Catching cereal killers: a multi-omics approach to disentangle yeast-Fusarium interactions in the phyllosphere

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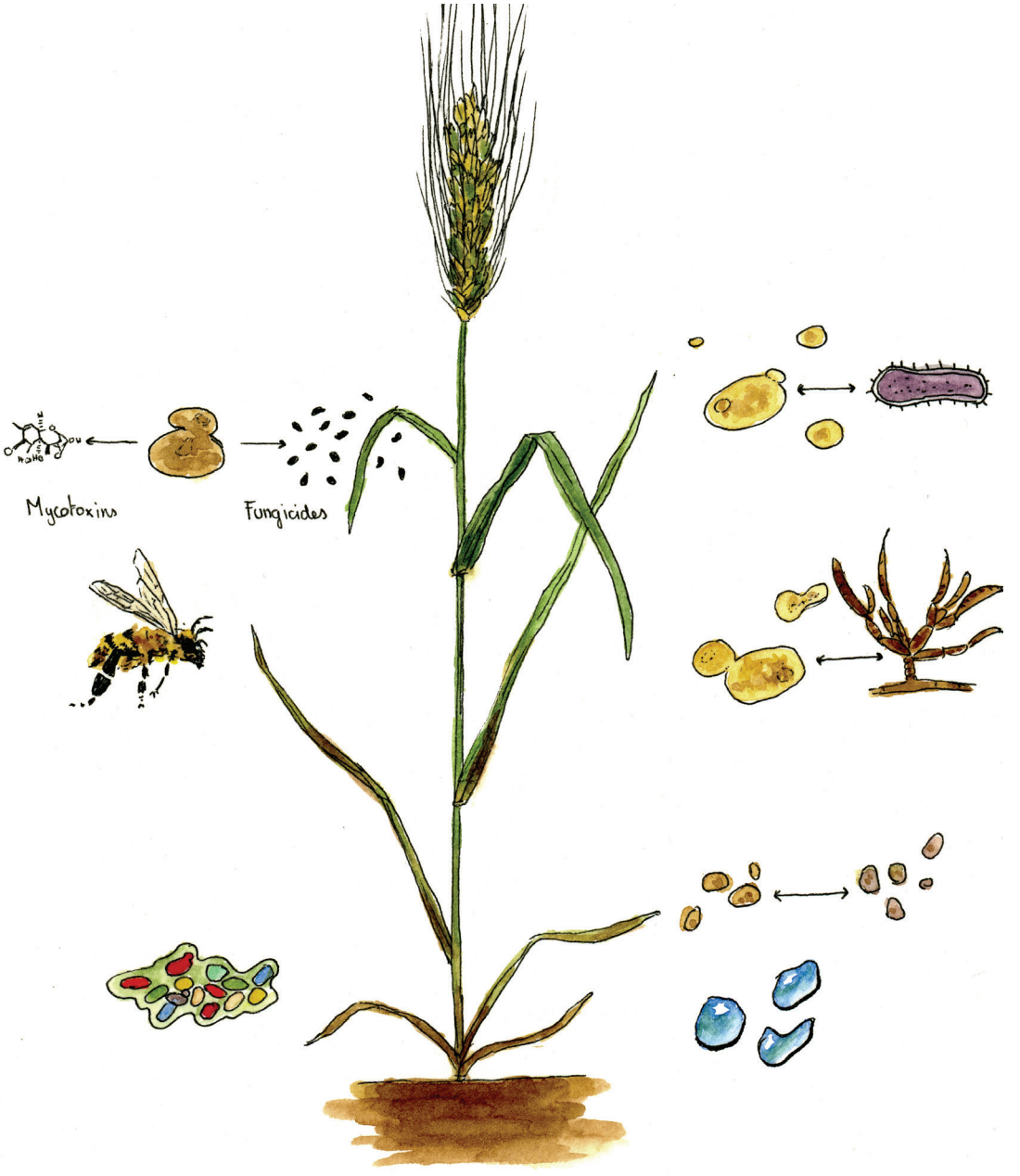
Mycotoxins
Fungicides

Chapter 2

Ecology and functional potential of phyllosphere yeasts

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Abstract

The phyllosphere environment, i.e. the aerial parts of plants, harbors a rich microbial life of which yeasts constitute a considerable part. Yeasts are versatile microorganisms that can withstand extreme abiotic conditions, yet little is known about their natural roles in the phyllosphere environment. Current knowledge on yeasts stems primarily from industrial and medical research on the model yeasts *Saccharomyces cerevisiae* and *Candida albicans*, which can be found on plant surfaces and tissues. Here, we explore the diversity, dynamics, interactions and genomics of yeasts associated with plant leaves and how tools and approaches developed for model yeasts can be exploited to disentangle the ecology and natural functions of phyllosphere yeasts. A first genomic survey exemplifies that we have only scratched the surface of the functional potential of phyllosphere yeasts.

Keywords

Environmental yeasts, adaptive traits, functional characterization, plant-microbe interactions, omics

Diversity and dynamics of phyllosphere yeasts

The phyllosphere, the aboveground parts of the plant, is colonized by bacteria, filamentous fungi, protists, viruses and yeasts. Here, the term yeast refers to budding yeasts and fungi with a yeast-like growth stage [Box 1]. Much of the current, yet limited knowledge of the diversity of phyllosphere yeasts comes from studies using cultivation-based approaches[1–3]. Earlier studies based on phenotypic characterization primarily discriminated between pink- and white-pigmented yeasts, classified as *Sporobolomyces* and *Cryptococcus*, respectively[4]. The introduction of Sanger sequencing enabled more thorough discrimination of yeasts based on sequencing of the **internal transcribed spacer** (ITS) and of the **D1/D2 region** (see Glossary). Culture-dependent studies have shown that yeasts comprise a diverse community on plant surfaces as well as in internal plant tissues, often reaching densities of on average 10^3 - 10^5 colony forming units (CFU) per gram of leaf, while bacteria typically reach densities of 10^7 CFU per cm^2 leaf[5]. However, comparison across different studies makes it difficult to estimate the densities of bacteria, filamentous fungi and yeasts in the phyllosphere. An earlier study on the phyllosphere of sugar beet plants showed that the densities of yeasts, filamentous fungi and bacteria reached up to 10^{10}, 10^7 and 10^{11} CFU per g of dry leaf[6], respectively, suggesting that yeasts are highly abundant on leaves. Investigations on the natural spatial distribution of yeasts in the phyllosphere are still needed. An earlier culture-independent study using **fluorescence in situ hybridization** (FISH) probes started advancing our knowledge on this topic. By following the natural distribution of yeasts on apple leaves over two years, the authors showed that higher cell densities of the yeast-like *Aureobasidium* were found over the midveins and smaller veins, compared to interveinal areas[7].

