to one of its own cell wall proteins. The cell wall of *S. cerevisiae* contains polysaccharides such as β-glucan and chitin, along with proteins that bind to several other proteins (Fig. 4.1) (Shibasaki et al. 2008a). α-Agglutinin is one of the most convenient and simple anchoring proteins in this system, although Cwp1, Flo1, and other cell wall proteins can also be

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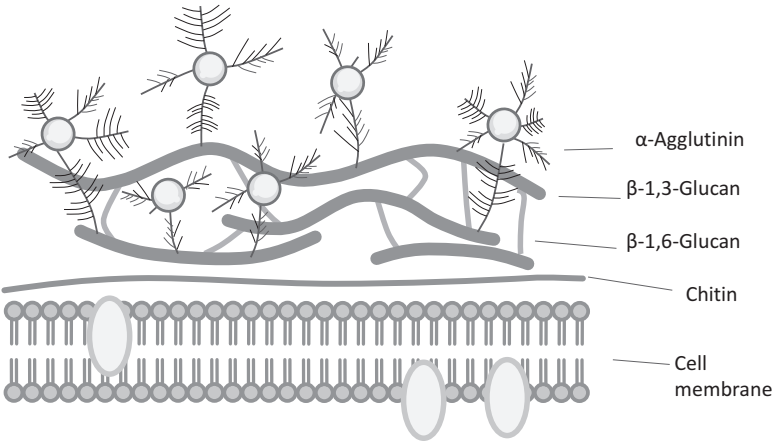

Fig. 4.1 Structure of the yeast cell surface

used for a yeast molecular display system. The gene fusion construct results in the production of a hybrid protein on the cell surface that is exposed to the exterior of the cell (Fig. 4.2). A yeast cell displaying such a foreign protein on its surface has been termed "arming yeast" (Shibasaki et al. 2009; Shibasaki and Ueda 2009).

4.1.2 Biomedical Application of a Molecular Display Yeast System

Several researchers have studied and developed molecular display systems for biomedical applications. For instance, antibody display systems were created by the combination of the antibody-binding protein Staphylococcal protein A. The Z-domain generated from the B-domain of protein A has been used as a scaffold for the purification of immunoglobulin (Ig) molecules because it is able to specifically bind to the Fc portion of Ig (Shibasaki et al. 2006). Binding of IgG onto the surface of the yeast cell via the displayed Z-domain was demonstrated and was suggested to serve as a useful tool in enzyme-like immunoassays (Nakamura et al. 2001). This Z-domain-displaying yeast has also been investigated as a potential purification tool for the convenient production of proteins in a medium. The Z-domain could efficiently recover the secreted Fc-fusion protein in cultivation medium on the yeast cell surface, which could then be quickly purified (Shibasaki et al. 2007). As another example of an IgG display system, Lin et al. (2004) produced and evaluated catalytic antibodies on the yeast cell surface. Moreover, the yeast display system has been gradually recognized as a candidate therapeutic antibody screening system (Doerner et al. 2014). In addition, an artificial antibody such as "Affibody"

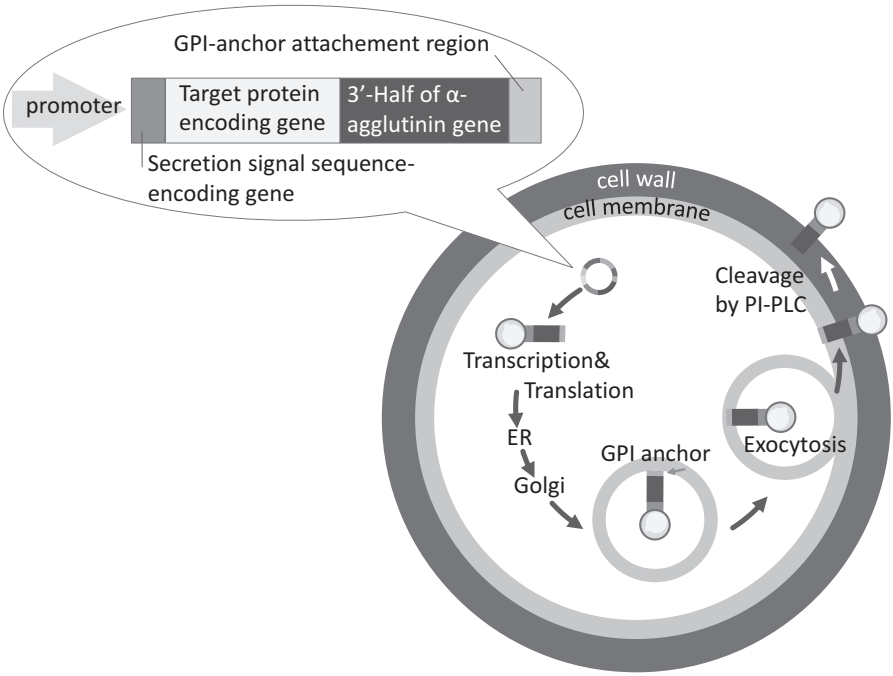

Fig. 4.2 Genetic construction of molecular display on the cell surface of *S. cerevisiae*

molecule represents an attractive target molecule to be displayed in the next generation of yeast display systems (Ståhl et al. 2017, Shibasaki et al. 2014b).

This same system has also been applied as a hormone receptor. The ligand-binding region of estrogen receptor was displayed on the yeast cell surface, which could be successfully used to detect the concentration of estrogen and estrogen-like compounds (Yasui et al. 2002). Furthermore, G-protein-coupled receptor (GPCR), somatostatin receptor, and glucagon-like peptide-1 receptor were effectively displayed on the yeast cell surface in their active forms. This GPCRs display system was demonstrated to serve as a useful method for screening novel ligand or agonists (Hara et al. 2012; Shigemori et al. 2015).

Thus, molecular display technology has been developed for a variety of biomedical applications. Wide adoption of these arming yeasts is expected to facilitate the efficient discovery of novel bioactive compounds or pharmaceutical antibodies. The next stage of this field is focused on applying molecular display technology in which the entire arming yeast cell is used as the therapeutic agent itself beyond a screening tool for drug discovery.

4.2 Molecular Display System as a Convenient Tool for Vaccine Development

4.2.1 Candidiasis

Infectious diseases have been a constant source of suffering throughout human history. Fungal diseases are among the most ubiquitous infectious diseases, affecting people worldwide. Although most fungi are not harmful to humans, several species can pose a serious threat to human health.

Candidiasis is an infectious disease that is mainly caused by the fungus *Candida albicans* as well as some other *Candida* species (Bassetti et al. 2016). Several types of candidiasis occur when the host immunity is compromised by AIDS, cancer treatment using chemicals, or the administration of immunosuppressants for organ transplantation. The current treatment options for candidiasis are limited and unsatisfactory, mainly because of delayed diagnosis and the lack of reliable methods to detect *Candida* inside the body of a patient (Noble and Johnson 2007; Arendrup 2013).

Pharmacotherapy of candidiasis often involves the administration of caspofungin, micafungin, anidulafungin, and amphotericin B. However, mutants of *Candida* with reduced susceptibility to these drugs have recently emerged (Arendrup and Patterson 2017). In addition, the undesirable side effects of chemical drugs against *Candida* species represent a severe clinical problem (Tsuda et al. 2011). Therefore, infection prevention via vaccination against *Candida* species is expected to be a complementary approach to pharmacotherapy in combating candidiasis. The yeast molecular display system has been applied toward this goal as a convenient method to prepare an antigen that can be used for candidiasis vaccination (Shibasaki et al. 2013).

4.2.2 Display and Evaluation of Antigens

Administration of the glycolytic enzyme enolase 1 protein (Eno1p), one of moonlighting proteins (Jeffery 1999), of *C. albicans* to mice showed protection against infection with *C. albicans* (Xin et al. 2008; Laín et al. 2007); therefore, Eno1p was chosen in a trial for the display of antigens against candidiasis in vaccine development. If the arming yeast displaying Eno1p could induce an immune response against *C. albicans*, it could be developed into an oral vaccine that could be administered widely in a noninvasive manner (Fig. 4.3).

In the development of an Eno1p-displaying yeast system, the *ENO1*gene was amplified and introduced into a plasmid vector for molecular display (Shibasaki et al. 2001). This plasmid included the gene encoding, the cell surface anchoring protein α-agglutinin, and a multi-cloning site for introducing any gene encoding the foreign protein or peptides to be displayed. The surface display of Eno1p was confirmed using fluorescent microscopy after staining of the fluorescent-labeled

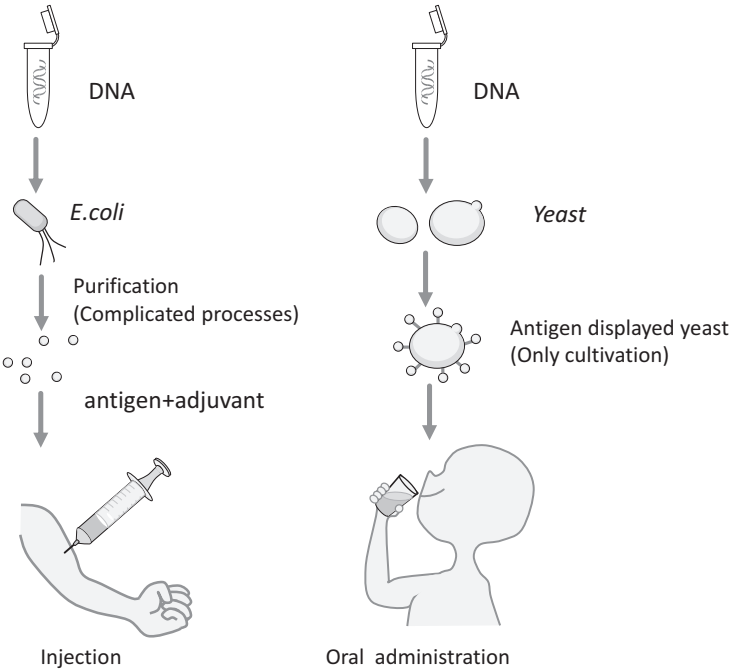

Fig. 4.3 Concept of an oral vaccine produced by a molecular display system

antibody (Fig. 4.4). In general, the vector used in a yeast cell surface display system contains a peptide tag sequence for the rapid detection of gene expression so that the displayed protein can be detected by the combination of an antibody against this tag (primary antibody) and an antibody labeled with a fluorescent reagent (secondary antibody). This Eno1p-displaying yeast cell was then orally administered to mice. At the ninth week after administration of the arming yeast, an increase in the IgG titer in the serum was confirmed (Fig. 4.5). A lethal dose of *C. albicans* was then injected into the Eno1p-displaying yeast-administered mice. At the fifth week after *C. albicans* challenge, 60% of the Eno1p-displaying yeast-administered mice were alive, whereas all of the mice given the control yeast died of the infection (Fig. 4.6).

To compare the performance and utility of the yeast display system with those of other types of display systems, a *Lactobacillus* display system was applied for Eno1p display. The *Lactobacillus* display system has also been well studied for the display of various proteins, including antigenic proteins (Adachi et al. 2010). Oral administration and evaluation were conducted in the same manner as described for the yeast display system. Over the same time period, only 10% of the mice treated with Eno1p-displaying cells were alive when using the *Lactobacillus*system, despite adjusting for the same protein amounts administered with both systems. The most likely reason for this difference in survival using the yeast and *Lactobacillus* systems is the adjuvant effect of β-glucan in the yeast cell (Rodríguez et al. 2009).

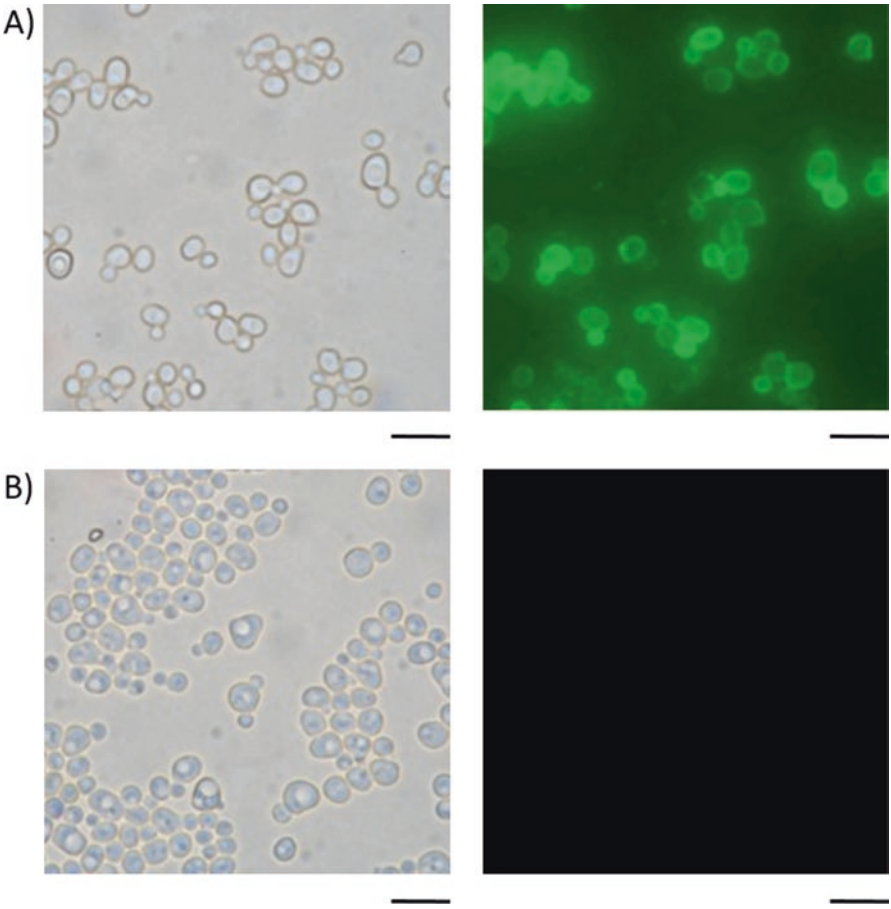

Fig. 4.4 Confirmation of molecular display on the yeast cell surface with an immunofluorescent micrograph. (**A**) Eno1p-displaying cell, (**B**) control yeast cell. Left, light field micrographs; right, immunofluorescent micrographs. Bars = 5 μm

4.2.3 Proteomic Analysis to Find an Optimal Display Antigen

An appropriate protein for vaccine development is one that is not only a suitable target but also exerts high immunogenicity and a good therapeutic effect. Thus, with the goal of finding a better antigenic protein against *C. albicans*, proteomic analysis was performed. Virulence-related proteins in *C. albicans* were comprehensively screened using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system equipped with a long monolithic column (Aoki et al. 2013). Because candidiasis is accompanied by severe symptoms once *C. albicans* gets into the bloodstream, comprehensive screening of proteins induced by serum was expected to reveal the most suitable antigens for vaccine development. Based on this concept,

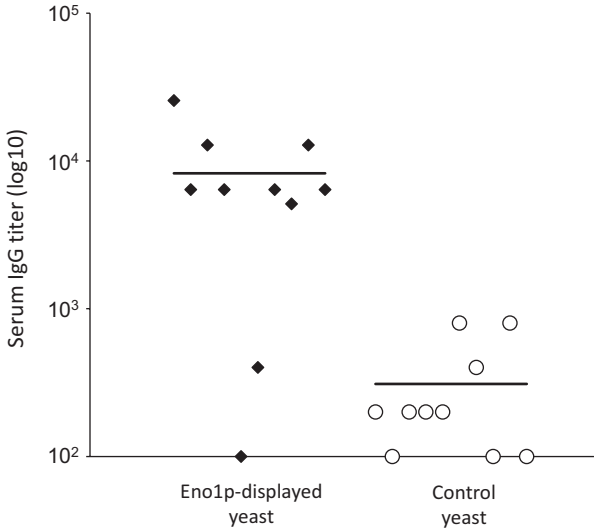

Fig. 4.5 Antibody response after yeast cell administration. Circles, serum of mice after administration of Eno1p-displaying yeast; diamonds, serum of mice after administration of control yeast

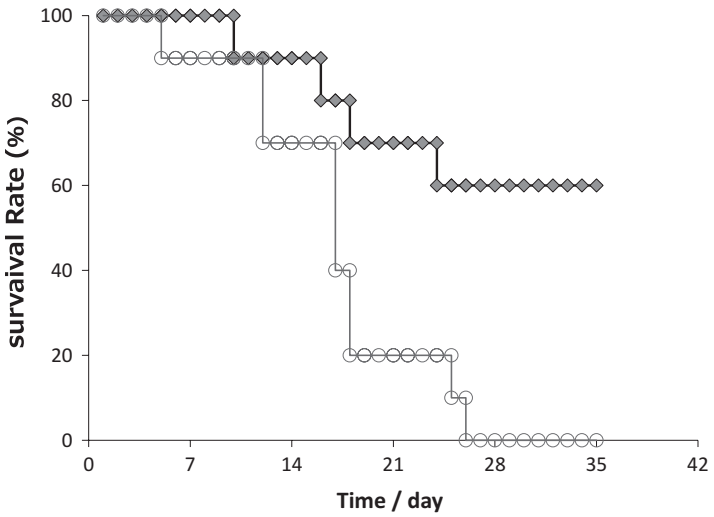

Fig. 4.6 Survival rates of mice administered an oral vaccine developed with a yeast molecular display system. Circles, administration of yeast (host cell); diamonds, administration of yeast displaying Eno1p

C. albicans cells were incubated in yeast peptone dextrose medium (YPD series) or YPD + fetal bovine serum (FBS series). Proteins extracted from these cells were then evaluated by LC-MS/MS, and a total of 1024 proteins were identified and quantified (Shibasaki et al. 2014c). Among these proteins, 28 were specifically

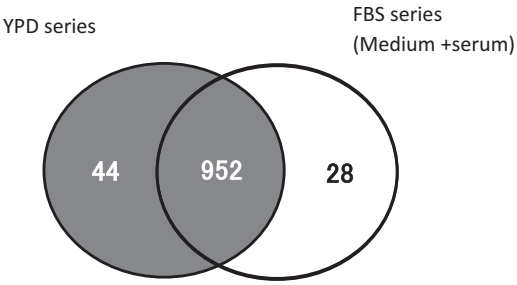

Fig. 4.7 Summary of proteomic analyses of *C. albicans* in serum. A total of 1024 proteins were identified, including 44 YPD-specific and 28 FBS-specific proteins

found in the FBS series (Fig. 4.7), including Mdh1, which showed upregulated expression in this serum-existing condition. Thus, the *MDH1* gene was cloned, and the purified Mdh1 protein was prepared. To validate its performance for vaccine development, Mdh1p was administered to mice via a subcutaneous or intranasal route. Five weeks after challenging the mice with a lethal dose of *C. albicans*, the Mdh1p-administered mice showed a 100% survival rate, whereas the mice administered Eno1p (as a control) in the same manner showed only a 10% survival rate (Shibasaki et al. 2014a). Thus, Mdh1p is considered to be a powerful candidate protein to be displayed for oral vaccine development. This investigation suggests that both immunogenicity and virulence should be considered when screening for an appropriate antigen with proteomic analyses.

4.3 Conclusion

S. cerevisiae is generally recognized as safe organism and is thus suitable for the preparation of oral vaccines using a molecular display system without the need for antigen purification or an adjuvant (Shibasaki and Ueda 2016). The yeast molecular display system shows great potential as a convenient tool to protect against various infectious diseases in a noninvasive manner. Combining the molecular display system with proteomic analyses (Shibasaki et al. 2016; Kitahara et al. 2015) can provide a powerful approach for creating effective antigen-displaying cells in the next generation of vaccine development (Shibasaki and Ueda 2017).

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Chapter 5
Trichoderma, a Factory of Multipurpose Enzymes: Cloning of Enzymatic Genes

Roshan Lal Gautam and Ram Naraian

5.1 Introduction

Trichoderma is a most prevalent, industrially culturable, and well-studied fungal genus belonging to the family Hypocreaceae, which currently comprises more than 200 genetically defined species (Atanasova et al. 2013). *Trichoderma* is a filamentous fungus that is able to produce and secrete a wide range of extracellular hydrolytic enzymes used for plant cell wall degradation (Bech et al. 2014) and thus is considered as a factory of multipurpose enzymes. The genus *Trichoderma* contains filamentous Ascomycetes that are widely used in industrial applications because of their high secretory capacity and inducible promoting characteristics (Mach and Zeilinger 2003). In biocontrol, *Trichoderma* genes can confer beneficial features to plants, mainly in the control of plant diseases, through being functionally expressed in plants (Lorito et al. 1998; Hermosa et al. 2012). Enzyme-synthesizing genes have a major part in the biocontrol process by regulating some signals and lead to the secretion of some enzymes or proteins that aid the destruction of pathogens (Sharma et al. 2011). *Trichoderma* species are the best choice because of their greater significance in the production of industrial enzymes and recombinant proteins (Kubicek and Penttila 1998; Penttila 1998).

Genomic studies reveal that *Trichoderma* sp. contains many valuable genes with a great variety of expression patterns, which allows these fungi to be used in many applications such as biocontrol agents, plant growth promoters, and environmental adaptation against extreme conditions, such as drought, salt, and heavy metal tolerance (Singh et al. 2018). Fungi of this genus also have a potential to degrade a wide range of the lignocellulosic biomass, including recalcitrant and highly toxic phenolic compounds (Saili et al. 2014). This fungus is known to inhibit the growth of

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other fungal pathogens by synthesizing antifungal antibiotics and cell wall-degrading enzymes including chitinases, proteases (Kumar et al. 2009), cellulase, amylase, lipase, and pectinase (Bhale and Rajkonda 2012). The ligninolytic system of *Trichoderma* has an extracellular enzymatic complex that includes manganese peroxidase and a copper-containing phenoloxidase known as laccase (Ruiz-Duenas and Martinez 2009). The versatility of enzyme production has led to the cloning of genes; in this reference, genes encoding chitinase and glucanase from *Trichoderma atroviride* have been cloned and expressed in rice, which showed increased resistance to blast disease (Liu et al. 1994). In the present chapter we have focused on the specific potentials of *Trichoderma* under different aspects including enzymes produced by *Trichoderma*, ample multitask applications of enzymes, and finally the cloning of genes in substrate hosts.

5.2 *Trichoderma*

The *Trichoderma* genus is a group of cosmopolitan fungi, frequently present in all types of soils, manure, and decaying plant tissues (Alexander 1961). This particular versatile fungal genus *Trichoderma* belongs to the Deuteromycotina, and they are probably anamorphic species belonging to the ascomycetous order Hypocreales (Alexopoulos and Mims 1979). Approximately 200 *Trichoderma* species optimally grow in various ecosystems (Hermosa et al. 2012), such as terrestrial and forests. The most versatile species of the genus *Trichoderma* include *T. harzianum, T. viride, T. atroviride, T. asperellum, T. virens, T. longibrachiatum, T. hamatum, T. pseudokoningii, T. reesei, T. arundinaceum, T.* koningiopsis, *T. cremeum, T. longipile, T. citrinoviride, T. spirale, T. crissum, T. ovalisporum, T. koningii, T. polysporum, T. saturnisporum,* and *T. gamsii*. Members of the *Trichoderma* genus are generally found in natural habitats such as on plant root surfaces where they control plant root diseases (Hasan et al. 2014). *Trichoderma* spp. have an excellent biocontrol and growth-promoting potential for many crop plants (Verma et al. 2007; Bai et al. 2008; Savazzini et al. 2009). *Trichoderma* spp. have the ability to directly influence the growth of bacterial and fungal pathogens in the rhizosphere through several pathways (Leelavathi et al. 2014), mainly by the secretion of enzymes toxic to the pathogens (Benitez et al. 2004). Therefore, species of *Trichoderma* are considered to be the most successful biofungicides in agricultural use, and because of this more than 60% of the industrial biofungicides worldwide are prepared with *Trichoderma*-based formulations (Verma et al. 2007). Promising *Trichoderma* isolates work with different mechanisms of action of direct parasitism, such as competition for nutrients, stimulation of plant health, and inducers of plant systemic resistance against various pathogens (Harman et al. 2004a; Anees et al. 2010; Woo et al. 2014; Jain et al. 2015; Rai et al. 2016). At present several *Trichoderma*-based formulations are commercially available in the market with various names. A biofungicide formulated with *T. harzianum*, named Trichodex, is much used to control soil-borne and

phyloplane pathogens. In addition, several other formulations, such as Binab-T, Glio-gard, and Root-shield, are also used systematically to control different soil-borne pathogens, which severally cause damping-off and root rot in growing plants (Gashe 1988; Samuels 1996). Moreover, species of *Trichoderma* are well known to produce a large number of antibiotics, such as trichodermin, trichodermol, harzianum A, and harzianolide (Claydon et al. 1991; Dickinson et al. 1995).

5.3 *Trichoderma:* A Factory of Enzymes

Species of *Trichoderma* very efficiently produce a large number of industrially important extracellular enzymes, many of which are experimentally utilized in several fields of biotechnology (Kunamneni et al. 2014). With the characteristic of very rapid growth, fungal cell factories produce about 40–50% of the value of industrial enzymes (Paloheimo et al. 2016). In this reference, *Trichoderma* are known to be highly efficient producers of numerous extracellular enzymes (Bhale and Rajkonda 2012) including cellulase, pectinase, chitinase, protease, lipase, and amylase. These enzymes produced by *Trichoderma* can degrade plant cell walls; being capable of attacking each of the major polymeric components of cell walls; these are characterized as cellulolytic, hemicellulolytic, pectolytic, or proteolytic (Wheeler 1975). *Trichoderma* currently occupies the position of "fungal cell factories" through having produced industrially important enzymes during the past 40 years (Tolan and Foody 1999; Merino and Cherry 2007).

5.3.1 Pectinase

Pectinase (E.C. 3.2.1.15) is known as a collective name of enzymes that break down pectin, a cementing polysaccharide material found in plant cell walls. The group of enzymes that constitute pectinases are pectin methylesterases, polygalacturonase, pectin lyases, pectate lyases (Djordjevic et al. 1986; Pifferi et al. 1989), and pectozyme. Therefore, pectinase constitutes a complex enzymatic system catalytically responsible for the breakdown of pectic substances (Ahamed et al. 2003) that include protopectin, pectic acid, pectinic acid, and pectin (Kashyap et al. 2001). Several species of *Trichoderma* produce pectin-degrading enzymes (Haltmeier et al. 1983) that are useful in many industries for the extraction, clarification, and liquefaction of fruit juices (Nazia et al. 2003). In addition, pectic enzymes have been shown to have a significant role in plant pathogenesis and microbial decomposition of plant materials (Rombouts and Pilnik 1980). Pectinases are helpful for improving the viscosity of fruit juice drops and the pressing ability of the pulp wherein the jelly structure disintegrates and the volume of fruit juice is easily obtained with higher yields (Tapre and Jain 2014).

5.3.2 Cellulase

Cellulase (E.C. 3.2.1.4), one of the most important and highly demanded cellulolytic enzymes in the global enzyme market, is produced by fungi growing saprobiotically on complex lignocellulosic material (Kalsoom et al. 2019). Cellulase is an enzyme complex that acts synergistically to decompose organic cellulose and which includes endoglucanases (1,4-β-D glucanglucanohidrolase), exoglucanases (exo-1,4-β-D-glucan cellobiohydrolase), and β-D glucosidases (Lee et al. 2008). The endoglucanase randomly breaks the internal O-glycosidic bonds and consequently forms glucan chains of variable lengths; the β-glycosidases act specifically on the β-cellobiose disaccharides and liberate glucose (Bayer et al. 1994; Singh 1999). Exoglucanase acts as an exoenzyme on crystalline cellulose and releases cellobiose as a main product (Ilmen et al. 1997). Cellulases are composed of independently folding, structurally, and functionally discrete units called domains (Henrissat et al. 1998). The catalytic domains of cellulases have been categorized into several families based on the variable amino acid sequences and crystalline structures (Henrissat 1991). In view of their function, the enzymatic hydrolysis of cellulosic materials is correlated with the level of cellulose crystallinity (Weimer and Weston 1985). Mycoparasitism assists *Trichoderma* sp. to antagonize plant pathogens through nutrient competition, secretion of extracellular hydrolytic enzymes, and antifungal secondary metabolites such as diketopiperazines, sesquiterpenes, polyketides, and peptaibols (Saxena et al. 2015; Oda et al. 2015).

5.3.3 Xylanase

Xylanases (E.C. 3.2.1.8) are hemicellulolytic enzymes that degrade xylan, a hemicellulose. The xylans are a complex group of polysaccharides with a backbone of β-(1-4)-linked xylose units (Scheller and Ulvskov 2010). Xylanases are responsible for the hydrolysis of xylan; first, they attack the internal chain linkages, subsequently releasing xylosyl residues by endwise attack of the xylooligosaccharides (Mohamed et al. 2013). β-1,4-Endoxylanase hydrolyzes the β-1,4-glycosidic bonds in xylan chains via a double displacement mechanism in a random manner (Jeffries 1996). Fungi are widely used as the major source of xylanases, and their xylanolytic systems have been well studied (Polizeli et al. 2005; Van den Brink and de Vries 2011). The filamentous fungus *Trichoderma reesei* produces extracellular xylanase that digests xylan into its simple monomers (Lopez et al. 2011). Xylanases produced by *Trichoderma reesei* Rut C-30 were found to be highly effective in the hydrolysis of ammonia-treated dwarf elephant grass (Colina et al. 2003). In one study, xylanase production using *Trichoderma inhamatum* showed the highest values of activity with different agro-industrial wastes such as wheat bran, corn cob, oat bran, and sugar cane bagasse, respectively (de Oliveira da Silva and Carmona 2008). Therefore, the fungal genus *Trichoderma* is one of the most recognized sources of industrial hydrolytic enzymes (Xiong et al. 2005).

5.3.4 Chitinase

Chitinases (E.C. 3.2.1.14) are a heterogeneous group of enzymes that break the glycosidic bonds of chitin by catalyzing hydrolytic reactions (Nicol 1998). Chitinases are classified into the hydrolyzing enzyme families 18 and 19 of glycosyl hydrolases (Bhattacharya et al. 2007) based on the characteristics of N-terminal sequence, enzyme localization, isoelectric pH, signal peptides, and the inducers (Kumar et al. 2018). Chitin is a biopolymer of *N*-acetyl-D-glucosamine (Gooday 1991) present in the inner layer of the fungal cell wall that inhibits the germination and growth of fungal hyphae (Lorito et al. 1998). Chitinases are a successful and effective tool for absolute degradation of the mycelia or conidial walls of phytopathogenic fungi (De la Cruz et al. 1995). *Trichoderma* chitinase has a major function in cell wall degradation because most phytopathogens possess chitin as a cell wall constituent (Smitha et al. 2014). A dual role of *Trichoderma* chitinase is recognized: inhibiting fungal growth through cell wall digestion and releasing pathogen-borne elicitors that induce further defense reactions in the host (Dana et al. 2006). In one study it was found that the chitinase from the *T. harzianum* isolate 1051 remarkably affected the cell wall of *Moniliophthora (Crinipellis) perniciosa* (De Marco et al. 2000).Therefore, chitinase-producing organisms combined with β-glucanases or cellulases establish an agriculturally effective biocontrol system against both phytopathogenic and soil-borne fungi (Irene et al. 1994) as a biological fungicide. Chitinases are highly produced by fungi, yeasts, bacteria, plants, and in insects (Huang et al. 2005). *Trichoderma* is the most potent filamentous fungus, producing vast amounts of chitinase with good activity. Extracellular chitinase produced by *Trichoderma virens* is important with respect to growth rate, sporulation, antibiotic production, and colonization efficiency on cotton roots and growth in soil (Baek et al. 1999).

5.3.5 Lipase

Lipases (triacylglycerol lipases; E.C. 3.1.1.3) are enzymes with the biological function of catalyzing the hydrolysis of fats and oils, converting them into fatty acids and glycerol (Kim et al. 2002; Ohnishi et al. 1994). The natural substrates of lipase are triacylglycerols, which have a very low water solubility (Ghosh et al. 1996). The lipase enzymes are found naturally in plants, animals, yeast, fungi, and bacteria (Boonmahome and Mongkolthanaruk 2013). However, filamentous fungi are believed to be the best source of extracellular lipase that can be exploited for their mass production at a large scale (Sharma et al. 2016). Lipases have important roles of lipid metabolism in eukaryotes such as fat digestion, reconstitution, and adsorption and also lipoprotein metabolism (Kumar and Ray 2014). Lipase from other *T. harzianum* strains (Rajesh et al. 2010) has demonstrated favorable kinetics for its industrial uses such as biodiesel production and environmental applications

(Toscano et al. 2013). In view of their potential application, lipases are well-known industrial enzymes that have the third highest demand, after protease and amylases, in the global market (Ulker et al. 2011).

5.3.6 Protease

Proteases (E.C. 3.4.21.112) constitute a large group of enzymes that catalyze the hydrolysis of peptide bonds in the polypeptide chains of proteins (de Souza et al. 2015). Proteases are also called peptidase or proteinase, defining the proteolytic function (Hedstrom 2002). On the basis of catalytic function, proteases are classified into two major groups: exopeptidases and endopeptidases. Proteases that break peptide bonds at either the N- or C-terminus of polypeptide chains are known as exopeptidases and those which cleave peptide bonds within the polypeptide chain are categorized as endopeptidases (Lopez-Otin and Bond 2008). A broad group of microorganisms such as bacteria, fungi, yeast, and actinomycetes are known to synthesize these enzymes (Madan et al. 2002). Fungal proteases have attracted much attention by researchers because fungi can grow on substrates of very low cost and can secrete large amounts of enzymes (Anitha and Palanivelu 2013). Proteases have remarkable roles in the interaction of *Trichoderma* with fungal phytopathogens (Fan et al. 2014), with a critical role in mycoparasitism (Dou et al. 2014). The potential of *Trichoderma harzianum* protease to digest the cell wall of *Moniliophthora (Crinipellis) perniciosa* indicates that this is involved in the antagonistic phenomenon occurring between two fungi (De Marco and Felix 2002). *Trichoderma harzianum* and other saprotrophic fungi primarily secrete only subtilisin-like proteinases (Chandrasekaran and Sathiyabama 2014). Proteases from fungi variably have either a nutritive role or specific roles in cell metabolism or in pathogenicity, often as virulence factors (Raju et al. 1994; Haq et al. 2006). The protease enzymes also break hydrolytic enzymes into peptide chains and further into amino acids, thereby destroying their ability to act on plant cells (Hasan et al. 2014). These enzymes differ widely in several properties such as substrate specificity, active site, and catalytic mechanism, and work differentially under mechanical stress, chemical environment, pH, and temperature for stability and activity (Nirmal et al. 2011). Fungal proteases are exploited for applications in various industries such as production of detergents, leather, pharmaceuticals, and food and in wastewater treatment (Racheal et al. 2015).

5.3.7 Amylase

Amylases (E.C. 3.2.1.0) are the enzymes that digest starch (Takata et al. 1992). Functionally, they catalyze the hydrolysis of starch into its low molecular weight sugar molecules (Singh et al. 2016) such as amylose, amylopectin, and glycogen.

The term amylase jointly refers to α-amylase, β-amylase, and γ-amylase. α-Amylase hydrolyzes α-1,4-glucosidic bonds in starch, amylopectin, and glycogen in an endo pattern and consequently forms simpler products (Mohamed et al. 2014). β-Amylase catalyzes the breakdown of a second α-1,4-glycosidic bond, cleaving two glucose units (maltose) at a time (Saini et al. 2017). However, γ-amylase cleaves α-(1,6)-glycosidic linkages, and also attacks the last α-(1,4)-glycosidic linkage at the non-reducing end of amylose and amylopectin, yielding glucose (Singh et al. 2011). *T. harzianum* α-amylase A3 works optimally in acidic environments; thus, it is in high demanded for use in the starch industry (Negi and Banerjee 2009). Ca^{2+} enhances the activity of *T. harzianum* α-amylase A3 as much as 145% (Mohamed et al. 2011).

5.3.8 Ligninolytic Enzymes

Ligninolytic enzymes represent a group of oxidizing enzymes degrading the organic plant polymer lignin. The ligninolytic extracellular enzymes are classified as phenol oxidases and heme peroxidases (Falade et al. 2017), including manganese peroxidase (MnP) and laccase. Manganese-dependent peroxidases and laccase are lignin-degrading enzymes with great potential for industrial applications (D'Souza et al. 2012).

5.3.8.1 Manganese Peroxidase

Manganese peroxidases (MnP; E.C. 1.11.1.13) are the most abundant group of extracellular ligninolytic enzymes, present in white-rot fungi (Hatakka 1994). These enzymes, secreted by *Trichoderma*, potentially remove phenol from wastewater (El-Shora et al. 2017). Manganese peroxidases basically oxidize Mn^{2+} to Mn^{3+} in the presence of H_2O_2 and organic acid chelators such as lactic acid (Goudopoulou et al. 2010). Mn^{3+} is complexed with dicarboxylic organic acids for successive oxidation of several phenolics that are obtained from lignin (Wariishi et al. 1989).

5.3.8.2 Laccase

Laccases (benzenediol:oxygen oxidoreductase; E.C. 1.10.3.2) are unique metalloenzymes that belong to the multicopper oxidase family (Cazares-Garcıa et al. 2013). Laccase is a blue copper oxidase that contains four copper atoms per molecule bound in the catalytic center and catalyzes the four-electron reduction of oxygen to water (Giardina et al. 2010; Jorenek and Zajoncova 2003). Laccases have a wide substrate specificity and because of that they have gained much attention from researchers for many industrial and environmental applications (Fernandes et al. 2008; Moldes et al. 2008). The efficiency of crude and partially purified *Trichoderma*

harzianum WL1 laccase for the decolorization of synthetic dyes (rhodamine 6G, erioglaucine, and trypan blue) with complex aromatic structures has been extensively evaluated (Sadhasivam et al. 2009).

5.4 Cloning of Genes into Bacterial and Yeast Hosts

Several genes of *Trichoderma* are known for the molecular synthesis of ligninolytic, cellulolytic, hemicellulolytic, pectinolytic, chitinolytic, and lipolytic enzymes. The production of these enzymes takes place because of concerned genes and their accessory elements present in the genome of particular fungal species. The expression of these genes is known to be dependent on culture condition, available inducers, and ultimately the host or the fungal species being cultured. Gene cloning has recently been adopted for studying the structure, function, and overexpression of several genes synthesizing enzymes (Ahmad et al. 2005). The strategy of gene cloning was found to be very efficient for enhanced production of enzymes such as cellulases (Ahmad et al. 2005). In the following section here we have presented several major cloning studies conducted for several enzymes independently. Most of the workers isolated genes from different *Trichoderma* sp. that they have cloned into several hosts such as bacteria and yeast for their efficient expression.

5.4.1 Cloning of Trichoderma *Chitinase Genes*

Several successful attempts regarding the cloning of chitinase genes from *Trichoderma* sp. to many suitable bacterial and yeast strains have been conducted. Both exo and endo types of chitinases have been cloned and characterized by various workers from different *Trichoderma* sp. and artificially transferred into plant systems; these have shown tolerance to various biotic as well as abiotic stresses (Pandian et al. 2018).These cloning studies resulted in overexpression of chitinase genes. Several major studies are listed in Table 5.1.

5.4.2 Cloning of Trichoderma *Cellulase Genes*

Expression of cellulase genes is generally induced by cellulose, its derivatives (e.g., cellobiose, sophorose), lactose, and the monosaccharide L-sorbose (Hirasawa et al. 2018). Multiple transcription factors involved in the regulation of cellulase expression in *T. reesei* have been identified (Portnoy et al. 2011), including *Xyr1*, *Ace2*, *Ace3*, and *Cre1* (Zhang et al. 2018). However, the inducers have their limitations for achieving a satisfactory amount of celluloses for proper supply before demand. The increased market demand for cellulase has challenged laboratories and their leaders

Table 5.1 Several important studies for the cloning of chitinase genes from *Trichoderma* into suitable bacterial and yeast hosts

Trichoderma species	Gene	Host	References
T. asperellum	*Chi*	*Agrobacterium tumefaciens*	Zhang et al. (2016)
T. asperellum strains TN42, CH2, SH16, PQ34	*chi42*	*Escherichia coli* TOP10	Loc et al. (2011)
T. virens UKM1	*Cht2*	*Pichia pastoris*	Al-Rashed et al. (2010)
T. atroviride	*chit33*	*E. coli*	Matroudi et al. (2008)
T. reesei PC-3-7	*Chi46*	*E. coli*	Ike et al. (2006)
T. atroviride P1	*Ech30*	*E. coli*	Klemsdal et al. (2006)
T. reesei PC-3-7	*Chi46*	*E. coli*	Ike et al. (2006)
T. aureoviride M	*ech42*	*Saccharomyces cerevisiae* H158	Song et al. (2005)
T. asperellum	*chit36Y*	*E. coli* JM109, *P. pastoris* GS115	Viterbo et al. (2002)
T. harzianum	*chit33*	*E. coli*	Limon et al. (1995)

Table 5.2 Major studies attempting the cloning of the cellulase gene from *Trichoderma* into efficient yeast and bacterial hosts

Enzymes	*Trichoderma* species	Gene	Host	References
exo-β-1,3-Glucanase	*T. asperellum*	*tag83*	*R. solani*	Marcello et al. (2010)
Cellobiohydrolase	*T. viride*	*cbhI, cbhII*	*S. cerevisiae*	Song et al. (2010)
β-1,4-Endoglucanase	*T. reesei QM6a*	*egI*	*A. niger D15*	Rose and van Zyl (2002)
Glucan 1,3-β-glucosidase	*T. atroviride*	*gluc78*	*R. solani*, *P. ultimum*	Donzelli et al. (2001)
β-1,4-Glucanase	*T. reesei*	*egl3*	*S. cerevisiae*	Gietz et al. (1992)

for a low-cost supply of cellulase with rapid production. As is very well known, the genomes of most *Trichoderma* strains carry genes for cellulase production. Several workers have attempted to clone the cellulase gene into efficient bacterial and yeast strains for enhanced production of cellulase. Some of these major studies of cellulase gene cloning are compiled in Table 5.2.

5.4.3 *Cloning of* Trichoderma *Protease*

Because the protease secreted by *Trichoderma* has an efficient role in biocontrol activities, it is the most widely studied. A protease (*Prb1)* specifically produced by *Trichoderma harzianum* under simulated mycoparasitic conditions that was purified

and characterized (Geremia et al. 1993) was found to have improved biocontrol activity against *Rhizoctonia solani* (Flores et al. 1997). The gene for an extracellular serine protease *(tvsp1)* was cloned from *T. virens* (Pozo et al. 2004) and its overexpression consequently improved the protection of cotton seedlings against *R. solani*. Moreover, a serine protease gene (*tvsp1*) that was cloned from *Trichoderma virens* established a biocontrol agent effective against soil-borne fungal pathogens (Pozo et al. 2004). In a different study the aspartic protease gene *TaAsp* from *T. asperellum* T4 was cloned and expressed in a *Pichia pastoris* host (Yang et al. 2013). Therefore, cloning and expression of protease genes as conducted in many studies are listed in Table 5.3).

5.4.4 *Cloning of* Trichoderma *Xylanase Genes*

Trichoderma reesei also produces xylanase to digest hemicellulose into xylose (Zhang et al. 2018). *Xyn1* gene expression is initiated by cellulosic and xylanosic carbon sources (Mach et al. 1996), but xyn3 expression responds to cellulosic carbon sources only (Xu et al. 2000b). Several studies related to the cloning of *Trichoderma* xylanase are listed in Table 5.4).

Table 5.3 Several important studies attempted for the cloning of protease genes from *Trichoderma* into bacterial and yeast hosts

Enzymes	*Trichoderma* species	Gene	Host	References
Aspartic protease	*T. asperellum ACCC30536*	*Asp55*	*E. coli BL21*	Dou et al. (2014)
Aspartic protease	*T. asperellum T4*	*TaAsp*	*Pichia pastoris*	Yang et al. (2013)
Serine protease	*T. harzianum*	*SL41*	*S. cerevisiae*	Liu et al. (2009)
Serine protease	*T. virens*	*tvsp1*	*E. coli*	Pozo et al. (2004)

Table 5.4 Several important studies attempted for the cloning of xylanase genes from *Trichoderma* into suitable bacterial, fungal, and yeast hosts

Trichoderma species	Gene	Host	References
T. reesei Rut C-30	*Xyn2*	*Pichia pastoris*	He et al. (2009, 2019)
T. reesei Rut C-30	*xyn2*	*Escherichia coli* BL21	Jun et al. (2008)
T. harzianum E-58	*xyn2*	*E. coli* DH10B	Ahmed et al. (2007)
T. reesei PC-3-7	*xyn3*	*E. coli* JM109	Ogasawara et al. (2006)
Trichoderma strain SY	*Xyl*	*E. coli*	Min et al. (2002)
T. reesei QM6a	*xyn2*	*Saccharomyces cerevisiae* Y294	la-Grange et al. (2001)
T. reesei QM 9414	*xyn1, xyn2*	*Schizosaccharomyces pombe*	Okada et al. (1999)
T. reesei	*XYN2*	*Saccharomyces cerevisiae*	la-Grange et al. (1996)
T. reesei ALK02721 ALK02221	*xln2*	*Trichoderma reesei*	Saarelainen et al. (1993)

5.5 Application of Enzymes Produced by *Trichoderma* sp.

Many *Trichoderma* species or, more precisely, many individual *Trichoderma* strains have various important applications in industry and human life (Druzhinina et al. 2006). *Trichoderma* provides diverse, profitable bioproducts used in industrial applications, including foods, textiles, and pulp and paper (Mtui 2012). The use of enzymes in the textile industry meets the current requirements for environmentally friendly processes (Puranen et al. 2014). *Trichoderma* sp. probably have significant wide-scale use in the remediation of pollutants in soils and waters (Harman et al. 2004b).

5.5.1 Application of Cellulase

Cellulases of *Trichoderma* have potential application in several industries. For the carbon sources, different types of substrate such as wheat bran, rice straw, corn straw, sugarcane bagasse, corn cob, and sunflower hulls are used by *Trichoderma* to produce cellulolytic enzymes. These enzymes have significant roles in many biotechnological and industrial applications including the production of bioethanol, pulp and paper, textiles, brewing, food, animal feed, and the agricultural industry (Table 5.5). However, the cost of commercial cellulase is estimated to be too high for industrial application (Lynd et al. 2008). The cellulase enzyme is highly used for the saccharification of the different cellulosic substrates. The employment of waste materials for the production of fuels and chemicals adds economic value and reduces environmental impacts (Pereira et al. 2008). Recently, because of the constant increase in oil prices the significance of biofuel production from lignocellulosic biomass as an alternative energy source has been intensified, and efficient conversion of lignocelluloses to biofuel is gaining much interest (Bak et al. 2009).

5.5.2 Application of Chitinase

Chitinase from *Trichoderma* is widely used in many applications such as the pharmaceutical, food, pest management, waste recycling, leather, and agricultural industries (Table 5.6). These enzymes are also widely used as biocontrol agents. The chitinase of *Trichoderma* is potentially used in agriculture as a biological control agent because of its properties as a biopesticide and biofertilizer (Urbina-Salazar et al. 2019).

Table 5.5 Applications of cellulase produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. reesei NCIM 1186	Agricultural weed, *Prosopis juliflora* Pods	Production of biofuels	Jampala et al. (2017)
T. harzianum strain HZN11	Sweet sorghum bagasse	Production of bioethanol	Bagewadi et al. (2016)
T. harzianum	Glycerol, sugarcane bagasse	Biodiesel manufacturing	Delabona et al. (2016)
T. reesei CEF19	Rice straw	Production of bioethanol	Kumar et al. (2014)
T. viride, *T. reesei*	Wheat bran, root crush	Textile, pulp, brewing, food, and agriculture industry	Pirzadah et al. (2014)
T. harzianum SNRS3	Rice straw	Biobutanol production	Rahnama et al. (2014)
T. asperellum RCK201	Corn cob, wheat straw, and sugarcane bagasse	Saccharification of lignocellulosic biomass, biofuel production	Raghuwanshi et al. (2014)
T. reesei	Ramie powder, bamboo, rice-straw, corn-straw, corn cob and cotton stem	Biorefinery	Xiong et al. (2013)
T. harzianum IOC 3844	Sugarcane bagasse	Ethanol production	Rocha et al. (2013)
T. viride	Sugarcane bagasse	Food, animal feed, textile, pulp and paper industries, and especially production of bioethanol	Lan et al. (2013)
Trichoderma sp. IS-05	Wheat bran	Bioethanol production	Andrade et al. (2011)
T. reesei Rut C 30	Sunflower hulls	Enzymatic saccharification, ethanol production	Sharma et al. (2004)

5.5.3 *Application of Lipase*

Fungal lipase production has potential biotechnological and industrial applications. Lipases of *Trichoderma* are widely used in biodiesel production and the detergent industry (Table 5.7). These industries take advantage of the ability of lipases to catalyze the hydrolysis of long-chain triacylglycerols of low water solubility at the lipid–water interface, and also to carry out reactions of esterification, alcoholysis, or acidolysis in microaqueous conditions (Berglund 2001). Fungal enzymes have been chosen for industrial applications because of their safe status and ease of cultivation (Jaeger et al. 1999).

Table 5.6 Application of chitinase produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. harzianum, *T. atroviride*	Chitin, glucans	Pharmaceutical, food, and agronomic industry, biocontrol of phytopathogenic fungi and in pest control	Urbina-Salazar et al. (2019)
T. virens	Shrimp waste	Recycling of chitin wastes	Rachmawaty and Madihah (2016)
T. viride	Coffee husks	Pharmaceuticals, food, feed, and leather industries	Karthikeyan et al. (2014)
T. asperellum UTP-16	Wheat bran	Biocontrol of pests and diseases as growth-promoting factors, in food and feed, medicine, and waste management	Kumar et al. (2012)
T. virens UKM1	Shrimp waste	Biocontrol agent	Abd-Aziz et al. (2008)
T. harzianum Rifai T24	Chitin, corn steep solid	Biocontrol agent	El-Katatny et al. (2001)

Table 5.7 Applications of lipase produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. lentiforme ACCC 30425	Olive oil	Detergent industry	Wang et al. (2018)
T. harzianum	Wheat bran	Biodiesel production	Toscano et al. (2013)
T. harzianum	Castor oil cake, sugarcane bagasse, olive oil	Production of biodiesel	Coradi et al. (2013)

5.5.4 Application of Pectinase

The major application of the enzyme pectinase includes fruit juice extraction, clarification, and liquefaction, and it is also used in the textile industry (Table 5.8). The low-cost onion skin waste is most efficiently used as the major substrate for the production of fungal pectinase, which is subsequently used in fruit juice clarification and other applications (Ismail et al. 2016).

5.5.5 Application of Protease

Trichoderma produces proteases, which have important functions including biocontrol, production of antimicrobial compounds, control of plant diseases, and nutrition requirements. Several workers have studied the production of protease using efficient *Trichoderma* strains growing on a variety of lignocellulosic and other substrates (Wu et al. 2017) (Table 5.9).

Table 5.8 Application of pectinase produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. viride EF-8	Egyptian onion (*Allium cepa* L.) skins	Fruit (apple, lemon, orange) juice clarification	Ismail et al. (2016)
T. viride	Agro-industrial waste, orange peel	Textile and juice industries	Irshad et al. (2014)
T. harzianum	Citrus peel	Extraction, clarification, and liquefaction of fruit juices	Nazia et al. (2003)

Table 5.9 Application of protease produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. asperellum GDFS1009	Rose Bengal agar including streptomycin and chloramphenicol, potato dextrose broth	Production of antimicrobial compounds, agricultural biocontrol	Wu et al. (2017)
T. harzianum T334	Glutamine and yeast extract medium	Controlling plant diseases, antagonists against plant pathogens	Szekeres et al. (2004)
T. harzianum isolate 1051	Bactodextrose agar	Controls cocoa plant witches' broom disease	De Marco and Felix (2002)
T. harzianum CECT 2413	Yeast extract, potato dextrose agar	Mycoparasitism regulation; hydrolyzes proteins to free amino acids	Delgado-Jarana et al. (2002)

5.5.6 Application of Amylase

Amylases are known for the broad range of industrial applications such as detergents, textiles, food, paper, and the saccharification of lignocellulosic complex substrates. Recently, Marques et al. (2018) produced amylase on cornmeal or soybean meal by *Trichoderma harzianum* ALL 42 for the preparation of chick feed. Abdulaal (2018) also reported the use of *Trichoderma* for its use in detergents, food, textiles, and the paper industry. Several applications of amylase are shown in Table 5.10.

5.5.7 Application of Xylanase

Trichoderma xylanase is well imported for several usages at a large scale, including biofuel production, pharmaceuticals, bioremediation, biobleaching, development of broiler chick feed, crop production, and the pulp and paper and leather industries. Several important applications established by particular studies are listed in Table 5.11.

Table 5.10 Application of amylase produced by *Trichoderma* sp. grown on basal substrate medium

Trichoderma species	Substrate	Application	Reference
T. harzianum ALL 42	Cornmeal or soybean meal	Development of broiler chick feed	Marques et al. (2018)
T. pseudokoningii	Potato starch	Detergents, food, textiles, paper industry	Abdulaal (2018)
T. harzianum	Mandarin orange peel	Reduction in pollution and in saccharification industry	Mohamed et al. (2011)
Trichoderma sp. KUPM0001	Sago hampas	Ethanol production	Shahrim et al. (2008)
T. harzianum 1051	Bactodextrose agar, potato starch	Antagonistic activity against witches' broom disease in cocoa	de Azevedo et al. (2000)

Table 5.11 Application of xylanase produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	Reference
T. harzianum ALL 42	Cornmeal or soybean meal	Development of chick feeds	Marques et al. (2018)
T. reesei NCIM 1186	Agricultural weed, *Prosopis juliflora* Pods	Production of biofuels	Jampala et al. (2017)
T. viride	Wheat bran	Pharmaceuticals, food, feed, and leather industries	Karthikeyan et al. (2014)
T. reesei SAF3	Wheat bran	Biobleaching	Kar et al. (2013)
T. reesei RUT C-30	Beechwood xylan	Pulp and paper industry	Gamerith et al. (1992)

5.5.8 Application of Manganese Peroxidase

Manganese peroxidase (MnP) produced by *Trichoderma* spp. is a highly versatile enzyme, with many industrial applications exploited for the removal of phenol from wastewater, antagonistic action, production of enzyme from agricultural wastes, detoxification of pollutants, and in the pulp paper industry. The potential applications of versatile MnP from *Trichoderma* are mentioned in Table 5.12.

5.5.9 Application of Laccase

Laccase enzymes produced by *Trichoderma* have extensive applications in diversified disciplines such as dye decolorization, waste detoxification, bioremediation, delignification, biobleaching, and the pulp paper industry. Laccases are most in

Table 5.12 Application of manganese peroxidase (MnP) produced by *Trichoderma* sp. grown on several basal substrates

Trichoderma species	Substrate	Application	References
T. asperellum	Barley straw	Utilization of agricultural wastes for enzyme production and valuable products	Abdel-Ghany et al. (2018)
T. harzianum	Czapek's yeast extract	Removal of phenol from wastewater	El-Shora et al. (2017)
T. pseudokoningii	Milled wheat straw	Detoxification of pollutants, pulp and paper industry	Acharya et al. (2013)
Trichoderma sp.	Coffee pulp	Detoxification of agro-industrial residuals with a high phenolic content	Mata et al. (2005)
Trichoderma sp.	Wheat straw	Antagonistic action	Savoie and Mata (1999)

Table 5.13 Application of laccase produced by *Trichoderma* sp. grown on basal substrate medium

Trichoderma species	Substrate	Application	Reference
T. harzianum	PDA supplemented with guaiacol	Bioremediation, detoxification, dye decolorization	Ranimol et al. (2018)
T. muroiana IS1037	Rubber wood dust	Biobleaching, pulp and paper industry	Jaber et al. (2017)
T. harzianum strain HZN10	Wheat bran	Delignification of biomass	Bagewadi et al. (2016)
T. viride Pers. NFCCI-2745	Coconut husk	Bioremediation phenolic compound-rich industrial effluents	Divya et al. (2014)
T. harzianum ZF-2	Wheat straw powder, soybean meal	Dye decolorization	Gao et al. (2013)
Trichoderma sp.	Rice straw	Detoxify phenolic compound	Assavanig et al. (1992)

demand and considered as one of the most promising enzymes for future industrial applications (Xu et al. 2000a). Several studies related to the application of *Trichoderma* laccase are compiled in Table 5.13.

5.6 Conclusions

Based on the critical review of the literature related to the several enzymes produced by *Trichoderma*, it can be concluded that this genus is a very fast growing fungus. *Trichoderma* has a strong capability to grow under very moderate conditions. The enzymes produced by this fungus (pectinase, cellulase, xylanase, chitinase, lipase, protease, amylase, manganese peroxidase, laccase) can be exploited in various

industrial and domestic applications related to human welfare. The strong machinery of genes responsible for synthesizing the enzymes can be successfully cloned into other potential hosts (bacterial, yeast) for efficient production of enzymes.

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Chapter 6
Recent Advances in Molecular Approaches for Mining Potential Candidate Genes of *Trichoderma* for Biofuel

Richa Salwan, Anu Sharma, and Vivek Sharma

6.1 Introduction

Fungi are key players in the global carbon cycle due to their heterotrophic life cycle. The broad nutritional range can be observed from their ability to produce enzymes and bioactive metabolites which grant them access to huge diversity of carbon sources in different ecological niches (Martin et al. 2011). Filamentous fungi, in particular belonging to the genus *Trichoderma* (*Ascomycota*), are known for their nutritional versatility. *Trichoderma reesei* is used for the production of commercial cellulolytic enzymes for biofuels and other industries. Trichoderma species such as *T. harzianum, T. viride, T. virens, T. atroviride, T. koningii, T. saturnisporum, T. hamatum and T. asperellum* are also used as biocontrol agents, due to their antagonistic potential and ability to tolerate fungal toxins (Mukherjee and Kenerley 2010; Sharma et al. 2013, 2016a, b, 2017a; Sharma and Shanmugam 2012; Geistlinger et al. 2015; Druzhinina et al. 2018). Besides this, the enzymes and antibiotics produced by filamentous fungi are used in food, detergent, pulp, and feed industries (Tsang 2014). The strains of *T. asperellum*, *T. viride* Pers. NFCCI-2745, and *T. viride* strain FRP3 are explored, for the bioremediation of polycyclic aromatic hydrocarbons (Zafra et al. 2015), phenolic pollutants (Divya et al. 2014), and glyphosate (Arfarita et al. 2013) in agricultural fields.

The production of biofuel from agricultural biomass is presently considered a major focus of research due to its renewable nature. For this, the cellulose-containing biomass is initially hydrolyzed to sugars by chemical or enzymatic processes. The pretreatment followed by fermentation through consolidated bioprocessing (CBP)

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or direct microbial conversion (DMC) either by one or a consortium of microbes is the preferred choice nowadays (Lynd et al. 1999). For CBP-based approach, efforts are concentrated on anaerobic bacteria involved in conversion of cellulose to ethanol (Fig. 6.1). However, few filamentous fungi belonging to *Aspergillus* (Skory et al. 1997), *Fusarium* (Singh and Kumar 1991), *Monilia* (Gong et al. 1981), *Neurospora* (Deshpande et al. 1986), and *Rhizopus* (Skory et al. 1997) do not need strict anaerobic conditions and still can directly ferment cellulose to ethanol. Among *Trichoderma* strains, *T. reesei* is widely explored for various glycosyl hydrolases at industrial level.

To completely understand the molecular basis of *Trichoderma* strains responsible for the effective biodegradation of lignocellulose, they have been studied using transcriptomics (Bailey et al. 2006; Alfano et al. 2007; Bae et al. 2011; Morán-Diez et al. 2012), proteomics (Marra et al. 2006; Segarra et al. 2007; Shoresh and Harman 2008), and metabolomic approaches (Bae et al. 2011; Brotman et al. 2012). The identification of molecular components being expressed or transcribed or translated during biomass degradation is of paramount importance. In comparison to genomic data, the transcriptomic data which represents a complete set of RNA population of an organism reveal active components involved in response to external environmental

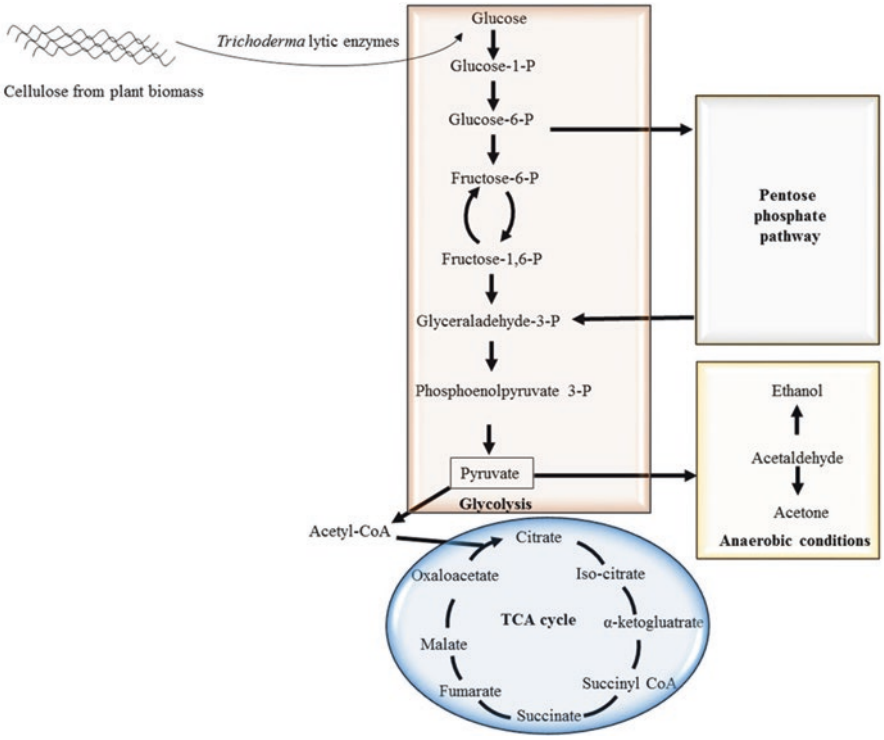

Fig. 6.1 Metabolic pathway of biofuel production from agricultural biomass using *Trichoderma* and anaerobic microbial strains

or internal developmental stimuli. A large number of techniques such as EST libraries, subtractive cDNA libraries, microarray, transcriptomics, and translatome-based studies have been done for the identification and characterization of genes/transcripts involved in differential functions. In contrast to microarray-based approach, DNA sequence-based methods are capable of directly determining the target DNA sequence. The advancements in high-throughput and automated DNA sequencing approaches provide new avenues for accurate mining of transcriptomes, compared to Sanger sequencing of cDNA or EST libraries which are relatively of low throughput, expensive, and not quantitative. In parallel, the improvements in DNA technologies such as metagenomics and meta-transcriptomics have made it possible to determine dynamics of microbial soil communities and target the required transcripts from unculturable microbes in a better and accurate way (Buée et al. 2009; Öpik et al. 2009; Jumpponen et al. 2010).

The availability of genomic data has further strengthened the adaptive lifestyle of *Trichoderma* strains. A comparative genomic data of *Trichoderma* species has helped in unraveling the genomic expansion and evolutionary mechanisms associated with its broad host range. Now, the comparative genomic studies revealed a better understanding of the metabolic and regulatory processes involved in the saprophytic or symbiotic lifestyles of *Trichoderma* (Martin et al. 2011). Here in this book chapter, we have discussed the role and recent developments in molecular techniques in mining the genome/genes of *Trichoderma*.

6.2 Molecular Approaches for the Identification of Target Gene/Proteins of *Trichoderma*

The diversity in lifestyle of *Trichoderma* strains demands expression of various genes and secretion of proteins responsible for diverse functions. The studies based on genomic, transcriptomics, and proteomics tools help to understand the molecular basis of physiological and ecological behavior of *Trichoderma* to perform under diverse conditions including growth in the presence of cellulose, lactose, and other conditions (Fig. 6.2). A brief discussion of molecular tools for the differential expression of transcripts/proteins and their characterization is given in Table 6.1.

6.3 Expressed Sequence Tags (ESTs)

ESTs initially applied for screening of cDNA library of the human brain are an efficient tool which offers a rapid way of characterizing sets of genes expressed in a time-stage-specific manner (Adams et al. 1991). These ESTs are also used for developing molecular markers. ESTs represent unique DNA sequences derived either from total RNA or poly (A) RNA under specific conditions and are cloned

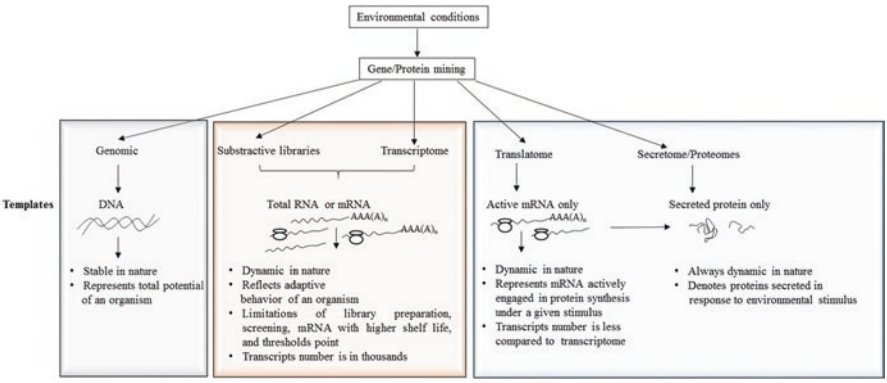

Fig. 6.2 Overview of genomic and proteomic approaches for mining genes

and sequenced randomly from cDNA libraries. EST data is often deposited as single-pass read in a database. A typical EST is sufficiently long (300–1000 bp DNA) and capable of identifying the expressed gene. The EST number of a gene serves as an indicator of its expression level. For example, the gene expressed at higher level indicates more ESTs represented in cDNA library. Since ESTs often represent only partial profiles of the gene expression, therefore, they have limitations of data redundancy. Additionally, for housekeeping genes, the large number of ESTs tends to ignore transcripts expressed at low level (Knox 2004). In fungi, differential RNA display was first used for the identification of genes involved in symbiotic association. In *Trichoderma*, ESTs and cDNA microarray-based studies have been used for the differential identification of genes involved in plant interaction. Based on ESTs and cDNA microarray studies, an insight on *T. reesei* gene expression related to tricarboxylic acid cycle and electron transport chain has provided valuable insights in its role and limitation in ethanol fermentation. Moreover, the experimental findings also revealed how acetaldehyde can lead to acetate biosynthesis instead of ethanol and hence prevent the regeneration of NADH required for anaerobic metabolism (Chambergo et al. 2002).

6.4 Genome Sequencing

Thousands of ESTs have proven its enormous usefulness in delineating the genes and their expression patterns, however EST studies lack a significant proportion of the genes expressed at low levels or expressed for a short period. Moreover, EST studies do not depict accurate pictures of the genome such as gene organization and their regulation and repeatedness at genome-wide level and their syntenic relationships. For gaining insights into these features, studies employing genome sequencing are required. Since the publication of *Phanerochaete chrysosporium* genome in 2004, over 400 genomes of filamentous fungi have been completed or are in a pro-

Table 6.1 Examples of genomics and proteomics tools used in mining genes of *Trichoderma* strains for biofuel or plant biomass degradation

Sl. no.	*Trichoderma* species/strains	Techniques	Functions	References
1.	*T. reesei* strain QM 9414	Expressed sequence tag and cDNA microarrays	Identified the programming of the tricarboxylic acid cycle and electron transport chain encoding genes during the oxidation of pyruvate	Chambergo et al. (2002)
2.	*Trichoderma* strain A10	Category I CBP organisms	*Trichoderma* strain A10 fermented microcrystalline cellulose and glucose to ethanol	Stevenson and Weimer (2002)
3.	*T. reesei*	Shotgun sequence and expressed sequence tags (ESTs)	Profiling of carbohydrate-active enzymes involved in the hydrolysis of plant cell wall polysaccharides	Martinez et al. (2008)
4.	*T. reesei* QM9414, mutant form of *T. reesei* QM6a	Protein abundance in multiplexed samples (PAMUS)	The study revealed how cellulose induces biosynthesis of cellulases and the optimum concentrations of each secreted enzyme during cellulosic substrate utilization.	Adav et al. (2013)
5.	*T. reesei* wild-type CICC40360 wild and *T. reesei* recombinant strain HJ48	Transcriptional profiling	Comprehensive curation and analysis of fungal biosynthetic gene clusters for natural products	Li et al. (2016)
6.	*T. reesei* QM6a and QM9414	PacBio RSII and Illumina sequencing platform	Genome reannotation of a model fungus can provide a better blueprint for biotechnological and industrial applications	Li et al. (2017)
7.	*T. harzianum* IOC-3844	BAC library and BAC end sequencing approach	Rapid selection of genes and genomic regions associated with biomass conversion	Crucello et al. (2015)
8.	*T. reesei*	Whole-genome-based metabolic modeling using CoReCo	A metabolic model of *T. reesei* for simulating the performance of the production strain	Castillo et al. (2016)
10.	*T. reesei*	Genome wide and RNA sequencing on carbon sources using Illumina HiSeq 2000	Using stoichiometric metabolic model revealed novel insight into the metabolism of protein production	Pakula et al. (2016)

(continued)

Table 6.1 (continued)

Sl. no.	*Trichoderma* species/strains	Techniques	Functions	References
11.	*T. hamatum* strains YYH13 and YYH16	Genome sequencing using Illumina HiSeq sequencing platform	Revealed genome of *T. hamatum* strains have potential to serve as a model organism for cellulase production	Cheng et al. (2017)
12.	*T. reesei* RUT-C30 and CL847 strains	Two-dimensional electrophoresis followed by MALDI-TOF or LC–MS/MS	Identified proteins involved in biomass degradation	Herpoël-Gimbert et al. (2008)
13.	*T. reesei* Rut C-30	Two-dimensional followed by MALDI-MS and LC–MS/MS	Enzyme production by *T. reesei* is carbon source-dependent, and the lactose and xylose induce cellulolytic and xylanase production	Jun et al. (2013)
14.	*T. reesei* QM6a, QM9414, RUT C30, and QM9414MG5 strains	SDS-PAGE followed by LC–MS/MS	Higher production of hydrolytic protein expression at acidic pH	Adav et al. (2011)
15.	*T. reesei* QM6a and Rut C30	iTRAQ labeling and LC–MS/MS	Role of lignocellulolytic proteins in the secretome of *T. reesei* wild-type and mutant RutC30 is dependent on both nature and complexity of different lignocellulosic carbon sources	Adav et al. (2011)
16.	*T. reesei*	High-throughput genomic and proteomic differential secretome (2D-DIGE) analysis	Identified new players in cellulose degradation such as accessory proteins with non-catalytic functions secreted in different carbon sources	dos Santos Castro et al. (2014)
17.	*T. asperellum* S4F8 and *T. reesei* Rut C30	SDS-PAGE and label-free quantitative shotgun LC–MS/MS	Identified higher hemicellulase and β-glucosidase activities for S4F8 and also identified proteins which are exclusive or common between two strains	Marx et al. (2013)
18.	*T. reesei*	Blue native electrophoresis LC–MS/MS and 2D-BN-PAGE zymography	Role of multienzymatic complexes grown in media supplemented with either lactose or galactose	da Silva et al. (2015)

(continued)

Table 6.1 (continued)

Sl. no.	*Trichoderma* species/strains	Techniques	Functions	References
19.	*T. reesei*	Protein purification	Responsible for the saccharification of polymeric cellulosic substrates	Ogunmolu et al. (2017)
20.	*T. reesei*	Machine learning approaches like multiple kernel learning (MKL), pairwise kernels, and kernelized structured output prediction in the supervised graph inference framework	Identify completely novel candidate secretion proteins conserved in filamentous fungi having unique secretion capabilities	Kludas et al. (2016)

cess to be sequenced (http://genomeonline.org). To explore the fungal biodiversity, 1000 Fungal Genomes Project (www.1000.fungalgenome.org), initiated by the Department of Energy, USA, in collaboration with Joint Genome Institute, aims to sequence at least two species from each family of kingdom fungi (Fig. 6.2).

Presently, genome of several *Trichoderma* species has been sequenced (https://genome.jgi.doe.gov/programs/fungi/index.jsf). The genome of *T. atroviride*, *T. virens*, and *T. reesei* ranges from 31 to 38.8 Mbp in size with 11,865 genes for *T. atroviride* and 12,518 genes for *T. virens* and 9143 genes for *T. reesei*. The comparative studies for gene families showed 46 expanded families, whereas only 26 were expanded in *T. atroviride* and *T. virens*. The first genome of *T. reesei* QM6a strain was obtained using next-generation sequencing (NGS) (Martinez et al. 2008), and draft genome QM6a-v2.0 was publicly made available (Joint Genome Institute of the US Department of Energy; https://genome.jgi.doe.gov/Trire2/Trire2.home). The QM6a-v2.0 draft genome lacks complete genomic information, due to technical limitations and assembly errors in NGS technology. Finally, the complete genome of QM6a was done using third-generation DNA sequencing (Li et al. 2017). Another technique was also deployed which offers reads of up to 60 kb and capable of performing telomere-to-telomere information for seven chromosomes and additional single mitochondrial genome based on single molecule real-time (SMRT) by Pacific BioSciences (PacBio). The high-quality data was then used for correcting sequencing errors in long reads and resulted in only <0.0024% low error rate in the final genome draft. PacBio-based studies revealed that QM6a mitochondrial genome was identical to the QM9414 mitochondrial genome (42,139 bp; NC_003388.1) (Chambergo et al. 2002). No sequence ambiguity in the complete QM6a genome sequence was found, compared to several ambiguous areas, and over 48,252 of unidentified bases was reported in draft version V2.0 of QM6a genome. Moreover, for the sequencing and reannotation using third-generation approach of the QM6a, complete genome sequence revealed several biologi-

cally important genes missing in the previous QM6a-v2.0 genome sequencing (Li et al. 2017).

6.5 Whole-Genome Stoichiometric Metabolic Models

To fully understand the interconnection between metabolic pathways of an organism, over 1483 whole-genome-based metabolic reaction networks have been predicted. These BioModelsdatabase (Juty et al. 2015) based on whole-genome stoichiometric metabolic models has been used for representing*Escherichia coli* (Orth et al. 2011), *Bacillus subtilis* (Henry et al. 2009), *Aspergillus niger*, *A oryzae*, *A. nidulans* (David et al. 2008; Vongsangnak et al. 2008; Andersen et al. 2008), *S. cerevisiae* (Herrgard et al. 2008), *Pichia stipitis* and *P. pastoris* (Caspeta et al. 2012), and *Arabidopsis thaliana* (Poolman et al. 2009). In addition, the Path2Models branch of the BioModels database representing 112,898 models for metabolic, 27,531 models for non-metabolic, and 2641 models for whole-genome metabolism has also been explored (https://www.ebi.ac.uk/biomodels-main/). Using a novel computational approach, the metabolic network models were constructed using CoReCo and integrating sequence data for 49 important fungal species at the genome scale in a probabilistic framework (Pitkänen et al. 2014).

For HJ48 and CICC40360 strains, these genes were predominantly grouped into pathways such as ER [PATHWAY: tre04141], tryptophan metabolism [PATHWAY: tre00380], lysine degradation [PATHWAY: tre00310], glycerolipid [PATHWAY: tre00561], glycerophospholipid metabolism [PATHWAY: tre00564], glycolysis/gluconeogenesis [PATHWAY: tre00010], pentose phosphate pathway [PATHWAY: tre00030], citrate cycle (TCA cycle) [PATHWAY: tre00020], base excision repair [PATHWAY: tre03410], and DNA replication [PATHWAY: tre03030]. Statistical analysis revealed enrichment of differentially expressed genes involved in protein processing in the endoplasmic reticulum (ER) [PATHWAY: tre04141], although the differentially expressed genes were enriched for the pentose phosphate pathway [PATHWAY: tre00030], glycolysis/gluconeogenesis [PATHWAY: tre00010], TCA cycle [PATHWAY: tre00020], DNA replication [PATHWAY: tre03030], and base excision repair [PATHWAY: tre03410], but this enrichment was not statistically significant (FDR $\leq$0.05) (Pitkänen et al. 2014).

6.6 Transcriptome Analysis

Due to the stable nature of genome, it is difficult to validate the functional annotation of genomes. Therefore, whole transcriptome-based sequencing to identify the role of new genes is emerging as a key tool in assigning function to unannotated genes. In this context, comparative high-throughput transcript profiling is extensively explored to understand the differential gene expression of an organism under

different conditions (Tsang 2014). For example, the differential expression analysis of total 3222 genes with q value <0.005 and log2 (fold change) >1 for HJ48 and CICC40360 strains of *T. reesei* revealed upregulation of 2106 genes and downregulation of 1116 genes. Similar analysis for putative functions of genes using GO Seq based on common biological properties is commonly used to deduce the role of different genes in metabolic pathways such as biological and molecular functions in a cellular component. The differentially expressed transcripts are classified using KEGG database. RNA sequencing also known as transcriptomic technology is also used in *T. reesei* for cellulase production for obtaining a global view of the gene expression for biomass degradation. The transcriptional profiling revealed that 22 glycoside hydrolases and 3 cellulases are encoding genes in response to 3 different carbon sources (Chen et al. 2014). RNA-seq provides transcriptional profiling of putative biomass-degrading genes in response to various carbon sources, and three genes encoding cellulases have been characterized under different cultivation conditions using real-time PCR (Chen et al. 2014) (Fig. 6.2).

