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Investigation of the indigenous fungal community populating barley grains: Secretomes and xylanolytic potential☆

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ABSTRACT

The indigenous fungal species populating cereal grains produce numerous plant cell wall-degrading enzymes including xylanases, which could play important role in plant-pathogen interactions and in adaptation of the fungi to varying carbon sources. To gain more insight into the grain surface-associated enzyme activity, members of the populating fungal community were isolated, and their secretomes and xylanolytic activities assessed. Twenty-seven different fungal species were isolated from grains of six barley cultivars over different harvest years and growing sites. The isolated fungi were grown on medium containing barley flour or wheat arabinoxylan as sole carbon source. Their secretomes and xylanase activities were analyzed using SDS-PAGE and enzyme assays and were found to vary according to species and carbon source. Secretomes were dominated by cell wall degrading enzymes with xylanases and xylanolytic enzymes being the most abundant. A 2-DE-based secretome analysis of *Aspergillus niger* and the less-studied pathogenic fungus *Fusarium poae* grown on barley flour and wheat arabinoxylan resulted in identification of 82 *A. niger* and 31 *F. poae* proteins many of which were hydrolytic enzymes, including xylanases.

Biological significance: The microorganisms that inhabit the surface of cereal grains are specialized in production of enzymes such as xylanases, which depolymerize plant cell walls. Integration of gel-based proteomics approach with activity assays is a powerful tool for analysis and characterization of fungal secretomes and xylanolytic activities which can lead to identification of new enzymes with interesting properties, as well as provide insight into plant-fungal interactions, fungal pathogenicity and adaptation. Understanding the fungal response to host niche is of importance to uncover novel targets for potential symbionts, anti-fungal agents and biotechnical applications.

1. Introduction

Over 200 species of microorganisms populate the surface of barley grains, including fungi, yeast and actinomycetes [1]. The composition of the microbial population varies significantly according to developmental stage of the grains, environmental factors, as well as post-harvest storage conditions. The initial colonizers of cereal grains after ear emergence are bacteria, which are replaced by yeasts and eventually by fungi after anthesis [2]. Fungi can have severe effects on the quality of the grains, due to e.g. discoloration, reduced germination, and the production of mycotoxins [3,4]. The fungi colonizing the grains can be categorized as field or storage fungi. The field fungi are those colonizing the developing and mature grains on the plant, with major genera comprising *Alternaria*, *Cladosporium* and *Fusarium*, which typically require high moisture content [3,4]. The storage fungi become more abundant on and within the stored grains, where the moisture level has decreased, these are primarily *Aspergillus* and *Penicillium*. Some of the fungi are known pathogens, e.g. *Fusarium*. Contamination of grains by fungal mycotoxins e.g. aflatoxin and ochratoxin, poses a critical hazard to food safety, human and animal health [5]. A characteristic of the invading fungi is the secretion of a collection of enzymes, including xylanases, polygalacturonases, pectate lyases and lipases, which play important roles in nutrient acquisition, host colonization, virulence and ecological interaction [6,7]. The availability of complete fungal

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genome sequences and advances in-omics techniques have significantly contributed to a better understanding of plant-fungus interactions, fungal pathogenicity and defense mechanisms in plants. Gel-based proteomics has enabled detailed analysis of several fungal secretomes [8–11]. *Aspergillus oryzae*, traditionally used in production of fermented foods, was found to produce a combination of cell wall degrading enzymes when grown on wheat bran, including β-glucosidases, α-mannosidases, cellulases and xylanases [12]. The secretome of *A. niger* grown with xylose and maltose was characterized with identification of about 200 proteins and reported to be strongly influenced by the culture conditions and available nutrient source [13]. Secretomes of *F. graminearum* grown on a variety of media, including isolated plant cell walls, wheat and barley flour, contain numerous secreted enzymes such as xylanases, cellulases, proteinases and lipases, depending on the nutrient source [11,14,15]. An obvious key challenge, however, is the further analysis of the function and regulation of the identified fungal proteins, including xylanases.

Although plant responses to fungal attack have been studied mainly in the context of single plant-fungus interactions, plants in the field are exposed to a diverse community of microorganisms, and rely for general protection on proteins and defense molecules produced at the interface with the environment. For example, wheat bran tissues contain numerous oxidative stress and defense-related proteins and inhibitors, as well as proteins that improve tissue strength to hinder pathogen entry [16]. Previously, we investigated the plant-microbe interface by analysis of the surface-associated proteome of barley grains [17] and found this to be dominated by plant proteins with roles in defense and stress-response. However, numerous proteins from the populating microbiota were also identified including fungal and bacterial proteins involved in polysaccharide degradation [17]. Grain-surface-associated xylanase activity was of microbial origin, and xylanases were identified from the fungi *Verticillium dahlia*, *Cochliobolus sativus* (the teleomorph of *Bipolaris sorokiniana*) and *Pyrenophora tritici-repentis* (anamorph of *Drechslera tritici-repentis*) [17], all of which are known to be present on grasses or cereals. To gain more insight into the ability of the fungi to produce xylanases, we isolated and identified fungi from the surface of barley grains and analyzed their secreted proteins and xylanolytic activities. Moreover, to better understand adaptation of the fungi to varying carbon sources, the secretomes of two fungi (*Aspergillus niger* and *Fusarium poae*) grown on barley flour and wheat arabinoxylan to mimic the natural growth substrates, were characterized.

