

## Symposium contribution/Contribution à un symposium

## Functional genomic approaches in cereal rusts

GUUS BAKKEREN<sup>1</sup>, XIAO SONG<sup>1</sup>, VINAY PANWAR<sup>1</sup>, ROB LINNING<sup>1</sup>, XIBEN WANG<sup>2</sup>,  
CHRISTOF RAMPITSCH<sup>2</sup>, BRENT McCALLUM<sup>2</sup>, JOHN FELLERS<sup>3</sup> AND BARRY SAVILLE<sup>4</sup>

<sup>1</sup>Agriculture and Agri-Food Canada, Pacific Agri-Food Research Center, Summerland, BC, V0H 1Z0, Canada

<sup>2</sup>Agriculture and Agri-Food Canada, Cereal Research Center, Winnipeg, MB, R3T 2M9, Canada

<sup>3</sup>USDA-ARS-HWWGRU, Manhattan, KS 66506, USA

<sup>4</sup>Trent University, Forensic Science Program, Peterborough, ON, K9J 7B8, Canada

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**Abstract:** Cereal rust fungi are pathogens of major importance to agriculture, threatening cereal production worldwide. Targeted breeding for resistance, based on information from fungal surveys and population structure analyses of virulence, has been effective. Nevertheless, breakdown of resistance occurs frequently and continued efforts are needed to understand how these fungi overcome resistance and to determine the range of available resistance genes. The development of genomic resources for these fungi and their comparison has released a torrent of new ideas and approaches to use this information to assist pathologists and agriculture in general. The sequencing of gene transcripts and the analysis of proteins from haustoria has yielded candidate virulence factors among which could be defence-triggering avirulence genes. Genome-wide computational analyses, including genetic mapping and transcript analyses by RNA sequencing of many fungal isolates, will predict many more candidates. Functional assays, such as leaf infiltration using *Agrobacterium* for delivery of cloned fungal effectors, are being developed. This will allow the screening of wheat germplasm for novel resistance genes for breeding. Comparative analyses have also revealed fungal virulence genes, providing fungal targets for disease control in host-produced RNAi approaches.

**Keywords:** avirulence, effector, *P. graminis*, *P. striiformis*, *P. triticina*, *Puccinia* species, secretome, wheat leaf rust

**Résumé:** Les champignons responsables de la rouille chez les céréales sont des agents pathogènes très importants sur le plan de l'agriculture, et ils menacent la production céréalière partout dans le monde. La sélection ciblée pour la résistance, basée sur les données des enquêtes sur les maladies fongiques et les analyses de la virulence relativement à la structure des populations, s'est avérée efficace. Néanmoins, il arrive souvent que la résistance s'érode et il faut alors redoubler d'efforts pour comprendre comment ces champignons la brisent ainsi que pour caractériser la gamme disponible de gènes de résistance. Le développement des ressources génomiques, entre autres, relatives à ces champignons a fait surgir un torrent de nouvelles idées et de méthodes sur les façons d'utiliser cette information au profit des pathologistes et de l'agriculture en général. Le séquençage des transcrits des gènes et l'analyse des protéines des haustoriums a produit des agressines probables parmi lesquelles il y aurait des gènes d'avirulence qui induisent des réactions de défense. Les analyses bio-informatiques à l'échelle du génome entier, y compris la cartographie génétique et l'analyse des transcrits par séquençage de l'ARN de plusieurs isolats fongiques, décuplera les possibilités. Des essais fonctionnels, comme l'infiltration d'*Agrobacterium* dans les feuilles pour y produire des clones d'effecteurs fongiques, sont au stade du développement. Cela permettra le criblage du germoplasme de blé pour y déceler des gènes de résistance originaux pour la sélection. Les analyses comparatives ont également permis de détecter des gènes de virulence fongiques qui offraient des cibles à la lutte contre les maladies chez les hôtes capables de déclencher les mécanismes de silençage de l'expression génique (interférence ARN).