Similarly, transcriptomic study in the presence of glucose for *T. reesei* strains showed minor change in the behavior of genes related to glycolytic pathway (Chambergo et al. 2002). Experimental study also revealed upregulation of initial glycolytic pathway in HJ48 compared to CICC40360. For transcripts related to TCA pathway, two transcripts were found downregulated in HJ48 strain. Such case studies are helpful in understanding the role of pyruvate pathway in fermentation (Huang et al. 2016). During cellulose metabolism, complex sugar chains are ultimately converted into ethanol via a number of metabolic pathways including glycolysis, subsequent fermentation, and the pentose phosphate pathways. RNA-seq has been used to investigate the differentially expressed genes in CICC40360 and HJ48 strains of *T. reesei* for ethanol production (Huang et al. 2014; Häkkinen et al. 2014). Transcription profiling coupled to RT-qPCR has been used for genome-wide identification of candidate genes and the expression profile of various transcripts of *Trichoderma* against different host plants and fungal pathogens (Pakula et al. 2016; Sharma et al. 2017b).

6.7 Quantitative Secretome Analysis

The proteome constitutes a set of proteins produced by the cell or organism under given conditions (Wilkins et al. 1996a, b), whereas the secretome represents a subset of the proteome of extracellular nature responsible for saprophytic, parasitic interaction with plant or plant pathogen suppression activities (Wilkins et al. 1996a, b). Proteomics studies have enhanced our scientific understanding on diverse lifestyle of *Trichoderma* species (De Mojana et al. 2018). Live cells and their enzymes with eco-friendly nature, high specificity and stability, low input costs, as well as low waste generation offer vast biotechnological applications to synthesize bio-based products, also known as white biotechnology (Wilkins et al. 1996a, b; De Oliveira and De Graaff 2011; Bianco and Perrotta 2015; De Mojana et al. 2018).

The secretome of saprophytic filamentous fungi comprises a dynamic set of proteins including carbohydrate-active enzymes (CAZymes) which can hydrolyze plant polysaccharides into value-added products such as fermentable sugars and several other applications (Figs. 6.1 and 6.2).

The genomic and other databases have revolutionized the way in which mass spectrometry can be used to analyze molecular mass of the proteins. So far, the qualitative details of several proteins including their annotations are available in the databases. However, approaches which demand staining or labeling of proteins allow to perform parallel comparative studies on protein expression under different conditions. Conventional 2D approach involving excision of stained spots followed by mass spectrometry has several limitations like proteins present in abundance mask the proteins present in low amount, leading to poor reproducibility and unreliable results (Issaq and Veenstra 2008). The isotope labeling of proteins in shotgun proteomic methods has been discovered to overcome the quantification-associated difficulties using gel-based approaches (Wu et al. 2005). Another technique known as stable isotope in cell culture (SILAC) involves metabolism-based incorporation of specific labeled amino acids into proteins, and two types of amino acids are used (12C -light' and 13C -'heavy) (Ong et al. 2002). The proteins in both samples are combined and identified by MS due to mass shift of the particular peptides. Based on the ratio of peak intensities in the MS spectrum, the relative protein abundance is determined. Although SILAC is an effective technique, it offers disadvantages like use of endogenous labeling. Therefore, alternate techniques involving cysteine-specific protein-based labeling known as "isotope-coded affinity tags" (ICAT®) is designed for comparative studies (Gygi et al. 1999). The specificity for cysteine residues of ICAT reduces the sample complexity. However, its major limitation involves peptides which lack cysteine residues. Another alternate known as isobaric tagging technique can overcome a major drawback of isotope tagging. In isobaric tags, the same peptide from comparative studies appears as a single peak in the MS which decreases the complexity of the data in comparison to isotopic labeling strategies. Here, iTRAQ isobaric tags for relative and absolute quantification developed by Applied Biosystems can detect up to 4–8 samples (Ross et al. 2004; Choe et al. 2007). In iTRAQ, tags react with primary amine group of peptides to make them labeled, and hence it is possible to even determine posttranslational modifications (Fig. 6.3).

The secreted protein includes transporting proteins, the cell surface receptors, and the extracellular proteins. The secretome of filamentous fungi grown in the presence of lignocelluloses is mainly reported to contain a plethora of carbohydrate-active enzymes (CAZymes) which includes several glycoside hydrolases, glycosyltransferases, polysaccharide lyases, and carbohydrate esterases which play a vital role in degrading or modifying glycosidic bonds. Additionally, enzymes classified as auxiliary activities (AA) or lytic polysaccharide monooxygenases (LPMO) have also been reported. The protein secretion by *T. reesei* can yield over 100 g/l of cellulase in industrial production (Cherry and Fidantsef 2003). The secretome of an organism is influenced by the biological features of microorganisms, and it varies in response to external or internal stimuli (Papagianni 2004). Both the quan-

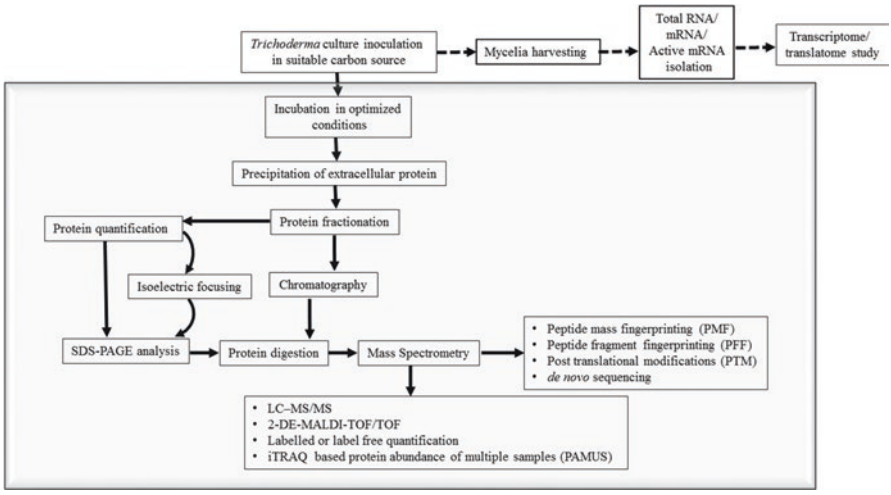

Fig. 6.3 Strategic plan for the characterization of proteasome in *Trichoderma*

tity and type of enzymes produced by the fungal secretome are highly influenced by the carbon source. The lignocellulose-based substrates in *Trichoderma* induce the production of cellulases and hemicellulases where glucose is the sole carbon source (Aro et al. 2005; De Mojana et al. 2018). The role of new protein can be monitored based on sequence similarity search. Still, the molecular function provides limited information about the cellular function of a protein.

Proteomics-based studies are one of the most promising tools in understanding the enzyme composition in the secretome, in response to plant biomass breakdown or plant fungal pathogen attack. The modulation of fungal secretome for different events is quite interesting and can reveal the adaptive behavior of *Trichoderma* strains. 2D gel electrophoresis (2-DE) and MS are powerful tools for studying secretome. In this approach, isoelectric focusing based on pI followed by electrophoretic separation (based on molecular weight) separates hundreds of proteins in a single gel. The separated proteins can be used for the identification with either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI)-based MS (Karpievitch et al. 2010). The limitations of 2D include sensitivity, range of pH strip, and problems of separating hydrophobic proteins (Rogowska-Wrzesinska et al. 2013). In recent studies, the 2-DE approach has replaced alternate methods, where gel-free methodologies involving tandem mass spectrometry (MS/MS) coupled with liquid chromatography (LC) are being used (Rogowska-Wrzesinska et al. 2013). In bottom-up approach, prior to LC–MS/MS, proteins present in the sample are enzymatically digested and then identified based on the fragmentation patterns (Cox and Mann 2011; Mann et al. 2013; Zhang et al. 2013). Alternatively, top-down approaches use intact proteins for isoform determination and understanding posttranslational modifications (PTM). LC–MS proteomics is replaced by 2-DE-MS mainly (De Mojana et al. 2018), whereas bottom-up strategy

is limited due to sample fractionation, ionization, and fragmentation (Mann et al. 2013). The peptide mass fingerprint can be used to identify proteins from primary sequence databases (http://www.matrixscience.com/search_intro.html) (Fig. 6.3).

The chromatographic techniques involving reverse phase (RP), affinity or ion exchange (IE), and size exclusion chromatography are also explored either individually or in combination with mass spectrometric studies. In chromatographic fractionation, the mass of each intact protein is determined and then subsequently used for posttranslational modifications and other studies (Shi et al. 2004; Karpievitch et al. 2010). Native electrophoresis-based method uses polyacrylamide gels such as clear-native PAGE and Blue-native PAGE for the determination of quaternary structures and multi-subunit complexes of enzymes for proteomics studies. Native PAGE-based 2-DE can also be performed for better resolution of the protein complexes (Weiland et al. 2014). Based on native PAGE analysis, the multienzyme complexes have been studied in filamentous fungi for their synergistic functions (Borin et al. 2015; Gomes et al. 2017; da Silva et al. 2012). In both top-down and bottom-up approaches, the protein is identified by comparative study of in silico peptide or peptide fragments against sequence databases of peptide fragment fingerprinting and peptide mass fingerprinting (Listgarten and Emili 2005; Mann and Kelleher 2008) (Fig. 6.3).

To identify and characterize the diversity and quantity of adaptive and dynamic nature of secretome in response to stimulus, high-throughput LC–MS/MS techniques are being employed for *Trichoderma* species. A better understanding on lignocellulose targeting enzymes against wild-type and mutant *T. reesei* strains in the presence of carbon sources such as carboxymethyl cellulose (CMC) has been reported using LC–MS/MS techniques. A significant better and label-free quantitative representation of hydrolytic enzymes with exponentially modified protein abundance index (emPAI) was achieved by trypsin-mediated digestion of gel pieces and LC–MS/MS in acidic conditions. An alternate method for the estimation of protein abundances in multiplexed samples (PAMUS) comprising eight different secretomes of *T. reesei* QM6a and Rut C30 grown on glucose, starch, cellulose, and a mixture of starch and cellulose has been assessed. Here, protein abundance in the multiple sample has been estimated using exponentially modified protein abundance index (emPAI) method (Adav et al. 2013).

iTRAQ-based quantitative secretome analysis of both the mutant strain RUT-C30 and wild-type *T. reesei* strains QM6a identified a total of 636 secreted proteins, and 230 of them were quantified (Mann and Kelleher 2008; Adav et al. 2013). A combination of iTRAQ labeling approach and emPAI allows quantification of every identified protein under diverse conditions. Using the same approach, 63 cellulolytic and 34 hemicellulolytic enzymes have been identified and estimated in the secretome of *T. reesei* RUT-C30 and QM6a strains. A comparative study also revealed that the secretome of *T. reesei* RUT-C30 is inhibited by breakdown products of starch through feedback inhibition. *T. harzianum* biomass degrading secretome analysis using a combination of SDS-PAGE, LC–MS/MS, and 2-DE-MALDI-TOF/TOF in the presence of cellulose led to the identification of proteins which included xylanases, exo- and endo-glucanases, galactosidases, ara-

binofuranosidases, and chitinases (Do Vale et al. 2012). 2D fluorescence difference gel electrophoresis (2D-DIGE) coupled with LC–MS/MS identified several accessory proteins including cell wall glucanosyltransferase, SSCRP, ceramidase family proteins, CIP1, isoamyl alcohol oxidase, and amidase in the presence of sophorose which are otherwise not related to glycoside hydrolase (Fig. 6.4).

The secretome analysis of fungal culture grown in the presence of cellulose as carbon source showed the production of xyloglucanase CEL74a, polysaccharide monooxygenase, and xylanases in addition to classical cellulases. The secretome analysis of *T. asperellum* S4F8 and *T. reesei* RUT-C30 in the presence of SCB as carbon source revealed higher GH proteins, hemicellulases, and β-glucosidases in *T. asperellum* as compared to *T. reesei* RUT-C30. The comparative SDS-PAGE and label-free LC–MS/MS-based techniques reported a total of 174 GH families' proteins which were common to both species. For *T. harzianum* strain P49P11, the shotgun LC–MS/MS analysis of secretome displayed 32 proteins, belonging to 24 different GHs and 4 carbohydrate-binding module (CBM) proteins (Delabona et al. 2012a, b). The secretome analysis of *T. harzianum* T4 and *T. harzianum* IOC 3844 revealed the cooperative action of CAZymes and non-catalytic proteins for effective hydrolysis of plant biomass was observed using 2-DE-MALDI-TOF/TOF and gel-free shotgun LC–MS/MS (Francieli et al. 2011). Still, lack of verified annotations and the requirement of amino acid sequence similarity cutoffs limit its use (Dos Santos Castro et al. 2014). Similarly, the secretome analysis identified three putative

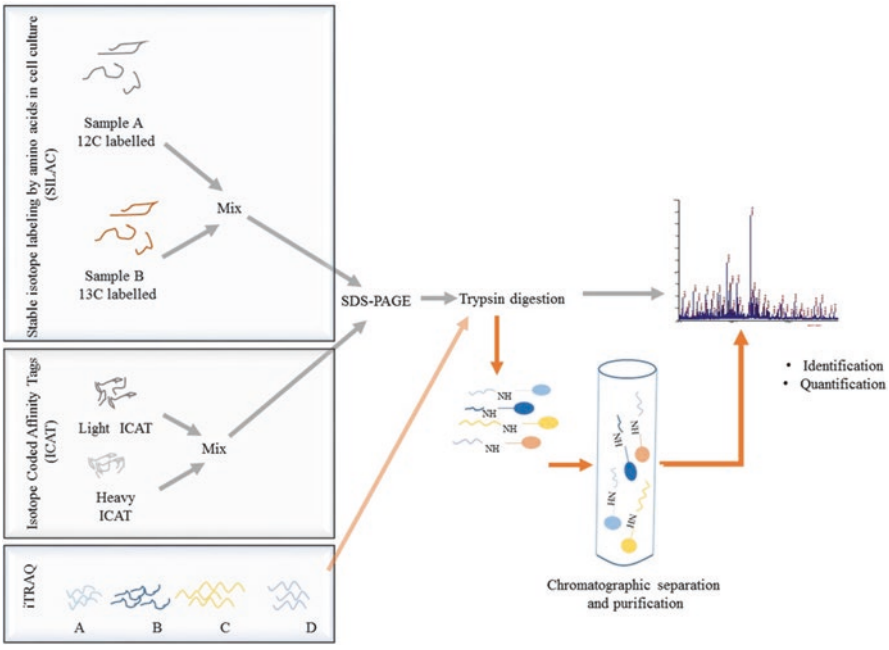

Fig. 6.4 Overview of proteomics approaches in protein identification and quantification

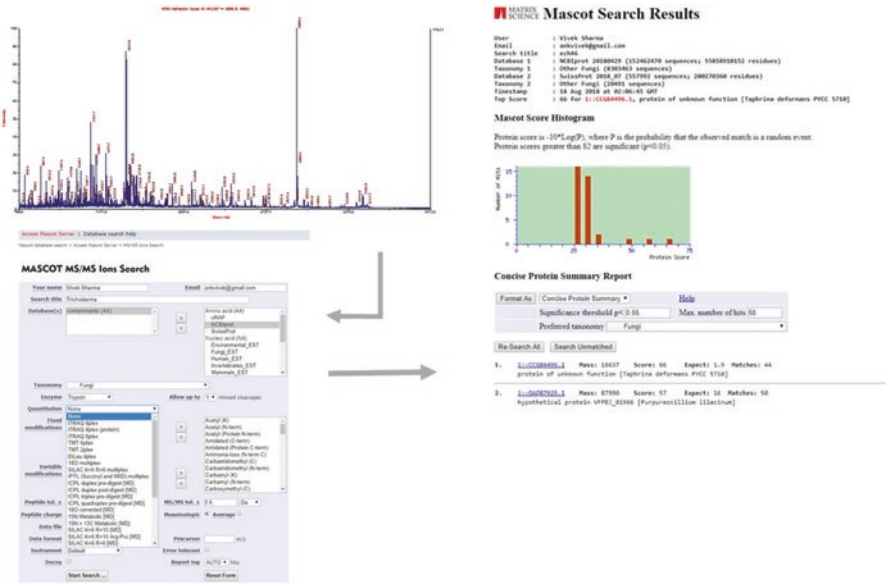

Fig. 6.5 Example of Mascot search results for extracellular protein of *Trichoderma*

complexes in *T. harzianum* secretome using BN-PAGE technique where native proteins bound to Coomassie Brilliant Blue maintains the integrity and prevents aggregation and negative charge acquisition due to denaturing 2D electrophoresis. The two-dimensional zymogram analysis revealed higher accuracy of spot identification. LC–MS/MS and homology-driven studies revealed interesting information about the formation of complexes by cellulolytic, hemicellulolytic, and chitinase enzymes (Fig. 6.5).

In the last decades, machine learning methods have emerged as a choice to identify the proteins and overcome the sequence similarity requirement of annotation. Recent developments in advanced computational methods have extended their role beyond basic sequence comparison. A number of complex machine learning methods such as cross-species cluster co-conservation and link propagation have been developed for predicting protein interaction networks, metabolic network reconstruction across the species, and predicting physical and functional role of protein–protein interaction and more specific biological networks (Kludas et al. 2016).

6.8 Translatome

The transcript expression at genomic level provides systematic monitoring of genes, and the high-throughput techniques have remarkably increased the transcriptomics data. Compared to stable nature of genome, the transcript profiling is dynamic and

regulated both at time and space level. The transcriptomic response may represent a collective response of absolute transcripts and transcripts actually involved in adaptive response through a subset of mRNA population actively translated. Thus, both the nature of mRNA and the environmental stimuli greatly modulate the translation process. The translation of mRNA in central dogma reprograms the cell activities by protein synthesis, and in a cell, the amount of total RNA and protein often shows a high amount of variation (Fig. 6.2).

Therefore, studies based on integrated omics approaches offer vital importance in accurate understanding of translational response. Studies based on translatome approach which target only active mRNAs can be vital tools in bridging the gap between proteomics and transcriptomics data and addressing the bottlenecks by accurate characterization of actively engaged mRNA population (Sharma et al. 2017a, b). Although ESTs, whole-genome, and transcriptome-based approaches have been instrumental in gene mining of *Trichoderma* during interaction with plants and plant pathogens, applications of these tools for mining active candidate genes are least explored. The mRNA and protein amounts do not correlate perfectly in native or engineered systems (Tian et al. 2004; Jayapal et al. 2008; Vogel and Marcotte 2012; Payne 2015). Therefore, the studies at posttranscriptional levels are of paramount importance in elucidating the functional role of a gene (Picard et al. 2013). The translatome, an approach targeting mRNA population engaged with ribosomes, is quite useful for the accurate identification of active mRNA population. Originally deployed in oocytes and embryos (Terman 1970; Gurdon et al. 1971), it has emerged as a key tool in gene characterization. Therefore, considering the background noise in transcriptome-related studies and other associated artifacts in the identification of differential expressed transcripts, translatome focusing only on active mRNA populations is a valuable tool (Picard et al. 2013; Yanguez et al. 2013; Piccirillo Ciriaco et al. 2014; King and Gerber 2016; Meteignier et al. 2017; Sharma et al. 2017a, b).

Experimental studies based on gene behavior at translational level are prominent tools for understanding the translational regulation in an adaptive response of the host (Halbeisen and Gerber 2009; Spriggs et al. 2010). The translatome-based studies help in the identification of key regulatory factors at translational level over the pools of genome-wide translated mRNA (Zupanic et al. 2013). Beside this, the translatome studies are of immense importance in determining the ribosome number on active mRNA molecule (Koritzinsky and Wouters 2007; Thomas and Johannes 2007; Picard et al. 2013). Three methods such as polysomal and ribosomal profiling and ribosome affinity purification (RAP) are used for translatome-based studies. The first approach involving polysomal profiling discovered in the 1960s separates the actively translated mRNAs bound to ribosomes by sucrose gradient centrifugation, and mRNAs are used for Northern blot or RT-qPCR, cDNA microarray, and RNA-seq analysis (Karginov and Hannon 2013; Spangenberg et al. 2013). On the other hand, ribosomal profiling initially used in *Saccharomyces cerevisiae* identifies the location of ribosomes at codon level or nucleotide level (Ingolia et al. 2009). The benefits of this technique involve the acquisition of information with respect to the position of the ribosomes on translated mRNA. Here, the deep nucleo-

tide sequencing site in mRNA fragment is protected from the action of RNase I treatment by ribosome (Ingolia et al. 2012). The polysomal and ribosomal profiling demands relatively large sample size to obtain enough RNA for analysis. The RAP discovered in *S. cerevisiae* captures monosomes and polysomes using antiFLAG affinity resin (Inada et al. 2002). The coupling of the RAP transcriptome analysis provides better insights and deep approximation of the translated mRNA (Halbeisen and Gerber 2009; Jiao and Meyerowitz 2010).

6.9 Functional Genomics View of Biosynthetic Gene Clusters

Synthetic biology and genetic engineering-based approaches can help in mining cryptic gene clusters in fungi (Wiemann and Keller 2014). These techniques can also activate transcription of silent gene clusters in natural hosts (Bouhired et al. 2007; Mao et al. 2015; Mattern et al. 2015) as well as the expression of uncharacterized gene or gene clusters in heterologous hosts (Chang et al. 2013; Heneghan et al. 2010; Kealey et al. 1998; Pel et al. 2007; Richter et al. 2014; Sakai et al. 2012; Yin et al. 2013). The recent expansion in synthetic biology tools such as identification of biosynthetic gene clusters (Medema et al. 2011), expression of multigene pathways, and refactoring of gene clusters has helped in regulating the expression of gene cluster which is otherwise cryptic under lab conditions (Unkles et al. 2014). In the modern era of continuous generation of DNA sequencing data and computational biology approaches, different biosynthetic gene clusters (BGC) can be identified based on homology and chemical modulation of their encoded products (Medema and Fischbach 2015; Pi et al. 2015; Throckmorton et al. 2015). Several gene clusters have been characterized, and new gene clusters can be identified in the genomes based on homology to previously characterized gene clusters (Cimermancic et al. 2014; Inglis et al. 2013; Medema and Fischbach 2015; Li et al. 2016).

6.10 Future Directions and Conclusions

Functional assignment of a gene to a primary metabolic network can be augmented based on genomic and proteomic approaches. These approaches help us to understand the behavior of an organism which can be explored for human welfare. The underlying mechanisms that led *Trichoderma* strains to grow on plant biomass and expand its nutritional range has vital importance in industrial and agricultural sectors. The technological advancements help us to understand the function of a gene or protein. The advances in next-generation genome sequencing and proteomics tools have played a vital role in understanding the lifestyle of different organisms to their varied habitat and also offer advantages of removing the ambiguity, errors, and other limitations of the previous generation tools. Similarly, a massive shift in

genome sequencing will lead to a better understanding of the biological and ecological roles of *Trichoderma* genes and proteins for biotechnological applications. The multifactorial role of *Trichoderma* offers tremendous scope to understand the genomic plasticity across different species in investigating the adaptive signatures under various ecological conditions. Keeping in view the adaptive lifestyle of *Trichoderma* species as plant symbionts to its potential in biofuel, it is tempting to directly compare the genomic- and proteomic-based analysis. Studies based on integrated techniques will play a pivotal role and provide unprecedented discoveries related to key enzymes common across the species of *Trichoderma* with fundamental role in biomass degradation for developing value-added products such as biofuel.

The use of whole-genome sequencing, microarray, subtractive libraries, and complete transcriptome analysis using new and in-depth RNA sequencing (RNA-seq) technologies and proteomic approaches such as iTRAQ has already led to the discovery of several genes and proteins with novel functions, for diverse biotechnological applications. Whole-genome stoichiometric metabolic models and complex system biology approaches have played remarkable roles in understanding the metabolism of an organism. The developments of classified expression databases such as filamentous fungal gene expression databases (FFGED; http://bioinfo.townsend.yale.edu) can provide a collective and collaborative platform for further research. Such databases are capable of comparing and correlating diverse experimental data and identifying candidate genes automatically and effectively (http://bioinfo.townsend.yale.edu/index.jsp).

Lack of knowledge on the primary metabolism of filamentous fungi is a major bottleneck in filling the gaps. Being fully benefitted from the recent genomic revolution, it is imperative to systematically catalog biosynthetic gene or gene clusters – associated metadata for diverse applications. Earlier approaches were focused on inferring differential genes, gene neighbors and their clusters, and their phylogenetic profiling. Recently, methods such as protein–protein interaction (PPI) networks for depicting physical interactions have been developed. In parallel, the conclusions derived from high-throughput techniques such as genomic, gene expression data, and proteomics analysis are of paramount importance to understand the role of a gene or its product in any given process. To understand the possible role and conditions involved in the transcription of gene and its translation to protein through gene expression and its exoproteome's correlation with the transcriptome, it is of absolute interest to understand the role of active candidate genes. Use of recent molecular approaches for identification and the quantitative estimation followed to automatic machine coupling and then their genome shuffling will provide a detailed insight of the active candidate genes in complex biological system in which are responsible for adaptive behavior of *Trichoderma* strains or species.

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Chapter 7
Genetically Modified Microbes for Second-Generation Bioethanol Production

Saurabh Singh, Anand Kumar Gaurav, and Jay Prakash Verma

7.1 Introduction

The present world economy is highly dependent on the stocked natural resources of the Earth, which are being used for the production of fuel, electricity, and other needs. The very high level of fossil fuel consumption has generated a high level of pollutants in the atmosphere, with the scenario being worse in urban areas. Because the level of greenhouse gases in the Earth's atmosphere has drastically increased, bioethanol has received worldwide interest. Bioethanol is a major second-generation biofuel. The global market for bioethanol has entered a phase of rapid, transitional growth. Many countries around the world are shifting their focus toward renewable sources for power production because of depleted crude oil reserves. The trend is extending to transport fuel as well. Most of the environmentally aware countries across the globe consider biomass for its economic utilization, and have directed state policies regarding the same, to meet future energy demands and also to meet carbon dioxide reduction targets. The primary focus is on reducing the emissions and thereby complying with the Kyoto Protocol for specified targets and also meeting energy demands. As well as the production of bioethanol, lignocellulosic biomass is also used in the production of both power and heat through combustion. Petroleum-based fuels can be replaced by bioethanol and other biofuels if biomass materials such as sugarcane bagasse, corn stover, switchgrass, and algae are effectively utilized. As a matter of fact, lignocellulosic biomass is the most abundant biomass present on the surface of the Earth. Among biomass sources, agricultural wastes are the most plentiful and cheapest, especially wheat straw, which is the

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most plentiful in Europe and is second worldwide after rice straw. As well as wheat, several other crops produce plentiful waste such as corn stover, sugarcane bagasse, and rice straw.

Second-generation biofuels are those fuels that are produced from waste lignocellulosic biomass. Agricultural farms produce an abundance of lignocellulosic biomass that is considered waste. Food materials in the form of lignocellulose compose less than one third of the total lignocellulosic biomass produced on agricultural farms, which indicates the amount of waste produced as lignocellulosic biomass. Apart from waste produced in the agricultural fields, an abundance of lignocellulosic waste is produced in the forests and on uncultivated lands. Leaf litter in the urban environment, for example, is typically treated as a waste. Although the leaf litter in forest areas becomes decomposed and is processed in an environmentally sound manner, urban leaf litter tends to be burnt for disposal. The burning and disposal of lignocellulosic waste in an environmentally unsound manner tends to increase the load of atmospheric pollutants. In many urban areas, a problem of smog conditions arises with the excessive burning of this waste. The waste on agricultural fields when burnt adds to this effect by several fold.

In this chapter we discuss different forms of microbes used in the production of bioethanol along with their modification at genetic level to enhance performance. Major emphasis is given to the fungal microbes as they are the major types involved in industrial production of bioethanol. Genetic modifications carried out in fungal strains, mainly *Trichoderma reesei*, are discussed in detail in this chapter.

7.2 Second-Generation Biofuel Production from Fungal Strains

Second-generation biofuel production typically consists of three major steps: pretreatment, enzymatic hydrolysis, and fermentation (Gupta and Verma 2015). Pretreatment is the step in which the waste undergoes different types of treatment to loosen the cellulose fibers from the other components, lignin and hemicellulose. The main aim of the pre-treatment process is to cause disruption of the lignocellulosic matrix and remove lignin from the complex of cellulose and hemicellulose, thereby facilitating the hydrolytic enzymes to bring about effective degradation. One of the common pre-treatment process currently in prevalence is the steam explosion, although the severity of this process generates several by-products that hinder further steps (Alvira et al. 2010; Jurado et al. 2009). An alternative to avoid these problems is the use of biological pre-treatments, which present additional advantages as being cheaper, safer, less energy consuming, and more environmentally friendly. Bio-pre-treatment is a term used by Salvachúa et al. (2011) for the pre-treatment of lignocellulosic waste by the application of microbes. The fungal strains provide a very good solution for this purpose. Many fungal strains such as *Poria subvermispora* and *Irpex lacteus* are used for bio-pre-treatment.

7.3 Fungal Strains and Bioethanol

Agricultural waste increases in direct proportion with agricultural food crop production. The produced waste, if utilized efficiently, can provide an alternative to the dwindling oil reserves. The production of bioethanol from fungal strains is seen as a very good approach for alternative biofuel production. Fungal strains, in general, are better and more efficient than bacterial strains for the conversion of lignocellulosic biomass into bioethanol. As can be seen from the literature, many fungal strains are available that can be employed at three important steps of bioethanol production from lignocellulosic biomass. In the biological pre-treatment stage, microbes, usually rot fungus species, are used for the degradation of lignin and other hemicellulosic compounds. These fungal strains include brown rot fungi, white rot fungi, and soft rot fungi, which bring about the degradation of lignin, hemicellulose, and some amount of cellulose (Sánchez 2009).

7.3.1 Fungi for Biological Pre-treatment

Biological pre-treatment, which mainly involves fungal strains to bring about the degradation of compounds other than cellulose in the feedstock, is an eco-friendly process. Lignin degradation in nature is found to be caused by a very few microorganisms, mainly basidiomycetes. According to a study by molecular clock analyses, it is suggested that lignin degradation originated around the end of the Carboniferous period, which also coincides with decrease in the rate of organic carbon burial (Floudas et al. 2012). Biological pre-treatment has an advantage over chemical pre-treatment because it avoids the production of acid by-products, thereby preventing the inhibition caused by such by-products. The use of fungal strains for biological pre-treatment is not feasible industrially because of its slow rate of action in comparison to other methods of pre-treatment such as physical pre-treatment and chemical pre-treatment. Many known strains of white rot fungus, including *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercoreus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnabarinus*, and *Pleurotus ostreatus*, are widely used for the biological pre-treatment of agricultural waste (Shi et al. 2008). Among these, *P. chrysosporium* is considered a good candidate for the degradation of lignin. Lignin, which is chemically very strong, is connected by three dimensions, making it a very strong polymer. White rot fungi thus help in reducing the lignin content of lignocellulosic material as they produce very strong lignin-degrading enzymes, thus providing a perfect delignified substrate for other subsequent uses such as biofuel production (Rodrigues et al. 2008). The ability of white rot fungus to selectively degrade lignin from the lignocellulosic biomass, producing cattle feed as well as a fuel production source, makes it a perfect candidate for biological pre-treatment. White rot fungus are the only organisms capable of substantial lignin degradation apart from brown rot and ectomycorrhizal species

(Floudas et al. 2012). One of the articles also suggests that a biorefinery coupled with a mushroom production plant can help in two ways, in sustainable biofuel production as well as fostering rural economy (Kalia and Purohit 2008). White rot fungi have other added advantages for consideration as a good pre-treatment agent as they have a nonspecific and nonstereoselective enzyme system, formed by lignin peroxidases and manganese-dependent peroxidases (Levin et al. 2008). Lignin peroxidases work by interacting directly with nonphenolic lignin structures, as they are themselves strong oxidants. Lignin peroxidases cannot penetrate the pores of strong lignocellulosic material. Manganese-dependent peroxidases, on the other hand, enter this strongly bound lignin matrix by producing small diffusible strong oxidants. Feruloyl esterase, an enzyme that brings about the conversion of ferulic acid and *p*-coumaric acid to hemicellulose, constitutes a key enzyme in the delignification process. This enzyme is found to show synergism with cellulases, xylanases, and pectinases, and thus it does not hinder other processes (Hermoso et al. 2004). The use of white rot fungus for the pre-treatment of wheat straw for 10 days resulted in a better process in three ways: reduction in acid loading for hydrolysis, thereby resulting in the increase of fermentable sugars, and last, reduction in amount of fermentation inhibitors (Kuhar et al. 2008).

Research on brown rot fungus is very limited and their characteristics have not been much explored, such as the ability of decolorizing wastewater and biosorption of heavy metals. Dey et al. (1994) studied the production of manganese peroxidase, which causes delignification of rice straw and has the ability to decolorize dye. Dey at al. also observed that the fungus *Polyporus zosteriformis* produced not only Mn peroxidase, acid protease, α-amylase, and lignin peroxidase, with maximum activities of 40, 8300, and 4200 U l^{-1} and 50 nkat l^{-1}, respectively, but also brought about 99% decolorization of Congo red dye in 9 days with 18.6% lignin removal in 3 weeks from rice straw (Dey et al. 1994). During the decay of lignin caused by brown rot, a chemical alteration in lignin takes place. A nuclear magnetic resonance (NMR) study of spruce decay by brown rot showed loss of methoxy groups, and cleavage of lignin β-*O*-4 linkages in the decay of birch (Pandey and Pitman 2003). In the same study, it was found that in the wood decay caused by the white rot *Phanerochaete chrysosporium*, the lignin content decreased along with the xylan content with the progression of decay. In *Coriolus versicolor*, lignin degradation was preferred over carbohydrate degradation. Brown rot fungus is the most prevalent wood-decaying fungus in coniferous forests, with a significant role in the conversion of wood into coarse debris with soil organic matter (Blanchette et al. 1994), whereas white rot fungi are known to cause degradation of lignin along with cellulose, with some having the ability to selectively degrade lignin (Eriksson et al. 1990). The mechanism through which the decay of the lignin by brown rot takes place in thought to be Fenton chemistry for the production of hydroxyl anions. During the process, methoxy carbon removal from lignin takes place with the production of an aromatic hydroxyl-rich product. The process is also thought to be brought about by the oxidation of aliphatic side chains of lignin (Agosin et al. 1989).

Phenolic compounds that are produced by fungi function as ferric iron chelators and sources of electrons for iron reduction (Kerem et al. 1999). The form of these low molecular weight reactants, which are small in size, makes it easier to penetrate the lignocellulosic matrix, thereby bringing about its degradation (Jellison et al. 1997).

7.3.2 Microbes for the Hydrolysis of Polysaccharides

The pre-treatment process is followed by the hydrolysis of the pre-treated substrate in the presence of hydrolytic enzymes. The main cellulose-degrading fungal species include *Trichoderma, Penicillium*, and *Aspergillus* (Galbe and Zacchi 2002). In general, cellulolytic bacteria are observed to produce lesser amounts of cellulase in comparison to the cellulolytic fungus. Among these, the best-known producer of cellulase enzyme has been found to be *Trichoderma*, which is known to produce a complex mixture of cellulase enzymes. The cellulase system consists of three different types of enzymes: endoglucanases, cellobiohydrolases, and β-glucosidases. A common aspect noticeable in this complex is that all these enzymes have specificity to hydrolyze β-1,4-glycosidic bonds. Also, these enzymes work in tandem and have a synergistic effect on the substrate to cause degradation. The enzyme β-glucosidase causes the cleavage of cellobiose formed by the hydrolysis carried out by endoglucanases and cellobiohydrolases. The endoglucanases bring about the degradation of the cellulose to cellodextrins, and the cellobiohydrolases subsequently convert cellodextrins into cellobiose. Cellobiose, a disaccharide, is then converted into glucose in the presence of the β-glucosidase enzyme (Gupta and Verma 2015). The process is supposed to happen in tandem, and accumulation of any of the products inhibits the activity of the respective enzymes because of product inhibition. Therefore, it becomes necessary for cellulose-degrading microbial species to produce all three specific types of enzymes in appropriate amounts to bring about the complete degradation of lignocellulosic waste or pre-treated cellulosic feedstock. The maximum activity of cellulase enzyme occurs at temperatures around 50° ± 5 °C at a pH of 4–5 (Saddler and Gregg 1998). Apart from that, the residence time of the enzymes over that substrate also are important in the production of greater amounts of glucose.

Many known fungal strains such as *Sclerotium rolfsii*, *Phanerochaete chrysosporium*, and *Aspergillus* spp., as well as *Trichoderma* and *Penicillium*, produce cellulase in large amounts. The use of *Trichoderma reesei* for the production of cellulase enzyme at the industrial level for application in different uses is credited to its ability to produce very high amounts of cellulase enzyme, up to about 100 g l^{-1} (Viksø-Nielsen 2008). The amount of enzyme production is also dependent on the type of inducer used in the cultures as a carbon source. It is observed that sophorose, which is a molecule containing two glucose units, linked by a β-1,2-linkage, induces maximum production of the cellulase enzyme (Mandels et al. 1962; Nisizawa et al. 1971).

In bacterial strains, *Bacillus* sp. are generally used for the production of cellulase enzyme on an industrial level: these are widely used in the textile industries for several purposes such as dye decolorization and preparation of fibers. Their use is preferred because of their broad range of growth conditions. Their maximum activity is obtained at a pH of 7, at which more than 85% of activity occurs (Jung et al. 1996).

In some anaerobic bacteria, a specialized mechanism is present to bring about the effective degradation of cellulosic materials, called cellulosomes (Béguin et al. 1987; Behera et al. 2014). Cellulosomes form as an alternative for the inability of anaerobic bacteria to effectively penetrate the cellulosic material: first found in *Clostridium thermocellum*, these were observed as large protuberances on the surface with scaffoldin protein and attached enzymatic subunits (Bayer et al. 1983, 1998; Fontes and Gilbert 2010; Lamed et al. 1983). These cellulosomes are an assembly of enzymatic subunits that aid in the successful hydrolysis of cellulosic material to monomer sugars. Apart from their ability to degrade cellulosic material, they are also found to possess the ability of gene regulation, and thereby helping in the production of new cellulosomal subunit.

7.4 Genetically Modified Microbes for Bioethanol Production

Genetically modified microbes have revolutionized the microbe-based industry. The prevalent genetic modification process is the gene cloning process, wherein a desired gene of interest from a selected microbe is inserted into a host microbe, typically *Escherichia coli*, with the help of a suitable vector (Fig. 7.1).

The industrial production of cellulase from different microbial strains is aimed at low cost and high enzyme production levels. The use of natural isolates for the production of industrially significant enzyme levels is not practical. Therefore, to enhance the existing levels of enzyme production in the naturally occurring fungal isolates, the microbial strains are genetically modified. The process of genetic modification in the fungal strains is best done by cloning cellulase enzyme genes or specific enzyme genes. The process can be summarized in the following steps: identification and isolation of the desired enzyme gene by digesting chromosomal DNA with restriction endonucleases and DNA fragments of a suitable range ligated with the digested material. The obtained product, the ligation mixture, is used for the production of the transformed strains. A fact of significance in the cloning of the cellulase gene is that its expression is regulated at the transcriptional level, which has been confirmed by differential hybridization studies (Messner and Kubicek 1991). The end product inhibition to the enzyme by glucose can also be explained at this level. It is also seen that glucose repression of cellulase occurs at this level. The addition of sophorose to the glucose-based cultures caused transcription activity to stop (Ilmen et al. 1997).

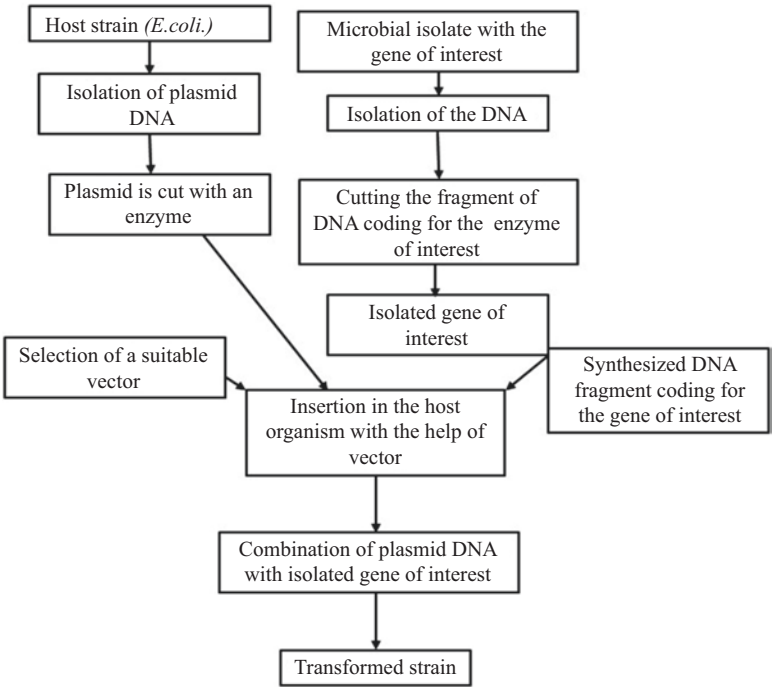

Fig. 7.1 Typical process of gene cloning and preparation of genetically modified microbes

7.4.1 Trichoderma reesei *and Its Application in Bioethanol Production*

The use of *Trichoderma reesei* in the bioethanol industry has long been prevalent . It is used for the production of beer, wine, and cheese owing basically to its hydrolyzing and fermentation capabilities. The idea of cellulase secretion from *Trichoderma reesei* has been explored with the possibility of a phenomenal change in the biofuel industry (Jeffries and Lindblad 2009). Research and development and the understanding of cellulase enzyme activities in detail have been possible through this microbial strain (Reese 1956; Mandels and Reese 1957, 1960). The first eukaryotic cellulase to be cloned was cellobiohydrolase CBH1, which was cloned from the same strain (Shoemaker et al. 1983; Divne et al. 1994). It has also been established that simply boosting the gene of interest cannot enhance the overall activity cellulose-degrading ability of the microbe. To overcome this, a fusion gene approach strategy can be used in which the encoded protein is also used as an expression enhancer apart from the promoter and terminator region of the gene of interest (Bischof et al. 2016). This strategy is a result of the research program at the Natick and Rutgers University in which increasing the efficiency of the extracellular protein of a *Trichoderma* strain QM6a by 20 fold through mutagenesis was studied.

Later, the QM6a was found to be a sterile female by Seidl et al. (2009), resulting in the isolation of a novel strain, RUT-C30, which currently serves as the prototype for the hyperproduction of cellulase enzyme and is available in the public domain (Mandels et al. 1971). The titers of the enzyme reach a level of about 30 g/l with lactose used for the cellulase-inducing substrate (Durand et al. 1988).

With the advances in technology and the need to improve the industrial production of the enzyme, techniques and methods were explored for continuous improvement. Today, high-speed atomic force microscopy has enabled us to visualize cellulose degradation by cellobiohydrolase CBH1/CEL7A on the cellulose surface (Igarashi et al. 2011). With nearly all the possibilities being explored in the transformation, these techniques were considered as the best option and had become available by the early 1990s (Gruber et al. 1990). Kuhls et al. (1996) found that *Hypocrea jecorina* is the sexual form of *Trichoderma reesei*, which opened new possibilities in the field of cellulase production. Whole genome studies of *Trichoderma reesei* began in 2003 with the first transcriptomic study by Foreman et al. (2003) of the gene expression of *T. reesei*: the DNA microarray-based cDNA library corresponds to more than 5000 different transcripts of the *T. reesei* genome. Subsequent studies on genome analysis led to the discovery of many potential factors associated with cellulase production: nucleocytoplasmic transport, vacuolar protein trafficking, and mRNA turnover (Bischof et al. 2016; Kubicek 2013; Le Crom et al. 2009). Presently, about 80% of ethanol production through the cellulase enzyme utilizes *T. reesei* strains (Bischof et al. 2016). One of the major setbacks observed in the secretions of the *T. reesei* strain formulation was the lack of sufficient β-glucosidase activity as it is bound to the fungal cell walls (Messner et al. 1990; Ryu and Mandels 1980). Genetically modified fungal strains are found to secrete lower levels of β-glucosidase (Lynd et al. 2002; Merino and Cherry 2007). Inefficiency in producing higher levels of this enzyme means that there is inefficient saccharification at the last step, whereby cellobiose is to be converted into glucose. Subsequently, cellobiose inhibition makes a significant difference in the final production of glucose. The addition of β-glucosidase in the reaction vessel can increase the production of glucose from cellobiose, which results from the combined effects of endoglucanases and cellobiohydrolases (Sørensen et al. 2013).

Of the many factors involved in the degradation of cellulose, C2H2 type transcription factor CRE1, which mediates carbon catabolite repression, is the major one (Bischof et al. 2016). In the presence of favorable carbon sources such as glucose, it shuts down the transcription of its target genes. However, the hybridized fungal strains show considerable increase in the amount of production of the cellulase enzyme.

In cloning the β-glucosidase gene from fungal strains, Takashima et al. (1998) cloned a novel fungal β-glucosidase gene (bgl4) with its homologue (bgl2), isolated from *Humicola grisea* and *Trichoderma reesei*, respectively. The results obtained showed the recombinant *H. grisea* BGL4 enzyme to be thermostable. Significant levels of β-glucosidase activity were obtained in both strains, and significant β-galactosidase activity was also seen in recombinant *H. grisea* BGL4.

7.4.2 Genetically Modified Bacteria for Bioethanol Production

The use of bacterial strains for bioethanol production is less explored in comparison to the use of fungal strains. Some bacterial species show enzyme activity of one of the many enzymes required in the cellulase system. Bacterial strains that degrade cellulosic material can be found naturally in locations such as the gut of ruminants, the gut of insects such as wood borers, and also some bacteria that inhabit extreme environments (Kawai et al. 1987; Honda et al. 1987, 1988; Srivastava et al. 1999). In the past, many studies have involved genetic modifications in microbes to enhance cellulase enzyme production. *E. coli* (JM83), a transformed strain, produced results of 1.51 U/ml for endoglucanase as well as 0.32 U/ml for β-glucosidase (Srivastava et al. 1999). In another study, *E. coli* RI, the transformant, was produced with the help of genes isolated from a thermophilic anaerobe NA10. As a significant finding, it showed maximum activity at 80°C, which can be very beneficial in reducing the steps involved in bioethanol production and thus assist cost-effective production, because the boiling point of ethanol is 78°C (Honda et al. 1988). In this study, the transformant endoglucanase activity was found to be 307 U/mg protein and exoglucanase activity to be 0.2 U/mg protein.

7.5 Conclusions

Research on different microbes for cellulose degradation found no single natural isolate that has good activity of all three enzymes: endoglucanase, exoglucanase, and β-glucosidase. Therefore, efficient production of glucose from a lignocellulosic substrate is hindered. The use of genetically modified microbes can help in the formation of new hybrid strains capable of producing all three enzymes in good amounts and thus allow the efficient production of glucose. Apart from the pretreatment processes, which should be standardized, the use of genetically modified microbes for the production of glucose from lignocellulosic biomass can be useful. The genetic modification in fungi has mostly been carried out with *Trichoderma reesei* variants. The use of fungal strains for this purpose has yielded better results in comparison with bacterial strains. However, bacterial strains offer many other advantages such as the diversity of forms present in the environment and ease of culturing. The use of extremophilic microbes for the purpose of cellulose degradation has also opened up new possibilities in this field.

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Chapter 8
Fungal Bioengineering in Biodiesel Production

Shubhra Tiwari, S. K. Jadhav, Gauri Dutt Sharma, and Esmil Beliya

8.1 Biodiesel and Its Advantage

The exploitation of fossil fuel and their impact on the environment have led to seek an alternate and sustainable source of energy. Biofuels may emerge as a new source of renewable energy. Biodiesel is an alternative fuel made from biological sources. It is an attractive energy source due to its environmental benefits. Biodiesel is produced by the process of transesterification. It can be extracted from algae, bacteria, and fungi. Fungi have great capacity to accumulate the lipids intracellularly. Due to its environmental benefits and renewable resources, biodiesel has become more lucrative in recent years. Microbial oils may serve as a potential feedstock for the production of biodiesel which need further research. Biodiesel, a liquid fuel, can be obtained from biological materials such as vegetable oil and animal fats. These contain free fatty acids, phospholipids, sterols, water and other impurities. Many vegetable oils have been explored for the production of biodiesel such as palm oil, soybean oil, sunflower oil, coconut oil, and rapeseed oil (Shay 1993). The advantage of biodiesel over fossil fuel is of its low toxicity, renewability, and rapid degradation more in comparison with diesel fuel. To use biodiesel, there is no need for engine modifications (Romano and Sorichetti 2011). Biodiesel fuel has drawn attention globally as a blending part of fuel diesel in vehicles (Demirbas 2009).

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8.2 The Raw Material for Biodiesel Production

There are various raw materials which are used for biodiesel production. Two types of plant oils, i.e., edible and nonedible, have been used for the biodiesel production.

8.3 Edible Plant Oils

Currently, biodiesel has been predominantly produced from edible vegetable oils and considered as the first generation of biofuel. Biodiesel is chiefly produced from soybean in the United States, rapeseed in Canada, sunflower in Europe, palm in Southeast Asia, and jatropha in India. Due to competition with edible oils, the large-scale production of biodiesel has been of great concern (Refaat 2010). The use of edible oil for production is mainly carried out by biodiesel producers which are the European Union, the United States, Brazil, and Indonesia. The worldwide largest biodiesel producers are the United States and Brazil; they produced 6 and 4.3 billion liters of biodiesel in 2017, respectively (Fig. 8.1) (https://www.statista.com/statistics/271472/biodiesel-production-in-selected-countries). In addition to overcome edible oil, researchers have sought some other renewable resources for biodiesel production (Ahmia et al. 2014).

8.4 Nonedible Plant Oils

As nonedible plantoils are not consumed by human due to its toxic components, these plants can be cultivated on wasteland, which provides a better choice for biodiesel production (Ahmad et al. 2011). Jatropha, karanja, tobacco, mahua, neem, and castor are important nonedible oil plants.

8.5 Microalgae

Microalgae are also used as raw material for biodiesel production and are called third-generation biofuel resource. Due to rapid biomass production and a good source of high oil content, the microalgae have been recognized as good sources for biodiesel production (Rengel 2008).

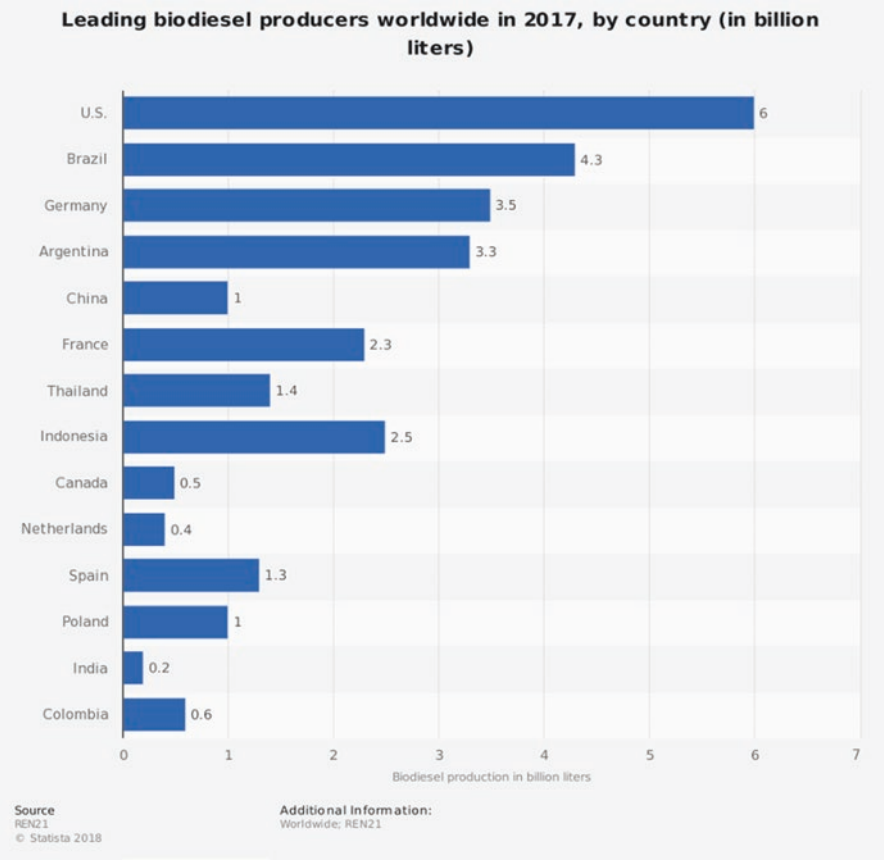

Fig. 8.1 Leading biodiesel production worldwide in 2017 (www.statista.com)

8.6 Animal Fat

The tallow, choice of white grease or lard, fish fat (in Japan), and chicken fat are an example of animal fats, which are used to produce biodiesel. In comparison with plant crops, these fats frequently offer a commercial benefit.

8.7 Biodiesel Production

Due to free fatty acids, phospholipids, sterols, water, odorants, and other impurities, the oil cannot be used as fuel directly. To overcome some chemical modification like pyrolysis, microemulsion, dilution, and transesterification have been done. Biodiesel is the mono-alkyl esters of long-chain fatty acids derived from renewable

feedstocks. It is composed of fatty acid methyl esters which are formed by the triglycerides in vegetable oil by transesterification process.

8.8 Transesterification

It is also known as alcoholysis, in which the displacement of alcohol takes place (Srivastava and Prasad 2000). This process has been used for the reduction of high viscosity of triglycerides. The process is called as methanolysis if methane is used in this process. Transesterification is a reversible reaction which is continued by mixing the reactants in the presence of a catalyst. The function of catalyst is to accelerate the reaction. It may be a strong acid or base.

The molar ratio of alcohol to oil, catalyst concentration reaction temperature, and reaction time are the main factors that affect the process of transesterification. Transesterification consists of a number of reversible and consecutive reactions. The triglyceride molecule is removed in the form of glycerin during the process. The triglycerides are broken stepwise into diglycerides and monoglyceride and finally converted into methyl esters and glycerol (Fig. 8.2).

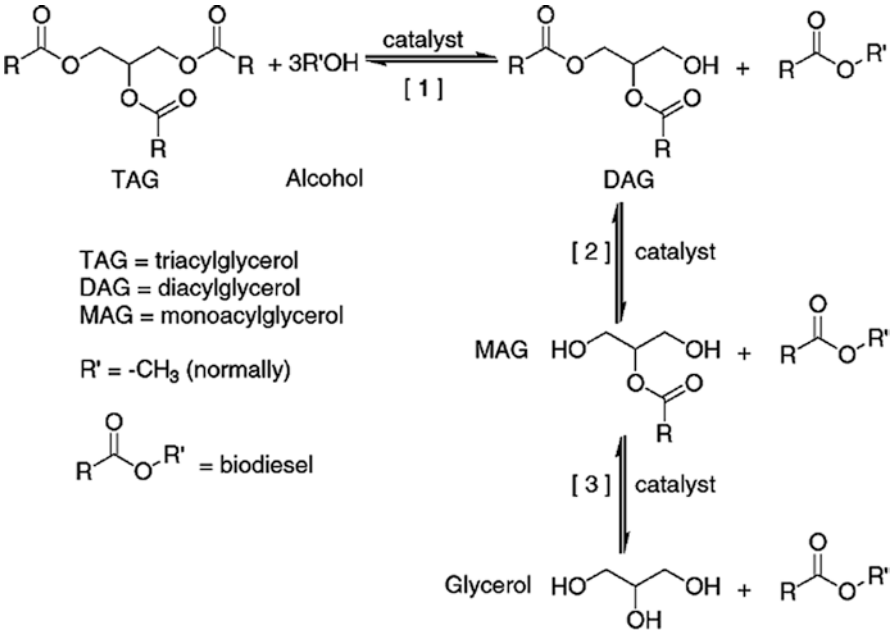

Fig. 8.2 Process of transesterification for biodiesel production (Gasahw et al. 2015)

8.9 Biodiesel Production by Lignocellulosic Biomass

Lignocellulosic biomass is the most attractive and largest biomass resource in the world that can be used as a raw material for the economic production of microbial oils. The production of ethanol and butanol has been done successfully. The major lignocellulosic biomass resources come from the residues generated by agricultural, forest, and industrial sources (Yousuf 2012). Lignocellulosic biomass can be categories into two groups (Fig. 8.3).

The use of agro-industrial residues has attracted the attention of researchers worldwide to overcome the limitations of traditional feedstock. This residual substrate is served as the nutritional source for microorganisms that accumulate lipids intracellularly. Lignocellulosic biomass is the cheapest and promising raw material to produce microbial oil from agrowaste (Huang et al. 2009; Dai et al. 2007). Lignocelluloses are complex in nature; they need pretreatment to release simple sugar. Various pretreatment techniques, physical, chemical, and biological methods, are used, depending on the properties of the substrate, pretreatment may be. Physical pretreatment involves the milling, grinding, steam explosion, sonication, etc. Chemical pretreatment can be done by acid or base hydrolysis (Lenihan et al. 2010; Taherzadeh and Karimi 2008). Enzymes produced by a variety of microorganisms are capable of breaking down the lignocellulosic materials to sugars, and it is considered as biological pretreatment.

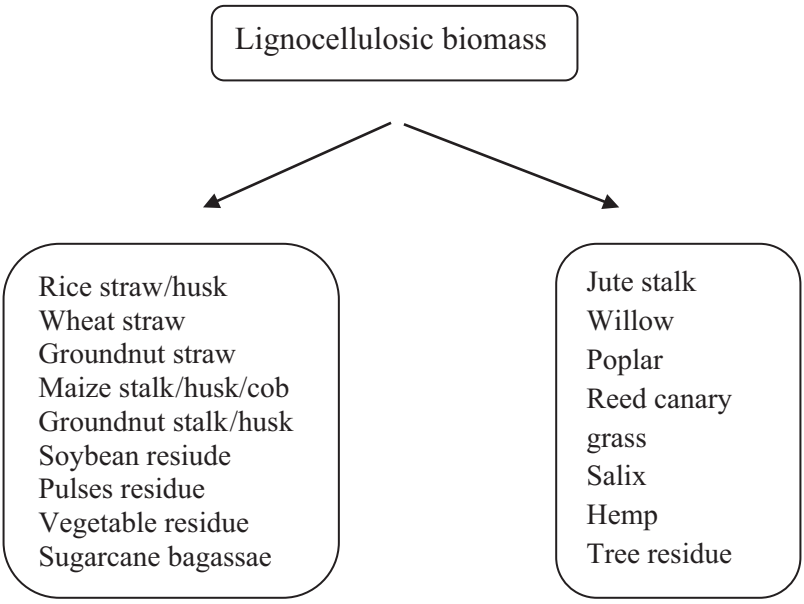

Fig. 8.3 Showing different lignocelluloses biomass for biodiesel production

8.10 Role of Fungi in Biodiesel Production

To overcome the negative impact of the biodiesel production from plants or animal resources, the use of microbes which can be easily grown can yield large amounts of lipids, like triglyceride; they have been considered as the most promising solutions (Tao et al. 2006). An oleaginous microorganism contains more than 20% of lipids of its dry weight, and it produces intracellular lipid (Mutanda et al. 2011; Meng et al. 2009). The production of microbial oil has many advantages over vegetable oils and animal fats due to fast growth of microbes, and oil requires less effort (Li et al. 2008). Several oleaginous yeasts and microalgae had been reported to accumulate as single cell oil (Aggelis and Sourdis 1997). Some lipid-accumulating yeasts are *Rhodotorula graminis* (Guerzoni et al. 1985), *Lipomyces starkeyi* (Holdsworth and Ratledge 1988), *Rhodosporidium toruloides*, *Cryptococcus albidus*, *Trichosporon pullulan*, and *Yarrowia lipolytica* (Liu et al. 2000; Liang et al. 2006). The oil accumulation in fungi depends on the carbon, nitrogen ratio, temperature, pH, oxygen, and concentration of trace elements and inorganic salt.

8.11 Advantage of Fungal Oil for Biodiesel

In microbial oil production, its cost depends mainly on the species of fungus chosen for cultivation and lipid concentration within cells. The biomass and oil productivity of oleaginous microorganisms are higher than plant oil productivity. The lipid content may be less due to slight change in the amount of sunlight. The use of low-cost and effective alternative feedstocks such as wastewater, municipal, and lignocellulosic wastes can improve the economics of single-cell oil production. In terms of a fast growth rate and productivity, oleaginous microbes are the choice for biodiesel production, as edible and nonedible oil plant farming needs lots of space and maintenance. The independence of weather provides an advantage for the microbe cultivation (Thevenieau and Marc-Nicaud 2013).

8.12 Fungal Bioengineering for Biodiesel Production

Fungal bioengineering is a part of genetic engineering that aims to either increase or decrease any metabolic product or any change in the microorganism for the production of a specific enzyme to enhance the production of the desired product. By this technique, the modification is done at the genetic level in the metabolic pathway. The desired gene can be inserted or knocked out according to the need. There are two approaches to increase the production of the biodiesel or desired product. The first way is changing the expression of specific genes to overcome specific rate-limiting steps in the target pathway that result in decreased catabolism of target

compounds combined with inhibition of competitive pathways. The second way is to change the expression of regulatory genes that controls structural genes downstream of multiple biosynthesis genes. In the context of biodiesel or biofuel production, genetically modified organisms are in focus for the production of green fuel (Verpoorte and Memelink 2002). In the transformation of microbes into the desired cell factories with high efficiency of biodiesel production, genetic engineering plays a key role. In gene cloning, RNAi technology is one of the best techniques used for bioengineering of biodiesel-producing fungi and also the genetic manipulation of lignocellulosic-degrading enzymes for the production of lipid. Bioengineering stability of engineered strains and methods are an important issue. The modification of lipid degree of fatty acid unsaturation or chain length of fatty acid is the main challenge, which all are regulated by different enzymes. Fungal bioengineering involves the cloning of the gene of an enzyme, their transgenic expression, and modification of genes to achieve high microbial recombination (Kalscheuer and Steinbuchel 2003).

A number of genetic tools have been developed to transform plasmids, knock out genes, and develop both episomal and integrative expression cassettes to enable metabolic engineering approaches for the bioengineering of fungi. For biodiesel production, microbial fatty acid metabolism is of great interest for the production of biodiesel. The microorganism has been modified in various aspects by overexpression of key fatty acid and TAG biosynthesis enzymes for high-level fatty acid production (Beopoulos et al. 2011). The overexpression of three fatty acid biosynthesis genes, namely, ACC1, FAS1, and FAS2, could increase the lipid accumulation by 17%. It was reported that the deletion of glycerol 3 phosphate dehydrogenase gene (GUT2) in yeast *Y. lipolytica* can increase the lipid synthesis manyfold (Runguphan and Keasling 2014). Due to deletion of GUT2, the formation of dihydrogen acetone phosphate (DHAP) is prevented from glycerol-3-phosphate which is the cause of the overexpression of glycerol-3-phosphate dehydrogenase1 (GPD1) and synthesis of more lipids. For the improvement of biodiesel feedstock, some other lipid biosynthetic pathway genes have been explored. They are acetyl-CoA carboxylase (ACC), diacylglycerol acyltransferase (DGA), D9-desaturase (D9), and ATP citrate lyase (ACL) genes regulating various steps in the lipid synthetic pathway. Presently, the oleaginous fungus *Mortierella isabellina* has also been reported for intracellular lipid production (Ruan et al. 2013, 2014). The successful bioengineering of the fungi to produce biodiesel requires more research and a better understanding of the strain improvements.

8.13 Conclusion

There is a need to focus more on alternative substitute of the fossil fuel and green source of energy. For biodiesel production, microbial oil might become one of the potential feedstocks due to renewability, low cost, and fast growth rate. Biodiesel obtained from fungal strain by employing cost-effective experimental approach can serve as one of the alternative platforms to compensate the rising energy crisis.

Genetic modifications through bioengineering can open new doors for the performance improvement of microorganisms producing oils. Biodiesel can provide an alternative solution to the environmental pollution caused by fossil fuel combustion and meet the increasing world energy demand.

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Chapter 9
Bioengineering Fungi and Yeast for the Production of Enzymes, Metabolites, and Value-Added Compounds

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9.1 Introduction

Fungi have traditionally been exploited due to their metabolic capabilities related to their ecological niches and adaptive strategies that include the production of enzymes, organic acids, and secondary metabolites, among others, to interact with substrates and other organisms. Also, some physiological and morphological characteristics such as their mechanisms for propagation and conservation as well as their remarkable secretory efficiency have played an important role in their habitat distribution.

Fungal bioengineering has played a crucial role in biotechnology since these microorganisms are able to produce diverse metabolites of great interest in different industrial sectors. The emergence of fungal genomics, proteomics, transcriptomics, and metabolomics research associated with the comprehensive analyses of genes, proteins, and metabolites ha allowed the efficient manipulation of genes of interest, greatly facilitating their expression and optimization. Genetic-editing methods such as CRISPR/Cas9, DNA recombinant technology, and metabolic engineering have collectively generated a robust and versatile fungal bio-environment for the production, in a very cost-effective manner of a wide array of value-added compounds such as alkaloids, terpenoids, flavonoids, polyketides, non-ribosomal peptides,

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biofuels, extracellular enzymes, polymers, and others. On the other hand, since growth and metabolite production are greatly affected by several parameters including cultivation medium, inoculum, pH, temperature, aeration, agitation, and other; it is particularly important to carefully select the bioprocessing strategies to efficiently produce the desired value-added compounds.

The yeast *Saccharomyces cerevisiae* has had a prevalent position in biotechnology that continues up to this date (Mattanovich et al. 2014; Kavscek et al. 2015). Further, there is an increasing number of several other yeasts that could be used more advantageously, to avoid, for example, the losing of carbon atoms in the production of ethanol since *S. cerevisiae* is a "crab tree-positive" yeast (Vieira Gomes et al. 2018). Among these "nonconventional" yeasts, the most frequently used are *Pichia pastoris* (*Komagataella* spp.), which adds fewer glycosylations on recombinant proteins, can use methanol as its sole carbon source, and secretes proteins at higher rates (Moser et al. 2017; Prielhofer et al. 2017); *Yarrowia lipolytica* for its ability to use hydrocarbons as carbon source and the synthesis of fatty products (Goncalves et al. 2014); *Kluyveromyces lactis* for its efficient production of recombinant proteins (Rodicio and Heinisch 2013); *K. marxianus* for its ability to grow and efficiently produce ethanol and other products at 45 °C (Nonklang et al. 2008; Gombert et al. 2016); etc. Yeast has been used for the production of therapeutical proteins (Kim et al. 2015) and other high-value products (Dai et al. 2015). Yeast has been also used for the synthesis of isoprenoids (Vickers et al. 2017), and numerous metabolic engineering studies have been performed to improve the yield of second-generation biofuels such as butanol isomers – up to ~16 mg/g glucose (Generoso et al. 2015; Mans et al. 2018). *S. cerevisiae* and particularly *P. pastoris* can also be used advantageously for the production of membrane proteins (Routledge et al. 2016; Dilworth et al. 2018). An important application is the production of biopharmaceuticals. Many of these have been produced in yeast and approved for human use: Insulin, glucagon, growth factors, enzymes, blood factors, and vaccines (Wang et al. 2017a). Because of the numerous literature available regarding the biotechnological applications of yeasts, this review will mainly focus on the genetic manipulation methods and the applications of filamentous fungi.

9.2 Genetic Manipulation of Yeasts and Filamentous Fungi

Yield improvement always requires physiological and genetic manipulation through mutagenesis or genetic engineering. For this purpose, the preferred species for biotechnological applications are those with rather small, known, and annotated genomes. The average genome size and number of genes are approximately 36.9 Mb and 11129.45, and 46.48 Mb and 15431.51 for Ascomycota and Basidiomycota, respectively (Mohanta and Bae 2015).

S. cerevisiae efficiently integrates DNA fragments at genomic sites that present homology to its flanking ends (Rothstein 1991). This characteristic has been key to allow for the easy manipulation of yeast for decades now (Sherman 2002; Duina et al. 2014). Several integration plasmids have been developed with increasing

advantageous features over the years (Guldener et al. 1996), and even with the availability of CRISPR/Cas9-based methodology, the design and development of such plasmids continue up to this date (Amen and Kaganovich 2017). The powerful applications of CRISPR/Cas9 in yeasts have added versatility to the yeast system as a biotechnological tool (Ryan and Cate 2014; Enkler et al. 2016; Kang et al. 2016; Walter et al. 2016; Weninger et al. 2016; Stovicek et al. 2017; Schwartz and Wheeldon 2018). *S. cerevisiae* also offers a wide array of cloning and expression plasmids, promoters, methods, and resources available that greatly facilitate their use in multiple applications (Sikorski and Hieter 1989; Gnugge and Rudolf 2017). A particularly useful strategy is the expression of recombinant proteins at the yeast surface (up to 10^5 copies per cell), which allows their displaying and interaction with other proteins or surfaces (Konning and Kolmar 2018). Importantly, such strategy could also be applied to enzymes, for various synthetic biology purposes (Tanaka and Kondo 2015; Konning and Kolmar 2018). On the other hand, Evolutionary engineering is a powerful strategy applied for the identification of strains with desired phenotypes. Evolutionary engineering can be also applied advantageously in yeast for the production of fuels and chemical products, with obvious important industrial applications. These processes might require the growing of cells for several generations (~50 to >100 generations) under a particular selective condition, to allow development of a particular evolved phenotype such as high temperature or chemical tolerance, synthesis of an essential micronutrient, faster sedimentation, etc. (Mans et al. 2018).

Otherwise, genetic manipulation of filamentous fungi is usually based on the transformation of strains, for which an exogenous DNA (with the gene of interest) is incorporated to a competent cell through a shuttle vector carrying also a suitable gene marker and flanking sequences. Once inside, gene rearrangement relies on two independent molecular mechanisms: homologous recombination (HR) for gene addition or replacement or, more frequently, ectopic integration based on nonhomologous end joining (NHEJ) (Fincham 1989; Krappmann 2007). The exogenous DNA should be expressed, maintained, and replicated in order to improve the phenotypic trait (Ruiz-Díez 2002). In practice, the delivery of DNA into the cell occurs at very low frequency, and to overcome this, protoplast-mediated transformation is the most commonly used method. Because there is no standardized unique protocol, each transformation strategy should be frequently optimized even when working with the same species or strain (Li et al. 2017). Modern strategies like CRISPR/Cas9 gene-editing techniques for knock-in and knockout of genes also require the use of protoplasts.

9.2.1 Mutagenesis

Conventionally, strain improvement for filamentous fungi was carried out using physical and/or chemical agents, to introduce random or directed changes in DNA. Although mutations can arise spontaneously at low frequency (1×10^{-10} to 1×10^{-5}/generation), the use of mutagens allows either better metabolic performance

or a higher number of harmful and lethal mutations (Parekh et al. 2000). Subsequent random or rational screening is important to select the desired mutants.

Several physical and chemical agents had been used for a long time to induce random mutations. The physical agents employed include ionizing radiation (γ, X-rays), which produces DNA breakage and causes deletions or structural changes; and UV rays, which dimerize thymines and might cause deletions, frameshifts, and base transversions (Parekh et al. 2000). Microwave radiation (nonionizing) has also been tested for strain improvement (Li et al. 2010). Some of the chemical agents used are base analogues that produce impairing, base deamination, and mainly AT→GC and GC→AT transitions (Parekh et al. 2000). Among these, the alkylating agents have a particular considerable effect in DNA causing GC→AT transitions, and because of their acute toxicity, most of them (nitrosoguanidine and mustard agents) are either prohibited or their use is highly regulated and restricted. Among other chemicals used, caffeine causes frameshift mutations, and it is effective in some bacteria and fungi. Also, hydrogen peroxide produces single- and double-stranded DNA breaks and base transversions. Finally, intercalating agents like ethidium bromide and acridine dyes cause frameshifts and deletions (Parekh et al. 2000).

The combined effect of physical and chemical mutagens has allowed the improvement of biotechnological important strains like *Trichoderma reesei* Rut C-30 (Peterson and Nevalainen 2012), *Penicillium chrysogenum* (Ziemons et al. 2017), and other fungi (Demain and Adrio 2008).

9.2.2 Genetic Transformation of Filamentous Fungi

Genetic transformation is the most fully applied technique for DNA transportation inside the cell and genetic modification in fungi for biotechnological purposes, which includes targeted integration of genes, site-directed mutagenesis, multicopy integration of genes, overexpression of genes, cassettes for the conditionally expression of genes, and introduction of heterologous genes (Turgeon et al. 2010).

9.2.2.1 Protoplast-Mediated Transformation

Because the fungal cell wall has a complex chemical structure, it prevents the uptake of DNA. In this sense, the common procedure before transformation includes the removal of the cell wall by enzymatic methods to obtain protoplasts from germinating spores, germinating tubes, or young mycelia.

Several factors should be considered in order to optimize the yield of protoplast formation and transformation efficiency:

- Selection of the starting material, which might comprise spores (conidia, basidiospores), germ tubes, or young mycelia, depending on the particular species.

- The digestion of the cell wall using an enzyme cocktail could represent the critical step, since the enzymatic activity even depends on the batch of production. Enzymes that degrade fungal cell wall include mainly β-glucanases, cellulases, chitinases, pectinases, proteases, xylanases, and L-glucuronidase, all available as commercial preparations from *Trichoderma harzianum*, *T. viride*, *R. solani*, *Aspergillus niger*, *Basidiomycetes*, *Helix pomatia*, and others.
- Osmotic stabilizers are very important to prevent the lysis of protoplasts during the enzymatic treatment. Sorbitol, sodium chloride, mannitol, sucrose, potassium chloride, and magnesium sulfate are the most commonly used stabilizers. Even the selection of a stabilizer depends on the fungal species, but sorbitol is the most commonly used. Once stabilized, the protoplast can be frozen for storage. Additionally, the regeneration medium for protoplasts must contain an osmotic stabilizer, and the recovery of transformants can be improved through a two-layer culture in which the bottom layer contains lower concentration of stabilizer than the top one (Ruiz-Díez 2002).
- Use of polyethylene glycol (PEG) and calcium ions is known to increase the uptake of DNA and constitutes an effective strategy for protoplast transformation. Low-molecular-weight PEG (PEG ·3000 or 4000) facilitates DNA adhesion onto cell membranes, while calcium ions increase the membrane permeability through the formation of pores or channels.
- Protoplast-mediated transformation is also highly influenced by the temperature, and the use of low (ice) or room temperature improves the transformation efficiency when PEG is used.
- Selectable markers are necessary for the recovery of transformants, especially because the filamentous fungi show very low frequency of transformation. For this purpose, there are several dominant (drug resistance) and auxotrophic markers (Meyer et al. 2010). Selection markers that confer drug resistance are preferred when there is no previous information about the genotype of the manipulated strain (Olmedo-Monfil et al. 2004). However, when using industrial strains and taking into account both biosafety and regulations, the auxotrophic or nutritional markers are commonly used.
- Either lineal or circular double-stranded plasmid can be used for transformation. The integrative plasmids usually contain long homology sequences and lack autonomous replication sequence, to allow the stable integration of the gene of interest into the genome.

As shown in Fig. 9.1 and described below, protoplast formation allows the genetic manipulation of fungi through several different methods.

