

Commentary

Cereal immunity against powdery mildews targets RNase-Like Proteins associated with Haustoria (RALPH) effectors evolved from a common ancestral gene

Pietro D. Spanu

Department of Life Sciences, Imperial College London, United Kingdom.

Email: p.spanu@imperial.ac.uk

Tel: +44 (0) 207 5945384

Total word count:

Number of Figures: 1

Number of Tables: 0

Number of Supporting Information: 0

18 Introduction

19 Plants have evolved highly complex ways for detecting non-self entities. This is driven by the need to
 20 protect themselves against potential pathogens, parasites or herbivores (Boller & Felix, 2009). One
 21 of the pillars that support plant immunity are “resistance” (*R*) genes. The existence of *R* genes
 22 controlling disease was first established right after rediscovery of Mendelian genetics at the beginning
 23 of the twentieth century (Biffen, 1905). Many *R* genes determine highly specific recognition of so-
 24 called “avirulence” (*Avr*) factors in the pathogens. In recent decades, our knowledge of *R* and *Avr*
 25 genes has progressed significantly, even if a mechanistic understanding of recognition is still
 26 incomplete. This understanding is complicated by the observation that many of the products encoded
 27 by *Avr* genes actually play significant roles in contributing to virulence and pathogenicity – that is
 28 they are “effectors” of disease (Jones & Dangl, 2006). In some plant-pathogen interactions, the key
 29 players in this game are well known. But that is not the case for the very common and agronomically
 30 significant powdery mildews. In these diseases information is very skewed in favour of the hosts:
 31 whilst several *R* genes have been cloned and characterised in some key crops (notably barley
 32 (*Hordeum vulgare*) and wheat (*Triticum aestivum*)) (Zhou *et al.*, 2001; Srichumpa *et al.*, 2005),
 33 understanding avirulence in the fungi that cause powdery mildew is poorly understood, and until
 34 recently was limited to two enigmatic transposon-like *Avr* genes in barley powdery mildew (Ridout *et al.*,
 35 2006; Sacristán *et al.*, 2009). This situation is now rebalanced with the publication of three
 36 seminal papers over the past year (Bourras *et al.*, 2015; Lu *et al.*, 2016; Praz *et al.*, this issue).

37 Cloning cereal powdery mildew secreted avirulence effectors

38 Through truly Herculean efforts of researchers in two of the main laboratories involved in cereal
 39 powdery mildew research, we now have positive identification of several additional *avr* genes in
 40 *Blumeria graminis*: *AvrPm3^{a2/f2}* and *AvrPm2* in the *forma specialis tritici*, which infects wheat
 41 (Bourras *et al.*, 2015; Praz *et al.*, this issue), and *Avr_{a1}* and *Avr_{a13}* in the *forma specialis hordei*, which
 42 infects barley (Lu *et al.*, 2016). Additionally, one gene (*SvrPm3^{a1/f1}*) has been identified that
 43 effectively suppresses recognition of one of the *Avr* proteins in wheat (Bourras *et al.*, 2015). In this
 44 commentary, I argue that, contrary to what is stated in the original papers, four out of the five genes
 45 are clearly phylogenetically related. In fact, they are most likely to have derived from a single
 46 ancestral gene encoding a ribonuclease (RNase). More of this later.

47 One of the reasons that make the work leading to the cloning of these *Avr* genes impressive is that *B.*
 48 *graminis*, like all powdery mildew fungi, are obligate pathogens: they can only be grown on live
 49 hosts. The strategies taken by the two research groups combine several independent “orthogonal”
 50 approaches that were dependent on the new genomic resources the powdery mildew community has
 51 developed over the past decade (Spanu *et al.*, 2010; Wicker *et al.*, 2013). In essence, the wheat