**Mots clés:** avirulence, effecteur, *P. graminis*, *P. striiformis*, *P. triticina*, *Puccinia* spp., rouille brune, sécrétome

Correspondence to: G. Bakkeren. E-mail: guus.bakkeren@agr.gc.ca

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## Introduction

Cereal rust fungi are pathogens of major importance to agriculture, threatening cereal production worldwide. In Canada, wheat is the largest crop and wheat leaf rust (WLR), caused by *Puccinia triticina* Eriks. (*Pt*), is one of the most serious diseases. Annual yield losses in wheat due to leaf rust were estimated from 5–20% or approximately \$88 million annually for the period 2001–2005 (McCallum *et al.*, 2007). The combined annual losses in wheat due to various rusts (*Pt*; stem rust caused by *P. graminis* Pers. f. sp. *tritici* Eriks. or *Pgt*; and stripe rust caused by *P. striiformis* Westend. f. sp. *tritici* Eriks. or *Pst*) are estimated at \$200 million in Canada (Manitoba AFRI [Agriculture, Food and Rural Initiatives], disease bulletin). Even a moderate reduction of rust disease incidence on cereals would have a major impact on the economics of cereal production. The development of wheat cultivars genetically resistant to stem and leaf rust has been an important and successful aspect of wheat breeding in Canada and has saved producers from catastrophic losses (McCallum & DePauw, 2008).

Rust surveys, race structure analyses and targeted breeding for disease have contributed greatly over the last 60 years to national and international efforts to maintain crop production levels in the presence of these pathogens. However, targeted breeding has led to repeated introductions of resistance genes, monoculture production, and consequently the evolution of new races of fungi. The incredible adaptability of these fungi seems to stem from an enormous genetic fluidity, which can result in genomic rearrangements and mutations within isolates or populations, and their huge population sizes. It is imperative that we learn more about the molecular basis of this fluidity, the genetic variation that exists among the ‘species’ and lineages in nature, and how this affects the basic biology of the pathogen–host interaction. Studies on genetic variability provide insight into changes in the rust fungus populations and predict new, potentially catastrophic introductions. They will also identify factors important in the host–pathogen interaction, such as fungal virulence genes or host response elements, which can have predictive value useful for surveys and breeding programmes, permitting the development of more durable defence strategies.

The emergence of novel, highly virulent, wheat stem rust isolates from East Africa in the late 1990 s (Ug99 and derivatives) has proven to be a real threat to wheat production worldwide. In the beginning of this millennium, it became clear that scientists needed to act quickly and that new insights into the biology and genetic make-up of these fungi were needed urgently. In the era of large genomic projects, it was thought that one way

of advancing knowledge was to sequence the genomes of these fungi; this had proven very insightful in other systems and several small rust fungal projects looked promising. A large-scale project was initiated and the first *Pgt* genome sequence was released in 2007. Expressed Sequence Tags (ESTs) to support gene discovery and genome annotation were generated (Zhong *et al.*, 2009). Analysis of these resources and comparison to those of the poplar rust fungus, *Melampsora larici-populina* Kleb., has recently been described (Duplessis *et al.*, 2011). Currently, the genomes of several other *Pgt* isolates are being sequenced for comparative analyses (C. Cuomo, L. Szabo, J. Ellis, *Puccinia* Group Sequencing Project). Our efforts to generate genomic resources for *Pt* included an EST database (Hu *et al.*, 2007b; Xu *et al.*, 2011) and resulted in a collaborative project to sequence the genome of this fungus as well (C. Cuomo, J. Fellers, L. Szabo, G. Bakkeren, *Puccinia* Group Sequencing Project). A *Pt* draft genome sequence was released in November of 2009; efforts to improve the assembly are underway and three more *Pt* genomes have been sequenced, including two from the parents of a mapping population (McCallum *et al.*, 2004; *Puccinia* Group Sequencing Project). A *Pst* genome project is also underway at the Broad Institute (C. Cuomo, S. Hulbert, X. Chen, *Puccinia* Group Sequencing Project) and a partial genome was recently released (Cantu *et al.*, 2011). With the generation of these genomic resources and their comparative analyses ([http://www.broadinstitute.org/annotation/genome/puccinia\\_group/MultiHome.html](http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html)), the groundwork has been laid for numerous studies.

However, to translate the vast amount of generated genomic, transcriptomic and proteomic information for use in biological studies and plant pathology, functional approaches are needed. Can we genetically transform these fungi for candidate gene studies? Can we identify candidate secreted effectors, i.e. virulence factors, many of which could represent the large repertoire of known avirulence genes which interact with resistance genes and trigger defence? Can we design functional assay systems for such effectors allowing for the screening of germplasm in search of novel sources of resistance? Could inventories of such avirulence genes allow for the design of diagnostic assays to assist with rust surveys? Could identified fungal pathogenicity and virulence genes be targets for disease control?