9.2.2.2 Restriction Enzyme-Mediated Integration (REMI)

Random mutagenesis could also be achieved when a restriction enzyme is added during the transformation of protoplasts, spores, or mycelia to produce double-stranded breaks (DSBs) at its recognition sites in the genome. As the transforming

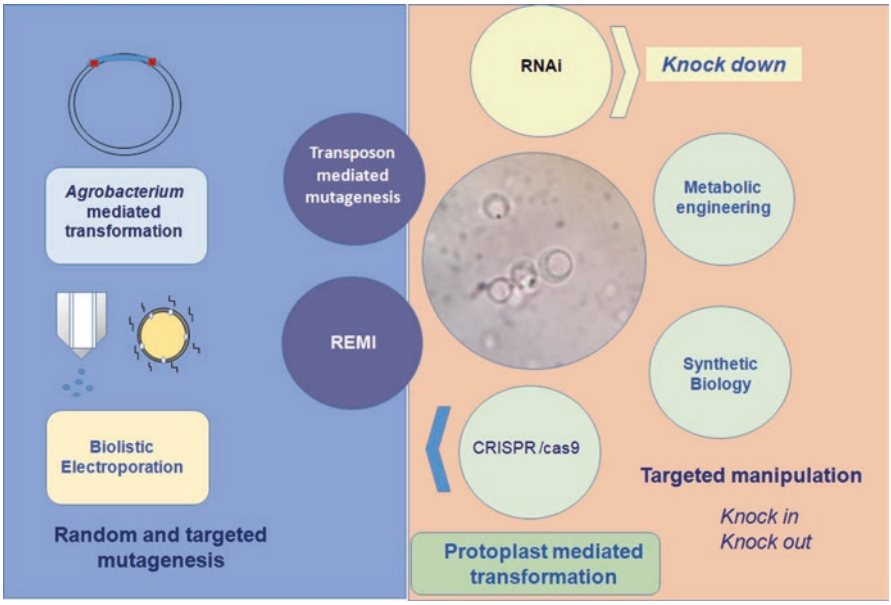

Fig. 9.1 Overview of the main genetic manipulation techniques in filamentous fungi. Legend: Apart from *Agrobacterium*-mediated transformation or biolistic and electroporation, most of genetic manipulation in fungi requires protoplast transformation. Random or targeted mutagenesis (light blue), knockdown (light yellow), or targeted genome engineering (melon) could be achieved by choosing adequate technique

DNA is treated with the same enzyme, more random integration events occur after DSB repair mainly by nonhomologous end joining (NHEJ). This procedure increases the transformation efficiency in many filamentous fungi, depending on the type and concentration of the restriction enzyme used (Vos et al. 2015). If plasmid rescue is desirable, the restriction sites must be known, and the genomic DNA should be digested with a restriction enzyme that does not cut the vector. In this case, the insertion site can be found by inverse PCR (iPCR) and thermal asymmetric interlaced PCR (TAIL-PCR) (Wang et al. 2017a, b).

9.2.2.3 Transposon-Mediated Mutagenesis

Mobile genetic elements like transposons are present in the main groups of filamentous fungi (*Zygomycetes*, *Ascomycetes*, and *Basidiomycetes*). Transposable elements (TEs), as in other eukaryotes, include class I (RNA) and class II (DNA) (Daboussi and Capy 2003; Fávaro et al. 2005). The TEs could be used as a very efficient method for discovering new genes and identifying gene function via high-throughput mutagenesis in filamentous fungi.

Also, both endogenous and engineered heterologous transposons can be used indistinctly without using a high-efficiency transformation method (Jiang et al.

2013). Known natural transposons, like *Vader* from *Aspergillus niger* (Hihlal et al. 2011) and *impala* (Fávaro et al. 2005), can be used for random mutagenesis in several species (Daboussi and Capy 2003).

9.2.2.4 *Agrobacterium*-Mediated Transformation (AMT)

AMT has been a widely used approach for fungal transformation, for more than two decades (De Groot et al. 1998). Through the engineering of vectors, AMT allows the integration of any marker or gene of interest in the genome without protoplast preparation, and it is successfully employed in *Ascomycetes*, *Basidiomycetes*, *Zygomycetes*, and other groups. Additionally, spores, germlines, or mycelia could be used as the starting materials for co-transformation.

Genetic manipulations using AMT include random mutagenesis (knockout), targeted genome modification (*knock-in*, replacements, overexpression, complementation), and random as well as knockdown strategies with RNAi (Frandsen 2011). The gene of interest is randomly inserted in the host DNA mostly as a single copy, which contributes to its stability. However, the frequency of transformation depends on the concentration of acetosyringone, the time and temperature of co-culturing, and the fungal inoculum.

Different vectors and technologies are available for T.DNA delivery in fungi, from plant binary vectors to cloning technology: Gateway system, Golden Gate assembly, and BIBAC system for vector design (Idnurm et al. 2017)..

9.2.2.5 Electroporation

Exogenous DNA can be incorporated to spores, fungal cells, or protoplasts by inducing the reversible formation of micropores in the cell membranes using an electric shock with the appropriate field intensity. Different parameters need to be considered, including electric field intensity, capacitance, pulse duration, and frequency with regard to the electroporator, as well as pH, temperature, and the concentration of the exogenous nucleic acid (Li et al. 2017).

Unlike bacteria and yeast, only a very low number of transformants are obtained when using electroporation of intact cells of filamentous fungi. For most species of fungi, to improve the frequency of transformation, an enzymatic treatment of cells walls is needed in combination with the electric field. However, this treatment might cause the decreasing of cell viability because of irreversible damages in the membranes. Another option is the use of protoplasts, especially for genera that have thick walls, and high chitin and chitosan contents. By using this approach, it is possible to achieve a twofold increase in the transformation efficiency. Alternative modifications that allow improvements in the efficiency of the electroporation method include the addition of PEG, dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) (He et al. 2016).

Despite its rather low efficiency, electroporation represents a simple, fast, and cheap procedure for the transformation of filamentous fungi (Rivera et al. 2014).

9.2.2.6 Biolistic

Biolistic (biological ballistics) consists in the bombardment at high speed of microparticles (usually made by gold, tungsten, or platinum) coated with DNA to impact spores or hyphae. When a microparticle enters the cells, the DNA is released and could be inserted randomly in the host genome. Suitable conditions for using a gene gun to transform fungi are the use of an inert gas (He) with a pressure between 500 and 2000 psi and an acceleration of micro-projectiles to speeds of 400 m/s or more, in partial vacuum (~30 mm Hg) (Rivera et al. 2014).

The main advantages of this method are related to its simplicity, non-requirement of a vector or specific sequences for DNA delivery, non-dependence on the electrophysiological properties of the cell, and also that avoids the use of protoplasts. However, as is the case for other physical approaches, the transformation efficiency is very low. Additionally, the integration of the DNA could occur as multiple copies in the genome, thus increasing the probabilities of gene silencing, low stability, or altered genetic expression (Rivera et al. 2014).

Even with these considerations, the biolistic method has been applied successfully in several filamentous fungi, such as *Aspergillus*, *Trichoderma*, and others (Su et al. 2012).

9.2.2.7 RNA Interference (RNAi)

Despite the technical limitations for efficient genetic manipulation, gene expression can be controlled through RNA interference (RNAi) in a post-transcriptional level to disrupt translation (gene knockdown). In natural conditions, RNAi is induced by foreign nucleic acid sequences (RNA viruses and transgenes) (Weld et al. 2006). Previously, a dicer complex cleaves dsRNA to produce a small interference RNA (siRNA), which hybridizes with the target RNA (based on sequence similarity) and guides it to a silencing complex (RISC) that degrades the target messenger RNA (Salame et al. 2011). Engineered RNAi could be used for the knockdown of genes as a tool for functional genomics in fungi. It contains an inverse repeat of the target sequence that folds up into a "hairpin" to produce a double-stranded structure (Weld et al. 2006).

RNA-based knockdown is especially useful when multiple copies of the gene of interest are present in the genome or when the deletion of the target gene causes lethality. However, the main limitations of this method are related to the stability of the silenced strain and the possibility of an incomplete and/or reversible silencing (Meyer 2008; Salame et al. 2011).

9.2.2.8 CRISPR/Cas9-Mediated Transformation

Gene edition technology, especially the CRISPR/Cas9 system, has emerged as a promising and versatile tool for genetic manipulation. The CRISPR/Cas9 technique relies on a RNA-guided system, in which the Cas9 nuclease is directed by a single chimeric (gRNA) guide RNA (containing a 20 nt region, called the *protospacer*, which is complementary to the target site in the DNA), to produce a break in a double-stranded DNA. Additionally, to induce the break, another short-sequence "NGG" (protospacer adjacent motif (PAM)) is located 3–5 bp downstream of the site to be cut.

The break could be repaired by nonhomologous end joining (NHEJ) pathway generating indel (random insertions and deletions) or frameshift mutations and premature stop codons. Advantageously, if there is a co-transforming gene sequence, the editing complex could repair the DNA by homologous recombination (HR), thus allowing gene targeting (Nødvig et al. 2015; Deng et al. 2017).

The main strategies to introduce the editing cassette or genes include transformation or integration in the host genome of DNA encoding Cas9 and the sgRNA, the use of a plasmid to express them, transformation of DNA encoding Cas9 and subsequent transformation of in vitro-transcribed sgRNAs, and incorporation of the editing complex CRISPR/Cas9/sgRNA ribonucleoprotein (RNPs). The transformation could be achieved via different methods that involve protoplasts and PEG, *Agrobacterium*-mediated transformation (AMT), electroporation, or a biolistic approach. Cas9 and sgRNA-mediated traGene edition has been successfully used for single-gene disruption, marker-based single-gene replacement and precise editing, marker-free precise editing, multiple gene disruption, or replacement in different species of *Aspergillus*, *Trichoderma*, *Ustilago*, *Alternaria*, *Fusarium*, *Phytophthora*, and others, for several applications (Schuster and Kahmann 2019).

The system has the advantages of its simplicity and the high specificity of the editing complex. Once implemented in the host, it is possible to introduce changes in another gene by modifying the sgRNA spacer sequence, as well as silencing several genes simultaneously by transforming the cell with different sgRNAs, along with Cas9 (Hsu et al. 2014). Nevertheless, the most interesting advantage is that it allows marker-free deletions by using transient expression from plasmids that can self-replicate only under antibiotic pressure (Vicente Muñoz et al. 2019).

Practical applications include the use of CRISPR/Cas9 in a very promising approach, to extend the substrate range of an organism, expanding its capacity to grow on a variety of carbon sources such as biomass-derived sugars (de Paula et al. 2019). Although the application of CRISPR/Cas9 technology in filamentous fungi is currently still in its infancy compared to other microorganisms, especially bacteria, this tool has already been successfully used in fungi engineering, for several biotechnological applications (Shi et al. 2017).

9.2.2.9 Metabolic Engineering and Synthetic Biology

Modern approaches for bioprocess optimization are strongly supported by genetic engineering for strain improvement, as well as the use of functional genomic technologies to produce quantitative data from different biological phenomena. On this basis, it is possible to adjust the chemical and physical conditions in order to obtain maximal production of the desired compound. In synthetic biology, information about structural and gene-coding sequences and regulatory elements allow the assembly of genetic circuits, biosensors, and different devices, including microorganisms. Complementary to his approach, metabolic engineering seeks the optimization of cellular processes or metabolic pathways to produce a desired compound from a substrate that is, preferably, cheap and simple (García-Granados et al. 2019). Further, for the implementation of synthetic biology approaches, several aspects need to be taken into account, such as the modulation of transcription factors controlling carbon and nitrogen metabolisms to alter the metabolic flux and the skewing of both primary and secondary metabolites (Park et al. 2017).

Collectively, the filamentous fungi are a very attractive source of genetic and regulatory sequences, genes that code for enzymes, and others. Additionally, these organisms are considered as excellent chassis for the development of cell factories, following an engineering approach for metabolic pathway redesign. The main strategies to engineer filamentous fungi for industrial biotechnological purposes include the use of single modules and genetic or metabolic design. For filamentous fungi, the synthetic promoters as well as CRISPR/Cas9 editing have been successfully employed, for different applications (Martins-Santana et al. 2018).

Recently, a novel synthetic expression system for heterologous production in filamentous fungi and yeast has been developed, which uses a synthetic transcription factor. This system consists in two expression cassettes, one with a core promoter to allow the basal expression of a synthetic transcription factor (sTF), which in turn stimulates the transcription of a second cassette with a coding region (gene of interest, GOI). Strong expression of heterologous genes could be achieved by including upstream binding sites (BS) for the transcription factor and selected core promoters. This system allows fine-tuned metabolic pathway optimization in multiple hosts if genetic stability is achieved in order to reduce the probability of recombination among the repetitives TF-binding sites (Rantasalo et al. 2019).

9.3 Engineering Fungi for Biotechnological Applications

9.3.1 Enzymes

Fungi are the most important biological sources of enzymes, producing nearly 60% of commercial enzymes (native or recombinant) (Østergaard and Olsen 2011). Filamentous fungi, such as *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* play important roles in the agricultural, food, and pharmaceutical industries

due to their high rate of protein secretion (Shi et al. 2017). Additionally, these strains are considered as generally recognized as safe (GRAS) because they have been used for a long time for the production of food enzymes for humans and animals (Sewalt et al. 2016).

Interestingly, the genus *Aspergillus* alone provides more than 25% of all industrial enzymes, and other important fungal producers include *Trichoderma*, *Penicillium*, *Rhizopus*, and *Humicola*, accounting for the production of 20% of the industrial enzymes (Østergaard and Olsen 2011). The main applications of industrial enzymes include specialized enzymes (mainly proteases, amylases, and cellulases that are used in the manufacturing of detergents, starch, textiles, leather, paper and pulp, and personal care items), enzymes for the food industry (e.g., proteases, lipases, pectinases, and other enzymes that are used for the manufacturing of dairy products, wine and juice, fats, and oils; for brewing or baking), and enzymes for animal feeding uses (like cellulases, phytase, xylanases, and β-glucanases used to improve fiber digestibility) (McKelvey and Murphy 2017).

Classical genetic manipulation approaches like random mutagenesis have been employed for many years to improve strains for enzyme production. For example, the *Trichoderma reesei* wild-type strain QM6a was treated with irradiation of conidia to obtain the mutant QM9414, which exhibits at least two-fold more cellulase production while remaining as a catabolite-repressed strain. Further strategies of mutagenesis combining physical and chemical agents simultaneously or sequentially were carried out to obtain improved cellulolytic strains like QM6a, M7, and NG14 (catabolite de-repressed strains). Finally, after UV mutagenesis and screening for increased resistance to 2-deoxyglucose (2DG), the RUT-C30 strain was isolated as a hypercellulolytic, hypersecretory, and catabolite de-repressed strain. *T. reesei* RUT-C30 is considered as a paradigm for developing processes for the production of cellulolytic enzymes. Genomic approaches have revealed the cellular, genetic, and molecular mechanisms underlying the hyperproducing capability for cellulases of RUT-C30, which also exhibits higher rates of protein secretion (Peterson and Nevalainen 2012).

Post-genomic approaches for engineering *T. reesei* are based in metabolic engineering for the manipulation of carbon catabolite repression (CCR), nutrient sensing, chromatin remodeling (Gupta et al. 2016), and the establishment of a regulatory network for genes encoding cellulolytic and xylanolytic enzymes (Shida et al. 2016). For example, an engineered *T. reesei* strain was developed using multiplexed CRISPR/Cas9 and a synthetic expression system (using the inducible cbh1 promoter), to produce high amounts of highly pure lipase B from *Candida antarctica* (Rantasalo et al. 2019). Synthetic biology approaches are using artificial transcription factors to express cellulase genes constitutively (Zhang et al. 2018b), minimal transcription activators (constructed with one native DNA-binding domain (DBD) and one transcriptional activation domain), and new regulators (like the VIB-1 family regulatory protein) (Zhang et al. 2018a, b).

On the other hand, *Aspergillus* displays a wide array of enzymes including α-amylases, glucoamylases, glucose oxidase, lipases, proteases, phytases, amyloglucosidases, pectinases, cellulases, etc. that are used in the food, starch processing,

textile, and paper industries (Brandl and Andersen 2015; Mojsov 2016). The bioengineering strategies for *Aspergillus* started with classical mutagenesis. The most prominent example is the development of the glucoamylase-producing strain *A. niger* NRRL 3122 generated by classical mutagenesis (Baker 2006). Other independent examples include (Teotia et al. 2016) the three-fold increase of extracellular phytase production by *A. niger*, achieved applying two UV treatments and resistance to 50 μg/mL hygromycin B as the selection method (Chelius and Wodzinski 1994); the treatment of *A. niger* with UV and nitrous acid (NA) to obtain, among several mutants, the strain NAII with 2.53 times higher lipase activity compared to the parental strain; and the use of UV irradiation (254 nm) and N-methyl-N′-nitro-N-nitrosoguanidine in three sequential and combinatorial treatments to enhance the production of pectic acid-degrading enzymes, from *A. sojae* ATCC 20235 and mutants of *A. sojae* CBS 100928. The latter example produced the improved strain M3, which showed at least 1.7 times increased activity than the wild strain *A. sojae* ATCC 20235. Further, two more cycles of UV treatments generated mutant DH56 with 2.4-fold enhanced polygalacturonase production (Heerd et al. 2014).

Other representative examples are the improvement of an *Aspergillus* sp. strain for the production of a raw starch-digesting enzyme by solid-state fermentation, which was achieved by two repeated sequential exposures to γ and UV irradiations and four repeated treatments with N-methyl-N′-nitrosoguanidine. The mutant strain *Aspergillus* sp. XN15 demonstrated 19.4 times greater activity than the wild-type strain (Vu et al. 2010). For the production of cellulases, the spores of a strain of *Aspergillus niger* were subjected to UV irradiation and treated with ethylmethane sulfonate (EMS). The isolated EMS5 mutant exhibited 30–50% enhanced cellulase production over the parent/wild-type strain (Reddy et al. 2017). The strain *A. niger* DS03043, called GAM strain for the DSM company, is a glucoamylase overproducer derived by classical mutagenesis from the original strain *A. niger* NRRL3122 (van Dijck et al. 2003).

Furthermore, classical strategies can be integrated with omics tools to obtain genetically engineered strains for the homologous expression of enzymes A recent example is the combined use of random mutagenesis of the strain *A. fumigatus* LMB35Aa (Paul et al. 2017) with treatments with DES (diethyl sulfoxide) and hydrogen peroxide. Among the isolated mutant strains, LMB-35Aa-6I and LMB-35Aa-6II showed 1.8-fold increases in enzymatic activity at pH 8.4 (96 hours), and LMB-35Aa-12II showed a 3.2-fold increase in the activity at pH 9.4. (72 hours). Transcriptomic analysis of the best cellulase-producing strain revealed that the cellulose catabolic process had been enriched in this mutant, and further, a three-fold higher expression of the non-characterized endoglucanase gene (*afu7g0154o*) at 37 °C (Fig. 9.2, Angel Zhang, Yvette Ludeña, and Gretty K. Villena, unpublished work).

The advent of genetic engineering has allowed the improvement of strains by increasing the copy number of genes using knock-in strategies, the modulation of gene expression through the use of strong promoters, knockout of unwanted genes, manipulation of metabolic pathways, and the development of fungal strains for the

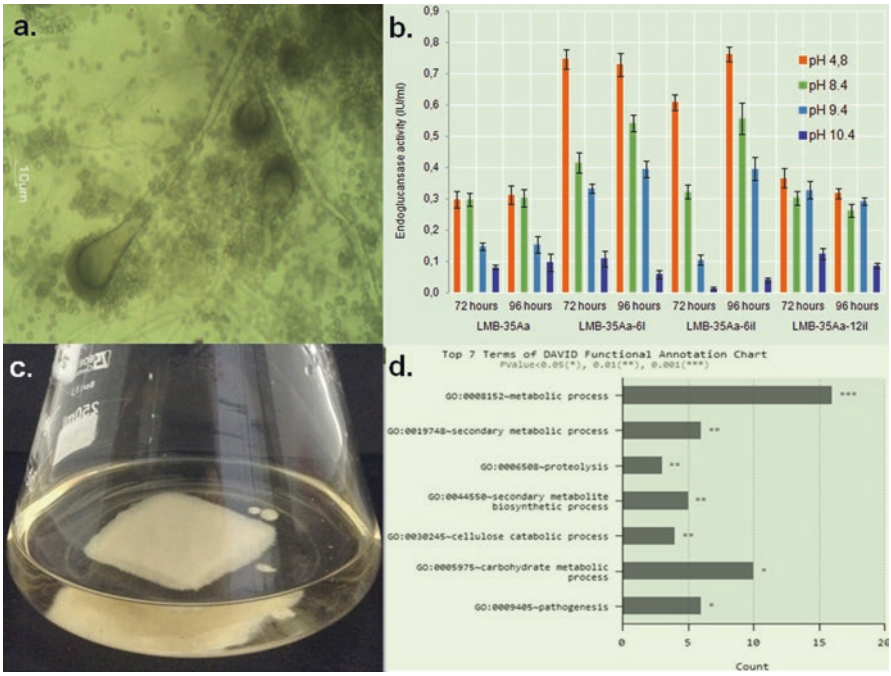

Fig. 9.2 Random chemical mutagenesis for improving cellulase production in *Aspergillus fumigatus LMB-35Aa.* Legend: (**a**) Wild strain LMB35Aa was treated with diethyl sulfoxide and hydrogen peroxide to obtain mutants LMB35Aa-6I, LMB35Aa 6II, and LMB35Aa-12II, which have better endoglucanase activity at neutral–alkaline pH range. (**b**) Cultures were grown as biofilms, (**c**) and transcriptomic gene ontology enrichment revealed that cellulose catabolic process was induced in the strain LMB35Aa-12II (**d**) (Angel Zhang, Yvette Ludeña and Gretty K. Villena, unpublished work)

production of heterologous proteins. The main fungal hosts for the production of heterologous and engineered gene products belong to the genus *Aspergillus (A. niger*, *A. awamori*, *A. oryzae*), followed by *T. reesei* (Nevalainen 2001). Some important factors that could limit the heterologous production of enzymes and proteins include codon usage frequencies, proteolytic processing, undesired proteolysis, protein folding, glycosylation, missorting (Nevalainen 2001), availability of promoters (the most commonly used are *gla*A and *alcA/alc*R) and selectable markers, signal peptide optimization, and the efficiency of protein secretion (Gomez et al. 2016). In this regard, *Aspergillus* strains show advantages related to the attainment of correct folding of some eukaryotic proteins because of their adequately introduced post-translational modifications including glycosylations, proteolytic cleavages, and disulfide bond formation (Dimarogona and Topakas 2016).

The GAM strain of DSM Industrial has been utilized as a host for the production of different enzymes. For the large-scale production of phytase, multiple copies of the phytase *phy*A gene from *A. niger* NRRL3135 were integrated under the control of the host promoter *gla*A ans, and two commercial strain were obtained: *A. niger*

DS25956 and DS27301. Following a similar procedure, the commercial production of xylanase was achieved with the improved strain *A. niger* DS26538, which contains multiple copies of an endo-1,4-b-xylanase gene from *A. niger* DS16813. Further engineering strategies were applied to establish the strain DS03043 as DSM Industrial's primary host for the production of other enzymes, such as the deletion of multiple copies of the *gla*A gene (Δ *gla*A loci) and the inactivation of the major protease pepA (van Dijck et al. 2003).

Furthermore, genes that code for many cellulases (endoglucanases, cellobiohydrolases, bacterial cellulases), auxiliary enzymes LMPOs (lytic polysaccharide monooxygenases), hemicellulases (glucanohydrolases, xylanases, arabinanases, xylosidases, arabinofuranosidases, mannanases, feruloyl esterases, glucuronoyl esterases), lignin-modifying enzymes (manganese peroxidase, lignin peroxidase, versatile peroxidase, dye-decolorizing peroxidase, glyoxal oxidase, laccases, pyranose dehydrogenase, cellobiose dehydrogenase from *Trichoderma*, *Aspergillus*, *Penicillium*, *Fusarium*, *Geotrichum*, *Trametes*, and *Coprinus*, among other fungal species as well as bacteria and microbiomes), and others have been successfuly expressed in *Aspergillus* species (Dimarogona and Topakas 2016). Additionally, pancreatic phospholipase A2, prochymosin, egg-white lysozyme, triglyceride lipase, aspartyl protease, and chymosin have been produced in *A. niger*, *A awamori*, and *A. oryzae* (Fleißner and Dersch 2010). Importantly, a recombinant strain of *Aspergillus oryzae* producing an aspartic proteinase from *Rhizomucor miehei* has been used commercially, for cheese production (Adrio and Demain 2003).

Transcriptomic analyses were performed on *Aspergillus nidulans* recombinant strains producing alpha-arabinofuranosidase (AbfA), beta-glucosidase (BglC), and thermophilic mannanase (Tp-Man5), finding that the heterologous genes *abfA* and *bglC* were highly expressed, while *tp-man5* was not. Interestingly, thirty differentially expressed genes were found to be common to all the recombinant strains, suggesting that these changes represent a general response to the expression of heterologous genes (Calzado et al. 2018).

More recently, an in silico analysis of the *Aspergillus niger* GSMM genome-scale metabolic network model was performed to elucidate the reaction equations and gene–protein associations required for the strain improvement and process optimizations for glucoamylase production (Lu et al. 2017). For the heterologous expression of the same enzyme, a systematic approach to study protein secretion in *A. niger* was done by placing the *gla*A gene under the control of the tunable Tet-On system. This study demonstrated that the levels of post-Golgi carriers heavily depend on and correlate with the levels of expression of the *gla*A gene (Fiedler et al. 2018).

In addition, advances in the use of CRISPR/Cas9 technology for engineering filamentous fungal systems have facilitated the development of engineered strains of *Aspergillus* and other species (Donohoue et al. 2018). A mutagenesis approach based on the CRISPR/Cas9 system with plasmids expressing the gene-encoding Cas9 nuclease and single-guide RNAs for the mutagenesis of target genes was proved in *A. oryzae* with mutation rates of 10–20% (Katayama et al. 2016). An optimized vector harboring an AMA1-based autonomously replicating plasmid and

the drug resistance marker *ptr*A were used allowing an improved mutation efficiency of 50–100%. Besides, co-transformation with the genome-editing plasmid and a circular donor DNA enabled marker-free and multiplex gene deletion/integration in *A. oryzae* (Katayama et al. 2019). Likewise, for marker-free CRISPR gene editing in NHEJ-deficient strains of *Aspergillus*, gene targeting can be done with single-stranded oligonucleotides to introduce specific point mutations and gene deletions with about 100% efficiencies. This system works efficiently in *A. nidulans*, *A. niger*, and *A. oryzae* (Nødvig et al. 2018).

Another interesting approach to optimize CRIPSR/Cas9 technology is being developed. A simple method for gene targeting that provides selectable, iterative, and ultimately marker-free generation of genomic deletions and insertions has been tested in *A. niger.* These studies were initially performed targeting the phenotypic marker *albA* and subsequently to validate the approach, by targeting the *glaA* and *mstC* loci, achieving 100% gene-editing efficiency across all three loci (Leynaud-Kieffer et al. 2019).

Otherwise, a protocol for the knockout of the pigment synthesis (*pks*P) gene in conidia (as selection marker) and the knock-in of endoglucanase genes (from *Trichoderma reesei*, *Talaromyces wortmanii*, or *A. niger*) in *A. fumigatus* LMB35Aa is being developed to overproduce neutral–alkaline cellulases for the textile industry (Fig. 9.3) (Jhon S. Benites and Gretty K. Villena unpublished work).

Finally, a modified CRISPR/Cas9 system for *A. niger* with a plasmid derived from the pAN7-1 series and an easy-to-switch "ribozyme–gRNA–ribozyme (RGR)" element was used for the overexpression of the *gla*A gene and downregulation of *agd*F, via a one-step knockout/knock-in (Zhang et al. 2019). As for other species, a CRISPR/Cas9 system was applied in *Myceliophthora*, a thermophilic biomass-degrading filamentous fungus, to increase cellulase production (Liu et al. 2017). Several cellulases, including exoglucanases and endoglucanase in *N. crassa*, had their expressions increased by a transcriptional factor (clr-2) regulated by CRISPR/Cas9 (Matsu-ura et al. 2015). These enzymes will be applied in consolidated bioprocessing (CBP) approaches for industrial applications.

9.3.2 Organic Acids

Filamentous fungi are the most prominent biological sources for organic acid production. Since 1917, *Aspergillus niger* is well known as the best citric acid producer, which is used in the food industry as acidulant and flavor additive, due to its taste, palatability, and low toxicity (Tong et al. 2019). Word production of citric acid in 2007 was at about 1.6 million tons, and market estimates in 2014 were at about $2.6 billion and supposed to increase up to $3.6 billion by 2020. Nowadays, China is the major producer with approximately 60% of the world production. By the middle of the twentieth century, mutagenesis strategies with UV radiation, X-rays, and chemicals were performed in order to increase the citric acid yield (Cairns et al. 2018). Recently, a system metabolic approach that utilizes multilevel omics data

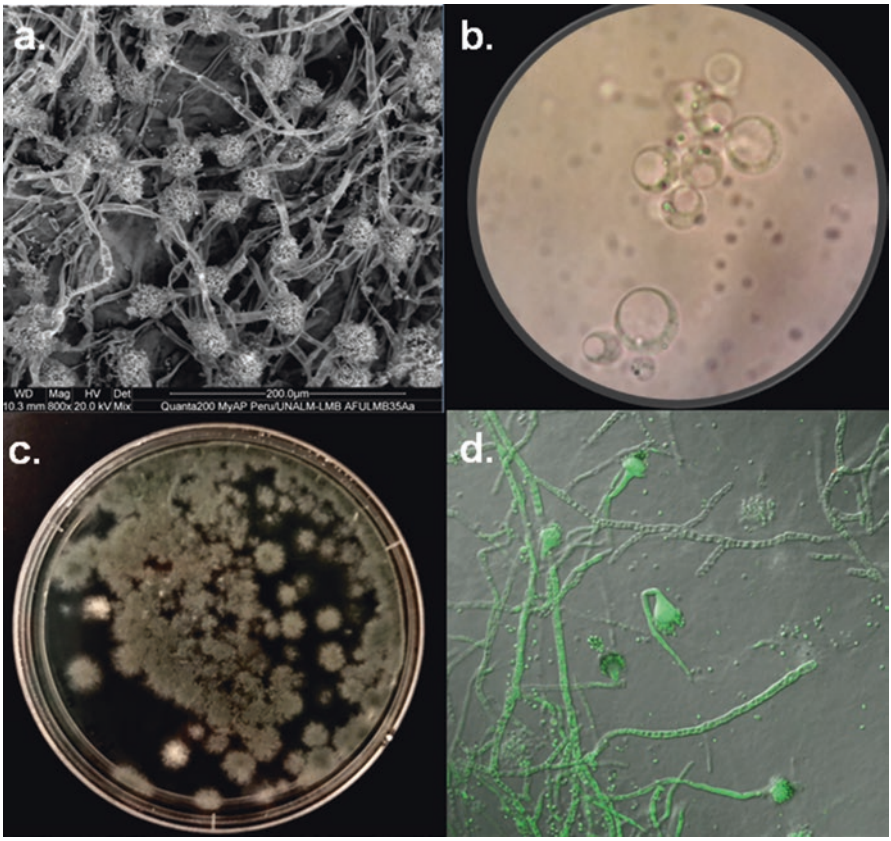

Fig. 9.3 Genome editing by CRIPSR/Cas9 of the *Aspergillus fumigatus* LMB LMB35Aa for the knock-in of cellulase genes. (**a**) *Aspergillus fumigatus* LMB35Aa was used as the model strain for gene edition. For that, (**b**) protoplast were prepared and transformed with a vector harboring the Cas9 gene and guide RNA for the knockout of the *pks*P gene to obtain albino colonies (**c**). A co-transformation with an endoglucanase and GFP reporter genes showed the correct insertion. (**d**) Confocal-laser scanning microscopy shows the mycelium and conidia of the edited strain, which are expressing GFP (Jhon S. Benites and Gretty K. Villena unpublished work)

was proposed to engineer *A. niger* as a highly optimized cell factory for industrial citric acid production. The strategies focused in some key aspects such as improvement of substrate utilization, avoidance of by-product synthesis, removal of negative feedback effects, enhancement of the precursor supplement, improvements in transport efficiency of substrates and citric acid, optimization of NADH regeneration, increasing robustness and resistance to stress, and optimal morphology regulation. All these could be accomplished using engineered promoters, transcription factors, and transcription regulation through CRSIPRi/CRSIPRa system or RNAi (Tong et al. 2019).

Other organic acids with industrial applications include gluconic acid, itaconic acid, kojic acid, malic acid, and oxalic acid, which are also produced in *Aspergillus*

species. Kojic acid is mainly produced by *A. oryzae* but also by other *Aspergillus* species (*A. flavus*, *A. tamarii*) and *Penicillium* (Yang et al. 2017).

Furthermore, heterologous expression of key genes has been reported in order to improve organic acid production through metabolic engineering strategies. For malic acid production in *Aspergillus oryzae* NRRL 3488, the overexpression of the pyruvate carboxylase and malate dehydrogenase genes and the gene coding for the C4-dicarboxylate transporter led to a yield of 69% of the theoretical maximum, which is the highest reported for any microbial system (Brown et al. 2013; Wakai et al. 2017). Enhanced succinic acid production in *Aspergillus saccharolyticus* was achieved by heterologous expression of the NADH-dependent fumarate reductase gene (*frd*) from *Trypanosoma brucei* (Yang et al. 2016; Wakai et al. 2017).

The strain *A. oryzae* LDH-871, which simultaneously causes saccharification from starch (with its native amylases) and fermentation of lactate, was obtained by genetic engineering by disruption of the endogenous gene and its replacement with the bovine L-lactate dehydrogenase gene (Wakai et al. 2014).

Another engineered *A. niger* strain was obtained by expressing a synthetic metabolic pathway to produce L-ascorbic acid from D-galacturonic acid. Two enzyme genes from the L-ascorbic acid synthetic pathway from *Euglena gracilis* (EgALase) and *Malpighia glabra* (MgGALDH) and the gene coding for the unspecific L-gulono-1,4-lactone lactonase (smp30) from the animal L-ascorbic acid synthetic pathway were introduced into *A. niger,* and expressed under the control of a host inducible promoter. This strategy allowed the construction of a cell factory that produces L-ascorbic acid from pure D-galacturonic acid or pectin-rich biomass in a consolidated bioprocess (Kuivanen et al. 2016).

Also in *A. niger*, the CRISPR/Cas9 system was used to obtain an engineered strain containing both disrupted galactaric and D-galacturonic acid catabolism and expressing a heterologous uronate dehydrogenase gene (Kuivanen et al. 2016). Galactaric acid (GA) is a dicarboxylic acid that can be produced by the oxidation of D-galacturonic acid, the main constituent of pectin. Mold strains can be engineered to perform this oxidation by expressing the bacterial enzyme uronate dehydrogenase. GA is used as a chelator in skin care products and has the potential application in polymer synthesis as a chemical platform.

According to the biorefinery concept, fungal cell factories are increasingly required to efficiently utilize different carbon sources for the production of organic acids from renewable biomass. For this reason, several metabolic engineering strategies should be implemented to develop newer and more robust aspergilli-based factories (Yang et al. 2016).

Finally, polyunsaturated fatty acids could be produced by molecular breeding of natural producing fungi like *Mortierella alpina*, which synthetizes linoleic (LA) and oleic acid (OA). Heterologous expression of the desaturase enzyme gene from *Coprinopsis cinerea* caused a five-fold increase in LA production, and further, by suppressing the native desaturase gene expression by RNAi, the accumulation rate of OA was as high as 68% of the total fatty acids. These transformants could be used as hosts for the production of other fatty acid derivatives (Sakamoto et al. 2017).

9.3.3 Secondary Metabolites

Secondary fungal metabolites include different chemical families like alkaloids, isoprenoids, non-ribosomal peptides, and polyketides (Khan et al. 2014), which have different applications as antibiotics, cholesterol-lowering drugs, therapeutic compounds, pigments, mycotoxins, and others. Of these, about 22% are produced by filamentous fungi. The main antibiotics include the natural penicillin G and the biosynthetic penicillin V with a combined market of $4.4 billion, many semisynthetic penicillins; and the semisynthetic cephalosporins, with a $11 billion market (Adrio and Demain 2003).

The best antibiotic producer is the genus *Penicillium*, a good example of the classical mutagenic approach to improve the fungal strain to achieve 4000-times higher titers than the original parental strain (Peberdy 1986). *P. chrysogenum* NRRL 1951 was the parental strain and together with its descendants have been improved with strong mutagenic treatments resulting in overproducing strains, most of them with multiple-copy number of the penicillin biosynthesis gene cluster (as many as 50 copies of the cluster). Nevertheless, the strain P2niaD18, a nitrate reductase-deficient derivative with increased penicillin production, has only two copies of the cluster, which suggests that it is not the limiting factor. Instead, the wide domain regulatory factors in trans involved in the biosynthesis of penicillin are the important targets for future strain improvement (Ziemons et al. 2017). *P. chrysogenum* also produces a broad range of secondary metabolites. Metabolic pathways for biosynthesis, regulation, and transport of secondary metabolites are commonly arranged in gene clusters including a core of biosynthetic genes (BGCs) of polyketide synthases (PKSs), non-ribosomal peptide synthetases, and terpene synthases (Guzmán-Chávez et al. 2018), which can be identified and mined from genome sequences (Hillman et al. 2017). Different strategies have been implemented to activate the expression of cryptic BGCs in a targeted manner that includes genome editing to improve the homologous recombination pathway, ribosome engineering and heterologous expression, and refactoring (especially with CRISPR/Cas9 to introduce specific tailoring enzymes) (Guzmán-Chávez et al. 2018). Additionally, it is important to consider that heterologous expression and host engineering might activate silent genes. This was the case for *A. nidulans*, which could express a silent gene cluster of *Aspergillus terreus* that synthesizes asperfuranone, an anticancer drug candidate (Hillman et al. 2017).

Non-ribosomal peptides (NRP) are a group of secondary metabolites synthesized in fungi by NRP synthetases (NRPSs). The NRPS enzyme genes are organized as gene clusters in the genomes, and most of them stay silent until the fungal-specific regulator of secondary metabolism, LaeA, allows their expression. Therefore, LaeA is a common target for the identification of novel gene clusters and metabolites (Soukup et al. 2016). Other strategies for the bioengineering of fungal strains include the iterative reprogramming of enzymatic modules to produce “unnatural” cyclo-oligomer depsipeptides with various chain lengths (Leitão and Enguita 2014; Soukup et al. 2016).

A*spergillus* strains are suitable hosts for the heterologous production of secondary metabolites. *A. oryzae* is one of the two preferred strains used because of its limited endogenous secondary metabolism, and the other is *A. nidulans* because of the robust genetic toolbox available for this microorganism (Anyaogu and Mortensen 2015).

Regarding the production of pigments and antioxidants, heterologous expression of a cluster from *Monascus purpureus* has been reported, with a combination of the integration of a biosynthetic gene cluster and the overexpression of the global regulator *lae*A under the control of the constitutive P-pgk promoter to produce monacolin K and terrequinone in *A. oryzae* (Sakai et al. 2012). Another similar approach to overexpress *lae*A used the *A. nidulans alc*A promoter in *Monascus pilosus* to produce monacolin K and pigment, and to enhance the antioxidant activity of *Monascus*-fermented rice (Lee et al. 2013; Wakai et al. 2017).

Terpenoids, another family of secondary metabolites from higher fungi, display a wide range of biological activities, acting mainly as anticancer, cytotoxic, and anti-inflammatory agents. Exogenous biosynthesis of terpenoids via a synthetic biology approach remains an interesting challenge. After elucidation of the biosynthetic pathways, the syntheses of a few important sesquiterpenes, diterpenes, and triterpenes in heterologous hosts have been reported (Xiao and Zhong 2016).

Statins are another group of metabolites produced by fungi that became successful drugs to keep lower cholesterol levels in blood, decreasing the risks of atherosclerosis. Natural statins like lovastatin and compactin produced by *Aspergillus terreus* and *Penicillium citrinum* are used for the synthesis of other semisynthetic statins (Barrios-González and Miranda 2010). In order to improve the biotechnological statin production, classical mutagenesis strategies as well as heterologous production have been employed. A metabolic reprogramming approach was used to produce the active drug pravastatin by dual-step fermentation and a biotransformation process in *Penicillium chrysogenum* DS50662. This strategy involved the introduction of the compactin synthetic pathway to improve production levels and a new cytochrome P450 (P450 or CYP) from *Amycolatopsis orientalis* (CYP105AS1) to catalyze the final compactin hydroxylation step (McLean et al. 2015).

Besides, through synthetic biology applications, biosynthetic modules from secondary metabolic processes can be rationally engineered and combined to produce either new or known compounds. In this regard, it is of great interest to specially consider cryptic genes that constitute an unexplored source of new chemicals and drugs. The main advantages of this approach are the possibility of designing and combining different building blocks to produce primary drug scaffolds for further enzymatic or chemical modifications, and to design of cell factories that produce metabolites with lower production costs when compared with chemical synthesis (Leitão and Enguita 2014). Remarkably, in *A. fumigatus*, the CRISPR/Cas9-mediated single-nucleotide insertion in the polyketide synthase gene of the trypacidin biosynthetic pathway allowed reconstitution of the producing capability of a nonproducing strain (Weber et al. 2017). The trypacidin drug has antiprotozoal activity.

New computational tools for genomic and metabolomic studies allow the identification of novel secondary metabolites as well as the utilization of genome-scale

metabolic models (GEMs) and, on this bases, the design of fungal cell factories for the production of secondary metabolites (Nielsen and Nielsen 2017). In one such example, a meta-analysis of several transcriptomic data has allowed the establishment of the co-expression networks for 9579 genes (~65%) in the *A. niger* genome. Subsequent experimental validation has identified at least four transcription factors (VelC, StuA, MjkA, and MjkB) that are involved in secondary metabolite synthesis, which were used to activate production of multiple natural products (Schaepe et al. 2018).

9.4 Engineered Fungi for Value-Added Bioproducts from Lignocellulosic Biomass

Lignocellulose biomass, the most abundant renewable organic resource on Earth, is a potential feedstock to obtain value-added products (Lynd et al. 2005). This biomass consists of three main components: lignin, cellulose, and hemicellulose (Li et al. 2015; Gupta et al. 2016). An efficient bioconversion of lignocellulose material is made by filamentous fungi, which can produce several products of great biotechnological interest during this process. However, large-scale conversion of lignocellulose to useful products is still challenging due to the heterogeneity and recalcitrance of the lignocellulosic materials, especially lignin (Balan 2014).

Despite the recalcitrance of lignin, its biotechnological exploitation to produce a value-added product is very important for the development of lignocellulosic biorefineries (Gupta et al. 2016). Current uses of lignin include the manufacturing of phenol–formaldehyde adhesives, concrete admixture, vanillin, and polyurethane foams, among others (Li et al. 2015). The production of enzymes such as peroxidases and laccases for the fungal degradation of lignocellulosic biomass is also a possibility to aid in the generation of value-added products (Li et al. 2015).

With respect to cellulose, another component in the lignocellulosic biomass, it is not an easily degradable biopolymer, despite being formed by glucose units interconnected by 1 → 4 glycosidic bonds. The hydrolysis of glucose from cellulose biomass could result in fermentable products of industrial interest, for example, propanol, acetone, succinic acid, ethanol, lactic acid, as well as hydroxymethylfurfural (De Bhowmick et al. 2018).

Importantly, cellulose processing can also result in nanocellulose (nanofibers and nanocrystals), a promising material for reinforcing cement and plastics, and in the manufacturing of sensors and protheses. Nanocellulose presents highly desirable characteristics for various industrial sectors: biodegradability, accessibility, low cost, biocompatibility, hydrophilic character, high mechanical strength, low weight, and large surface area. The procedure to obtain nanocellulose requires the enzymes cellobiohydrolases and endoglucanases to break down the crystalline structures and the amorphous sites of cellulose (Dufresne 2013). These enzymes are produced by filamentous fungi whose genomes can be edited to enhance their production as described above.

Another lignocellulose biomass component is hemicellulose, a complex polysaccharide chain that includes arabinoxylan, xyloglucan, xylan, glucomannan, and glucuronoxylan, found in plant cell walls. Sugars such as xylose are released during the hydrolysis of hemicellulose and can be converted into xylitol, a sugar substitute, or used in the generation of bioethanol or biohydrogen (Dondelinger et al. 2016). Arabinose, another pentose sugar in hemicellulose, can also be used to produce value-added chemicals like furfural (Menon and Rao 2012), which is a basic material for the synthesis of furan resins and Nylon 6 polymers.

However, to efficiently hydrolyze lignocellulosic biomass into useful products, mainly three steps are required. In the first step, physical, chemical, or biological methods are applied to destroy the structure of lignocellulose. This pretreatment is necessary to increase the enzymatic digestibility of cellulose. After the pretreatment, lignocellulolytic enzymes are used to degrade cellulose and hemicellulose into simple sugars, as well as to allow the action of saccharifying enzymes.

Industrial lignocellulolytic enzymes are mainly produced by filamentous fungi and yeasts, which can be engineered using molecular tools to obtained microorganisms with high enzyme-producing abilities, at low cost (Mohagheghi et al. 2014).

In addition to the modifications of gene transcription and the engineering of protein secretion processes by molecular tools, the engineering of mycelial morphology is also applied to reduce the viscosity in submerged cultures. This strategy was successfully tested through to *gul-1* gene disruption in *N. crassa* (Lin et al. 2018). In this sense, the engineering of the substrate utilization pathway can also be applied to enable the growth in an inexpensive medium such as lignocellulosic biomass, thus lowering the enzyme production costs (Ellila et al. 2017).

The most recent advance in this area is the development of a microorganism capable of consolidated bioprocessing (CBP). This process is used when the production of hydrolytic enzymes, the lignocellulose breakdown, and microbial fermentation are made in one single step to further lower the biofuel production cost (Kawaguchi et al. 2016; Jiang et al. 2018). The main strategy to develop a CBP microorganism is to introduce cellulolytic ability into non-cellulolytic, ethanol-producing organisms (Lynd et al. 2005). In this context, a very attractive CBP organism candidate is *S. cerevisiae*, the yeast known as the major producer of ethanol (Walker 1998). Thus, in this yeast the genes coding for endoglucanase of *Trichoderma reesei* and the β-glucosidase of *Saccharomycopsis fibuligera* were introduced, constructing a recombinant strain with the ability to grow on phosphoric acid-swollen cellulose as its sole carbon source and produce ethanol (Den Haan et al. 2007).

Based on this study, another recombinant yeast strain was developed in which cellobiohydrolases, β-glucosidases, and endoglucanases were assembled into a trifunctional minicellulosome through cohesin and dockerin. This recombinant *S. cerevisiae* exhibited a higher cellulolytic activity due to the enzyme–enzyme and enzyme–substrate synergistic effects (Wen et al. 2010; Du et al. 2011).

S. cerevisiae has also been engineered to degrade and utilize xylan, one of the major polysaccharide chains present in hemicellulose. Different hemicellulases from *Trichoderma reesei*, such as endoxylanase, β-xylosidase, acetylxylan esterase,

α-D-glucuronidase, and α-L-arabinofuranosidase, were heterologously expressed and secreted by *S. cerevisiae* (Tabañag et al. 2018).

In addition to *S. cerevisiae*, other yeasts such as *Moesziomyces antarcticus* and *M. aphidis* were screened for their ability to produce xylanolytic enzymes to produce ethanol when grown on D-xylose and xylan from wood, without exhibiting cellulolytic activity (Faria et al. 2019). Other chemicals besides ethanol can be produced from these engineered yeasts such as recombinant inulinase produced from *Aspergillus kawachii* in *Pichia pastoris*. This recombinant acid-stable enzyme has potential applications in industrial processes since it hydrolyzes inulin to fructose, in a single enzymatic step (Chesini et al. 2018).

Through gene silencing methods for metabolic engineering, some ethanologenic white-rot fungus such as *Phlebia* sp. exhibited the capacity to produce other chemicals besides ethanol (Motoda et al. 2019). In this context, the ethanologenic fungi *Aspergillus terreus* and *Neurospora crassa* were used for the heterologous expression of cis-aconitic acid decarboxylase enzyme to synthesize itaconic acid (IA), suggesting that these fungi might be considered a good choice for chemical production from lignocellulosic materials (Zhao et al. 2018).

IA, also known as methylene succinic acid, is a saturated dicarboxylic organic acid and has a large number of industrial applications such as the production of plastics, adhesives, elastomers, or coatings; making it an alternative to existing polymers (El-Imam and Du 2014). Further, IA can also be used in detergents, shampoos, pharmaceuticals, herbicides, and thickeners in lubricating grease preparation (Hegde et al. 2016).

In addition to fungal genetic modification, the modulation of the cell metabolic machinery through the culture medium can be a consolidated bioprocess, according to what was observed for *Fusarium oxysporum*, which was cultured with complex nitrogen sources to produce different proteins (da Rosa-Garzon et al. 2019). Therefore, this current approach that combines genetic modification methods and the use of alternative culture medium could be advantageously used to manipulate many biological processes for fungal engineering. However, the successful engineering demands a complete and thorough understanding of the biological processes in order to be able to introduce the effective genetic modifications (Yadav et al. 2018).

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Chapter 10
Fungal Production of Prebiotics

S. A. Belorkar

10.1 Introduction

Artificial sweetnesrs has gained momentum since the 1980s. The term was coined to indicate a group of healthier and low-calorie food items synonymously called as functional foods. The mild sweet taste these food items provide to the consumers is due to their oligosaccharide nature. A variety of oligosaccharides has been tagged under the banner of functional foods. Of all the oligosaccharides, fructooligosaccharide has successfully gained special attention. The health industry has a constant rise in the demand of such functional foods. This functional food has gained recognition and popularity as prebiotic food items. A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" according to Gibson and Roberfroid (1995). The health-conscious consumers are finding the benefits of prebiotics unavoidable, reflecting a high increase in their demand. The great beneficial impact of these prebiotic oligosaccharides is due to their indigestible nature (Nacos et al. 2006; Nabarlatz et al. 2007; Moreno et al. 2017). The commercialization of oligosaccharides (OS) as prebiotics is popularized due to their low-calorie intake benefit to the consumer. The major health benefits like artificial sweetness, low-calorie value, and prebiotic effect of these oligosaccharides have attracted industries like food, pharmaceuticals, and upcoming health-promoting industry.

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10.2 Prebiotics: A Historical Account

The founding member which initiated the study on oligosaccharide was inulin also known as oligofructose. Since 2000, synthetic fructooligosaccharides have also attracted investigators. The studies were mainly focused on fructooligosaccharides resulting in data which supported its classification as an established functional food ingredients (Roberfroid and Delzenne 1998; Roberfroid et al. 1998).

Initial elaborate studies on these two basic prebiotic, viz., inulin and FOS, elaborated their presence in many fruits and vegetables. The most common sources are wheat, onion, banana, garlic, and leeks (Lee et al. 1995). Prebiotics include fructans (inulin and fructooligosaccharides (FOS)), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO), maltooligosaccharides (MOS), xylo-oligosaccharides (XOS), soybean oligosaccharides (SOS) (raffinose and stachyose), lactosucrose (LS), and lactulose (Macfarlane et al. 2008; Roberfroid and Slavin 2000), although some of them are only recognized as potential prebiotics. The functional oligosaccharides include fructooligosaccharides (FOS) (Chen et al. 2000; Belorkar and Gupta 2016), glucooligosaccharides (GOS), isomaltooligosaccharides (IMO), soybean meal oligosaccharides (SMO), mannan oligosaccharides (MOS), galactooligosaccharides, gentiooligosaccharides, isomaltulose, lactosucrose, maltooligosaccharides (MO) (Fric 2007), xylooligosaccharides (XOS) (Moure et al. 2006), pectin-derived acidic oligosaccharides (pAOS) (Li et al. 2010), and cyclodextrins (Astray et al. 2009) summarized in Fig. 10.1.

10.3 Oligosaccharides-Accorded FOSHU Status

On the basis of chemical structure, oligosaccharides are classified as carbohydrate with low molecular weight comprising 3–10 sugar molecules in the chain. Their characteristics are in between monosaccharides and polysaccharides (Weijers et al. 2008). These oligosaccharides offer resistance to digestion as a virtue of its anomeric C atoms (C1 or C2) of the monosaccharide units. The conformation of the osidic bonds offers non-susceptible characteristics toward the hydrolytic activity of human digestive enzymes (Roberfroid and Slavin 2000).

These nondigestible oligosaccharides often abbreviated as NDOs act as dietary fibers and prebiotics due to their physicochemical and physiological properties. NDOs have potential to flourish the microbes in the gut. The gut microflora ferment these NDOs to release energy and products like metabolic substrates, lactic, and short-chain carboxylic acids as end products of fermentation (Quigley 2010). The NDOs promote the proliferation of probiotics (Qiang et al. 2009; Saminathan et al. 2011).

Of all the oligosaccharides shown in Fig. 10.1, many have gained recognition as “foods for specified health use” (FOSHU) in Japan since 1991. The global market has now given acceptance to NDOs classified under functional food. The present

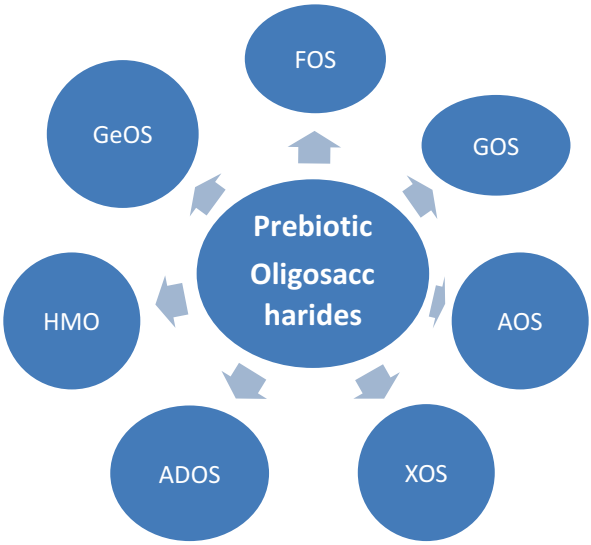

Fig. 10.1 Types of oligosaccharides. FOS fructooligosaccharides, GOS galactooligosaccharides, XOS xylooligosaccharides, AOS arabinooligosaccharides, ADOS algae-derived marine OS, HMO human milk oligosaccharidesl, GeOS gentioligosaccharides

market of these functional food is US $27 billion and expected to show a hike of 8.5–20% growth in its demand. In 2005 market for functional foods was estimated to be US $73.5 billion. The global prebiotics market is expected to grow around 12.7% in the next 8 years, so manufacturers are developing new alternatives to obtain sustainable and efficient processes for application on a large scale (Mano et al. 2018). With the increase in the health awareness of the consumer, there is a boom in the health market. Until 2003, countries like the United States, Europe, and Japan witnessed market for functional foods and supplements. In Canada, the health market was sluggish due to the impact of Natural Health Products Regulations (2004). The demand for functional food has a promising market in China, India, and Latin America.

10.4 Sources of Prebiotic

Functional oligosaccharides are found in varying concentrations in milk, honey, sugarcane juice, soya bean, lentils, mustard, fruits, and vegetables such as onion, asparagus, sugar beet, artichoke, chicory, leek, garlic, artichoke, banana, rye, barley, yacon, wheat, tomato, and bamboo shoots (Mussatto and Mancilha 2007).

Functional properties of oligosaccharides from different sources vary due to their variable monomers, degree of polymerization, and osidic bonds. *The important fungal sources of varied oligosaccharides are summarized in* Table 10.1.

Table 10.1 Fungal sources of few prebiotic oligosaccharides

Prebiotic oligosaccharides	Fungal sources	
Fructooligosaccharides	*Aspergillus oryzae* *Aureobasidium*	Kurakake et al. (2010) Castro et al. (2017) Xie et al. (2017
Galactooligosaccharides	*Aspergillus oryzae*	Aehle (2004)
Xylooligosaccharides	*Aspergillus foetidus Aspergillus brasiliensis*	Chapla et al. (2012) Menezes et al. (2017)
Arabinooligosaccharides	*Penicillium purpurogenum*	Vilches et al. (2018)
Glucooligosaccharides	*Trichoderma harzianum*	Giese et al. (2011)
Maltooligosaccharides	Cell wall of *Paecilomyces* spp. *Saccharomyces cerevisiae Ganoderma lucidum*	Dimitroglou et al. (2010) Eseceli et al. (2010)

10.5 Fructooligosaccharides

The studies on the FOS intensified due to promising results of these oligosaccharides on overall health of the consumer. Experimental proofs indicated the following health-promoting characteristics envisaged in these prebiotic molecules.

10.6 Natural Occurrence

FOS are available in natural food items. *The details of their concentrations are given in the following* Fig. 10.2.

The varied length of fructose polymers is the reserve carbohydrates in plants. The fructose oligomers are reported to be derivatives of sucrose. The enzyme mechanism for FOS synthesis was well studied in plants like *Beta vulgaris* (Allen and Bacon) and *Helianthus tuberosus* (Edelman et al. 1963; Edelman and Dickerson 1996; Edelman and Jefford 1968). The occurrence of FOS in animal product like honey is also well established. *The sources of prebiotic fructooligosaccharides are summarized in* Fig. 10.3.

The synthetic tool for FOS is the Ftase enzyme. The enzyme is produced by plants and microbes. In microbes bacteria, yeast and mold are extensively reported to be ftase producer. Fungi predominate among all groups as potent Ftase producer.

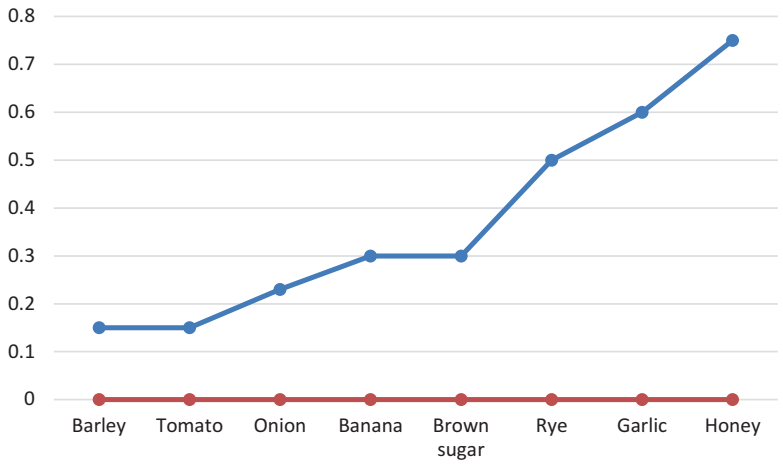

Fig. 10.2 Occurrence of FOS in natural food sources (Flamm et al. 2001)

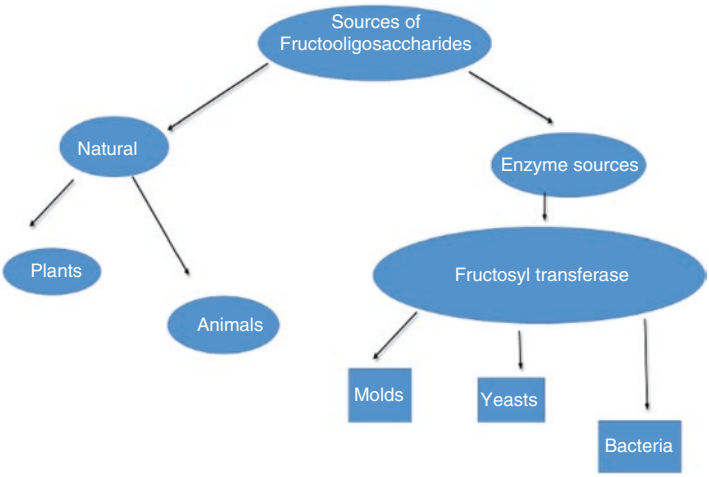

Fig. 10.3 Sources of prebiotic FOS

The comparison of Ftase efficacy of all the reported sources reveals fungal enzymes to be more dependable. The enzyme yield from plant sources is poor with seasonal dependency as a major drawback. Fungal enzyme has robust yield and exhibits tolerance toward industrial parameters like temperature and pH (Yun 1996). In comparison with plant sources, fungal enzymes are thermostable and have comparably larger size.

10.7 History

Since 1980, research was intensified for a potential Ftase with high yield of FOS. Fungal enzyme were more dependable, and the initial study of Hidaka was successfully accomplished using *A. niger*. FOS production by *Aureobasidium* species was also reported. Black yeast *A. pullulans* was reported to produced Ftase befitting industrial usage (Jung et al. 1989).

Apart from *A. niger*, *A. pullulans* novel fungal sources like *Scopulariopsis brevicaulis* was also screened for Ftase production (Takeda et al. 1994). Reports indicated a variety of products which depended mainly on enzyme source and thereafter its mechanism.

10.7.1 GRAS Status

The initial FOS produced was launched Meiji Seika Pharma, Co., Japan, during 1984. The FOS then did not have acceptance as well as recognition in European Market. The potential of FOS in health market triggered many companies to bag GRAS status to it as a product. The health market witnessed multiple applications for permission to use FOS in food items in Japan, Korea, and Europe region during 1996. Presently the FOS has gained the GRAS status from the US Food and Drug Administration.

A. oryzae, a well-known mold in traditional koji preparation, is reported to be an Ftase producer. This mold has a well-established role in preparation of traditional fermented food items. *A. oryzae* is also a source of many industrial enzymes in the food industry. Due to prolonged use of the mold in food item preparation, this fungus has been accorded GRAS status (Gomi 1999). Thus industries depend solely on fungal sources of enzymes for commercial FOS preparation.

10.7.2 About the Enzyme

10.7.2.1 Whether Hydrolase or Transferase?

The enzyme utilized for FOS synthesis has a controversy in its nomenclature. Initially this transferase activity of this enzyme was accidently observed during invertase study at high sucrose concentration (Bealing and Bacon 1953; Bearing 1953; Strathof et al. 1986). Some workers therefore use the term β-fructofuranosidase, recognized as hydrolases with E.C. 3.2.1.26 number. Another set of workers accept its designation as transferase and refer it as fructosyltransferase with E.C. 2.4.19 as its designation.

10.7.2.2 Mechanism of Synthesis of FOS in Fungi

$$\text{Sucrose substrate} \xrightarrow{\text{Hydrolysis}} \text{G} + \text{F} \xrightarrow{\text{Transferase}} \text{G} - \text{F} - \text{F} + \text{G}$$

The reaction mechanism in *A. pullulans* was reported by Jung et al. (1989). The fungal enzymes catalyze a reversible primary step. However, the transfer reaction is irreversible. The Ftase studied from various fungal sources are reported in Table 10.1. The initial study on purification of Ftase from *Aspergillus niger* ATCC 20611 was reported by Hirayama et al. (1989). The study elucidated purification fold of 51.6 and MW 340 kDa. Optimum pH and temperature reported were 5.0–6.0 and 50–60 °C, respectively. A low MW of 81–168 kDaFtase was reported by L'Hocine et al. (2000) from *Aspergillus niger* AS0023 as a novel source. The attempts of ftase purification from various fungal sources are successfully accomplished. The initial step used is ammonium sulfate precipitation followed by column chromatography. The commonly used stationary phases were CM Sepharose, DEAE- Cellulose, Sephadex-G, DEAE- Sepharose, etc. *The enzyme purification attempts made in the past decade has been summarized in* Table 10.2.

Metal ions also have critical effect on Ftase activity. CaCl2, EDTA, MgCl2, CoCl2, MnCl2, SDS, Tween-80, 2-Mercaptoethanol, Ba2+, Mg2+, and Ca2+ are reported to be activators. Hg2+, Ag2+, Ni2+, CuSO4, and Zn2+ are reported to be inactivators for the Ftase enzymes.

10.7.3 Nature of Products Formed

Table 10.3 provides the fungal sources of Ftase enzyme reported. Until 2000, few researchers had worked on fungal sources including *Aureobasidium pullulans*, *Aspergillus japonicas*, *A. niger*, *A. sydowi*, *Claviceps purpurea*, *Fusarium oxysporum*, *Penicillium frequentans*, *P. spinulosum*, *Phytophthora parasitica*, *Scopulariopsis*, *Brevicaulis* and *Saccharomyces cerevisiae*, (Yun 1996), *P. citrinum* (Hayashi et al. 2000), *A. foetidus* (Wang and Rakshit 2000), and *A. phoenicus* (Van Balken 1991). Fructosyltransferase has been reported to be produced by several

Table 10.2 Fungal sources of Ftase studied for purification

Fungus	Source of enzyme	References
Aspergillus niger	Extracellular	L'Hocine et al. (2000)
Aspergillus aculeatus	Commercial enzyme	Ghazi et al. (2007)
Aspergillus pullulans	Intracellular	Lateef et al. (2007)
Aspergillus niger	Intracellular	Nguyen et al. (2005)
Aspergillus pullulans	*Intracellular*	Lateef et al. (2007)
Aspergillus niger	Extracellular	Belorkar (2018)
Candida sp.	Extracellular	Hernalstein and Maugeri (2010)

Table 10.3 Fungal sources of Ftase

Microorganism	Reference
Penicillium purpurogenum	Dhake and Patil (2007)
Aureobasidium pullulans CCY 27-1-94	Vanda'kova´ et al. (2004)
Aspergillus oryzae CRF202	Sangeetha et al. (2005)
Aspergillus japonicusTIT-KJ1	Chien et al. (2001)
Aureobasidium pullulans KCCM12017	Shin et al. (2004)
Aureobasidium pullulans CFR77	Sangeetha et al. (2004a, b)
Aspergillus niger IMI303386	Nguyen et al. (2005)
Aspergillus japonicas TIT90076	Chen (1995)
Aspergillus japonicum JN19	Wang and Zhou (2006)
Aureobasidium pullulans CFR77	Lateef et al. (2007)
Aspergillus japonicus TIT 90076	Chen and Liu (1996)
Aureobasidium pullulans KFCC10524	Yun et al. (1997)
Penicillium citricum FERM P-15944	Hayashi et al. (2000)
Aspergillus foetidus NRRL337	Wang and Rakshit (1999)
Aureobasidium pullulans	Dake and Kumar (2012)
Aspergillus niger	Hendro and Toharisman (2008)
Aspergillus niger AS0023	L'Hocine et al. (2000)
Aspergillus niger	Rajoka and Yasmeen (2005)
Aspergillus stallus	Belorkar et al. (2015)
Syncephalastrum racemosum Cohn	Patil and Butle (2014)

microorganisms. Research has been done using intracellular and extracellular enzymes with promising results. The highest potential of Ftase production has been consistently shown by fungi. *The very common types of products formed during Ftase catalysis are depicted in* Fig. 10.4. It is evident from Table 10.1 that *Aspergillus* sp. has the highest potential of Ftase production.

10.8 Ftase Production

The enzyme production has been attempted by both submerged and solid-state fermentations. Most of liquid culture techniques employed sucrose as a carbon source for enzyme production with exceptions like Wang and Rakshit's experiment (1999) where Maltose was used as carbon source. The effect of sucrose concentration was reported to be the most critical factor for Ftase production. Experiments varying sucrose concentrations from 10 g to 280 g/L have been reported (Dhake and Patil 2007; Yun et al. 1992). Majority of experiments reported were carried out in flasks. On the contrary, Wang and Rakshit (2000) used bioreactors for Ftase production. The general outlay of enzyme production is given in Fig. 10.4. Almost all experiments were conducted at incubation temperatures of 30 °C and initial pH of the production medium within range of 4.0–6.0. Experimental variations were included to assess the impact of experimental conditions on Ftase production. Substrate feed-

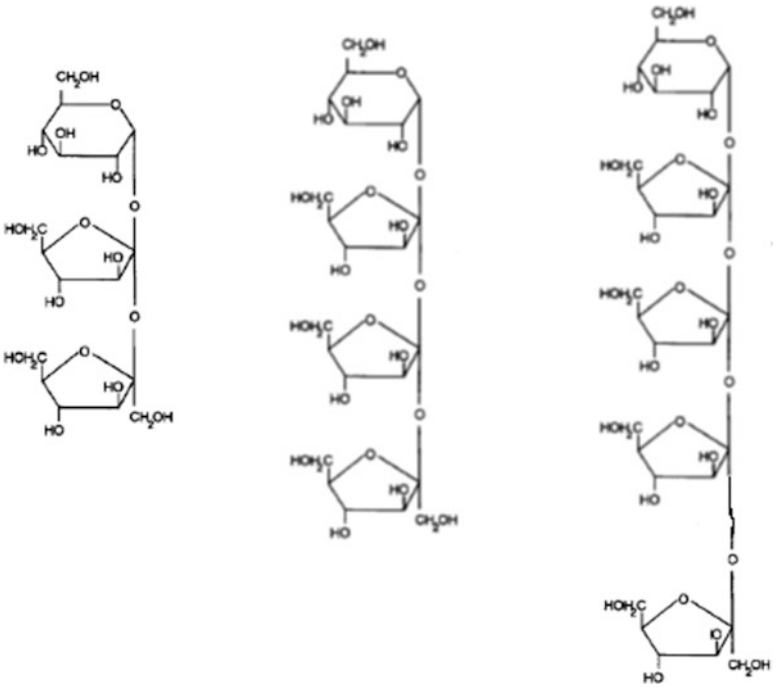

Fig. 10.4 Kestose, nystose, and N-fructosylnystose

ing intermittent in the incubation period was reported to enhance the Ftase production (Dhake and Patil 2007). Experimental designing has also been implemented for optimization of Ftase production (Vandakova et al. 2004). Designing of experiments was mainly targeted to understand the influence of carbon sources, nitrogen sources, and additives on Ftase production.

Due to the physicochemical production of fungal Ftase, they ware accorded as founder tool for commercial by production of FOS. The first commercial FOS produced was *A. niger* marketed under Neosugar label by Meiji Seika, Co., in Japan. Cheil Foods and Chemicals Co. in Korea succeeded marketing FOS produced by immobilized *A. pullulans. The general layout of crude enzyme production is given in* Fig. 10.5

10.9 Production of FOS by Fungi

The general layout of FOS production is given in Fig. 10.6. The FOS production process was executed by using various enzyme forms, immobilized cells, immobilized enzymes, and free enzymes as well. Table 10.4 depicts various fungi reported

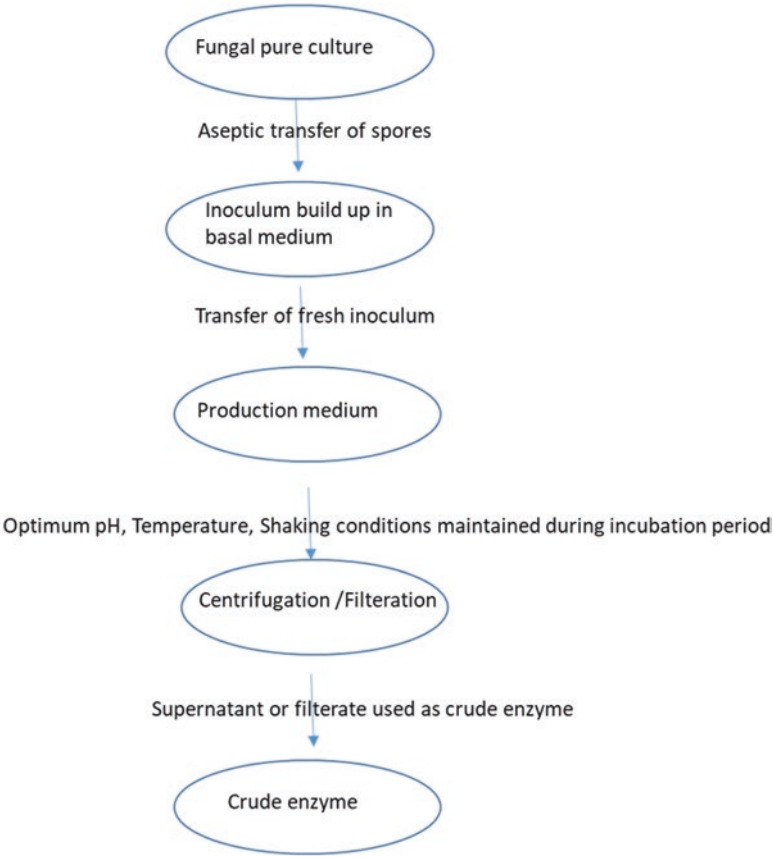

Fig. 10.5 Schematic representation of crude Ftase enzyme

to produce FOS. Many process designs have been proposed by authors to achieve optimum FOS product.

Recycling of the fungi pellets of *A. oryzae* was successfully executed by Sangeetha et al. to improve FOS production. The production was studied using flasks by various researchers (Chein et al. 2001; Shein et al. 2004; Sangeetha et al. 2004a; Hayashi et al. 2000). FOS production was optimized in bioreactors by Katapodis et al. in 2004. The temperature at which FOS production was carried out are in range of 280 C to 300 C. The pH range reported for optimum FOS production was 4.0–6.0. Substrates invariably used were high sucrose concentration. Reports of use of molasses have also been recorded. Variation in sucrose concentration was found to be critical for FOS production. Sucrose from 400 g/l (Chein et al.) to 700 g/l (Hayashi et al. 2000) has been reported.

Screening of important parameters was performed using Plackett–Burman design. The parameters investigated to optimize FOS production were pH of the reaction mixture and reaction time (Sangeetha et al. 2002). In the past Yun (1990) reported to produce FOS by immobilized cells of *Aureobasidium pullulans*. Stirred tank reactors were used for FOS production.

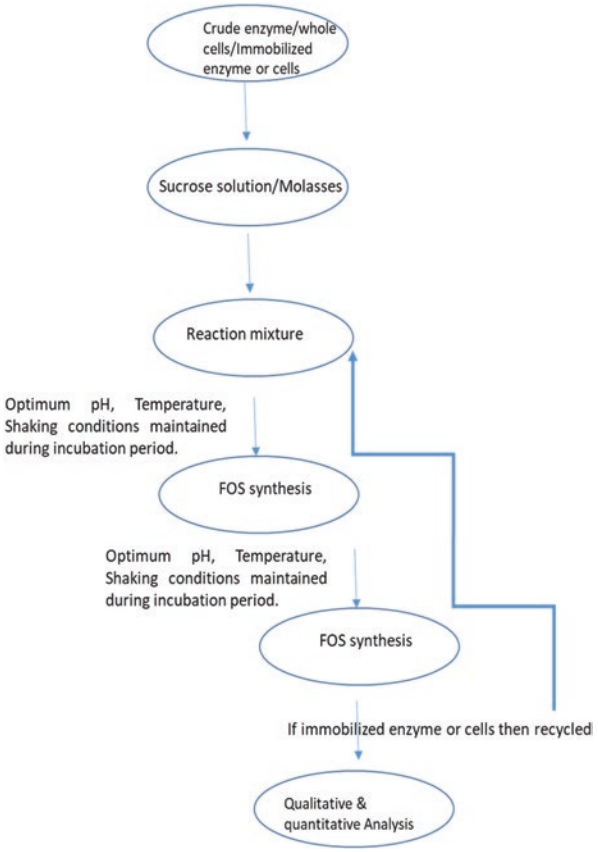

Fig. 10.6 Schematic representation of FOS production

Table 10.4 Fungal sources of fructooligosaccharides

Organism	Source	pH/temperature/ incubation period	FOS yield (%)	Authors
Aureobasidium pullulans	Intracellular	5.0/9 h/55 °C	59%	Lateef et al. (2007)
Aspergillus oryzae	Extracellular	55 °C/18 h	50–54%	Mable et al. (2007)
Aspergillus sp.	Whole cells	5.5–6.0/60 °C	376 g/l	Fernandez et al. (2003)
Aspergillus japonicas	Ssf	28 °C/48 h	128.7 g/l	Mussatto and Teixeira (2010)
Aspergillus phoenicus	Intact cells	55 °C/pH-8.0	300 g/l	Balken et al. (1991)
Schwanniomyces occidentalis			101 g/l	Álvaro-Benito et al. (2007)
Aureobasidium pullulans	Extracellular	5.0/30 °C/24–48 h	62%	Yoshikawa et al. (2008)

A large-scale production of FOS method was developed by Sangeetha et al. in 2005. Scaling up of the production process was performed. The process was applicable for commercial use. Immobilization of fungal mycelium was attempted by Chein et al. (2001). Cells were reported to be stable for long-term operations. Other authors utilized crude enzymes from *Aureobasidium pullulans*. The initial step was enzyme production followed by its use in FOS production yield 62%. An alternative reaction was attempted in combination with commercial glucose isomerase which increased the yield to 69% (Yoshikawa 2008). Álvaro-Benito et al. (2007) reported a yeast *Schwanniomyces occidentalis* producing novel extracellular novel Ftase producing selectively 6-kestose as product.

Mussatto and Teixeria (2010) utilized corncobs, coffee silverskins, and cork oak as solid substrate for FOS production. The fungi used was *A. japonicas*. Results indicated optimum FOS production of 128.7 g/l by coffee silverskins accompanied by 71.3 U/l of significant FOS production. The coupled synthesis was industrially significant. Fernandez et al. (2003) used *Aspergillus* sp. as an isolate from soil as whole cells to catalyze FOS production. Maximum FOS production was reported to be 376 g/dm-3. Optimum conditions reported were 5.5 to 6.0 pH and temperature of 600C. Mabel et al. in (2007) reported 50–54% of FOS production utilizing *A. oryzae* derived Ftase. Lateef et al. reported 59% of FOS by using Intracellular Ftase of *Aureobasidium pullulans*. 59% of FOS was produced within 9 h of reaction time.

10.10 Structural Analysis and Quantification of Oligosaccharides

Earlier quantification of oligosaccharides in plants and food items was accomplished by a multicenter validation ring test, later adopted as Association of Official Analytical Chemists method 997.08: well known as “fructans in food products, ion exchange chromatography method” by Roberfroid.

Analytical methods are important to determine the physicochemical properties and help to assess functionality of oligosaccharides. Thus, it is important to elucidate structural, number of monosaccharides, types of bonds, their sequence, branching patterns, and anomeric configuration.

Initial studies were dependent on thin-layer chromatography (TLC) which furnished the number of monomeric units or the length of the oligomer. Later HPLC became an efficient tool for analysis of FOS both quantitative and qualitative. Mass spectrometry emerged as another promising alternative for its analysis, accuracy, versatility, and sensitivity. Mass spectrometry and UV spectrophotometry combined with artificial neural networks (ANN) (Dias et al. 2009).