powdery mildew *Avr* effector genes were identified and cloned by a combination of genome-informed map-based cloning coupled with a genome-wide association (Bourras *et al.*, 2015; Praz *et al.*, this issue). In contrast, the barley powdery mildew *Avr* genes were cloned by a complex transcriptome-wide association study of a large set of isolates originating from very widely dispersed locations across the globe (Lu *et al.*, 2016). In both cases, successful attempts were made to validate experimentally the functional identity of the candidate genes. Heterologous expression of the wheat powdery mildew genes induces a clear necrosis in *Nicotiana benthamiana* when co-expressed with the corresponding “cognate” resistance genes; conversely, the barley powdery mildew *Avr* proteins determine specific responses when expressed in barley genotypes carrying the cognate *R* genes. Both sets of experiments were driven by transient expression of T-DNA-based constructs delivered by *Agrobacterium*. The barley powdery mildew *Avr* proteins are also active in transgenic *Arabidopsis thaliana* plants that heterologously express the correct barley *R* genes. Taken together, these experiments not only corroborate functionally the gene identification, but also demonstrate a significant conservation of the fundamental signalling pathways underpinning immune responses in plants; a conservation that goes back at least as far as the dicot-monocot divergence (ca. 150 MYA).

Many cereal *Avr* proteins are RALPHs

One intriguing observation is that three out of the four *Avr* genes identified in these studies, as well as the suppressor of avirulence in the wheat powdery mildew pathogen (*SvrPm3^{av/f1}*), encode proteins with features resembling catalytically inactive RNases. That is, they are part of the superfamily of Rnase-Like Proteins associated with Haustoria: i.e. the so-called “RALPH” effectors. The existence of this large superfamily was examined in great detail in a comprehensive study on *B. graminis* f.sp. *hordei* Candidate Secreted Effector Proteins (CSEPs) (Pedersen *et al.*, 2012). Meanwhile it has been shown that expression of two RALPH encoding genes in barley powdery mildews is necessary for full development of virulence: they appear to function indeed as effector proteins (Pliego *et al.*, 2013).

The fact that so many of the cereal powdery mildew *Avrs* are RALPHs may be due to the fact that a) they represent a large part of the overall CSEP complement (~25% of the barley powdery mildew CSEPs are RALPHs) and b) many of them are expressed at very high levels in the infected plant (Pedersen *et al.*, 2012). In fact, these are some of the most abundant fungal proteins found specifically in infected epidermis: For example, *Avr_{a1}* /CSEP0008 was the first protein identified as BEC1001 (Blgr_ctg_01259) in a proteomic survey of the *B. graminis* proteins found specifically in infected barley epidermal tissues (Bindschedler *et al.*, 2009). It is therefore perhaps not surprising that host *R* genes evolved to target abundant proteins produced by *B. graminis* in host cells. Alternatively, these proteins might be targeted by *R* protein since they exert key functions during fungal pathogenesis. We reported a very high signal of diversification selection pressure operating on some of the CSEP families, including Family 21 of the *B. graminis* CSEPs (see Fig. 4 in (Pedersen *et al.*, 2012)) that are

homologous to the *B. graminis* f.sp. *tritici* AvrPm2 (Clade 1 in Fig. 6, Praz *et al.*, this issue); these observations are congruent with a strong selective pressure to diversify and “escape” recognition imposed by host R proteins.