## The essence of plant–microbe interactions

‘A fine balance of protein interactions will determine whether the parasite is successful at establishing a feeding relationship (leading to further colonization and

reproduction), or unsuccessful and instead being detected by and stimulating host defenses' (Holub & Cooper, 2004). Given this general observation, there are basically two ways of altering this balance and combating pathogens: (a) target the pathogens by affecting their 'armour' (pathogenicity or virulence genes, or even essential pathogen-specific house-keeping genes) thereby weakening them so as to prevent host damage or pathogen propagation such as sporulation or (b) improve the resistance of the host which could include priming or sensitizing the host's defence potential and/or increasing the perception of the pathogens, for example through introgression of resistance genes recognizing certain fungal factors.

Plant resistance to most non-adapted pathogenic microbes is achieved through preformed physical barriers and, if such microbes succeed in gaining access to the interior through stomata or wounds, through subsequent biochemical barriers. Resistance is generally triggered through host recognition of specific 'molecular patterns'. These have been referred to as Pathogen-Associated Molecular Patterns or PAMPs, and the corresponding resistance is named PAMP-triggered Immunity or PTI. It often involves specific host receptors (Jones & Dangl, 2006). Large-scale genomic projects will reveal many PAMPs that plants could respond to. They will also identify host response elements that can be used to boost resistance through breeding or genetic engineering. However, true pathogens, apart from possessing a range of tools (lytic enzymes, toxins, appressoria) for plant penetration, have adapted to initial host defences by suppressing them. Over the last 5 years, a picture is emerging of the underlying molecular basis: pathogens overwhelm their hosts with so-called 'effectors', often small secreted proteins (SSPs) which have co-evolved to target host components involved in defence, but also, in the case of biotrophs when the relationship has been established, to divert nutrients (Voegelé & Mendgen, 2003; Hogenhout *et al.*, 2009; Stergiopoulos & De Wit, 2009; Ali & Bakkeren, 2011). As a counter-measure, during an evolutionary arms-race, plant hosts have fine-tuned resistance genes whose protein products recognize certain pathogen factors to initiate defence. These defence triggers are often the effectors (in many cases SSPs) and the products of the traditional avirulence or *Avr* genes, genetically superimposed on the pathogen's basic ability to infect (Stergiopoulos & De Wit, 2009). This form of resistance has been named Effector-Triggered Immunity or ETI (Jones & Dangl, 2006) and has been the basis for many breeding programmes to obtain genetic resistance. It has also often been less durable and easy to overcome by the pathogen by simply changing (mutating or deleting)

the corresponding effector so it is no longer recognized by the *R* genes to trigger defence.

Inhibiting pathogen infection and development has traditionally been accomplished through the use of (large quantities of) fungicides, most of which are not very specific or discriminatory. However, 'smart', very specific 'bio-fungicides' could be developed if targets specific to these fungi and not to their host or other microbes were known, such as essential pathogenicity factors. These 'fungicides' could then be produced in antagonists as a form of biocontrol, or in plants using transgenic approaches in which case they become part of the genetic make-up of the plant to provide cheap and intrinsic protection. Still, ETI, based on the traditional introgression of resistance genes recognizing *Avr* genes, will remain a very valuable defence mechanism. In fact, large-scale genomics is already providing insight into the repertoire of and variability among fungal effectors. High variability among related effectors indicates that they are under selection pressure, possibly evading recognition by host components such as the resistance proteins. These effector variants are therefore good candidates to probe a wider host gene pool to identify novel and possibly more effective resistance genes. When identified, such resistance genes with novel specificities can then in turn be used for gene pyramiding through conventional breeding or genetic engineering approaches. The key to success to more effective ETI-based resistance would lie in identifying more effective resistance genes that can recognize fungal effectors least prone to changes or deletion by the pathogen because they serve important (virulence) functions, i.e., cause a large fitness penalty when changed.

Several 'pathosystems' (a pathogen interacting with its host plant) have been studied in great detail because of the (relative) ease with which one or both partners can be manipulated at the genetic and molecular level and have therefore become model systems. *Arabidopsis thaliana* (Heyhn) is a model plant for which several well-researched pathogens have been described: an apoplastic bacterium *Pseudomonas syringae* pv. *tomato* Okabe (Hou *et al.*, 2009), necrotrophic fungi (*Botrytis cinerea* Pers. and *Alternaria brassicicola* Schwein.) (Glazebrook, 2005; Choquer *et al.*, 2007) and an obligate biotrophic oomycete (*Hyaloperonospora parasitica* Pers.) (Baxter *et al.*, 2010). In these systems, genetic analyses of both host and pathogen, as well as their interactions, have led to the discovery of many genes and a nascent understanding of the molecular basis of host-pathogen interactions. Over the last few years, complete genome sequences of all partners involved, and large-scale, genome-wide analysis of the expression and regulation of genes implicated in these interactions, has added tremendously to our