Community assembly in the phyllosphere is based on stochastic colonization (e.g. due to wind, rain or insects) and selection by the host plant[8]. Culture-dependent and independent studies on yeast dynamics provided further insight into factors affecting the abundance and diversity of phyllosphere yeasts. For example, higher yeast CFU numbers were detected on senescing sugar beet leaves as compared to mature and immature leaves[6]. Moreover, mature and immature leaves showed little fluctuation in the abundance of yeasts over the season. Most of the fundamental knowledge of yeast population dynamics, however, stems from studies on natural fermentation by yeasts living on the skin of grapes[9]. In this system, a number of factors such as nutrients (i.e. sugar concentration and nitrogen source), temperature and oxygen availability, influenced the population dynamics and metabolic activity of yeasts[10].

Cultivation-independent studies, using **restriction fragment length polymorphisms** (RFLP) analysis combined with **amplicon sequencing** of the ITS and D1/D2 regions and **metagenomics**, have advanced our knowledge on factors impacting yeast community assembly in the phyllosphere. No plant genotype effects have been observed for yeast communities in different studies on tomato leaves[11,12]. Moreover, yeasts showed a higher relative abundance than filamentous fungi in the leaves of different tomato genotypes. Another study investigating the microbial community composition of four different wild tomato species growing in two different geographical locations during two consecutive years showed that the epiphytic fungal communities (filamentous fungi and yeasts) remained stable across the samples[12]. Additionally, a persistent core of OTUs (i.e.

across host species and geographical origin) consisting of 10 bacteria, 7 yeasts and 12 filamentous fungi was found for 85% of the samples, including the yeasts *Filobasidium*, *Aureobasidium*, *Vishniacozyma* and *Cryptococcus*. Conversely, studies on *Mussaenda pubescens* leaves across different locations showed that fungal community composition was strongly influenced by host genotype, and weakly by geographic distance[13]. *Cryptococcus* and *Erythrobasidium* yeasts were the most dominant Basidiomycota genera in this study. Studies on cereals have shown that fungicide application and geographic location affected the diversity of yeasts on the leaves of wheat, barley, oat, rye and triticale, albeit to a lesser extent[14]. Interestingly, location explained yeast diversity of younger leaves, whereas the cultivar better explained the diversity of yeasts on older wheat leaves.

Box 1. Taxonomy and diversity of yeasts

Yeasts are usually described as single-celled eukaryotic members of the fungus kingdom. The term yeast includes several unrelated fungal lineages that have a unicellular phase in their life cycle. This lifestyle is spread throughout the fungal kingdom and, therefore, yeast is not considered a taxonomic unit[94]. Yeasts are placed in two separate phyla, namely Ascomycota and Basidiomycota[87]. The Ascomycota phylum comprises the 'true yeasts' (subdivision Saccharomycotina, e.g. *Saccharomyces cerevisiae*) and the 'fission yeasts' (subdivision Schizosaccharomycetes, e.g. *Schizosaccharomyces pombe*)[94] which, primarily divide by budding and binary fission, respectively. During budding division, a new cell is formed through a bud from the parent cell, whereas during the binary fission a single cell is divided into two equal daughter cells. The Basidiomycota phylum includes dimorphic filamentous fungi (e.g. *Aureobasidium pullulans*, *Candida albicans*) that display yeast-like and filamentous growth stages.

Phyllosphere yeast communities have also been shown to follow seasonal dynamics. For example, a cultivation-dependent survey of the yeast distributions on leaves and flowers of 25 different plant species grown over five consecutive years significantly changed over the year. The relative abundance of ascomycete yeasts gradually increased on leaves during the transition from spring to autumn and then decreased in winter[15]. *Cryptococcus albidus* and *Rhodotorula mucilaginosa* were primarily found on leaves, whereas *Metschnikowia* sp. predominated the nectar-yielding flowers, representing more than 50% of the community[15]. More recent studies using cultivation-independent approaches showed that *Aureobasidium*, followed by *Cryptococcus*, was the most abundant genus on leaves and fruits of four different plum cultivars in the early fruit maturation period (May), whereas *Metschnikowia*, a yeast attracted to high-sugar contents, was the most abundant genus in the late fruit maturation period (July)[16]. Interestingly, yeast abundance on leaves and fruits in the month of July, during fruit maturation, followed a cultivar-specific manner, which is hypothesized to be caused by the changing sugar content. This seasonal dynamics of yeasts on the leaves and flowers is likely related to fluctuating temperatures and specific nutrients secreted or leaking from the plant during the different developmental stages and seasons[3,15,17]. Indeed, studies on mature grapes showed that

the yeast population reached 10^3 and 10^5 cells/g, with higher densities (approximately one log) found on damaged berries with higher concentrations of sugars and other nutrients[18]. Higher densities of *Saccharomyces cerevisiae* were also described for damaged grape berries. Despite its extensive association with human-related environments, such as for wine fermentation, the natural abundance of *S. cerevisiae* is found to be low on undamaged grape berries[19]. This species is naturally found in different ecological niches, often in changing environments and subjected to limiting nutrient availability, such as the bark of oak tree stems[20–22].

In addition to host species and environmental factors, other biotic factors such as inter- and intra-specific as well as cross-kingdom interactions can impact the dynamics of yeasts in the phyllosphere. This was exemplified in the study by Sapkota et al (2017) who showed, via network analysis, a negative correlation between the yeasts *Sporobolomyces*, *Dioszegia*, *Cystofilobasidiaceae* with the pathogen *Zymoseptoria tritici* on wheat leaves[23]. Recent computational analyses coupled with colonization assays provided insights into the interconnectivity of specific microbial taxa in the *Arabidopsis* phyllosphere[24]. The filamentous oomycete *Albugo*, the yeast *Dioszegia* and the bacterium *Caulobacter* were highly interconnected and impacted microbial community structure on the leaves of *Arabidopsis*. In addition, *Dioszegia* was found to inhibit the growth of *Caulobacter*, possibly due to spatial competition and direct antagonism. Also plant chemical cues, such as hormones and other metabolites, may inhibit or promote the growth of specific yeasts. To date, however, little is known about yeast-yeast interactions in the phyllosphere as well as their interactions with other members of the phyllosphere (Figure 1, Key Figure). Below we highlight several interactions between yeasts and other (micro)organisms in the phyllosphere.