A common *ur*-RALPH

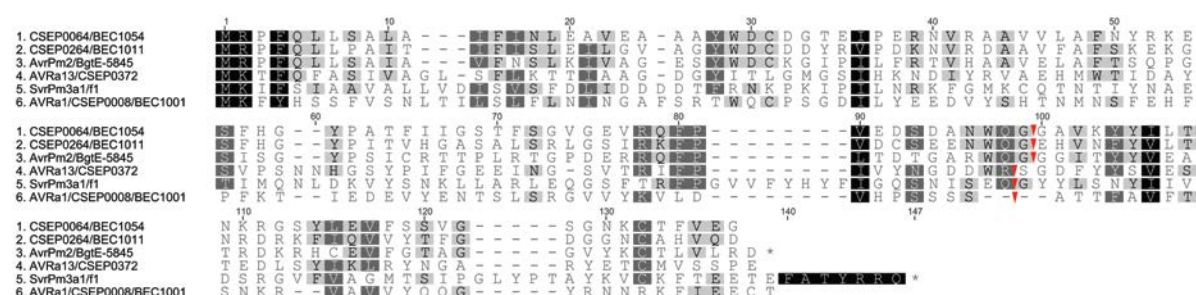
The identification of the *B. graminis* Avr and Svr proteins as four RALPH effectors leads to an interesting conclusion. In the original paper describing the barley powdery mildew CSEPs, we noticed that a very large number of RALPH effectors have a single intron in what appears to be the same relative position (Pedersen *et al.*, 2012). The most parsimonious explanation for this is that all these RALPHs have evolved from a single RNase or RNase-like ancestor, a hypothetical *ur*-RALPH. Here, I have compared the cDNA sequences of all of the RALPH Avrs and the Svr with their genomic DNA sequences. All four genes contain a single intron, and all are in the same relative position to the ones found previously (Fig 1, the sequences of CSEP0064 and CSEP0264 are shown as a comparison), and that includes the wheat powdery mildew RALPHs, too. I therefore suggest that, contrary to what was stated (Lu *et al.*, 2016), these Avr effectors are both structurally and phylogenetically related. This is in spite of the fact that the primary protein sequence has diverged so much as to hide “homologies” that are detectable by simple sequence comparisons. Evidently the *ur*-RALPH must have been present prior to the divergence of the barley and wheat powdery mildews, a fact that is also supported by the presence of homologous RALPH families such as Family 21/Clade1 (Praz *et al.*, this issue).

What next?

The exciting results reported in the last few months promise to open new avenues of research into the evolution of the cereal powdery mildew Avr – R genes and may help to define in detail the mechanisms of how the respective proteins work (Lu *et al.*, 2016; Praz *et al.*, this issue). In spite of research into host proteins that interact with some of the RALPH effectors (Pennington *et al.*, 2016), their mode of action to promote virulence in the absence of corresponding R genes is so far entirely hypothetical (Spanu, 2015) and remains to be elucidated. Likewise, how these effector proteins are targeted and internalised into the plant cell cytoplasm is unclear: this may be dependent on the so-called “YxC” motif that is conserved in many *B. graminis* CSEPs (Godfrey *et al.*, 2010). Intriguingly, Praz *et al.* (this issue) have noted the presence nearby YxC of a motif reminiscent the RXLR-dEER signature that is associated with translocation of oomycete effectors in their host cells; however, while the processes of internalisation in the host plant cells for oomycete effectors is still controversial (Petre & Kamoun, 2014), those which underpin the internalisation of fungal secreted effectors is completely unknown. Clearly, all these are now highly rich research seams to mine: I look forward to new nuggets emerging from this work.

Figure Legend

Figure 1 Sequence alignment of three Avr proteins (AvrPm2, Avr_{a13}, Avr_{A1}), one suppressor of avirulence (SvrPm3^{a1/f1}) and two RALPH effectors (CSEP0064, CSEP0264) from the cereal powdery mildew *B. graminis*. The proteins were aligned using ClustalW (Blosum; gap open cost: 10; gap extend cost: 0.1; Geneious V5.6 software). Amino acids with similar properties are highlighted on a grey scale (black: highest similarity). The positions of the single intron detected in each gene, by direct comparison of cDNA and genomic sequences are shown by red arrows. The observation that the intron appears to be in the same relative position suggests a common phylogenetic origin of all the genes.