understanding and to the significance of these model systems. In a more-relevant agricultural setting, research on the rice (*Oryza sativa* L.) – blast fungus (*Magnaporthe (Pyricularia) oryzae* Couch & Kohn) (Wilson & Talbot, 2009) and the barley (*Hordeum vulgare* L.) – powdery mildew (*Blumeria graminis* DC. f. sp. *hordei* Em. Marchal (Glawe, 2008) interactions has progressed recently as a result of knowledge gleaned from model systems and the recent generation of pathogen genome sequences. However, the large size of cereal genomes has slowed sequencing efforts of host genomes. The rice and corn genome sequences are now available and those of barley and wheat and will be available in the near future; the rice and corn genomes as well as the genome of the switchgrass (*Brachypodium distachyon* L.) a model for cereals and grasses, are already available (Feuillet *et al.*, 2011). Therefore, research on the cereal-rust fungal pathosystems, although far from representing model systems, is getting a boost because of the recent advancements made in generating genomic resources.

### Genomic data mining

Understanding organismal interactions requires in-depth knowledge of both partners, and in the case of plant pathology, increased effort is needed to study the pathogens themselves in order to understand the disease process. Over the last 25 years, forward genetic mutational screens have discovered many pathogen genes involved in pathogenicity and virulence in various bacterial, fungal and oomycete systems and have provided insight into the molecular basis of several plant diseases. This functional data, combined with information from the many genome projects, has led to the compilation of searchable databases such as the Plant–Host Interaction Database (PHI-Base) (Baldwin *et al.*, 2006; Winnenburg *et al.*, 2006, 2008) and others classified for certain functions and Gene Ontologies (Korves & Colosimo, 2009; Torto-Alalibo *et al.*, 2009). The increased knowledge base has made it easier to employ reverse-genetic techniques to study the effect of gene deletions, or more recently, suppression of expression of genes using RNA interference (RNAi), of certain candidate pathogenicity or virulence homologs in other pathogens. These approaches, however, have been rather challenging in biotrophic pathogens that are often refractory to molecular genetic manipulation, such as the (cereal) rust fungi.

A way forward for such pathogens has been a gene discovery approach based on brute-force sequencing of random cDNA clones picked from libraries generated from mRNA populations isolated from specific life cycle or infection stages of the organisms. Such

Expressed Sequence Tags (ESTs) and their subsequent computational analyses, which includes gene annotation and comparisons to available databases, has led to the discovery of many sequences with homology to pathogenicity or virulence genes in other pathogens. In the cereal rust fungi, EST collections contributed to gene discovery and stage-specific expression analyses for wheat leaf rust (*Pt*, Thara *et al.*, 2003; Zhang *et al.*, 2003; Hu *et al.*, 2007b; Xu *et al.*, 2011), wheat stem rust (*Pgt*, Broecker *et al.*, 2006; Zhong *et al.*, 2009; Duplessis *et al.*, 2011), wheat stripe rust (*Pst*, Ling *et al.*, 2007; Zhang *et al.*, 2008; Ma *et al.*, 2009; Yin *et al.*, 2009) and ryegrass crown rust, *Puccinia coronata* Corda f. sp. *lolii* Brown (Dracatos *et al.*, 2006). These cereal rust resources have revealed candidate pathogenicity and virulence genes, some of which were shown to be specifically expressed upon plant infection, i.e. in haustoria, and/or were homologs of such infection-specific factors in other rusts, such as in the bean rust fungus, *Uromyces viciae-fabae* (Pers.) Schroet. (Hahn & Mendgen, 1997; Jakupovic *et al.*, 2006) or the flax rust fungus, *Melampsora lini* (Ehrenb.) Lev. (Catanzariti *et al.*, 2006).

Currently, computational analyses of the generated genome sequences, including exhaustive database searches, have added many more candidate pathogenicity and virulence genes to the growing list (Soderlund, 2009; Seidl *et al.*, 2011). However, large numbers of genes seem specific to *Puccinia* species and without known homologs or functions, could represent pathogenicity factors.