More recent techniques like the fast atom bombardment (FAB) technique, electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) are springing up as better options for FOS analysis (Cmelik and Chmelik 2010). MALDITOF-MS and NMR are some other well-known methods for structure analysis of oligosaccharides (Okada et al. 2010).

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Chapter 11
Fermentative Production of Secondary Metabolites from Bioengineered Fungal Species and Their Applications

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11.1 Introduction

Industrial-scale production of enzymes, polysaccharides, pigments, vitamins, lipids, and glycolipids has been increasing. Use of fungi for commercial-scale production of abovementioned products through fermentation has become a trend for the last two decades. Besides the use of fungi for industrial fermentation for production of biotechnologically important products, fungi are exceptionally used in carrying out biotransformation processes for production of fine chemicals including isomers (Adrio and Demain 2003; Cocaign et al. 2013). The developments in omics techniques have unfastened the new possibility in research. Advancements in recombinant DNA technology and understanding of genomic information made a way to exploit the full potential to engineer fungi for production of enzymes and secondary metabolites. The understanding of the genes responsible for specific metabolite aids in finding its underexpression or overexpression under known experimental conditions. This increased the utilization of fungi for industrial fermentation in recent times (Adrio and Demain 2003). Metabolically engineered fungal strains not only increase the titer yield of product but also result in formation of new products, novel enzymes, and other secondary metabolites having economic value (Macheleidt et al. 2016). Earlier in industries, secondary metabolite production using fungal

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strains is mostly carried out under submerged (SmF) conditions; this is mainly because the scale-up processes were simple when compared to scale-up in SSF (Robinson 2001). Recent days production of secondary metabolites through SSF has been tremendously increased because the economically efficient processes have been optimized to produce them through SSF (Li et al. 2014; Glassey and Ward 2015). In this chapter we have discussed about the advantages of SSF over SmF for production of secondary metabolites using fungal strains, especially bioengineered fungi. Genomic overview of bioengineered fungi and application of metabolite produced through fermentation has been discussed in this chapter.

11.2 Submerged Fermentation (SmF) and Solid-State Fermentation (SSF)

Solid-state fermentation is the biotechnological process which involves growth of microorganisms in the near absence of water or lower level of moisture content. Solid support matrix is used as the growth media for SSF. The solid materials used for this fermentation process act as source of nutrients and also as support matrix for the microorganisms. The substrates or support materials used for SSF are agro-industrial wastes (sugarcane bagasse, cassava bagasse), lignocellulose materials (straws, plant materials, wood shavings), deoiled oil seed cakes (coconut oil cake, soybean cake, groundnut oil cake), and cereal grains such as rice, wheat, barley, and corn. The matrix used in SSF varies based on their physiochemical properties including their chemical composition, particle size, porosity, water absorbing/holding property (water activity, a_w), and mechanical resistance. The water activity of the matrix plays an important role in SSF, because sufficient amount of water is required for microbial growth (Gervais and Molin 2003; Glassey and Ward 2015). The microorganisms adhere to the solid matrix having minimal moisture content and start to utilize them for their growth. The substrate utilization rate in SSF is comparably slower than SmF, because the microorganisms grow slowly and also take longer fermentation time. The particles used in SSF as solid media have less liquid phase and more of gas phase exists between them. SSF is best suited for the microorganisms which require less water for their growth. Even though the SSF provides good adequate microenvironment for the growth of various microbial flora including bacteria, yeast, and fungi, it is best suited for fermentation involving filamentous fungi. The filamentous fungi, like *Aspergillus* sp., *Penicillium* sp., and *Trichoderma* sp., have the competence to grow on the surface of low moisture containing substrates, and also they penetrate through the particles and utilize the nutrients (Mukherjee et al. 2012). Fermentative production of antibiotic penicillin was considered to be more economical under SSF than SmF, because of high yield of product. Similarly, production of several antimicrobial compounds and secondary metabolites was economical under SSF than SmF (Robinson 2001).

11.3 Secondary Metabolites as Bioactive Compounds

The secondary metabolites produced by various microbial strains were found to possess antibiotic, antiviral, antifungal, and anti-yeast activities. The fungal strains were found to be a rich source for these secondary metabolites. Among them, strains which are of marine origin have gained an important role as a source of biologically active secondary metabolites (Fatima et al. 2016; Hasan et al. 2015). Penicillins G and V, well-reported antibiotics produced by *Penicillium* sp. Griseofulvin, a halogenated polyketide, have been reported to be produced by few *Penicillium* sp. acting as inhibitor of tubulin polymerization. Nonribosomal peptide-based metabolites pneumocandin B and echinocandin B are β-1,3 glucan synthase inhibitors produced by *Glarea lozoyensis* and *Aspergillus pachycristatus*. Few polyketides like lovastatin and compactin were synthesized by *Monascus purpureus*, *Penicillium citrinum*, and other *Penicillium* sp., which inhibit conversion of mevalonate into cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Other secondary metabolites, viz., mycophenolic acid, gibberellic acid, kojic acid, fusidic acid, ergotamine, ergometrine, etc., produced by various fungal strains find application in food, pharmaceutical, agrochemical, and cosmetic industries (Jůzlová et al. 1996; Zhang et al. 2015; Hasan et al. 2015).

11.4 SSF for Production of Secondary Metabolites from Fungal Species

Many fungal strains have been reported to produce industrial enzymes, organic acids, flavors, and antibiotics through SmF and SSF. Among all the fungal strains reported till date for the industrial-scale production of several metabolites through fermentation, *Aspergillus* sp., *Penicillium* sp., and *Trichoderma* sp. are predominantly studied (Mukherjee et al. 2012; Sherkhane et al. 2017; Son et al. 2018; Cocaign et al. 2013). These filamentous fungal species have competence to produce large quantities of industrial enzymes, antibiotics, and volatile metabolites, and also they were reported for high product yield at low fermentation cost using cheaper raw materials. It has been well reported that the production of secondary metabolites through SSF possesses several biotechnological advantages over producing them under SmF. The advantages include higher fermentation productivity under low cost, lower catabolic repression, and higher yield of end products (Zhang et al. 2015). Coumarin- and terpenoid-based metabolites have been recently reported from *Aspergillus oryzae* KCCM 12698 through SmF and SSF. The metabolite profile of fungal strains cultivated under SSF and SmF was reported to differ based on the culture conditions. For the abovementioned strain, coumarin-based metabolite production was found to be predominant under SSF, but the same species was found to secrete more of terpenoid-based metabolites

under SmF (Son et al. 2018). Several antimicrobial compounds were produced by fungal eupenicinicols from *Eupenicillium* sp. LG41; tetracycline, aflatoxin, and cyclotetracycline from *Aspergillus*; and mycotoxins from *Aspergillus niger* and *Penicillium viridicatum* (Li et al. 2014). *Monascus* species was reported to produce various metabolites including mevinolin, lovastatin, and ankalactone and several other antimicrobial compounds (Jůzlová et al. 1996). Monacolin K (lovastatin) production by fungi *Monascus purpureus* 9901 through kinetic modeling revealed that the cell growth, substrate utilization, and product formation were rapid under SSF than SmF (Zhang et al. 2015).

11.5 Regulators/Regulatory Gene Targets for Improving Fungal Metabolite Production

Filamentous fungi are known to produce a cornucopia of biologically active compounds (Hoffmeister and Keller 2007). With the improved sequencing methodologies and sequenced fungal genomes, they are found to possess many more biosynthetic pathways than were already exploited. Genome mining and epigenetic approaches have been employed to elucidate the mechanism of regulation of SM synthesis (Brakhage and Schroeckh 2011). Fungal genome analysis reveals the facts that there are two highly conserved genes, namely, PKS (polyketide synthases) and NRPS (nonribosomal peptide synthetases), putatively involved in the synthesis of SMs and that most of these genes are found in clusters. Challenges in accessing these bioactive compounds are the inability to cultivate the producing strains in vitro and the gene clusters being silent under in vitro conditions (Hertweck 2009), until their activator for induction is identified (Brakhage et al. 2008). Pel et al. (2007) could not detect any mRNA of the anticipated SM gene clusters under standard growth conditions of *Aspergillus niger* CBS 513.88.

11.5.1 Mechanism of Regulation of Biosynthesis of Fermentation Products of Fungi

Microbes have various regulatory pathways that check the levels of metabolites produced, preventing them from overproducing and secreting them into the surroundings, in order to compete with other life forms in nature and to be energy efficient. However, industrial fermentation process runs on the opposite path. It requires overproducing strains that can further be deregulated (by enhancing positive regulation and bypassing and/or removing negative regulation) to make it produce huge quantities of the desired commercial product (Sanchez and Demain 2002). Fermentation involves production of materials by the action of various enzymes that must act in coordination to make the whole process cost-effective. At any given time, it is important for the cells to produce only the necessary enzymes and in right amounts for the formation of desired products. Once the product is

synthesized in desired quantities, the enzymes should be downregulated, and their activity has to be controlled, thus achieving optimal utilization of cell's energy resources. However, certain control systems that modulate cell metabolism are induction by substrate, nutritional regulation, and feedback regulation. The essence of fermentation lies in modifying such metabolic pathways to overproduce a desired metabolite (Sanchez and Demain 2002).

11.5.1.1 Regulation by Induction

Nature exposes microbes to dynamic physical and chemical environment at all times. To adapt themselves, they respond to these stimuli by varying their metabolism within a given limit. Absence or presence and if present, the concentration of a metabolite or enzyme would solely depend on the presence of compounds called inducers in the environment, which turns on the cell's metabolism and produces the compounds just as needed. An inducer helps translate a gene into a protein by binding with a repressor that is prebound to DNA at the operator and removes it to facilitate RNA polymerase binding. β-Galactosidases used for production of lactose-free milk products, glucose oxidase used for diagnostic purposes, and glucose isomerase employed in high fructose corn syrup production are some of the examples of inducible enzymes (Barker 1980). Multiple inducers may also regulate a single gene as in the case of xylanase III gene (Xyn III) induced by Avicel, sophorose, or sorbose in *Trichoderma reesei* PC-3-7, a filamentous fungi (Xu et al. 2000). Many times, these inducers are the substrates in which the produced enzymes work, as in case of tryptophan for tryptophanase (Botsford 1975), xylose for glucose isomerase (Sanchez and Smiley 1975), and hydantoinase for the production of d-*p*-hydroxyphenylglycine (Hartley et al. 2001). Industrial fermentation requires constitutive mutants that produce the product without the control of the inducers (Demain 1971, 1988; Hartley et al. 2001), adding great value to the process by lowering the cost of production (Demain 1971; Parekh et al. 2000).

Many pathways have also been reported to have amino acids as inducers of SM production. Few such examples are the use of methionine for the synthesis of cyclase, expandase, and δ-(L-α-aminoadipyl)-L-cysteinyl-L-D-valine synthethase by *Acremonium chrysogenum* in the cephalosporin pathway (Zhang et al. 1987), tryptophan for the production of dimethylallyl tryptophan synthetase in the biosynthesis of alkaloids in ergots (Krupinski et al. 1976), glutamate for the production of penicillin G (Lara et al. 1982), and α-aminoadipate for δ-(L-α-aminoadipyl)-L-cysteinyl-L-D-valine synthesis and also biosynthesis of penicillin, by the fungi *Penicillium chrysogenum* (Hönlinger and Kubicek 1989) and phenylalanine as an inducer for the formation of benzodiazepine alkaloid (Luckner and Nover 1977). SM production is also found to increase with the inducing amino acid levels inside the cells as in the case of penicillin overproducing strain of *P. chrysogenum* that showed higher level of α-aminoadipate pool and decreased rate of conversion of α-aminoadipic acid into lysine (Jaklitsch et al. 1986; Casqueiro et al. 1999). Branched amino acids have been reported to induce biosynthesis of nikkomycin by an unidentified mechanism (Zahner and Kurth 1982).

11.5.1.2 Nutritional Regulation

Catabolite Repression by Carbon Sources

Fermentative production of different metabolites by microbes requires a variety of carbon sources like glucose, sucrose, corn starch, and molasses to list a few, as a substrate for growth. Nevertheless, they sometimes have negative effect that limits production, a condition called carbon catabolite regulation. This phenomenon chiefly guarantees coordinated and consecutive utilization of available carbon sources, based on those that supply energy readily, when a complex of carbon sources is available. Also, the production of enzymes necessary for degrading other carbon sources is subdued until the primary source is spent. An example of SMs and enzymes regulated by carbon catabolite regulation includes repression of α-amylase production by glucose and acetate in *Aspergillus oryzae* (Barbesgaard et al. 1992), wherein glucose also acts as an inducer for the same enzyme astonishingly (Carlsen and Nielsen 2001). Another example pertains to the production of β-lactam ring containing antibiotic cephalosporin C repressed by glucose in *Acremonium chrysogenum*. Here, glucose blocks the mRNA formation of two genes, *pcbC* and *cefEF*. Surprisingly, another strain that produced more cephalosporin C had good amount of *pcbC* transcripts, indicating that deregulation of carbon source repression and strain improvement is interlinked (Jekosch and Kuck 2000). Not just glucose but its analogues were also reported to have a role in overproducing pyruvate by a fungus *Torulopsis glabrata* (Miyata and Yonehara 1990), pectinase and citric acid by *Aspergillus niger* (Kirimura et al. 1992; Loera et al. 1999), and glucoamylase by *Aspergillus terreus* (Ghosh et al. 1991).

Another way of increasing SM production in case of repressive carbon sources is to limit its concentration in the fermentation process by a fed-batch approach. This approach depends on the rate of feed supplied and cultivation kinetics that are case specific (Agrawal et al. 1989). The advantage with this system lies in the limiting concentration of the source that prevents suppressive effect over the product. One classical example of such a production is that of penicillin, where excess glucose leads to acid accumulation and increased oxygen demand than that can be supplied by the aeration system of the fermentor. However, limited glucose levels may also lead to increase in the pH of the system and insufficient biomass formation due to the use of organic nitrogen as a carbon source. Thus, for the commercial production of penicillin, there must be an optimization of concentrations of glucose and nitrogen sources to limit oxygen requirement and growth rate (Stanbury et al. 1995). Production of cellulase by *Trichoderma reesei* is also controlled by fed-batch mechanism (Neway 1989).

Regulation by Nitrogen Sources

Microbes rely on a wide variety of nitro compounds for their growth as nitrogen sources, but not all of them are effective. Generally, ammonia, asparagine, and glutamine are considered good, while urea and proline are considered poor sources

of nitrogen. As already discussed for carbon sources, microbes also developed highly coordinated regulatory mechanisms that allow them to utilize energy-efficient nitrogen source first from the complex of sources available and simultaneously repress production of enzymes that are not required at that instance. Regulation of nitrogen sources is particularly important in fermentation technology as it affects the enzymes involved in primary and secondary metabolite production, affecting cell growth as well as product formation (Merrick and Edwards 1995; Shapiro 1989). Uricase enzyme used in estimation and removal of uric acid, nitrate reductase used in determination and elimination of nitrates from water, proteases used in the dairy products and detergents, N-carbamoylamino acid amidohydrolase and hydantoinase are some examples of enzymes repressed by ammonium salts (Hartley et al. 2001; Hawker et al. 1992; Kole et al. 1988; Marzluf and Fu 1988; Oestreicher and Scazzocchio 1993).

Nitrogen sources like ammonium salts that favor growth have been reported to have negative effect on SM production. In order to compensate, complex media would be supplemented with a protein source like soybean meal, and defined media would be fortified with amino acids like proline that gets slowly metabolized, for improved SM production. Very little literature is available on the mechanism of inhibition of ammonium and certain amino acids on fermentative production of SMs. Neomycin and kanamycin production are shown to have negative effect due to the presence of ammonium, but nitrate and some amino acids have inducing effect (Shapiro 1989). Also, no unanimity is found in the literature regarding the effect of ammonium on the production of aminoglycosides, as few report to obstruct but others have shown to stimulate. Dihydrostreptomycin production was shown to be tripled by entrapping ammonium using magnesium phosphate in the broth (Omura and Tanaka 1984). Contrastingly, Inoue et al. (1983) showed that ammonium stimulated streptomycin production.

Nitrogen-rich media is shown to affect enzyme synthesis for metabolism and also utilization of other nitrogen sources present in the broth. To avoid such effect on SM production, many approaches have been reported. Most frequently used one is the genetic manipulation. Mutant strains that are not affected by nitrogen sources, those that are resistant to growth inhibition in the presence of ammonium or amino acid analogues, and those that overproduce at high ammonium levels were isolated (Sanchez and Demain 2002).

Regulation by Phosphorus

Inorganic phosphorus is usually the main growth-limiting factor of natural ecosystems. Living organisms, therefore, evolved a wide range of mechanisms to utilize phosphorus optimally. Inorganic phosphate often regulates production of SMs and enzymes in fermentation conditions. Not much has been studied about the regulation of phosphate in SM production, but it is putatively involved in affecting enzymes like kinases and phosphatases (Krebs and Beavo 1979). In *Dictyostelium discoideum*, morphological differentiation is found to be controlled by phosphates

through protein kinases (Leichtling et al. 1984). Phosphate is also reported to control pathways that do not involve phospho- or dephosphorylations, as in the case of red polyketide pigment formation in *Monascus* sp. by enzyme inhibition (Lin and Demain 1993). Phosphate also represses various antibiotic synthetases, where orthophosphate is not at all involved, as seen in β-lactam synthetases repression in *Acremonium chrysogenum* that produces cephalosporin C (Martin 1989). Thus, phosphate-sensitive production has to be carried out at low concentrations of phosphate, which is usually less than that required for growth, for high productivity. Another strategy to overcome growth anomalies in microbes is the use of classical genetics, where phosphate-resistant mutants are isolated and employed in fermentation. This approach has been employed for anthracycline production (Segura et al. 1997) and a non-polyenic macrolide antibiotic formation (Gesheva 1994).

11.5.1.3 Feedback Regulation

Feedbackregulation is the approach where metabolic pathways self-regulate their product formation. It is accomplished by any one of the two means: feedback inhibition (controlling the existing enzyme activity) and feedback repression (blocking the enzyme synthesis). Feedback regulation of pathways involved in primary and secondary metabolism in living systems is well established. Amino acids, vitamins, and nucleotides are some of the commercially important primary metabolites whose production is regulated by feedback mechanism. This regulation gets perplexed due to the presence of branched pathways in making multiple products as in aspartate family amino acids that exhibit allosteric inhibition (Kikuchi et al. 1999). A lot of SMs like ergot alkaloids, tylosins, antibiotics, etc. either inhibit or subjugate their own production. Examples include repression of acetyltransferase in kanamycin biosynthesis, arylamine synthetase in chloramphenicol production, and inhibition of *O*-methyltransferases in synthesis of indolmycin, alkaloids, mycophenolic acid, and puromycin.

Improvements in the field of genetic engineering led to better strategies than classical screening for mutant strains. Metabolic engineering, an amalgam of microbial genetics, genetic engineering, and cell biology, can also be employed in overproducing desired metabolites (Nielsen 2001). Tools for gene manipulation and cell function analysis are the basis for successful engineering of metabolome. For enzymes regulated by feedback mechanism, it would be beneficial to improve the reaction that follows the production of inhibitory metabolite in the cascade, to increase the yield of the product. This technique is successfully applied by Nielsen and Jørgensen (1995) who achieved regulation of acyl-CoA: isopenicillin acyltransferase enzyme that converts isopenicillin N into penicillin G by *P. chrysogenum* (Nielsen and Jørgensen 1995). Another strategy can be the introduction of genes insensitive to feedback regulation of enzymes. Amino acids (Ikeda and Katsumata 1992; Eggeling and Sahm 1999), antibiotics (Khetan and Hu 1997), and carotenoids (Miura et al. 1998; Shimada et al. 1998) are few more examples of products obtained by using metabolic engineering to improve the yield of fermentation.

11.6 Fermentative Production of Secondary Metabolites by Bioengineered Fungal Strains

Fungi are well-known for their ability to produce SMs. However, their produce cannot fulfill the requirements of the industry and society. Thus there is a need to produce these commercially important metabolites in large quantities from potent strains at reasonable fermentation conditions. A step toward this approach is taken by Richter et al. (2014) in expressing a nonribosomal peptide synthetase ESYN of *Fusarium oxysporum* heterologously in *Aspergillus niger* that exhibits antiviral, antimicrobial, and antineoplastic characteristics. The *esyn1* gene, whose product catalyzes enniatin family peptide synthesis, was controlled by a tunable hybrid promoter called Tet-on promoter that works in bacterial and fungal systems and that turns on during the exponential phase in the growth of *A. niger*. By optimizing the feeding conditions, high yield was achieved by them. They could also establish a strain of *A. niger* that also constitutively produced D-HIV dehydrogenase, which utilizes α-ketovaleric acid pool in the cell, to make the precursor of enniatin, along with the ESYN (Richter et al. 2014).

Gerke and Braus (2014) reviewed the potential of engineering the fungal strains for their ability to produce novel SMs. Their discussion is on the potential of mutant strains whose conserved eukaryotic regulatory complexes called COP9 signalosome and the velvet complex, which has a link with the development and SM production, are impaired to make the fungi produce the SMs. Flaws in these complexes alter the development of the fungi and regulation of SM production (Richter et al. 2014). Polyketide metabolites, lovastatin, and geodin were produced by the engineered strains of *Aspergillus terreus*, and their produce was improved by Askenazi et al. (2003) by generating transcriptional profiles and identifying specific promoters that control the synthesis of these commercial metabolites.

In 2006, Ro et al. produced high titers of an antimalarial drug precursor artemisinic acid from engineered *Saccharomyces cerevisiae* by altering mevalonate pathway, a novel cytochrome P450 monooxygenase and amorphadiene synthase from the plant *Artemisia annua*. Artemisinic acid, thus produced, is an extracellular product, making the purification simple and cost-effective (Ro et al. 2006).

Sakai et al. (2008) constructed a heterologous expression system in *Aspergillus oryzae* that consists of citrinin synthesizing gene cluster through a cosmid vector that can shuttle between *Aspergillus* and *E. coli*, from *Monascus purpureus*. The transformed clones were further gene-edited by incorporating additional copies of an activator gene, *ctnA*, which is regulated by *A. nidulans* trpC promoter, improving the yield by 400-fold (Sakai et al. 2008). Xue et al. (2017) constructed a recombinant *Pichia pastoris* having citrinin polyketide synthase gene *pksCT* from *Monascus purpureus* and phosphopantetheinyl transferase gene *npgA from Aspergillus nidulans*. They could successfully produce citrinin by methanol induction after assembling its complete biosynthetic pathway (Xue et al. 2017). Van Dijk and Wang (2016) also reviewed on the heterologous expression of SMs in the host *Aspergillus nidulans* (van Dijk and Wang 2016). Most recently, He et al. (2018)

reviewed SM pathway reconstruction in addition to gene editing to discover novel compounds, understand their pathways, and improve their yield (He et al. 2018).

11.7 Application of Fungal Secondary Metabolites

11.7.1 Antifungal Metabolites

A vast number of fungi are known to produce compounds that are useful in defense from other fungal species, thus known for their antifungal activity. *Trichoderma* spp. are known to possess fungicidal and fungistatic properties putatively as they could produce cell wall degrading enzymes and different SMs. It has also been proven that some of these species induce either a local or a systemic defense response in agricultural crops like cotton, cucumber, bell pepper, tobacco, and lettuce (Harman et al. 2004; Yedidia et al. 2003). They are also been found to be widely employed in producing commercial enzymes that recycle cellulosic wastes (Cutler et al. 1999).

Chrysophanol, an anthraquinone produced by *Trichoderma aureoviride* isolates (de Stefano et al. 1999), displayed antifungal activity against *Cryptococcus neoformans*, *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes* (Agarwal et al. 2000). *Trichoderma virens* is found to yield a novel daucane sesquiterpene or carotane that is usually found in plant families like Compositae and Umbelliferae, which also showed similar activity against different yeasts including *Candida albicans* (Watanabe et al. 1990) and dermatophytes.

A pyrone compound 6-pentyl-2H-pyran-2-one, first discovered to be produced by *Trichoderma viride* (Collins and Halim 1972) and later commercially produced by *Trichoderma harzianum* (Claydon et al. 1987) and *Trichoderma koningii* (Simon et al. 1988), is proved to have good activity against *Rhizoctonia solani* and, to some extent, against *Fusarium oxysporum* (Scarselletti and Faull 1994). Another analogue of this pyrone, a volatile compound, 6-(1′-pentyl)-2H-pyran-2-one, was also shown to have similar activity against *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Penicillium* spp., and *Candida albicans* (Claydon et al. 1987; Parker et al. 1997). Hill et al. (1995) patented two hydro-derivatives of this pyrone, namely, massoilactone and δ-decanolactone, for their alleviating ability of plant disease symptoms caused by *Botrytis* and *Phytophtora* spp. (Hill et al. 1995). They were also shown to inhibit growth of *Candida albicans*, *Aspergillus niger*, and also *Staphylococcus aureus* by Kishmoto et al. (2005). Another related compound viridepyronone produced by *T. viride* has inhibitory activity on *Sclerotium rolfsii*, a fungal plant pathogen (Evidente et al. 2003). A set of complex pyranes called koninginins A and B produced by *T. harzianum* (Almassi et al. 1991), D produced by *T. koningii* (Dunlop et al. 1989), E produced by *T. harzianum* (Ghisalberti and Rowland 1993) and *T. koningii* (Parker et al. 1995), and G produced by *T. aureoviride* (Cutler et al. 1999) were all shown to have antagonistic effect on the growth

of *Gaeumannomyces graminis* var. *tritici* fungus (Almassi et al. 1991; Ghisalberti and Rowland 1993). Growth of plant pathogens like *Phytophthora cinnamomi*, *Fusarium oxysporum*, *Bipolaris sorokiniana*, *Rhizoctonia solani*, and *Pythium middletonii* was also affected by koninginin D (Dunlop et al. 1989).

A group of steroidal antibiotics called viridins have been shown to possess antifungal activity and also inhibitory activity on certain enzymes involved in cell signaling (Hanson 1995). Viridin, an antifungal compound found to be produced initially by *T. virens* by Brian and McGowan (1945), is also shown to be produced by *T. koningii* (Berestetskii et al. 1976), *T. viride* (Golder and Watson 1980), and *T. virens* (Singh et al. 2005). This compound forbids spore germination in *Fusarium caeruleum*, *Colletotrichum lini*, *Stachybotrys atra*, *Penicillium expansum*, *Aspergillus niger*, *and Botrytis allii* (Brian and Mcgowan 1945; Ghisalberti 2002). A similar alcohol, viridiol, expressed by *T. viride* and few *Gliocladium* sp., is demonstrated to have phytotoxic effect too along with antifungal properties (Howell and Stipanovic 1994; Moffatt et al. 1969). Variants of the same compound, demethoxyviridin, also displayed similar properties (Aldridge et al. 1975). Cole et al. (1975) also demonstrated phytotoxicity of demethoxyviridiol produced by *Nodulisporium hinnuleum* (Cole et al. 1975). Wortmannin and 11-desacetoxywortmannin, initially obtained from *Penicillium* sp., were elucidated to have fungicidal activity by Brian et al. (1957) and Haefliger and Hauser (1973), respectively. Viridiofungins, a class of metabolites with citrate group in them, produced by *T. viride* (Harris et al. 1993; Mandala et al. 1997), are a broad range of fungicides against *Aspergillus*, *Cryptococcus*, and *Candida* sp. (Harris et al. 1993).

Dickinson et al. (1989) isolated harzianopyridone, a pyridine ring containing compound from *T. harzianum* that inhibited growth of *Rhizoctonia solani*, *Botrytis cinerea* (Dickinson et al. 1989), *Pythium ultimum*, and *Gaeumannomyces graminis* var. *tritici* (Vinale et al. 2006). Vinale et al. (2006) also found that a compound T22azaphilone, produced by two strains of *T. harzianum*, had inhibitory effect on *Pythium ultimum*, *Rhizoctonia solani*, and *Gaeumannomyces graminis* var. *tritici*.

Metabolites possessing butenolide ring, like the harzianolide obtained from various strains of *T. harzianum* (Almassi et al. 1991; Claydon et al. 1991; Ordentlich et al. 1992), its dehydro-derivative, and T39butenolide isolated by Vinale et al. (2006) from a commercial *T. harzianum* strain (Vinale et al. 2006), were all found to show antagonistic effect on the growth of *Gaeumannomyces graminis* var. *tritici* (Almassi et al. 1991; Vinale et al. 2006). Seiridin and isoseiridin obtained from *Seiridium cardinale* (Evidente et al. 1986; Sparapano et al. 1986) are found to be phytotoxic (Lorenzo Sparapano 1995). Trichothecenes, mainly obtained from genus *Fusarium*, are mycotoxic for humans and animals, causing symptoms like vomiting, diarrhea, intestinal problems, and immune system impairment (Hussein and Brasel 2001). Bisvertinolone, a fermentative product of *T. longibrachiatum* (Abe et al. 1998a, b; Andrade et al. 1992), is an antifungal metabolite that acts by inhibiting β-(1,6)-glucan biosynthesis (Kontani et al. 1994).

Gliotoxin is a diketopiperazine compound produced by *T. viride* (Brian 1944) that exhibited antifungal properties to *Rhizoctonia solani* (Jones and Pettit 1987). A close relative of it, gliovirin, is produced from *Gliocladium virens* (*T. virens*)

(Stipanovic and Howell 1982) and is effective against *Pythium ultimum* (Howell and Stipanovic 1983). Ergokonins A and B, two oxygenated derivatives of ergosterol, i.e., fungal sterol, produced from *T. koningii* (Reichenbach et al. 1990; Augustiniak et al. 1991), possess inhibitory effect on yeast and mycelial fungi, which has been patented by Reichenbach et al. (1990). It was also shown that ergokonin A is nearly ten times more effective than ergokonin B (Reichenbach et al. 1990). Vicente et al. (2001) also showed that ergokonin A is effective against *Aspergillus* and *Candida* sp. and inactive against *Fusarium*, *Cryptococcus*, and *Saccharomyces*. A 3,4-dihydro-8-hydroxy-3-methylisocoumarin compound, mellein, obtained initially from *Aspergillus* sp. (Burton 1950; Sasaki et al. 1970) and later from *T. aggressivum*, is active against *Agaricus bisporus* (Krupke et al. 2003).

11.7.2 Antineoplastic Metabolites

Emodin, an anthraquinone of *Trichoderma viride* (Slater et al. 1967), is an antineoplastic agent (Wu et al. 2006; Huang et al. 2006). Trichodermamides A and B, modified dipeptides, were initially isolated from marine *T. virens*. Liu et al. (2005) and Capon et al. (2005) later isolated these compounds from *Aspergillus unilateralis* and *Spicaria elegans*. Trichodermamide B exhibited substantial in vitro cytotoxicity against the human colon carcinoma HCT-116 cell lines, whereas trichodermamide A demonstrated less cytotoxicity on three different cancer cell lines A-549, HL-60, and P388 (Garo et al. 2003; Liu et al. 2005).

Viridin, viridiol, demethoxyviridin, demethoxyviridiol, wortmannolone, and virone were all shown to inhibit phosphatidylinositol 3-kinase (Dodge et al. 1995), thus used in the treatment of PI 3-kinase-dependent pathways, including neoplasms in humans (Ghisalberti 2002). Viridiofungins were also shown to inhibit farnesyl transferase, an important enzyme involved in the farnesylation of Ras protein, thus capable of treating cancer (Meinz et al. 1993). Mandala et al. (1997) also demonstrated cytotoxic effect of these viridiofungins on HeLa cells at concentrations as low as nanomoles.

Trichodenones, natural cyclopentenones, produced by *T. harzianum* isolated from the poriferan *Halichondria okadai* gathered in Japan (Amagata et al. 1998), displayed cytotoxic effect on cultured P388 cells. Harziphilone isolated from *T. harzianum* was found to be cytotoxic to murine cancer cell line M-109 at micromolar concentrations (Qian-Cutrone et al. 1996). Trichodermin, first purified from T. viride (Godtfredsen and Vangedal 1964) and later produced from *T. sporulosum* and *T. polysporum* (Adams and Hanson 1972) and *T. reesei* (Watts et al. 1988), was found to be a cytotoxic metabolite against several cell lines (Choi et al. 1996). Corley et al. (1994) showed that a culture of *T. harzianum* produced harzianum A, a compound that displayed cytotoxicity on HeLa and HT1080 cell lines (Lee et al. 2005). Heptelidic acid chlorohydrin isolated from a strain of *Acremonium* exhibited cytotoxic effect against some human cancer cell lines as demonstrated by Kawashima et al. (1994).

11.7.3 Antibacterial Metabolites

Wu et al. (2006) showed that emodin can act as an antimicrobial agent (Wu et al. 2006). It also displayed bacteriostatic effect on Gram-positive bacteria like *S. aureus* (Chukwujekwu et al. 2006). Trichodermaol, another related compound, also showed antibacterial activity on *Streptococcus aureus* and *Bacillus subtilis* (Adachi et al. 1983). Tamura et al. (1975) isolated isonitrile trichoviridin from *T. koningii* (Tamura et al. 1975); however, Yamano et al. (1970) have already patented its isolation from *T. viride* and its antibiotic properties (Yamano et al. 1970).

An antimicrobial compound trichosetin, with considerably good activity against *Bacillus subtilis* and *Staphylococcus aureus*, was isolated from dual culture of *T. harzianum* and *Catharanthus roseus* callus (Marfori et al. 2002). A homologue of trichosetin, named equisetin, isolated from *Fusarium* sp. is an antibiotic against Gram positives (Burmeister 1976). Cissetin, isolated from the fungus OSI 50185, is very active against many Gram positives and, in particular, manyfold active than trichosetin and equisetin against penicillin-resistant *Streptococcus pneumoniae* (Boros et al. 2003). *Staphylococcus aureus* was shown to be inhibited by two analogues of gliovirin isolated from *T. longibrachiatum* (Nakano et al. 1990). Various peptaibols synthesized by different *Trichoderma* sp. are found to possess antimicrobial activity against Gram-positive organisms (Reino et al. 2008). Berg et al. (2004) demonstrated that a cyclonerodiol derivative called lignoren, isolated from *T. lignorum*, showed reasonable antimicrobial activity against *Mycobacterium smegmatis*, *Bacillus subtilis*, *Sporoblomyces salmonicolor*, *Rhodotorula rubra*, and *Pseudomonas aeruginosa* (Berg et al. 2004).

Koningic acid, a sesquiterpene heptelidic acid, isolated from culture filtrate of *Chaetomium globosum*, *Gliocladium virens*, and *Trichoderma viride*, had displayed activity against anaerobic bacteria, particularly against *Bacteroides fragilis* (Itoh et al. 1980). Pentalenolactone, produced by several *Streptomycetes* strains, is found to be active against a wide range of organisms including Gram positives, Gram negatives, pathogenic, saprophytic fungi, and also protozoans (Cane and Sohng 1994).

11.7.4 Therapeutic Metabolites

Ali et al. (2004) showed that the anthraquinone, emodin, had psychotherapeutic effect (Ali et al. 2004). Hydroxy lactone called R-mevalonolactone is isolated from *T. harzianum* OUPS-N115 strain obtained by Amagata et al. (1998). This compound is found to assist metabolism of cholesterol in aged skin on application, thus a potential candidate as an antiaging skin cosmetic (Yamashita 2000).

Takashima and Wataya (1999) showed that trichodermol acts against malarial parasite. Trichodimerol, reported from *T. longibrachiatum* (Andrade et al. 1992), *Penicillium chrysogenum* (Warr et al. 1996), and *Trichoderma* USF-2690 strain (Abe et al. 1998a, b), prominently inhibits lipopolysaccharide- induced tumor

necrosis factor-α production, thus potentially being a new lead for the treatment of septic shock (Mazzucco and Warr 1996). Derivatives of trichodimerol, demethyl trichodimerol (Abe et al. 1998a, b), bisorbibetanone (Abe et al. 1999) produced by *Trichoderma* USF-2690, bisorbicillinol (Abe et al. 1998a, b), bisorbibutenolide, and bisorbicillinolide (Abe et al. 1998a, b), are all found to be antioxidants (Reino et al. 2008). Hebbar and Lumsden (1998) showed that gliotoxin has antibacterial, antiviral, and immunosuppressive characteristics.

Compactin, also called mevastatin, produced by *Penicillium brevicompactum* (Brown et al. 1976), *T. pseudokoningii*, and *T. longibrachiatum* (Endo et al. 1986), is a cholesterol-lowering agent (Endo 1985; Goldstein et al. 1979). Monacolin K (lovastatin), isolated from fungi like *Monascus*, *Aspergillus*, or *Verticillium*, and simvastatin, a synthetic relative of lovastatin, are leading lipid-lowering drugs available worldwide (Jones 1990). Tanaka et al. (1998) have shown that koningic acid has inhibitory activity in vitro toward human malarial parasite *Plasmodium falciparum*. Amplification of DNA viruses like HSV-1 and HSV-2 that cause herpes simplex is proved to be inhibited by pentalenolactone (Cane and Sohng 1994). Acorenone-B, a spiro-sesquiterpenic compound, exhibited inhibitory action on multidrug-resistant microbes like *Staphylococcus aureus* SA2 (Reino et al. 2008).

11.7.5 Enzyme Inhibitors and Ion-Channel Modulators

Emodin is found to have inhibitory activity against enzymes like monoamine oxidase (Fujimoto et al. 1998) and tyrosine kinase (Jayasuriya et al. 1992; Kumar et al. 1998). A carotane or daucane sesquiterpene and its oleic ester derivative called L-735,334 isolated by Lee et al. (1995) act as calcium-activated potassium channel modulators (Ondeyka et al. 1995; Lee et al. 1995).

Harzianic acid, a compound with pyrrolidinedione ring, produced by *T. harzianum* (Sawa et al. 1994) and its derivatives demethylharzianic acid and homoharzianic acid obtained from *T. harzianum* F-1531 (Kawada et al. 2004) were shown to be the inhibitors of protein phosphatase type 2A. Two other compounds, melanoxadin and melanoxazal, obtained from *Trichoderma* ATF-451 strain inhibited formation of melanin in *Bombyx mori*'s larval hemolymph. Also, melanoxazal displayed intense inhibition of mushroom tyrosinase enzyme (Hashimoto et al. 1995; Takahashi et al. 1996).

Westerberg et al. (1976) demonstrated the inhibitory activity of trichodermin on the elongation and termination of protein synthesis (Westerberg et al. 1976). Burmeister (1976) presented the potential of equisetin, and Singh et al. (1998) established the activity of phomasetin obtained from *Phoma* sp. as inhibitors of HIV-1 integrase enzyme. Cyclonerodiol, first reported from *Trichothecium* by Nozoe et al. (1970) and *Gibberella fujikuroi* by Pitel et al. (1971), exhibited inhibition in sodium channels of frog skeletal muscles.

Statins are found to control cholesterol biosynthesis by inhibiting HMG CoA reductase enzyme (Jakobisiak and Golab 2003). Koningic acid is found to be able to vary ATP production by inhibiting D-glyceraldehyde-3-phosphate dehydrogenase by Endo et al. (1985), Sakai et al. (1988), and Kato et al. (1992). Pentalenolactone is also shown to have the same potential as disclosed by Cane and Sohng (1994).

11.7.6 Other Metabolites

A pyrone compound, 6-pentyl-2H-pyran-2-one, identified in *T. viride* (Collins and Halim 1972) and later produced from *T. harzianum* (Claydon et al. 1987) and *T. koningii* (Simon et al. 1988), is a flavoring agent with the aroma of coconut, used in confectionery (Claydon et al. 1987). Other compounds like 3-octanone, 3-octanol, and 1-octen-3-ol produced by different *Trichoderma* sp. produce mushroom flavor (Combet et al. 2006). Methyl ketone, produced by the strain *T. viride* TS (Janssens et al. 1992), is used as a flavor enhancer in cheese making (Hagedorn and Kaphammer 1994; Patton 1950).

Harzianolide is found to promote plant growth by its auxin-like activity on the stems of *Pisum sativum,* and 6-*n*-pentyl-6*H*-pyran-2-one is shown to affect growth of the seedlings of *Brassica napus* and *Lycopersicum esculentum*, *indicating their role in regulation of plant growth and activation of defense response in these plants* (Vinale et al. 2008). Trichosetin is shown to inhibit growth of root and shoot in plants like *Vigna radiata*, *Oryza sativa*, *Capsicum frutescens*, *Lycopersicum esculentum*, and *Medicago sativa* in seedling growth assays (Marfori et al. 2003).

11.8 Conclusions

Production of secondary metabolites from fungal always finds immense capacity in industrial fermentation, due to the yield and economically feasible processes. The metabolites produced by them give important lead for future drug discovery and development. Elucidation of the molecular mechanism behind the secondary metabolites production will provide a deeper insight into the regulatory control and biosynthesis of these metabolites under controlled conditions. In the future, combination of genetic engineering and process development will further enhance the production of these metabolites under industrial-scale fermentation.

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Chapter 12
Recent Progress on *Trichoderma* Secondary Metabolites

Younes M. Rashad and Ahmed M. Abdel-Azeem

12.1 Introduction

Trichoderma Pers. (Ascomycota, Sordariomycetes, Hypocreales, teleomorph *Hypocrea* Fr.) species are frequently found on dead wood and bark, on other fungi, and in soil and living as endophytes within healthy plant roots, stems, and leaves (Al-Askar et al. 2012, 2014; Mukherjee et al. 2013; Salem and Abdel-Azeem 2014).

Trichoderma plays a significant role in various ecosystems (Klein and Eveleigh 1998). Species of this genus are commonly found in soil, on decaying wood, and occasionally on other fungi. They are important for humans due to their applications in the production of enzymes, antibiotics, and heterologous proteins for food, feed, textile, and biofuel industries (Liu et al. 2016; Darmasiwi et al. 2016; He et al. 2018), biocontrol of plant pathogens (Saber et al. 2017; El-Sharkawy et al. 2018; Swain et al. 2018), and treatment of water or soil pollutions (Harman et al. 2004). Nevertheless, some species are causal agents of green mold disease in mushroom cultivation (Komoń-Zelazowska et al. 2007) or even as opportunistic human pathogens (Kredics et al. 2003; Sandoval-Denis et al. 2014). Many taxa are treated as agents for improving seed germination and nutrient use efficiency and breaking of seed dormancy, as well as source of transgenes and herbicides, and are long known to improve plant growth through the production of certain secondary metabolites (Zeilinger et al. 2016; Patle et al. 2018).

Taxonomy of *Trichoderma* traces back to 1794 when the genus was established. Its species concepts were problematic due to their similarities in culture characteristics. The first serious attempt to establish clear species concepts was made by Rifai

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(1969) who divided the genus into nine "species aggregates" based on morphology. In the following years, Bissett (1984, 1991a, b, c) conducted detailed studies and divided *Trichoderma* into five sections based also on morphological features, which was later proved to be insufficient to differentiate species (Kindermann et al. 1998; Kullnig-Gradinger et al. 2002). With development of molecular techniques, DNA sequences were applied in exploring the phylogenetic relationships among species of the genus (Kindermann et al. 1998). Species of the genus have been divided into 17 clades, as well as scattered independent lineages, according to phylogenetic information (Zhu and Zhuang 2014; Jaklitsch and Voglmayr 2015; Qin and Zhuang 2016a). Previous studies showed also that fungi in the same clades may share certain similar morphological features in addition to sequence data.

Among these clades, the *Trichoderma* clade is the largest and comprises more than 70 species. The Harzianum, Longibrachiatum, and Viride clades are among the largest and most diverse clades of the genus. The Harzianum clade includes several morphologically cryptic but biologically distinct species. Recently 15 species were recently added to the clade (Chen and Zhuang 2017a), although relationships among species of the clade need further research. Chen and Zhuang (2017b) introduced seven new species of the genus *Trichoderma* belonging to the Viride clade from soil samples of different regions in China. In 2018, Zhang and Zhuang introduced three new species during their field surveys in central and southwestern China, namely, *T. acremonioides*, *T. rugosum*, and *T. subalni*, which are located, respectively, in the Viride, Longibrachiatum, and Harzianum clades. Up to now, about 295 *Trichoderma* species have been described based on combined analyses of phylogenetic and morphological data (Bissett et al. 2015; Montoya et al. 2016; Qin and Zhuang 2016a, b, c; Qin and Zhuang 2017; Chen and Zhuang 2017a, b; Zhang and Zhuang 2017, 2018).

Trichoderma spp. display antimicrobial activity against an important number of bacteria, yeasts, and filamentous fungi (Vizcaíno et al. 2005), in which numerous and varied secondary metabolites (SMs), such as peptaibols, gliotoxin, gliovirin, polyketides, pyrones, and terpenes, may be involved (Vinale et al. 2006, 2009; Xiao-Yan et al. 2006). Considering the structures of *Trichoderma* antibiotic molecules present in nature, two main types have been distinguished: (1) low-molecular-weight and volatile metabolites, including simple aromatic compounds, some polyketides such as pyrones and butenolides, volatile terpenes, and isocyanate metabolites, all of them relatively nonpolar substances with a significant vapor pressure, and (2) high-molecular-weight polar metabolites, including peptaibols and diketopiperazine-like gliotoxin and gliovirin compounds, which may exert their activity through direct interactions by means of contact between *Trichoderma* spp. and their antagonists (Sivasithamparam and Ghisalberti 1998; Szekeres et al. 2005; Reino et al. 2007).

Over the years, numerous *Trichoderma* SMs have been isolated and their structures determined in analytical studies. More than 120 *Trichoderma* SMs structures have been reported (Sivasithamparam and Ghisalberti 1998; Reino et al. 2007). However, quantifying the exact number of SMs produced by *Trichoderma* spp. is not an easy task since they can produce more than 1000 compounds, depending on the strain, the environmental conditions, and the sensitivity of the detection method.

In recent years, information about the biosynthetic pathways of *Trichoderma* SMs and aspects regarding fungal metabolism and ecological interactions have begun to emerge from genetic and genomic studies. As reported for other fungi (Yu and Keller 2005; Bayram et al. 2008), heterotrimeric G-proteins and MAPKs affect the production of *Trichoderma* antifungal metabolites (Reithner et al. 2005, 2007); and the *vel1* velvet gene has also been implicated in the regulation of gliotoxin biosynthesis and other SMs genes, as well as conidiation in *T. virens* (Mukherjee and Kenerley 2010). Bioinformatics analysis of the three available *Trichoderma* genomes has revealed that the mycoparasitic species *T. atroviride* and *T. virens* are enriched in SMs-related genes as compared with *T. reesei*, a biomass-degrading species (Martinez et al. 2008; Kubicek et al. 2011; Baker et al. 2012; Mukherjee et al. 2012b). These latter and other recent studies have allowed progress in the field of *Trichoderma* SMs to determine the role of these compounds in three-way complex interactions between plants, phytopathogens, and *Trichoderma* (Viterbo et al. 2007; Velázquez-Robledo et al. 2011; Malmierca et al. 2012; Mukherjee et al. 2012b).

Hermosa et al. (2014) concluded that many SMs remain to be discovered in *Trichoderma* since this fungal genus includes multiple species that are highly adapted to different ecological niches and their SMs diversity seems to be limitless. Modifying growth conditions, manipulating regulatory factors, and using new mass spectrometry techniques should allow the discovery of novel molecules. Few SMs regulator proteins have been identified, and far from being a bottleneck, this has encouraged studies aimed at understanding SMs gene organization, transcription, and production. Many *Trichoderma* SMs isolated in the past were not investigated for a wide range of biological activity, and perhaps most of them need to be re-evaluated using new scenarios to explore novel applications. The development and use of acute, sensitive, and specific analytical methods would be fruitful in the screening of *Trichoderma* SMs, and for our understanding of their biosynthetic machinery in light of the expanding knowledge of microbial genetics and the information acquired from the genomes of this sequenced genus. However, the cooperation of scientists from several different disciplines is essential if we are interested in understanding the function of genes in SMs production. This may will lead to the discovery of novel compounds or pathways, which in turn may reveal important new aspects for many human applications, including pharmaceutical and agri-biotechnological uses.

A wide variety of SMs produced by *Trichoderma* was applied in agricultural, industrial, and pharmaceutical uses (Saravanakumar et al. 2015, 2018; El-Sharkawy et al. 2018; He et al. 2018). *Trichoderma* SMs exhibit diverse biological activities which enable their producers of *Trichoderma* spp., directly and indirectly, to perform multifunctional roles and prevail in different ecosystems. For example, fungi that inhabit the polar (Arctic and Antarctic) areas produce a variety of SMs in order to survive in the extreme and severe environmental conditions (Tripathi et al. 2018). Kamo et al. (2016) reported 11 known bioactive compounds in addition to another new one which produced by *T. polysporum* (OPU1571) isolated from Arctic region. However, production of these SMs is species- or in some cases strain-dependent and

may require special conditions and/or specific stimulator(s) (Zeilinger and Schuhmacher 2013). These SMs include terpenes, peptaibols, siderophores, pyrones, polyketides, gliotoxin, gliovirin, and isocyanate metabolites (Zeilinger et al. 2016). In this regard, more than 1000 of bioactive compounds have been reported to be produced by the members of *Trichoderma* genus (Neumann et al. 2015).

Biosynthesis of the SMs is controlled by gene clusters encoding a set of synthetic enzymes (such as nonribosomal peptide synthetases, polyketide synthases, and terpene synthases), accessory enzymes (such as oxidoreductases), and/or transporters and transcription factors (such as xylanase promoter-binding protein 1) (Bansal and Mukherjee 2016a, b; Derntl et al. 2017). Revealing of the whole genome sequence of some *Trichoderma* taxa, viz., *T. reesei*, *T. atroviride*, and *T. virens*, enriches the understanding about the SMs-related gene regulation (Druzhinina et al. 2011; Mukherjee et al. 2013). Nevertheless, most SMs' biosynthesis-related genes are silent under normal lab conditions which impedes the detection of new SMs (Wiemann and Keller 2014). To overcome this issue, use of SMs regulators is needed (Derntl et al. 2017).

Trichoderma spp. produce a variety of SMs for varied purposes which comprise competition, mycoparasitism, antibiosis against other microorganisms and predators, induction of microbial growth, communication with plant and other organisms, plant growth regulation, induction of plant resistance, protection against extreme environmental conditions, and as signaling or effectors molecules (Contreras-Cornejo et al. 2016; Venturi and Keel 2016; Abdalla et al. 2017; Saber et al. 2017; El-Sharkawy et al. 2018). However, some SMs pose toxic potentiality, while others have therapeutic and pharmaceutical uses (Vargas et al. 2014; Saravanakumar et al. 2016, 2018). In this chapter, we will discuss the recent progress on different SMs produced by members of genus *Trichoderma* for various purposes. Names and activities of some newly identified SMs are given in Table 12.1 with supporting references.

12.2 Antimicrobial Activity

Members of *Trichoderma* genus have been extensively studied as biocontrol agents against phytopathogens and pests due to their antagonistic potentialities (Liu et al. 2016; Kamo et al. 2016; Debbi et al. 2018). In this regard, El-Sharkawy et al. (2018) reported the biocontrol of wheat stem rust disease using isolates of *T. harzianum* HL1 and *T. viride* HL5. The anti-spore germination effect of their SMs (3-deoxy-d-mannonic acid, 5-hydroxymethylfurfural, succinic acid, 1,2,3-propanetriol monoacetate, 1,3-dihydroxyacetone, lactic acid, butanoic acid, and valeric acid) was recorded against the uredospores of *Puccinia graminis*. The antifungal activity of SMs of *T. harzianum* WKY1 against the mycelial growth and spore germination of *Colletotrichum sublineolum*, the causative of sorghum anthracnose, was also reported (Saber et al. 2017). Swain et al. (2018) reported a novel strain of *T.*

Table 12.1 Newly identified secondary metabolites from *Trichoderma* spp

Activity	Compound name	Species name	Reference
Antifungal	Nonribosomal peptides SF1	*T. velutinum* ACR-P1	Sharma et al. (2016b)
	Nonribosomal peptides SF4		
	1-(4-Bromobutyl)-2-piperidinone	*T. asperellum* GDFS1009	Wu et al. (2017)
	2,2,6,6-Tetramethyl-4-piperidinone		
	6-Pentyl-2H-pyran-2-one		
	2,6,10-Trimethyl-tetradecane		
	2,6,10-Trimethyl-pentadecane		
	Oxalic acid, butyl 6-ethyloct-3-yl ester		
	Trichokonin VI	*T. pseudokoningii* SMF2	Zhao et al. (2018)
	1, 2-Benzenedicarboxylicacid, bis (2-methylpropyl) ester	*T. longibrachiatum* T6	Zhang et al. (2018)
	1, 2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester		
	3-Deoxy-d-mannoic acid	*T. harzianum* HL1	El-Sharkawy et al. (2018)
	5-Hydroxymethylfurfural		
	1,2,3-Propanetriol monoacetate		
	1,3-Dihydroxyacetone		
	Succinic acid		
	Valeric acid		
	Butanoic acid		
	Lactic acid		
Antibacterial	Trichaspside A	*T. asperellum* cf44-2	Song et al. (2018)
	Trichaspside B		
	7,10-Epoxycycloneran-3,11,12-triol		
	11-Hydroxy-9-harzien-3-one		
	Trichocitrin	*T. citrinoviride* cf-27	Liang et al. (2016b)
	Nafuredin		
	Citrostadienol		
	Citrinovirin		

(continued)

Table 12.1 (continued)

Activity	Compound name	Species name	Reference
Precursors for antimicrobial compounds	Acetamide Diethylamine Ethylene glycol Ethanolamine o-Toluic acid Ethylamine Glycine	*T. asperellum* GDFS1009	Wu et al. (2017)
Cytotoxic	Tandyukisins E	*T. harzianum* OUPS-111D-4	Suzue et al. (2016)
	Tandyukisins F		
Anticancer	16-Methylheptadecanoic acid methyl ester	*Hypocrea lixii* TSK8	Saravanakumar et al. (2015)
Antioxidant	Hydrophobin HFBII	*T. reesei*	Khalesi et al. (2016)
Antihypercholesterolemic	Lovastatin	*T. viride*	Rashid et al. (2014)
Mycotoxin, mutagenic, fetotoxic, teratogenic	Alternariol 1′-hydroxy-9-methyl ether (1)	*Trichoderma* sp. Jing-8	Solhaug et al. (2015)
Mycotoxin, antimalarial, anticancer	Gliotoxin	*T. virens*	Vargas et al. (2014)
Mycotoxin	Trichothecene (trichodermin)	*T. arundinaceum* *T. brevicompactum*	Shentu et al. (2013) Sharma et al. (2016a)
Plant growth promoter	Indole-3-acetic acid	*T. harzianum* WKY1	Saber et al. (2017)
	Chrysophanol	*T. harzianum* ETS-323	Liu et al. (2016)
	Ethylene	*T. atroviride*	Contreras-Cornejo et al. (2015)
	Harzianolide	*T. harzianum* SQR-T037	Cai et al. (2013)
	β-Acoradiene β-Cubebene β-Cedrene β-Bisabolene β-Himachalene γ-Himachalene	*T. aggressivum* *T. asperellum* *T. harzianum* *T. longibrachiatum* *T. pseudokoningii* *T. viride*	Lee et al. (2016)
Coconut aroma	Aroma6-pentyl-α-pyrone	*T. viride* EMCC-107	Fadel et al. (2015)
Aroma compound	δ-Octalactone γ-Nonalactone γ-Undecalactone γ-Dodecalactone δ-Dodecalactone		

(continued)

Table 12.1 (continued)

Activity	Compound name	Species name	Reference
Antiplatelet aggregation	Herquline B	*T. pinophilus* F36CF	Vinale et al. (2017)
Antiproliferative	O-Methylfunicone		
	Harziaphilic acid	*T. harzianum* M10	Vinale et al. (2012)
Siderophore	Ferrirubin Ferricrocin Coprogen B Dimerumic acid		

erinaceum isolated from tree barks in India as a biocontrol and biofertilizer agent for rice crop. This isolate significantly inhibited growth of the phytopathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii*, and *S. oryzae* in vitro and *R. solani* and *Helminthosporium oryzae* under field conditions. A novel isolate of *T. asperellum*, designated GDFS1009, shows potent antifungal activity against the phytopathogens *Fusarium oxysporum* f. sp. *cucumerinum* Owen and *F. graminearum*; the causal agents of *Fusarium* wilt of cucumber and stalk rot of corn, respectively, was also reported by Wu et al. (2017). This isolate exhibited high inhibition rate against both of the two pathogens in vitro and in the greenhouse. In addition, precursors for antimicrobial compounds such as acetamide, diethylamine, ethylene glycol, ethanolamine, o-toluic acid, ethylamine, and glycine, as well as a set of antimicrobial SMs such as 1-(4-bromobutyl)-2-piperidinone, 2,2,6,6-tetramethyl-4-piperidinone, 6-pentyl-2H-pyran-2-one, 2,6,10-trimethyl-tetradecane, 2,6,10-trimethyl-pentadecane, and oxalic acid, butyl 6-ethyloct-3-ylester were reported to be produced by *T. asperellum* GDFS1009. Molecularly, in the *T. asperellum* GDFS1009 genome, genes encoding for mycoparasitism-related enzymes (chitinase, glucanase, and protease) and induced resistance elicitors (endopolygalacturonase, Epl protein, hydrophobin, polygalacturonase, swollenin, and xylanase) were detected. All of these detected factors are contributed in the antagonistic nature of *T. asperellum* GDFS1009 against the two tested *Fusarium* pathogens. In general, the antagonistic mechanisms exerted by *Trichoderma* members include production of antimicrobial SMs (antibiosis), mycoparasitism, competition with other microorganisms for space or nutrients, and/or induction of the plant resistance against the invaded pathogen (Abdel-Fattah et al. 2007; Qualhato et al. 2013; Sadykova et al. 2015; Arseneault and Filion 2017; Zhang et al. 2018).

On the other hand, development of multidrug-resistant pathogens as a result of the inadvisable use of antibiotics and different climatic changes increases the demand to discover novel antimicrobial SMs (Garcia-Solache and Casadevall 2010; Chaudhary 2016). Biomedical activities of SMs produced by *Trichoderma* spp. have been studied in the recent years as new alternatives to the common antibiotics (Sadykova et al. 2015; Saravanakumar et al. 2018). Various new SMs with antimicrobial activities have been identified from *Trichoderma* spp. Nonribosomal peptides (NRPs) are a group of antimicrobial peptides produced by a wide range of

microorganisms, among them, the so-called peptaibiotics which produced mainly by *Trichoderma* spp. More than 1297 peptaibiotic sequences have been reported in the literature (Neumann et al. 2015). The produced NRPs showed a variety of bioactivities including antifungal, antibacterial, antiviral, anticancer, antiparasitic, and immunosuppressant (Panizel et al. 2013; Zhao et al. 2018). In addition, they have been used as bioagents against phytopathogenic fungi (Degenkolb et al. 2015). In this regard, two NRPs, belonging to subfamilies SF4 (medium chain of amino acid residues) and SF1 (long-chain amino acid residues), were reported to be produced by the new psychrotrophic isolate *T. velutinum* ACR-P1 (Sharma et al. 2016b). This isolate showed an aggressive antifungal activity in vitro against the phytopathogenic fungi *F. oxysporum*, *Verticillium dahliae*, *Alternaria alternata*, and *Colletotrichum capsici* (Sharma et al. 2017). Zhao et al. (2018) reported another antimicrobial peptaibol, trichokonin VI, produced by *T. pseudokoningii* SMF2. This peptaibol exhibited antifungal activity against the gray mold of moth orchid caused by *Botrytis cinerea*. Eight new peptaibols belonging to the trichorzianine family were also isolated from the sponge-associated isolate of *T. atroviride* NF16 (Panizel et al. 2013). The isolated peptaibols showed moderate antibacterial activity. Zhang et al. (2018) identified four inhibitory SMs from the culture filtrate of *T. longibrachiatum* T6. Of them, two compounds (1,2-benzenedicarboxylicacid, bis(2-methylpropyl) ester and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester) showed highly fungicidal activity against *Valsa mali*, the causal agent of valsa canker of apple, at 200 μg mL^{-1}. Moreover, *Trichoderma* spp. play an important role in inhibiting mycotoxin-producing fungi and reducing their toxin biosynthesis. Consumption of the mycotoxin-contaminant foods results in many health issues; sometimes it may be carcinogenic for humans and animals. Braun et al. (2018) reported an antifungal (mycoparasitic) activity of *T. harzianum* MRI349 against the mycotoxin-producing fungi *Aspergillus carbonarius* and *A. flavus* and inhibition of their mycotoxin biosynthesis at the transcriptional level. The venomous effects of members of *Fusarium* genus are attributed to their production of mycotoxins which cause dangerous health issues such as fumonisins, produced by *F. moniliforme*, which cause neurotoxicity, leukoencephalomalacia, hepatotoxicity, and liver cancer (Antonissen et al. 2014).

Fumonisin B1 toxin (FB1) is a well-known inhibitor of sphingolipid biosynthesis through inhibiting ceramide synthase (CS) enzyme (Heidtmann-Bemvenuti et al. 2011). In a recent study, Sharma et al. (2018) indicated that FB1 inhibited CS in yeast (*Saccharomyces cerevisiae*) but not in *T. guizhouense.* FB1 has been found to compete the substrate for the binding sites of the CS resulting in an inactive state of the enzyme. However, in case of CS from *T. guizhouense*, no competition for the substrate binding site was observed.

New bioactive SMs have been reported also from marine-derived *Trichoderma* spp. including peptides, terpenes, aromatics, and polyketides (Zhu et al. 2015; Zhang et al. 2017a; Blunt et al. 2017). Song et al. (2018) identified ten terpenes from the culture filtrate of the *T. asperellum* cf44-2, an endophytic fungus of the marine alga *Sargassum* sp. Of the isolated SMs, trichaspin, trichaspsides A and B (bisabolane derivatives); 9-cycloneren-3,7,11-triol, 11-cycloneren-3,7,10-triol, and 7,10-epoxycycloneran-3,11,12-triol (cyclonerane sesquiterpenes); and

11-hydroxy-9-harzien-3-one (harziane diterpene) were identified. Four SMs of them exhibited antibacterial activity against five aquatic pathogenic bacteria (*Vibrio parahaemolyticus*, *V. anguillarum*, *V. harveyi*, *V. splendidus*, and *Pseudoalteromonas citrea*). Trichocitrin, a new antibacterial diterpene, was also extracted from the culture of *T. citrinoviride* cf-27, an endophyte of the marine brown alga (Liang et al. 2016b). In addition, another four known SMs (nafuredin, 5-hydroxy-2,3-dimethyl-7-methoxychromone, 24-methylenecycloartanol, and citrostadienol) were also produced by *T. citrinoviride* cf-27. Of them, citrostadienol and nafuredin showed antibacterial potentiality against *Escherichia coli*. Citrinovirin, a novel norditerpene, was isolated and identified from the culture of *T. citrinoviride* cf-27 in addition to cyclonerodiol, 3-(2-hydroxypropyl)-4-(hexa-2E,4E-dien-6-yl) furan-2(5H)-one, and 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone (Liang et al. 2016b). Citrinovirin exhibited antibacterial activity against the pathogenic bacteria *Staphylococcus aureus* and a biotoxicity against the marine zooplankton *Artemia salina* and the phytoplankton *Chattonella marina*, *Heterosigma akashiwo*, and *Prorocentrum donghaiense* (Liang et al. 2016a). On the other hand, trichodiene, non-phytotoxic volatile organic compound produced by two isolates of *T. harzianum* (T34–5.27 and E20–5.7), exhibited a negative effect on the biocontrol activity against the common bean insect pest *Acanthoscelides obtectus*. Application of the trichodiene producer isolates led to an increment in the daily insect emergence and increased the attraction capacity of insects more than their parental strains on the treated bean plants (Rodríguez-González et al. 2018).

12.3 Therapeutic Activity

Various SMs produced by *Trichoderma* spp. have shown eminent therapeutic potency against different human diseases. Tandyukisins E and F, new decalin derivatives produced by *T. harzianum* OUPS-111D-4, showed cytotoxic activity against the cancer cell lines P388, HL-60, and L1210 (Suzue et al. 2016). In addition, 16-methylheptadecanoic acid methyl ester produced by the marine *Trichoderma* (*Hypocrea lixii* TSK8) exhibited a significant anticancer activity against two human cancer cell lines: oral cancer (KB) and skin carcinoma (A431). The inhibitory concentrations against KB and A431 cancer cells were 18.75 ± 0.12 and 37.5 ± 0.42 mg mL^{-1}, respectively (Saravanakumar et al. 2015). Class II hydrophobin (HFBII) is another bioactive compound produced by *T. reesei* and poses antioxidant and ACE (angiotensin I-converting enzyme)-inhibitory activities. Their ACE-inhibitory activity was reported at ≥0.5 mg mL^{-1}, while their antioxidant activity at 0.01–0.40 mg mL^{-1} (Khalesi et al. 2016). Cariaco et al. (2018) reported the immunomodulatory and antimalarial effect of the ethanolic extract of the culture filtrate produced by the isolate *T. stromaticum* against *Plasmodium falciparum* NF54 in infected human red blood cells and in a mouse model of experimental cerebral malaria. One of the various therapeutic benefits of SMs produced by *Trichoderma* spp. is their role as a cholesterol-lowering agent (e.g., lovastatin and compactin). Lovastatin is an anti-hypercholesterolemic drug (a competitive

inhibitor of hydroxy-methylglutaryl-CoA reductase, the first enzyme in the cholesterol biosynthesis). Of 17 fungal isolates screened for lovastatin production, Rashid et al. (2014) reported a great capability for its production (72.9 μg g^{-1}) by *T. viride*.

On the other hand, some SMs produced by *Trichoderma* spp. are hazardous to human health such as mycotoxins. In this regard, Zhang et al. (2017b) isolated a novel natural mycotoxin from culture filtrate of *Trichoderma* sp. Jing-8 which was identified as alternariol 1′-hydroxy-9-methyl ether (1). This mycotoxin may show mutagenic, fetotoxic, teratogenic characteristics or results in DNA damage (Solhaug et al. 2015). One of the most important epidithiodioxopiperazine-type mycotoxins produced by *Trichoderma* spp. is gliotoxin (GT). It was isolated as a secondary metabolite of *T. virens* (Vargas et al. 2014). In spite of the widely use of GT-producing *T. virens* isolates in agriculture as biocontrol agents, GT production has been reported during infection with *A. fumigatus* (aspergillosis) in human lungs and sera, and in mice. Moreover, it is considered as a virulence determinant of this pathogen. GT contributes to the infection process by suppressing the activity of immune cells (neutrophils) or phagocytes (Scharf et al. 2016). Recent studies suggest the use of GT as a diagnostic biomarker for aspergillosis infection in neutropenic patients (Sugui et al. 2017). However, at concentrations below the cytotoxicity level, GT may have some therapeutic uses such as antimalarial and anticancer drug (Hubmann et al. 2017, 2018). Trichothecene (trichodermin) is another sesquiterpenoid mycotoxin produced by *T. arundinaceum* and *T. brevicompactum*. It may be naturally accumulated at potentially harmful concentrations in cereals and grains (Shentu et al. 2013; Sharma et al. 2016a). Their modes of action include inhibition of protein synthesis in the 60S ribosomal subunit and in mitochondria, production of free radicals which cause oxidative stress, induction of ribotoxic stress, cell proliferation, and cytotoxicity responses in animal cells (Sharma et al. 2016a).

12.4 Plant Growth Promotional Activity

Numerous plant growth promoters have been reported to be produced by members of genus *Trichoderma* (Zeilinger et al. 2016). Saber et al. (2017) reported a high production (138.9 μg mL^{-1}) of the phytohormone indole-3-acetic acid (IAA) by the isolate *T. harzianum* WKY1 which improved the plant growth of sorghum. Chrysophanol is another anthraquinone secondary metabolite produced by *T. harzianum* ETS-323 which significantly stimulated the growth of cabbage seedlings and induced the production of the photosynthesis-related proteins in their leaves. In addition, the sucrose transport-related genes were overexpressed in the chrysophanol-treated cabbage leaves (Liu et al. 2016). In another study, the plant hormone ethylene produced by *T. atroviride* was found to be involved in the root system alterations (root architecture and biomass) in *Arabidopsis* seedlings. It affected auxin signaling through the central regulator CTR1 and enhanced activity of mitogen-activated protein kinase 6 (Contreras-Cornejo et al. 2015). More recently, the plant growth-promoting activity of the endophytic fungi has received special attention. The growth-promoting mechanisms include enhancement of

1-aminocyclopropane-1-carboxylic acid (ethylene precursor) or production of plant hormones such as IAA, gibberellins, auxins, and cytokinins (Patle et al. 2018). In this regard, the endophytic fungus *T. gamsii* NFCCI 2177, isolated from lentil roots in the Indian Himalayan Region, was found to pose a potential growth-promoting activity on maize, soybean, wheat, and lentil. Moreover, it showed phosphate-solubilizing capacity and ammonia production ability (Rinu et al. 2014). Martínez-Medina et al. (2014) studied the inducing effects of *T. harzianum*, *T. ghanense*, and *T. hamatum* on the phytohormone profile with plant growth-promoting potential (zeatin, zeatin riboside, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylic acid, and abscisic acid) on melon plants. An induction in auxin and reduction in cytokinins and abscisic acid content were recorded. The study showed a great association of auxin trigger with plant growth improvement by *Trichoderma*. In contrast, in another study, the non-hormone-producing isolate *T. harzianum* T-22 (in axenic growing media) enhanced the plant growth by regulating hormone biosynthesis genes in the *Prunus* rootstock (Sofo et al. 2012). Harzianolide is another recently identified SM from *T. harzianum* strain SQR-T037 which poses a remarkable plant growth-promoting activity. Treatment of tomato seedlings with harzianolide as purified compound or in the crude extract significantly enhanced their growth by 2.5-fold. In addition, it exhibited a systemic resistance inducing activity against phytopathogenic fungi (Cai et al. 2013). In a recent study, of 20 *Trichoderma* isolates screened for volatile-mediated plant growth promotion activity, 9 isolates belonging to *T. aggressivum*, *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride* significantly enhanced overall plant growth and total chlorophyll content in *Arabidopsis thaliana*. A total of 141 unique volatile compounds were identified including hydrocarbons, alcohols, ketones, aldehydes, alkanes, alkenes, esters, aromatic compounds, heterocyclic compounds, and various terpenes. Moreover, plant growth-promoting isolates were found to produce terpenes such as β-acoradiene, β-cubebene, β-cedrene, β-bisabolene, β-himachalene, and γ-himachalene (Lee et al. 2016).

On the other hand, *Trichoderma* spp. play another crucial indirect role in enhancing plant growth through alleviating ecological stresses leading for crop yield improvement. Zaidi et al. (2018) reported that application of *T. harzianum* strain S2 in addition to the best management practices of International Rice Research Institute enhanced the grain yield of rice under rainfed conditions, recording 4.91 and 4.81 t ha^{-1} during 2013 and 2014, respectively. Role of *Trichoderma* spp. in mitigating salinity stress on crop plants was also studied. Yasmeen and Siddiqui (2017) reported that seedlings of rice and maize treated with the endophytic fungus *T. harzianum* (Th-6) and grown under salinity conditions (50, 100, and 150 mM NaCl) exhibited better growth and higher relative water content and stomatal conductance compared to the Th-6-untreated plants. In addition, the Th-6-treated seedlings showed higher content of the photosynthetic pigments, proline content, and higher activity of catalase and superoxide dismutase enzymes. Moreover, in a recent study, it was found that the root

exudates from tomato plants exposed to different stress factors acted as chemoattractants of the soil fungus *T. harzianum* T-22 (Lombardi et al. 2018).

Solubilization of sparingly soluble minerals is another mechanism by which *Trichoderma* spp. may promote plant growth (enhance nutrient uptake) through production of mineral-solubilizing metabolites such as organic acids (acidification), siderophores (chelation), ferric reductase (redox), and phytase (hydrolysis). Li et al. (2015) reported the isolate *T. harzianum* SQR-T037 as a mineral-solubilizing fungus with the ability to solubilize phytate, Fe_2O_3, CuO, and metallic Zn as well as produce organic acids such as lactic acid, citric acid, tartaric acid, and succinic acid.

12.5 Biotechnological Activity

A lot of studies that address the biotechnological applications using members of *Trichoderma* genus have been done in the last few years. One of the most interesting applications in this field is the green synthesis of metallic nanoparticles based on *Trichoderma* species which provides an eco-friendly, economic, effective, and low-energy method for nanoparticle production. Their capability to produce various types of SMs and enzymes makes them good candidates for biosynthesis of metallic nanoparticles. These SMs such as peptaibols, siderophores, polyketides, terpenoids/steroids, nonribosomal peptides, and pyrones contain many phenolic groups that have been oxidized and convert to quinones donating electrons which act as reducing and stabilizing agents through the biosynthesis process (Mukherjee et al. 2012a). Qu et al. (2017) reported a success biosynthesis of gold nanoparticles of diverse shapes using the biomass of the newly isolated strain *Trichoderma* sp. WL-Go. These biogenic nanoparticles have a significant potential for decolorization of different azo dyes. In this regard, different species of *Trichoderma* were used also in the biosynthesis of gold nanoparticles of various sizes and shapes such as *T. asperellum* (Mukherjee et al. 2012a), *T. harzianum* (Tripathi et al. 2014), and *T. viride* (Mishra et al. 2014). Silver nanoparticles were also biosynthesized using *T. harzianum* (Guilger et al. 2017). The produced silver nanoparticles exhibited a strong antifungal activity against mycelial growth and sclerotia germination of the white mold fungus *Sclerotinia sclerotiorum*, which attack many crops. Silver nanoparticles were biosynthesized also using *T. longibrachiatum*, *T. harzianum*, and *T. viride* and showed significant inhibitory effect against the fungal growth of the phytopathogenic fungi: *F. verticillioides*, *F. moniliforme*, *Penicillium brevicompactum*, *H. oryzae*, and *Pyricularia grisea* (Elamawi et al. 2018). Saravanakumar and Wang (2018) reported also a biosynthesis process of silver nanoparticles of size ranged between 15 and 25 nm using *T. atroviride* which showed antibacterial, antioxidant, and cytotoxicity activities. In another study, selenium nanoparticles were also biosynthesized using culture filtrate, cell lysate, and crude cell wall of *T. asperellum*, *T. harzianum*, *T. atroviride*, *T. virens*, *T. longibrachiatum*, and *T. Brevicompactum* producing nanoparticles of size ranged between 49.5 and 312.5 nm (Nandini et al. 2017). The biosynthesized selenium nanoparticles inhibited growth, sporulation,

and zoospore viability of the pearl millet downy mildew fungus (*Sclerospora graminicola*).

Moreover, *Trichoderma* spp. have another interesting biotechnological application represented in their capability to degrade the highly toxic, mutagenic, and carcinogenic azo dyes produced from textile, food, plastic, printing, leather, and cosmetic industries and disposed into the ecosystem as wastewater. A noteworthy degradation, up to an efficiency of 99.2%, of azo dyes in the real textile effluent using *T. tomentosum* under non-sterile conditions was reported (He et al. 2018). Remarkable enzymatic activities of manganese peroxidase and lignin peroxidase were recorded during the azo dye decolorization process revealing their possible involvement in this process.

One of the most important biotechnological applications of *Trichoderma* spp. is their role as potential bioremediators in the eco-friendly metal removal technology. Their strategies in this regard can be categorized into four groups: biosorption, bioaccumulation, biovolatilization, and phytobial remediation (Tripathi et al. 2013). Recent researches showed a remarkable metal tolerance as well as a high capacity to accumulate these metals from the surrounding polluted environment by *Trichoderma* spp. Some isolates of *Trichoderma* spp. pose multiple tolerances to more of one kind of heavy metals. Their tolerant responses may differ in case of single than multiple metal ions; synergistic or antagonist interactions may take place between these metal ions (Siddiquee et al. 2015). In this regard, Nongmaithem et al. (2016) screened 14 *Trichoderma* isolates for their biosorption potential of nickel and cadmium through metabolically mediated pathways of uptake. Of the evaluated isolates, three (MT-4, UBT-18, and IBT-I) showed a high potential of nickel tolerance (up to 200 ppm), and three (MT-4, UBT-18, and IBT-II) exhibited a significant tolerance of cadmium (up to 150 ppm). Total removal of nickel was observed at concentrations up to 40 ppm. The biosorption activity of an organism is attributed to the ion-exchange capacity of their SMs which contain functional groups such as hydroxyls, carbonyls, carboxyls, sulfonates, amides, imidazoles, phosphonates, and phosphodiester (Pradhan et al. 2007). Many of these functional groups exist in SMs produced by *Trichoderma* spp. Biovolatilization is another alternative strategy exerted by *Trichoderma* spp. in the metal bioremoval technology. This strategy includes an enzymatic transformation of the metalloid compounds into their volatile derivatives (i.e., biomethylation). With regard to this, three mercury-resistant isolates of *T. virens*, isolated from the San Joaquin region in the State of Querétaro, México, were found to grow at high $HgCl_2$ levels ranged between 100 and 200 mg L^{-1} and catalyze mercury volatilization and accumulation. Of them, *T. virens* strain 20-D exhibited the highest Hg volatilization potential (213.04 μg m^3), while their mycelium accumulation of mercury was 18.5 μg g^{-1}. Interestingly, inoculation of $HgCl_2$-treated *A. thaliana* plants with these isolates significantly reduced Hg concentration in their roots and rosette leaf tissues recording 75% and 50% reduction, respectively (Hernández-Flores et al. 2018). Moreover, the bioremediation role of *Trichoderma* spp. extends also to their biodegradation potential for organic contaminants. Andreolli et al. (2016) isolated a fungal isolate (*T. longibrachiatum* Evx1) which has the ability to degrade diesel fuel and polycyclic aromatic

hydrocarbons as well as decolorize polynuclear organic dyes. Their biodegradation potential for the C12–40 hydrocarbons reached 54.2%.