Multipartite interactions between yeasts and other phyllosphere members

Yeast-yeast interactions

Toxin production by yeasts is a well-known mechanism underlying yeast-yeast interactions that can confer a competitive advantage under limited nutrient availability. Killer yeasts produce and secrete extracellular toxic proteins (killer toxins) that are lethal to other sensitive yeasts competing for the same space and/or carbon sources. The ability to produce killer toxins appears to be widespread in nature and has been described for *Pichia*, *Sporobolomyces*, *Rhodotorula*, *Candida*, *Metschnikowia*, *Debaryomyces*, *Aureobasidium* among others[26–28]. Killer toxins have been described to play a role in intra- and inter-specific interactions[27]. To date, however, the mechanisms underlying these interactions are largely unknown and the majority of studies on the ecology of killer yeasts is limited to *in vitro* experiments and mathematical models.

Yeast-plant interactions

Previous studies showed that *Pseudozyma churashimaensis*, isolated from the leaves of pepper plants, provided plant protection against the bacterial pathogen *Xanthomonas*

axonopodis and a number of plant viruses by eliciting plant defenses[29]. The underlying mechanisms by which *P. churashimaensis* induces resistance remains to be investigated. For *Hanseniaspora opuntiae*, genome-wide transcriptome analysis of *Arabidopsis* revealed transcriptional reprogramming of plant defense responses against the fungal pathogen *Botrytis cinerea* involving genes in the **jasmonic acid/ethylene (ET/JA) signaling** pathways[30]. Several other yeast species, including *Rhodosporidium paludigenum*, *Metschnikowia fructicola* and *Candida oleophila*, have shown to protect fruits against a broad range of pathogens by inducing systemic resistance[31–34]. Proteomic and transcriptomic analysis of grapes and apple fruit tissues inoculated with *Yarrowia lipolytica* revealed differential expression of proteins involved in defense-responses mediated by a crosstalk between the salicylic acid (SA) and ET/JA pathways[35,36].

Several studies have been initiated to unravel the underlying mechanisms for the growth-promoting effects of yeasts on plants. For example, the yeast-like fungus *Ustilago esculenta* altered *Arabidopsis* root architecture (e.g. increased the number of lateral roots and reduced primary root elongation) by modifying auxin-regulated gene expression[37]. Knowledge on phyllosphere yeasts with deleterious effects on plant growth and health is still largely elusive. Studies on the *Arabidopsis* phylloplane identified two potential yeast pathogens belonging to the genera *Taphrina* and *Protomyces*, which produce indolic compounds and induce an auxin response *in planta*[1]. Members of these genera have a yeast-like growth stage and are known to cause plant diseases such as tumors and gall symptoms in flowers, stems and leaves.

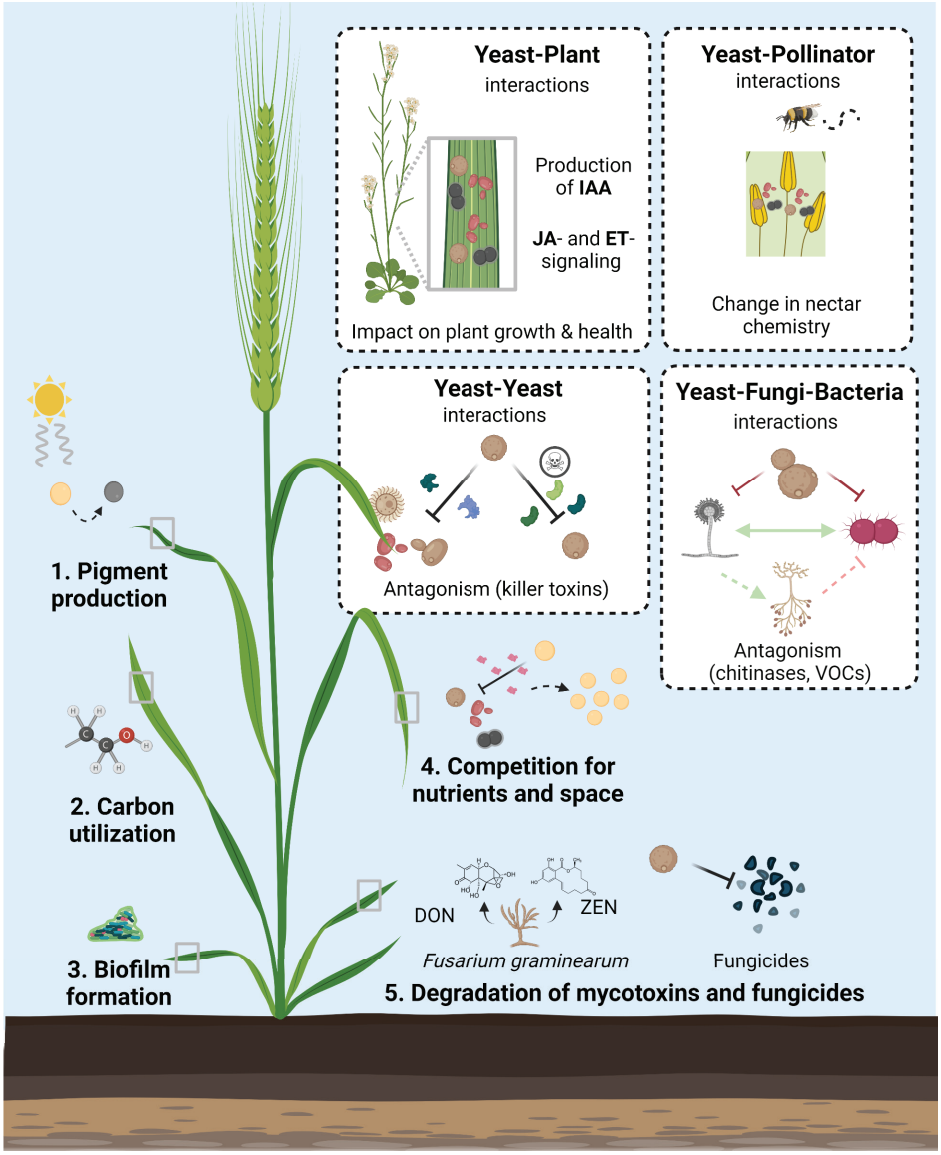
In addition to leaves and flowers, floral nectar serves as a microhabitat for several yeasts. Yeasts can alter nectar chemistry, including sugar content and pH, and concomitantly affect interactions between plants and pollinators[25]. For example, nectar inoculated with the yeast *Metschnikowia reukaufii* was shown to be visited more frequently and for a longer period of time by foraging bumblebees than sterile nectar[38,39]. Yeasts have also been involved in the attraction of beetles that feed on fermented plant sap via the production of specific volatiles[40]. Yeast-insect associations appear to be widespread in nature and have been previously described in several studies[41–44].