### The search for a pathogen's 'Achilles' heel'

Among the many candidate pathogenicity and virulence genes that could be potential targets for disease control, research on microbial pathogenesis worldwide seems to be focused on effectors (Hogenhout *et al.*, 2009). This is not surprising since they seem to be primarily responsible for suppressing host defence. Such effectors are therefore likely candidates for disease control if they are genetically reasonably stable in the population, have a major function and lack functionally redundant proteins. However, it appears that there are often paralogs of such genes – related family members that have evolved from each other sometimes because they possess avirulence functions and are therefore under selection pressure to change. Such redundancy in function is common (Catanzariti *et al.*, 2006; Yin *et al.*, 2011). Computational analysis of sequenced genomes can predict likely secreted proteins, the so-called 'secretome', based on the presence of signal peptides, predicted localization, and for some organisms, specific amino-acid motifs (Kamoun, 2006; Link & Voegelé, 2008; Mueller *et al.*, 2008; Desvaux

*et al.*, 2009; Choi *et al.*, 2010; Godfrey *et al.*, 2010; Joly *et al.*, 2010). Many SSPs have also been predicted in the available EST and genome resources of rust fungi (Yin *et al.*, 2009; Duplessis *et al.*, 2011; Xu *et al.*, 2011; Saunders *et al.*, 2012).

### Transcriptomics

Expression or induced expression of genes is a likely indication of the need for the products they encode. Gene expression analysis of pathogens during the infection cycle is therefore a good guide for the involvement of potential pathogenicity and virulence genes, especially in combination with the computational gene function predictions mentioned. For cereal rust fungi, custom microarrays (Duplessis *et al.*, 2011), EST/cDNA arrays (Bakkeren, unpublished raw data), relative, normalized EST coverage in cDNA libraries and quantitative real-time PCR of candidate genes (Thara *et al.*, 2003; Zhang *et al.*, 2003; Broeker *et al.*, 2006; Hu *et al.*, 2007b; Ma *et al.*, 2009; Yin *et al.*, 2009; Dong *et al.*, 2010; Xu *et al.*, 2011) have yielded information on genes expressed during infection. New sequencing technologies, such as RNA sequencing, have also been employed for cereal rust fungi. This technique involves the large-scale random sequencing of total cDNA to achieve deep coverage of many genes by millions of small tags; the number of tags per gene correlates with the level of its transcription. This way, differential transcriptomes have been generated for *Pgt* and *Pt*, comparing resting urediniospores and urediniospores germinated over water, and during wheat infection (Cuomo, Szabo, Ellis, Fellers, Bakkeren *et al.*, [http://www.broadinstitute.org/annotation/genome/puccinia\\_group/MultiHome.html](http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html)). We have embarked on a large-scale RNA deep-sequencing approach to reveal variability among effectors expressed during wheat infection, by comparing races having evolved under selection pressure in agricultural settings and among historical isolates.

### Proteomics

Similar to transcript profiling, proteomics aims to generate a genome-wide profile of proteins produced during a specific life-cycle or interactive stage of an organism. It is also feasible to perform comparative and quantitative proteomics by comparing profiles between different developmental stages. It can be argued that identifying proteins is a more definite indication of required functions than revealing transcripts. However, one drawback of proteomics is the need for relatively large amounts of protein and thus low-abundant proteins will likely be missed compared with the likelihood of revealing rare

transcripts by RNAseq. Currently, for cereal rust fungi, we have generated a partial proteome, focusing on isolated haustoria from *Pt*-infected wheat (Song *et al.*, 2011). Over 260 proteins were identified by searching a database of translated *Pt* ESTs and *Pt* genome-predicted proteins (a partial set since a draft genome was used). Among the proteins were many predicted pathogenicity and virulence factors. Approximately 50 proteins were predicted to be secreted among which at least six had an effector protein signature. With improved haustorial isolation protocols, gel-free, column-based reverse HPLC protein fractionation and concentration techniques, it is anticipated that more proteins, including ones that are expressed in low amounts, will be revealed. The anticipated complete *Pt* genome sequence and derived comprehensive *in silico*-predicted protein complement will drastically improve protein identification. Of major interest is the extra-haustorial matrix, a structure surrounding the haustoria thought to be the traffic hub for protein and nutrient exchange between fungus and wheat cytoplasm (Voegelé & Mendgen, 2003). It has recently been shown that some effectors localize to the extrahaustorial matrix in the flax rust pathosystem (Rafiqi *et al.*, 2010).

