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Cereal powdery mildew effectors: a complex toolbox for an obligate pathogen

Salim Bourras[1], Coraline R Praz[1], Pietro D Spanu[2] and Beat Keller[1]

Cereal powdery mildews are major pathogens of cultivated monocot crops, and all are obligate biotrophic fungi that can only grow and reproduce on living hosts. This lifestyle is combined with extreme host specialization where every mildew subspecies (referred to as *forma specialis*) can only infect one plant species. Recently there has been much progress in our understanding of the possible roles effectors play in this complex host–pathogen interaction. Here, we review current knowledge on the origin, evolution, and mode of action of cereal mildew effectors, with a particular focus on recent advances in the identification of *bona fide* effectors and avirulence effector proteins from wheat and barley powdery mildews.

Addresses
[1] Department of Plant and Microbial Biology, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland
[2] Department of Life Sciences, Imperial College London, Imperial College Road, SW7 2AZ London, United Kingdom

Corresponding author: Bourras, Salim (s.bourras@botinst.uzh.ch)

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Introduction

Powdery mildews are agronomically important fungal pathogens infecting a wide range of monocot and dicot crops. Cereal powdery mildew diseases are caused by only one species, *Blumeria graminis*, which can be divided into several subspecies, corresponding to highly specialized pathogens infecting only one specific crop species. These are sometimes referred to as '*forma specialis*' (f.sp., literally forms belonging to one host species). For instance, *Blumeria graminis tritici* (*B.g.* f. sp. *tritici*), *B.g. hordei*, and *B.g. secalis*, can only grow on wheat, barley, or rye respectively [1••,2,3]. All cereal mildews are obligate biotrophs, meaning that they can only grow and reproduce on living host tissue. Considering the fact that this lifestyle is combined with extreme host specialization, it was proposed that highly complex and yet poorly understood mechanisms regulate host–pathogen interactions in cereal mildews [4••,5••]. Whole genome sequencing of the wheat and barley powdery mildews revealed on the one hand a drastic reduction of the gene content compared to other ascomycete pathogens, and on the other hand an expansion of the putative effector gene complement [4••,5••]. In this context, we propose that cereal powdery mildews provide a highly informative system to study the molecular role of effectors in pathogen virulence and host adaptation, based on a case of extreme host specialization. This review aims at providing a concise overview of current knowledge in effector biology of cereal mildews. In particular, we summarise advances in effector gene identification and functional characterization. Finally, possible ways for accelerating effector gene function discovery in cereal mildews will be discussed.

Genome-wide identification of candidate effectors in cereal mildew genomes

In powdery mildews, putative effectors have been identified using whole genome sequencing, and large-scale analysis of the fungal proteome, based on *a priori* criteria defining groups of protein coding genes that eventually constituted the putative effectors. Thus, Candidate Secreted Effector Proteins (CSEPs) from wheat and barley powdery mildew were defined as predicted secreted proteins (i.e. including an N-terminal 'signal peptide') that did not have trans-membrane domains, and did not have homologues in non-mildew fungi [4••,5••]. Based on these criteria, initial sets of 472 and 437 CSEPs were identified in the barley and wheat powdery mildew genomes, respectively. There have been continuous efforts to improve effector gene prediction in mildews which has resulted in the identification of larger sets of 722 and 734 CSEPs in the barley and wheat powdery mildew genomes, respectively [6••,7].

In addition to genome information, powdery mildew effectors were also mined in the proteome of *B.g. hordei*, by identifying proteins that are found in fractions enriched with isolated haustoria [8] or in infected barley epidermis devoid of epiphytic fungal material [9,10]. In the latter work, to generate a group of proteins named Blumeria Effector Candidate (BEC) proteins, the criterion of excluding homologs present in non-mildew fungi

was not used. This has led to the identification of effector proteins such as BEC1005 and BEC1019, which are necessary for full virulence in barley powdery mildew, and are broadly conserved in ascomycete fungi [11,12]. Interestingly, there is nearly complete overlap between the set of barley powdery mildew CSEPs and the BECs; that is, there are only five BECs which are not included in the CSEP set [13].

