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Review

The secretome of the maize pathogen *Ustilago maydis*

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ABSTRACT

Ustilago maydis establishes a biotrophic relationship with its host plant, i.e. plant cells stay alive despite massive fungal growth in infected tissue. The genome sequence has revealed that *U. maydis* is poorly equipped with plant cell wall degrading enzymes and uses novel secreted protein effectors as crucial determinants for biotrophic development. Many of these effector genes are clustered and differentially regulated during plant colonization. In this review, we analyze the secretome of *U. maydis* by differentiating between secreted enzymes, likely structural proteins of the fungal cell wall (excluding GPI-anchored proteins) as well as likely effectors with either apoplastic or cytoplasmic function. This classification is based on the presence of functional domains, general domain structure and cysteine pattern. In addition, we discuss possible functions of selected protein classes with a special focus on disease development.

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1. Introduction

The fungus *Ustilago maydis* is the cause of smut disease in maize. *U. maydis* is a biotrophic pathogen that requires living host tissue for completion of its infection cycle. To establish an infection, haploid *U. maydis* cells must mate on the leaf surface as it is the resulting dikaryon which represents the infectious form. The dikaryon shows filamentous growth and is able to differentiate infection structures (appressoria) when receiving appropriate signals on the leaf surface. Appressoria are unmelanized and are thus unlikely to function through turgor accumulation as is the case in other plant pathogenic fungi (Tucker and Talbot, 2001). Penetration is direct and is likely mediated by plant cell wall degrading enzymes, which must be secreted locally at entry sites. Upon penetration the host plasma membrane invaginates and tightly encases the invading hyphae. Initially, hyphae are found intracellularly in epidermal cells. At later time points they enter mesophyll tissue where they can be found intracellularly as well as in the apoplast. The apoplastic form develops huge aggregates and differentiates into spores (Banuett and Herskowitz, 1996; Doehlemann et al., 2008; Snetselaar and Mims, 1994). Fungal development in plant tissue is accompanied by the formation of large tumors in which plant cells divide and enlarge, presumably in response to fungal signals. In order to colonize plants *U. maydis* must either evade or suppress plant defence responses, must acquire nutrients in a compartment which is not particularly rich in carbon and nitrogen supply presumably by redirecting the metabolism of the host to the site of infection and must manipulate the plant cell cycle to create the environment for its proliferation. The fungal molecules responsible for these processes are currently unknown. While a wealth of data describes aspects of fungal development, signal transduction, morphology and gene regulation (see recent reviews by (Lee et al., 2003; Perez-Martin et al., 2006; Steinberg, 2007a,b,c,d) insights into the molecules which shape the biotrophic phase came only recently when the genome sequence was analyzed (Kämper et al., 2006). In contrast to necrotrophic plant pathogenic fungi *U. maydis* was found to contain only a small set of 33 plant cell wall degrading enzymes, which is in line with the limited amount of damage inflicted on the host. Interestingly, the genome was found to contain 12 gene clusters encoding three or more secreted proteins of unknown function whose expression in most cases was significantly upregulated during the tumor stage (Kämper et al., 2006). Five mutants carrying deletions in individual gene clusters were affected in virulence with phenotypes ranging from hyper virulent to complete absence of disease symptoms. This illustrated that these clustered genes code for novel effectors with crucial roles during pathogenesis. Current work is focused on elucidating whether these effectors have an apoplastic function or whether they may be translocated to the host cell and exert a cytoplasmic or nuclear function there. This would be similar to RXLR-motif containing secreted proteins of oomycetes which were shown to function inside plant cells (Kamoun, 2007; Morgan and Kamoun, 2007; Whisson et al., 2007). The secreted effector

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proteins of *U. maydis* do not possess an RXLR-motif nor do they share a recognized conserved domain that could substitute for this motif. In this regard they are similar to Avr proteins and other effector proteins from rust fungi that are secreted from haustoria and end up inside plant cells (Catanzariti et al., 2006; Dodds et al., 2004; Kemen et al., 2005). In this review, we catalog the secretome of *U. maydis* in proteins with enzymatic and non-enzymatic functions, respectively, establish subcategories based on computational approaches and speculate on the function of specific proteins during fungal development.

2. The secretome of *U. maydis*

The published annotation of secreted proteins in *U. maydis* was based on a combination of SignalP and ProtCom to generate data that were comparable to *M. grisea* (Dean et al., 2005; Kämper et al., 2006). Through this analysis 426 proteins were considered to be secreted through the classical signal sequence dependent route (Kämper et al., 2006). As this analysis failed to include secreted proteins like Mig1 and Mig2 that were experimentally shown to be secreted (Basse et al., 2002; Basse et al., 2000) we have now used a less stringent selection described in Bioinformatic Analyses. To avoid redundancy with another review in this issue, the 39 GPI-anchored proteins included in this compilation (Supplementary Tables 1 and 2) are not discussed here. In addition, 6 proteins were manually excluded to be secreted based on functional studies of orthologues in other systems (Supplemental Tables 1 and 2). Based on similarity, secreted proteins with likely enzymatic function and with likely non-enzymatic function were defined and are discussed separately below.