## Functional genomics – strategies for functional analysis of rust fungal genes

### Genetic transformation

Without reliable genetic transformation and gene deletion techniques, functional analysis in the cereal rust fungi is a challenge indeed. Using particle bombardment, transient expression in germinating *Pt* and *Pgt* urediniospores and seemingly stable integration in *Pt* was obtained (Schillberg *et al.*, 2000; Webb *et al.*, 2006). However, because of the difficulty of performing a genetic analysis through crosses, verification of stable integration with the obtained phenotype could not be confirmed. Recently, stable genetic transformation of *M. lini* was achieved using *Agrobacterium tumefaciens* in an *in planta* selection system based on suppression of an avirulence gene by RNAi (Lawrence *et al.*, 2010). Currently, no avirulence genes have been cloned from cereal rust fungi, and the transformation system developed for *M. lini* would likely not be suitable for the testing or deletion of genes involved in pathogenicity or virulence since its selection is based on overcoming the host resistance response. We have been pursuing the use of cell-penetrating peptides (CPPs; Chugh & Eudes, 2008; Qi *et al.*, 2011) for the introduction of DNA into *Pt* urediniospores and have obtained positive marker gene ( $\beta$ -glucuronidase) expression (Song & Bakkeren, unpublished raw data). In general, therefore,

it seems that the introduction and stable integration of nucleic acids into the genome of rust fungi is feasible; the bottleneck is the fact that these obligate biotrophic fungi are difficult (Williams *et al.*, 1966; Kuck & Reisener, 1985) if not impossible to culture *in vitro*, making selection for transformants very difficult. Urediniospores can be germinated and maintained for a few days on agar or water surfaces, allowing for possible selection or enrichment. For example, germination of urediniospores can be suppressed with 40  $\mu\text{g mL}^{-1}$  hygromycin B (Song & Bakkeren, unpublished raw data). Selection *in planta* is feasible, as was mentioned above, and possible alternative selection pressures could be imposed by watering or spraying plants with hygromycin B or glufosinate-ammonium if the proper resistance was introduced in the fungus as a selectable marker (hygromycin B resistance or the *bar* gene, respectively).

#### Heterologous expression

As an alternative to genetic transformation of *Puccinia*, we have explored the possibility of using a heterologous pathosystem. EST database screening identified a MAP kinase, PtMAPK1, a homolog to two MAPKs, Ubc3/Kpp2 and Kpp6, in the corn smut fungus and model pathogen, *Ustilago maydis* (Hu *et al.*, 2007b). When *Ubc3/Kpp2* is deleted in *U. maydis*, mating and subsequent pathogenic development is impaired (Mayorga & Gold, 1999; Muller *et al.*, 1999), whereas  $\Delta Kpp6$  mutants are impaired for invasive growth in corn tissue; double mutants are essentially non-pathogenic (Brachmann *et al.*, 2003). We showed as a proof-of-concept, that the *PtMAPK1* rust fungus homolog, when the coding sequence was expressed from an *Ustilago*-specific Hsp70 promoter, could complement both these mutations, including the double deletion mutant, and restore mating and pathogenicity (Hu *et al.*, 2007a). Interestingly, when *PtMAPK1* was expressed from its own endogenous rust promoter, it was still able to partially complement a  $\Delta Ubc3/Kpp2$  mutant, indicating that (certain) *Pt* promoter elements are recognized by the *Ustilago* transcription machinery. This was corroborated further when we constructed *Pt*-specific plasmids having the coding sequences for hygromycin B resistance or the mCherry fluorescent protein transcribed from *Pt*-specific Hsp70- or actin- promoter and terminator signals. These constructs could genetically transform *U. maydis* when selected for on hygromycin B and showed red fluorescence typical of mCherry production (Bakkeren, unpublished raw data). These results indicate the feasibility of using *Ustilago* species for heterologous expression and hence functional analysis of *Pt* genes.

#### Assays in whole plant or protoplasts

Predicted secreted proteins could reside on the outer walls of fungal hyphae or haustoria, be embedded in the extra-haustorial matrix, or be delivered on the plant surface (before penetration), in the apoplastic space or in the cytoplasm inside the host cell. In any case, such proteins likely interact with host cell components, resulting in some response. These fungal proteins are therefore candidates for testing in the plant environment. Expressed under the control of strong constitutive plant promoters such as the CaMV 35S, the maize ubiquitin or rice actin promoters (Himmelbach *et al.*, 2007), candidate *Puccinia* genes can be introduced in wheat leaf tissue by *Agrobacterium* infiltration or particle bombardment. When marker genes are present on such constructs, such as  $\beta$ -glucuronidase or fluorescent proteins (confocal) microscopy can be performed to analyse the reaction in cells having received the transforming DNA. This can be combined with histochemical staining and/or chemical assays specific for certain reactions (production of phenolics, NO and/or reactive oxygen species, callose production, etc.). Of course, in such tests, these fungal proteins are assayed for activity without the presence and hence active delivery by the pathogen.