Yeasts in multipartite interactions

The antagonistic activity of yeasts against bacteria and filamentous fungi is long known and conferred via different mechanisms, including competition for nutrients and space, secretion of enzymes, toxin production, secondary metabolite production (volatile and diffusible) and mycoparasitism[45]. A recent functional characterization of the antagonistic activity of *Metschnikowia pulcherrima* demonstrated the involvement of pulcherriminic acid[46]. A random point mutation in a gene encoding an **ortholog** of the *S. cerevisiae* SNF2 protein, compromised the synthesis of the cyclodipeptide pulcherriminic acid, leading to partial loss of the antifungal activity by this yeast[46]. Mass spectrometry confirmed the presence of the pulcherrimin precursor and new pulcherriminic acid precursors in the wildtype, which were absent in the mutant strain. In another study, coupling of genome sequencing, genetic characterization and transcriptome analysis revealed the involvement of the lysozyme GH25 in antagonism by the phyllosphere yeast *Moesziomyces bul-*

Key Figure

Representation of adaptations and interactions of yeasts in the phyllosphere



Yeasts colonize the surfaces and internal tissues of plants, including leaves, flowers, and fruits (the phyllosphere). Different abiotic and biotic factors influence the abundance of yeasts in the phyllosphere. Different physiological and genetic adaptations of phyllosphere yeasts to (a)biotic stresses are depicted and include: pigment production, flexible carbon utilization, biofilm formation, competition for nutrients and space, and degradation of mycotoxins and fungicides. Interactions with their host plants, other members of the phyllosphere (yeasts, bacteria, fungi, pollinators), may affect the abundance and diversity of yeasts. Figure designed with BioRender (https://biorender.io), not to scale.

latus towards the oomycete pathogen *Albugo laibachii*[47]. Further functional characterization of this enzyme will be needed to elucidate if and how the enzyme directly interferes with the growth and development of the pathogen.

In the next section, we highlight the different adaptive traits that are proposed to influence the abundance and diversity by facilitating their colonization and competition with other microorganisms in the phyllosphere.

Lifestyle of phyllosphere yeasts: adaptation to a harsh environment

The phyllosphere is a harsh and dynamic environment where yeasts are exposed to a number of different (a)biotic stresses, including low nutrient availability, UV radiation, temperature and humidity oscillations, as well as toxic compounds (**mycotoxins** and fungicides)[48]. The phyllosphere is also considered a 'short-lived environment' for yeasts inhabiting flowering and shedding plants[8,49]. Therefore, the lifecycle of microorganisms living in the phyllosphere of these plants might be shorter compared to those living on evergreens. Below we describe key adaptations which have been proposed to facilitate the colonization and survival of yeasts in the phyllosphere (Figure 1). In Box 2 we elaborate on the proposed molecular mechanisms underlying these adaptations.

Carbon and nitrogen metabolism

Versatile carbon utilization plays an important role in microbial adaptation to the phyllosphere where microorganisms compete for nutrients and space. Reduced C1-compounds, such as methane and methanol, are abundant and released by most plants and can be utilized by methylotrophic microorganisms as the sole carbon source[50]. Methanol is released via different mechanisms by plants, primarily via cell wall pectin demethylesterification via pectinmethylesterases (PMEs), as well as via demethylation of nucleic acids and proteins. The latter process, however, is believed to be negligible (reviewed in [50–52]). Not surprisingly, the phyllosphere microbial communities often harbour a variety of methylotrophs comprising bacteria, fungi and archaea[12,51]. Compared to methylotrophic bacteria, the ecology of methylotrophic yeasts is still largely unknown and only a few genera have been described to utilize methanol, which include *Candida*, *Pichia*, (*Komagataella*) *Ogataea*, *Hansenula* and *Kuraishia*[53]. The biochemistry and cell biology of methylotrophic yeasts have been investigated for *Candida boidinii*, *Pichia pastoris* and *Hansenula polymorpha* [Box 2], but for the other genera/species the underlying mechanisms remain unknown. Whereas methanol is used as carbon source by eukaryotic methylotrophs, methylamine is used as nitrogen source. Quantitative analyses of the growth of wild-type *Candida boidinii* and an atg11 mutant strain (scaffold protein that mediates selective autophagy) on *Arabidopsis* leaves showed a shift in nitrogen source, with nitrate and nitrate reductase (Ynr1) being essential for colonization of young leaves, and methylamine for colonization of older and wilting leaves[54].

Biofilm formation

The majority of phyllosphere microorganisms live in multicellular communities (biofilms) attached to the surfaces of leaves and fruits[55]. Biofilms play a role in stress resistance, providing a competitive advantage[56,57]. Research on biofilm formation by *Candida* and *Saccharomyces* species has greatly advanced our understanding of the molecular and genetic basis of biofilm development in yeasts[55,58]. Different imaging methods, e.g. scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), have been employed to visualize biofilm formation by pathogenic *Candida in vivo*[59,60]. At the molecular level, different transcriptional regulators have been identified that coordinate biofilm formation in *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis*[61]. Large-scale screening of 4,961 *S. cerevisiae* mutants revealed the involvement of **peroxisomes** in biofilm formation[62]. Furthermore, biofilm formation by the biocontrol yeast *Pichia kudriavzevii* coincided with enhanced antagonistic activities against the fungal pathogens *Botrytis cinerea* and *Colletotrichum gloeosporioides* as well as with tolerance to heat and oxidative stress[57]. To date, however, biofilm research of beneficial plant-associated yeasts lags far behind that of medically important yeasts. Hence, further efforts should be made to visualize the spatial arrangements of yeasts on plant surfaces and to disentangle the underlying mechanisms of biofilm formation in the phyllosphere.