Production of aroma compounds as natural food additives or flavors is another important industrial application of *Trichoderma* spp. Various studies have reported production of SMs by *Trichoderma* species such as the unsaturated lactone 6-pentyl-α-pyrone, the volatile compound responsible for the coconut-like aroma in their cultures. In this regard, Fadel et al. (2015) reported the production of coconut aroma 6-pentyl-α-pyrone as a major volatile compound in addition to δ-octalactone, γ-nonalactone, γ-undecalactone, γ-dodecalactone, and δ-dodecalactone by *T. viride* EMCC-107 cultivated as a solid-state fermentation using sugarcane bagasse. The produced coconut aroma was characterized and evaluated for its impact on the overall odor profile. Microbial aromatic compounds were also produced by *T. viride* using solid-state fermentation of *Pandanus tectorius* fruits as a substrate. Using GC/MS system, 27 aromatic compounds were detected in the culture filtrate including alkene hydrocarbons, alcohol, amide, and monoterpene aldehid (Darmasiwi et al. 2016).

12.6 Microbial Communications

Root microbiome refers to various microbial communities surrounding and inhabiting the plant roots. Different bidirectional interactions occur between these microbial communities with each other in the soil, i.e., antagonism or synergism on one side and between them and the plant roots on the other side. Effect of root microbiome on the plant is ranging from enhancing their health, development, and stress tolerance to parasitizing their roots. In the same time, growth of root microbiome is positively or negatively affected by the root exudates. Moreover, some researchers consider the root microbiome as a secondary genome which provides the plant with microbial SMs (Rout and Southworth 2013). The high species diversity and richness in the root microbiome result in intra- and interspecies communications between their members. SMs play the key role in implementing these communications as chemical signals. In spite of the large number of SMs produced by these microorganisms, little are known about their activities and biosynthesis. Moreover, genome mining of a single organism shows an inconsistency between the number of the genes involved in the secondary metabolism and the known SMs (Craney et al. 2013). Interestingly, recent researches showed that the interspecies communication between microbiome members may result in inducing silent gene clusters to produce novel SMs by other species (Netzker et al. 2015). Many researchers have studied co-cultivations of two or more organisms in order to trace novel SMs (Bertrand et al. 2014; Marmann et al. 2014). In this regard, harziaphilic acid, a novel SM, with antiproliferative effect on colorectal carcinoma cells, was isolated and fully characterized from the fungal co-culturing of *T. harzianum* M10 (producer) and the endophyte *Talaromyces pinophilus* F36CF (elicitor). Furthermore, upregulation of siderophores (ferrirubin, ferricrocin, coprogen B, and dimerumic acid) for both

fungi was also reported in this mixed culture. Production of microbial siderophores in the soil has a growth-promoting effect on the plants (iron solubilization), while it has a suppressive effect on other soil microorganisms (iron competition) (Vinale et al. 2012). On the contrary, biosynthesis of 3-O-methylfunicone and herquline B by *T. pinophilus* F36CF was downregulated in the presence of *T. harzianum* M10 compared with the single culture (Vinale et al. 2017). In a recent study, co-culturing of *T. hamatum* (CMB-MF030) and *Chaunopycnis* sp. (CMB-MF028) led to production of chaunopyran A, a rare class of 2-alkenyl-tetrahydropyran, through activation of the silent gene clusters encoding for it. In addition, this mixed culturing resulted also in methylation of pyridoxatin (antifungal agent) converting it to its non-antifungal form (Shang et al. 2017). Wu et al. (2018) reported also enhancement of specific amino acid production as well as antimicrobial compounds from the mixed culture of *Bacillus amyloliquefaciens* ACCC11060 and *T. asperellum* GDFS1009 compared with their single cultures. Co-culturing of a microorganism and plant was also reported. In this regard, co-cultivation of *A. thaliana* seedlings with *T. virens* Tv29.8 and *T. atroviride* IMI 206040 significantly promoted the overall plant growth specially the lateral roots and root hairs through IAA production in both normal and saline conditions (Contreras-Cornejo et al. 2014). Triggering of abscisic acid, L-proline, and ascorbic acid and enhanced elimination of Na+ through root exudates was also reported for the co-cultivated seedlings. In contrast, in an axenic co-culture of the two isolates *T. harzianum* T-22 and BOL-12QD with the *Chenopodium quinoa* cv. Kurmi and Maniqueña real, a significant inhibitory effect by the two isolates was recorded on the plant growth, specially the lateral root development, as well as chlorophyll and betalain contents (Rollano-Peñaloza et al. 2018). In another study, volatile SMs produced by *T. asperellum* IsmT5 (6-pentyl-α-pyrone) negatively affected the plant growth of *A. thaliana* inhibiting primary root, leaf size, and fresh weight (Kottb et al. 2015). In axenic cultures, nature of the *Trichoderma*-plant interactions depends on the compatibility between the *Trichoderma* strains and the plant genotypes.

12.7 Conclusions and Outlook

Numerous bioactive SMs, produced by members of genus *Trichoderma*, have been discovered each year which have different agricultural, biotechnological, and therapeutic applications in our life. In spite of the immense ability of *Trichoderma* spp. to produce these SMs, our knowledge about their biosynthesis, regulating genes, bioactivities, and elicitors is still limited. Genome mining of members of genus *Trichoderma* is essential to improve our understanding for their obscure secondary metabolism, help in discovery of new SMs, and generate mutant strains with massive capacity to produce biotechnologically and therapeutically important SMs. Molecular studying of the bidirectional *Trichoderma*-plant communications and their signaling molecules is emerging also as an essential need for the best field exploitation of these beneficial fungi. Furthermore, accelerated technical progress

at molecular level in the identification and biodiversity studying tools will lead to the discovery of novel species of genus *Trichoderma*.

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Chapter 13
Fungal Genes Encoding Enzymes Used in Cheese Production and Fermentation Industries

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13.1 Introduction

The applicability of microorganisms such as Fungi in biotechnological areas is potentiated by numerous advantages, including ease of handling, short culturing time, and ease of use in large-scale production. Fungal sources for alternative enzyme production have been thoroughly studied because of the importance of their secondary metabolites in the biotransformation process. Among different techniques that are applicable in fungal enzyme production, DNA recombinant technology has gained more attention wherein fungi act as a host. Intracellular and extracellular enzymes secreted by fungi are vital in developing the specific textures and flavors of cheese (Mohanty et al. 1999). The following section details the fungal

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genes encoding enzymes with their applicability in the cheese manufacturing industry. The industrial enzyme market is known to be a dynamic market approaching approximately $1 billion annually as enzymes become a commodity chemical that increases the productivity and efficiency of reaction rates (Pearce 1997). The market volume for enzymes fluctuates with industrial type and use, but the biocatalytic molecules are widely used in the food industries such as dairy, meat, fruits and vegetables, bakery, and brewery. Moreover, the enzymes produce very few by-products and are in general nontoxic compared to metal catalysts. During the initial stage of cheese production, fungi have a limited role, but in the ripening stage their function is vital. Extracellular and intracellular enzymes secreted by fungi aid in carbohydrate utilization and protein and lipid metabolism. The hydrolysis of a cheese matrix into smaller units by utilizing enzymes in energy metabolism is central in understanding the importance of fungi in dairy industries. This chapter analyzes deeply the biocatalysts secreted by fungi and their encoding genes in optimizing cheese production (Ghosh et al. 2009). The chapter also defines the extracellular enzymatic reactions and intracellular metabolic pathways of the enzymes encoded by fungal genes via glycolysis, gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. Regarding the nutritional aspect of cheese, some secondary metabolites produced by fungi can be harmful to consumers. With the increasing concern about the nutritional quality of food as well as human health, it is important to utilize genetic engineering techniques as remedial action for overcoming these shortcomings. Hence, the chapter also elaborates the gene tailoring and heterologous regulation of fungal genes found in cheese and their industrial applications (Nevalainen et al. 2005).

13.2 Enzymes in the Cheese Production and Fermentation Industries

Enzymes are typically proteins that act as catalysts to expedite most of the chemical reactions within a cell (Tymoczko et al. 2002). Similar to all catalysts, enzymes are able to reduce the activation energy of a reaction while increasing the reaction rate. Therefore, these biological macromolecules have a vital role in metabolism and function commercially; specifically, in food production at an industrial scale such as in the fermentation industries, cheese production, and meat industry. The most important use of enzymes, however, is in the fermentation and dairy industries for product development and even quality measurements (Humbert and Alais 1979; Pollock 1979). Thus, our focus is on the enzymes used in the manufacturing processes of the fermentation and cheese industries. Cheese is a dairy product that is enriched with specific texture and a wide range of flavors and is a popular food among consumers (Bachmann 2001). The main raw material for cheese is usually

the milk of cows, buffalo, goats, or sheep (Barlowska et al. 2011), which contains proteins, basically caseins. Casein is the key protein in the manufacturing process of cheese: liquid milk is converted into a semi-solid by different steps related to various enzymes. The making of cheese is also supported by the activities of enzymes present in the milk itself (FAOSTAT 2017).

13.2.1 Enzymes in Cheese Production

All the enzymes used in the production process of cheese can be categorized into two principal groups: endogenous and exogenous enzymes. Endogenous enzymes refer to the enzymes present in the milk itself. These enzymes, notably plasmin, lipoprotein lipase, acid phosphatase, and xanthine oxidase, are subsidiary in the cheese production process (Table 13.1). Exogenous enzymes include rennet, lipases, lysozyme, catalase, glucose oxidase, and β-galactosidase: their function is connected with the starter culture and secondary microflora (Law and Mulholland

Table 13.1 Enzymes used in cheese production (Products and 1994 n.d.)

	Enzyme	Function	Reference
Endogenous enzymes	Plasmin	Important for cheese ripening and benefit the quality of dairy products, hydrolyze caseins, specifically kappa casein, which stabilizes micelle formation preventing coagulation	
	Lipoprotein lipase	Hydrolysis of fatty acids from triglycerides and phospholipids in the milk; also involved in the biosynthesis of milk fat and support flavor generation	
	Acid phosphatase	Help stabilize the cheese feed by dispersing protein and emulsifying fat	
Exogenous enzymes	Rennet	Used to coagulate milk, destruction of whey proteins	Law and Goodenough (1995)
	Lipases	Used to break down milk fats and give characteristic flavors to cheeses	
	Lysozyme		
	Catalase	Preserve natural milk enzymes that are beneficial to the end product and flavor development of the cheese, inhibit the bacterial cultures that are required for the actual cheese production	
	Glucose oxidase	Used to remove oxygen from packed cheese	
	β-Galactosidase	Improve the taste of lactose-hydrolyzed milk; cold-active β-galactosidase is used for producing lactose-free cheese	

1995) in the production of enhanced flavors. Amino acids and peptides are important in the development of cheese flavor, whereas the peptidase enzyme from lactic acid bacteria (LAB) is important in the natural flavor development of the cheese. Recently, most cheese manufacturers have been trying to use the recent advances in recombinant DNA technology to improve the starter culture bacteria and their key enzyme activities (Hui et al. 2004). The manufacturing of cheese is always regarded as a technique of preserving milk by the means of its nutritional value in a safe and palatable form of added value (Law and Goodenough 1995). Traditionally, some varieties of cheese required an extended maturation time, but this prolonged maturation period is impractical at the industrial scale for reasons of cost. The maturation process of cheese usually involves a slow and controlled breakdown of nutrients (fat, protein, carbohydrates) of the curd by starter bacteria added by the industry. Further, a secondary flora is added to the cheese according to the type. Understanding the reactions involved in the process is essential to accelerate the cheese ripening by using different enzymes, cultures (ex-lactic cultures), and other additives in hard cheese production (Law and Goodenough 1995). New impetus has been given to the search for 'ripening technology' by the introduction of lactic cultures, which are selected for phage resistance.

13.2.2 Other Fermentation Industries

The major food-related fermentation industry is brewery and wine making. The worldwide production of breweries was expected to be 197.3 billion liters in 2013 (Barth et al. 2014): China is recognized as the world largest producer (50 billion liters), followed by USA (22.4 billion liters), Brazil (13.55 billion liters), Germany and Russia. In the brewery industry, the most common product is beer, which starts with malt fermentation. The end product can be further fermented into other products based on the alcoholic percentage. Small or larger yeasts are used to produce different types of beer recipes (Marshall 2002). Brewers believe that endogenous enzymes contribute to the flavor profile and overall acceptance of the product (i.e., fermentability, filterability, foam, clarity, flavor) (Bamforth 2009). Involvement of the enzymes within the brewery process in the first stage of the malting process is summarized in Table 13.2. In wineries, the enzymes also have a key role in the wine making process. Many of the enzymes involved in the process come from the grape itself, the indigenous microflora on grapes. Sometimes, the activity of these enzymes is not efficient and commercial enzymes are needed (Mojsov 2013). Pectinases, glucanases, xylanases, and proteases improve the clarification and processing of wine. Glycosidase releases varied aromas from the precursor compounds. Urease aids in the reduction of ethyl carbamate formation, and glucose oxidase helps in the reduction of alcohol levels.

Table 13.2 Enzyme applications in brewing

Brewing step	Enzyme involved	Action
Yeast fermentation	α-Amylases	Barley starch hydrolysis during malting process, or in high adjunct mashes, or containing raw barley grain (unmalted), by *Aspergillus* spp., *Bacillus* spp. Heat-stable exogenous α-amylases (from *Bacillus* spp.) during mashing can reduce the wort starch problem to the lautering step and sparging
Mashing/ fermentor	Glucoamylases	Participate in saccharification (the last step of starch degradation) for releasing glucose units
Filtration aid/ prevention of haze	β-Glucanase	Hydrolyze β-glucans in beer mashes, but this enzyme in unmalted barley is used as an adjunct, is not sufficient to hydrolyze it. Hence, addition of exogenous β-glucanases is required
Mashing	Glucanases, hemicellulases, xylanases	Cell wall breakdown: Applied mainly if poorly modified malt or unmalted adjuncts, such as barley, wheat, or oat are used. In this case these enzymes have corrective action Xylanases eliminate pentosans, especially when using wheat as adjunct
Mashing	Proteinase	Production of free amino nitrogen, especially in high adjunct mashes
Fermentor	α-Acetolactate decarboxylase	Enhance maturation, by converting α-acetolactate into acetoin, before it can be converted into diacetyl
Filtration and beer haze of stored beer	Mixture of cysteine proteinases	Elimination of haze-forming components (proteins, or polypeptides and polyphenols), improving beer stabilization
Stored beer	Prolyl endopeptidase	Elimination of haze-forming polypeptides; potential value in producing beer for celiacs
Packaged beer	Glucose oxidase/ catalase	Elimination of oxygen

Reproduced from Hui et al. (2004), with permission of CRC Press

13.3 Cheese Types and Their Associated Fungi

The presence of fungi or mold in a food is a sign of contamination, but some food products use fungi as a component of the product in the manufacturing process, which is becoming common (e.g., blue cheese, bread). This use is mostly specific to the dairy industries, especially in cheese production, as most of the physical and chemical characteristics of the end product are highly correlated with the microflora being added (Beer et al. 1984; Horne 1998). This chapter details the use of fungi in manufacturing different cheese types. Cheese can be categorized into three main groups: soft, hard, and blue (McSweeney et al. 2004) (Table 13.3). Soft cheese is ripened for a short time; its varieties include Feta, Brie, and Camembert. Hard cheese, ripened for various lengths of time, includes varieties ranging from semi-hard to hard and includes Cheddar, Gouda, Muenster, Parmesan, and Romano. Last,

Table 13.3 Types of cheese

Type	Well-known varieties
Soft	Cream cheese, Brie, Neufchâtel
Semi-soft	Havarti, Munster, Port Salut
Medium-hard	Gouda, Edam, Jarlsberg, Cantal, Kashkaval/Caşcaval, and Swiss-style cheeses such as Emmental and Gruyère
Semi-hard	Cheddar, Colby, Monterey Jack, Edam, Gouda
Hard	Grana Padano, Parmesan, Pecorino

Fig. 13.1 Camembert cheese. (Source: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/penicillium-camemberti)

blue cheese, ripened by green molds and with a blue-veined appearance, includes Gorgonzola, Roquefort, and Stilton among commercial blue cheeses.

Cheese is also classified broadly as soft-ripened cheese, washed-rind cheese, and blue cheese (Fox et al. 2000), with the presence of mold being an important feature. In soft-ripened cheese, the structure is comparatively firm and chalky in texture before exposure to the molds for aging. The mold generally used is *Penicillium camemberti*, which forms a flexible white crust and subsidizes the smooth and soft texture with intense flavors (Fig. 13.1). Brie and Camembert, the most famous soft-ripened cheese types, are made by allowing the white mold to grow on the outer surface of cheese for a few days or weeks. Cheese made from goat milk is sometimes treated with white mold or sometimes with blue mold (Fox et al. 2004).

Washed-rind cheese is ripened inwardly with white molds that enhance the softness of the end product. The way of treating differs, however, as this cheese is periodically cured in a brine solution (Leclercq-Perlat et al. 2004; Harbutt 2009) or in a mold-bearing agent such as beer or wine brandy, and with spices that can provide their surfaces with a reddish-orange hued smear originating from the bacterium *Brevibacterium linens*. This specific class of bacteria is able to impart distinctive flavors and pungent odors while producing a firm flavorful rind around the cheese (Dufossé et al. 2005; Deetae et al. 2007; Roth et al. 2010). Washed-rind cheese is included in soft cheese, such as Limburger, and in semi-hard and hard cheese such as Appenzell categories (Fig. 13.2).

Fig. 13.2 Limburger cheese. (Source: https://www.lafromagerie.co.uk/limburger)

Fig. 13.3 Munster (Meunster) cheese. (Source: http://thecheesestoreandmore.com/muenster-cheese-orange-rind-per-pound/)

Smear-ripened cheese, such as Munster and Port Salut, is usually ripened by in a solution treated with *Brevibacterium linens*, *Debaryomyces hansenii*, or *Geotrichum candidum* (Mounier et al. 2017), which can enhance the strong flavor during maturation. Some cheese makers occasionally use older cheese to transfer the microflora to the new cheese instead of using commercial starter cultures. A significant orange or pink color can be observed on the exterior surface of this cheese type. The manufacturing process includes washing to ensure the uniform growth of the desired fungi and also to prevent contamination and the growth of other, undesired molds (Fig. 13.3).

In blue cheese, the fungus *Penicillium roqueforti* or *Penicillium glaucum* (Fig. 13.4) is inoculated in the cheese during production while the cheese is in the form of loosely pressed curds (SERATLIĆ et al. 2011; Diezhandino et al. 2015). The most renowned blue cheese varieties are Roquefort, Gorgonzola, and Stilton. This cheese has distinct blue veins that give the assertive flavor, color, texture, and aroma profile and thus the name blue cheese (Fox et al. 2004).

Fig. 13.4 Blue cheese. (Source: https://www.winemag.com/2017/03/30/feeling-blue-for-cheese/)

13.4 Fungal Enzymes for Cheese Production

During cheese fermentation and ripening stages, filamentous fungi and yeasts produce an array of enzymes that are of great importance in cheese production. These enzymes include intracellular and extracellular enzymes that are essential for lactose and lactate utilization and protein and lipid metabolism (Ghosh et al. 2009). Extracellular enzymes are involved in the breakdown of the cheese matrix macromolecules through processes such as hydrolysis of sugars, degradation of proteins, and breakdown of lipids (Sinsabaugh 1994). After the macromolecules are broken down into smaller units, they are transported into the cheese microorganisms where they are utilized in the central metabolism for energy production (Fig. 13.5). In this metabolism, the intracellular enzymes have a major role through several cycles and pathways including glycolysis, gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (Lessard et al. 2014).

13.4.1 Lactose and Lactate Utilization

Lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB) perform the fermentation of lactose into lactate, which acts as a main carbon source in surface-ripened cheese (Leclercq-Perlat et al. 1999). However, in some yeasts and molds, a β-galactosidase-encoding LAC4 gene is present that briefly enables the utilization of lactose through its breakdown into galactose and glucose (Sreekrishna and Dickson 1985). The galactose is further metabolized through glycolysis, the TCA, and the pentose phosphate pathway. For effective utilization of lactate by yeasts and molds, the expression of the genes JEN1, DLD1, and CYB2 is essential. JEN1 encodes for a lactate transporter, and DLD1 and CYB2 encode for lactate dehydrogenase enzymes that metabolize lactate to produce sugars through

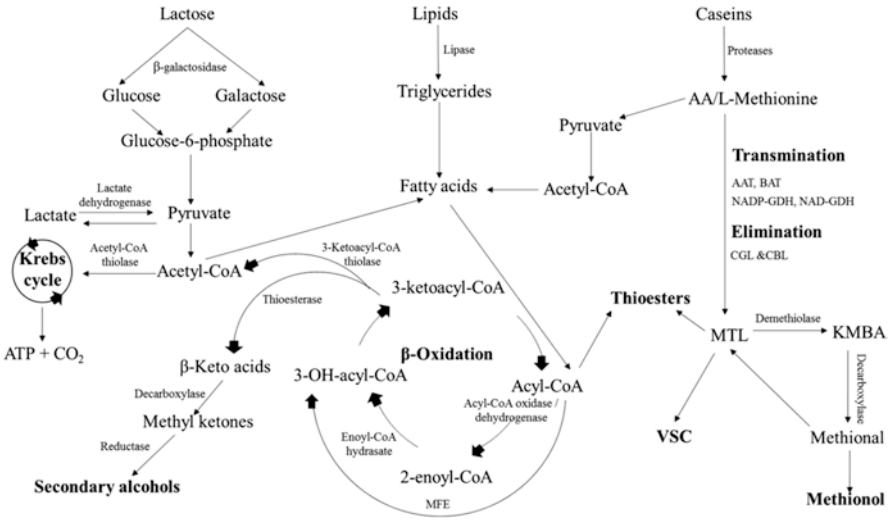

Fig. 13.5 Cheese metabolic pathway for energy utilization and formation of flavor compounds. MTL methanethiol, KMBA 2-keto-4-methylthio butyric acid, VSC volatile sulfur compounds, AA amino acid, MFE multifunctional enzyme, AAT aromatic aminotransferases, BAT branched-chain aminotransferases, NADP-GDH NADP-glutamate dehydrogenase, NAD-GDH NAD-glutamate dehydrogenase, CGL cystathionine-γ-lyase, CBL cystathione-β-lyase; lactose and lactate utilization

gluconeogenesis (Casal et al. 1999; Lodi et al. 1999; Cholet et al. 2007). Sugars from lactate metabolism are further metabolized into ATP and CO_2 by the enzymes phosphoenolpyruvate kinase and fructose-1,6-bisphosphate through gluconeogenesis and the glycolysis metabolic pathway, respectively. Phosphoenolpyruvate kinase and fructose-1,6-bisphosphate are encoded by the genes PEPCK and FBP, respectively (Lessard et al. 2014). After lactose and lactate utilization as a carbon source, the remaining energy sources in cheese at this point are proteins and lipids.

13.4.2 Protein Metabolism

The enzymes metallopeptidases and serine peptidases are the key proteases observed in cheese yeasts and fungi during ripening, particularly in *Penicillium roqueforti* and *Penicillium camemberti* (Lessard et al. 2014). These enzymes include (i) aspartic protease, which hydrolyzes β-caseins and αS1-caseins; and (ii) metalloprotease, an extracellular serine carboxypeptidase that hydrolyzes proteins to release amino acids and metalloaminopeptidase. Apart from yeasts and molds, other sources of proteases include milk, rennet, LAB, and NSLAB. These enzymes are typically detectable in cheese after 21 days of ripening (Lessard et al. 2014). After the exhaustion of lactose and lactate as a carbon source, the expression of peptidase genes

initiates proteolytic activity (Mounier et al. 2007). Through proteolysis, casein is degraded, followed by the subsequent catabolism of peptides and amino acids to produce amines, ammonia, keto acids, carbonyls, alcohols, hydrogen sulfide, methanethiol, and thioesters. These compounds contribute to flavor development in cheese during ripening. In addition, proteolysis also contributes to changes in the texture of cheese and de-acidification. Aminotransferases and lyases are other essential enzymes for amino acid metabolism during ripening. Therefore, the expression of their corresponding genes is necessary for the metabolism of amino acids such methionine. In *Yarrowia lipolytica* and *Geotrichum candidum*, expression of the amino acid transporter gene GAP1 initiates the amino acid metabolism by aiding their transport into and within the cells (Cholet et al. 2007). The genes CGL and CBL encode for cystathionine-γ-lyase and cystathionine-β-lyase, respectively, which metabolize methionine (Demarigny et al. 2000). In addition to those enzymes, branched-chain and aromatic aminotransferases initiate the transamination of methionine to produce methanethiol, which produces a sulfur aroma in cheese (Bonnarme et al. 2001). The genes BAT1, BAT2, ARO8, and ARO19 encode for branched-chain and aromatic aminotransferases in *Y. lipolytica, G. candidum*, and *Kluyveromyces lactis* (Cholet et al. 2007; Mansour et al. 2008). In *G. candidum*, the enzyme NAD-glutamate dehydrogenase encoded for by the gene GDH generates NADH, ammonia, and alpha-ketoglutarate through the transamination of L-methionine (Miller and Magasanik 1990; Rosenberg and Altemueller 2001; Cholet et al. 2007). Another dehydrogenase found in *G. candidum* is isopropylmalate dehydrogenase, encoded for by the gene LEU1. Other compounds including keto-γ-methylthiol and butyric acid are formed through the Ehrlich pathway (Lessard et al. 2014)

13.4.3 Lipid Metabolism

Lipid metabolism is another major biochemical process in cheese ripening that also contributes to the flavor, color development, and texture variation observed in cheese (Maldonado et al. 2017). Lipases hydrolyze the triglycerides into di- and monoglycerides, free fatty acids, and glycerol; in addition, fatty acids, methyl ketones, lactones, esters, aldehydes, and alcohols are released either by free fatty acid metabolism or cell wall-related lipid metabolism. Methyl ketone precursors and methyl ketones in cheese include 2-heptanone and 2-nonanone, 2-pentanone and 2-decanone; secondary alcohols include 2-heptanol, 2-nonanol, and 2-pentanol (Sørensen et al. 2011). Lipolytic activity in cheese is mainly caused by yeast and fungal lipases, secreted by *P. roqueforti*, *P. camemberti*, *Y. lipolytica*, and *G. candidum* (Lanciotti et al. 2005; Boutrou and Guéguen 2005); examples include extracellular lipases, intracellular lipases, and acidic and alkaline lipases. Genes encoding for lipases that have been observed in *Y. lipolytica* and *G. candidum* include LIP1, LIP2, LIP7, and LIP8 (Bertolini et al. 1994; Fickers et al. 2005; Kumura et al. 2019). Fatty acids are catabolized through β-oxidation to produce acetyl-CoA,

which is fed into the TCA cycle to produce ketone bodies; another acetyl-CoA molecule is recycled into the β-oxidation cycle (Trotter 2001; Maggio-Hall and Keller 2004). Fungi such as *P. camemberti* possess two β-oxidation pathways, mitochondrial and peroxisomal β-oxidation pathways, whereas yeasts such as *G. candidum* possess only the peroxisomal β-oxidation pathway. Therefore, after mitochondrial β-oxidation of fatty acids in *P. camemberti*, the acetyl-CoA molecule produced is directly catabolized by a peroxisomal multifunctional enzyme (MFE) in the peroxisomal β-oxidation pathway (Lessard et al. 2014). The β-oxidation pathway is optimized through the MFE (Table 13.4).

Table 13.4 Enzymes and their encoded genes in metabolic pathways of cheese fungi

Purpose	Enzyme/protein; pathway	Genes	Microorganism	Reference
Lactate metabolism	Lactate transporter	JEN1	*Yarrowia lipolytica, Penicillium camemberti, Debaryomyces hansenii, Saccharomyces cerevisiae, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014)
	D-lactate dehydrogenase	CYB1	*Y. lipolytica, D. hansenii*	Lessard et al. (2014)
	L-lactate dehydrogenase	CYB2	*Y. lipolytica, P. camemberti, Geotrichum candidum*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015)
	D-lactate dehydrogenase	DLD1	*G. candidum, P. camemberti, Y. lipolytica, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015)
	Lactate dehydrogenases	DLD2	*G. candidum, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015)
Lactose metabolism	β-Galactosidase	LAC4	*Kluyveromyces lactis, P. camemberti, Kluyveromycesmarxianus*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Lactose permease	LAC12	*K. lactis, P. camemberti, Kluyveromycesmarxianus*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
Galactose metabolism	Galactose catabolism	GAL1, GAL7, GAL10	*G. candidum, D. hansenii*	Monnet et al. (2016)
	UDP-glucose 4 epimerase	GALE	*P. camemberti*	Lessard et al. (2014)

(continued)

Table 13.4 (continued)

Purpose	Enzyme/protein; pathway	Genes	Microorganism	Reference
Amino acid metabolism	Amino acid transporter	GAP1	*Y. lipolytica, G. candidum*	Lessard et al. (2014), Castellote et al. (2015), Monnet et al. (2016)
	Aromatic aminotransferases	ARO8	*Y. lipolytica, G. candidum, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015)
	Aminotransferase	ARO19	*Y. lipolytica*	Cholet et al. (2007), Lessard et al. (2014)
	Aminotransferase	BAT1, BcAt	*Y. lipolytica, G. candidum, K. lactis*	Cholet et al. (2007), Lessard et al. (2014)
	Branched-chain amino acid aminotransferase	BAT2, ArAt	*Y. lipolytica, G. candidum, K. lactis, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014)
	NAD+-dependent glutamate dehydrogenase	GDH2	*G. candidum*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Cytoplasmic aspartate aminotransferase	AAT2	*G. candidum*	Lessard et al. (2014), Castellote et al. (2015), Monnet et al. (2016)
	Pyrroline-5-carboxylate dehydrogenase; proline catabolism	PUT1, PUT2	*G. candidum*	Monnet et al. (2016)
	Glycine metabolism	GCV1, GCV2	*G. candidum, D. hansenii*	Monnet et al. (2016)
	Isopropylmalate dehydrogenase	LEU1	*G. candidum*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Transcriptional activator of amino acid biosynthesis	GCN4	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)
	De novo synthesis of NAD+ from tryptophan	BNA2, bNA7	*G. candidum*	Lessard et al. (2014), Castellote et al. (2015), Monnet et al. (2016)

(continued)

Table 13.4 (continued)

Purpose	Enzyme/protein; pathway	Genes	Microorganism	Reference
Proteolysis	Proteases	PEP4 & PRC1	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)
	Proteases; degradation of unfolded proteins	LHS1 & UBC6	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)
Lipid metabolism	Extracellular lipase Lip2p	LIP1	*G. candidum*	Fickers et al. (2005), Bertolini et al. (1994)
	Extracellular lipase Lip2p	LIP2	*Y. lipolytica, G. candidum*	Fickers et al. (2005), Bertolini et al. (1994)
	Triacylglycerol hydrolase	LIP7 & LIP8	*Y. lipolytica*	Fickers et al. (2005), Bertolini et al. (1994)
Central metabolism	E1β-subunit of the PDH complex	PDB1	*Y. lipolytica, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Pyruvate decarboxylase isozymes; pyruvate catabolism	PDC1	*Y. lipolytica, D. hansenii, K. marxianus*	Cholet et al. (2007), Lessard et al. (2014)
	Acetolactate synthase	ILV6	*Y. lipolytica, D. hansenii*	Cholet et al. (2007), Lessard et al. (2014)
	E1α-subunit of the PDH complex	PDA1	*Y. lipolytica, D. hansenii, K. marxianus*	Cholet et al. (2007), Lessard et al. (2014)
	Phenylpyruvate decarboxylase; pyruvate catabolism	ARO10	*D. hansenii*	Cholet et al. (2007), Lessard et al. (2014)
	Acetyl-CoA synthetase; ethanol and acetate catabolism	ACS1	*G. candidum*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015)
	Oligopeptide transporter; TCA cycle	OPT2	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)
	Glycogen catabolism	GDB1 & SGA1	*G. candidum*	Lessard et al. (2014), Castellote et al. (2015), Monnet et al. (2016)

(continued)

Table 13.4 (continued)

Purpose	Enzyme/protein; pathway	Genes	Microorganism	Reference
	F1F0 ATP synthase; production and consumption of ATP	ATP1, ATP2, ATP5 & ATP7	*G. candidum*	Cholet et al. (2007), Lessard et al. (2014), Castellote et al. (2015), Monnet et al. (2016)
	Gluconeogenesis and glycolysis	MTH1	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)
	Phosphoenolpyruvate carboxyl kinase; gluconeogenesis	PEPCK	*G. candidum, P. camemberti*	Lessard et al. (2014), Monnet et al. (2016)
	Fructose-1,6-bisphosphatase; gluconeogenesis	FBP	*G. candidum, P. camemberti*	Lessard et al. (2014), Monnet et al. (2016)
	Isocitrate lyase; glyoxylate cycle	ICL	*G. candidum, P. camemberti*	Lessard et al. (2014), Monnet et al. (2016)
	Malate synthase; glyoxylate cycle	MAS	*G. candidum, P. camemberti*	Lessard et al. (2014), Monnet et al. (2016)
	3-ketoacyl-coA thiolase; TCA cycle	KAT	Molds	Lessard et al. (2014), Monnet et al. (2016)
	Perixosomal multifunctional enzymes: β-oxidation	MEF	*P. Camemberti*	Lessard et al. (2014), Monnet et al. (2016)
	Cystathionine-β-lyase	STR3, CBL	*D. hansenii, Y. lipolytica*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Cystathionine-γ-lyase	CYS3, CGL	*D. hansenii, Y. lipolytica*	Cholet et al. (2007), Lessard et al. (2014), Monnet et al. (2016)
	Ribosomal protein	RPL10	*G. candidum*	Lessard et al. (2014), Monnet et al. (2016)

13.5 Mechanism of Fungal Genes Encoding Enzyme-Mediated Cheese Fermentation

Several studies analyzed the gene expression profile of cheese yeast and molds during ripening using meta-transcriptomics analysis (Cholet et al. 2007; Lessard et al. 2014; Castellote et al. 2015; Monnet et al. 2016). A fourfold increase in fungal gene transcripts associated with primary biochemical reactions including the metabolism of carbohydrates, lipids, and proteins, and secondary biochemical reactions including the catabolism of amino acid and fatty acids, are observed in *Lactococcus lactis*, *Kluyveromyces lactis*, *Geotrichum candidum*, and *Debaryomyces hansenii* between day 5 and day 35 during ripening (Dugat-Bony et al. 2015). Generally, functional and metabolic gene expression is affected by the availability of specific substrates corresponding to the enzymes and pathways regulated by the genes. Lactose and lactate metabolism-associated genes are abundant at ripening because of the initial abundance of lactose. As lactose and lactate become depleted as a carbon source, proteolytic-associated gene expression commences and is followed by lipolysis-associated genes. Toward the end of cheese ripening, gene transcripts related to amino acid and fatty acid catabolism are abundant, whereas the genes associated with translation are observed to decline, indicating reduced translational activity by microorganisms (Castellote et al. 2015). Environmental factors also have an impact on gene expression during ripening. An increase in temperature has been observed to improve expression of genes associated with protein metabolism, lipid metabolism, and amino acid and fatty acid catabolism, thereby cutting short the ripening time (Monnet et al. 2016). Other factors including salt content and pH are observed in *Saccharomyces* sp. and lactic acid bacteria (Xie et al. 2004). In reblochon-style cheese, gene transcripts of *G. candidum* are high throughout ripening whereas *D. hansenii* gene transcripts increase as ripening progresses (Monnet et al. 2016).

The genes associated with lactose metabolism are upregulated, with expression of the β-galactosidase LAC4 gene only being observed within the first days of ripening in molds. The galactose metabolism-associated gene GAL10 is overexpressed during this time as well, and its transcripts are low throughout ripening, although in yeasts the expression of GAL10 and JEN1 is upregulated with ripening time. Transcripts of the lactate transporter gene JEN1 are present through the ripening period while the lactate dehydrogenase DLD1 gene transcripts decrease throughout ripening (Lessard et al. 2014). As lactate becomes abundant, the gluconeogenesis- and glyoxylate cycle-associated genes PEPCK, ICL, FBP, and MAS are upregulated, and they gradually decrease throughout ripening in both yeasts and molds. At the end of ripening in yeast, lactate is fully exhausted as a carbon source; however, the genes associated with its catabolism are not downregulated at this point (Castellote et al. 2015). This observation is an indicator that cheese yeasts lack a mechanism to regulate the transcription of lactate metabolism-associated genes (Lessard et al. 2014; Monnet et al. 2016).

The transcripts of amino acid catabolism-associated genes AAT and BAT along with GDH1 transcripts are present at the beginning of ripening and increase with time. In molds, GDH1 transcripts are observed within the first 21 days of ripening, whereas AAT and GDH2 expression is upregulated with time followed by a gradual decrease. The CGL gene is expressed toward the end of ripening in yeasts. Similarly, in molds the expression of CGL, CBL, AAT, GDH1, and BAT is upregulated following the same trend (Castellote et al. 2015; Monnet et al. 2016). This high level of expression of genes associated with amino acid metabolism indicates an increase in the degradation of amino acids toward the end of ripening. With this increased degradation of amino acids, an increase in ammonia is also noted (Monnet et al. 2016), which is caused by the high availability of free amino acids in the cheese at this point in ripening, with glutamate being the most abundant (Rosenberg and Altemueller 2001).

Most genes associated with lipid metabolism are upregulated during the first 35 days of ripening and downregulated thereafter (Monnet et al. 2016). However, some genes associated with fatty acid catabolism in the TCA cycle are active throughout days 56 and 77. In yeasts, the expression of acetyl-CoA thiolase is observed throughout ripening, as is true for thioesterase expression in molds. Lipase expression is present in molds during the first day but is minimal. With ripening time, the overexpression of lipase-associated genes in yeasts, the upregulation of expression of enoyl-CoA-hydratase associated gene in molds, and the expression of MFE and acyl-CoA synthase-associated genes are recorded in both molds and yeasts. Halfway through the ripening period, the gene transcripts related to fatty acid metabolism enzymes such as enoyl-CoA-hydratase, acyl-CoA synthase, and 3-ketoacyl-CoA thiolase increase within the cheese matrix. Beyond that, the transcripts of enoyl-CoA-hydratase and 3-ketoacyl-CoA thiolase are still detectable in molds, while the lipase-associated gene expression is still evident in both yeast and molds. The gene associated with F1F0 ATP synthase is downregulated at this point, suggesting a decline in ATP production and consumption at the end of ripening (Castellote et al. 2015; Monnet et al. 2016).

13.6 Fungal Gene Tailored Strains for Enhanced Cheese Fermentation

The primary aim of this section is to provide an overview for the immensely important fungal gene tailored strains for enhancing the flavor and quality of cheese in the fermentation process. Genetics is one of the most applicable tools for addressing quality enhancement in foods. Genetic approaches for addressing limitations in fermentation, which involve the alteration of genes, are discussed in this chapter with an emphasis on cheese fermentation (Table 13.5). Cheese is a complex biological ecosystem and acts as a harbor for diverse communities of microorganisms. Among these, fungi have a vital role. The important fungi found in cheese are *Penicillium*

Table 13.5 Fungal gene tailored strains commonly found in cheese fermentation with their desirable secondary metabolites and genes

Fungi species	Secondary metabolite of interest	Tailored gene cluster	Reference
Penicillium roqueforti	Mycophenolic acid	*mpaA, mpaB, mpaC, mpaDE, mpaF, mpaG* and *mpaH*	Del-Cid et al. (2016)
	Roquefortine C	*rds, rdh, rpt,* and *gmt*	Kosalková et al. (2015)
	Second dimethylallyltryptophan synthase	DmaW2	Kosalková et al. (2015)
	Andrastin A	*adrA, adrC, adrD, adrE, adrF, adrG, adrH, adrI, adrJ* and *adrK*	Rojas-Aedo et al. (2017)
	PR-toxin	*prx1, prx2, prx3* and *prx4*	Hidalgo et al. (2014, 2017)
	Mycophenolic acid, roquefortine C, andrastin A	*sfk1*	Torrent et al. (2017)

sp., *Cladosporium* sp., *Mucor* sp., *Geotrichum* sp. (yeast), and *Trichoderma* sp. (Sørensen et al. 2011; Hymery et al. 2014).

The filamentous fungus that is widely used in the blue cheese ripening process is *Penicillium roqueforti*. Addition of this microorganism during the cheese production process forms a blue-veined or spotted appearance in the final product. *P. roqueforti* also improves the organoleptic properties of different types of blue cheese. Among several secondary metabolites produced by *P. roqueforti*, the meroterpenoid compound, a mycophenolic acid, is considered to be a contaminant in cheese production (García-Estrada and Martín 2016). The bioinformatics approach has identified a genomic region of approximately 24.4 kbp that contains seven gene clusters involved in the biosynthesis of mycophenolic acid. These identified clusters have been named *mpaA*, *mpaB*, *mpaC*, *mpaDE*, *mpaF*, *mpaG*, and *mpaH* (Cheeseman et al. 2014; Del-Cid et al. 2016). A dramatic reduction of mycophenolic acid production has been observed after silencing each of the aforementioned genes.

A study by Kosalková et al. (2015) has revealed that the entire 16.6-kb region of the roquefortine gene cluster shows 98–99% identical organization and nucleotide sequences among three strains of *Penicillium roqueforti* isolated from blue cheese in the UK, USA, and France. These identified clusters are found with four genes: *rds*, *rdh*, *rpt*, and *gmt*. The *rds* gene encodes roquefortine dipeptide synthetase, the *rdh* gene encodes roquefortine D-dehydrogenase, the *rpt* gene encodes roquefortine prenyltransferase, and the *gmt* gene encodes methyltransferase. Roquefortine C (Fig. 13.6) interferes with RNA synthesis and interacts with P450, thereby acting as a neurotoxic agent in mice and chickens. This secondary metabolite is not considered to pose health consequences in humans but is considered as a contaminant in blue cheese. Therefore, silencing of *rds* or *rpt* genes by RNAi strategy has been evaluated to reduce roquefortine C production by 50% in roquefortine biosynthesis (García-Estrada et al. 2011; Ries et al. 2013; Ávalos et al. 2014).

A different study has elucidated the silencing of a second dimethylallyl tryptophan synthase (DmaW2), which is a different form of roquefortine prenyltransferase of *P. roqueforti* (Fernández-Bodega et al. 2017). This silencing of DmaW2 suppressed the formation of indole alkaloids that are involved in the biosynthesis pathway of isofumigaclavine A, which eliminates the possible toxicity of these secondary metabolites in cheese production (Polonsky et al. 1977). One of the secondary metabolites from *P. roqueforti* is andrastin A, the meroterpenoid compound identified as an antitumoral compound (Rojas-Aedo et al. 2017). The 29.4-kbp gene cluster in the genomic region involved in the biosynthesis of andrastin A in *P. roqueforti* has been sequenced and annotated. The genome region named *adr* contains ten genes: *adrA*, *adrC*, *adrD*, *adrE*, *adrF*, *adrG*, *adrH*, *adrI*, *adrJ*, and *adrK*. The RNA-mediated gene silencing of all ten genes has resulted in significant reductions in andrastin A production, confirming that all ten genes are involved in the biosynthesis of andrastin A. The overproduction of andrastin A is an interesting factor in the cheese production process because of its antitumoral activity. Another potent mycotoxin produced by *P. roqueforti*, termed PR-toxin (Fig. 13.7), is considered to be another contaminant in blue cheese. The *ari1* gene containing four gene clusters from *P. roqueforti* is identified as responsible for the production of PR-toxin. Silencing of the four genes *prx1*, *prx2*, *prx3*, and *prx4* has resulted in a 65–75% reduction in PR-toxin biosynthesis (Hidalgo et al. 2014, 2017; Dubey et al. 2018).

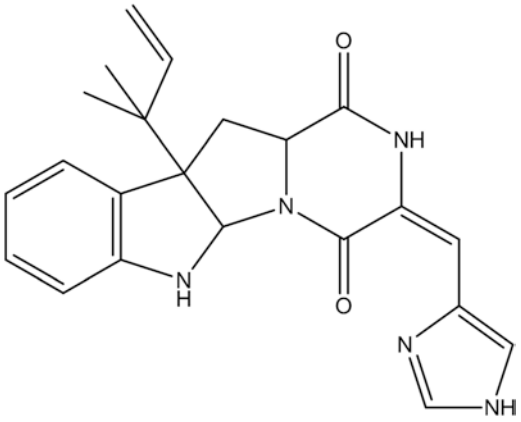

Fig. 13.6 Chemical structure of roquefortine C

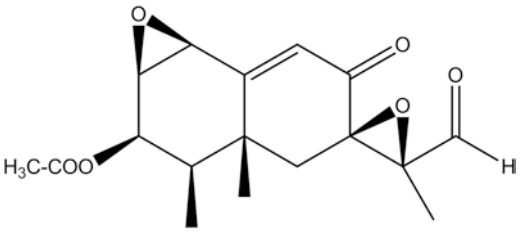

Fig. 13.7 Chemical structure of PR-toxin

The suppressor of four kinase (*sfk1*) genes was originally discovered from *Saccharomyces cerevisiae*, identified to be involved with thermal stress resistance. The RNAi silencing of the *sfk1* gene in *P. roqueforti* has been identified to deplete the production of undesirable secondary metabolites found in cheese production such as mycophenolic acid, roquefortine C, and andrastin A (Torrent et al. 2017). Microbial contamination of food and feed with filamentous fungi can result in production of undesirable secondary metabolites whose consumption could lead to drastic health implications in both humans and animals. Today public concerns about food safety and quality have increased, with demands for hygienic and quality-enriched foods. Therefore, fungal gene tailored strains would be a key strategy in enhancing quality in food products such as cheese, as just discussed.

13.7 Heterologous Regulation of Fungal Proteins for Cheese Production

The natural ability of filamentous fungi to secrete a large amount of proteins has gained attraction for their use as hosts in heterologous regulation (Table 13.6). By producing a large amount of enzymes, the filamentous fungi are vital in industry (Nevalainen et al. 2005). The production of heterologous protein is one of the main interests in academic and industrial sectors. Therefore, this section discusses the protein expression and secretion of fungi with regard to heterologous regulation.

Table 13.6 Fungi and their heterologous catalysts

Strain/genotype	Characteristics/phenotype	Heterologous protein of interest	References
Yarrowia lipolytica			
PO1d, E150	Amplification of LIP2	Lipase	Pignede et al. (2000)
	Hybrid promoter for *XPR2* gene	Bovine prochymosin	Madzak et al. (2000)
Po1h	Overexpression of *Saccharomycopsis fibuligera*	Acid protease	Yu et al. (2010a)
Po1h	Amplification from *Saccharomycopsis fibuligera* A11 genomic DNA	Acid protease	Yu et al. (2010b)
22a-2	*AP1* gene transformation	Acid protease	Yu et al. (2013)
Saccharomyces cerevisiae			
LHDP1	Transformation with plasmid YEplac181-LAC4bearing the LAC4 gene	β-Galactosidase	Becerra et al. (2004)
	Lipase II gene of *Geotrichum candidum*	Lipase	Vernet et al. (1993)

Yeast carries a number of genetically vital advantages in regulating the effective secretion and performance of heterologous proteins. *Yarrowia lipolytica* is a non-conventional yeast found in cheese, capable of producing secondary metabolites and enzymes such as lipase with considerable industrial potential (Brígida et al. 2014). Lipid metabolism is an important part of cheese production as it helps enhance cheese flavor by releasing volatile fatty acid compounds. Amplification of *LIP2* in *Y. lipolytica* has been demonstrated to increase lipase production, which is important industrially (Pignede et al. 2000). The *XPR2* gene encodes the production of large amounts of an alkaline extracellular protease in the industrial yeast *Y. lipolytica*. The industrially complex regulation of this gene is hindered by the *XPR2* promoter. Through remedial action by using a hybrid promoter devoid of extracellular proteases, an increase in the heterologous secretion of bovine prochymosin, an acidic milk coagulant, is proved (Madzak et al. 2000).

Rennet is a commercially important enzyme in cheese production that is used in the initial stages of cheese production for milk coagulation and separation into solid coagulants. The current vegetarian trends for cheese manufacturing have created a need for alternatives sources of rennet other than a calf's or goat's stomach. In this regard, a rennet substitute has been discovered for the cheese industry (Yu et al. 2010a). The acid protease of *Saccharomycopsis fibuligera* is cloned in *Y. lipolytica* and overexpressed. The resultant recombinant acid protease is purified and the positive transformation confirmed by milk clotting. Another study has separated the protease structural gene from *Saccharomycopsis fibuligera* A11 and cloned it into the multiple cloning site of the surface display vector pINA1317-YlCWP110 expressed in the yeast *Y. lipolytica* (Yu et al. 2010b). The cloned *Y. lipolytica* displayed the extracellular acid protease activity that is applicable in milk clotting.

An additional study explained the transfer of the acid protease gene (*AP1* gene) from *Saccharomycopsis fibuligera* A11 to the native acid protease gene in *Y. lipolytica* and its applicability as an acid protease in cheese manufacturing industry (Yu et al. 2013). Genetic engineering techniques have facilitated *Saccharomyces cerevisiae* to produce the heterologous enzymes that are important in lactose metabolism (Becerra et al. 2004). As lactose is the key sugar present in milk, thus lactose metabolism is vital in dairy manufacturing. Most cheese manufactures want to reduce the downstream cost of production by utilizing the major by-product whey in cheese manufacturing. Therefore, the heterologous production of *Kluyveromyces lactis* β-galactosidase in lactose-containing media by *Saccharomyces cerevisiae* recombinant strain LHDP1 is a valuable resource in dairy industries such as cheese production (Guimarães et al. 2010; Domingues et al. 2010).

In cheese, *Geotrichum candidum* secretes lipases that are important in developing flavor, as discussed earlier under lipid metabolism. The lipase II gene of *Geotrichum candidum* has been amplified into *Saccharomyces cerevisiae* and thus it secretes lipase into the medium. This discovery is useful in producing high levels of biocatalysts with reduced production costs (Vernet et al. 1993; Kademi et al. 2003). The strains of *Penicillium camemberti* CECT 2267 and *P. roqueforti* NRRL 849 are proved to have important biotechnological properties such as resilient performance relative to growth, protoplast regeneration, and transformation (Chávez

et al. 2010). Numerous fungi are major sources of secreting biocatalysts for cheese production, hence, much attention is bestowed on isolating specific gene from cheese fungi and amplifying them in other organisms. However, studies on heterologous regulation of fungi for cheese industry are limited, and more research is needed.

13.8 Conclusion

Cheese is a dairy product that is consumed worldwide. It has a complex ecosystem as it harbors heterogeneous microorganisms. Among microorganisms, fungi have a vital role in cheese ripening. The diverse fungal species are responsible for different types of cheese in characteristic appearance, texture, and flavor. In the metabolism of the cheese matrix components, the fungi have a central role in proteolysis and lipolysis: these processes start during the middle of the ripening and continue to the end. The ripening time takes months to year depending on the cheese variety. Therefore, regulation and understanding of gene expression within fungi in cheese would help reduce cheese ripening times, thereby saving production time and cost. Moreover, some secondary metabolites from fungi in cheese are toxic, and remedial actions are required through encoding genes to remove or reduce these toxic secondary metabolites. Gene tailoring and heterologous regulation are remedial actions that can enhance the quality and safety of cheese. Fungi from cheese have commercially important genes that are helpful in the production of industrial biocatalysts. However, the studies on gene regulation for utilization in cheese production are still limited, calling for continued research.

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Chapter 14
Unraveling the Potentials of Endophytes and Its Applications

M. Nandhini, A. C. Udayashankar, Sudisha Jogaiah, and H. S. Prakash

14.1 Introduction

Endophytes are microbes which colonize symptomlessly the living, internal tissues of the host (Petrini 1991). The definition given by Quadt-Hallmann et al. (1997) is endophytes are those that can be isolated from surface-disinfested plant tissue or extracted from within the plant and that do not visibly harm the plant. Endophytes readily colonize all niches in plants, coming through soil, wind, air, or water. They colonize the internal tissues of plants without causing any visible signs of infection (Wilson 1995). Endophytes were majorly studied in temperate region plants, whereas the studies were drawn out to tropical and crop plants as well to isolate diverse endophytes from the environment. Endophytes have been found to inhabit tissues of roots, stems, branches, twigs, bark, leaves, petioles, flowers, fruit, and seeds.

Based on earlier studies, it is estimated that nearly all plants including mosses, liverworts, and ferns colonize one or more endophytic fungi (Zou and Tan 2001), as such, it is projected that there are about one million endophytic fungi that possibly inhabit plants (Dreyfuss and Chapela 1994). The total amount of endophytic fungi represents about 65% of all estimated 1.5 million fungal species (Hawksworth 2001). This indicates that endophytic fungi are major groups of fungal diversity. Some studies have revealed that a majority of endophytic fungi are not host specific; instead, they have a wide range of hosts and that the abundance and dominant species on each host plant may be different. The colonization rate and interspecies

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diversity may vary with different parts of host plants and also correlate with the age of the hosts and seasons (Sun et al. 2008). Interspecies variation of endophytic fungi at different parts of the host plant is largely attributed to variation in physiological conditions and texture difference of host tissue (Aly et al. 2010). The endophytes have been shown to exhibit organ and tissue specificity due to their adaption to the altered physiological environment in different plant tissues. Variations in the endophytic profile can be caused by different parameters such as seasonal changes, stresses on the host plant, plant organs, etc. (Mocali et al. 2003). It is noteworthy that intraspecies diversity of endophytic fungi within the same part of the host plant is also abundant. The distribution and diversity of endophytes are done by culture-based study based on the colonization frequency. A culture-based study of ten sea grass species revealed that *Aspergillus terreus* was the most dominant species in rhizomes of the sea grasses (Venkatachalam et al. 2015).

The transmission of endophytes is a mixed strategy (both vertically and horizontally) (Saikkonen et al. 2004). Endophytes are an enthralling group of microbes that are associated with tissues of terrestrial and aquatic plants and have attracted the attention of mycologists, ecologists, pharmacists, industrialists, and plant pathologists with their varied functions (Bacon et al. 2004).

Endophytes are capable of synthesizing secondary metabolites as similar to the host plant in which they reside. The genetic transfer from the host to the endophyte in evolutionary time has directed to the integration of the pathway genes of the host into the endophyte (Tan and Zou 2001). Till date there is no evidence for the horizontal transfer of genes coding for secondary metabolites between a plant host and its endophytic associate. In effect, the mechanism underlying the production of plant secondary metabolites by an endophyte remains inexplicable.

Gundel et al. (2017) have reported the trans-generational effect of vertically transmitted endophytes which changes the phenotype and fitness of the host plant. Trans-generational effects could arise due to the presence or absence of endophytes, endophyte load, genotype, or species composition or also because of endophyte priming of the plant. These endophytes are assumed to disrupt the ecology of plants, by enhancing the ability of host plants to endure and resist abiotic and biotic stresses through different mechanisms that are only moderately understood (Kogel et al. 2006).

14.2 Methods of Endophyte Identification and Diversity

The molecular identification and their phylogenetic relationships are carried out by ITS and 16S rDNA amplification. Xiong et al. (2013) had isolated 81 endophytic fungi from *Taxus x media* and grouped into 8 genera based on the ITS rDNA sequence. Even though ITS regions appear to have several advantages for defining the phylogenetic relationships and diversity of the organisms, their coding sequences

are highly conserved so that they contain too little parsimony information for the phylogeny of closely related species (Sogin et al. 1986; Nanney et al. 1989). A study revealed that morphologically distinct fungi *Pseudocyphellaria crocata*, *P. neglecta*, and *P. perpetua* could not be distinguished by ITS sequences (Summerfield and Eaton-Rye 2006).

Nuclear protein-coding gene sequences have been employed in previous studies of fungal endophytes to elucidate evolutionary relationships at the intraspecific and interspecific levels, through phylogenetic analysis of partial sequences representing orthologous intronic regions of β-tubulin, α-tubulin, actin, and elongation factor-1α (Craven et al. 2001). Among these genes, the beta-tubulin genes may be useful for investigating relationships between fungi at all taxonomic levels (Huang et al. 2009). Beta-tubulin genes exist in every cell nucleus responsible for encoding components of microtubules, which are major components of the cytoskeleton, mitotic spindles, and flagella of all eukaryotic cells (Fuller et al. 1987; Conner et al. 1989). Like the rDNA genes, the β-tubulin genes are involved in ancient eukaryotic functions, universally present in eukaryotes with constant function (Moores et al. 2002), and are among the most highly conserved eukaryotic proteins (Wade 2007). The transcription elongation factor (TEF) 1-alpha gene is usually present in a single copy and encodes the translation elongation factor that controls the rate and fidelity of protein synthesis (Baldauf and Palmer 1993). The encoded protein forms a ternary complex with aminoacyl-tRNA and GTP. Besides, it interacts with some cytoskeletal proteins, especially actin (Durso and Cyr 1994; Shiina et al. 1994). Molecular differentiation of *Fusarium* spp. with varied lifestyles based on TEF-1 alpha gene sequence analysis was done, and the study revealed that similarly named *Fusarium* species clustered together based on their lifestyles forming distinct clades, indicating that coding genes could be better used as a phylogenetic marker than noncoding ones (Barik and Tayung 2012).

The richness and distribution of endophytic fungi found in the coffee plantation were examined using the range diversity (RD) analysis by Saucedo-García et al. (2014). A combination of methods like ITS sequence analysis, colonization frequency analysis, similarity index analysis (Sorensen's index of similarity and Jaccard's index of similarity), and diversity analysis (Shannon–Weaver diversity and Simpson's diversity) were done by Qadri et al. (2014) to investigate the diversity of fungal endophytes.

Species diversity indices were measured with Shannon diversity index (H′), Shannon evenness index (J'), and Simpson diversity index (D) and were calculated for the evaluation of fungal species richness of endophytes isolated from *Vitex negundo* (Sunayana et al. 2014).

The phylogenetic diversity in 30 endophytic *Pestalotiopsis* strains from *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna*, and *T. chebula* was analyzed using restriction fragment length polymorphism (ITS-RFLP) and sequence analysis of the ITS region of ribosomal DNA (rDNA) (Tejesvi et al. 2009).

14.3 Role as Holobiont

The holobiont is comprised of the plant and its microbiome. The definition of holobiont as per Guerrero et al. (2013) is the genomic reflection of the complex networks of symbiotic interactions that link an individual of a given taxon with its associated microbiome. The plant holobiont is studied to show the communication and interaction between two partners which leads to a better performance of the plant (Gordon et al. 2013). The microbiome of the plant mainly is formed by the air microbiome, phyllosphere microbiome, seed microbiome, and soil microbiome. The majorly present endophytes in plants are from the soil microbiome which is due to the release of plant exudates which are rich in organic carbon compounds, leading to the accumulation of the plant microbiome belowground (Vandenkoornhuyse et al. 2015). The interaction between the host and microbiome is symbiosis or mutualism. The symbiotic mechanisms include nutritional dependencies, biofilm formation, molecular communications, enhanced dispersal of endophytes, etc. The competitive interactions in the microbiome include resource competition, contact-dependent competition, secretion of antimicrobial metabolites, emission of volatile organic compounds (VOCs), predation, etc. (Hassani et al. 2018).

14.4 Role in Secondary Metabolite Production

One of the beneficial roles is the production of secondary metabolites. Alkaloids, steroids, terpenoids, isocoumarins, quinones, flavonoids, phenylpropanoids, lignans, peptides, phenolics, aliphatics and volatile organic compounds, etc. are the range of metabolites produced by the endophytes (Kusari et al. 2013). It is hypothesized that the secondary metabolite synthesis genes are transferred to the endophytes, and hence the endophytes have the pathway genes for the synthesis of secondary metabolites. Previous reports have suggested that endophytes have developed genetic systems that allow the transfer of information between themselves and the host plant (Borges et al. 2009). Also, long-term coexistence with their hosts has resulted in a coevolutionary process through which these microorganisms have acquired interesting capabilities, such as powerful transformation. For instance, some endophytes can synthesize biologically active substances similar to the secondary metabolites produced by their hosts (Wang and Dai 2011).

The synthesis of host secondary metabolites by endophyte can be due to the fact that it can compete with other invading pathogens (Shweta et al. 2013). And also to provide plant defenses against pathogens and therefore the endophyte may increase fitness benefits through providing higher fitness to the host (Moussa et al. 2016). Directly or indirectly, entophytic *Bacillus* sp. exhibits suppression of broad spectrum of phytopathogens through production of secondary metabolites, namely, difficidin, polyketides, and bacillaene (Nakkeeran et al. 2019). Schulz et al. (2002) have reported that the metabolites produced by endophytes are higher in proportion

(51%) compared to their soil counterparts (38%). Helaly et al. (2018) have enumerated the diverse secondary metabolites synthesized from the endophytes of the genus *Xylariales* which comprised cytosporin F, pestaloficiol, chloropestolide, 2,3-dihydoxytetradecan-5-olide, etc. Numerous studies have been conducted to isolate the endophytes from the host plant, reporting their ability to produce secondary metabolites that have multiple applications such as antimicrobial, cytotoxic, anticancerous, antitumor, antioxidant, anti-inflammatory activities, etc. (Table 14.1).

Endophytes are found to influence the secondary metabolites of the host plant. The association of host–plant *Kadsura angustifolia* associated with endophytic fungus *Umbelopsis dimorpha* has led to the production of 18 different metabolites in *K. angustifolia* which has been reported for the first time in the plant (Qin et al. 2018).

14.5 Role in Pharma and Industry

Endophytes have been well-known to produce enzymes and secondary metabolites which are of prime importance for mankind. Endophytes use enzymes for the colonization of the host plant and thus have the potential to produce a wide range of enzymes such as cellulases, hemicellulases, laccases, chitinases, glucanases, xylanases, etc. The widespread applications of thermostable amylolytic enzymes from endophytes are investigated to improve the industrial processes of starch degradation. Endophytic bacteria such as *Microbacterium foliorum* isolated from *Phaseolus vulgaris* produce phytases that can be used in the animal feed industry (Costa et al. 2018). L-asparaginase used to treat acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma is produced in vitro by endophytic *Penicillium* and *Talaromyces* species isolated from *Tillandsia catimbauensis* (Silva et al. 2018). Numerous antibiotics such as ecomycins, pseudomycins, cryptocandin, echinocandins, and the pneumocandins have been isolated from endophytic fungi (Demain 1999). Several endophytes are known to have anti-insect properties. Nodulisporic acids are novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly. Naphthalene a major compound isolated from an endophytic fungus, *Muscodorvitigenus*, from a liana (*Paullina paullinioides*) acts against wheat stem sawfly and is used as an insect repellent (Daisy et al. 2002).

Hairy root cultures, induction of secondary metabolite production, and biotransformation are the different pathways in which the endophytes are used for the production of industrial and pharmaceutical products. The microbial transformation of cyclocanthogenol (CCG) by the endophytic fungus *Alternaria eureka* isolated from *Astragalus angustifolius* was carried out by hydroxylation, oxidation, epoxidation, O-methylation, ring expansion, and methyl migration (Ekiz et al. 2018). The industrial production of diosgenin by solid-state fermentation is carried out by Xiang et al. (2018) using the endophytic co-cultures of *Fusarium* sp. and *Curvularia lunata* isolated from *Dioscorea zingiberensis*.

Table 14.1 List of secondary metabolites isolated from endophytes

Endophyte	Host	Metabolite	Activity	Reference
Colletotrichum gloeosporioides	*Huperzia serrata*	Huperzine	Inhibitor of acetylcholinesterase	Kang et al. (2018)
Aspergillus versicolor	*Eichhorniacrassipes*	Aflaquinolone H	Cytotoxic activity	Ebada et al. (2018)
Phomopsis sp. and *Pestalotiopsis guepini*	Garlic	Trichodermin	Antifungal activity	Shentu et al. (2014)
Pichia guilliermondii	*Xanthium sibiricum*	Eupenicisirenins A and B	Antibacterial activity	Li et al. (2014)
Phomopsis sp.	*Miquelia dentata*	Camptothecin	Anticancer activity	Shweta et al. 2013
Aspergillus fumigatus	*Melia azedarach*	Fumitremorgin B, verruculogen	Antifeedant activity	Li et al. (2012)
Aspergillus fumigatus	*Sonneratiaapetala*	Talaperoxides	Cytotoxic activity	Li et al. (2011)
Cladosporium sp.	*Laggera alata*	Sterigmatocystin and secosterigmatocystin	Antimalarial activity	Matasyoh et al. (2011)
Chaetomium sp.	*Paris polyphylla*	5α,8α-Epidioxyergosta-6,22-dien-3β-ol	Antimicrobial activity	Zhao et al. (2010)
Talaromyces flavus	*Aegle marmelos*	Taxol	Anticancer activity	Gangadevi and Muthumary (2008)
Entrophosporainfrequens	*Quercus variabilis*	Brefeldin A	Antimicrobial activity	Wang et al. (2007)
Bacillus subtilis	*Nerium oleander*	Phenolics	Antioxidant activity	Huang et al. (2007)
Bartaliniarobillardoides	*Nothapodytes foetida*	Camptothecin	Anticancer activity	Amna et al. (2006)
Podospora sp.	*Erythrina crista-galli*	Phomol	Anti-inflammatory activity	Weber et al. (2004)
Eupenicillium sp.	*Crassocephalum crepidioides*	7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one	Antituberculous activity	Kongsaeree et al. (2003)
Geotrichum sp.	*Spondias mombin*	Bioactive fraction	Antimicrobial activity	Rodrigues et al. (2000)

14.6 Role in Agriculture

The major challenge in the agriculture sector is to raise the agricultural productivity per unit of land to meet the growing food demand. One-quarter of soils worldwide face degradation resulting in changes in the soil quality and microbial diversity due to agricultural intensification and urbanization (Lal 2015). Incorporating beneficial microbiomes into agricultural systems provides the potential to improve the effectiveness of crop plant production, whereas the beneficial microbial communities in modern agriculture have been underutilized (Nagaraju et al. 2012a, b; Jogaiah et al. 2013, 2018). The crop microbiome, plant phenotype, and environment affect the crop yield. Microbes common in challenging environments are expected to protect yield under stresses such as drought, heat, salinity, heavy metals, disease, and herbivory (Busby et al. 2017; Satapute et al. 2019). Commercially endophytes are used as bio-fertilizers, herbicides, insecticides, biocontrol agents, etc. Some commercially used biopesticides are Galtrol, Mycostop, Ecofit, SoilGard, etc.

14.7 Role in Abiotic Stress Mitigation

Endophytes are used to overcome threats that lead to loss of crop yield, including plant stresses associated with unfavorable environmental conditions, such as drought, temperature extremes, or soil salinity, as well as biotic stress induced by plant pathogens and pests (Miliute et al. 2015). Abiotic stress mitigation by endophytes involves two mechanisms: (i) activation of host stress response systems soon after exposure to stress, allowing the plants to avoid or mitigate the impacts of the stress (Redman et al. 2002), and (ii) biosynthesis of antistress biochemicals by endophytes (Schulz et al. 2002). *Burkholderia phytofirmans* has shown to downregulate rbcL and COR 78 genes, accumulate pigments, and help in the induction of cold response pathway in response to cold stress (Su et al. 2015). Kavroulakis et al. (2010) reported the endophytic fungus *Fusarium solani* colonizes the tomato plant and helps in water stress management by the reduction in stomatal closure response to water stress and potentially improved water acquisition by roots and/or water conductance *in planta.* ACC deaminase expressing endophyte *Pseudomonas* sp. was observed to mitigate NaCl stress tolerance by reducing stress-related ethylene production which resulted in improved growth, photosynthetic performance, and ionic balance in tomato plants (Win et al. 2018). *Bacillus subtilis* and *Paenibacillus illinoinensis* enhanced the expression and activity of vacuolar H + -pumping pyrophosphatase required for the drought tolerance in pepper. The strains were found to stimulate a larger root system and enhanced the photosynthetic activity in leaves (Vigani et al. 2018).

14.8 Role in Plant Growth Promotion

Endophytes are gaining special attention due to their ability to promote the growth of host plants, control disease, and improve the fitness of the plant in adverse environmental conditions leading to increased yield. Endophytes are preferred due to their specificity to the host and are good candidates as bio-fertilizers and biocontrol agents since they can be transferred to next-generation plants through seeds (Rosenblueth and Martínez-Romero 2006).

Endophytes and plants live in symbiotic association, and the microbes play a major role in plant growth promotion, higher seed yield, and increasing resistance to biotic and abiotic stresses (Rai et al. 2014). They also produce various metabolites that can be used as eco-friendly agricultural products. The endophytic composition of the host plants varies according to the environmental conditions, geographic location, and seasons (Guo et al. 2008). Li et al. (2018b) evaluated the distribution of fungal endophytes in roots of *Stipa krylovii* across six vegetation types in the grassland of northern China and found that environmental parameters accounted for the variation in the communities than the vegetation type or geographical distance. All the plants examined to date have been known to be colonized by microbes (U'Ren et al. 2012). The endophytes are gaining attention as biological control agents as well as plant growth-promoting agents, as they could colonize the host efficiently and manage the plant diseases (Berg et al. 2005).

The endophytes promote the growth of the host plant by the production of phytohormones, siderophore, ACC deaminase, hydrolytic enzymes, etc. (Fig. 14.1).

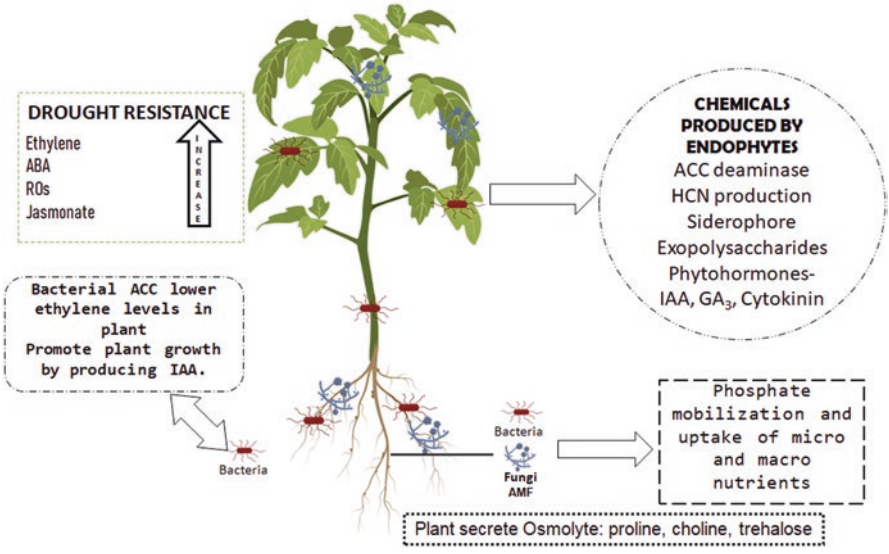

Fig. 14.1 Mechanism of enzymes, osmolytes, and phytohormones involved in drought mitigation, plant nutrition, etc. by endophytes (Patle et al. 2018)

Plant growth-promoting endophytic fungi *Aspergillus fumigatus* TS1 and *Fusarium proliferatum* BRL1 have been shown to produce gibberellins and regulate plant endogenous hormones on colonization in rice (Bilal et al. 2018). Ji et al. (2014) have reported 576 bacterial endophytic isolates from leaves, shoots, and roots of 10 rice cultivars and found that 12 isolates, when treated to rice seedlings, improved plant growth and increased height, dry weight, and antagonistic effects against fungal pathogens. Lubna et al. (2018) evaluated the endophytic fungus *Aspergillus niger* isolated from *Cannabis sativa* to improve the growth of rice. The fungi were observed to show growth-promoting traits such as the presence of siderophores, phosphate solubilization, and the production of indole acetic acid (IAA) and gibberellins and were further found to promote the growth of rice. The mechanism of growth promotion was attributed to the production of different forms of gibberellins and IAA by the endophyte. The presence of GA pathway genes (P50-1, P450-3, P450-4, ggs2, and des) was also confirmed using semiquantitative RT-PCR.

Several isolated bacterial endophytes have shown efficiency in antagonism or inducing resistance against the oomycete pathogen in crops such as grapevine, lettuce, sunflower, maize, etc. Endophytic *Bacillus asahii* isolated from cucumber has shown 42.1% control efficacy against the downy mildew disease in cucumber in field trails (Sun et al. 2008). Puopolo et al. (2014) have reported that *Lysobacter capsici* AZ78 produced a 2,5-diketopiperazine which showed antagonistic activity against *Plasmopara viticola* and *Phytophthora infestans*. Waqas et al. (2015) have reported the increased production of abscisic acid and jasmonic acid on colonization with *Paecilomyces formosus* to combat heat stress and improve plant growth in rice.

14.9 Disease Suppression by Endophytes

The endophytes display disease suppression either by competing with the pathogens for colonization or by directly antagonizing the pathogen by producing antimicrobial compounds or by inducing systemic resistance in the host by the production of defense-related enzymes. But to increase the specificity of endophytes, prospecting of microbes in the host for their growth promotion and disease protection abilities has been carried out in various crop plants (Table. 14.2).

Endophytes have been recognized as outstanding sources of novel bioactive compounds (Strobel and Daisy 2003). Their volatile organic compounds benefit host plants by providing additional lines of defense against pathogens (Macías-Rubalcava et al. 2010). Several metabolites showing antifungal activity have been isolated and characterized from endophytic fungi.

Mousa et al. (2015) isolated endophytic *Phoma* sp. from finger millet roots which showed antifungal activity against *Fusarium graminearum*. The antifungal compounds from the endophyte, viz., viridicatol, alternariol, and tenuazonic acid which showed anti-*Fusarium* activity, were identified using LC-MS and NMR.

Table 14.2 List of endophytes which suppress diseases in crops

Endophyte	Host	Activity	References
Penicillium brefeldianum	Melon	Antifungal and anti-nematodal activity against *Fusarium* wilt-root knot nematode complex	Miao et al. (2019)
Epicoccum nigrum	Potato	Induced resistance against the blackleg disease of potato caused by *Pectobacterium carotovora*	Bagy et al. (2019)
Epichloë festucae	Perennial ryegrass	Reduced the incidence of leaf spot disease caused by *Bipolaris sorokiniana*	Li et al. (2018a)
Aspergillus terreus	Tomato	Induced resistance against *Pseudomonas syringae* pv. tomato	Yoo et al. (2018)
Biscogniauxia sp.	Rice	Inhibited *Magnaporthe grisea* which causes blast disease in rice	Nguyen et al. (2018)
B. amyloliquefaciens	Tomato	Significantly decreased *Fusarium* wilt severity and enhanced tomato growth	Abdallah et al. (2017)
Pseudomonas putida	Black pepper	Induced expression of defense genes and PR proteins	Agisha et al. (2017)
Bacillus subtilis	Chickpea	Induced suppression of root rot caused by *Fusarium solani*	Egamberdieva et al. (2017)
Trichoderma hamatum	Pearl millet	Elicitation of glucanase, POX, PAL, and PPO	Siddaiah et al. (2017)
S. exfoliatus and *S. cyaneus*	Lettuce	Inhibited the mycelial growth of *Sclerotinia sclerotiorum* which causes lettuce drop	Chen et al. (2016)
Bacillus oryzicola	Rice	Induced systemic resistance against bacterial blight (*Xanthomonas oryzae*) disease	Chung et al. (2015)
Penicillium citrinum and *Aspergillus terreus*	Sunflower	Regulated the hormones involved in plant defense against the stem rot caused by *Sclerotium rolfsii*	Waqas et al. (2015)
Paraconiothyrium variabile	Maize	Antagonistic activity against *Fusarium oxysporum* and suppresses the production of mycotoxin	Prado et al. (2015)
Trichoderma asperellum	Cacao	Controlled vascular streak dieback disease caused by *Ceratobasidium theobromae*	Rosmana et al. (2015)

Yang et al. (2013) isolated endophytic *Paenibacillus xylanilyticus*, *Paenibacillus polymyxa*, and *Bacillus subtilis* from seedling, squaring, and boll-setting stages of cotton. The combined application of three endophytic bacteria was found to control the effects of *Verticillium dahliae* which causes *Verticillium* wilt of cotton.