Origin and evolution of mildew effectors

The Erysiphales, that is the fungi causing powdery mildews, are an ancient monophyletic group which are estimated to have originated within the Leotiomycetes over 120 million years ago (Mya) [14]. The closest sister group is the non-pathogenic Myxotrichaceae, while all extant Erysiphales are obligate biotrophic pathogens of plants. It is reasonable to assume that effector proteins played essential roles in the early evolution of the powdery mildew fungi. One good example are the genes encoding for effector proteins with a predicted fungal ribonuclease-like (RNase-like) three dimensional structure similar to the ribonuclease T1 from *Aspergillus phoenicis* [6••]. The genes encoding the so-called RNase-like effectors have a single intron in a strictly conserved position [15]. Remarkably, both RNase-like effectors and the conserved intron are present in *Erysiphe necator* the agent of grapevine powdery mildew, which is thought to have diverged from the cereal mildew fungi in the late Cretaceous, over 70 Mya [Spanu and Dry, unpublished results]. Therefore, it is likely that this class of mildew effectors accounting for ca. 10–15% of all predicted mildew CSEPs and represented in six out of the 20 largest mildew effector families [6••,7] derived from a single ancestor similar to the canonical fungal RNase T1 [16]. In a recent study, Menardo and colleagues found a new class of CSEPs with structural homologies with the MD2-related lipid-recognition (ML) domain [7], which is predicted to be involved in binding to specific lipids (IPR003172) [17]. Notably, the gene family encoding for ML-like CSEPs is conserved in distinctly different lineages of grass powdery mildews, hinting this novel class of mildew effectors may also be derived from a common ancestor [7].

Cereal powdery mildew effector families are under stronger diversifying selection than non-effector genes, and show extreme levels of sequence variation and gene turnover [7], while the predicted structures appear conserved (Figures 1 and 2a) [18]. Essentially, only the protein sequence encoding the predicted signal peptide is highly conserved among members of the same family. Immediately after the putative cleavage site, sequence homology is reduced to a conserved variant of the YxC motif [6••], and the position of a few amino acids including residues of putative structural importance such as cysteines and prolines (Figure 2a). This high level of sequence divergence might be a result of a strong diversifying selection pressure imposed by the host immune system. Indeed, several RNase-like effectors from wheat, barley, and rye powdery mildews interact with nucleotide-binding leucine-rich repeat (NLR) immune receptors from the host, or suppress such a recognition, suggesting they are likely under selection to evolve [19••,20,21••,22••].

Mode of action of cereal mildew effectors

The first insights into the mode of action of cereal mildew effectors were largely based on transcriptomics and proteomics approaches in *B.g. hordei* [8–10,13]. In particular, RNA-seq monitoring of barley powdery mildew transcripts during the early stages of host infection revealed a two-step mode of action: a first wave of CSEP transcripts accumulated during host cell entry (12 hour), and a second wave of transcripts accumulated at the stage of haustorium formation (24 hour) [24•]. Similarly, high induction of CSEPs at the haustorial stage (48 hour) is also observed in wheat powdery mildew (Figure 2b; [23]). These results substantiate the importance of candidate effectors for mildew virulence, and suggest there are different subsets of CSEPs fulfilling distinct biological functions depending on the developmental stages of the fungus during host colonization. Indeed, Host Induced Gene Silencing (HIGS) [25••] of 21 individual barley powdery mildew effector genes resulted in significant reduction of host penetration and haustorium formation, further supporting the essential contribution of mildew CSEPs to the establishment of host infection [11,26•,27•,28]. Pliego and colleagues also showed that HIGS downregulation of 50 haustorially expressed barley mildew effectors resulted in highly variable effects on fungal virulence, ranging from a significant increase to a significant reduction of haustorium formation depending on the targeted effector gene [11]. This data suggests that some CSEPs are probably dispensable, while others such as BEC1054 and BEC1011, whose HIGS downregulation resulted in 60–70% reduction of haustorium formation, are acting as *bona fide* (i.e. true) effectors that are essential for mildew virulence [11].

There is evidence that mildew effectors interfere with components of host basal metabolism and host immunity, with prominent examples being CSEP0055 [26•], BEC3, BEC4 [29•], CSEP0105, CSEP0162 [27•], and BEC1054 [30•] from *B.g. hordei*, and $SvrPm3^{a1/f1}$ [19••,20] from *B.g. tritici* (Figure 3). Ahmed and colleagues showed that the sequence unrelated CSEP0105 and CSEP0162 both interact with the stress related small heat shock protein chaperones HSP16.9 and HSP17.5 from barley [27•]. We propose a possible mode of action of mildew CSEPs based on functional redundancy among effectors (Figure 3). In an approach combining pull-down assays from barley protein extracts and experimental validation by yeast-2-hybrid, Pennington and colleagues showed that BEC1054 physically interacts with a barley pathogen-related-5 (PR5)