Pigment production

Yeasts produce several carotenoids (red, orange and yellow pigments), including β-carotene, torulene, astaxanthin and torularhodin. Microbial pigments have been implicated in absorption of radiation, regulation of temperature and protection against toxins[63–65]. The occurrence of pigmented yeasts in the phyllosphere is presumably the result of selection pressure imposed by UV radiation. Remarkably, dark-pigmented yeasts were more abundant in the Arctic whereas light-pigmented yeasts were more abundant in the Tropics[63]. Dark-pigmented yeasts overheated when irradiated at ambient temperatures, whereas non-pigmented yeasts were relatively unaffected. In contrast, dark-pigmented yeasts had an advantage when exposed to cold temperatures compared to the non-pigmented yeasts. These findings suggest that pigment production can be seen as a proxy for the geographical distribution of yeasts. The protective role of pigments in UV protection has been described for the yeasts *Rhodotorula mucilaginosa*, *Cystofilobasidim capitatum* and *Sporobolomyces ruberrimus*. Higher concentrations of carotenoids, in particular torularhodin, were found to enhance UV tolerance in *R. mucilaginosa*[64]. In addition to UV tolerance, pigment production provides a physical barrier against toxic compounds[65]. For example, albino mutants of *Aspergillus* spp. were more sensitive to reactive oxygen species, whereas pigmented *Cryptococcus neoformans* was less susceptible to hypochlorite[66]. A number of yeast pigments was also associated with antimicrobial activity. For example, the red pigments torulene and torularhodin produced by *Rhodotorula* glutinis and *Sporobolomyces* sp. showed antagonistic activity against *Bacillus cereus* and *Alternaria citri*[63,66,67].

Degradation of toxins, heavy metals and fungicides

Mycotoxins, heavy metals and fungicides are widespread in nature and agriculture and

Box 2. Mechanisms involved in the adaptation of yeasts to life in the phyllosphere

Carbon and nitrogen utilization.

The yeasts *Pichia pastoris* and *Candida boidinii* have been the major focus to unravel the mechanisms involved in sensing and utilization of methanol as a carbon source in the phyllosphere environment. Changes in methanol emissions appear to follow the plant circadian rhythm as well as plant defense and developmental processes, increasing during the night and/or after wounding and decreasing during the day and with leaf aging[10]. The Wsc cell-surface protein family (Wsc1 and Wsc3) is involved in sensing methanol and transmitting the signal to methanol-inducible genes through Rom2 in *Pichia pastoris*[95]. Knock-outs of several methanol-inducible genes in *Candida boidinii* identified the essential proteins[96]: i) alcohol oxidase (AOD, e.g. MOD1 and MOD2[97]) which oxidizes methanol to formaldehyde, ii) dihydroxyacetone synthase (DAS) which fixes the toxic intermediate formaldehyde to xylulose 5-phosphate, and iii) formaldehyde dehydrogenase (FLD) which yields NADH (reviewed in[98]). Another important aspect is the localization of these enzymes in peroxisomes, membrane-bound organelles encoded by PEX genes, together with the peroxiredoxin PMP20[96]. Nitrogen is available in different forms in the phyllosphere (e.g. ammonium, nitrate, methylamine) and essential for plant growth and development[54]. Studies on nitrogen metabolism have mainly focused on *Hansenula polymorpha* and C. *boidinii* since *S. cerevisiae* is unable to utilize nitrate. Nitrate and methylamine are secondary nitrogen sources that need to be reduced to primary sources such as ammonium and glutamine. Nitrate is reduced to nitrite by nitrate reductase (Ynr1), and further to ammonium by nitrite reductase (Yni1), whereas methylamine is converted to ammonium by the peroxisomal enzyme amine oxidase (Amo1). Methylamine also induces the methanol-induced genes AOD1, FLD1, FGH1 and FDH1 since the intermediate compound of methylamine reduction is formaldehyde[99].

Resistance to environmental stresses.

S. cerevisiae has been used to study a plethora of mechanisms to cope with fluctuating and hostile environments. One important protein family for this adaptation is flocculins, encoded by the FLO genes[100]. During biofilm formation, this gene enables *S. cerevisiae* to switch from unicellular to multicellular growth. This specific type of growth shields cells from the stressful environment, and consequently, increases resistance against antimicrobial compounds[62]. Genome-wide screening of *S. cerevisiae* identified six single gene mutants (SNF2, YHR134W, THR1, LMS1, YAF9 and YBR099C) involved in UV resistance which had not previously been described[101]. In addition to these, more than 30 other genes were found to be involved in UV resistance in *S. cerevisiae* using random mutagenesis, however the underlying molecular mechanisms are currently unknown[63]. Pigments have also been described to protect yeasts against UV radiation. In this context, several genes involved in pigment production have been identified in *Rhodotorula* sp., including two carotenoid synthesis-related genes (phytoene synthase (PSY1) and phytoene dehydrogenase (CRTI)), and three other key genes (phytoene synthase (crtB), lycopene cyclase (crtY) and phytoene desaturase (crtI))[102].

impact yeast colonization and survival in the phyllosphere. Mycotoxins, such as aflatoxins, zearalenone and deoxynivalenol produced by leaf-infecting filamentous fungi, are typically found on leaves of cereals[68]. Yeasts have been shown to absorb mycotoxins into the outermost layer of their cell wall[69] as well as to remove toxic compounds (e.g. arsenic, cadmium and mercury) and other metals from the environment[70]. For example, *Aureobasidium* can utilize inorganic sulfur as well as absorb and detoxify copper[65,71]. In addition to mycotoxins and heavy metals, phyllosphere yeasts are able to degrade fungicides[72,73]. Higher relative abundance of yeasts in the wheat phyllosphere subjected to different fungicide treatments were found, most notably *Sporobolomyces* but also *Udeniomyces* and *Cryptococcus* species[73] (Box 2).

Collectively these studies give insights into the different adaptations employed by a few yeast genera that likely facilitate their survival in the phyllosphere. Whether these traits are widespread in other yeast genera/species and if they favor their colonization in the phyllosphere remains to be investigated. These versatile traits have led to enhanced interest in fundamental research in eukaryotic cells and biotechnological applications. For example, *S. cerevisiae* serves as the model organism for biology of eukaryotes as well as a machinery for a vast variety of industrially interesting enzymes. In the last section, we will explore techniques extensively used for this yeast species and how we could learn from these approaches to understand the ecology and functional roles of phyllosphere yeasts.