One attractive set of fungal proteins for testing encompasses the effector-type proteins (SSPs). Avirulence proteins are effectors triggering resistance reactions and this is often manifested by a programmed (hypersensitive) cell death, involving several distinctive biochemical changes (ROS production, DNA laddering, collapse of cell organelles and general breakdown of cellular integrity). In other pathosystems, the delivery of avirulence proteins in host (leaf) tissues by means other than by the original pathogen (such as *Agrobacterium* infiltration, particle bombardment, viral expression systems), results often in visible necrosis indicative of the HR, if the matching *R* gene is present. Depending on the proteins, the N-terminal signal peptide needs to be omitted. In one assay, a  $\beta$ -glucuronidase-expressing control construct is co-bombarded together with an avirulence gene-expressing test construct and compared with a leaf area close-by that received only the control construct (sometimes using a double-barrelled particle gun). If expression of the avirulence gene produces statistically significantly fewer blue-staining foci compared with the control, this is likely the result of an avirulence-triggered HR if this is reproducible in a cultivar/*R*-gene specific manner (Mindrinos *et al.*, 1994). In some cases, the avirulence protein-*R* protein interaction can produce an HR when both are introduced in a different (related) plant species. Recently, another delivery system to test for avirulence activity of effectors was

developed. *Pseudomonas syringae* pv. *tomato* (*Pst*) isolate DC3000, non-pathogenic to *Arabidopsis thaliana*, was used to deliver, through its Type III Secretion System, an effector from the oomycete *Hyaloperonospora parasitica*, pathogenic on this host, by linking it to a signal-peptide motif from a *Pst* avirulence protein (Rentel *et al.*, 2008). This construct uses GateWay™ cloning/recombineering technology for the insertion of any gene of interest and we have used this in a cereal-infecting *Pseudomonas* species, successfully expressing several *Ustilago* and *Puccinia* candidate effectors (Song & Bakkeren, unpublished raw data). Recently, several other *Pseudomonas* species (*P. syringae* and *P. fluorescence*) were used to show delivery of candidate proteins into wheat and barley leaf tissue (Yin & Hulbert, 2011).

Many microbial effectors have been shown to suppress defence responses in plants. This is believed to be their main function whereas the avirulence function, the triggering of host defences, is an unintended 'side effect'. We have also used the *Pseudomonas*-delivery system to successfully demonstrate suppression of barley defence responses triggered by the *Pseudomonas* species, by expressing an *U. hordei* (Pers.) effector (Song, Ali & Bakkeren, unpublished raw data).

Several of the mentioned assays can also be developed for the introduction of test constructs into protoplasts or cell suspension cultures, rather than into whole leaves. This has the advantage of easier visualization of potential reactions, and cleaner results for biochemical reactions, such as ROS production, electrolyte leakage, etc. or quick molecular tests for the expression of diagnostic genes, such as PR genes. Such systems and assays will similarly identify the activity of fungal gene products after delivery into the cells through electroporation, particle bombardment, polyethylene glycol (PEG) or CPPs, of constructs expressing them. To test effectors for entry into such cells, they can also first be produced in other organisms such as in *E. coli*, yeast (*Pichia pastoris*) or insect cells/baculo virus expression systems, and then subsequently purified and added to the protoplasts or cell cultures. Techniques to obtain viable barley and wheat protoplast cultures have been established in our laboratory (Cervantes & Bakkeren, unpublished raw data; in collaboration with T. Xing, Carleton University, and D. Gaudet and A. Laroche, AAFC, Lethbridge, AB).