2. Materials and methods

2.1. Barley grains and growing sites

Barley grains from six cultivars (Barke, Cabaret, Frederik, Quench, Scarlett, Simba) harvested in 2009–2011 were obtained from Sejet Plant Breeding, Horsens, Denmark (9° 50′ 51.32″ E, 55° 51′ 29.27″ N, 34 m). Each cultivar was grown in three plots in the same field in a fully randomized block design and grains from different plots were mixed to eliminate location effects. Quench and Simba were also grown at another site in Denmark, Koldkærgaard (10°04′ 40.3″ E, 56° 18′ 28.1″ N, 39.99 m) in 2010 and 2011. The cultivar Himalaya harvested in 2003 in Pullman, WA, USA was also analyzed. Danish spring (March–May) and summer (June–August) of 2009 were characterized with mean temperature of 8.3 °C and 16.2 °C with 652 and 700 h of sunshine, and 119 and 217 mm of precipitation. Spring and summer of 2011 had mean temperature of 8.1 °C and 15.9 °C with 301 and 573 h of sunshine, and 172 and 321 mm of precipitation, respectively (Danish Meteorological Institute). According to the Danish soil classification system, soil at both growing sites Sejet and Koldkærgård were assigned a JB number of five, which denotes a coarse sandy clay texture.

2.2. Isolation of fungi from barley grains

Fungi were isolated and identified by direct plating of 20–35 grains on (i) potato dextrose agar (PDA [18]), (ii) malt extract agar (MEA) and (iii) MEA Oxoid [19]. For species identification, the isolated fungi were cultivated on a range of different media, including dichloran glycerol (18%) agar (DG18 [20]), dichloran rose bengal yeast extract sucrose agar (DRYES [21]), vegetable juice water (V8 [22]) and Czapek Dox oprodione dichloran agar (CZID [23]). DG18 and DRYES were incubated at 25 °C in the dark, while V8 and CZID plates were incubated in alternating light and dark cycles at 20–23 °C. For black fungi such as *Alternaria* species, V8, DRYES and potato carrot agar (PCA) were used. For *Fusarium* species, PDA, YES (yeast extract sucrose agar [18]), and SNA (Synthetischer Nährstoffarmer agar) were used. For *Penicillium* species, MEA, YES, CYA (Czapek yeast extract agar [19]), and CREA (Creatine sucrose agar; [18]) were used, while for *Aspergillus* section *Aspergillus* species, CYA, CYA20S (CYA with 20% (w/v) sucrose [19]), CZ (Czapek Dox agar [18]), DG18 and YES, were used. These cultures were incubated for 7 days at different temperatures and alternating dark and light cycles. Fungi were identified based on typical colony form under a stereomicroscope (lower magnification and perception of depth) and conidia morphology with light microscope (higher magnification). The percentage of kernels infected with each identified fungal species was calculated.

2.3. Cultivation of fungi on solid medium

The fungi were cultivated in medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) agar and 1% (w/v) wheat arabinoxylan (WAX) as carbon source [24]. The fungi were grown at 25 °C for 7 days.

2.4. Liquid medium

Fungi from densely covered agar plates were used to inoculate 8 mL liquid medium composed of 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) asparagine and 0.5% (w/v) KH_2PO_4 supplemented with either 1% (w/v) WAX or finely ground barley flour as carbon source into 50 mL tubes [25]. The fungi grew on the surface of the medium, and the proteins were secreted into the medium to break down nutrients. Negative controls composed of medium and WAX or barley flour were included. The samples were incubated for 7 days at 25 °C. Subsequently, culture supernatants were collected by centrifugation at 3200*g* for 30 min at 4 °C.

2.5. Washing procedure to extract the surface-associated grain proteins

A washing procedure was implemented that effectively extracts the surface-associated proteins from grains [26] in 25 mM sodium acetate pH 5.0 containing 0.02% (w/v) sodium azide under agitation for 8 h at room temperature. The washing liquids containing extracted proteins were filtered through MN 615 filter paper (Macherey-Nagel, Dueren, Germany) and assayed for xylanase activity.

2.6. Agarose plate assay for detection of xylanase activity

Agarose gels containing dyed substrate (0.1% (w/v) Remazol Brilliant Blue-dyed WAX (Megazyme), 1% (w/v) agarose, 0.2 M sodium citrate-HCl pH 4.8) were prepared in petri dishes. Five microliters of supernatant from fungal liquid cultures were added to 2 mm diameter wells punched into the plates and incubated overnight at room temperature. Xylanase activity appeared as clearing zones around the wells. The assay was used for an initial screen of all fungal isolates grown on barley flour and WAX, and was subsequently repeated for fungal strains grown on WAX, with similar results (not shown).

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