Unexplored genomic and functional potential of phyllosphere yeasts

Insights and approaches from model yeasts

Saccharomyces cerevisiae has been traditionally employed in food and fermentation processes and, in the past decades, explored for the production of numerous enzymes, biopharmaceutical proteins, and biofuels. *Saccharomyces cerevisiae* was the first completely sequenced eukaryotic genome[74,75]. Since then, it has been used as a model organism for investigating the molecular and metabolic processes of eukaryotes. Genome databases, genome-wide collections of mutant libraries as well as protein interactome collections for *S. cerevisiae* have allowed a high-throughput functional characterization[76,77]. For example, an extensive collection of knock-out mutants covering 96% of the yeast genome as well as genome-wide engineering with single-nucleotide precision are currently available[78]. A recent systematic analysis using 4,000 single- and 30,000 double mutants generated a gene-gene interaction map for *S. cerevisiae* under 14 environmental conditions, which included alternative carbon sources, osmotic and genotoxic stresses as well as exposure to different bioactive compounds[77,79,80]. In addition, large-scale population genomic analyses explored the phenotypic diversity of 1,011 *S. cerevisiae* isolates from different ecological and geographical origins[81]. These resources and tools are highly instrumental for metabolic engineering, systems- and synthetic biology of eukaryotic microorganisms. Hence, these approaches and tools represent an excellent basis for investigating the genomic potential of environmental yeasts, including phyllosphere yeasts[82].

Advances in genome-editing tools for non-*Saccharomyces* isolates have been facilitated by the development of the **CRISPR/Cas9 system**. This system has been successfully adapted not only to *S. cerevisiae*[83] but also to other yeast species used in biotechnology and cellular biology research, such as *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Komagataella phaffii* (formerly *Pichia pastoris*) and *Schizosaccharomyces pombe* (reviewed in[84]), and more recently, also to environmental yeasts such as *Aureobasidium pullulans* and *Pseudozyma flocculosa* isolated from leaves[85,86]. The production of flocculosin, a rare antifungal glycolipid, has been described for *Pseudozyma flocculosa* and was proposed to be involved in the antifungal activity of *P. flocculosa* against powdery mildew. Functional characterization of this compound has been previously hampered by the inability to genetically manipulate this species. CRISPR-Cas9 system allowed the generation of a mutant specifically altered in flocculosin biosynthesis[86]. Interestingly, however, these mutants did not lose their ability to inhibit the fungus, suggesting that this compound was not the prime responsible for the antifungal activity of *P. flocculosa*.

Genomic and functional potential of phyllosphere yeasts

Since the genome of *S. cerevisiae* was sequenced, the number of yeast genome studies has increased considerably, although the number of sequenced genomes from environmental yeasts is still limited. Here we advocate coupling 'omics-based approaches to functional analysis to explore the genetic and functional repertoire of phyllosphere yeasts. Although research on model yeasts has provided significant insights into the yeast molecular, cellular and metabolic processes (see above), little is still known on how phyllosphere yeasts cope with the adverse environmental factors listed above. For example, environmental yeasts are limited by low-nutrient availability, in contrast to the lifestyle of *S. cerevisiae* and other conventional yeasts used in fermentation studies which thrive in very rich culture conditions. Phyllosphere yeasts likely use different metabolic routes and regulatory mechanisms for substrate utilization and thus, further efforts should be made to investigate their genetic and metabolic potential under natural conditions.

To illustrate the yet untapped potential of phyllosphere yeasts, we performed a literature search (Web of Science) on available articles on yeasts. Over 294.000 articles were found with the search term 'yeasts', with the majority of these articles belonging to the categories biochemistry, biotechnology and cell biology. A total of 19.658 articles was found in ecology-related categories, such as Environmental Science, Plant Science, Agronomy and Ecology. This simple search illustrates the current knowledge gap of yeast ecology. Currently, 2.521 genomes are publicly available for true yeasts (NCBI genome, true yeasts Saccharomycotina) with approximately 50% belonging to *Saccharomyces* species. We further searched for available genomic data using yeasts inhabiting the wheat (*Triticum aestivum*) phyllosphere (including leaves, spikes, stems, grains and internal tissues) as an example. Wheat is one of the oldest and most valuable cereal crops in the world (FAO) and, together with rice, the most studied crop with an extensive number of microbiome studies currently available (Web of Science, April 2022). A total of 688 articles was found using the search term 'wheat microbiome', 318 articles for the 'wheat microbiome bacteria' and less than 10% using 'wheat microbiome yeast' (28 articles) or 'wheat microbiome archaea' (26 articles), another group of microorganisms often overlooked in microbiome studies (Web of Science, April 2022). Since the majority of microbiome studies does not

make a distinction between filamentous fungi and yeasts, this literature search most likely missed other studies on yeasts. To compensate for this, we performed an additional literature search using the combination 'wheat' and the names of each of the 186 yeast genera (true yeasts and yeast-like fungi) described so far based on the latest edition of the taxonomic study 'The Yeasts'[87]. Out of these 186 genera, 34 genera comprising 45 species were found for the wheat phyllosphere (Table 1). To have insight into the current publicly available genomic data for the phyllosphere yeasts as well as their genomic potential, we then searched for genome sequences of yeast species isolated from the wheat phyllosphere. For the 45 yeast species found in this literature search, whole genome sequences were available for 37 species (approximately 80%) at NCBI Genome (https://www.ncbi.nlm.nih.gov/datasets/genomes/). Genera such as *Rhodotorula*, *Aureobasidium* and *Candida* have the highest number of sequenced genomes. Additionally, 8 out of the 45 species are largely unexplored (< 10 articles on Web of Science), with no information about their functional potential, industrial use or pathogenicity. For isolates of environmental origin, the available studies primarily describe their antagonistic activities but the underlying mechanisms are still largely unknown. The majority of studies involving functional characterization includes the yeast species *Aureobasidium pullulans*, *Candida albicans*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima*, *Pseudozyma flocculosa* and *Saccharomyces cerevisiae*. These findings illustrate that the functional characterization of environmental yeasts still lags behind that of the model yeast *S. cerevisiae* and other yeasts of medical and industrial importance.