Bacillus subtilis strain isolated from wheat was found to exhibit high antifungal activity against *Gaeumannomyces graminis* var. *tritici* which causes take-all disease in wheat. Field experiments showed that endophyte-inoculated plants were found to

give 55.3% protection against take-all disease compared to uninoculated controls (Liu et al. 2009).

A total of 60 different endophytic bacteria were isolated from *Cymbopogon citratus*, *Azadirachta indica*, *Phyllanthus emblica*, *Boerhavia diffusa*, *Boerhavia repens*, *Pisum sativum*, *Sorghum bicolor*, and *Parthenium hysterophorus*. Among the endophytes, *Pseudomonas fluorescens* and *Bacillus* sp. showed the highest protection of 68% and 63%, respectively, against downy mildew disease in pearl millet (Chandrashekhara et al. 2007).

The diverse group of endophytes is proven to be useful in nutrient-poor environments and when plants are under stress due to drought and pathogen attacks (Rodrigues et al. 2000; Saikkonen et al. 2004). Irrespective of plant host or endophyte genera, symbiosis has resulted in increased plant biomass production and reduction of disease in plants (Rodrigues et al. 2000).

14.10 Endophytic Bioengineering

Endophytic microorganisms during their long coevolutionary process with their hosts have developed several important and novel characteristics. In order to sustain a stable symbiosis, endophytic microorganisms secrete varieties of extracellular enzymes that contribute to colonization and growth. All these precise enzymes, under certain conditions, could be exploited. Currently, more and more composite chemical reactions are being replaced by reasonable and pollution-free enzymatic reactions. Bacteria have been extensively used in bioengineering, but endophytic microorganisms have not been fully utilized. Hence, great efforts to expand endophyte resources could bring us a diversity of benefits, such as novel and effective bioactive compounds that cannot be synthesized by chemical reactions. It is noteworthy that, similar to the secondary metabolites produced by host plants, endophytes can synthesize biologically active substances, after long-term coexistence with hosts. This could help us to gather many important drug compounds such as paclitaxel and camptothecin in limited time (Wang and Dai 2011).

Endophytes are important pool of genetic diversity and a significant resource for the innovation of novel bioactive secondary metabolites. Endophytic microorganisms are a prosperous resource of natural products displaying a broad spectrum of biological activities (Strobel 2003; Tan and Zou 2001; Strobel et al. 2004), and the phytochemistry of endophytes continues to augment in importance. The reported natural products from endophytic microbes comprise antibiotics, anti-pathogens, immunosuppressants, anticancer compounds, antioxidant agents, and other biologically active substances. Bioactive natural products maintain an enormous impact on current medicine, in spite of a focused interest on synthetic products. Around 60% of the new drugs registered during 1981–2002 by the FDA as anticancer, antimigraine, and antihypertensive agents are either natural products or based on natural products (Newman et al. 2003).

There are several applications of endophytes for the production of novel metabolites. First, because of over-collection of wild plants, natural sources of traditional medicines are in short supply. It has been reported that many endophytes could produce substances of potential use to modern medicine, including gentiopicrin, taxol, cryptocin, pentaketide alkaloids, etc. (Yin et al. 2009; Gangadevi and Muthumary 2008; Li et al. 2000; Brady and Clardy 2000; Barros and Rodrigues 2005). Improving existing drugs by modifying them with endophytes is another way to exploit novel metabolites. For example, camptothecin is a potent antineoplastic agent, but it is compromised in therapeutic applications due to its very low solubility in aqueous media and high toxicity. An endophytic fungus from *Camptotheca acuminata* produces camptothecin (i), 9-methoxycamptothecin (ii), and 10- hydroxycamptothecin (iii) (Kusari et al. 2009). Compounds (ii) and (iii) are two important analogues of compound (i) with lower toxicity and potential anticancer efficacy.

As a result of gene recombination through the evolutionary process, endophytes have developed the biochemical ability to generate compounds similar or identical to those produced by their host plants. Bioactive natural products from endophytes have huge prospective as the resource of novel medicinal and agricultural products and methods to assist the identity of appropriate natural products from this source are required. This aspect adds further weight to the conservation of plant biodiversity and greater organization in the collection and cataloguing of endophytic microorganisms worldwide (Zhang et al. 2006).

Recent progress in the molecular biology of secondary metabolites offers a better insight into how the genes for these bioactive compounds of endophytic microorganisms are organized. There has been an improved understanding of biosynthetic pathways to some bioactive endophytic compounds by chemical and biochemical means (Young et al. 1998). Endophytes are relatively poorly investigated group of microorganisms. The association between endophytes and their hosts merits improved quantitative analysis, particularly at the molecular and genetic levels. The cloning of the genes of endophytic metabolites has opened up attractive screening possibilities for the direct identification of endophytic strains (Wang et al. 2004; Haarmann et al. 2005).

14.11 Conclusion

Endophytes play an important role in plant health and also help mankind by providing industrial and pharmaceutical applications. The research conducted on the diversity and potential of endophytes has tapped just 1% of the total endophytic population present in the environment. They untapped resources that need further investigation and deep understanding of the underlying mechanisms in plant growth promotion, disease control, and their effective utilization in agriculture. The commercialization of endophytes as bio-fertilizers, herbicides, and biocontrol agents has to be taken a step further to achieve sustainable agriculture.

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Chapter 15
The Role of Fungi and Genes for the Removal of Environmental Contaminants from Water/Wastewater Treatment Plants

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15.1 Fungal Classification

Globally fungi are generally categorized as microfungi and macrofungi according to their fruiting bodies size. The fruiting bodies of macrofungi are visible to the naked eye, due to its diameter of 1 mm (e.g., mushrooms). Furthermore the microfungi cannot be seen with naked eye due to its microscopic fruiting bodies (e.g., *Penicillium*). Reproduction takes place through spore formation. Most fungal spores are different in color and shape, and their size ranges from 2 to 20 lm [22]. Fungi are more basically categorized by their type of reproduction (both asexual and sexual) and the nature of their multicellular or multinucleate hyphal filaments. Traditionally, true fungi are categorized into five taxonomic divisions. The characteristics of each division are given in Table 15.1.

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Table 15.1 Physical characteristics of the most important fungal divisions

Sl. no.	Physical characteristics	Division
1	Common basidiomycetes include mushrooms, puffballs, and toadstools. Visible features of the fungi are the propagative structures. Sexual reproduction includes the basidiospore development on club-shaped cells recognize as basidia	*Basidiomycota*
2	Comprise more than 32,000 species of unicellular (yeasts) to multicellular fungi. Asexual reproduction in yeasts takes place by budding while sexual by forming a sac/ascus	*Ascomycota*
3	Fungi in this division produce zoospores proficient for movement via a fluid medium by means of simple flagella	*Chytridiomycota*
4	Fungi included in this group also lack the main stage (i.e., sexual reproduction) or whose perfect stage is as yet undiscovered. Reproduction most frequently takes place by conidia or conidia-like spores. Various forms of this division are pathogenic in nature affecting humans, wildlife, or floras	*Deuteromycota*
5	In *Zygomycota* the hyphae have long multinucleate cell rather than one nucleus per cell, haploid hyphae that consist of their mycelia. Asexual reproduction takes place by means of spores produced in stalked sporangia	*Zygomycota*

15.2 Water/Wastewater Contamination

The continuous seeking of human toward industrialization and comfort life is leading to the environmental contamination and consequently deterioration of human health.

15.3 Role of Fungi in Wastewater Treatment

Diverse group of microorganisms are present in wastewaters, which become scattered in different phases of the sludge and wastewater management procedure at wastewater treatment plants (WWTPs) (Teixeira et al. 2013). Among the microorganisms, filamentous fungi were detected in sewage and air at WWTPs formerly (Korzeniewskaa et al. 2009). Fungi are eukaryotic organisms which are different in shape and size (Gravesen et al. 1994). They are unicellular like yeast and most of them are filamentous. Some of these microorganisms live as a saprophyte and grow on dead organic materials, and others are either facultative or obligate parasite. Because fungi are heterotrophic microorganisms, they have the ability to break down organic matters by secreting some degradative enzymes (Tran et al. 2013; Haritash and Kaushik 2009). These enzymes can be produced extracellularly or intracellularly to enhance the absorption of organic molecules into the fungal cell. These organic molecules can be used as both energy and carbon sources for the

growth and division of fungal cells (Sankaran et al. 2010). The degradative enzymes are regulated by various groups of catabolic genes.

Among the major problems that faced the bacterial wastewater treatment plant is the generation of large quantities of sludge which is mainly bacterial biomass (Sankaran et al. 2010). The generated sludge is characterized by low value and it requires high-cost for treatment before disposal. The recent strategy suggested to overcome this problem is to cultivate fungi as a source of different valuable biochemicals. So, integration of valuable sources recovery with wastewater remediation may lead to an economically workable solution for wastes management. From this point of view, the use of fungi in wastewater treatment can be an attractive strategy as fungi can utilize the organic waste as a feed to generate economic fungal byproduct with concomitant wastewater remediation (Sankaran et al. 2010). Filamentous fungi offer a diverse prospect. In food industries several filamentous fungi are often cultivated as a source of valuable products such as biochemical and protein with relatively costly substrates such as molasses or starch (Barbesgaard et al. 1992). Mycological cultivation plays an important role in the conversion of wastewater organic materials into readily harvestable mycological biomass, which is further used as a source of animal food and possibly human diet (Guest and Smith 2002). So the use of filamentous fungi is an effective stategy for treatment of highly contaminated wastewater.

Removal and detoxification of pollutants can be achieved by physical, chemical, or biological means (Ryan et al. 2005). However, a biotechnological approach is widely adopted due to its cost-effectiveness, higher efficiency, and generation of nontoxic value-added products. Thus, the fungal process not only offers a solution to wastewater remediation but also provides an opportunity for byproduct recovery (Fountoulakis et al. 2002). One of the several advantages of fungal process is the enzyme-mediated activity that provides solution to the treatment of waste streams containing hazardous or xenobiotic organic pollutants. The enzymes are produced during all phases of the fungal life cycle and are present even at low pollutant concentrations (Ryan et al. 2005). Fungal biomass secretes specific and nonspecific extracellular enzymes that have attracted the attention of researchers working on degradation of complex high-molecular-mass organic pollutants.

15.4 Heavy Metal/Metal Ion Bioremediation

The main source of heavy metal pollution is industrial wastewaters during metal processing as well as other pollutant routes. Any industrial activity using metals has a metal disposal problem (Das et al., 2008) [1]. Nature of heavy metals is persistent and non-biodegradable; therefore, it is very difficult to purify the environmental compartments (soil and water body) from these toxic pollutants.

Heavy metals can be divided into essential metals such as zinc, iron, manganese, and copper and nonessential metals such as lead, mercury, nickel, and cadmium (Grąz et al., 2011) [2]. Cadmium and lead are included among the major pollutants

because of their high toxicity (Salinas et al., 2000, Blaudez et al., 2000, Carrillo-González and González-Chávez, 2012, Jaeckel et al., 2005) [3–6]. The main reasons for release of cadmium to the environment are mine tailing, effluents from textile, tannery, leather, galvanizing and electroplating industries, as well as cadmium batteries. Biomagnification of cadmium in nature and its migration through drinking water, food, and air to human body cause severe health effects like kidney damage, bronchitis, and cancer (Salinas et al., 2000) [3]. So, there is an urgent need to remove these toxic pollutants from the environmental compartments by using effective remediation methods.

Conventional treatment systems have many disadvantages including insufficient metal sequestration, high reagents and/or energy requirements, high costs, and generation of toxic sludge or other waste products that require disposal. Restoring metals in an efficient and economical procedure has necessitated the use of different options in metal-separating methods. Literatures showed that bioaccumulation of metals by organisms has been successful to some extent (Salinas et al. 2000) [3]. Bioremediation of heavy metals from aqueous environment by bacteria, fungi, algae, and plants is the most promising method for complete and safe removal; it is also a cost-effective strategy.

Fungal cell has a great ability to entrap heavy metal ions into its cell structure and subsequently adsorb it on the binding site that located in the fungal cells structure (Brierley 1990; Gadd 1988). This mode of heavy metal uptake is independent on the viability of fungal cells or biological metabolic cycle and is called biosorption or passive uptake. On the other hand, the process of which the heavy metals pass through the cell via cell membrane and participate in the metabolic cycle is so called active uptake or bioaccumulation (Dönmez and Aksu 1999; Malik 2004). The passive uptake (biosorption) of heavy metals is noneffective method for wastewater treatment because they are limited by many factors that decrease the potential of biosorption such as pH, temperature, and the complete saturation of active sites and functional groups of fungal cell structure with heavy metals. So that, the biosorption mode does not achieve any detoxification of heavy metals (Malik 2004). Under such situation, the application of viable microbial cells is a more favorable option for heavy metal removal due to continuous metabolic uptake of metals after physical absorption. After that, the metals diffused into the cells during detoxification get bound to chelating agents or intracellular proteins before being incorporated into vacuoles and other intracellular sites (Malik 2004; Saunders et al. 2001). These processes are often irreversible and ensure less risk of releasing metal back to the environment (Gekeler et al. 1988). The major limitations of active uptake of heavy metal by fungal cells are the requirement of carbon and energy sources, external supplementation, and sensitivity of some fungal strains to high metal/salt concentration. Table 15.2 summarizes the bioremediation of heavy metals by different fungi species.

The walls of fungal biomasses are composed of macromolecules (chitosan, chitin, glucan, phospholipids, lipid), which comprise amino groups (R_2NH, $R\text{-}NH_2$), carboxyl groups (RCOOH), phosphates, melanin, lipids, hydroxides (OH-), and sulfates ($R\text{-}OSO_3$-) (Kapoor et al. 1999). Those functional groups are metal sorption

Table 15.2 Fungi used for heavy metal removal from wastewater and aqueous solution

Heavy metals biosorption/removal	Fungi	References
Cadmium, nickel	*Trichoderma atroviride* strain F6	Babich and Stotzky (1977)
Copper, lead	*Aspergillus niger*, *Trichoderma asperellum*, *Penicillium simplicissimum*	Iskandar et al. (2011)
Cobalt	*Trichoderma*, *Aspergillus*, *Mortierella*, *Paecilomyces*, *Penicillium*, *Pythium*, and *Rhizopus*	Ross and Townsley (1986)
Zinc, lead	*T. harzianum*, *F. phyllophilum*	Ozsoyet al (2008)
Arsenic	*Trichoderma* (FA-06)	Ashida (1965)
Copper, zinc, cadmium	*Trichoderma atroviride*	Tsekova and Todorova (2002)
Zinc	*Trichoderma atroviride*	Yazdani et al. (2010)
Zinc, barium, iron	*Trichoderma atroviride*, *Mortierella exigua*	Karcprzak and Malina (2005)
Copper	*Trichoderma viride*, *Aspergillus niger*, *Penicillium spinulosum*	Delgado et al. (1998), Anand et al. (2006)
Copper, zinc, cadmium	*Trichoderma atroviride*	López Errasquín and Vázquez (2003)
Lead and cadmium	*Aspergillus niger*, *Penicillium*, *Alternaria*, *Rhizopus*, *Monilia*, *Trichoderma*	Zafar et al. (2007)
Chromium (VI)	*Rhizopus arrhizus*	Niyogi et al. (1998)
Copper and zinc	*Aspergillus niger*	Price et al. (2001)
Chromium (VI) and iron (III)	*C. vulgaris* and *R. arrhizus*	Sag et al. (1998)
Lead	*Rhizopus nigricans*	Zhang et al. (1998)
Zinc	*Rhizopus arrhizus*	Zhou (1999)
Copper	*Phanerochaete chrysosporium*	Sing and Yu (1998)
Copper	*Aspergillus niger*	Modak et al. (1996)
Lead	*Aspergillus niger* (strain 4)	Meyer and Wallis (1997)
Lead (II)	*Phellinus badius*	Matheickal and Yu (1997)
Heavy metals	*Aspergillus niger*	Kapoor and Viraraghavan (1998a, b)
Gold and silver	*Aspergillus niger*	Gomes et al. (1998)

sites (Kapoor and Viraraghavan 1997; Javanbakht et al., 2014). Moreover fungi eliminate metals principally by chemisorption (ion exchange), adsorption, complexation, micro-precipitation, chelation, physical adsorption, and coordination (Kapoor and Viraraghavan 1997; Long et al., 2019).

In some filamentous fungi, there are many strategies to overcome the toxicity of heavy metals:

1. Production of oxalate by brown-rot and white-rot fungi. The oxalate secretion process is stimulated under Cu(II) and Cd(II) stress (Clausen and Green 2003; Jarosz-Wilkolazka and Gadd 2003) and leads to the formation of insoluble

metal-oxalate crystals that is thought to prevent toxic metal ions from entering fungal cells (Jarosz-Wilkolazka and Gadd 2003).

2. Production of extracellular mucilaginous materials (ECMM) or bio-emulsifiers with high metal-binding capabilities. Cu(II), Pb(II), and Zn(II) could trigger ECMM production by *Curvularia lunata* (Paraszkiewicz et al. 2009, 2010). Importantly, the pullulan production by *Aureobasidium pullulans* was stimulated by Ni(II) and Cd(II) exposures (Breierová et al. 2004). Additionally, the ratio of ECMM in the *Trametes versicolor* and *Gloeophyllum trabeum* biomass increased when they are exposed to Cu(II) (Vesentini et al. 2006).
3. Production of a soil glycoprotein called glomalin by the arbuscular mycorrhizal fungi *Glomus* and *Gigaspora* species (Wright et al. 1996), which possess a remarkable capability to sequester Cu(II) (Gonzalez-Chavez et al. 2004). Also, chitin and melanin can also take part in metal biosorption (Gonzalez-Chavez et al. 2004).

Glutathione GSH plays a critical role in fungal heavy metal tolerance and oxidative stress defense as well (Jozefczak et al. 2012; Bellion et al. 2006); however, the overexpression of GSH increases the toxic metal/metalloid tolerance (Pócsi 2011). The transgenic plants *Arabidopsis thaliana* could accumulate and tolerate Cd and As when they are stimulated by yeast γ-glutamylcysteine synthetase GSH1 and garlic phytochelatin synthase AsPCS1 (Guo et al. 2008). It is worth noting that recombinant GSH overproducing yeast strains have also been engineered using self-cloning modules containing an intracellular expression vector with GSH1 and GSH2 biosynthesis genes in *Pichia pastoris* (Fei et al. 2009) or the GSH1 gene in *Saccharomyces cerevisiae* (Pócsi 2011; Wang et al. 2009). This approach may be limited particularly with Hg because the overexpression of Hgt1p GSH transporter in *S. cerevisiae* leads to elevation of the intracellular GSH level and subsequently results in the induction of cell toxicity (Pócsi 2011).

The stimulation and overexpression of phytochelatin into the fungal cell could enhance the fungal strain to be more tolerance to metal ion. In addition the heterologous expression of *Saccharomyces pombe*, red alga, higher plant, or nematode phytochelatin synthases provides Cu(II), Cd(II), Sb(III), and As(III) resistance to *S. cerevisiae* (Osaki et al. 2008).

It was mentioned that the low molecular mass metal chelator proteins that are termed metallothioneins exhibit a great affinity toward Zn(II), Cd(II), as well as Cu(II) (Wysocki and Tamás 2010). Furthermore, the overexpression of metallothionein PiMT1 gene in *Piciai volutus* complemented the Cd(II) and Cu(II) hypersensitivity of metallothionein-deficient yeast strains and even enhance the Cu(II) tolerance of the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Bellion et al. 2007).

Cu/Zn-superoxide dismutase (Cu/Zn-SOD) plays an important role in the improvement of metal and oxidative stress tolerance of fungi. This enzyme is mainly expressed by Sod1p gene with co-expressed with the Cu(II)-chaperone Ccs1p (Ferreira et al. 2014). In the absence of either Ccs1p overexpression or high-dose Cu(II) supplementation, the cells showed symptoms of emerging oxidative stress

and shortened chronological life span (Harris et al. 2005). The genetically engineered deep-sea yeast *Cryptococcus liquefaciens* strain N6 exhibited fourfold higher activity compared to Sod1p of baker's yeast *S. cerevisiae* when it was cloned with Cu/Zn-superoxide dismutase (Kanamasa et al. 2007).

In filamentous fungi, intracellular siderophores play a critical role in keeping excess of iron in a thermodynamically inert state (Pócsi 2011). In baker's yeast, iron homeostasis is regulated by the Aft1p and Aft2p transcriptional activators (Pócsi 2011; Johnson 2008).

It was supposed that the major metal/metalloid stress response regulator of baker's yeast when it was exposed to Cd(II), As(III), Sb(III), Se(III), and Hg is the bZIP-typ transcriptional factor Yap1p as well as Yap2p (Wysocki and Tamás 2010; Hirata et al. 1994). According to Azevedo et al. (2007), Yap1p and Yap2p transcription factors share a common Cd(II)-sensing domain. Considering other bZIPs, Yap5p is involved in the regulation of the Fe homeostasis via the regulation of CCC1 encoding the vacuolar iron transporter Ccc1p (Li et al. 2008), and Yap8p plays a pivotal role in the regulation of As(III) detoxification (Haugen et al. 2004; Wysocki et al. 2004).

15.5 Hydrocarbon Bioremediation

Petroleum is a natural resource confined in large deposits in the Earth crust. Accidental petroleum spills alter the impacted environment and trigger the development and implementation of remediation strategies for cleaning up the polluted sites. Oil spills became an international concern in 1967, when ~120,000 tons of crude oil was released by the Torrey Canyon supertanker into the English Channel. This first large-scale oil spill forced UNO's International Maritime Organization to create in 1973 the International Convention for the Prevention of Pollution from Ships MARPOL with the aim of designing emergency protocols and strategies toward oil spills. Since then, there have been a number of significant marine oil spills, even only the emblematic spills usually alert the public opinion. Oil spills are difficult to avoid during petroleum processing and delivery. So, the contamination of water with petroleum hydrocarbon became a serious problem that threaten the biological live.

Petroleum is composed by three main hydrocarbon fractions. Paraffin is usually the most abundant fraction and contains linear and branched aliphatic hydrocarbons. Naphthenes are alicyclic hydrocarbons composed by one or more saturated rings with or without lateral aliphatic branches. The aromatic fraction is composed by hydrocarbons containing at least one aromatic ring. Hydrocarbons can possess from few up to >60 carbons. A higher molecule size correlates with a higher boiling point. Petroleum-derived products are obtained by fractional distillation, by which different fractions are enriched according to its boiling range (Speight 2015).

Many fungal species are known to have the ability to degrade persistent pollutants (Hesham et al. 2017). The majority of studies have been focused on the on

biodegradation of hydrocarbons by white-rot fungi (Hesham et al. 2017; Lee et al. 2014). Fungi have a diversity of tactics to counter with numerous contaminated composites such as persistent organic pollutants (POPs) including polycyclic aromatic hydrocarbons (PAHs) and pesticides (Herzig et al., 2019). These procedures comprise enzymatic practices such biomineralization and bioadsorption as well as biodegradation and biotransformation facilitated by enzymatic structures (Nunes and Malmlöf, 2018). The specific structure of the cell wall such as chitosan or chitin is mediated by bioadsorption (Gadd 2009). In some species of fungi, including *Phoma* sp. UHH 5-1-03, biosorption addicted to fungal mycelia plays a significant role in the removal of 17α-ethinylestradiol, bisphenol A, and triclosan, until reach to the equilibrium (Hofmann and Schlosser 2016).

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of two or more fused benzene rings. These compounds are widely distributed in the environment and formed during the combustion of organic molecules (Kim et al. 2013). PAHs discharged from various activities, such as combustion of fossil fuels including coal, shipping, use and disposal of petroleum products, agricultural burning, and use of wood-preserving products, are persistently hazardous pollutants to the environment (Hadibarata and Teh 2014). Through the industrial wastewater discharges, PAHs are distributed in the marine environment and finally bind to particulate matter of the sediments (Abdel-Shafy and Mansour 2016). Low-molecular-weight (LMW) PAHs (composed by two to three aromatic rings) are predominant in petrogenic sources and can be introduced into water through municipal and urban runoff and oil spills, discharge from tanker operations, etc. High-molecular-weight (HMW) PAHs (composed by four to six aromatic rings), such as pyrene and BaP, are most important in pyrogenic sources and are introduced to the aquatic environment mainly in the form of exhaust and solid residues. These two types of PAH compounds are classified as priority pollutants according to the US Environmental Protection Agency (EPA) and have been accumulating in sediments due to their limited water solubility and high affinity for particulate matter (Cai et al. 2009; Kim et al. 2013).

Once petroleum hydrocarbons reach the aquatic environment, damage can be the result of several causes. Primary biological impact is due to the blocking effect of oil layer to water, nutrients, oxygen, and light access that lead to death of aquatic flora and fauna. Cytotoxic and mutagenic effects of hydrocarbons are behind the long-term pollution consequences (Baboshin and Golovleva 2012).

The main hydrocarbon degraders, are microorganisms which include bacteria, filamentous fungi, and yeasts (van Beilen and Funshoff 2007; Wentzel et al. 2007), are used to overcome low bioavailability of PAHs.

Microorganisms possess evolved mechanisms to activate hydrocarbons, generating metabolic intermediates that funnel to central metabolic pathways. By oxidizing these substrates, microorganisms can take advantage in nutrient-limited niches. Addition of one or two hydroxyl groups to the hydrocarbon skeleton seems to be the ubiquitous first step during aerobic catabolism (Fig. 15.1). So, microbial degradation of hydrocarbons by bacteria, filamentous fungi, and yeasts can be considered as an attractive biotechnological alternative for achieving possible mineralization of

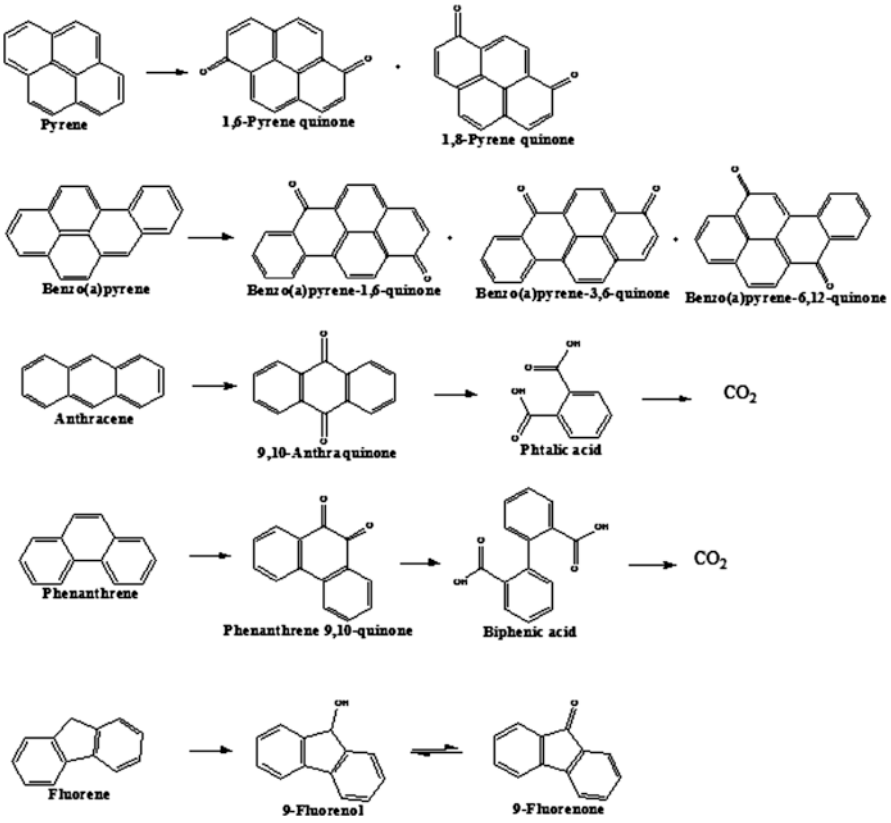

Fig. 15.1 Degradation of polycyclic aromatic hydrocarbons by ligninolytic fungi (Kadri et al. 2017)

pollutant 12 and its transformation into less toxic products with greater solubility in water (Lee et al. 2014; Fuentes et al. 2014).

Many fungi metabolize polycyclic aromatic hydrocarbons with enzymes that include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450, and epoxide hydrolase. The products include trans-dihydrodiols, phenols, quinones, dihydrodiol epoxides, and tetraols, which may be conjugated to form glucuronides, glucosides, xylosides, and sulfates. The fungal metabolites generally are less toxic than the parent hydrocarbons. Cultures of fungi that degrade polycyclic aromatic hydrocarbons may be useful for bioremediation of contaminated soils, sediments, and waters. The following white-rot fungi are the most extensively studied PAH degraders: *Phanerochaete chrysosporium* (Barclay et al. 1995; Brodkorb and Legge 1992), *Pleurotus ostreatus* (Vyas et al. 1994), and *Trametes versicolor* (Vyas et al. 1994; Boonchan et al. 2000). These fungi are able to degrade some five-benzene-ring PAHs and detoxify PAH-polluted soils and sediments due to the production of extracellular lignin-degrading enzymes. Figure 15.1 shows Oxidation of polycyclic

aromatic hydrocarbons by ligninolytic fungi. Nonlignolytic fungi, such as *Cunninghamella elegans*, *Penicillium janthinellum*, and *Syncephalastrum* sp., can transform a variety of PAHs, including pyrene, chrysene, and benzo[a]pyrene, to polar metabolites (Kadri et al. 2017).

The key enzymes in hydrocarbon degradation pathways are oxygenases, which catalyze the addition of molecular oxygen to the substrate (Fuentes et al. 2014). Monooxygenase- and dioxygenase-encoding genes play a key role in hydrocarbon biodegradation by fungi and bacteria. These genes are characterized by their wide phylogenetic distribution as well as high sequence divergence (Iwai et al. 2011; Fuentes et al. 2014); these genes become overexpressed after input of hydrocarbons in the medium or environment. The expression of different ring hydroxylating dioxygenase (RHD) genes increased in the presence of polycyclic aromatic hydrocarbons (PAHs) (Fuentes et al. 2014).

Levels of RHD genes changed significantly in environment during bioremediation and after addition of aromatic hydrocarbons. Additionally, alkane monooxygenase (alk)-encoding genes showed different dynamics at soil and seawater (Yergeau et al. 2012) where alkB gene copy number increased up to 100-fold in less than 1 week after pollution (Sei et al. 2003). Genes encoding enzymes catalyzing downstream reactions seem to behave in a similar way as RHD genes (Hesham et al. 2014). For example, levels of catechol 2,3-dioxygenase *xylE* gene from (methyl) toluene degradation pathway correlate with degradation rates in hydrocarbon-polluted environment. A positive correlation between hydrocarbon degradation rate and functional *alkB*, *xylE*, and *nahAc* gene abundance was observed (Salminen et al. 2008). Therefore, catabolic gene quantification can be an adequate approach for monitoring bioremediation processes (Fuentes et al. 2014).

PAHs could be degraded by fungi with cytochrome P450 (P450) monooxygenases and with soluble extracellular enzymes such as manganese peroxidase, lignin peroxidase, and laccase (Peng et al. 2008). Of these, the CYP52, CYP53, and CYP504 P450s, known to be involved in hydrocarbon degradation (Črešnar and Petrič 2011), had higher relative abundances in cliff sample than river water one. Fungal manganese peroxidases and laccases were detected with similar abundances in both metagenomes (Črešnar and Petrič 2011). Under anoxic conditions aromatic compounds are metabolized through alternate pathways, including fumarate addition,O_2-independent hydroxylation, and carboxylation. Genes for benzylsuccinate synthase (bssABCDE), ethylbenzene dehydrogenase (EB_dh), ATP-dependent class I benzoyl coenzyme A (CoA) reductase (brcABCD), and ATP-independent class II benzoyl-CoA reductase (bamBCDEFGHI) were found in HR_M at low frequencies but not in cliff (Wong et al. 2015).

Hydrocarbon degradation pathways expand the microbial metabolic versatility and the carbon source range for growth. In alkane and aliphatic hydrocarbon degradation, successive oxidations produce carboxylic acids that can be degraded by the β-oxidation pathway. In PAH degradation, metabolic intermediates are channeled into central aromatic routes such as catechol, gentisate, and protocatechuate pathways.

15.6 Antibiotics

Antibiotics are used globally instead of their conventional use in medicine. Antibiotics are commonly used in research experiments, genetic engineering, crop production, aqua culture, animal breeding, and fish farming (Dietze et al. 2005; Yanong 2006). Since the extensive usage of antibiotics, the microorganisms present in the waste have a good opportunity for developing resistance to the antibiotics. Due to the unsuccessful handling, or inappropriate disposal, antibiotics are released into the aquatic ecosystem via wastewater discharge, and as a result sulfamethoxazole, tetracycline, sulfamethazine, trimethoprim, ciprofloxacin, and erythromycin have been noticed in numerous wastewater treatment plants (WWTPs) which release their treated wastes to both ground and surface waters (Karthikeyan and Meyer 2006). Wastewater treatment plants (WWTPs) are one of the major hotspots for spreading antibiotic-resistant microorganisms (Baquero et al. 2008; Manaia et al. 2011). During the biological treatment of wastewater that effluent from variuos sources, the continuous contacting between bacteria and antibiotics (even at very low concentration) leads to increase and spread of antibiotic resistance (Da Silva et al. 2005; García-Galán et al. 2011; Lucas et al. 2016). Antibiotics resistance genes(ARGs) are considered the danger that threaten the public health within the twenty-first century as mentioned by the World Health Organization (WHO) (2014). Few articles have discussed the removal of antibiotics and antibiotics resistance genes (ARGs) from the environment (Gao et al. 2012; Lucas et al. 2016; Xu et al. 2015; Rodriguez-Mozaz et al. 2015).

Five ARGs, bla_{TEM} (resistance to β-lactams), *qnrS* (reduced susceptibility to fluoroquinolones), *ermB* (resistance to macrolides), *sulI* (resistance to sulfonamides), and *tetW* (resistance to tetracyclines), were detected and quantified using qPCR assays in wastewater samples (Lucas et al. 2016). The treatment of these samples with fungal strain *Trametes versicolor* ATCC 42530 resulted in decreasing the copy number and the expression of these. It had been showed that the *ermB* and *tetW* genes completely disappeared both after treatment with *Trametes versicolor* ATCC 42530 and in the non-inoculated control bioreactor (Gao et al. 2012; Rodriguez-Mozaz et al. 2015).

The copy number of bla_{TEM}gene exhibited a marked decrease during fungal treatment (Lucas et al. 2016). On the other hand, the copy numbers of bla_{SHV} and *sulI* genes, increased by thousandfold and tenfold, respectively in both fungal and control bioreactors; however, the increase was significantly lower ($p < 0.05$) in the fungal bioreactor than in the control bioreactor.

There is a correlation between the copy number of antibiotic-resistant genes and the occurrence of antibiotics in the environment (Lucas et al. 2016). The concentration of *ermB* gene decreased by three orders of magnitude in the presence of macrolides (even higher concentrations) (Lucas et al. 2016).

The tetW gene disappeared totally in both bioreactors, even though tetracycline antibiotics were hardly removed along the treatment (29% and 26% removal in the fungal and the control bioreactors, respectively). The concentration of this gene has

also been reported to decrease by three or four orders of magnitude in presence of small amounts of tetracycline antibiotics.

The concentration of b-lactam antibiotics in raw wastewater was quite high (c.a.10 mg L^1) although removal in both bioreactors was very efficient, reaching values close to zero. Nevertheless, levels of bla_{TEM} and bla_{SHV} along the treatment were quite different. In the fungal bioreactor, the bla_{TEM} gene also disappeared (100% removal), in agreement with previous studies (Rodriguez-Mozaz et al. 2015), whereas ARG concentration in control bioreactor did not undergo noteworthy change after treatment. In contrast, the bla_{SHV} gene increased in both bioreactors almost to the same extent, in agreement with the assumption that ARGs increase is favored by the presence of selective agents, such as antibiotics (Allen et al. 2010). The hypothesis here is that despite the decrease in the concentration of b-lactams in both bioreactors, the concentration was high enough to exert a selective pressure; however further studies are required to understand the relationship between the evolution of the bla_{TEM} and bla_{SHV} genes and the concentration of b-lactam antibiotics, including the exposure to sub-therapeutic concentrations.

The concentration of sulfonamides and the sulI gene increased in both bioreactors, whereas in another study in an urban WWTP (Lucas et al. 2016; Rodriguez-Mozaz et al. 2015), both antibiotics and the gene decreased their concentrations. These positive correlations between the gene and antibiotics are in line with the classical knowledge about the emergence of antibiotic resistance (Allen et al. 2010).

The relationship between the *qnrS* gene and quinolones showed a similar trend to that found between the bla_{SHV} gene and b-lactams. An increase of the *qnrS* genes was observed, whereas the antibiotic decreased. Quinolones are the most abundant group in wastewater, and therefore, despite their depletion, they may exert enough selective pressure to increase the gene concentration. Some studies have also suggested that qnr genes may have other functions (e.g., regulation of cellular DNA-binding proteins) in addition to the antibiotic resistance that contribute to its spread (Wang et al. 2004).

15.7 Trace Organic Contaminants (TrOCs)

Trace organic contaminants (TrOCs) include diverse groups of chemicals such as pharmaceuticals and personal care products (PPCPs), surfactants, pesticides, and industrial chemicals. Due to their ineffective removal by conventional wastewater treatment processes, these TrOCs commonly existed in the aquatic environment including groundwater and surface water and even seawater (Sui et al. 2015). The majority of these compounds is biologically active even at trace concentrations (in the range of few ng/L) and can impose detrimental impacts on aquatic environment as well as on human health (Gavrilescu et al., 2015; Pal et al., 2014) [4, 5]. Synthetic hormones can induce endocrine-disrupting effects on aquatic lives (Vandenberg et al., 2012; Chen and Ying, 2015) [6, 7]. Excessive exposure to a nonlethal dose of antibiotics may result in the development of antibiotic-resistant genes in bacteria

which became an emerging concern for human health according to the World Health Organization (de García et al., 2013; Camargo et al., 2014; Rizzo et al., 2013; Nazaret and Aminov, 2014) [8–11]. Bioremediation processes are environmentally friendly and cost-effective (Zhang et al., 2011; Benner et al., 2013; Hai et al., 2007; Hai et al., 2014b) [12–14]. The conventional activated sludge and membrane bioreactor processes can efficiently remove bulk organics, nutrients, and pathogens. However, certain groups of TrOCs such as pharmaceutical and personal care products (PPCPs) with strong electron-withdrawing functional groups are poorly removed by the conventional microbial treatment processes (Alturki, 2013; Hai et al., 2014a; Hai et al., 2014b) [2, 15–17]. Thus, effective treatment strategy to remove TrOCs from wastewater is urgently needed.

Pharmaceutical and personal care products (PPCPs) represent a wide group of TrOCs used for human and veterinary medicine and as fragrances in perfumes and other household products. These types of compounds are considered to be emerging pollutants due to their recalcitrant nature (Kosjek et al. 2007; Ternes et al. 2006). Previous studies detected PPCP concentrations in the environment in the range of nanograms per liter (ng/l) to micrograms per liter (lg/l) (Suárez et al. 2008).

Several treatments have been proposed for PPCP removal. Conventional physicochemical processes, advanced oxidation processes (AOPs), membrane filtration, and activated carbon demonstrated remarkable removal efficiencies for degradation of certain compounds, including tranquilizers, fragrances, and anti-inflammatory drugs, whereas other compounds, such as anti-epileptics, showed less efficient degradation (Ikehata et al. 2006). An emerging technology for the effective degradation of PPCPs involves the application of white-rot fungi. These microorganisms are capable of degrading lignin and several persistent pollutants. This ability is related to the secretion of oxidative enzymes, such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Marco-Urrea et al. 2009). Specifically for ligninolytic fungi, Marco-Urrea et al. (2009) studied the ability of four white-rot fungi, *Trametes versicolor*, *Irpex lacteus*, *Ganoderma lucidum*, and *Phanerochaete chrysosporium*, to degrade carbamazepine, ibuprofen, and clofibric acid. This study demonstrated that after 7 days of incubation, ibuprofen was degraded by all four strains, while carbamazepine and clofibric acid were much more recalcitrant; only T. versicolor attained significant degradation of both of these compounds. The use of oxidative enzymes to oxidize PPCPs in vitro has been demonstrated to remove the estrogenic activities from genistein, bisphenol A, nonylphenol, estrone (E1), 17b-estradiol (E2), estriol (E3), and ethinyl estradiol (EE2) (Cabana et al. 2007).

Extracellular enzymes of white-rot fungi (WRF) are characterized by their capacity to degrade the complex structure of lignin, WRF, and lignin-modifying enzymes which have been investigated recently for the degradation of a broad spectrum of PPCPs (Cruz-Morató et al., 2013; Yang et al., 2013a; Nguyen et al., 2013) [24–27]. The potential of WRF for the removal of PPCPs has been investigated mostly in batch mode. There are only a few studies on continuous flow reactor configurations (Rodriguez Porcel et al., 2007; Cruz-Morató et al., 2013; Yang et al., 2013a; Modin et al., 2014; Hai et al., 2013) [24, 26, 28–30].

Three different types of extracellular lignin-modifying enzymes, namely, lignin peroxidases (LiPs), laccase, and manganese-dependent peroxidases (MnPs), are secreted by WRF. The main difference between laccase and peroxidases is the electron acceptor where oxygen and hydrogen peroxide are the respective electron acceptors (Lundell et al., 2010; Guillén et al., 2000) [38, 39]. Not every WRF species produces all three enzymes, and combination of major lignin-modifying enzymes varies from one WRF species to another. Even the secretion pattern of enzymes varies within a WRF species. For instance, different strains of *Trametes versicolor* has been reported to secrete all three enzymes, but laccase is the main enzyme secreted by the strain ATCC 7731 (Bending et al., 2002; Yang et al., 2013b; Nguyen et al., 2014) [40–42]. In addition, composition of growth medium and culture conditions can influence the secretion of a specific enzyme. Degradation of some pollutants such as phenolic compounds, peptides, and organic acids by WRF may result in the formation of low-molecular-weight mediators which can enhance the spectrum of compounds degraded by WRF (Marco-Urrea et al., 2010; Pointing, 2001; Guillén et al., 2000) [37, 39, 43]. Based on the secretion patterns of enzymes, WRF can be categorized as (Hatakka, 1994) [44] (i) MnP-laccase group such as *T. versicolor*, *Pleurotus ostreatus*, *Dichomitus squalens*, and *Panus tigrinus*; (ii) LiP-laccase group such as *Phlebia ochraceofulva*; and (iii) LiP-MnP group such as *Phanerochaete chrysosporium*. In addition to the extracellular enzymes, intercellular enzymes may play an important role in the degradation of xenobiotics. Intracellular cytochrome P450 enzyme system has been observed to play a vital role in the degradation of some PPCPs such as chlorinated hydrocarbons and polycyclic aromatic hydrocarbons (Golan-Rozen et al., 2011; Marco-Urrea et al., 2006; Marco-Urrea et al., 2008) [45–47]. Cytochrome P450 is a group of monooxygenases which can degrade PPCPs by catalyzing a number of reactions such as heteroatom oxygenation, dehalogenation, and hydroxylation (Bernhardt, 2006) [48].

15.8 Bioremediation of Synthetic Dyes

Large volume of water and chemicals are used in textile industries during wet processing. Diverse chemical substances are used in different composition, extending from inorganic composites to polymers and organic products (Banat et al. 1996). The occurrence of dyes in very low concentrations in wastewater is extremely visible and objectionable (Nigam et al. 2000). Commercially more than 100,000 dyes are available globally with over 7×10^5 ton of dye stuff being produced yearly (Meyer 1981; Zollinger 1987). Moreover dyes are resistant to be degraded when they exposed to water, light, and numerous chemicals due to their chemical structure and composition (Puvaneswari et al., 2006). Different types of fungi belonging to *Ascomycota* and *Basidiomycota* were isolated from wastewater of textile dye plant. *Ascomycota* contain fungal strains, e.g., *Verticillium*, *Colletotrichum*, *Fusarium*, and *Paecilomyces variotii*, with potent dye-degrading enzymes such as manganese-dependent peroxidases, ligninases, and laccases (Shanmugapriya et al.,

2019). Recently, the laccases have attracted substantial interest for biotechnological solutions (Theerachat et al., 2019). *Fusarium oxysporum* utilize the β-ketoadipate pathway to degrade aromatic compounds (Porri et al., 2011). Also, we detected three members of *Tremellaceae* known to carry enzymes, e.g., laccases, particularly suitable for degradation of aromatic compounds (Puvaneswari et al., 2006; Theerachat et al., 2019). Furthermore, *Basidiomycota* include several white-rot fungi, such as *Bjerkandera*, *Trametes versicolor*, and *Pleurotus ostreatus*, which have displayed a capability to degrade different dyes (Anastasi et al., 2010). Some species also produce several types of lignolytic enzymes such as laccase, lignin peroxidase, and manganese peroxidase used in various applications in industry today to degrade pulp, dyes, and other xenobiotics (Theerachat et al., 2019). It can be noted that the white-rot fungi group carries several ligninolytic enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, and laccase. We note that these enzymes degrade phenolic and aromatic substrates through radical reactions with H_2O_2 under aerobic conditions (Shanmugapriya et al., 2019). Moreover these enzymes are also used for the degradation of lignin (Shanmugapriya et al., 2019). Among the dyes the major class of commercially produced azo dyes are not freely degraded by microorganisms, but these can be degraded by *P. chrysosporium* (Paszczynski and Crawford 1995). Further fungi such as *Inonotus hispidus*, *Hirschioporus larincinus*, *Coriolus versicolor*, and *Phlebia tremellosa* have also the ability to decolorize dye-containing effluent (Banat et al. 1996).

Lignin-modifying enzymesLME (MnP, LiP, Lac) are produced in multiple isoforms and encoded by gene families with complex regulation. Nutrient levels, mediator compounds, and required metal ions (Mn^{2+} for MnP, Cu^{2+} for Lac) affect transcription of respective genes. Judicious manipulation of the chemical environment may allow the production of an adequate mixture of LME giving good decolorization without side products; however, this approach is not optimal. Gene amplification and expression in appropriate hosts could be promising for abundant production and affordable price of LME, as is already the case with laccases used commercially in the pulp and paper industry. Further potential benefits of genetically improved LME could be extended to substrate range, catalytic activity, and stability for industrial application of LME (Wesenberg et al. 2003).

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Chapter 16
DNA Barcoding for Species Identification in Genetically Engineered Fungi

Meghna Singh and Neha Singh

16.1 Introduction

Fungi are the most diverse living form on earth. The diverse range of fungi includes molds, yeasts, mushrooms, etc.; all these forms differ in their life cycles, metabolisms, morphogenesis, and ecologies. Around 1.5–5 million species of fungi are present on earth. These versatile organisms are known to survive varied temperature on earth (Choi and Kim 2017). Fungi are heterotrophic in nature; they survive on the decaying matter and absorb the nutrients from their chitin cell wall. Fungi are really important for their products used for biochemicals, plastics, fertilizer, and fuel from biowastes. Other than this, they are also used in the maintenance of crop yields, for production of food ingredients, as antibiotics, and for finding new drug candidates, antimicrobials, and biological medicines (Richards et al. 2017). Earlier mycologists used morphological features as one of the methods to define fungal species. The morphological (phenetic or phenotypic) concept is the classic approach in which units are defined on the basis of morphological characteristics and ideally by the differences among them. There is another polythetic concept that is based on a combination of characters, although each strain does not have the same combination. The ecological concept, which is based on adaptation to particular habitats rather than on reproductive isolation, is often used for plant pathogenic fungi. The biological concept, which was developed before the advent of modern phylogenetic analysis, emphasizes gene exchange (i.e., sexual and parasexual reproduction) within species and the presence of barriers that prevent the cross-breeding of species.

Other than life cycle complexity, fungus also contains complex genomes. The average genome size of fungal species ranges from about 2 to 180 million nucleotides, while the predicted number of proteins coded is 2–35 thousand proteins. Presently, whole genome sequences of around 400 fungal species are available

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publically. The "1000 Fungal Genomes Project" was initiated in 2014 by the Joint Genome Institute of the US Department of Energy conducting a 5-year project to sequence more than 1000 fungal genomes from across the Fungal Tree of Life, filling in gaps and sequencing at least 2 reference genomes from at least 500 recognized families of fungi (Grigoriev et al. 2013). This project has been critical to increase the amount of fungal genomes available online in reference databases. Late in 2017, the scientific milestone of having free access to more than 1000 fungal genomes for public consult and research has been achieved.

The fungus offers a wide range of complex systems and products due to which fungi carry a great potential in terms of their biotechnological use and product discovery. Therefore, it is extremely important to understand more deeply about fungi and their diversity. The role of evolution is really important in describing the patterns exhibited by the "kingdom" fungi. It also helps in decoding the ancestral features associated with primitive fungal forms which may at some point help to harness the potential of fungi (Araujo and Sampaio-Maia 2018).

Fungi are heterotrophic eukaryotes with most members containing thick cell walls made of chitin and cellulose. They play essential roles in nutrient cycling, environmental protection, plant and animal health, and human welfare such as relating to food security and infectious diseases. Similar difficulties and challenges have been found at the molecular level, and, therefore, it was not possible to define a synapomorphy for this "kingdom" (Nagahama et al. 2011; Richards et al. 2017). It is quite difficult to differentiate between various fungal species given the amount of changes that happen during the course of life cycle and re-culturing. Almost complete biodiversity of kingdom fungi is being revealed majorly due to advancements in molecular biology techniques, better genotyping methods, and introduction of whole genome sequencing. Having a rapid and precise identification of fungal specimens to species is critical in many fields including conservation and sustainable use of biodiversity, ecological monitoring, prevention and control of fungal pathogens, quarantine control of exotic species, and human health.

16.2 DNA Barcoding in Genetically Engineered Fungi

DNA barcoding concept provides an effective and broad identification system which is based on sequence diversity in short and well-defined gene regions. The taxonomical tool "DNA barcode" was first proposed by Hebert et al. (2003a). The method uses short DNA sequences or DNA barcode for the biological identification of species. An ideal DNA barcode must possess characteristics of easy retrievable with single primer pair, agreeable to bidirectional sequencing and effective discrimination among species. The basic work process is described in Fig. 16.1. The competent discriminatory power is given by the difference between inter- and intraspecific distances known as "DNA barcoding gap." Various researchers have used DNA barcoding method for the identification of species integrating it with the

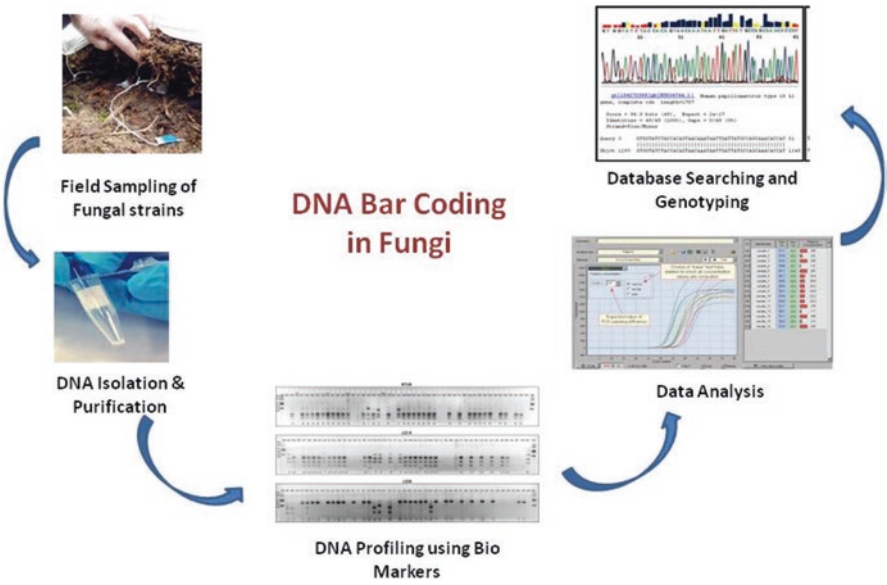

Fig. 16.1 Basic process used for DNA barcoding in fungi

classical taxonomical approach based on molecularization, computerization, and standardization of taxonomy (Kane and Cronk 2008; Newmaster et al. 2008; Casiraghi et al. 2010; Sahare and Srinivasu 2012; Vohra and Khera 2013).

Once the barcoding is done, standard or reference barcodes must be derived from expertly identified vouchers deposited in biological collections with online metadata and validated by available online sequence chromatograms. Interspecific variation should exceed intraspecific variation (the barcode gap), and barcoding is optimal when a sequence is constant and unique to one species (Hebert et al. 2003a). Ideally, the barcode locus would be the same for all kingdoms.

In addition, barcode sequences can be used to identify cryptic species within previously described fungal species and in helping biodiversity studies. The importance of DNA barcoding in fungi is revealed by the fact that fungi were part of the first DNA barcode initiative and conferences in London, UK, 2005. At the 6th International Barcode Conference in Guelph, Ontario, Canada, in 2015, there were 16 presentations on fungal barcoding, covering different groups of fungi from edible mushrooms to human fungal pathogens and from indoor air samples to lichenform fungi (Adamowicz 2015).

In comparison to plants and animals, fungi are often considered as a simple organism. However, they exhibit great diversity in morphology, ecology, and life cycles (Alexopoulos et al. 1996; Ainsworth et al. 2008). Morphologically, fungi can range from microscopic single-celled yeasts to filamentous molds and macroscopic multicellular mushrooms. Ecologically, they can be found in every habitable niche on earth, from the bottom of oceans to the stratosphere, from the equator to the

Arctic and Antarctic regions, and from rivers and lakes to deserts. Reproductively, they can propagate both asexually and (or) sexually in a variety of structures such as conidiophores and mushroom fruiting bodies, often producing sexual and (or) asexual spores that can survive extreme environmental stresses and disperse long distances (Alexopoulos et al. 1996; Ainsworth et al. 2008; Xu et al. 2005). Their extensive variations in size, shape, and cultivability mean that the challenges in studying fungal diversity overlap with many of those faced by researchers in other fields. For example, yeast researchers use similar standards and share the challenges of bacteriologists to obtain viable cultures to describe new species (Kurtzman et al. 2011). Mushroom researchers often document species from dried wild fruiting bodies and face problems comparable with those of plant biologists (e.g., Singer 1986). The very different and varied criterions used by fungal taxonomists make it a more rigorous process to discriminate various species and compare them among different groups of fungi (Alexopoulos et al. 1996; Kurtzman et al. 2011; Singer 1986).

16.3 Molecular Methods Used for DNA Barcoding in Fungi

The fungal DNA barcode is based on the identification of specific markers. There are various methods used for DNA barcoding. The most widely used ones are as follows:

16.3.1 DNA Base Composition

The GC content of any species is measured from melting temperature of the melting curve of DNA. The differences in melting curves are found to be characteristic to various species (Guého et al. 1997). The GC content of known fungal species has been established, and it is found that the difference greater than 2% of GC content in fungi indicates different strains. In case of two ecologically related strains, about 1% difference in GC content is acceptable (Guého et al. 1992).

16.3.2 DNA Hybridization

The DNA-DNA hybridization method compares the speed of heteroduplex formations among the strains. The hybridization greater than 80% signifies same species while less than 20% proves nonidentical. The range in between this indicates the subspecific entities (Xu 2006; Mitchell et al, 1997).

The DNA hybridization-based methods involve multiple steps like selection, cloning, and chemical labeling of sequences specific to the target organism. These sequences are then used as probes to detect RNA or DNA of the pathogen in extracts or tissue squashes of plant material. The process may involve immobilization and detection of nucleic acid on a membrane or utilize a microplate format similar to that used in immunoassays (Umek et al. 2001). The development and use of nucleic acid hybridization assays to detect and identify plant pathogenic fungi have been limited, although species-specific DNA probes have been developed toward several *Fusarium species*, including *F. culmorum*, *F. graminearum*, and *F. avenaceum* (Knoll et al. 2002). However, hybridization is a relatively insensitive method, and there are few instances where this method has been used to detect fungi directly in extracts from plant tissues or other samples. Hybridization assays are sufficiently sensitive for the identification of fungi cultured from plant tissue, but this, of course, incurs the problems associated with selection during isolation and greatly increases the time required to complete the analysis (Mostafa et al. 2012).

16.3.3 AFLP (Amplified Fragment Length Polymorphisms)

AFLP is a powerful method for fingerprinting of fungal strains and for generating a large number of dominant markers for the analysis of genetic crosses (Vos et al. 1995). AFLP is mostly performed in two PCR steps. The first step is the pre-amplification step that uses unlabeled primers with single selective nucleotide in the primer. After the first step, the reaction mixtures are diluted for second PCR amplifications. In the second amplification, additional selective nucleotides are often added to enhance specificity. The selective second step often uses fluorescently or radioactively labeled primers (Xu 2006). In the study of entomopathogenic fungi, the AFLP relies on the initial digestion of the entire genomic DNA with restriction enzymes into fragments of variable size. Specific adaptors are then attached to the sticky ends, and the fragments are amplified by PCR with primer pairs that anneal to the adaptors. The fragment sizes can be scored on polyamide gels, or if they are labeled with fluorescent tags, the fragments can be sized more objectively on a capillary sequencer. This fingerprinting method is more reproducible and has recently been used to characterize genotypes of *Beauveria bassiana* and *Metarhizium anisopliae* (de Muro et al. 2003, 2005; Inglis et al. 2008). The AFLP technique has several powerful advantages over the other methods. Many more fragments can be generated and analyzed in a simple reaction. It can detect restriction site variations as well as insertions and deletions within a genomic region. Different enzymes and/or selective extension nucleotides can be used to create new sets of markers. In addition, the fragments are stable and highly reproducible since they are amplified with two specific primers under stringent conditions (Xu 2006). AFLP has been used to develop a species-specific assay for *A. ochraceus* that did not cross-react with other *Aspergillus* or *Penicillium* species tested (O'Brian et al. 2003).

16.3.4 RFLP (Restriction Fragment Length Polymorphism)

Among all the electrophoretic methods, restriction fragment length polymorphism is very significant for taxonomic studies. It involves digesting DNA samples with a panel of restriction enzymes. The digestion pattern and fragment generation can be tabulated and compared within species. Besides this phenetic trees can also be constructed for distinguishing fungal species. Mitochondrial DNA (mtDNA) comparisons were first used in RFLP for taxonomy typing. Besides mitochondrial DNA the mtSSU rDNA is also used by few researchers. mtDNA is generally indicative of differences somewhat below the species level, but in groups where microspecies are currently distinguished, such as in the dermatophytes, the differences seem to correspond to teleomorph species.

16.3.5 RAPD (Random Amplified Polymorphic DNAs)

RAPD analysis has attracted a lot of attention after its advent during the 1990s (Williams et al. 1990). In RAPD analysis, genomic or template DNA is primed at a low annealing temperature (30–38 °C) with a single short oligonucleotide (ten bases) in the PCR. Multiple PCR products of different electrophoretic mobility are typically generated (Williams et al. 1990). RAPD analysis detects two types of genetic variations: (i) in the length of DNA between the two primer binding sites and (ii) in sequence variation at the priming regions. Nucleotide substitutions in the region of PCR primer binding, particularly at the 3′ ends, can prevent binding of the primer to the DNA template. As a result, this band will be missing in a PCR reaction. Similarities in banding profiles among strains (i.e., the number and mobility, but not the density of the bands) can be calculated and used to infer strain relationships. When multiple primers are screened, RAPD analysis can be very sensitive to detect variation among isolates that cannot be observed using other methods (Xu 2006). RAPD markers have become popular because this PCR technology is relatively easy to implement (Williams et al. 1990) and necessitates small amounts of genomic DNA (Paplomatas 2006). The greater importance of RAPDs to plant pathologists is it can be assayed using very small amounts of fungal biomass, making them an ideal tool for obligate biotrophs such as rusts and mildews. Because a large number of amplicons can be screened in a relatively short period of time, RAPDs are especially useful in differentiating clonal lineages for fungi that reproduce asexually. Furthermore, RAPD data are easy to interpret because they are based on amplification or non-amplification of specific DNA sequences (amplicons), producing a binary dataset that is easy to enter into a spreadsheet for analysis (McDonald et al. 1997). Although technically fast and simple, there are some disadvantages to RAPD. RAPD analysis can detect minute variation among strains because even a single nucleotide mismatch in the priming region may prevent annealing and the absence of a characteristic band on gels. Small differences in any

aspect of PCR conditions that affect binding of the primer may have similar effects; consequently, RAPDs are sensitive to the vagaries of the testing procedure. This problem can be minimized if strains under study are treated identically. When multiple strains are compared, the same PCR buffer, the master mix (includes all four nucleotides, primers, appropriate ions, and DNA polymerase), and the same thermal cycler and PCR running program should be used at the same time (Xu et al. 1999, 2000a). Despite their drawbacks, RAPDs are powerful tools that are especially useful for fungi that are obligate parasites or that have a population structure composed of clonal lineages. PCR-based genetic markers that can detect more than two alleles and that exhibit codominance, such as SCARs and microsatellites (Groppe and Boller 1997), are likely to replace RAPDs as studies of fungal population genetics become more sophisticated. As advanced PCR-based markers become available, it may become possible to amplify specific DNA sequences from soil or root samples and make a direct assessment of the genetic structure of populations of soilborne fungi without first making pure cultures. But if a fungus can be cultured readily on artificial media, then RFLPs offer many advantages for population genetics studies (McDonald et al. 1997). Random amplified polymorphic DNA (RAPD) analysis has been used by a large number of workers to study variability within and between species, including many *Fusarium* species (*F. graminearum*, *F. cerealis*, *F. crookwellense*, *F. venenatum*, *F. torulosum*, *F. sambucinum*, *and F. proliferatum*) (Carter et al. 2002). RAPDs are also applied to the carnation wilt fungal pathogen *Fusarium oxysporum* f. sp. *dianthi*, and they were able to identify specific binding patterns that were subsequently used as probes to distinguish between races of the pathogen (Manulis et al. 1994). In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers (Shi et al. 1995); *Alternaria* species pathogenic to crucifers could be differentiated on the basis of RAPD profiles (Sharma and Tewari 1998).

16.3.6 SSR (Simple Sequence Repeats) and ISSR (Inter-simple Sequence Repeats)

SSRs also known as microsatellites are a powerful tool in taxonomic and population genetics studies. SSR or ISSR techniques originally used to assess genetic diversity of plants or animals are now widely used in measuring fungal diversity (Liang et al. 2005; Guo 2010). Van der Nest et al. (2000) and Geistlinger et al. (2015) developed SSR markers for *F. oxysporum* and *Trichoderma*, respectively, to assess their genetic diversity. Barve et al. (2001) estimated the genetic variations in *F. oxysporum* f. sp. *ciceri* populations using oligonucleotide probes and restriction enzymes. Intraspecific genetic diversity among fungal endophytes of temperate pasture grasses was determined by developing SSR markers. Earlier, nuclear microsatellite markers for *Epichloë* sp. have been developed by Moon et al. (1999). De Jong et al. (2003) studied EST-derived simple sequence repeat (SSR) markers for pasture grass

endophytes which revealed high level of polymorphism between *Neotyphodium* and *Epichloë* sp. and low level of polymorphism within *Neotyphodium coenophialum* and *N. lolii*. To compare population dynamics between *E. festucae* and its host plant *F. rubra*, microsatellite markers have been developed (Crautlein et al. 2014). ISSR markers are effective tools for studying population genetic characteristics and microbial diversity. Endophytic fungi *Phialocephala fortinii* and a nonsporulating mycelium having the same allozyme phenotypes were differentiated on the basis of ISSR analysis (Grunig et al. 2001; Guo 2010). ISSR markers are effective tools for studying population genetic characteristics and microbial diversity. SSR markers have also been used to estimate the relationship among endophyte, its host, and their geographical origin. Genetic diversity and population structure of *Alternaria alternata* recovered from *Pinus tabulaeformis* studied by using SSR marker revealed no relationship between genotypes of *A. alternata* and host tissue ages (Guo et al. 2004). Similarly, SSR markers were developed to study genetic diversity, and gene flow between populations of endophytic fungi *Lasiodiplodia theobromae* exhibited no evidence of host specificity. Also, there was a very high gene flow between populations of *L. theobromae* from different hosts (Mohali et al. 2005). In another study, 18 endophytic fungi *Guignardia mangiferae* strains from different host species were characterized using ISSR-PCR. The results showed that isolates did not correspond to the host species or the geographical origin (Rodrigues et al. 2004). The SSR or ISSR techniques are fast, low in cost, and easy to perform. It is similar to RAPD except the amplification conditions (especially annealing temperature) are more stringent and longer primers are required in this analysis. Since microsatellites evolve and mutate more rapidly than other areas of genome, SSR markers are more reproducible and robust than RAPD markers (Liang et al. 2005; Guo 2010). SSR markers are more informative in revealing variation among closely related species (Aradhya et al. 2001), hence are considered as an ideal tool for studying genetic variation among endophytes.

16.4 DNA Markers Used for Fungal Characterization

The best DNA barcode selection is based on some predefined criteria such as universality of primers, feasibility, and species level resolution. The ideal DNA barcode is selected by screening the best possible candidate gene and testing it on all taxa and species of targeted group (Consortium for the Barcode of Life 2007). Table 16.1 enlists some of the widely used biomarkers and their primer sequences.

The various loci were identified as probable DNA barcodes of fungi with suitable primary barcode characteristics:

1. *Ribosomal DNA region*: The ribosomal DNA (rDNA) genes are the widely used loci in the molecular level study of fungi due to high stability and variability in conserved and diverse regions (Hibbett 1992; Lutzoni et al. 2004; Begerow et al. 2010). The ribosomal DNA (rDNA) cistrons constituted of 18S (ITS1) smaller

Table 16.1 Some of the top listed loci and primer sequences used for fungal DNA barcoding

Locus	Primer name	Oligonucleotides (5′ ➔ 3′)	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS-1	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS2	GCTGCGTTCTTCATCGATGC	White et al. (1990)
ITS-2	ITS3	GCATCGATGAAGAACGCAGC	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
Nlsu	LROR	ACCCGCTGAACTTAAGC	Site.biology.duke.edu/fungi/mycolab/primers.htm
	LRS	TCCTGAGGGAAACTTCG	Site.biology.duke.edu/fungi/mycolab/primers.htm
TEF1α	EF1-1018F	GAYTTCATCAAGAACATGAT	Stielow et al. (2015)
	EF1-1620R	GACGTTGAADCCRACRTTGTC	Stielow et al. (2015)
60S L10	60S- 506F	GTYGAYTTCAAYGTYCC	Stielow et al. (2015)
	60S- 908R	CTTVAVYTGGAACTTGATGGT	Stielow et al. (2015)
PGK	PGK-533F	GTYGAYTTCAAYGTYCC	Stielow et al. (2015)
	PGK-533R	ACACCDGGDGGRCCGTTCCA	Stielow et al. (2015)
TOP1	TOP1-501F	ACTGCCAAGGTTTTCCGTACHTACAACGC	Stielow et al. (2015)
	TOP1-501R	CCAGTCCTCGTCAAWGACTTRATRGCCCA	Stielow et al. (2015)
LNS2	LNS2-468F	GGCGATGTGCTGAACATGATCGGHCGWGAYTGGAC	Stielow et al. (2015)
	LNS2-468R	CGGTTGCCRAAKCCRGCATAGAAKGG	Stielow et al. (2015)
TEF3	EF3-318SF	TCYGGWGGHTGGAAGATGAAG	Stielow et al. (2015)
	EF3-3538R	YTTGGTCTTGACACCNTC	Stielow et al. (2015)

Adapted from Schoch et al. (2012) and Stielow et al. (2015)

subunit, 5.8S genes, and 28S (ITS2) larger subunits have been effectively used as markers. Because, these are highly conserved, occur in multiple copies up to 200 per haploid genome, thus designed as "universal primers" (Yao et al. 1992; Bruns et al. 1991). The 18S gene is readily used in phylogenetic analysis because of its irregular conserved and variable regions at different levels of generality (Goes-Neto et al. 2002). The 28S gene comprises of conserved region with considerable sequence variation (600–900 bp) that accounts for the large variation in fungal size (shown in Fig. 16.2). It is most extensively used as a primary DNA barcode for fungi for the identification and phylogenetic studies (Schoch et al. 2012) because of the genetic variation in 18S and 28S segments, easy modification, and resolution in most of fungal groups. The ITS region is a successful primary biomarker in fungal species except yeasts, where the 18S gene became the standard for identification (Raja et al. 2017). Many fungal species such as Basidiomycota, Sclerotium, *Laetiporus*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, and *Fusarium* showed intra-isolate variation in ITS region leading to the development of secondary biomarkers (Wang and Yao 2005; Okabe et al. 2001; Lindner et al. 2011; Vydryakova et al. 2012; Pryor and Michailides 2002; Skouboe et al. 1999; Schubert et al. 2007; O'Donnell and Cigelnik 1997). Several studies have examined the implications of ITS region for identification of fungal plant pathogen using high-throughput sequencing (Sharma et al. 2015; Feau et al. 2009). For species-rich Ascomycete genera with these shorter amplicons, including the mold genera *Cladosporium* (Schubert et al. 2007), *Penicillium* (Skouboe et al. 1999), and *Fusarium* (O'Donnell and Cigelnik 1997), the ITS often has insufficient variation to unequivocally identify species. For these genera, a second barcoding gene is necessary for precise species identification. The secondary markers were developed by the association of ITS with 18S and 28S genes, respectively.

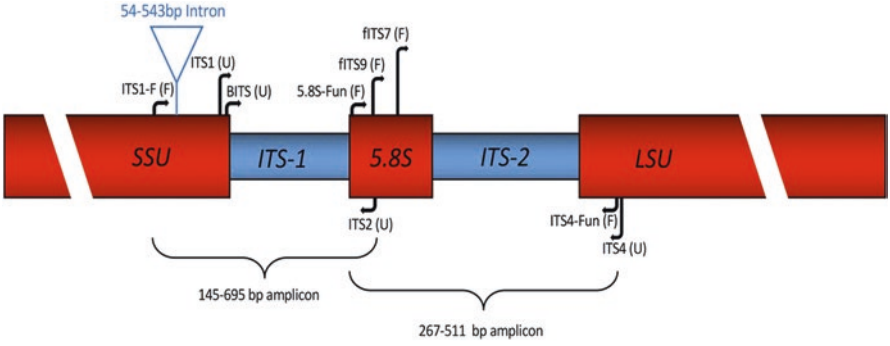

Fig. 16.2 Fungal nuclear ribosomal ITS primer map. Blocks in red are the SSU (18S or small subunit nuclear rRNA gene), 5.8S (also a structural RNA gene), and LSU (28S or large subunit nuclear rRNA gene). The blue triangle above the SSU gene indicates the type I self-splicing intron insertion site. (Source: Taylor et al. (2016a))

2. Cox1 (CO1): The cytochrome c oxidase subunit 1 (Cox1), a mitochondrial gene, is the accepted universal DNA barcode for all groups of animals including fungi by Consortium for the Barcode of Life (Hebert et al. 2003a, b; Schindel and Miller 2005). The fungal CoX1 gene is a mitochondrial gene with variable length from 1584 bp to 22 kbp, with possible barcode regions from 642 bp to 12.3 kbp, due to different introns size. It is a simple gene and devoid of gaps in absence of introns, so accurate species identification can be achieved for the sequence with 650 bps or less (Meusnier et al. 2008). A genetic map of mitochondrial genome is shown in Fig. 16.3. It has been extensively used in the phylogenetic studies of fungi such as oomycete genus *Phytophthora* (Kroon et al. 2004; Martin and Tooley 2003). The Cox1 was successfully used as DNA barcodes in *Penicillium* (Seifert et al. 2007); oomycetes (Robideau et al. 2011); Basidiophora, *Plasmopara*, and *Plasmoverna* (Thines 2007); *Fusarium* (Gilmore et al. 2009); Ascomycota (Santamaria et al. 2009); and *Aspergillus niger* (Geiser et al. 2007). The Cox1, as a DNA barcode, was doubtful due to unresolved alpha taxonomy and complex *Arbuscular mycorrhizal* (AM) fungi. It is also a difficult marker for mushrooms, where introns frequently occur in the barcode region

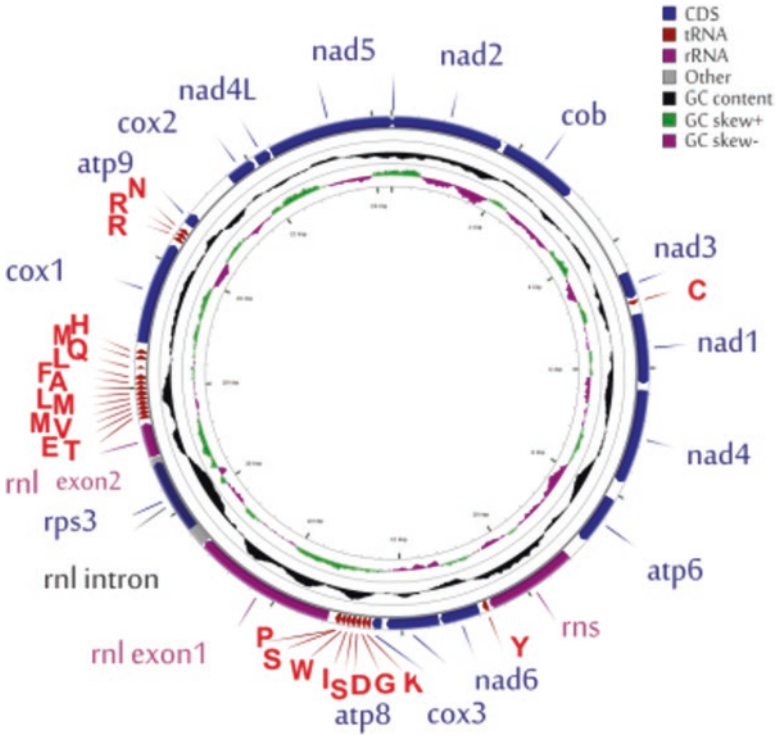

Fig. 16.3 A typical circular map of a mitochondrial genome: the mtDNA of *Trichophyton tonsurans* (sequence available by Broad Institute). Map generated by *M. Ntertili* using CGView Server. (Source: http://mitofun.biol.uoa.gr/)

(Vialle et al. 2009). Cox1, as barcoding marker, is useful in sequencing, aligning, and amplifying the relatively short fragment with uniform length using degenerate primers and in phylogenetic studies. Cox1 as marker of DNA barcode gives a tough fight to already establish ITS marker for DNA barcoding in fungi. Cox1 was first used in the Canadian Barcode of Life Network Project in 2003–2004. Cox1 was not used in Assembling the Fungal Tree of Life probably because of reports of introns, some of them mobile, in fungal mitochondrial genes.

3. *Tubulin genes*: The tubulin (tub) coding genes, associated with ITS, are used in the identification of fungi belonging to some clades, such as Eurotiales and Hypocreales (Samson et al. 2014; Montoya et al. 2016; Raja et al. 2017). The beta-tubulin gene sequences contain 3.5-fold more phylogenetic information than the small subunit (SSU) rRNA gene; thus it has been reported as an ideal marker for analysis of deep level phylogenies and for complex species groups (Thon and Royse 1999).

4. *The AFTOL genes* (*RPB1*, *RPB2*, *nucLSU*, *nucSSU*, *mtSSU*, *TEF1α*, *and mtATP6*): The multi-laboratory consortium AFTOL (Assembling the Fungal Tree of Life) selected six genetic markers – the large and small ribosomal subunits (LSU, SSU), ribosomal polymerase B1 and B2 (RPB1, RPB2), translation elongation factor 1-α (TEF1), and the small subunit of the mitochondrial ribosomal operon (mtSSU) as the probable DNA barcode. The RNA polymerase II second largest subunit (RPB2) and the transcription elongation factor 1 alpha (TEF1) are important single-copy protein-coding genes (Froslev et al. 2005; Estrada et al. 2010).

   The translation elongation factor 1-α (TEF) gene encodes for protein translation machinery and used in phylogenetic analysis. This gene has successfully elaborated large information in case of *Fusarium* and Lepidoptera (). It shows high level of sequence polymorphism compared to intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin, and histone H3.

   The RPB1 and RPB2 genes encode for the assembly platform for factors that regulate transcription initiation, elongation, termination, and mRNA processing. The RPB2 has been thought to be well conserved and present in a single-copy gene in fungi. James et al. (1991) reported that more than 85% identical amino acids exist in the 12 highly conserved regions of RPB2. Thus, it is frequently used in phylogenetic analysis.

   Nuclear ribosomal genes (nucLSU and nucSSU) are present in multiple copies and often used for taxonomic and phylogenetic analyses in eukaryotes. These genes were successfully examined for phylogenetic analysis in genus *Cordyceps* by Nikoh and Fukatsu (2001). The conserved mitochondrial genes (nad2, nad4, cox1, cox2, and cox3) in the lichenized fungi *Usnea* and other relatives were supposed to exhibit the best estimate phylogeny with obligate symbiotic partners (Funk et al. 2018).