#### Search for novel *R* genes

Some of the functional assays for effectors described above will be useful in large-scale screening of wheat or other progenitor/cereal germplasm, cultivars and breeding lines. Large numbers of candidate effectors have

been identified in computational analyses of cereal rust fungus genomes and EST databases. In analogy with other pathosystems, including flax rust (Catanzariti *et al.*, 2006), we can reasonably assume that many effectors have avirulence functions. In *P. triticina*, we also begin to find molecular markers correlating with avirulent phenotypes in genetic populations segregating for over 14 avirulence genes (McCallum *et al.*, 2004). The RNA sequencing projects will reveal variation among effectors and correlation with defeated *R*-genes will also identify possible candidates. Such candidate effectors can easily be cloned in the various constructs for expression and testing in the mentioned assays. Upon screening of germplasm, a positive interaction based on the molecular or visible reaction set up for that particular assay, will indicate the presence of a matching *R* gene in that line. If that line belongs to a segregating population, the assay can become part of the screening tool to select for the *R* gene in breeding programmes to facilitate pyramiding resistance genes. If direct molecular interactions between the avirulence and *R* proteins are demonstrated, as predicted by the 'receptor-ligand model' and demonstrated to often occur in cereals (Catanzariti *et al.*, 2010), biochemical tools can be used to isolate the *R* gene. Such *R* gene can then be introduced in various elite breeding lines via genetic engineering.

The genomes of the rust fungi analysed thus far seem to have many repeats and transposable elements and are therefore prone to frequent changes (Duplessis *et al.*, 2011; Fellers & Bakkeren, unpublished raw data). The effector repertoire of a given isolate seems large: an initial analysis revealed more than 750 and 1000 in the partial *Pgt* and *Pt* genomes (Duplessis *et al.*, 2011; Xu *et al.*, 2011; Joly & Bakkeren, unpublished raw data) and is likely more diverged among races and populations. However, inventories of specific effectors with avirulence functions can possibly allow for the design of diagnostic assays, likely based on PCR-specific primers or spotted arrays, to complement rust surveys in the near future.

#### Candidate gene suppression by RNAi

The techniques outlined above can reveal candidate pathogenicity and virulence factors, some of which will be pathogen-specific and essential for its development: a pathogen's 'Achilles' heel'. Such factors are obvious targets for disease control. One other way of testing the function of candidate cereal rust fungal genes in pathogenicity is by suppressing their expression through 'gene silencing'. The production of inhibitory molecules of anti-sense orientation to the transcribed mRNA will result in complementary duplexes which will

be cut by an endogenous DICER enzyme into small, 22–26 base-paired double-stranded pieces. These will be amplified and target the original mRNA transcripts for destruction thereby preventing protein production and/or the activity of that gene. Introduction of a so-called hairpin, a fold-back of two complementary parts of the sequence of the gene transcript, will have the same effect. The suppressing ‘signal’, likely the dsRNAi pieces, can easily spread to neighbouring cells.

We have developed this idea for *P. triticina*, but with a twist. Whereas gene silencing normally is used for the suppression of genes within the same organism or within the cells’ cytoplasm (in the case of targeting of viruses), we hypothesized that this inhibitory signal, possibly the dsRNAi molecules themselves, could be produced in the host and affect fungal gene expression through uptake, possibly via the haustoria. We selected three fungal genes judged essential for disease development based on previous studies, the mentioned *PtMAPK1* (Hu *et al.*, 2007a), a calcineurin regulatory subunit CNB1 (Cervantes-Chávez *et al.*, 2011) and a cyclophilin with no ‘off-target’ sequences in the wheat host. RNAi constructs were introduced into susceptible wheat cultivar ‘Thatcher’ by the *Barley stripe mosaic virus* (BSMV) vector or via *Agrobacterium tumefaciens* infiltration. After 5 days, when dsRNAi molecules were produced in the wheat plants, these were challenged with *Pt* resulting in a severe reduction of fungal development and reduced sporulation (Panwar & Bakkeren, unpublished raw data). Microscopic observations of fungal development in suppressing plants revealed possible differential effects caused by the suppression of the different genes. This might allow the study of the function of particular rust fungus genes during infection, even in the absence of a genetic transformation system. Recently, the BSMV approach was shown to successfully suppress the expression of fungal genes and disease in the powdery mildew fungus (*Blumeria graminis*)–barley interaction and was termed ‘host-induced gene silencing’ or HIGS (Nowara *et al.*, 2010). It was also explored for the wheat–stripe rust *Pst* interaction, but since several effectors were targeted with likely redundant functions, no effect on disease suppression was seen (Yin *et al.*, 2011). Nevertheless, the targeting of effectors with avirulence functions using this approach should allow for a powerful selection in cultivars harbouring the matching *R* gene.

## Summary

The vast amount of generated genomic, transcriptomic and proteomic information is fast revealing insight into the biology of the cereal rust fungi. We have described

several functional approaches to translate this information into strategies that will be useable for applications in plant breeding and general crop protection. We rely heavily on the novel large-scale sequencing technologies and computational analyses, but will be able to develop strategies for more durable crop protection.

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