Tools and approaches successfully used in studies investigating the biotechnological potential of yeasts could also be exploited for advancing our understanding of the ecology of phyllosphere yeasts. For example, CRISPR/Cas9 has so far been applied to 5 out of the 45 yeast species found in the wheat phyllosphere (*Pseudozyma flocculosa*, *Debaryomyces hansenii*, *Aureobasidium pullulans*, *Saccharomyces cerevisiae* and *Candida albicans*[84,86,88,89]). In addition to its successful application for genome editing of *Candida albicans* and *Saccharomyces cerevisiae*[84], CRISPR/Cas9 has also been successfully used to unravel the involvement of flocculosin in the biocontrol activity of *P. flocculosa*[86], and part of the molecular mechanism underlying extremophilic properties of *D. hansenii*[89]. For *Aureobasidium*, this technique has proved to be 40% more efficient compared to mutagenesis based on homologous recombination with donor DNA[88]. Overall, the application of CRISPR-Cas9-mediated mutagenesis has shown to be precise and applicable in yeasts. In combination with the large availability of whole genome sequences, more efforts should be put into the optimization of this method for different yeast species to investigate the molecular mechanisms underlying interactions and adaptations to the harsh life in the phyllosphere.

Table 1 Genomic and functional characterization of wheat phyllosphere yeasts. Wheat (Triticum aestivum), one of the oldest and most cultivated cereals, has been used as the model plant for this literature search. The species listed below were isolated from wheat leaves, grains, spikes, straws and inner tissues. Species indicated in bold refer to those with assembled genomes available at NCBI. Functional characterization and tools used to unravel the underlying mechanisms are indicated when available (NA: not available). The studies used for the functional charaterization were expanded to different plant species (i.e. not exclusively isolated from wheat). When no functional characterization was available for environmental isolates, we included approaches and tools used for industrial and clinical isolates. Source refers to the origin of the yeast strain described in the study used for functional characterization (E: environemental isolates, I: industrial isolates, C: clinical isolates).

Species	Phyllosphere compartment	No. of genomes (aver. size)	Functional characterization (approaches/tools)	Source	Refs
Ascomycota					
Aureobasidium pullulans, A. proteae	Leaves, spikes, grains	73 (28.3 Mb)	Genome mining for biotechnological potential and stress tolerance (antiSMASH); volatilome (SPME-GC-MS); comparative proteomics (MALDI-TOF/TOF); biocontrol potential (bioassays); optimization of genome editing (CRISPR/Cas9); degradation of fungicides (bioassays)	E, I	72, 88, 103, 104
Arxula terrestris	Grains	0	NA	NA	105
Candida albicans, C. sake, C. pelliculosa, C. silvicola	Leaves, grains	73 (14.2 Mb)	Comparative genomics; genome-wide transcriptomics; gene editing (CRISPR/Cas9); fungicide resistance (bioassays), biofilm formation (bioassays, microscopy), virulence (mutant libraries)	E, I, C	59, 60, 84, 103, 106
Clavispora lusitaniae	Grains (Qu)	31 (44.5 Mb)	Starter culture of rice wine wheat (ribosomal innic spacer analysis)	E, I	107
Debaryomyces hansenii	Leaves, grains	12 (12.1 Mb)	Production and antimicrobial activity of killer toxins inhibiting filamentous fungi and bacteria (SDS-PAGE, bioassays); mechanisms of haloand osmotolerance (immunoblotting, microscopy, CRISPR/Cas9); fungicide resistance (bioassays)	E, I	54, 74, 78, 79

Metschnikowia pulcherrima	Grains	1 (21.8 Mb)	Mechanisms of antagonistic activity against *Botrytis* caroliniana (whole-genome sequencing of wild-type and naturally occurring mutant strains, complementation, mass spectrometry)	E, I	46, 109
Pichia anomala	Leaves, grains	10 (14.15 Mb)	Mechanism of antagonistic activity against *Penicillium* roqueforti (wheat grain bioassay	E, I	106
Saccharomyces cerevisiae	Grains	1030 (12 Mb)	Production of killer toxins; optimization of genome editing (CRISPR/Cas9); biofilm formation (mutagenesis); model for eukaryotic biology; first completely sequenced eukaryotic genome; protein interactome collection (protein fragment complementation assay); UV resistance (random mutagenesis)	E, I, C	63, 75, 76, 78, 106, 110
Saccharomycopsis sp.	Grains (Qu)	14 (20 Mb)	Starter culture of rice wine wheat (metagenomics)	E, I	111
Saturnispora silvae	Grains	16 (9.3 Mb)	NA	NA	112
Schizosaccharomyces pombe	Grains	19 (12.8 Mb)	Hydrolyzation of maltose (in vitro screening)	I	113
Basidiomycota					
Bullera globispora	Leaves, grains	0	NA	NA	72, 114, 115
Bulleromyces albus	Grains	1 (19.4 Mb)	Identification of target decaprenyl diphosphate synthase-PDS genes for the production of coenzyme Q, essential for aerobic growth and oxidative phosphorylation (cloning)	I	105, 116
Cryptococcus tephrensis, C. laurentii, C. stepposus, C. albidus, C. humicolus	Grains	5 (20 Mb)	Resistance to fungicides (in vitro screening); mechanisms of antagonistic activity against powdery mildew, *B. cinerea*, P. *expansum* and A. niger (in vitro, in vivo and field experiments)	E	106, 112, 117, 118

Cutaneotrichosporon sp.	Grains	14 (19 Mb)	NA	NA	119
Cystofilobasidium capitatum	Inner tissue	1 (21.1 Mb)	Production of cold-active enzymes (SDS-PAGE); visualization of carotenoids and lipids formation and storage inside yeast cells (CLSM, FLIM)	I	120-122
Dioszegia hungarica, D. fristingensis, D. crocea, D. aurantiaca	Leaves, grains	2 (20 Mb)	Identification of inter- and intra-kingdom interactions (correlation network analysis), antagonistic activity against the bacterium Caulobacter (bioassays)	E	11, 13, 76, 77, 80
Filobasidium magnum, F. floriforme, F. wieringae	Grains	2 (32.2 Mb)	Mechanisms of radiation resistance (bioassays, membrane integrity assays, SEM)	E	112, 124
Golubevia pallescens	Grains	1 (27.8 Mb)	Mechanisms of antagonistic activity (mRNA-based systems approach)	E	105, 125
Guehomyces pullulans	Grains	0	NA	NA	105
Hannaella sinensis	Grains	0	Production of plant hormones and plant growth promoting traits (colorimetric assay, HPLC)	E	105, 126
Holtermanniella nyarrowii, H. takashimae	Leaves	1 (17.7 Mb)	NA	NA	115
Itersonilia pannonicus	Leaves, spikes, grains	0	NA	NA	11, 74, 75, 79, 85
Leucosporidiella fragaria, L. golubevii	Leaves	0	NA	NA	72
Malassezia restricta	Inner tissue, grains	5 (7.3 Mb)	Plant-pathogenic smut fungus for cereal plants (metagenomics)	E	105, 120
Microbotryum lychnidis-dioicae	Stem	23 (28 Mb)	Plant-pathogenic smut fungus for cereal plants (bioassays)	E	127