   The mt-ATP6 gene encodes the essential protein subunit of the enzyme ATP synthase. This gene provides phylogenetic resolution within fungal classes but

not at higher taxonomic levels. The ATP6 sequences were used to understand phylogenetic relationships between 27 taxa from the Boletales and 4 outgroup taxa. The analysis of the combined ATP6 and mtLSU rDNA sequences was more resourceful (Kretzer and Bruns 1999).

5. *Nonuniversal regions*: The nonuniversal regions such as ND6 (hypothetical protein), CAL (calmodulin), ACT (gamma-actin) or TUB2 (beta-tubulin 2) are frequently found in mitochondria (Carbone and Kohn 1999; Aveskamp et al. 2009; Lee and Young 2009; Verkley et al. 2014).

   The mt-ND6 gene provides information of a large enzyme complex in mitochondria known as active complex I NADH dehydrogenase 6 protein. This gene was considered as barcode candidates for Ascomycota due to their paucity of introns and to their length, above 400 bp (Santamaria et al. 2009).

   CAL (calmodulin) gene is intron less and encodes a protein of 148 amino acid residues. All calmodulins are composed of four similar calcium-binding domains. Actin gamma 1 gene encodes for cytoplasmic actin found in all cell types. It is one of the most conserved proteins and plays key roles in various cellular processes like cell growth regulation and motility of the components in the cells (Neveu 2007). β-Actin gene is important in gene expression studies of fungi (Rady and Shearer 1997). β-Tubulin gene encodes for the structural proteins of microtubules and other structural components in eukaryotes.

6. *Minichromosome maintenance complex*: The minichromosome maintenance proteins, MCM2 to MCM7, form DNA helicase heteromers, required essentially for initiation and elongation of DNA replication. These protein complexes are distributed along the chromosomes. The MCM7 is potential valuable single-copy protein-coding gene which contributes to multigene studies. Aguileta et al. (2008) demonstrated that the gene Tsr1 is required for rRNA accumulation during biogenesis of the ribosome (Gelperin et al. 2001), while MCM7 gene is a DNA replication licensing factor required for DNA replication initiation and cell proliferation (Moir et al. 1982; Kearsey and Labib 1998).

16.5 Tools Available for DNA Barcoding in Fungi

Wide arrays of tools are available for developing DNA barcodes. The sequence data retrieval followed by phylogenetic analysis is extensively accepted method. The three frequently used methods for phylogenetic analysis are maximum parsimony, maximum likelihood (Hillis et al. 1994; Felsenstein 1985), and Bayesian inference. The maximum parsimony develops the shortest tree based on minimum genetic events (Swofford et al. 1996; Farris 1970), the maximum likelihood method is based on the obtaining evolutionary relationship with different tree topologies (Ali et al. 2014), and the Bayesian inference is based on simulation technique called Markov chain Monte Carlo which explores the parameter-rich evolutionary relationship (Huelsenbeck and Ronquist 2001). The available tools used for fungal characterization and barcoding are described in Table 16.2.

Table 16.2 Latest tools for fungal DNA barcoding

Tool	Description	Source
BOLD	Storage, quality, and analysis of specimens and sequences to validate a barcode library	www.barcodinglife.com
Bayesian Evolutionary Analysis Sampling Trees (BEAST)	Used to analyze nucleotide and amino acid sequences, as well as morphological data	http://beast-mcmc.googlecode.com/
B	Sequence quality and contig overlap	http://www.nybg.org/files/scientists/dlittle/B.html
BioBarcode	Sequence based	http://www.asianbarcode.org
BioEdit	A fairly comprehensive sequence alignment and analysis tool and supports a wide array of file types and offers a simple interface for local BLAST searches	www.mbio.ncsu.edu/BioEdit/bioedit.html
BLOG	Data mining approach	http://dmb.iasi.cnr.it/blog-downloads.php
BPSI	Back propagation (BP) neural networks	zhangab2008@yahoo.com.cn
BRONX	Sequence identification incorporating taxonomic hierarchy	http://www.nybg.org/files/scientists/dlittle/BRONX.html
CAOS	Character based	http://sarkarlab.mbl.edu/CAOS
CBC Analyzer	Phylogenies based on CBC	http://cbcanalyzer.bioapps.biozentrum.uniwuerzburg.de/cgibin/index.php
CodonCode Aligner	Codon based	http://www.codoncode.com/index.htm
CLOTU	Amplicon and taxa data	http://www.mn.uio.no/ibv/bioportal/
DNA for Windows	A compact, easy to use DNA analysis program, ideal for small-scale sequencing projects	www.dna-software.co.uk/
EcoPrimers	Barcode markers and primer based	http://www.grenoble.prabi.fr/trac/ecoPrimers
Excali Bar	Calculate intra- and interspecific distances	http://datadryad.com/resource/doi:10.5061/dryad.r458n
Geneious	Provides an automatically updating library of genomic and genetic data for organizing and visualizing data. It provides a fully integrated, visually advanced toolset for sequence alignment and phylogenetics; sequence analysis including BLAST; protein structure viewing, NCBI, EMBL, PubMed auto-find, etc.	https://www.geneious.com/

(continued)

Table 16.2 (continued)

Tool	Description	Source
MAFFT	A multiple sequence alignment program for unix-like operating systems. It offers a range of multiple alignment methods, L-INS-i (accurate; for alignment of <~200 sequences), FFT-NS-2 (fast; for alignment of <~10,000 sequences), etc.	https://mafft.cbrc.jp/alignment/software/
FigTree	A graphical viewer of phylogenetic trees to display summarized and annotated trees produced by BEAST	http://tree.bio.ed.ac.uk/software/figtree/
Format converter v2.2.5	This program takes as input a sequence or sequences (e.g., an alignment) in an unspecified format and converts the sequence(s) to a different user-specified format	https://www.hiv.lanl.gov/content/sequence/FORMAT_CONVERSION/
Genetic Algorithm for Rapid Likelihood Inference (GARLI)	A program that uses genetic algorithms to search for maximum likelihood trees. It includes the GTR + Γ model and special cases and can analyze nucleotide, amino acid, and codon sequences. A parallel version is also available	https://www.its.hku.hk/services/research/hpc/software/GARLI
Hypothesis testing using phylogenies (HYPHY)	A maximum likelihood program for fitting models of molecular evolution. It implements a high-level language that the user can use to specify models and to set up likelihood ratio tests	http://www.hyphy.org
ITS2 Database	An exhaustive dataset of internal transcribed spacer 2 sequences from NCBI GenBank accurately reannotated. Following an annotation by profile Hidden Markov Models (HMMs), the secondary structure of each sequence is predicted. Also provides several tools to process ITS2 sequences, including annotation, structural prediction, motif detection, and BLAST search on the combined sequence-structure information. Moreover, it integrates trimmed versions of 4SALE and ProfDistS for multiple sequence-structure alignment calculation and neighbor-joining tree reconstruction. Together they form a coherent analysis pipeline from an initial set of sequences to a phylogeny based on sequence and secondary structure	http://its2.bioapps.biozentrum.uni-wuerzburg.de
jMOTU	Multiple operational taxonomic unit (MOTU) based	http://www.jmotu.com-about.com/
LV Barcoding	Locality-sensitive hashing-based	http://msl.sls.cuhk.edu.hk/vipbarcoding/

(continued)

Table 16.2 (continued)

Tool	Description	Source
Molecular Evolutionary Genetics Analysis (MEGA)	A Windows-based program with a full graphical user interface that can be run under Mac OSX or Linux using Windows emulators. It includes distance, parsimony, and likelihood methods of phylogeny reconstruction, although its strength lies in the distance methods. It incorporates the alignment program ClustalW and can retrieve data from GenBank	https://www.megasoftware.net/
MrBayes	A Bayesian MCMC program for phylogenetic inference. It includes all of the models of nucleotide, amino acid, and codon substitution developed for likelihood analysis	http://www.rbayes.sourceforge.net/download.php
ModelTest	Uses hierarchical likelihood ratio tests (hLRT) to compare the fit of the nested GTR (general time reversible) family of nucleotide substitution models. Additionally, it calculates the Akaike information criterion estimate associated with the likelihood scores	https://github.com/ddarriba/modeltest
Oligo Calculator	Online tool to find length, melting temperature, %GC content, and molecular weight of DNA sequence	http://biotools.nubic.northwestern.edu/
OFBG	Spp. discrimination using oligonucleotide frequencies	http://www.nbri.res.in/ofbg.php
OTUbase	Operational taxonomic unit (OTU) based	http://www.bioconductor.org/packages/release/bioc/html/OTUbase.html
Phylogenetic analysis by maximum likelihood (PAML)	A collection of programs for estimating parameters and testing hypotheses using likelihood. It is mostly used for tests of positive selection, ancestral reconstruction, and molecular clock dating. It is not appropriate for tree searches	http://abacus.gene.ucl.ac.uk/software/paml.html
Phylogeny.fr	A simple to use web service dedicated to reconstructing and analyzing phylogenetic relationships between molecular sequences. It includes multiple alignment (MUSCLE, T-Coffee, ClustalW, ProbCons), phylogeny (PhyML, MrBayes, TNT, BioNJ), tree viewer (Drawgram, Drawtree, ATV), and utility programs (e.g., Gblocks to eliminate poorly aligned positions and divergent regions)	https://www.phylogeny.fr/
PHYLIP	A package of programs for phylogenetic inference by distance, parsimony, and likelihood methods	http://evolution.genetics.washington.edu/phylip/phylipweb.html

(continued)

Table 16.2 (continued)

Tool	Description	Source
PhyML	A fast program for searching for the maximum likelihood trees using nucleotide or protein sequence data	www.atgc-montpellier.fr/phyml/
PAUP	PAUP has many options and close compatibility with MacClade. It includes parsimony, distance matrix, invariants, and maximum likelihood methods and many indices and statistical tests	https://paup.phylosolutions.com/
ProfDistS	Distance-based phylogeny on sequence-structure alignments	http://www.profdist.bioapps.biozentrum.uni-wuerzburg.de/
PTIGS-Idit	psbA-trnH intergenic spacer (PTIGS) based	http://psba-trnh-plantidit.dnsalias.org
MacClade	A computer program for phylogenetic analysis. Its analytical strength is in studies of character evolution. It also provides many tools for entering and editing data and phylogenies and for producing tree diagrams and charts	http://www.macclade.org/macclade.html
Neighbor-joining	For reconstructing phylogenetic trees from evolutionary distance data	https://www.sequentix.de/gelquest/help/neighbor_joining_method.htm
OBITools package	NGS data-based	http://metabarcoding.org/obitools
Q Bank	Identification and detection reference database	http://www.q-bank.eu/
RAxML	A fast program for searching for the maximum likelihood trees under the GTR model using nucleotide or amino acid sequences. The parallel versions are particularly powerful	https://sco.hits.org/exelixis/web/software/raxml/
Readseq	A tool for converting between common sequence file formats, particularly useful for those using various phylogenetic analysis tools	https://www.ebi.ac.uk/Tools/sfc/readseq
4SALE	A tool for synchronous RNA sequence and secondary structure alignment and editing	http://4sale.bioapps.biozentrum.uni-wuerzburg.de/
SAP	Bayesian phylogenetics	http://ib.berkeley.edu/labs/slatkin/munch/StatisticalAssignmentPackage.htm
Sequencher	The Premier DNA Sequence Analysis Software for Sanger and NGS Datasets	www.genecodes.com
Spider	Analysis of species identity and evolution	http://spider.r-forge.r-project.org/SpiderWebSite/spider.html
Tree analysis using new technology (TNT)	A fast parsimony program intended for very large datasets	www.zmuc.dk/public/phylogeny/tnt/

(continued)

Table 16.2 (continued)

Tool	Description	Source
TaxI	Distance-based method	axel.meyer@uni-konstanz.de
TaxonGap	Operational taxonomic unit (OTU) based	http://www.kermit.ugent.be/software.php?navigatieId=37&categorie Id = 17
Taxonerator	OTU and taxonomy data based	http://www.taxonnerator.com-about.com/
TreeView	Provides a simple way to view the contents of a NEXUS, PHYLIP, or other format tree files	www.treeview.net/
TrichOKEY	It is a program for the quick molecular identification of *Hypocrea* and *Trichoderma* on the genus and species levels based on an oligonucleotide DNA barcode	www.isth.info/tools/molkey/
VIP Barcoding	Vector-based software	http://msl.sls.cuhk.edu.hk/vipbarcoding/

Other computational methods for DNA barcoding are based on compensatory base changes (CBCs), operational taxonomic units (OTUs), DNA meta-barcoding, and locus-specific tools and techniques of neural network, machine learning, and data mining (Chatterjee and Das 2012; Bhargava and Sharma 2013; Meena et al. 2015). The compensatory base changes (CBCs)-based software, such as CBC analyzer, uses the process of mutations in paired structural site and has been successfully reported in rRNA ITS2 for the verification of closely related species (Coleman and Vacquier 2002; Wolf et al. 2005). The limitations of character-based methods can be easily resolved by distance methods. The molecular operational taxonomic units use conserved gene cluster as a representative of their origin genomes. The tools which use this method are jMOTU, Taxonerator, Taxon Gap, and CLOTU (Saitou and Nei 1986; Slabbinck et al. 2008; Jones et al. 2011).

DNA meta-barcoding is the process of identification of organisms from environmental sample such as soil, water, or air. These samples contain degraded DNA and required highly conserved and versatile primers with short barcodes. The tools used in meta-barcoding are ecoPrimers and OTUbase (Riaz et al. 2011; Beck et al. 2011).

16.6 Databases Available for DNA Barcoding in Fungi

The method based on the classification and identification of sequences in organisms allows the rapid and deep detection of diversity. The identification reality and reliability of the analyses depends on the informative databases and careful interpretation

of the data (Costello et al. 2013; Kõljalg et al. 2013; Lindahl et al. 2013; Nguyen et al. 2015; Hibbett et al. 2016). GenBank (Benson et al. 2015) is the most comprehensive and widely used sequence repository for the fungal identification, comprising millions of sequences from different fungal biomarkers. The Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2013) represents another bioinformatics platform for sequences. BOLD supplies tools for the storage, quality warranty, and analysis of specimens and sequences to validate a barcode library. To obtain a barcode status on BOLD, sequences must fulfill some requirements, such as voucher data, collection record, and trace files. In spite of its several advantages, unfortunately, the fungi remain considerably underrepresented in BOLD database.

The Web of Science (WoS) (Falagas et al. 2008) and PubMed are two of the currently major scientific literature databases due to their extensive set of publications. While WoS indexes most of the important journals on many areas of scientific research, PubMed is more limited in this aspect due to its specialization in biomedical literature. In fact, WoS was the biggest source of publications in our dataset, while BOLD was the database that contributed less. The major databases available for fungal barcoding are described in Table 16.3.

Many authors have sequenced closely related species to investigate the relationship of the taxa. Such studies have been carried out with larger genera such as *Penicillium*, *Fusarium*, and *Trichoderma*. Teleomorph and anamorph variation is not always congruent. The speed of evolution seems heterogeneous and is characterized by different rates of variation between groups. In general, about 2% intraspecific variability is maintained within species. Occasionally the use of ITS is problematic due to the occurrence of two different types in a single organism.

16.7 Limitations of DNA Barcoding

The DNA-based species identification process is based on genetic variation among species level. These variations may differ in between taxa and the causes are unknown. This leads in difficulty to differentiate between diverged and original species. Since there is no universal gene available for DNA barcoding, the process depends on the identification of taxonomically similar sequences resulting into complexity. The short size of DNA barcodes (approx. 500–1000 bp) limits the determination of the profound branches in phylogenies. However, there are some discrepancies over standard values of DNA barcoding. These differences directly challenge the traditional methods of species identification used earlier which were based on taxonomic identification or morphology-based taxonomy (Schindel and Miller 2005). Although with the increasing advent of technology in DNA barcoding and developing databases, it seems possible that DNA barcoding is going to become an unprecedented resource for taxonomy, systematic biology, disease identification, and diagnosis (Chase et al. 2007).

Table 16.3 Latest databases for fungal DNA barcoding

Database	Description	Source
AFTOL	ITS rDNA sequences	https://aftol.umn.edu/
Artemis	It is a free genome browser and annotation tool	http://www.anger-pathogens.github.io/Artemis
CBS-KNAW		
DnaSP	It is a software package for the analysis of DNA polymorphisms using data from a single locus	www.ub.edu/dnasp
Fungal barcoding	Provide up-to-date information on fungal barcoding	http://www.fungalbarcoding.org/
Fungal MLST Database Q Bank	Facilitate identification of agriculturally and medically important fusaria species	www.westerdijkinstitute.nl/fusarium/
FUSARIUM-ID	Database of partial translation elongation factor 1-alpha (TEF) DNA sequences	isolate.fusariumdb.org/guide.php
GBOL	Inventory and genetic characterization of animals, plants, and fungi in Germany	http://gbol.life/
GenBank	More than 100000 fungal sequences generated by conventional Sanger sequencing	https://www.ncbi.nlm.nih.gov/genbank
Geneious	It creates powerful, integrated, and visually appealing bioinformatics solutions, with a strong emphasis on ease of use and overall user experience	https://www.geneious.com/
ISHAM Barcoding	ITS and TEF1α DNA barcoding databases	http://its.mycologylab.org/
Phylogenetic Data Editor (PhyDE)	It is a system-independent editor for DNA and amino acid sequence alignments	www.phyde.de
RefSeq Targeted Loci (RTL)	It includes markers from archaea, bacteria, and fungi. Markers are represented by ribosomal RNA subunits in all three kingdoms and ITS DNA sequences in fungi	https://www.ncbi.nlm.nih.gov/refseq/targetedloci/
The DNA Data Bank of Japan (DDBJ)	It is a biological database that collects DNA sequences	https://www.ddbj.nig.ac.jp/
The European Nucleotide Archive (ENA)	It provides a comprehensive record of the world's nucleotide sequencing information, covering raw sequencing data, sequence assembly information, and functional annotation	https://www.ebi.ac.uk/ena
UNITE	A web-based database and sequence management environment for the molecular identification of fungi	https://unite.ut.ee/

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Chapter 17
Current Progress on Endophytic Microbial Dynamics on *Dendrobium* Plants

Surendra Sarsaiya, Jingshan Shi, and Jishuang Chen

17.1 Introduction

From the start of human civilization, medicinal plant parts, extracts, phytochemicals, and conventional botanical mixtures have been widely used for the healing of diverse human diseases (Dey and Mukherjee 2018). The orchids of the Orchidaceae family possibly originated 120 million years ago. It is the largest major family among angiosperms; further, the majority of this family is composed of highly evolved flowering plants, with approximately 25,000–35,000 species in 750–900 genera (Lam et al. 2015). *Dendrobium* is recognized as Shihu, broadly dispersed throughout China with 78 species, and approximately 30 species (of these 78 species) have been used as a chief medicinal and efficient food over many centuries, such as tea drinks or soup ingredients (Deng et al. 2017). *Dendrobium* plants are either lithophytic or epiphytic, frequently with aerial roots and buds (Ng et al.

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2012). Most *Dendrobium* plants are cross-pollinated, and within the same plant species, the maturity periods of pollen and stigma are frequently altered for additional ornamental cross-pollination (Yan et al. 2015). Numerous Orchidaceae, especially the stems of *Dendrobium* species, are widely used to supply remedial benefits through enhancing saliva secretion, reducing fever, and immunity enhancement (Jiangsu New Medicinal University 1986). This remedy is also useful for tonicity of the stomach, maintaining and enhancing the body fluid production level (Cui et al. 2014). Many bioactive parts of *Dendrobium* are well documented including bibenzyls, phenanthrenes, sesquiterpenes, and alkaloids (Ng et al. 2012; Zhang et al. 2017). It is an essential long-established Chinese medicine with efficient alkaloids with numerous pharmacological uses including immune regulation, antioxidation, antitumor, bacteriostasis, and hypoglycemia functions.

Dendrobium is typically propagated by extrication backbulbs and keikies, or by vegetative cuttings, but these are extremely slow and difficult methods that result in the survival of only a few propagules (da Silva et al. 2017). In fact, polysaccharides are one of the abundant components in *Dendrobium* species, and immunostimulatory action is one of the most vital biological actions of these polysaccharides (Deng et al. 2017; Yang et al. 2017).

Similar to root-inhabiting bacteria, endophytic microbes have mechanisms by which they support plant health and growth (Yi et al. 2013). Endophytic bacteria inhabit within the host plant tissue without unfavorably harming it. The impact of their bioactive compounds has been verified as positive in a wide variety of crops (Ryan et al. 2008). These compounds may contribute directly to plant growth by increased nutrient availability, fixation of biological nitrogen, and phytohormone production (Kim et al. 2011). Ultimately, they may also indirectly decrease microbial numbers that are dangerous to plant growth, and behave as biological control agents through antibiosis, competition, or systemic resistance stimulation (Yi et al. 2013). The quest for more capable endophytic microbial strains has been stimulated by the idea that these organisms generate a plethora of bioactive products (Wu et al. 2016).

Cultivable endophyte diversity occurred not only in the multiplicity of plant species occupied but also in the numerous taxa concerned, with the majority being linked with predominant organisms found in the soil (Mehnaz et al. 2009). Some bacteria such as *Bacillus pumilus* INR7 are distinctive examples for a biocontrol artefact that has been established using the ability of a single endophytic bacteria to provoke plant growth promotion (PGP) and induced systemic resistance (ISR) (Jeong et al. 2014). Mycorrhization could be an effective alternate way to recover the rooting and existence of *Dendrobium*. Photoautotrophic *Dendrobium* culture is a still unexplored aspect but could be a way to improve the efficacy of acclimatization events (da Silva et al. 2017).

In *Dendrobium* plant organs, cell culture technology has established countless approaches for making plant-specific beneficial metabolites, together with pharmaceuticals, acceptable chemicals, and coloring mediators. Efforts have been com-

pleted for the formation of cells with improved root cultures for assembly of value-added compounds (Cui et al. 2011). Protocorm, embryo, root, and shoot cultures are regularly applied for the production of industrial compounds (Naik et al. 2011). Bioreactor usage for large-scale production of cells and extrinsic roots has converted these into feasible cells for metabolite production (Cui et al. 2011), although numerous chemical as well as physical aspects need to be enhanced for effective metabolite production through bioreactor cultures (Lee et al. 2011; Lee and Paek 2012).

Now, denrobine, a major component of *Dendrobium* species, is increasingly drawing attention for its wide applications in healthcare, and the demand is ever increasing, but the wild resources of *Dendrobium* species cannot meet the market requirements. Thus, artificial cultivation of *Dendrobium* plants is extremely urgent (Li et al. 2017a, b, c).

17.2 Microbial Dynamics of *Dendrobium* Plants

The conventional approaches are not appropriate for preparing a bulky quality planting material. Several orchids can be spread sexually through seeds, but *Dendrobium* seeds are deficient in endosperm and nutritious substances, and it is usually difficult to produce these as wide-ranging plants in nature because seed germination requires symbiotic endophytic microbes. Whenever a new position of biological diversity is revealed and accessed, novel natural products are established (Fig. 17.1). The understanding that there are bulky, and typically unexplored, endophytic microbe clusters living inside the developed plants (endophytic fungi) led to

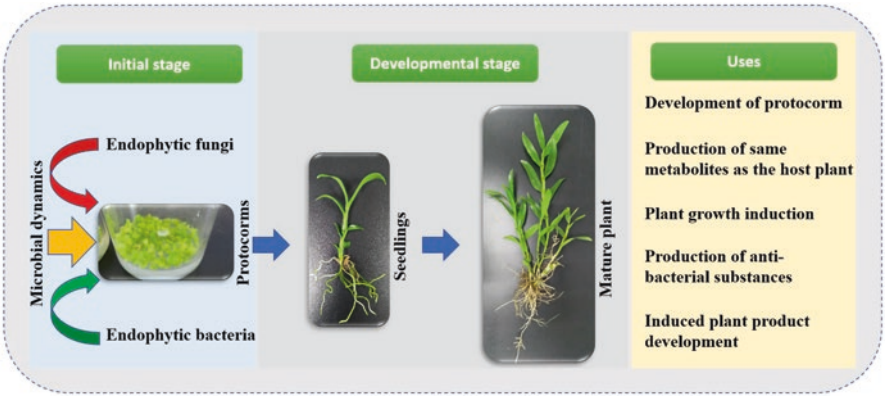

Fig. 17.1 Microbial dynamics of the *Dendrobium* plant for growth development and natural products

intensive discovery trials in both industrial and academic laboratories (Winarto et al. 2013).

17.2.1 Endophytic Fungi

Endophytic fungi associated with plants are fungal microbes that employ all or part of their development inhabiting healthy tissues of their plant hosts inter- or intracellularly, characteristically causing no superficial disease signs. These organizationally varied molecules have possible therapeutic importance, which has increased common interest in the transmission of endophytic fungi for the detection of novel metabolites and also specific novel antibiotics. The natural products that are typically "to blame" for the identification of endophytic fungi of plants as a vital source of accepted bioactive products include paclitaxel (taxol) because of the recognition of the taxol-forming fungal endophyte *Taxomyces andreanae* in 1993 (Stierle et al. 1993).

The fungal mycorrhiza has some incomparable characteristics in the life sequence of orchid plants (Singh et al. 2017). In the natural landscape, orchid linkage with many mycorrhizal fungi is vital for seed development because orchids lack endosperm and their seedling development requires nutrients from an external source. Mycorrhiza have significant effects on the development of the plant life cycle, and vegetative and propagative evolution (Hoysted et al. 2018). Sarsaiya et al. (2019a) have described a comprehensive evaluation of endophytic fungi for novel and critical insights on physiological, genomics, and functional analyses that provided novel information useful to overcome the growing challenges for *Dendrobium* development. On the other hand, Sarsaiya et al. (2019b) have reported for the first time that *Trichoderma longibrachiatum* caused leaf black circular spots on *Dendrobium nobile* in Guizhou Province, China.

Fungi allied with orchids are mostly contained in the genus *Rhizoctonia* (class Hymenomycetes, subdivision Basidiomycota). Nine orchid species that breed in Puerto Rico have a close association with as many as 108 *Rhizoctonia*-like fungi that include *Tulasnella*, *Thanatephorus*, and *Ceratobasidium* (Agustini et al. 2016). As Taylor and Bruns (1997) and Taylor et al. (2004) showed, 17 to 22 fungal species allied with photosynthetic orchids are in the Russulaceae family. Investigation on *Limodorum abortivum*, a natural orchid budding in Mediterranean areas, exhibited its relationship with fungi of the family Russulaceae (Gurlanda et al. 2006). Characterization of the orchid fungal mycorrhiza is very important when reviewing their association. Morphological characterization of mycorrhizal pure culture by means of special characters such as form, color, and colony pattern can only provide identification at the genus level; however, species-level identification is more available with the support of molecular identification. These molecular methods are simple because the DNA sequence that is required is not very complex. The small sequences such as internal transcribed spacer (ITS) among the small subunit (SSU) as well as large subunit (LSU) recombinant DNA can be used for species identification (Agustini et al. 2016).

Agustini et al. (2009) described that numerous orchid species in Papua, together with the *Calanthe* species of orchids, have symbiotic association with fungal mycorrhiza (Agustini et al. 2016). Orchidaceae genera are exceptional in numerous adaption characters such as the velamen root, symbiotic linking between fungi, and specific flowers that evolved strictly with insect pollinators (Hossain et al. 2013). The orchid roots together are attached with the aerial roots to the substrate; the substrate roots are linked with numerous endotrophic fungi (da Silva et al. 2015). Both nonmycorrhizal and mycorrhizal endophytes cooperate with their plant host in several biological actions. The fungi offer advantages to the orchid plants in various ecological aspects such as in low light (tuberous species and terrestrial rhizomatous species), in low-nutrient soil or spatial distribution, bark, development of siblings and seedlings, and on morphology (Rasmussen 1995). The association of orchids with mycorrhiza is a symbiosis type of cooperation wherein both the fungi and the plant are mutually promoted by each other; therefore, the relationship is considered mutualism. The mycorrhizae increase the plant contact with soil properties such as mineral ions and water and can support the continued growth of orchids in a dormant phase for many years. The fungi have multifunctional ability to produce wide-ranging plant growth development substances such as auxin (indole-3-acetic acid, IAA), gibberellic acid (GA3), abscisic acid, zeatin riboside, and zeatin (Table 17.1) (Liu et al. 2010; Parthibhan et al. 2017).

The various nonmycorrhizal fungi of orchids, also named endophytes, have also been purified in orchids from numerous tissues, including leaf, stem color, stem, rhizome, tubers, mature bulb, and even also from roots (Sarsaiya et al. 2019; Lin et al. 2020). Their mycelia have been described to secrete numerous plant growth-encouraging substances such as GA, auxin (IAA), and the vitamins B6, B2, and folic acid (Table 17.2) (Maor et al. 2004). Therefore, their symbiotic association for in vitro and in vivo seed germination and seedling development is described to support germination as well as fresh weight (Yang et al. 2008), although proven indications for the nutrient streams from fungal organisms to orchids are known only for carbon and nitrogen compounds (Stöckel et al. 2014); the transfer ensues either from the breakdown pelotons or from both living and breakdown forms of pelotons (Kuga et al. 2014). Because the symbiont has a strong impact on orchid growth, it is very important to study their fungal symbiotic relationships to develop new strategies for orchid conservation and better exploitation of their medicinal compounds (Parthibhan et al. 2017). The findings of Chen et al. (2011) showed that all ten plant species of *Dendrobium* were responsible for harboring endophytic fungi. The endophyte taxa *Acremonium*, *Alternaria*, *Ampelomyces*, *Cladosporium*, *Colletotrichum*, *Fusarium*, and *Verticillium* originated in most *Dendrobium* species.

The xylariaceous fungi occur frequently among the medically important *Dendrobium* plants, but their biological functions have remained uncertain up to the present. Rodriguez et al. (2009) revealed that xylariaceous fungi in the living world might be mutualistic with their plant host, even though the *Xylaria* association of mycorrhizal fungi in *Dendrobium* and other orchid plants has yet to be established. Furthermore, endophytic fungi show an imperative role in variable secondary metabolite production in their host orchid plants and may increase the concentrations of complete alkaloids and polysaccharides in therapeutically important

Table 17.1 Diversity of endophytic microbes with abundance and frequency on *Dendrobium* plants

Endophytic microbial taxa	Abundance/ frequency	Source	Reference
Fusarium oxysporum	22	*Dendrobiium officinale:* roots, stems	Jin et al. (2017)
Xylaria cubensis	21	*D. officinale:* roots, stems, leaves	Jin et al. (2017)
Colletotrichum spp.	12	*D. officinale:* roots, stems, leaves	Jin et al. (2017)
Pestalotiopsis spp.	07	*D. officinale:* roots, stems	Jin et al. (2017)
Alternaria spp.	06	*D. officinale:* roots, stems, leaves	Jin et al. (2017)
Aspergillus niger	06	*D. officinale:* stems, leaves	Jin et al. (2017)
Aspergillus flavus	06	*D. officinale:* roots, stems	Jin et al. (2017)
Trichoderma atroviride	05	*D. officinale:* roots, stems, leaves	Jin et al. (2017)
Cochliobolus spp.	03	*D. officinale:* roots, stems	Jin et al. (2017)
Cystobasidium slooffiae	02	*D. officinale:* roots	Jin et al. (2017)
Aureobasidium pullulans	02	*D. officinale:* roots	Jin et al. (2017)
Epicoccum sorghinum	02	*D. officinale: r*oots, stems	Jin et al. (2017)
Ceratobasidium spp.	NR	*D. lancifolium*	Agustini et al. (2016)
Guignardia endophyllicola	28	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Colletotrichum gloeosporioides	13	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Cladosporium sphaerospermum	02	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Fusarium solani	02	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Xylohypha spp.	02	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Colletotrichum spp.	01	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Curvularia brachyspora	01	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Fusarium nivale	01	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Pestalotiopsis spp.	01	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Scolecobasidium spp.	01	*D. crumenatum:* roots, stems	Mangunwardoyo et al. (2011)
Westerdikella spp.	01	*D. crumenatum: Roots, stems*	Mangunwardoyo et al. (2011)
Pestalotiopsis vismiae	01	*D. nobile:* stems	Yuan et al. (2009)
Colletotrichum species	10	*D. nobile:* stems, leaves, roots	Yuan et al. (2009)

(continued)

Table 17.1 (continued)

Endophytic microbial taxa	Abundance/ frequency	Source	Reference
Phomopsis amygdale	05	*D. nobile:* stems, roots	Yuan et al. (2009)
Phomopsis spp.	07	*D. nobile:* stems, roots	Yuan et al. (2009)
Clonostachys rosea	02	*D. nobile:* roots	Yuan et al. (2009)
Penicillium griseofulvum	07	*D. nobile:* stems, leaves, roots	Yuan et al. (2009)
Penicillium spp.	03	*D. nobile:* stems	Yuan et al. (2009)
Trichoderma chlorosporum	02	*D. nobile:* roots	Yuan et al. (2009)
Fusarium solani	02	*D. nobile:* roots	Yuan et al. (2009)
Fusarium proliferatum	06	*D. nobile:* stems, roots	Yuan et al. (2009)
Fusarium spp.	03	*D. nobile:* roots	Yuan et al. (2009)
Guignardia mangiferae	52	*D. nobile:* stems, leaves	Yuan et al. (2009)
Botryosphaeria spp.	02	*D. nobile:* stems	Yuan et al. (2009)
Xylaria spp.	25	*D. nobile:* stems, leaves, roots	Yuan et al. (2009)
Hypoxylon spp.	02	*D. nobile:* stems, leaves	Yuan et al. (2009)
Nemania spp.	01	*D. nobile:* stems	Yuan et al. (2009)
Rhizoctonia spp.	02	*D. nobile:* stems	Yuan et al. (2009)

Table 17.2 Effect of endophytic microbes on *Dendrobium* plant morphology and its development

Plant	Product	Microbes	Effect on plant morphology and development	Reference
Dendrobium nobile	Auxin (bioactive)	Bacterial species: *Azospirillum*, *Enterobacter*, *Streptomyces*, *Mycobacterium*, and *Bacillus pumilus*	Herb seed germination and its expansion	Tsavkelova et al. (2016)
D. friedericksianum	Not specified	Fungal species: *Trichosporiella multisporum*	Herb seed propagation	Khamchatra et al. (2016)

Dendrobium plants. Consequently, those secondary metabolites from xylariaceous taxa abundant in *Dendrobium* should be extensively discovered, and the association among the endophytic *Xylaria* strains and the eminence of *Dendrobium* as a medicine should be widely examined (Chen et al. 2013).

17.2.2 Endophytic Bacteria

Dendrobium plant-linked bacteria are generally known to have unquantified and often advantageous influences on plant growth because of nitrogen fixation, plant growth controllers, water uptake and mineral nutrition, and biosynthesis of bactericidal

and fungicidal constituents, therefore decreasing the amount of pathogens in the herbs (Li et al. 2017a, b, c). Endophytic bacteria populations, as a rich source of natural compounds as well as phytochemicals by means of promising therapeutic and agricultural utilization, have invited much consideration from investigators worldwide (Ryan et al. 2008; Bhore et al. 2013; Brader et al. 2014). Plant growth-promoting (PGP) bacteria is a complex, well-established phenomenon that is often accomplished by numerous probiotic traits revealed by the related bacterium, such as antagonism against plant pathogens (Haas and Défago 2005). The genera *Alternaria, Botrytis, Verticillium, Fusarium, Rhizoctonia*, and *Pyricularia* are dominant plant pathogens that contaminate a wide variety of plants, as well as vegetables, several crops, and fruit, causing important economic losses (Khush and Jena 2009; Williamson et al. 2007). In some other investigations, 22 endophytic stains showed antagonistic action in at least single plant pathogen analysis, signifying that these cultures have the possibility to be applied as biofungicides. Many procedures have been planned for antibiosis reaction, which could be arbitrated by the discharge of nonspecific or specific microbial metabolites, extracellular biocatalysts, or the formation of siderophores and other substances (Arora et al. 2008). Approximately all the analyzed antagonistic strains were able to yield biocatalysts such as protease and siderophores. The consequent plant pathogen elimination by potential antagonistic strains could be comparatively attributed to protease and siderophore production (Wu et al. 2016).

17.2.3 Physiology of Fungi in the Growth Induction of Dendrobium

The epiphytic *Dendrobium* species are greatly valued for their medically important bioactive substances, although their endophytic fungal populations are comparatively less well known (Chen et al. 2011). In *Dendrobium nobile*, Hyphomycetes, Coelomycetes, and Ascomycetes occurred as the most dominant endophytic fungi, showing complex tissue specificity and accumulation in the plant leaves, and subsequently in the stem as well as roots (Yuan et al. 2009). Two of the root fungal endophytes, *Trichoderma chlorosporum* and *Clonostachys rosea*, were found to have advantages for plant acclimatization and represented possible biocontrol and growth advancement characteristics correspondingly. In *D. loddigesii*, *Acremonium* and *Fusarium* occurred as the most dominant fungal isolates, indicating high assemblage and establishment in roots and thereafter into the leaves along with stems where a few isolates expressed antimicrobial as well as plant growth advancement (Chen et al. 2010). In the same way, several *Dendrobium* herb varieties, such as *D. fimbriatum*, *D. chrysanthum*, *D. officinale*, *D. devonianum*, *D. nobile*, and *D. thyrsiflorum*, have been widely documented to link with varied endophytic fungi with a high grade of assemblage and specificity (Hajong et al. 2013). These investigations suggested that the diverse endophytic fungi screened from *Dendrobium* plant species could have diverse applications (Parthibhan et al. 2017).

Several investigations have been devoted to the orchid–fungal interface and manipulation of its valuable substances. The additional sugar supply as an energy source provided from the fungi supports the *Dendrobium* plants to metabolize their stored food reserves for growth and development (Sarsaiya et al. 2020a, b). After the purpose of the linkage is fulfilled, the orchid still maintains the linkage with similar or different or various endophyte fungi in the roots and other organs (Smith and Read 2008). The nonmycorrhizal orchid endophyte fungi have been known for their manifold ecological functions and bioactive abilities (Yuan et al. 2009), although most of the investigations have recognized only identifications and characterizations of the many fungal endophytes. Thus, the roles of these nonmycorrhizal fungi are currently largely unknown (Parthibhan et al. 2017).

In orchid plants, leaf and stem endophytic fungi are reported to depress the mycorrhizal fungi development rate (Omacini et al. 2006). The root mycorrhizal fungi were not examined In *Dendrobium aqueum*, although the endophytic accumulation was relatively higher in the stems than in the leaf as well as the pseudobulb. The total fungal development rate was also complex on stem segments as related to leaf and pseudobulb parts, which is comparable to *Dendrobium nobile* (Yuan et al. 2009); in *Dendrobium loddigesii*, the leaves contain a greater quantity of endophytic fungi than the stem. This difference might occur because of the sterilant ($HgCl_2$) used; subsequently, the degree of external sterilization was described to greatly overturn repossession by the endophytic fungi (Hyde and Soytong 2008).

17.2.4 Endophytic Microbial Mechanism Responsible for Induced Product Development

Recent investigations showed that when *Dendrobium nobile* was inoculated with *Mycena* sp., and the isolated fungal mycorrhiza from the *Dendrobium officinale* roots sampled, its entire alkaloid percentage was significantly amplified (18.3%), suggesting that mycorrhizal fungi might encourage the biosynthesis of dendrobine. However, the association between host plant and the mycorrhizal fungus is still not very clear, which delays the application of mycorrhizal fungi. Therefore, considering the regulatory machinery of fungal mycorrhiza and refining the dendrobine percentage of *Dendrobium nobile* are of considerable importance.

HMGS and HMGR have been well recognized as equally vital key enzymes in the MVA pathway. Many investigations provided signs of a positive connection between sesquiterpene production and the expression of these two genes. Unexpectedly, this report does not indicate a specific relationship between these two genes and the dendrobine content, as shown by RNA-seq and qRT-PCR. These results suggested that the genes might not have an active function in the process of dendrobine biosynthesis infected by MF23 (Li et al. 2017a, b, c).

In the MVA pathway, the first enzyme, AACT, shrinks two molecules of acetyl-CoA to produce acetoacetyl-CoA in terpenoid backbone biosynthesis. PMK and MVD are crucial ATP-dependent enzymes in the MVA pathway that directly affect

isopentenyl diphosphate (IPP) biosynthesis, the building block of sesquiterpene skeletons. The three genes that encode the corresponding enzymes, AACT, PMK, and MVD, were highly expressed with increased dendrobine content. Previous reports confirmed that product accumulation of downstream reactions in the MVA pathway was enhanced in AACT-overexpressing transgenic plants. Therefore, AACT might take a similar role in the dendrobine biosynthesis induced by MF23. In addition, the increases in PMK and MVD expression levels in the model group improved the dendrobine content, which might be the result of increased IPP biosynthesis. In recent years, homologous genes of PMK and MVD have been cloned from *Amomum villlosum*, *Ginkgo biloba*, and *Eleutherococcus senticosus*. One report proved that PMK was highly expressed in the roots of *Aconitum heterophyllum* Wall where the aconite terpenoid alkaloids were synthesized and accumulated. Our results were similar to those in these reports just mentioned, but our work investigated the PMK expression level in *D. nobile* for the first time and was the first report on the effect of the MVD gene on regulating dendrobine biosynthesis, which provided a foundation for further research on MVD and PMK (Li et al. 2017a, b, c).

Sesquiterpene synthases accelerate the development of the sesquiterpene backbone from FPP53. Therefore, these enzymes are widely observed as the rate-determining regulatory phase in MVA pathways. In this study, the expression level of TPS21, a gene encoding sesquiterpene synthase, was negatively correlated with dendrobine biosynthesis. According to the structural features of its catalysate humulene, TPS21 was not the specific sesquiterpene synthase involved in dendrobine biosynthesis. Moreover, diverse reports have proved that different types of sesquiterpene synthases may exist in one plant, and these enzymes compete as they consume the common precursor, FPP, to synthesize different types of sesquiterpenes. Therefore, the low TPS21 expression level might cause more FPP to flux to the biosynthetic pathway of dendrobine, and TPS21 might affect dendrobine biosynthesis irregularly after plants are diseased by MF23. Post-modification enzymes, for example, cytochrome P450, *N*-methyltransferase, and aminotransferase, were important for all secondary metabolic plant pathways, together with alkaloid biosynthesis, hormones, signaling molecules, UV protectants, fatty acids, defense compounds, and pigments. It has been established that about one-half of the projected 19 enzymes in the taxol biosynthesis pathway were determined to be cytochrome P45059. Different studies revealed that by silencing genes encoding aminotransferases, the biosynthesis of relevant alkaloids in the opium poppy and *Camellia simensis* could be specifically concerned (Li et al. 2017a, b, c).

17.3 Application of Bacterial and Fungal Endophytes

17.3.1 Development of the Protocorm of Dendrobium

Dendrobium is among the very important ornamental orchids and is difficult to grow quickly by vegetative means. Propagation is usually performed by seed in vitro, but the difficulty is that this does not produce an unvarying crop and flower

color is not stable. The best results are obtained with the development of the protocorm. According to Restanto et al. (2016), using 20 g protocorm in a bioreactor for 8 weeks can produce 18,000 protocorms proficiently with 18,000 uniform orchid seedlings (Restanto et al. 2016).

Since the fungal endophytes that can produce taxol were described by Strobel and Daisy (2003), it was concluded that the appropriate endophytic fungi to ferment and synthesize wide-ranging active ingredients is one active way to resolve the resource deficiency of the few plant derivative compounds. Endophytic fungi, microbes that exist in host plant tissues and cause no deceptive destruction to the host plant throughout the observed phase in their growth cycle, are recognized to yield some intermittent and novel natural mediators with prominent pharmacological activities such as antitumor and antimicrobial (Wu et al. 2015).

An elicitor is a precise factor that can activate enzyme sites during plant biological metabolism, and elicitor stimulation can improve the metabolite content or even produce novel materials (Wang et al. 1999). Elicitors are categorized into two categories dependent on their origin, whether abiotic or biotic. Biotic elicitors have a natural origin and contain polysaccharides, glycoproteins, proteins, and cell wall fragments from bacteria, fungi, and plants. Abiotic elicitors have a nonbiological origin and include metal ions, UV light, and chemically defined compounds. Eliciting cultures with biochemical substances has been the most consistent method and a useful biotechnological means for assessing metabolite production in increased amounts (Radman et al. 2003). Some investigations have also used biochemical elicitors to encourage the production of bioactive substances (Sahu et al. 2013; Largia et al. 2015). By means of a volatile methyl ester of the herb hormone jasmonic acid, methyl jasmonate (MeJA) has been identified as a signaling molecule under biotic and abiotic stress. Some salicylic acid (SA) that caused an alternative pressure on signaling particles was lengthily investigated for its role in prompting plant resistance to pathogens (Rao et al. 2000). Up to the present time, however, MeJA or SA is an active elicitor utilized in various herb cultures (Sivanandhan et al. 2013; Largia et al. 2015; Wang et al. 2016).

17.3.2 The Production of Antibacterial Substances by Endophytes

The secondary metabolites formed by fungal endophytes are greater than those of other endophytic microbe categories, which might show moderate significance in the high occurrence of endophytic fungi isolation from plants (Zhang et al. 2006). With similar purpose, some fungal species appear to have advanced occurrence of isolation and consequently a relatively better means of determining an antibacterial compound formed by related species. The detection of an antibacterial consequence of a crude product of the broth culture or the mycelium is the primary step needed for the encounter of an original antibiotic. It often occurs that the separate substances, including some crude materials, do not have effective antibacterial action

themselves but performance synergistically when combined. The identification and structure elucidation of the strongest metabolite is vital in the expansion of an original antibiotic that could possibly be used in therapy.

Two new 10-oxo-10-*H*-phenaleno [1,2,3-de]-chromene-2-carboxylic acids, xanalteric acids I and II, and 11 known secondary metabolites were found from endophytic *Alternaria* sp. extracts, purified from the mangrove *Sonneratia alba* plant found in parts of China (Kjer et al. 2009). The two new substances, xanalteric acids I and II, exhibited feeble antibacterial activity against *Staphylococcus aureus*, with a minimum inhibitory concentration (MIC) of 250 and 125 g/ml, respectively. Altenusin showed comprehensive antimicrobial action against numerous impervious pathogens with MICs of 31.25–125 g/ml.

In a recent investigation of antimicrobial action of crude fungal extracts from endophytic fungi (from mangrove), Buatong et al. (2011) analyzed a total of 385 fungal extracts from 150 fungal endophytes by means of an antimicrobial screening method (a colorimetric microdilution procedure). They isolated fungal endophytes from leaves and branches of 12 mangrove species (*Avicennia alba*, *Aegiceras corniculatum*, *Avicennia officinalis*, *Bruguiera parviflora*, *Bruguiera gymnorrhiza*, *Lumnitzera littorea*, *Rhizophora mucronata*, *Rhizophora apiculata*, *Sonneratia caseolaris*, *Xylocarpus granatum*, *Scyphiphora hydrophyllacea*, and *Xylocarpus moluccensis*) screened from mangrove regions in Thailand in the south of Satun, Surat Thani, Songkhla, and Trang states. Ethyl acetate crude extracts were formed from ethyl acetate, culture broth, and hexane extract from the mycelia of fungi, with minimal bactericidal (MB) and minimal inhibitory (MI) concentrations against human pathogens. Ninety-two microbial isolates formed inhibitory substances. Most of the crude extracts (28–32%) repressed *Staphylococcus aureus* growth. Only 2 crude extracts repressed *Pseudomonas aeruginosa* growth, and none of the other extracts repressed *Escherichia coli*. The maximally active fungal extracts were purified from six distinct genera, including *Diaporthe*, *Acremonium*, *Hypoxylon*, *Pestalotiopsis*, *Phomopsis*, *Phomopsis*, and *Xylaria* recovered from *Rhizophora apiculate*, which presented the widest antimicrobial range with low MIC values of 8–32 g/ml against gram-positive bacteria.

Chromatographic extracts separated from cultures grew well in solid or liquid on rice media for the endophytic fungi *Ampelomyces* sp. recovered from the *Urospermum picroides* medicinal plant, which produced 14 natural compounds that were purified based on their ^{13}C- and ^{1}H-NMR as well as mass spectra and evaluation with earlier available data. 3-*O*-Methylalaternin, found from the *Ampelomyces* sp. extracts developed in liquid medium, and altersolanol A, from the fungi developed on solid rice media, showed antimicrobial activity against of the pathogens. 3-*O*-Methylalaternin showed activity with a MIC of 12.5 g/ml against *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *S. aureus*. Altersolanol A had an MIC of 12.5 g/ml against *S. epidermidis* and *E. faecalis*, and 25 g/ml against *S. aureus* (Yu et al. 2010). An analysis by Yagi et al. (1993) had previously found that altersolanol A prevents the growth of bacteria, especially gram positive, and *Pseudomonas aeruginosa* IFO 3080 when verified by means of the broth medium dilution technique (Radic and Strukelj 2012).

Aspergillus sp. was recovered from *Garcinia scortechinii* root samples, a small tree widespread in Malaysia that is frequently used by the native people for postpartum care and peptic ulcers (Ramasamy et al. 2010). Xanthones recovered from the plant host *G. scortechinii* had been found beforehand to prevent methicillin-resistant *Staphylococcus aureus* (Jain et al. 2019). The antimicrobial action of the crude extract of an ethyl acetate agar culture was tested against *Escherichia coli*, *Bacillus subtilis*, and *S. aureus* as well as *Micrococcus luteus* using a disc diffusion process (Radic and Strukelj 2012; Waghunde et al. 2017).

17.4 Endophytic Fungi as Producers of the Same Metabolites as the Host Plant

Some endophytic microbes can produce intermittent and vital bioactive compounds with properties similar to the distinctive properties of the herb host (Wu et al. 2007). This capability is of great importance in that it offers an alternative approach for the need to increase the yield of sluggishly growing and perhaps rare plants and also benefits preserving the world's always diminishing biodiversity. Furthermore, the higher production value of the phytochemical by manipulating microbial growth is easier and more cost-effective, leading to increased obtainability and abridged product marketplace prices (Waghunde et al. 2017). One of the greatest possibilities is the taxol-producing fungal endophytes, but specimen compounds of endophytic fungi with antibacterial action originally occurred in host plants have also been observed (Radic and Strukelj 2012).

Kusari et al. recovered the endophytic fungus identified as *Thielavia subthermophila* from the host plant *Hypericum perforatum* to examine the formation of hypericin, a naphthodianthrone consequential, and its precursor (Kusari et al. 2014). Both compounds confirmed antimicrobial action against several fungi and bacteria, including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella enterica*. *Eucommia ulmoides* Oliver is an outdated medicinal plant species widely used in China, and it is one of the chief rich sources of chlorogenic acid. This common medicinal source is in very limited supply currently because of the overexploitation of the native plant, which is now endangered in China. Chen et al. (2010) thought that by recovering a fungal endophyte from *Eucommia ulmoides* which would be suitable to produce a secondary metabolite similar to its host plant they could defend the host plant from extermination and find another way to yield its active ingredients to gratify the need. Chen et al. investigated recovering a fungal endophyte from *Eucommia ulmoides* Oliver and explored whether any of these fungal strains can produce chlorogenic acid (Chen et al. 2010).

Twenty-nine fungal endophytes were recovered on the basis of phenotypic character and were categorized into six major groups: N, B, S, C, A, E. From the antimicrobial action of chlorogenic acid, the recovered endophytic fungi were differentiated as probable makers of chlorogenic acid based on the antibacterial action of their extract. Most showed positive results for antibacterial action and were thus exam-

Table 17.3 Endophytic fungi producing metabolites with antibacterial activity

Endophytic fungi	Plant	Metabolite	Effective against	Method used	Reference
Fusarium solani	*Dendrobium loddigesii* Rolfe; stem, leaf, root	Ethyl acetate extract	*E. coli, Bacillus subtilis, Staphylococcus aureus*	Paper disc diffusion test	Chen et al. (2010)
Fusarium spp., *Phoma* spp., *Epicoccum nigrum*	*Dendrobium devonianum* Paxton; stem, root, *Dendrobium thyrsiflorum*; stem, root	Ethanol extract	*B. subtilis, S. aureus, E. coli*	Agar diffusion test	Xing et al. (2011)
Fusarium nivale	*Dendrobium crumenatum* Sw.	Secondary metabolites	*Candida albicans, C. tropicalis*	Kirby-Bauer disc test	Mangunwardoyo et al. (2011)

ined by high pressure liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC-MS), and liquid chromatography (LC)–MS. Chromatographic study indicated that strain B5 might be able to produce chlorogenic acid, even though the yield was comparatively low and was not quite appropriate for a corporate manufacturing level (Table 17.3) (Radic and Strukelj 2012).

17.5 Molecular Approach for *Dendrobium*-related Microbial Endophytes

The rapid growth in microbial molecular biology recently has not permitted outdated microbial examination approaches to be accompanied by the collection of unprecedented amounts of 16S rDNA information (Caporaso et al. 2012). The diverse procedures of molecular approaches, for instance, ribosomal DNA sequencing, single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), terminal restriction fragment length polymorphism (TRFLP), denaturing/temperature gradient gel electrophoresis (D/T-GGE), and novel barcoded amplicon pyrosequencing, are progressively prevalent in the studies of communities of microbes (Bakke et al. 2011). These methods are mostly reliant on 16S rDNA as well as suitable for the examination of microbial samples. Examination of the diversity of endophytic bacteria and their interaction with their host plant is still an identity important for the frontier of microbiology. As per the endosymbiosis concept, the rDNA of chloroplasts and mitochondria demonstrated a higher identity with bacterial rDNA (Rastogi et al. 2013).

Yu et al. (2013) described the universal bacterial primers likely to amplify segment of the chloroplast as well as mitochondrion 16S/18S rDNA, excluding that of endophytic rDNA from *D. officinale*, because the preponderant plant genome is

contained in the total genome DNA (Awasthi et al. 2020). It is hence unsuitable for known molecular microbial techniques, including SSCP, ARDRA, RAPD, T-RFLP, pyrosequencing, and DGGE/TGGE, all of which are based on the bacterial 16S rDNAs (Table 17.4).

17.5.1 Identification Approaches

Morphological identification of fungal endophytes is not as expensive as hitherto. For morphological or phenotypic characters, fungal identification, color of mycelia, number of nuclei/young hyphal vegetative cells, and the teleomorph morphology can be intact for species differentiation. However, it is typically hard to recognize orchid endophytic fungi at the species level and sometimes at the genus level because they will not sporulate readily in cultures or unless acquired for at least a few weeks (3–4 weeks) to months or years. Some fungi are still unidentified up to the present (Agustini et al. 2016).

Consequently, internal transcribed spacer (ITS) region molecular sequencing is generally chosen to identify and characterize fungal endophytes, whether alone or included in phenotypic identification. Henceforth, to preserve any wild orchid inhabitants and to reinstate the ex situ preserved plants to a natural habitat, data on their definite 76 fungal associates are vital (Parthibhan et al. 2017).

The molecular technique using DNA sequences was used to confirm the *Rhizoctonia* that was isolated. First, we used ITS rDNA sequences for the analysis, with results closely related to *Ceratobasidium* sp. (JX913817), which belongs to Basidiomycota, with 90–91% similarity. The 28S recombinant DNA sequences were analyzed at that time to confirm the identification of these orchid mycorrhizal fungi because of the low sequence similarity of ITS (Agustini et al. 2016).

17.5.2 Construction of Specific Primers

Many specific primers have been considered to distinguish bacterial and fungal endophytes in some plants. A primer 799f (*E. coli* numbering, 5′-AACMGGATTAGATACCCKG-3′) was considered that can increase maximum bacterial sequences with the elimination of chloroplast DNA (Yu et al. 2013).

17.6 Environmental Abiotic Factors Affecting *Dendrobium* Plant Products

Alkaloids are a type of secondary metabolite of plants with significant roles in defensive responses to ecological pressures. For instance, binary trauma induced an upsurge in indole alkaloid biological synthesis in the plant *Catharanthus roseus*.

Table 17.4 Specific primers, sequences, and tools used for the molecular identification of endophytes

Primer	Sequence	Software	Plant name	Microbes	Reference
Primer pairs: ITS1, ITS4	ITS1: (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)	BLAST, Clustal X	*Dendrobium nobile*	Fungi	Yuan et al. (2009)
Primer pairs: fC3/rM6-1, fC3/rM6-2, fC3/rM6-3, fC3/rM7, fC4/rM6-2, fC4/rM7, and fC4/rM6-3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′	BLASTN Algorithm, MEGA4.0	*Dendrobium officinale*	Bacteria	Yu et al. (2013)
Primer pairs: NL1/NL4 and ITS1/ITS4	5′-GCATATCAATAAGCGGAGGAAAAAG-3′/ 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and 5′-CTTGGTCATTTAGAGGAAGTAA-3′/ 5′-TCCTCCGCTTATTGATATGC-3′	Sequence Alignment Editor (Se-Al) v.2.0, CLUSTAL X v.1.8	*Lauraceae* and *Rutaceae*	Fungi	Ho et al. (2012)
Primer pairs: ITS1 and ITS4	5′-TCC GTA GGT GAA CCT GCG G-3′ and 5′-TCC TCC GCT TAT TGA TAT GC-3′	BLAST, Clustal X 2.1	*Dendrobium officinale*	Fungi	Jin et al. (2017)

Under deficiency conditions, the alkaloid quantity in the motherwort roots was improved to 1.7 fold more developed than the control sets, with a substantial change ($p < 0.01$). When infected by *Ceratocystis fimbriata*, several alkaloids in mango increased at different degrees of resistance to infection. Similarly, increased levels of MF23, as a foreign invader for *D. nobile*, might elicit plant defense, which is likely to include the alkaloid (dendrobine) biosynthetic pathway (Li et al. 2017a, b, c).

17.7 Conclusion and Future Prospects

Dendrobium plants have been widely used therapeutically as medicinal herbs and in nutraceutical production since early times in China. The previous three decades have seen growing study of the polysaccharides and their alkaloids isolated from *Dendrobium* herbs. Bioactivity including antitumor, immunomodulatory, antidiabetic, and antioxidant characteristics has been observed to originate from *Dendrobium* products. There are numerous important features of orchids that require immediate consideration and which will only be conceivable with microbial transformation. The major challenge for such investigations, however, is to produce large populations of orchids for assessment and analysis, as orchid plants are overall very sluggish and exclusive in growth. The identification and metabolic products of endophytic microbial species present extreme challenges for any plant pathologist. Endophytic microbial taxonomy has been continually varying for decades and continues to change, so assigning a name to a particular microbial species can be equally problematic as well as divisive.

It is significant to notice that producing the antibacterial constituents of these fungi has been accomplished in a test center, and more effort is required to isolate as well as purify these fungi with the aim to determine their structures and mechanisms. Molecular analysis is rapidly becoming the norm for classification and identification of endophytic microbial species because it offers the most reliable and consistent method for recognition of alterations between different groups. Pathogenicity tests can then be conducted to further characterize a species and its disease-causing capabilities. Once proof of identity and pathogenicity are established, separation procedures can be applied as needed to avoid the progress of disease into new areas. Therefore, further comparative investigations of chemical constituents among *Dendrobium* plants and its endophytic fungi are certainly desirable.

Because endophytic fungi are sought for in plants that have established use in traditional medicine or which grow in areas of great biodiversity, it is understandable that the discovery of many possibly useful metabolites can be expected. Concentrating on the examination of endophytic fungal range, relationships among endophytic fungi and their host herbs, looking for natural bioactive compounds thus formed, and refining the productivity of many potential natural substances, we can take primary advantage of genetic and molecular technologies and microbial

fermentation developments for the detection of much-needed antibiotics to control contagions caused by multidrug-resistant microbes. It is very clear that we have a similarly long path to accomplish the ultimate goal.

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Chapter 18
Understanding Its Role Bioengineered *Trichoderma* in Managing Soil-Borne Plant Diseases and Its Other Benefits

Santanu Sasidharan, Palistha Tuladhar, Shweta Raj, and Prakash Saudagar

18.1 Introduction

Trichoderma is the genus of a very versatile and opportunistic plant symbiotic fungus (Singh et al. 2018). This filamentous fungus is generally found in the agricultural soil and decaying soil; however, studies on the *Trichoderma* species of marine origins have also been conducted (Naher et al. 2014). This fungus is one of the most studied in the world because of its numerous applications in the industrial, agricultural, and clinical areas. And the researches on this family are on increase as with the increasing number of discoveries related to its use in human welfare (Mukherjee et al. 2013). It is generally asexual, but it has been recorded that *T. reesei* can undergo sexual development under experimental conditions (Mukherjee et al. 2013).

These fungi have a high proliferation rate and are prolific producers of secondary metabolites bioactive volatiles and nonvolatiles along with a huge arsenal of enzymes, antibiotics, and mycotoxins which have numerous applications and have aided the agricultural, industrial, and clinical area. It has a complex and dynamic relationship with the inhabitants of the rhizosphere (Mukherjee et al. 2013). It has a symbiotic relationship with the plants and helps in providing it protection against phytopathogenic diseases. Moreover, it helps in plant growth and development (Naher et al. 2014). It also performs bioremediation on the soil and improves its quality (Schuster and Schmoll 2010). They are also known to increase the tolerance of the plants against environmental stresses such as drought, high temperature, and salinity. Furthermore, it is used as a biopesticide or enhancer which is an alternative to the harmful chemicals used in the plant disease management. Also, the recombinant ones are used for industrial purposes for the production of the proteins (Naher et al. 2014).

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The classification had been difficult until the development of the DNA barcoding and study of the molecular structures. Still the classification of some of the most widely used species is not completely correct due to its overlapping characteristics (Mukherjee et al. 2013). Correct identification is necessary as to unleash the full potential of the species. Currently, the whole genomic sequence of seven species of *Trichoderma* genus is known as *Trichoderma reesei*, *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma virens*, *Trichoderma atroviride*, *Trichoderma longibrachiatum*, and *Trichoderma citrinoviride* (Singh et al. 2018).

However, all is not good. Recently, it has been discovered that *Trichoderma* species have been known to cause different diseases in animals and even humans; therefore, when used for the agricultural purposes, they must be tested (i.e., its ability to grow at 37 °C as this is the factor which contributes to its pathogenicity in humans) to assure the safety of its use (Mukherjee et al. 2013).

18.2 Diversity in *Trichoderma* sp.

For many years, *Trichoderma* sp. were considered to be a single species called *Trichoderma viride* because of their morphological similarity (Bisby 1939). Only in 1969, nine morphologically distinct species were formed to meet the need of the new discoveries and taxonomical demand (Rifai 1969). The accurate detection of species is difficult based on morphological features and availability (Druzhinina et al. 2005; De Respinis et al. 2010). In the twenty-first century, DNA methods and sequencing techniques brought more information on the different species of *Trichoderma* such as its identification (Hermosa et al. 2001; Atanasova et al. 2010; Samuels et al. 2010) and phylogenetic classification (Lieckfeldt 2000; Kullnig-Gradinger et al. 2002). The category *Trichoderma harzianum Rifai* included mycoparasites that could act on many aerial and soil-borne pathogens; hence, they are of economic importance (Gams and Meyer 1998). Due to a large number of mycoparasites existing in this genus, sometimes, there is a difficulty in identifying each of the strains due to character overlap. Events of evolution in the organisms that are antagonistic to the pathogens resulted in *Trichoderma* sp. being active against fungi from which they were isolated initially (Grondona et al. 1997). This, in turn, is a boon as they do not target non-specific organisms. Even though this species has been investigated for over 70 years and several genomes of this fungus have been sequenced, the commercial availability was explored only recently. The main *Trichoderma* strains that are marketed with potential applications include *Trichoderma harzianum*, *Trichoderma viride*, and *Trichoderma virens*. A brief list of genera that are affected by this plant pathogenic fungi contains *Armillaria*, *Botrytis*, *Chondrostereum*, *Colletotrichum*, *Dematophora*, *Diaporthe*, *Endothia*, *Fulvia*, *Fusarium*, *Fusicladium*, *Helminthosporium*, *Macrophomina*, *Monilia*, *Nectria*, *Phoma*, *Phytophthora*, *Plasmopara*, *Pseudoperonospora*, *Pythium*, *Rhizoctonia*, *Rhizopus*, *Sclerotinia*, *Sclerotium*, *Venturia*, *Verticillium*, and wood-rot fungi (Lumsden et al. 1995; Monte 2001).

Metabolically, *Trichoderma* shows diversity in utilization of substrates. There have been several approaches to group them according to their carbon utilization (Manczinger et al. 2002). *Trichoderma* also shows a species-specific preference for growth where they have evolved to utilize a range of sources like L-phenylalanine, N-acetylmannosamine, arabitol, trehalose, and sorbitol in *Trichoderma harzianum*; *Trichoderma atroviride* grows well in lactulose and maltotriose, *Trichoderma asperellum* on raffinose, dextrin, and D-glucose (Lopes et al. 2012). Diversity has also been observed in the secretion of secondary metabolites which are volatile antibiotics, water-soluble compounds, and peptaibols (Ghisalberti and Rowland 1993). The production varies according to the strain, specific compound, microbial presence, and balance between biosynthesis and biotransformation rates (Degenkolb et al. 2008; Vinale et al. 2009).

Even though the fungi are considered as natural and non-threatening to the general public, the assessment of these fungi's effect on nontarget organisms needs to be assessed before its commercial exploitation. The diversity in these fungi can be known only by using the contemporary sequencing analyses and metabolic diversity, which can just corroborate the identification. The diversity in the ITS sequences of various *Trichoderma* spp. is represented in the Fig. 18.1.

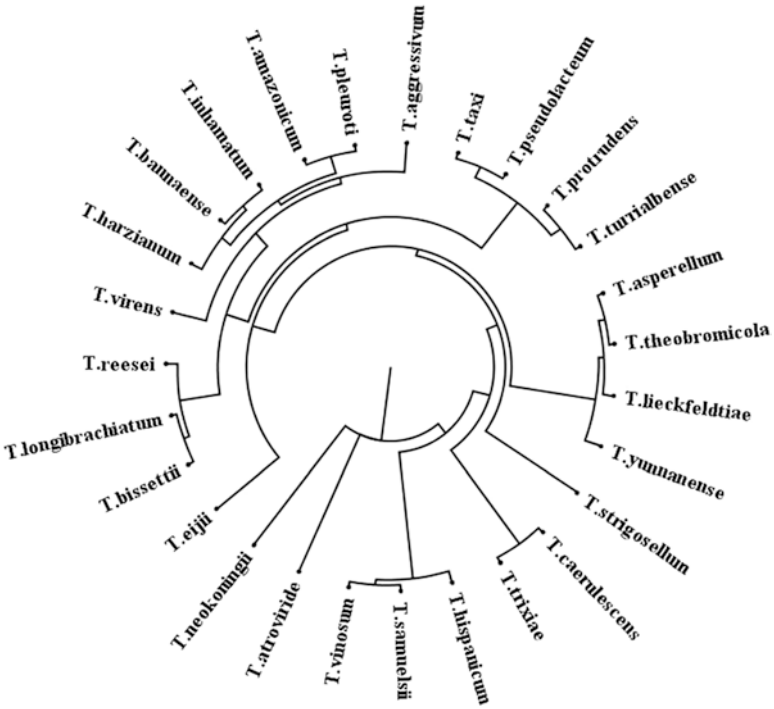

Fig. 18.1 Multiple sequence alignment and phylogenetic tree diagram of the ITS sequence of various *Trichoderma* spp. showing diversity and relationship between them

18.3 Bipartite Relationship Between *Trichoderma* and Plants

Trichoderma spp. share an opportunistic/facultative symbiosis with plants by growing in the rhizosphere. They draw the survival energy by penetrating and colonizing the plant roots and further derive sucrose and other nutrients from the plants. In return, they help the host plant by an immunity boost and improved photosynthetic abilities. The rhizosphere containing *Trichoderma* sp. provides a platform for the synchronized relation of transcriptomic, proteomic, and metabolic response in the plants (Brotman et al. 2012; Morán-Diez et al. 2012). This relation helps the plant to grow and resist pathogens efficiently.

18.3.1 Colonization of Roots by Trichoderma *sp.*

Trichoderma sp. has the ability to colonize roots both internally and externally. This ability has been attributed to various chemical signals that pass between both the roots and the fungi. The chemical signaling between the partners is poorly understood, but the sequence of interactions that follows has been studied in deep detail. The steps include attachment to the root, penetration of the root, and internal colonization of the roots. The fungi grow and stabilize inside the root by promoting the root growth using auxin secretion. The increase in root growth is directly proportional to the surface area colonized by *Trichoderma* sp. The genetic decoding of various genes has been done to understand the colonization in a better way. 1-Aminocyclopropane-1-carboxylate deaminase or ACCD gene regulation by *Trichoderma asperellum* in canola roots was studied by gene knockout method, and it was observed that the enzyme causes root growth and development (Viterbo et al. 2010). Deployment of small cysteine-rich hydrophobin-like protein has been found in *Trichoderma* sp. for attachment and anchoring to the roots. TasHyd1 and Qid74 from *T. asperellum* and *T. harzianum* are two such proteins that have been found to assist the attachment of the fungi (Viterbo and Chet 2006; Samolski et al. 2012). Another effector protein called tvhydii1 belonging to class II hydrophobin family has been discovered, whose overexpression increases root colonization and deletion reduces root colonization (Guzmán-Guzmán et al. 2017). *Trichoderma* sp. also secretes expansin-like proteins that are capable of binding to cellulose and loosening the structural order to make it more accessible for degradation by hydrolytic enzymes (Brotman et al. 2012; Morán-Diez et al. 2012). Once penetrated, the fungi can grow in large numbers in the epidermal layer and the outer cortex. Swollenin SWO1 is a major expansin from *T. reesei* used for saccharification of lignocellulosic substrates (Eibinger et al. 2016). To overcome the plant defense, the fungi (*T. koningii*) suppress the phytoalexin production during the root colonization period in *Lotus japonicus* roots (Masunaka et al. 2011).

18.3.2 Plant Growth Promotion

Trichoderma sp. produces many secondary metabolites, and koninginin A from *Trichoderma koningii* and 6-pentyl-alpha pyrone from *Trichoderma harzianum* are metabolites that act as plant growth regulators. *Trichoderma* sp. also decreases soil pH, produces citric acids and gluconic acid, and helps in minerals, phosphates, and micronutrient solubilization for better uptake by plants (Harman et al. 2004c; Vinale et al. 2008). Two strains, *Trichoderma harzianum* T22 and *Trichoderma atroviride* P1, were found to improve the growth of lettuce, pepper, and tomato plants under greenhouse and field conditions and demonstrated 300% increase in crop productivity. Treatment of plant seeds with *Trichoderma conidia* also increased the yield, showing that *Trichoderma* metabolism helps in plant growth (Benítez et al. 2004). *T. harzianum* treatment of tomato seeds accelerated the seed germination and seedling vigor and increased the salinity, osmotic, chilling, and heat stresses. The mechanism has been attached to the reduction of ethylene (ET) production (Gravel et al. 2007). Rhizosphere colonization of maize also displayed higher photosynthesis rates and CO_2 uptake when *T. virens* was added in calculated quantities (Vargas et al. 2009).

18.3.3 Systemic Induction of Plant Defense

The invasion by *Trichoderma* sp. is immediately responded by the plants using a string of reactions like rapid ion fluxes, oxidative bursts, callose deposition, and polyphenol synthesis. This bioagent can also promote uptake of nutrients, plant growth, and induction of plant responses to stresses caused by biotic and abiotic factors (Shoresh et al. 2010; Hermosa et al. 2012). The events that succeed these events assist the plants in acquiring tolerance to various pathogens. Various responses in the plant defense include chemical inducer signal molecules like salicylic acid (SA), jasmonic acid (JA), and methyl jasmonate (MeJA). The increase in tolerance is caused by salicylate and jasmonate/ethylene signaling (JA/ET) (Shoresh et al. 2010). SA and MeJA act to elicit various defense compounds like polyphenol, alkaloids, and pathogenesis-related proteins. The increase in tolerance by JA/ET signaling, termed as induced systemic resistance (ISR), is similar to the response triggered by plant growth-promoting rhizobacteria (PGPR). Increased dosage of SA can also induce SA-mediated systemic acquired resistance (SAR) which is similar to the response triggered by necrotrophic pathogens and also induce transcriptional reprogramming (Contreras-Cornejo et al. 2011; Salas-Marina et al. 2011; Yoshioka et al. 2012).

Many small molecules like xylanase and peptaibols like alamethicin and trichovirin II secreted by *Trichoderma* sp. illicit immune response in plants (Luo et al. 2010; Druzhinina et al. 2011). The Sm1/Ep11 is an ISR gene that codes for small

cysteine-rich hydrophobin-like protein from the cerato-platanin family, and the deletion of this gene from the fungi caused impairment in the ISR of maize (Djonović et al. 2006a, 2007b). The mechanism involves the monomeric form which is essential for the elicitation properties, whereas oxidative dimerization causes Sm1 to be inactive and downregulating ISR (Vargas et al. 2008). The family of cerato-platanin is highly conserved, and its structure and carbohydrate-binding properties suggest this mechanism (de Oliveira et al. 2011).

The generation of reactive oxygen species (ROS) such as hydroxyl (OH^-), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2) is regarded as the first line of defense mounted by the plants on the pathogens (Lehmann et al. 2015). The production indirectly controls the growth and development of the plants to destroy or bring down the biotic or abiotic stress. The ROS and its intermediates execute their response by a plethora of mechanisms like lignin cross-linking, cell wall reinforcement, hypersensitive response, and SAR development and by pathogen inhibition. Plants are immune to the ROS molecules and its intermediates due to the production of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and peroxidases. These enzymes in plants collectively detoxify the plant cells from ROS.

18.3.4 Influence of Soil Environment

The presence of *Trichoderma* in soil affects the availability of organic nutrients in the soil, and this, in turn, can affect the activities associated with *Trichoderma* (Hoitink and Boehm 1999; Wakelin et al. 1999). The main hindrance remains with the soil conducive environment that should complement the strain used (Leandro et al. 2007). Organic compost has been found to work best with *Trichoderma*, and the activity was found to be higher in the presence of the nutrients available from the compost. The nutrients found include carbohydrates from lignocellulose material breakdown, chitin, lipids, and other micronutrients (Hoitink et al. 2006). The damping-off of radish and crown caused by *Rhizoctonia* was significantly reduced in the presence of *T. hamatum* even though the potting mix contained high microbial content (Krause et al. 2001). In cucumber, the strain induced SAR and reduced the *Phytophthora* (leaf blight) significantly (Khan et al. 2004). All these studies were found to deliver better results in the presence of organic compost supplement soil mix rather than low microbial content soil. The hand-in-hand relations shared by *Trichoderma* sp. with soil microbes make them a favorable biocontrol agent in the field of organic farming. Root colonization with *Trichoderma* causes a shift in the microbial community. Colonization of maize roots with *T. harzianum* T12 reduced *Azospirillum* sp., but the *Pseudomonas* sp. population remained the same (Vázquez et al. 2000). There were large shifts in the other bacterial population when inoculated with BCA. *Armillaria mellea*, pathogen infection, was reduced significantly in grape wine when *T. atroviride* was applied (Savazzini et al. 2009). There have been changes in the effect of *Trichoderma* on the soil environment, and these have been found to be due to the induced resistance.

18.4 Bipartite Relationship Between *Trichoderma* and Pathogens

18.4.1 Mycoparasitism

Trichoderma possesses an ability to kill other fungi by parasitism, and this ability paved way for the success of the *Trichoderma* in the market (Mukherjee et al. 2012a). The ability of mycoparasitism is a complex sequential event that has three steps: recognition, attack and penetration, and finally killing of the host fungi (Benítez et al. 2004). Mycoparasitism is thus studied in detail today using *Trichoderma* as model organisms. *Trichoderma* possess the ability to detect the presence of fungi and parasitize them. This ability has many cellular processes and genes involved, making it a complex process. The sequence of events includes the production of exochitinases and chitinases to degrade the chitin cell wall, followed by secretion of glucanases, xylanases, N-acetylglucosaminidase, glucosidase, endoglucanase, amylase, and proteases. On sensing the presence of a pathogen far away, *Trichoderma* starts branching toward the pathogen (Chet et al. 1997). The process is supported by the presence of lectins, and once attacked, they form appropriate structures around the pathogens. It has also been found that the cell walls are degraded by chitinases and induce the production of exochitinases (Harman et al. 2004b). A mutation study on *T. atroviride* P1 strain showed that the expression of exochitinase (nagI) and endochitinase (ech42) was necessary for mycoparasitism (Vinale et al. 2008).

The mycoparasitism is switched on only when another fungus is sensed and the signals are sent to regulatory targets. The signal transduction pathway has been studied extensively, and they include G-dimeric protein and MAPK and cAMP pathway (Zeilinger and Omann 2007). MAPK TVK1/TmkA pathway is characterized in many species of *Trichoderma*, and they are important in regulating the output for efficient biocontrol (Mendoza-Mendoza et al. 2003; Reithner et al. 2007). As observed in other filamentous fungi, *Trichoderma* sp. also possesses three MAPK cascades: MAPKKK, MAPKK, and MAPK pathways for mycoparasitism and for its ability as a biocontrol agent (Kumar et al. 2010). The binding of signal molecules to G protein-coupled receptor leads to the cascade pathway. G protein-coupled receptor Gpr1 plays a role in the sensing of the host fungus and silencing the gene in *T. atroviride* causing unresponsiveness to host fungus (Omann et al. 2012). There are many other genes, approximately 20–30, that are involved in mycoparasitism and cascade pathways and are the major players in this relationship.