REFERENCES

- Biffen RH. 1905.** Mendel's laws of inheritance and wheat breeding. *The Journal of Agricultural Science* **1**(1): 4-48.
- Bindschedler LV, Burgis TA, Mills DJS, Ho JTC, Cramer R, Spanu PD. 2009.** *In planta* proteomics and proteogenomics of the biotrophic barley fungal pathogen *Blumeria graminis* f. sp. *hordei*. *Molecular & Cellular Proteomics* **8**(10): 2368-2381.
- Bourras S, McNally KE, Ben-David R, Parlange F, Roffler S, Praz CR, Oberhaensli S, Menardo F, Stirnweis D, Frenkel Z, et al. 2015.** Multiple avirulence loci and allele-specific effector recognition control the *Pm3* race-specific resistance of wheat to powdery mildew. *Plant Cell*. **27** (10): 2991-3012.
- Godfrey D, Bohlenius H, Pedersen C, Zhang Z, Emmersen J, Thordal-Christensen H. 2010.** Powdery mildew and rust fungal effector candidates share N-terminal Y/F/WxC-motif. *BMC Genomics* **11**(1): 317.
- Jones JDG, Dangl JL. 2006.** The plant immune system. *Nature* **444**(7117): 323-329.
- Lu X, Kracher B, Saur IML, Bauer S, Ellwood SR, Wise R, Yaeno T, Maekawa T, Schulze-Lefert P. 2016.** Allelic barley MLA immune receptors recognize sequence-unrelated avirulence effectors of the powdery mildew pathogen. *Proceedings of the National Academy of Sciences* in press.
- Pedersen C, Ver Loren van Themaat E, McGuffin LJ, Abbott JC, Burgis TA, Barton G, Bindschedler LV, Lu X, Maekawa T, Weßling R, et al. 2012.** Structure and evolution of barley powdery mildew effector candidates. *BMC Genomics* **13**: 694.
- Pennington HG, Gheorghe DM, Damerum A, Pliego C, Spanu PD, Cramer R, Bindschedler LV. 2016.** Interactions between the powdery mildew effector BEC1054 and barley proteins identify candidate host targets. *Journal of Proteome Research* **15**(3):826-39.
- Petre B, Kamoun S. 2014.** How do filamentous pathogens deliver effector proteins into plant cells? *PLoS Biology* **12**(2): e1001801.
- Pliego C, Nowara D, Bonciani G, Gheorghe D, Xu R, Surana P, Whigham E, Nettleton D, Bogdanove AJ, Wise R, et al. 2013.** Host-induced gene silencing based pathogen effector discovery in barley powdery mildew *Molecular Plant-Microbe Interactions* **26**(6): 633-642.
- Praz CR, Bourras S, Zeng F, Sánchez-Martín J, Menardo F, Xue M, Yang L, Roffler S, Böni R, Herren G, et al. this issue.**
- Ridout CJ, Skamnioti P, Porritt O, Sacristán S, Jones JDG, Brown JKM. 2006.** Multiple avirulence paralogues in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. *Plant Cell* **18**(9): 2402-2414.

- 164 **Spanu PD. 2015.** RNA–protein interactions in plant disease: hackers at the dinner table. *New*
 165 *Phytologist* **207**(4): 991-995.
- 166 **Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stuber K, van Themaat EVL, Brown JKM,**
 167 **Butcher SA, Gurr SJ, et al. 2010.** Genome expansion and gene loss in powdery mildew fungi
 168 reveal tradeoffs in extreme parasitism. *Science* **330**(6010): 1543-1546.
- 169 **Srichumpa P, Brunner S, Keller B, Yahiaoui N. 2005.** Allelic series of four powdery mildew resistance
 170 genes at the *Pm3* locus in hexaploid bread wheat. *Plant Physiology* **139**(2): 885-895.
- 171 **Wicker T, Oberhaensli S, Parlange F, Buchmann J, Shatalina M, Roffler S, Ben-David R, Dolezel J,**
 172 **Simkova H, Schulze-Lefert P, et al. 2013.** The wheat powdery mildew genome shows the
 173 unique evolution of an obligate biotroph. *Nat Genet* **45**: 1092-1096.
- 174 **Zhou F, Kurth J, Wei F, Elliott C, Valè G, Yahiaoui N, Keller B, Somerville S, Wise R, Schulze-Lefert P.**
 175 **2001.** Cell-autonomous expression of barley *Mla1* confers race-specific resistance to the
 176 powdery mildew fungus via a *Rar1*-independent signaling pathway. *Plant Cell* **13**(2): 337-
 177 350.
- 178