2.1. Genes encoding putative secreted enzymes

Using the bioinformatic criteria described above and in Bioinformatic Analyses 168 genes encode putative secreted enzymes. Seven of these were predicted to contain a GPI anchor and are therefore not discussed here (Supplementary Table 1). Three genes encode cytochrome P450 enzymes for which no extracellular functions have been described and these are also not discussed here. Using BlastP analysis the remaining genes were grouped by biological function (Table 1) and these functions are discussed below with respect to the pathogenic lifestyle of *U. maydis*.

2.1.1. Enzymes involved in modification of the plant cell wall

Unlike necrotrophic fungi, *U. maydis* does not massively degrade plant tissue. Localized degradation probably does occur during penetration, the passage from cell to cell and during fungal proliferation within tumor tissue when disappearance of the middle-lamella has been observed (Doehlemann et al., 2008). The recent genome analysis of *U. maydis* (Kämper et al., 2006) demonstrated that in contrast to other filamentous fungi, *U. maydis* contains a strongly reduced set of genes encoding plant polysaccharide degrading enzymes. This set is likely sufficient to weaken the plant cell wall to allow penetration. We also consider it possible that these fungal enzymes modify the plant cell wall to make it more flexible. This in turn might be necessary to accommodate the observed increase in cell size seen in tumor tissue. A more detailed analysis of the set of genes encoding putative plant cell wall modifying/degrading enzymes strengthens this hypothesis (see below).

A limited number of endoglucanases and endoxylanases are present in the *U. maydis* genome as well as several enzymes involved in removing side groups from xylan (acetylxylan esterase, α-L-arabinofuranosidase, arabinoxylan arabinofuranohydrolases) (Table 1), indicating that some hydrolysis of the cellulose and xylan chain will occur. In addition, endo- and exo-acting enzymes are present that act on pectin (polygalacturonase, pectin lyase, pectin methyl esterase), galactoglucomannan (endomannanase, α-galactosidase, β-mannosidase) and galactan (β-1,6-endogalactanase, β-galactosidase), respectively (Table 1). The maize cell wall consists mainly of cellulose and arabinoxylan, but small amounts of pectin and glucomannan have been reported (Carpita et al., 2001). The latter polysaccharides can aid in cross-linking the cellulose microfibrils and their hydrolysis would therefore weaken the cell wall structure. The low number of pectin- and galactomannan-active enzymes in the *U. maydis* genome could be a reflection of the small amount of pectin and galactomannan present in the host plant. Alternatively, it could be in the interest of the fungus to inflict only a partial degradation of the cell wall as this might increase its plasticity but not lead to full tissue collapse which could interfere with tumor development as well as metabolic reprogramming.

Previous studies have reported that extracts from apical meristem induced pectate lyase and cellulase activities in the diploid strain *U. maydis* FB-D12, while extracts from leaves induced xylanases and cellulases in the haploid strain *U. maydis* FB2 (Cano-Canchola et al., 2000). This demonstrated that different forms of *U. maydis* respond very differently to the presence of plant tissues, resulting in lytic enzyme mixtures that vary significantly in their range of activities (Cano-Canchola et al., 2000). Pectate lyase activity correlated with chlorotic spots that appeared after 3–4 days, while after 7–8 days anthocyanin formation and stunting of the plants occurred simultaneously with polygalacturonase activity. Tissue deformations in emerging gall areas coincided with cellulase activity at day 10–12, while teliospore production and maturation occurred at day 16–20 together with a second peak of pectate lyase and polygalacturonase activity (Cano-Canchola et al., 2000). Once the respective genes have been identified it will become possible to study their developmental mode of regulation.

Several putative lipases and four cutinases were detected in the genome (Table 1). These enzymes likely degrade plant lipids and the maize cuticle, respectively, which aids in partial degradation of the host cell walls. Lipids have also been proposed to represent one of the signals for filamentous growth of *U. maydis* in the host environment (Klose et al., 2004). Lipase activity has been detected in *U. maydis* as well as several other smut fungi in the presence of butter, Tween 80 or coconut oil (Donly and Day, 1984) (Katsivela et al., 1995). These lipases preferentially hydrolysed fatty acid ethylesters over triacylglycerols. The production of lipases during pathogenic development of *U. maydis* still remains to be studied.

The *U. maydis* genome also contains several lignin-related activities, specifically laccases and chloroperoxidase (Table 1). A laccase from *U. maydis* has been partially purified and characterized (Desentis-Mendoza et al., 2006). However, its estimated molecular weight does not fit any of the putative laccase polypeptides predicted from the genome. Lignin is only present in small amounts in maize cell walls (Gonzalez et al., 2004), but has a strong influence on the rigidity of the plant cell wall structure. Degradation of lignin is therefore expected to result in a more flexible cell wall.

2.1.2. Enzymes involved in degradation and utilization of other plant components

In addition to degradation of the plant cell wall, the range of genes encoding putative secreted enzymes in the *U. maydis* genome suggests that also other components of the plant cell are degraded (Table 1). This likely facilitates survival of *U. maydis* within the plant and may suppress the ability of the host to combat the fungus. A large set of extracellular proteases (Donly and Day, 1984) was detected in the genome sequence, which includes metalloproteases, aspartyl proteases, carboxy- and aminopeptidases, endo- and exo-proteases, and peptidyl peptidases (Table 1). Proteolysis plays an important role in different physiological contexts, including protein digestion, hormone maturation, germination, fertilization and other morphological processes (Holzer and

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