Naganishia albidosimilis, N. albida	Grains	5 (20.7 Mb)	Mechanisms of antagonistic activity against the fungal pathogens *Aspergillus flavus* and *Fusarium* proliferatum (bioassays)	E	112
Pseudozyma flocculosa	Leaves	2 (25.3 Mb)	Mechanisms of antagonistic activity against plant pathogenic fungi (CRISPR/Cas, bioassays; proteomics	E	86, 103
Rhodotorula glutinis, R. mucilaginosa, R. kratochvilovae, R. taiwanensis	Leaves, grains	110 (20.9 Mb)	Degradation of fungicides (bioassays); photoprotection activities by pigments (bioassays, HPLC-DAD)	E, I	64, 103, 112
Sporobolomyces roseus	Leaves, spikes, grains	1 (22.4 Mb)	Mechanisms of antagonistic activity against the fungal pathogens *Zymoseptoria tritici*, Cochliobolus sativus and *Penicillium expansum* via nutrient competition (bioassays)	E	11, 74, 75, 80, 91, 92
Tilletiopsis washingtonensis	Grains	1 (18.8 Mb)	Mechanisms of antagonistic activity against powdery mildews (bioassays)	E	105, 130
Trichosporon porosum	Straws	2 (25.8 Mb)	Production of triglycerol-lipids (enrichment culture and metagenomics)	I	131
Ustilago tritici	Grains	2 (19.5 Mb)	Plant-pathogenic smut fungus for wheat (bioassays)	E	132
Vishniacozyma victoriae, V. tephrensis	Leaves, spikes grains	1 (11.7 Mb)	Mechanisms of antagonistic activity against filamentous fungi; mycoparasitism (bioassays)	E	11, 75, 79, 80, 85

Concluding Remarks and Future Perspectives

Current research on the phyllosphere has focused primarily on bacteria and filamentous fungi, in particular plant pathogens. Here, we described a variety of traits that may contribute to the adaptive capacity of phyllosphere yeasts to the phyllosphere environment. Meta-omics studies have begun to describe the diversity and temporal dynamics of yeasts colonizing the leaves, flowers and fruits of several plant species. A future challenge will be to unravel the natural functions of phyllosphere yeasts for plant growth, development and health (see Outstanding questions). Coupling genomics, metabolomics and co-occurrence network analyses will allow to disentangle the interplay between yeasts, other microbial members in the phyllosphere and plants. To that end, designing experimental reductionist *in vitro* and *in planta* models is essential. Numerous tools and approaches generated in studies on model yeasts can now be exploited for environmental yeasts. Furthermore, it will be crucial to design new approaches to investigate the functions of phyllosphere yeast communities in their natural environment. A number of new approaches recently described for bacterial communities could be further exploited for yeast communities[90–93]. Enhanced fundamental knowledge of the mechanisms involved in the adaptive capacity of yeasts in the phyllosphere may in turn benefit the use of these yeasts in industry and agriculture.

Outstanding questions

- What is the spatiotemporal distribution of yeasts in the phyllosphere? How do they colonize the surface and interior tissues of leaves, fruits and flowers?
- What are the genetic, molecular, and chemical mechanisms by which plants shape the assembly of yeast communities in the phyllosphere?
- Which adaptive traits enable yeasts to colonize the harsh phyllosphere environment?
- How widespread is methylotrophy among phyllosphere yeasts?
- How abundant are yeast biofilms in the phyllosphere? How do yeasts orchestrate biofilm formation and how do these biofilms mediate the interactions between yeasts and plants?
- What are the molecular mechanisms underlying the interactions between killer yeasts and other members of the phyllosphere? What are the fitness costs for toxin production by killer yeasts in the phyllosphere?
- How abundant are toxin-degrading yeasts in the phyllosphere and what are the underlying molecular mechanisms? Can we exploit the production of mycotoxin-degrading yeasts for a more environment-friendly crop protection and decontamination of mycotoxins?

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Glossary

Amplicon sequencing: high-throughput culture-independent approach widely used for taxonomic delineation of prokaryotes (e.g. bacteria) and eukaryotes (e.g. filamentous fungi and yeasts), based on the sequencing of the 16S (bacteria), 18S (eukaryotes) and ITS (fungi) ribosomal regions.

Fluorescence in situ hybridization (FISH): technique using fluorescent probes that bind to DNA fragments and is used to localize in situ specific target DNA sequences and chromosomes. The presence or absence of the specific DNA sequences can be visualized with fluorescence microscopy.

Metagenomics: culture-independent analysis of whole genomes from microbial communities present in a sample. This approach is typically used to study microbial genomes from environmental and gut samples.

Internal transcribed spacer (ITS) region: spacer DNA situated between the small-subunit rRNA (16S) and large-subunit rRNA (28S). This region is primarily used for fungal taxonomic classification, and in combination with the D1/D2 region, for yeast characterization.

D1/D2 region: D1/D2 is a 600 bp domain of the large subunit of rRNA (LSU, 28S), widely used for yeast taxonomic characterization, often in combination with the internal transcribed spacers (ITS region).

Mycotoxins: secondary metabolites produced by cereal infecting fungal pathogens (*Aspergillus*, *Penicillium* or *Fusarium* species), which are harmful to human and animal health.

Peroxisomes: a membrane-bound organelle containing enzymes that play important roles in the metabolism and detoxification of reactive oxygen species.

Restriction Fragment Length Polymorphism (RFLP) analysis: technique used to identify intra- and interspecies variation based on different DNA sequence lengths (polymorphisms) resulted from the activity of restriction enzymes.

CRISPR/Cas9 system: this system includes the family of DNA sequences called 'Clustered Regularly Interspaced Short Palindromic Repeats' (CRISPR) and the enzyme Cas9, which is used for precise genome engineering. Cas9 cleaves specific strands of DNA guided by the CRISPR sequence.

Orthologs or orthologous genes: homologous genes are found in different organisms which evolved from a common ancestor.

JA- and ET-signaling: signaling pathways regulated by the plant hormones jasmonic acid and ethylene involved in plant growth, development and resistance to pathogens and insects.

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