18.4.2 Antibiotics and Secondary Metabolite Production

The survival of *Trichoderma* in the competitive environment of rhizosphere is tough, and the fungi employ its own army of enzyme and other metabolites (Vinale et al. 2008). The metabolites include volatile and nonvolatile compounds

like harzianic acid, tricholin, peptaibols, 6-pentyl-alpha-pyrone, massailactone, viridin, glisoprenins, gliovirin, heptelidic acid, etc. (Lumsden et al. 1992; Mukherjee et al. 2012b). Among these, gliovirin from *Gliocladium virens* was found to be an antibiotic that was found to inhibit *Pythium ultimum* and *Phytophthora*, but they were ineffective against *R. solani*, *T. basicola*, *B. thuringiensis*, and *P. fluorescens* (Howell and Stipanovic 1983). More than 100 metabolites have been derived from the fungi including antibiotics and amino acids with antibiotic activity. Few of them include gliotoxin, pyrones, alkyl pyrones, isonitriles, terpenes, polyketides, peptaibols, diketopiperazines, sesquiterpenes, polypeptides, and several steroids (Sivasithamparam and Ghisalberti 2014). The production of these varied metabolites has also been suggested to be used for the taxonomy of the *Trichoderma* sp. Among the peptides found in *Trichoderma*, the first antibiotic peptide or peptaibol to be characterized was paracelsin (Brückner et al. 1984). Peptaibols was first coined in 1992 by Bruckner. Peptabiotics or peptaibols involve a large group of non-ribosomal-dependent peptides possessing antibiotic activity. They are produced mainly by *Trichoderma* and *Hypocreales*. The mechanism of peptaibols involves the degradation of cell wall of fungal pathogens by creating pores/voltage-dependent ion channels and also synergistically provides resistance to plants. The gene responsible for peptaibols synthesis called peptaibols synthetase was cloned recently, and they catalyzed the formation of two different classes of peptaibols (Mukherjee et al. 2011). Aspereline A to F is a family of ten residue antibiotics that were produced by the fermentation of marine-derived *Trichoderma asperellum*. They were isolated from the Antarctic habitat. Aspereline exhibited anti-activity against bacteria and fungi pathogens like *Alternaria solani*, *Pyricularia oryzae*, *Staphylococcus aureus*, and *Escherichia coli*. Trichobrachins are another group of peptaibols of 11 residues that were isolated from *Trichoderma longibrachiatum* MMS151 sourced from blue mussels. They are categorized into three clusters, namely, trichobrachins A, B, and C. Apart from marine-derived peptaibols, several terrestrial ones include trichobrachins from terrestrial strain of *T. longibrachiatum*, trichofumins from *Trichoderma* sp., trichorovins from *T. harzianum*, trichorozins from *T. harzianum*, harzianins from *T. harzianum* and *Trichoderma pseudokoningii*, hypomurocins from *Hypocrea atroviridis*, Tv29-11-I/VI from *T. virens*, and hypojecorins from *T. parareesei*. Many other metabolites have been derived including amino acid derivatives like alkylated 5-ketoproline; dipeptides like trichodermamides A and B; cyclopeptides like cyclopeptide maculosins, atroviridetide, and trichoderide A; polyketides (synthesized by polyketide synthases); cyclopentatone like trichoderones A, B, and C; Anthraquinone like trichodermaquinone; xanthones like trichodermaxanthone; pyranone derivatives like trichopyrone; hexaketide sorbicillinoid derivatives like 6-demethylsorbicillin; octreotide derivatives like trichoharzin, trichodermaketones C and D, trichodermatides B, C, and D, and koninginins A, D, and E; tricyclic polyketides like 10,11-dihydrobisvertinolone, trichodermanones A, B, C, and D, 7-O-methylkoninginin, and trichodermaketones A and B; terpenoids like heptelidic acid cholorohydrin sesquiterpene, blazein, cerevisterol, and cholesta-7,22-diene-3β,5α,6β-triol; and hydroxylactone like harzialactones A and B (Ruiz et al.

2013). All these antibiotics and secondary metabolites contribute both resistances to plants against pathogens as well as help in the survival of *Trichoderma*.

18.4.3 Nutrient Competition

Root exudates and rhizosphere are sources of sugars, amino acids, iron, organic acids, and vitamins. The relationship between pathogen and *Trichoderma* also goes as far as the competition for nutrients. The fungi get rid of the pathogen by its capacity to grow a broad spectrum of substrate utilization and expand faster making it feasible as a control agent. In *T. harzianum*, it was observed that the fungi colonized blossom tissues and exclude *B. cinerea* from grapes (Gullino 1992). The competition for nutrients was also demonstrated in *T. harzianum* against *F. oxysporum* (Sivan and Chet 1989). The fungi can mobilize the nutrients efficiently and uptake them, thus proving a competitive environment for other microbes (Benítez et al. 2004). The presence of high microbial biomass also caused the fungi to shift from hyphal growth to sporulation, an event termed as "soil fungistasis" (de Boer et al. 2003). This event also reduced the efficacy of *T. harzianum*. *Trichoderma* sp. is naturally resistant to herbicides, phenolic compounds, and fungicides. The fungi also produce metabolites to arrest spore germination (fungistatic), kill cells (antibiosis), and modify the rhizosphere by acidifying the soil (production of organic acids like gluconic, citric, and fumaric acids) and do not let other microbes survive. Another mechanism of starvation includes iron uptake which is essential for fungi survival. *Trichoderma* sp. produces highly efficient siderophores capable of chelating iron and stopping the growth of fungi by starvation (Benítez et al. 2004). Siderophores bound to Fe^{3+} undergo reduction to Fe^{2+} catalyzed by ferric-chelate reductase. The Fe^{3+}-siderophore has to be released, and this is mediated by reductases or hydrolases.

18.4.4 Production of Lytic Enzymes

Hydrolytic enzymes are another group of weaponry deployed by the fungi against the pathogens. The trichodermal genome is coded with chitinases and glucanases. These enzymes partially degrade the cell wall causing parasitization. The evidence suggests that chit42/ech42 caused the production of chitinases, but the deletion of the genes was compensated by the reservoir of other genes (Druzhinina et al. 2011). There are over 5–7 distinct chitinases in *T. harzianum*. Glucanases comprise of cell wall lytic enzymes that play a role in mycoparasitism. Ech42 is induced during fungus-fungus interaction and solely uses colloidal chitin as its carbon source and degrades cell wall too. The system also has endochitinases (ech42 of 42 kDa) and two β-(1,4)-N-acetylglucosaminidases (73 and 102 kDa). β-glucanase-encoding gene tvbgn3 was deleted, and it caused reduction in the mycoparasitic ability (Djonović et al. 2006a, b). Co-expression of two β-glucanases

in *T. virens* caused increased biocontrol abilities toward *R. solani*, *P. ultimum*, and *Rhizopus oryzae* (Djonović et al. 2007a). Antibodies against the purified glucanases found that they are not related to each other and are probably encoded by two different genes. Glucanases have the capacity to lyse cell walls of yeast and fungi and bacteria. During mycoparasitism chitinases, glucanases, and proteases (Prb1/Sp1) are also secreted and play definitive roles (Viterbo and Horwitz 2010). Colonization of sclerotial structures of *T. virens* was also found to be due to the secretion of laccase (Catalano et al. 2011).

18.5 Tripartite Relationships Between *Trichoderma*, Plant, and Pathogens

The relationship between plant and pathogen and plant and *Trichoderma* has been studied, but the tripartite interaction between plant, pathogen, and *Trichoderma* has been least pored upon. Numerous plant defense factors, signaling molecules, and virulence and avirulence factors have been identified in plants. The simple partner relationship is easy to decode and study, but the analysis of a complex network of metabolites that interact between these three living systems is tough. Another difficulty in this analysis is to study the interactions in real time rather than studying it in vitro. The interactive talk between the three requires the quantification of gene expression occurring individually and, at the same time, in pairs of two and three. An even advanced study can be done where in situ study analysis of the compounds involves using fluorescent proteins. Even though the structural genomics has shed light on the identity and structure of genes expressed in plant-microbe interactions, proteomics has contributed more in terms of gene expression and protein profiles from various interactions (Lim and Elenitoba-Johnson 2004). One such study involved a three-way interaction study between plant, fungi, and pathogen. The proteomic approach was performed where the proteins were identified and characterized by trypsin digestion, mass spectroscopy, and in silico database analysis. The results showed that there was regulation of the interactions depending on various plant proteins. The presence of antagonist compounds regulated the response of a plant attack both quantitatively and qualitatively. The response mounted by the plant was strictly based on the *Trichoderma* and on the type of antagonistic protein. The proteome from the interaction of *T. atroviride* and soil microbes also showed homologies to fungal hydrophobins and to ABC transporters (Marra et al. 2006). A Gfp-based approach to study the interactions in situ involved the use of different biocontrol-related genes to influence the expression of living producers. The most interesting interactions involved the expression of CWDE genes in the presence of *R. solani* and *P. ultimum*. The Gfp-tagged mutants were found to fluoresce in the presence of host-fungal pathogen, purified colloidal chitin chitooligomers, and during the early phase. The host cell wall secretions of compounds were actively involved in mycoparasitism. The endo- and exochitinase involved in host hyphae degradation was also demonstrated (Lu et al. 2004). Differentially expressed proteins were found in *T. atroviride* proteome when the tripartite interaction between

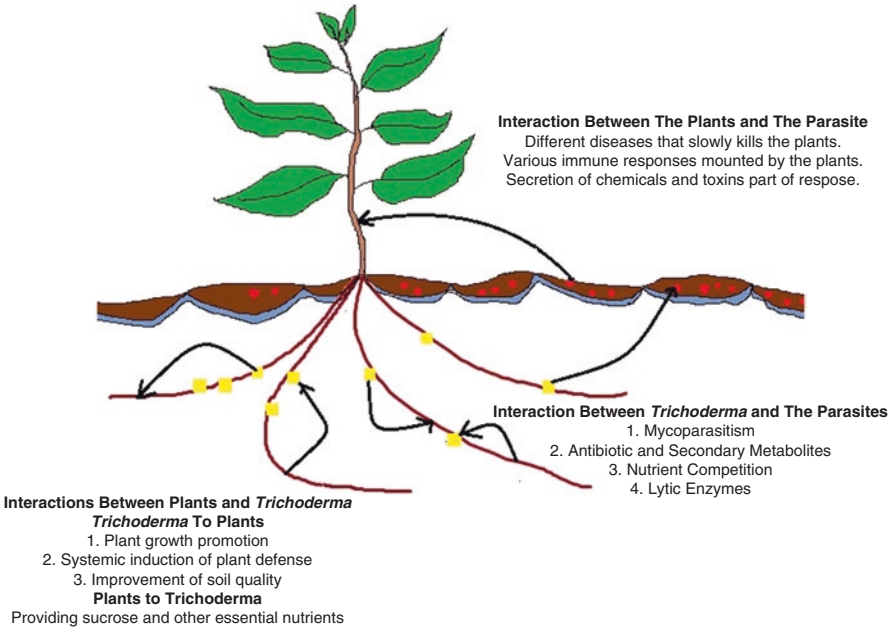

Fig. 18.2 The representation of tripartite relationships between *Trichoderma*, plant, and pathogen. The *Trichoderma* is represented in yellow squares, and the parasites are shown in red

pathogen *B. cinerea* and bean leaves was analyzed. The results suggested that the disease-related factors and plant proteome-specific proteins were regulated. Many homologues of PR proteins were also recorded, and conserved domains like nucleotide-binding site, leucine-rich repeats, SGS domains, Bet vI PR, and Barwin protein families were also found. In *M. grisea* proteome, the virulence factors such as cyclophilins were regulated in the plant and antagonist. The production of cell wall-degrading enzymes by *Trichoderma* when exposed to fungal phytopathogens like *Pythium ultimum* and *Rhizoctonia solani* has also been studied in deep (Keswani et al. 2013). The complexity of the study that is required and the need for a more integrated approach have to be addressed to understand the tripartite relationship much deeper. An illustration of the tripartite relationship is shown in Fig. 18.2.

18.6 Role of *Trichoderma* in Various Fields

18.6.1 Plant Health Promoter

Apart from fighting the pathogens, *Trichoderma* also boosts the health of plants. The fungi are opportunistic symbionts that can enhance the systematic resistance in plants as described above. The communication for improved communication is carried out by MAPK signaling, and consequently, the induction of systematic response takes place (Shoresh et al. 2006). The fungi also enhance root growth by occupying

the roots with the help of swollenin (Brotman et al. 2008). The rhizosphere interaction also protects the plants from toxic chemicals and creates a remarkable resistance to other pathogens. The fungi thus provide a solution for remediation of polluted waters and soil (Harman et al. 2004a).

18.6.2 Secondary Metabolites

Production of secondary metabolites in the ecological niche of the fungi is a necessity for the survival. The myriad of antibiotics produced by the fungi has been discussed above in the section. All these antibiotics and mycotoxins have received increased attention for development as potential drug molecules for various pathogenic bacteria. Various other organic compounds like terpenes, pyrones, polyketides, amino acids, and polypeptides produced by the fungi are also being tapped for their application in pharmaceutical and other analytical industries.

18.6.3 Biofuel, Pulp, and Textile Industry

Cellulases and other plant wall-degrading enzymes that are produced have been used in biofuel production (Rubin 2008). The enzyme cocktail that is extracted from *Trichoderma* decreases the cost of bioethanol production from cellulosic waste largely (Kumar et al. 2008). The cocktail has also proved its efficiency in pulp and paper industry and textile industry. Certain mutation and culture optimization have increased the production of the enzymes by providing cellulase, xylan, and plant polymer mixtures (Mach and Zeilinger 2003). The exploration of genomic sequences has revealed that all the enzyme cocktail enzymes lie within a small set of genes paving way for metabolic engineering to optimize it (Kubicek et al. 2009).

18.6.4 Food Industry

The food industry derives various food additives and related products from *Trichoderma*. Some of the products include β-glucanases for the brewing process, macerating enzymes for fruit juice production, livestock feed additives, and pet food (Blumenthal 2004). Some of the cellulases are used in baking, malting, and even in grain alcohol production. It is not only the enzymes that caught the food industry but also various metabolites. 6-Pentyl-α-pyrone was one of the first products to be isolated from *T. viride* which possesses a characteristic coconut-like odor and has antibiotic properties that can be implemented in the food industry (Oda et al. 2009). The compound is extracted around 7 g/L today from *T. atroviride*. The cell wall-degrad-

ing enzymes from *T. harzianum* were also suggested to be used in food preservation as antifungal agent, but the idea did not materialize much. Similarly, mutanase enzyme from *T. harzianum* can also be used in toothpaste for their ability to prevent the accumulation of mutan that causes dental plaque (Wiater et al. 2005).

18.7 Conclusion and Perspectives

A biocontrol agent is considered successful only when it manages to interact with the plant and pathogen equally and act accordingly. *Trichoderma* sp. successfully qualifies as a biocontrol agent where there is an impressive network of communication between various entities in the soil. The diversity in the *Trichoderma* sp. has revealed that the fungi have survived and evolved multiple mechanisms to form a symbiotic relationship with plants and defensive relationship with pathogens. The symbiotic relationship which includes root colonization, growth promotion, induction of systemic defense, and soil parameter variation has benefitted both the plant and the fungi in various ways. The relationship between *Trichoderma* and pathogen has been very defensive with various strategies including mycoparasitism, antibiotics, secondary metabolites, nutrient competition, and lytic enzymes being deployed at various stages to render the pathogen inactive and dead. The mechanism underlying these relations has been studied in detail, but most of the interactions still remain elusive. The expression of genes of *Trichoderma* is beneficial for the plants as it controls the diseases. The cross talks between the three subjects *Trichoderma*, plant, and pathogen have not garnered much attention among the scientists; nevertheless, there is a need to study this in more detail. The signaling pathways that connect these three, the defense induction, the development of each of the three in the presence and absence of the other, and the combinatorial effect on the three in the presence of biotic and abiotic stress, are potential areas that can unravel the complete potential of these fungi. The use of a completely deciphered *Trichoderma* sp. will certainly help in its usage as an effective biocontrol agent and its implementation in various fields.

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Chapter 19
Beyond Classical Biocontrol: New Perspectives on *Trichoderma*

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19.1 Introduction

Bioengineering, a term often used synonymously with "genetic engineering" or "biotechnology," is an emerging field that combines engineering principles with the study of biological systems. The end goal usually is to produce economically viable, biological products that can augment or replace those produced via synthetic chemistry or other "nongreen" technologies. The three most important genera of filamentous fungi used in biotechnology and bioengineering are *Aspergillu*s, *Penicillium*, and *Trichoderma*. All three of these major genera encompass species with a wide range of known applications in agricultural, industrial, and medical biotechnology, and each has been the subject of a recent scholarly monograph [for *Aspergillus*, see Gupta (2016); for *Penicillium* see Gupta and Rodriguez-Couto (2017); and for *Trichoderma* see Gupta et al. (2014)].

In this chapter, we will focus on the genus *Trichoderma*, with a particular emphasis on some less known aspects of its bioengineering potential. *Trichoderma* research has generated a voluminous scientific literature that has been reviewed, and reviewed well, by others. We will not attempt to revisit material on the industrial production of cellulases by *Trichoderma reesei* or their use in biofuel production from cellulosic waste. Nor will we cover the traditional use of *Trichoderma* formulations as biological control agents whereby *Trichoderma* species kill pathogens through multiple complementary mechanisms that include mycoparasitism; induction of systemic resistance in plants; competition and rhizosphere competence; secretion of siderophores; production of chitinases and glucanases; and the fungicidal action of toxigenic secondary metabolites and peptides. For excellent and

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comprehensive treatments of this material, see Samuels (1996), Howell (2003), Mukherjee et al. (2013), Gupta et al. (2014), and Samuels and Hebbar (2015).

Our goal here is to highlight understudied and underappreciated aspects of the uses of *Trichoderma* in biotechnology. In the first part of the chapter, we will focus on the use of *Trichoderma* volatiles to enhance plant growth or increase resistance to abiotic stress and the ability of many *Trichoderma* species to function as efficient biological fungicides at a distance through the emission of volatile organic compounds. In the second part of the chapter, we will shift our focus to the unexpected benefits of *Trichoderma* treatments on yields of desirable plant secondary metabolites by certain high-value medicinal, aromatic, and spice plant species. In our summary section, we will recommend promising avenues for future research that will enhance the bioengineering potential of *Trichoderma* as well as raise some important research questions.

19.2 Volatile Organic Compounds

Ecologists recognize that in many habitats, water is in short supply and that chemical signals can best be transmitted through the air (Bitas et al. 2013). It is well-known that *Trichoderma* is a successful rhizosphere inhabitant. The rhizosphere environment is well suited for volatile-mediated communication since the partners are spatially close to one another and because volatiles are more likely to accumulate and reach their threshold of activity in enclosed soil pockets than in aboveground atmosphere (Baily and Weisskopf 2012).

Work in our laboratory and others has shown that *Trichoderma* can have beneficial effects on plant growth without physical contact between plants and the fungus, relying solely on the transmission of signals in the atmosphere (Hung et al. 2013; Kottb et al. 2015; Lee et al. 2015, 2016, 2017). These beneficial effects are mediated by volatile organic compounds (VOCs), which are a large group of carbon-based substances with low molecular weight, low polarity, low boiling point, and high vapor pressure. Many of them are lipophilic (Herrmann 2010). They can be classified according to their boiling points (bp) into three groups: (1) very volatile organic compounds (bp <0 °C to 50–100 °C), (2) ordinary volatile organic compounds (bp 50–100 °C to 240–260 °C), and (3) semi-volatile organic compounds (bp 240–260 °C to 380–400 °C) (WHO 1989). Usually, however, they are classified based on their chemical properties as alkenes, acids, esters, ketones, thiols, and their derivatives (Piechulla and Degenhardt 2014; Hung et al. 2015). Almost all volatiles possess distinct odors, so outside the world of science, they are often described by their aroma properties with adjectives like “aromatic,” “earthy,” “fruity,” “floral,” “leathery,” “moldy,” and so forth (Sell 2006). Certain fungal volatiles are produced commercially for use as natural flavoring agents (Fratz and Zorn 2011). The human olfactory system can detect many VOCs in extremely low concentrations (McGann 2017).

It is well-known that *Trichoderma* spp. generate a diverse array of volatile organic compounds. As with other fungal species, the qualitative nature and quantitative amount of fungal volatile compounds detected vary enormously with the producing species and strain, nutrient source, available moisture, temperature, pH, light-dark status, and other environmental parameters (Zeppa et al. 1990; Korpi et al. 2009). Moreover, the purification, separation, quantification, and identification methods used for experimental analysis also affect the number and kinds of volatiles detected (Zhang and Li 2010; Hung et al. 2015).

A few specific examples, all referring to different published volatile profiles from *T. atroviride*, will illustrate how difficult it is to generalize from studies conducted using diverse strains, culture methods, and purification/detection methods. GC-MS analysis of *T. atroviride* strain IMI 352941 grown on a defined synthetic medium revealed 19 pyrone and dioxolane derivatives after pre-concentration of the fermentation broth on a C18 column, with 5,5-dimethyl-2*H*-pyran-2-one as the predominant pyrone (Keszler et al. 2000). Using strains of *T. atroviride* isolated from Czech soils, different media, and grown with light (conidiated) and dark (nonconidiated) regimes, GC-MS analysis revealed 33 volatile compounds after solid-phase microextraction (SPME). The amounts of 3-octanol, 3-octanone, 1-octen-3-ol, and toluene were higher in the headspaces of sporulating than non-sporulating cultures (Nemcovic et al. 2008). In yet another independently conducted study on *T. atroviride*, 25 volatile compounds were identified in the headspace of solid-grown cultures using SPME-GC-MS analysis. The detected VOCs included alkanes, alcohols, ketones, pyrones (6-pentyl-α-pyrone), furans, monoterpenes, and sesquiterpenes. Thirteen of the 25 identified volatiles were described from *Trichoderma* for the first time: α-phellandrene, β-phellandrene, α-terpinene, γ-terpinene, α-terpinolene, 2-n-heptylfuran, trans-p-menth-2-en-7-ol, α-bergamotene, β-farnesene, α-curcumene, α-farnesene, β-bisabolene, and nerolidol (Stoppacher et al. 2010).

Analyses of VOCs from other *Trichoderma* species reveal an even broader range of volatile profiles. For example, *T. harzianum* cultured in potato dextrose broth yielded 278 volatile compounds when identified by GC-MS using three different capillary columns with different nonpolar, medium polar, and high polar stationary phases. These VOCs encompassed alkanes, ketones, pyrones, furans, alcohols, monoterpenes, and sesquiterpenes (Siddiquee et al. 2012). A separate GC-MS analysis of VOCs produced by *T. viride* and *T. asperellum* revealed alkyl- and alkenyl-2*H*-pyran-2-ones and the new derivatives (*E*)-6-(pent-2-en-1-yl)-2*H*-pyran-2-one, 6-propyl-2*H*-pyran-2-one, and 6-heptyl-2*H*-pyran-2-one. The major compounds in the cultures of *T. viride* and *T. asperellum* were 6-pentyl-2*H*-pyran-2-one and (*E*)-6-(pent-1-en-1-yl)-2*H*-pyran-2-one, respectively (Wickel et al. 2013).

Interactions with other microbes also affect the VOC profile detected. For example, when different species and strains of *Trichoderma* and *Fusarium* are co-cultured, the VOCs these fungi produce cause a variety of interspecific effects that vary with the species and strains paired with one another (Li et al. 2018). *T. asperellum*, *T. harzianum*, *T. viride*, and *T. virens* all produced volatiles that inhibited growth of *F. oxysporum*. SPME-GC-MS analysis of headspace volatiles produced

by *T. harzianum* grown on potato dextrose agar revealed alcohols such as 2-methyl-1-propanol, 3-methyl-1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, and 2-phenylethyl alcohol (Li et al. 2018). In this analysis, fewer compounds were detected from *T. virens*. Both species produced 1-octen-3-ol, 3-octanone, and acetic acid (Li et al. 2018).

To recapitulate, VOCs are part of the extraordinary range of fungal metabolism. They occur in chemical families, all of which are easily transmitted through the atmosphere, which means they can act at a distance. In recent years, the volatiles produced by *Trichoderma* have become the subject of increased scrutiny due to their biological activities that include roles in both pathogen management and plant growth promotion, summarized in the sections below.

19.2.1 Fungicidal Action of Volatile Organic Compounds and "Trichofumigation"

Application of fungicidal synthetic chemicals is the most common strategy to control plant pathogens. Many of these chemicals have carcinogenic and toxigenic properties which, due to their long degradation periods, have a negative impact on the environment. Moreover, after years of continuous use, pathogens frequently develop genetic resistance necessitating the use of even higher amounts of problematic chemical fungicides (Bautista-Banos et al. 2006). Over the years, much of the interest in *Trichoderma* formulations surrounds their ability to suppress or kill plant pathogenic species through a variety of mechanisms that range from mycoparasitism to secretion of toxic metabolites (Vinale et al. 2008)

Moreover, there is increasing evidence that suites of volatiles emitted by a variety of fungal species can serve as chemical weapons against plant pathogens. One early and definitive demonstration of the fungicidal potential of fungal VOCs emerged from studies of the volatile mixtures emitted by *Muscodor albus* and related endophytes (Strobel 2006). When this fungus was grown in Petri plates in an enclosed incubator with other similarly cultured fungi, many of the other cultures died. The phenomenon was named "mycofumigation" and attributed to the combined action of the VOCs emitted by the growing endophyte (Strobel et al. 2001). We now know that laboratory co-cultivation of numerous isolates of *Trichoderma* with other fungi can lead to the inhibition or death of target strains, in the absence of physical contact between *Trichoderma* and the other species. This phenomenon is entirely comparable to the mycofumigation effects of the endophytic fungi isolated by the Strobel group. Thus, the exploitation of the fungicidal properties of certain *Trichoderma* volatile compounds promises an innovative and environmentally friendly approach for the reduction of unwanted fungal growth by what might be dubbed "Trichofumigation."

For instance, Bruce et al. (2000) investigated the inhibitory effects of VOCs from *T. aureoviride* against different wood-decay fungi. *T. aureoviride* was grown in low

nutrient media-containing amino acids such as phenylalanine, arginine, and glutamine. The VOCs produced by cultures grown on all amino acid combinations, but especially on phenylalanine and arginine, caused reduction in the growth of *Neolentinus lepideus* (also known as *Lentinus lepideus* or "the train wrecker" because of its ability to decompose old railroad ties). The medium with arginine alone or all amino acids together was effective in inhibiting growth of *Gloeophyllum trabeum*, a common brown rot species, while *Trametes versicolor* (also known as *Coriolus versicolor* or turkey tail) was least affected by the VOCs. In the same way, the VOCs produced by *T. aureoviride* and *T. viride* significantly inhibited the growth and protein production of wood-rotting basidiomycete *Serpula lacrymans*, a destructive dry rot, although the VOCs from *T. pseudokoningii* had no effect (Humphris et al. 2002).

In yet another example, the volatile compounds produced by two Egyptian isolates of *T. harzianum*, T23 and T16, when grown in dual culture, reduced mycelial growth of *Fusarium moniliforme* by 51% and 43%, respectively. The fungistatic effect was correlated with the presence of 6-pentyl-α-pyrone, and it was found that the effect was enhanced when the solid medium was supplemented with the compound (El-Hasan et al. 2007). Nevertheless, other studies involving different strains of *T. harzianum*, cultured in different ways, display fungicidal activity despite the absence of detectable 6-pentyl-α-pyrone. In this context, *T. harzianum* VOCs showed significant inhibition against the growth of *Fusarium oxysporum* f. sp. *cucumerinum*. Twelve volatiles were detected in the headspace of this strain, including pentadecane, α-cubebene, hexahydrofarnesol, pristane, verticillol, 2,4-di-tert-butylphenol, β-bisabolene, α-curcumene, lignocerane, nerolidol, biformen (6CI), and 2,6,10-trimethylundeca-5,9-dienal (Zhang et al. 2014).

Table 19.1 provides a list of published "Trichofumigation" studies in which it has been shown that exposure to volatile mixtures emitted by growing dual cultures of *Trichoderma* and selected plant pathogenic fungi inhibits or prevents growth of the pathogen. In most cases, it is not known which of the specific chemical compound or compounds from the *Trichoderma* VOC mixture is responsible for the fungicidal and fungistatic activity. However, as mentioned above, in several cases the "Trichofumigation effect" has been associated with strains that generate a single compound, namely, 6-pentyl-α-pyrone (also known as 6-n-pentyl-2H-pyran-2-one; IUPAC = 6-pentylpyran-2-one) (see Fig. 19.1) (Hanson 2005). This lactone has been known since the 1970s and has a distinct coconut odor (Collins and Halim 1972; Moss et al. 1975). In plate tests against *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici*, a strong correlation was found between strains of *T. harzianum* that inhibited growth of the pathogens and those that produced 6-pentyl-α-pyrone (Scarselletti and Faull 1994). Furthermore, this compound not only inhibited growth of *Fusarium*, but it lowered production of the mycotoxin deoxynivalenol by up to 80% (Cooney et al. 2001). Another interesting effect of 6-pentyl-α-pyrone is its nematocidal activity (Yang et al. 2012).

As a generally regarded as safe (GRAS) natural product, 6-pentyl-α-pyrone is used as a natural aroma/flavoring compound in the food and cosmetics industry, where it is also useful for its antimicrobial properties. Scaled-up fermentation

Table 19.1 Antifungal activity by *Trichoderma* volatile organic compounds ("Trichofumigation")

Trichoderma species	Target organism(s)	Reference
T. asperellum	*Fusarium solani* *Rhizoctonia solani*	Qualhato et al. (2013)
T. aureoviride	*Neolentinus lepideus* *Gloeophyllum trabeum*	Bruce et al. (2000)
T. aureoviride *T. viride*	*Serpula lacrymans*	Humphris et al. (2002)
T. brevicompactum *T. longibrachiatum* *T. virens*	*Fusarium oxysporum*	Anees et al. (2018)
T. ghanense *T. tomentosum*	*Rhizoctonia solani*	Qualhato et al. (2013)
T. gamsii	*Phoma herbarum* *Fusarium flocciferum* *Scytalidium lignicola* *Epicoccum nigrum*	Chen et al. (2016b)
T. harzianum	*Fusarium moniliforme* *Sclerotinia sclerotiorum*	El-Hasan et al. (2007)
T. harzianum	*Fusarium oxysporum*	Zhang et al. (2014)
T. harzianum *T. viride*	*Amylostereum areolatum*	Wang et al. (2019)
T. harzianum *T. viride*	*Fusarium oxysporum* f. sp. *radicis-lycopersici* f. sp. *lycopersici*	Li et al. (2018)
T. koningiopsis	*Phoma herbarum* *Fusarium flocciferum* *Scytalidium lignicola* *Epicoccum nigrum*	Chen et al. (2016a)
Trichoderma spp.	*Pyrenophora teres*	Moya et al. (2018)
T. spirale	*Corynespora cassiicola* *Curvularia aeria*	Baiyee et al. (2019)
T. viride	*Colletotrichum truncatum*	Bankole and Adebanjo (1996)

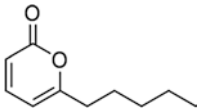


Fig. 19.1 6-amyl-α-pyrone, also known as 6-pentyl-2-pyrone and a number of other chemical names, is a volatile produced by many *Trichoderma* strains. It has a distinct coconut odor

methods have been developed for its commercial production by *T. harzianum*. The yield of these fermentations is limited because when the level of 6-pentyl-α-pyrone increases in the fermentation broth, it has a negative effect on *T. harzianum* growth, thereby limiting yields (Serrano-Carreón et al. 2002). Because the market price of natural aroma compounds is much higher than that of synthetic volatiles, various bioengineering strategies have been developed to circumvent this feedback constraint (Serrano-Carreón et al. 1993).

19.2.2 Enhancement of Plant Growth by Volatile Organic Compounds

Trichoderma VOCs can improve plant growth and enhance plant defenses against both biotic and abiotic stresses. In one study, using *Arabidopsis thaliana* as a model system, plants were grown in a shared atmosphere with *Trichoderma viride* in the absence of physical contact. After 4 weeks of exposure, *A. thaliana* increased total biomass by 45% and total chlorophyll concentration by 58%. A GC-MS analysis of the VOCs produced by *T. viride* found that isobutyl alcohol, isopentyl alcohol, and 3-methylbutanal were the most abundant compounds (Hung et al. 2013). In another study in which VOCs from *T. virens* stimulated *A. thaliana* growth and root development, it was shown that VOC-exposed plants accumulated jasmonic acid and hydrogen peroxide, with an enhanced expression of the jasmonic acid-responsive marker *pLox2:uidA* (Contreras-Cornejo et al. 2014).

The ability of *Trichoderma* to stimulate plant growth in the absence of physical contact has also been shown for *T. asperellum* and *T. atroviride.* In this context, when 7-day-old seedlings of *A. thaliana* were exposed to VOCs from 5-day-old culture of *T. atroviride*, after 2 weeks, plant size, biomass, and chlorophyll content were all increased. VOC profiles were composed of aromatics, alcohols, ketones, aldehydes, and alkenes (Lee et al. 2015). Similarly, *A. thaliana* plants exposed to VOCs of *T. asperellum*, without physical contact, showed a significant increase in trichome numbers; accumulation of defense-related compounds such as H_2O_2, anthocyanin, and camalexin; and increased expression of defense-related genes; 6-pentyl-α-pyrone was the main volatile detected in the headspace (Kottb et al. 2015).

The mixtures of VOCs produced by six different species of *Trichoderma*, including *T. aggressivum*, *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride*, increased the growth of *A. thaliana* and total chlorophyll content. *T. viride* VOCs also enhanced tomato seedlings growth and significant development of lateral roots. More than 141 unique volatile compounds were identified from these *Trichoderma* strains, including hydrocarbons, alcohols, ketones, aldehydes, alkanes, alkenes, esters, aromatic compounds, heterocyclic compounds, and various terpenes (Lee et al. 2016). Moreover, when *A. thaliana* was co-cultivated, in the absence of physical contact, with *T. viride*, *T. atroviride*, *T. longibrachiatum*, *T. citrinoviride*, *T. harzianum*, *T. koningii*, *T. koningiopsis*, *H. orientalis*, and *T. viridescence*, the fresh weights of shoot and roots, as well as the chlorophyll content of plants, were increased (Jalali et al. 2017). Control plants grown with salt stress (100 mM NaCl) had decreased size and chlorophyll content; however, when co-cultivated with *T. koningii*, *T. viridescence*, and *H. orientalis*, the VOCs emitted by these three *Trichoderma* species aided the plants in overcoming the stress (Jalali et al. 2017).

As with the fungicidal capacity of *Trichoderma* VOCs with respect to pathogens, most of the published research concerns volatile blends, and less is known about the individual bioactive compounds. It is of interest that 6-amyl-α-pyrone, depending on the concentration, will inhibit or promote seedling growth (Harman et al. 2004; Vinale et al. 2008).

19.3 The Impact of *Trichoderma* on Medicinal, Aromatic, and Spice Plants

Aromatic, medicinal, and spice plants have been used from prehistoric times to the present as botanical raw materials for cosmetic, culinary, perfumery, and/or therapeutic purposes (Sumner 2000; Yaldız et al. 2018). Currently, they are components of natural health foods and traditional medicinal products, as well as the starting materials for value-added ingredients such as essential oils, liquid extracts, powders, and resins (The International Trade Centre 2016). In recent years, there has been a growing demand for natural products, which in turn has led to increasing commercial production of aromatic and medicinal plants for mass-market consumption (Chandra and Sharma 2018). The global market is currently estimated to be around US$ 62 billion and is expected to grow at 15% annually, with Canada, China, Germany, India, and the United States as the largest exporters (Chandra and Sharma 2018; Gahukar 2018). To meet the increased market demand for these high-value natural plant products while not endangering their status as "organic" products, farmers are seeking methods to increase crop productivity without the use of synthetic pesticides and fertilizers. Commercial biofungicides containing *Trichoderma* preparations are well suited for this purpose. They provide a nature-friendly approach that can easily be introduced into organic systems of plant production (Kowalska et al. 2014).

The desirable plant products produced by aromatic, medicinal, and spice plants generally fall under the category of secondary metabolites, an enormous, chemically complex group that can be categorized into structurally similar families such as alkaloids, glycosides, polyphenols, and terpenes. Many of these natural products have been employed for centuries for their pharmacological properties in traditional Chinese medicine, Ayurvedic medicine, and other folk traditions (Chevallier 2001). There are often overlapping uses for crude preparations from a single plant species in food flavoring, aromatherapy, and medicinal purposes (Seidemann 2005). To give two of many possible examples, turmeric, a plant in the ginger family (Zingiberaceae), is used as a dye, a cosmetic, a spice, and a medicinal ingredient (Nair 2013). Similarly, species in mint family (Lamiaceae) are widely employed as flavorings, aromatherapy agents, and therapeutic agents (Lawrence 2006). Plants that produce perfumes, medicinals, and spices are higher in economic value than bulk agricultural crops such as soybean and corn, and plant strains that produce high concentrations of their desirable constituents are sought by specialty farmers.

The earliest and most numerous studies on the beneficial effects of the application of *Trichoderma* spp. on aromatic, medicinal, and spice plants are related to their protective effects against plant pathogens. One such example concerns ginseng, a mainstay of traditional Asian medicine, which produces steroid saponins known as ginsenosides. Ginseng is said to be an adaptogen, a term used in the alternative medicine community to describe a variety of tonics and folk medicine said to build up "vitality" and enhance general physical and mental health. Although modern medicine has provided insufficient research to determine if ginseng extracts actually

have the health effects attributed to them, the demand for ginseng root remains high in Korea and China as well as the western natural products market, and the plant has come increasingly under cultivation. A study of *Trichoderma koningiopsis* (strain YIM PH30002) showed that application of the fungus yielded significant growth inhibition of four root-rot pathogens of Chinese ginseng (*Panax notoginseng*): *Phoma herbarum*, *Fusarium flocciferum*, *Scytalidium lignicola*, and *Epicoccum nigrum*. This *Trichoderma* strain grew over each of phytopathogenic fungi with a coiling and twisting mycelium characteristic of mycoparasitism. In addition, this *Trichoderma* strain also produced at blend of at least ten kinds of volatile substances including alkanes, monoterpenes and arenes, heterocycles, and aldehydes, which likely contributed to the fungicidal effect (Chen et al. 2016a). Another strain of *Trichoderma* (*T. citrinoviride* PG87) isolated from Korean or Asian ginseng, *Panax ginseng*, exhibited antagonistic activity against six major ginseng pathogens. The pathogen control was attributed to high activity of the lytic enzyme endo-1, 4-β-D-glucanase (Park et al. 2019).

Several other studies attest to the ability of *Trichoderma* to control pathogens in aromatic medicinal and spice plants. Effective anti-pathogen effects have been shown against diseases caused by *Fusarium* spp. in *Hibiscus sabdariffa* (Parizi et al. 2012), *Zingiber officinale* (Zhang et al. 2017), *Withania somnifera* (Sharma and Trivedi 2010), and *Vanilla planifolia* (Sandheep et al. 2012); *Alternaria* spp. in *Cassia angustifolia* (Tagaram et al. 2015) and *Cuminum cyminum* (Jadeja and Pipliya 2008); *Puccinia thwaitesii* in *Justicia gendarussa* (Ragi et al. 2013); *Pythium aphanidermatum* in *Capsicum annuum* (Muthukumar et al. 2010); and even the hard-to-control nematode *Meloidogyne incognita* in *Mentha arvensis* (Pandey et al. 2011), *Platycodon grandiflorum* (Zhang and Zhang 2009), *Ocimum basilicum* (Tiwari et al. 2017), and *Withania somnifera* (Saikia et al. 2013).

19.3.1 Trichoderma Species as Elicitors of Secondary Metabolites in Medicinal, Aromatic, and Spice Plants

Although much of the published research focuses on the plant growth-enhancing properties of *Trichoderma*, increases in size and vigor, anti-pathogenic action, or improved ability to survive in the presence of abiotic stress, *Trichoderma* spp. can provide an additional, less well-understood benefit, namely, the upregulation of gene expression and subsequent yields of high-value secondary metabolites by aromatic, medical, and spice plants. Thus, the application of *Trichoderma* provides multivalent improvements, not just to the physiological pathways involved in shoot growth, root development, and flowering but also to expression of the gene pathways involved in the biosynthesis of economically valuable secondary metabolites. In this section, we review some studies that have measured the increases in the yield of such compounds.

Trichoderma and *Trichoderma* bioproduct applications are able to boost the production of specific biomolecules, such as colchicine in *Gloriosa superba* L. (Alice and Sundravadana 2012), menthol in *Mentha arvensis* L. (Ratnakumari et al. 2014), and tanshinone in *Salvia miltiorrhiza* Bunge (Ming et al. 2013). In basil, for example, *Trichoderma* treatments not only yielded an efficient control of the root-knot nematode *Meloidogyne incognita* but also enriched the essential oil production (Tiwari et al. 2017). In fact, in some medicinal species, *Trichoderma* spp. can be applied solely to improve secondary metabolites yield. In the case of the Chinese Sage (*Salvia miltiorrhiza*) treated with *T. atroviride*, more tanshinones (a class of anti-inflammatory and cytotoxic compounds used in traditional Chinese medicine) were produced in hairy roots due to the increased transcriptional activity of genes involved in the tanshinone biosynthetic pathway (Ming et al. 2013). Most of the overexpressed genes are common for all mono-, sesqui-, and diterpenes products. Table 19.2 provides a summary of the recently published studies on *Trichoderma* spp. effects on secondary metabolite production by medicinal, aromatic, and spice plants.

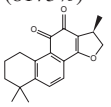
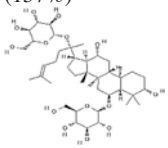
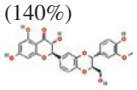
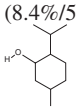
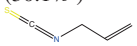
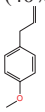
In summary, because of their multiple known mechanisms for enhancing plant growth, biocontrol strains of *Trichoderma* should be considered as a primary component of any integrated pest management program (Verma et al. 2007). With the high premium placed on "organic" methods for the production of natural products for the burgeoning health food and nutraceutical market, the application of *Trichoderma* formulations on high-value plants deserves increased research scrutiny. The specific use of *Trichoderma* spp. as biological elicitors for high-value natural products such as essential oils and pharmacologically active secondary metabolites represents a realistic target for future agronomic research in such specialty crops.

19.4 Conclusions and Future Prospects

There is current widespread use of *Trichoderma* spp. in agriculture, especially in organic farming. The use of *Trichoderma*, however, needs not be restricted to this production system since these biocontrol agents can be equally, or even more, effective than some chemical products. For example, the ability of *Trichoderma* application to suppress plant nematodes is remarkable. Given that chemical nematocidal agents are expensive, leave toxic residues in plants and soils, and are often ineffective in long-term suppression of nematodes (Zhang and Zhang 2009), *Trichoderma* treatments may be one of the few alternatives left for both organic and nonorganic farmers.

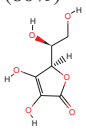
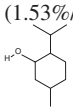
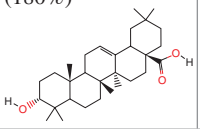
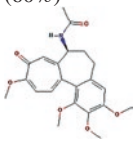
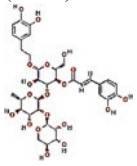
In this review, we focused on two understudied aspects of *Trichoderma* action. The section on VOC-mediated fungal plant interactions highlights the way that the presence of *Trichoderma* can have beneficial actions on plants without physical contact between the fungus and the plant. Biochemists and biologists perform most of their experiments in the aqueous phase, and therefore our general knowledge is

Table 19.2 Enhancement of secondary metabolites in selected aromatic, medicinal, and spice plants by exposure to *Trichoderma*

Trichoderma species (strain)	Plant species Latin name (common name)	Mode of application	Metabolites induced (increase[a, f])	Reference
T. atroviride (D16)	*Salvia Miltiorrhiza* Bunge (red sage or Danshen)	Extract of mycelium added into liquid half-strength B5 medium in hairy roots culture	Cryptotanshinone (8173%)[b] 	Ming et al. (2013)
		Extract of mycelium polysaccharide fraction added into liquid half-strength B5 medium in hairy roots culture	(6496%)	
T. citrinoviride (PG87)	*Panax ginseng* C.A. Mey (Asian or Korean ginseng)	Roots inoculated with 1 mL (1×10^6 spores/mL) in potato dextrose broth by dipping for 3 h	Ginsenoside (157%) 	Park et al. (2019)
T. harzianum (ATCC no. PTA-3701)	*Pogostemon cablin* Benth. (Patchouli)	Basal soil application of vermicompost containing the fungus	Essential oil (4.1%)	Singh et al. (2013)
T. harzianum (KHB)	*Silybum marianum* (L.) Gaertn (milk thistle)	50 mm plugs of medium with mycelium placed on the roots of each plantlet	Silymarin (140%) 	Hasanloo et al. (2010)
T. harzianum (NFCCI 2241)	*Mentha arvensis* L. (field mint)	Basal soil application (20 g per pot) of sorghum-sand mixture containing 2×10^7 CFU/g	Essential oil/menthol (8.4%/5.8%)[c] 	Ratnakumari et al. (2014)
T. harzianum (T22)	*Brassica juncea* (L.) Czern. (Chinese mustard)	Basal soil application (10 g/kg soil) of talc-based formulation containing 2×10^9 CFU/g	Seed oil (30.1%[c]) 	Ahmad et al. (2015)
T. harzianum (ThU)	*Mentha arvensis* L. (field mint)	Basal soil application of 2 g of mint biomass waste materials containing 2.0×10^8 CFU/g	Essential oil (23.8%)	Pandey et al. (2011)
T. harzianum (ThU)	*Ocimum basilicum* L. (Basil)	Basal soil application (2×10^6 spores/g soil) of mycelial material suspended in phosphate buffer	Essential oil/methyl chavicol[d] (40%/41.5%[e]) 	Tiwari et al. (2017)

(continued)

Table 19.2 (continued)

Trichoderma species (strain)	Plant species Latin name (common name)	Mode of application	Metabolites induced (increase[a, f])	Reference
T. longibrachiatum (T1)	*Allium cepa* L. (common onion)	Basal soil application (250 ml per pot) of conidial spore suspension (1×10^7)	Vitamin C (80%) 	Abdelrahman et al. (2016)
T. ovalisporum (NFCCI 2689)	*Mentha arvensis* L. (field mint)	Basal soil application (20 g per pot) of sorghum-sand mixture containing 2×10^7 CFU/g	Essential oil/ menthol (1.53%/4.24%[c]) 	Ratnakumari et al. (2014)
T. viride	*Calendula officinalis* L. (pot marigold)	Dried mycelia powder suspended in plant cell culture medium at a concentration of 0.5 mg/L	Oleanolic acid (180%) 	Wiktorowska et al. (2010)
T. viride	*Gloriosa superba* L. (flame lily, tiger claw)	Basal soil application (2.5 kg/ha) followed by spray (0.2%) of talc-based formulation	Colchicine (60%) 	Alice and Sundravadana (2012)
T. viride	*Teucrium chamaedrys* L. (wall germander)	Mycelial extract supplied at 0.05 mg/ml into cell suspension culture	Teucrioside (170%) 	Antognoni et al. (2012)

[a]Percentage of increase in relation to the control treatment
[b]Only the most significant increase is shown in table. Other compounds were also stimulated by the fungal extract
[c]Mean of two harvests
[d]Under salt stress conditions
[e]*T. harzianum* co-inoculation with *Bacillus tequilensis*
[f]Images of compounds retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov)

biased toward liquid-phase molecular interactions. Yet in soils, water is frequently limited. Aboveground, organisms need to communicate through the air. Volatile signal moves easily through the atmosphere and can accumulate to appropriate concentrations in soil pockets. There are numerous reports of demonstrable fungicidal effects of *Trichoderma* grown at a distance from a variety of economically destructive fungi, including a wide range of fungal plant pathogens. At low concentrations, VOCs emitted by *Trichoderma* are known to function in interspecific signaling and defense. There is abundant descriptive evidence that *Trichoderma* can not only enhance plant growth but also stimulate an increase in the production of valuable secondary metabolites. While these are valuable phenotypic observations, these findings are correlative, not causal.

A great deal of fundamental work remains to be done. How do plants perceive *Trichoderma* VOCs? Are there different receptor systems in roots and aboveground parts of plants? What kinds of molecular signaling pathways are triggered by volatile signals? How often, and how, do volatiles and other *Trichoderma* metabolites act cooperatively? What mechanistic effects are triggered by *Trichoderma* to increase plant secondary metabolite pathways? How closely do the effects we observe in protected, controlled laboratory environments reflect what occurs in the field? Until we have a better idea of which single compounds are the most active molecules in inducing observed phenotypic changes, it will be difficult to access the wealth of genomics and transcriptomics data now available in public databases.

A few researchers have started using the power of "omics" technologies to decipher the mechanisms by which *Trichoderma* exerts its many plant growth-promoting effects. For example, Abdelrahman et al. (2016) have used metabolic profiling to demonstrate that *T. longibrachiatum* can improve growth of onion plants and increase resistance to the pathogen *Fusarium oxysporum* by triggering plant production of a number of stress-responsive metabolites. Work in our laboratory, using a transcriptomics approach, studied plants treated with vapors of 1-decene. The expression of 123 genes was differentially affected, encompassing genes involved in cell wall modification, auxin induction, stress, and defense responses (Lee et al. 2019). Similarly, a transcript analysis of *Trichoderma* exposed to *Fusarium* volatiles indicated that the several genes, including those for chitinase- and subtilisin-like protease, as well as certain other genes, were upregulated (Li et al. 2018). The field is ripe for more such studies.

The genes responsible for the biosynthesis of some of the most important plant products used for aromas, spices, and traditional medicine are not well understood. Their respective elucidation remains an obstacle to maximizing our ability to use *Trichoderma* to increase yields of valuable plant secondary metabolites. Future experiments will need to address the specific genetically encoded signals that are involved in plant growth promotion, pathogen suppression, and induction of secondary metabolite biosynthetic pathways.

The genus *Trichoderma* has been called the "Swiss army knife" of agricultural biocontrol products (Lorito and Woo 2015). When all the species, strains, formulations, application systems, and beneficial outcomes are added up, the genus deserves this name. *Trichoderma* is a superstar among the beneficial microbes used in

agriculture. We believe that the time is right for more basic research on *Trichoderma* molecular biology so as to reach the full potential of this remarkable group of soil-inhabiting fungi in bioengineering.

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Chapter 20
Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR): Role and Mechanism of Action Against Phytopathogens

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20.1 Introduction

Plants in the environment face constant stress either it be biotic or abiotic. Biotic stress includes herbivores, pests and pathogens. Thus, plants developed a multilayer defence system to prevent the problem of biotic stress which includes the constitutive and the induced defence system. The thick outer covering of the plant body which prevents the entry of pathogen is the constitutive mechanism (Pandey et al. 2016). Plants by production of various compounds respond to the attack of herbivores or pathogens which in result reduces or inhibits the attack from the enemies. Plant responses occur in the organ where it was originally attacked (local response) and also in the distant plant parts which are unaffected (systemic response). One of these respond in systemic acquired resistance (SAR; induced systemic resistance, ISR) (Heil and Bostock 2002) (Fig. 20.1). On the other hand, Choudhary et al. have described SAR and ISR as two different forms of induced resistance based on their nature of elicitors and regulatory pathways. By exposing the plant to different microbes such as virulent, avirulent and non-pathogenic, SAR can be triggered, and for establishment of SAR, a time period is required depending on the plant and elicitors where PR protein and salicylic acid accumulation takes place. ISR involves plant growth-promoting rhizobacteria (PGPR); best known are the strains belonging to *Pseudomonas* (Choudhary et al. 2007). Azami-Sardooei et al. have also described induced resistance (IR) as a physiological state which enhances when plants are

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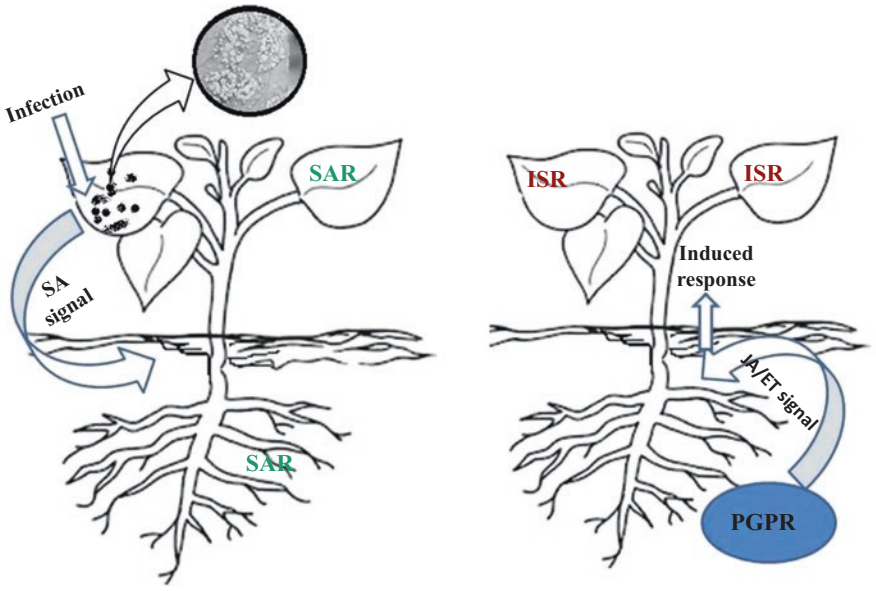

Fig. 20.1 Systemic representation of responses of SAR and ISR and their response mechanism

exposed to any kind of biotic and abiotic stimuli, and they have divided IR into two major types, viz., induced systemic resistance (ISR) and systemic acquired resistance (SAR). Conrath et al. have the opinion that ISR is not directly responsible for the defence mechanism of the plant; instead it just enhances the physiological state of the plant to react more efficiently to biotic stress (Conrath et al. 2006). Bakker et al. (2007) studied the ISR in plants inoculated with Pseudomonas bacteria and pathogens spatially and concluded that no direct interactions are possible between the two populations, suppression of disease development has to be plant-mediated. It is also suggested that plant protection can be enhanced by combining ISR and SAR as pathogens can be resisted both ways which will allow defence against a broad spectrum of pathogen than ISR/SAR alone (Choudhary et al. 2007).

Induced resistance was first described by Ross in the 1960s, based on his observation that the inducible response to TMV was not limited to the immediate vicinity of the necrotic lesions but also spreads to the other parts of the plants (Ross 1961; Van Loon 1997). The phenomenon SAR involves the accumulation of PR proteins, and it coincides with the plants' enhanced resistance for the subsequent attack of the pathogen (Penninckx et al. 1996). SAR is associated with a set of genes known as the SAR genes, but all genes responsible for defence response are not expressed during SAR. This particular spectrum of gene expression helps in distinguishing the SAR response from other resistant responses (Ryals et al. 1996). Systemic resistance induced by some non-pathogenic rhizobacteria is similar to pathogen-induced SAR (Van Loon et al. 1998).

It was seen that in many plant species, application of salicylic acid (SA) or its derivative acetyl salicylic acid induces the *PR* genes. Maiamy et al. also reported that in tobacco the concentration of endogenous SA increases 20-fold in the infected tissue and 5-fold in the uninfected ones. The rise in SA concentration induces the *PR* genes (Maiamy et al. 1990). In addition it was reported the increase of SA in the phloem sap of cucumber when infected by *Colletotrichum lagenarium* (Métraux et al. 1990).

In the recent years, the response of plant to pathogen attack is advancing to new horizons as new and advanced tools are implemented in biochemistry, cell biology, molecular biology and genetics across research disciplines (Somssich and Hahlbrock 1998). Molecularly increased expression of *PR* genes (pathogenesis-related genes) in the local and systemic tissue is the main characterisation of SAR. Van Loon first described PR proteins in the 1970s. He also recorded that different novel proteins get accumulated after tobacco is infected by TMV. He also stated that many PR proteins possess antimicrobial properties (Durrant and Dong 2004). The function of each protein in the defence response is not clearly defined. It is believed that the collective effects of many PR proteins result in SAR rather than a specific PR protein. On the onset of SAR, *PR* genes serve as useful molecular marker (Durrant and Dong 2004). According to Kinkema et al., SAR comprises of a broad-spectrum set of *PR* genes, and the key regulator of signal transduction leading to SAR is NPR1. NPR1 mutant fails to induce *PR* genes during systemic resistance, and nuclear localization of NPR1 is essential for induction of *PR* genes (Kinkema et al. 2000). Induced defence response in plants is triggered by microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) detected by the pattern recognition receptors (PRRs). This triggers Ca^{2+} and signalling cascades of mitogen-activated protein (MAP) kinase; flagellin/FLS2 and EF-Tu/EFR are two best characterised MAMP/PRR pairs (Boller and Felix 2009; Pandey et al. 2016). The first step for the plant response for the plant-microbe interaction is the recognition of MAMPs/DAMPs by the PRR. Flagellin-sensitive 2 (FLS2) is the best known PRR which identifies bacterial flagellin or the minimal epitope flg22 (Chinchilla et al. 2007; Sun et al. 2013; Zipfel 2014; Yuan et al. 2017). PRR-triggered immunity (PTI) can work against multiple microbes (either pathogenic or not) as PAMP are conserved. Along with PTI, plants are also dependent on ETI (effector-triggered immunity). ETI induces intracellular immune receptors which are mostly nucleotide-binding site leucine rich repeat (NBS LRR) (Zipfel 2014). PTI response typically consists of activating mitogen-activated protein kinase (MAPK) cascade. This results in the release of oxidative species which also deposits callose in the infection site, leading to defence-related gene expression (Altenbach and Robatzek 2007; Schwessinger and Zipfel 2008; Niu et al. 2016).

Some reports also suggest that application of brassinosteroids (BRs) externally increases the stress tolerance ability of the plants and helps in defence response. Xia et al. reported that cucumber when treated with BRs shows better tolerance to photo-oxidation and cold stress along with H_2O_2 accumulation and systemic induction of genes responsible for stress response (MAPK1 and MAPK2). Zhang et al. also demonstrated that brassinosteroids (BRs) induce defence-associated gene

expression in *Arabidopsis* when challenged by cucumber mosaic virus (CMV); they noticed that its increased levels enhance the tolerance against CMV (Zhang et al. 2015). Deng et al. also reported that local application of BRs induces systemic resistance to virus and overall decreases TMV accumulation in *Nicotiana benthamiana* (Deng et al. 2016).

Manipulation of the microbial population present naturally in the plant environment for induction of resistance in the plant against disease has a great scope of research in the present day. Induction of immunity in plants by the help of microorganism rather than using chemical pesticide is a natural, safe, effective and durable alternative (Pozo et al. 2002).

20.2 Pathways of SAR

20.2.1 Salicylic Acid Pathway

For inducing systemic acquired resistance in plants, salicylic acid has been proposed to act as an endogenous signalling response. It was first investigated in transgenic tobacco plant which possesses a bacterial gene encoding salicylate hydroxylase, which converts salicylic acid to catechol against tobacco mosaic virus. (Gaffney et al. 1993). As a defensive mechanism, plants produce phenolic compounds against various pathogen attacks which act as SAR. The salicylic acid-mediated SAR, investigation was done by using wheat cultivars "Karls 92 and lke" and with the used of synthetic elicitor viz; thiamine, riboflavin, 2,6-dichloropyridine-4 carboxylic acid (DCPCA), benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), methyl jasmonate and sodium salicylate (SS) in various concentration which spray on plants and found that BTH and DCPCA enhanced the phenolic content in plants (Ramos et al. 2017). Wittek and co-worker reported that on *Arabidopsis* application of folic acid or 7,8-dihydropteroate (DHP) precursor 7,8-dihydroneopterin (DHN) enhanced resistance against *Pseudomonas syringae* also activates local or systemic SA-dependent resistance (Wittek et al. 2015). Salicylic acid is not only directly involved in defence mechanism, but it also indirectly activates other biomolecules to act as SAR. Bernsdorff et al. investigated the relationships of the two immune regulatory plant metabolites which are salicylic acid (SA) and pipecolic acid (Pip) in *Arabidopsis thaliana* basal immunity to *Pseudomonas syringae*. During SAR, Pip orchestrates SA-dependent and SA-independent priming of pathogen responses in a flavin-dependent-monooxygenase 1 (FMO1)-dependent manner. The Pip/FMO1 signalling module acts for activation of SAR and also associated defence priming events in which SA amplifies Pip-triggered responses to distal tissue of SAR-activated plants (Bernsdorff et al. 2015). Nevertheless, Sun and other co-worker did some similar experiment to

analyse the role of transcription factors TGACG-binding factor 1 (TGA1) and TGA4 in regulating SA and Pip biosynthesis in *Arabidopsis thaliana* by quantifying the expression level of *systemic acquired resistance deficient 1* (*SARD1*) and *calmodulin-binding protein 60 g* (*CBP60g*) which encode two master transcription factors for plant immunity. Results showed that TGA1 and TG4 regulate Pip and SA by modulating the expression of *SARD1* and *CBP60g* (Sun et al. 2018).

20.2.2 Calcium Calmodulin Pathway

Calcium (Ca^{2+}) is also known for the activation of plant defence responses. Reports have suggested that Ca^{2+} signal is also involved in the activation of salicylic acid-mediated plant immune response through calmodulin (Du et al. 2009). Plants also produce numerous isoforms of calmodulin such as Gm-CaM-4 and Gm-CaM-4-5 which are two divergent calmodulin isoforms present in soybean (*Glycine max*) that have been reported to be involved in plant disease resistance (Park et al. 2004). It has been found that pathogen-induced CaM-binding TFs have been associated with plant defence responses by acting on homeostatic regulation by salicylic acid (SA), a defence-associated hormone in plants (Reddy et al. 2011; Pieterse et al. 2012; Aldon et al. 2018). Again, in *Arabidopsis* the production of SA in infected cells is enhanced by upregulation of the expression of ICS1 (isochorismate synthase 1) and EDS1 (enhanced disease susceptibility 1) genes, and expression of ICS1 and EDS1 is positively and negatively controlled by CBP60g and CAMTA3/AtSR1, respectively, two CaM-binding TFs (Pieterse et al. 2012; Du et al. 2009; Aldon et al. 2018).

20.2.3 Jasmonate and Ethylene Pathway

Defensin was found in Arabidopsis leaves affected by *Alternaria brassicicola* in both affected leaves and systemic leaves which increases the endogenous jasmonic acid concentration.The expression defensin gene induced by systemic pathogen gets reduced in the mutants ein2 and coi2 which blocks ethylene and methyl jasmonate response (Penninckx et al. 1996). Another transcription factor responsible for defence response in plant is *ethylene response factor 1 (ERF1). ERF1* can be activated either by ethylene or jasmonic acid, and both pathways are required simultaneously to activate *ERF 1* as mutant lines can block the expression of *ERF 1*. ERF1 is responsible for the downregulation of the PR genes during infection to induce defence response (Lorenzo et al. 2003).

It was again described that abscisic acid and jasmonic acid show antagonistic relationship which induces different defence mechanisms in plants. jin1/myc2 and aba2-1 mutant lines increased the resistance in *Arabidopsis* against *Fusarium oxysporum* (Anderson et al. 2004). Betsuyaku et al. (2018) demonstrated that jasmonic acid is antagonistic to SA and is triggered during ETI. They concluded that spatial distribution is responsible for activation of both phytohormone pathways (Betsuyaku et al. 2018). Three jasmonic acid oxidases (JAO) were identified that get induced during infection by *Botrytis cinerea. JAO2* enhances basal expression, while *jao2* mutants show antifungal resistance. Increased defence in *jao2* is dependent on *jasmonate response 1* (JAR1). In healthy and infected plant, jao2 mutant showed altered accumulation of several JAs (Smirnova et al. 2017). Additionally Luo et al. have described the combined function of JA/SA in defence mechanism of the plant in Melampsora larici-populina (Mlp). They also reported to have found a set of 943 genes identified as CRG (common responsive genes). It was also found that JA and SA regulate both growth and defence in the plants (Luo et al. 2019).

20.2.4 SAR During Pathogen Attack

Plants adopt various levels of defence mechanism during a pathogen attack. The outer hard layer of chitin and the cell wall act as the first layer of defence. Plants have also developed sophisticated mechanism of defence involving many signalling molecules like salicylic acid, jasmonic acid and ethylene. In the plant-pathogen interaction demonstrating the signalling pathways for disease, resistance has become the major area of interest (Pieterse et al. 2002). The transport of water and solutes from cell to cell is the vital point of systemic signalling in plants.

Azelaic acid (AzA), glycerol-3-phosphate (G3P) and salicylic acid (SA) are the signalling molecules during SAR. Lim et al. demonstrated that during SAR signalling, AzA and G3P show symplastic movement while SA moves through apoplast. They also showed that PD localising proteins (PDLP) 1 and 5 are required during SAR and have regulatory functions (Lim et al. 2016).

20.3 Molecular Basis of Induced Resistance

From the reviewed literature, it is made clear that plants during pathogen attack express pathogen-related genes (*PR* genes). In the past years, researchers had tried to identify the specific genes that are responsible for systemic resistance. Recent works also suggest that plant immunity is regulated by chromatin remodelling and DNA methylation (Luna et al. 2012).

During the onset of SAR in tobacco, expression of nine gene PR proteins families (PR-1 (PR-1a, PR-1b and PR-1c), PR-2 (PR-2a, PR-2b and PR-2c), PR-3 (PR-3a and PR-3b), PR-4 (PR-4a and PR-4b) and PR-5 (PR-5a and PR-5b), basic form of PR-1,

basic and acidic form of class III chitinase and β-1,3-glucanase, PRQ′) is found to be in high level (Ward et al. 1991). Pieterse et al. demonstrated that defence response is regulated differentially by *NPR1* depending upon the elicited signals (Pieterse et al. 1998). *Arabidopsis thaliana* progeny inoculated with *Pseudomonas syringae* pv. tomato shows resistance against (hemi)biotrophic pathogen *Hyaloperonospora arabidopsidis*. The SA-inducible defence genes expressed were *pathogenesis-related gene1*, *WRKY6* and *WRKY53* (Luna et al. 2012). Systemic resistance is induced by the AM fungus *Glomus fasciculatum* in tomato plants, the genes responsible for jasmonic acid biosynthesis and response are *OPR3* and *COII*, and PR1 is responsible for expression and response of salicylic acid (Nair et al. 2015). Pipecolic acid (Pip) is responsible for SAR and local resistance in many plants. Pip biosynthesis is facilitated by a critical enzyme encoded by *SAR-deficient 4 (SARD4)*. Along with *SARD4*, a SAR regulator *flavin-dependent monooxygenase1 (FMO1)* is required for enhanced resistance (Ding et al. 2016). *mildew resistance locus O (MLO)* gene family makes many plant species susceptible to powdery mildew. But *Arabidopsis MLO2* induces systemic resistance when challenged by *Pseudomonas syringae*. Although *MLO2* was not required for SA- or Pip-induced defence, it is necessary for proper induction of resistance in both SAR signals. *MLO6*, a close homolog, also has a less critical role in SAR (Gruner et al. 2018). *Arabidopsis* AP2, family protein involved in disease defence (APD1), is a member of AP2/EREBP superfamily that positively regulates SA biosynthesis and defence against virulent bacterial pathogens (Gautam and Nandi 2018). Pathogenesis-related (PR) proteins are associated with the development of systemic acquired resistance (SAR) against further infection enforced by fungi, bacteria and viruses. PR1a is the first PR-1 member that could be purified and characterised. Study shows that NtPR1a-overexpressing tobacco significantly reduced multiple of *R. solanacearum* and inhibited the development of disease symptoms compared with wild-type plants. Also, overexpression of NtPR1a activated a series of defence-related gene expression, including the hypersensitive response (HR)-associated genes *NtHSR201* and *NtHIN1*; SA-, JA- and ET-associated genes *NtPR2*, *NtCHN50*, *NtPR1b*, *NtEFE26* and Ntacc oxidase; and detoxification-associated gene *NtGST1*. *NtPR1a*-enhanced tobacco resistance to *Ralstonia solanacearum*, may be mainly dependent on activation of the defence-related genes (Liu et al. 2019).

Along with the different genes involved in the recent studies, it was revealed that microRNAs are also involved in triggering immunity in different species of plants. In *Arabidopsis* involvement of miR393 is seen suppressing auxin pathway to induce PTI response against bacterial pathogen (Navarro et al. 2006). miR160, miR393 and miR773 are also seen to have involvement in PTI in *Arabidopsis* (Li et al. 2010); miR482 and miR5300 in tomato (Ouyang et al. 2014) and miR6019 were reported to have significant role in ETI (Natarajan et al. 2018). It was reported by Natrajan et al. that in potato challenged by *Phytophthora infestans*, miRNA160 plays a crucial role. miR160 expression doesn't have direct effect on resistance, but it is required to trigger SAR (Natarajan et al. 2018).

20.4 Role in Defence

Systemic acquired resistance (SAR) is the defence response in the whole plant when they are exposed to any pathogen. SAR is not only important to resist disease but is also useful to recover from diseases that are formed. SAR is induced by different types of pathogens but mainly by those where necrosis takes place due to hypersensitive response of the plant.

It was also demonstrated that two PGPR strains, viz., *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90–166, show systemic resistance against *Fusarium oxysporum* f. sp. *cucumerinum* causing wilt in cucumber. Both PGPR strains were spatially separated from the pathogen. There were delayed development of disease symbol and reduced numbers of dead plants (Liu et al. 1995). Additionally Raupach et al. tested the effect of *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90–166 against cucumber mosaic virus (CMV) in cucumber and tomato and found that there were reduced number of virus-free plant and delayed development of disease symptoms (Raupach et al. 1996; Ramamoorthy et al. 2001). The inoculation of Pseudomonas fluorescens CHA0 in the root system of Arabidopsis thaliana ecotype Columbia; showed partial protection of leaves from *Peronospora parasitica*. This is determined by the extent of sporulation in the context of root fresh weight (FW) and it was found that spores per g of FW decreased in treated plants than the untreated ones (Iavicoli et al. 2003). Volatile organic compounds from rhizobacterial strains *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN973a have promising ISR and reduced disease severity of the pathogen *Erwinia carotovora* subsp. *carotovora* in *Arabidopsis* (Ryu et al. 2004). Nair et al. reported the systemic resistance of tomato plants colonised with *Glomus fasciculatum*, an AM fungus against *Alternaria alternata* (Nair et al. 2015). Additionally Choudhury et al. demonstrated that when *Rhizoctonia solani* causes lettuce bottom rot, nonribosomal secondary metabolites of *Bacillus amyloliquefaciens* FZB42 present in lettuce rhizosphere help in disease suppression by inducing plant defence gene (Chowdhury et al. 2015). Similarly it was also documented that *Micromonospora* isolated from root nodules of alfalfa when inoculated in the roots of tomato plants showed remarkable reduction in leaf infection by croorganisms were spatially separated which proves that *Micromonospora* induced systemic resistance against *Botrytis cinerea* (Hidalgo et al. 2015). Planchamp et al. also reported systemic resistance effect of *Pseudomonas putida* KT2440 against *Colletotrichum graminicola* in maize plants. Resistance observed was reduction in leaf necrosis and fungal growth infected by KT2440 (Planchamp et al. 2015). Salicylic acid induced systemic resistance shown in *Cucurbita* pepo against zucchini yellow mosaic virus (ZYMV) by delaying the appearance of disease severity, decreasing the percentage of dwarfism, improving the yield of infected plants and also showed the increasing SA concentration and peroxidase activity (Raie et al. 2017). Application of salicylic acid to the papaya seedlings showed no effect on the growth of the seedlings, but treated papaya seedlings showed increased tolerance to the papaya dieback pathogen *Erwinia mallotivora*, and 3 mM showed to be the best concentration to use as a SAR inducement (Bakar et al. 2018).

20.5 *Trichoderma* sp. Induced Resistance

It was reported that *Trichoderma harzianum* T39 was a remarkable biocontrol against *Botrytis cinerea*. Tomato, pepper, bean, lettuce and tobacco showed 25–100% reduction in grey mould symptoms when both microbes are spatially inoculated. Cucumber plants treated with *Trichoderma harzianum* show elevated levels of peroxidase and chitinase activities within 48 and 72 hours, respectively, and are believed to show induced systemic resistance (Yedidia et al. 1999). Cucumber plants grown in soil containing *Trichoderma asperellum* show 80% reduction of symptoms of angular leaf spot (by *Pseudomonas syringae* pv. *lachrymans*). It was further showed that mRNA of two defence genes, the lipoxygenase pathway gene and the phenylpropanoid pathway genes, was accumulated encoding hydroxyperoxide lipase (HPL) and phenylalanine ammonia lyase (PAL), respectively, that increased sixfold in bacterial inhibition in vitro (Yedidia et al. 2003). Similarly Martinez et al. recorded the elevation of PAL activity after 48 hours of application of *Trichoderma longibrachiatum* in the root system of melon. This induced accumulation of phytoalexin in leaves and reduced symptoms of powdery mildew caused by *Sphaerotheca fuliginea* (Yedidia et al. 2003). Yoshioka et al. reported that *Arabidopsis* root colonised with *Trichoderma asperellum* SKT-1 induces systemic resistance against *Pseudomonas syringae* pv. tomato. Both SA and JA/ET signalling pathways combine to trigger the ISR by cell-free culture filtrate of *Trichoderma* (Yoshioka et al. 2012). Similarly Lamdan et al. also reported that maize plant cultured with *Trichoderma virens* in the soil showed less severe infection by *Cochliobolus heterostrophus* compared to the untreated plants (Lamdan et al. 2015). *Trichoderma viride* reduces the oxidative stress in legumes (*Cajanus cajan*, *Vigna mungo*, *Vigna radiata*) due to pathogen attack. *T. viride*-induced resistance decreased the disease incidence by 7.52–15.40% against *Fusarium oxysporum* and 15.20–60.00% against *Alternaria alternata* (Rao et al. 2015). Plants grown on soil treated by solarisation and *Trichoderma harzianum* showed remarkable reduction of grey mould in cucumber, strawberry, bean and tomato and powdery mildew in cucumber caused by *Botrytis cinerea* and *Podosphaera xanthii*, respectively (Levy et al. 2015). Abdelrahman et al. (2016) demonstrated the accumulation of various abiotic and biotic stress metabolites in onion when treated with *Trichoderma longibrachiatum* and also showed resistance against oxidative stress and *Fusarium oxysporum* f. sp. cepa. *Trichoderma harzianum* T-aloe was tested in soya bean plants against *Sclerotinia sclerotiorum*, causing stem rot. T-aloe inhibited *S. sclerotiorum* with 51.2% efficiency in plate test and 56.3% in dual culture test. Treated plants showed increase in catalase, superoxide dismutase and peroxidise activity, while it showed decrease in H_2O_2 and superoxide radical in the leaves. In the leaves PR1, PR2 and PR3, the defence-related genes were also expressed (Zhang et al. 2016). Chickpea cultivar L550, susceptible to *Fusarium oxysporum* f. sp. *ciceris* (Foc), and Avarodhi, Foc resistant treated with *Trichoderma asperellum* T42 and *Pseudomonas fluorescens* OKC, showed significantly reduced wilting caused by Foc. Total phenolic content (TPC) also was increased in the treated plants

compared to the untreated ones (Kumar et al. 2017). Cucumber plants treated with *Trichoderma atroviride* TRS25 showed limited disease induction by *Rhizoctonia solani*. TRS25-induced resistance is dependent on SA derivatives such as methyl salicylate (MeSA), salicylic acid glucosylated conjugates (SAGC), ethylhexyl salicylate (EHS) and β-cyclocitral and other volatile organic compounds (VOC). Along with systemic resistance, TRS25 also promoted growth in the plants (Nawrocka et al. 2017). Jogaiah et al. studied the effect of *Trichoderma virens* (TriV_JSB100) on tomato cultivar Oogata-Fukuju against *Fusarium oxysporum* f. sp. *lycopersici*. They concluded that TriV_JSB100 spores cultured on barley grains and cell-free culture filtrate (CF) when inoculated in the tomato plants differentially induce SA and JA signalling pathways to induce defence against *Fusarium oxysporum* (Jogaiah et al. 2018). Similarly *Trichoderma asperellum* showed significant resistance against *Corynespora cassiicola* and *Curvularia aeria* that causes leaf spot disease in lettuce. Mycelial growth of *C. cassiicola* and *C. aeria* was reduced by *T. asperellum* by 83.79% and 85.71% in vitro. Cell-free culture filtrate of *T. asperellum* inhibits *C. cassiicola* and *C. aeria* by 50.38% and 53.97%, respectively. After 24 and 48 hours of treatment, β-1,3-glucanase, chitinase, peroxidise and polyphenol oxidase activities are found to be elevated (Baiyee et al. 2019).

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