The aspartic proteinase family of three Phytophthora species

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Abstract

Background: Phytophthora species are oomycete plant pathogens with such major social and economic impact that genome sequences have been determined for Phytophthora infestans, P. sojae and P. ramorum. Pepsin-like aspartic proteinases (APs) are produced in a wide variety of species (from bacteria to humans) and contain conserved motifs and landmark residues. APs fulfil critical roles in infectious organisms and their host cells. Annotation of Phytophthora APs would provide invaluable information for studies into their roles in the physiology of Phytophthora species and interactions with their hosts.

Results: Genomes of Phytophthora infestans, P. sojae and P. ramorum contain 11-12 genes encoding APs. Nine of the original gene models in the P. infestans database and several in P. sojae and P. ramorum (three and four, respectively) were erroneous. Gene models were corrected on the basis of EST data, consistent positioning of introns between orthologues and conservation of hallmark motifs. Phylogenetic analysis resolved the Phytophthora APs into 5 clades. Of the 12 sub-families, several contained an unconventional architecture, as they either lacked a signal peptide or a propeptide region. Remarkably, almost all APs are predicted to be membrane-bound.

Conclusions: One of the twelve Phytophthora APs is an unprecedented fusion protein with a putative G-protein coupled receptor as the C-terminal partner. The others appear to be related to well-documented enzymes from other species, including a vacuolar enzyme that is encoded in every fungal genome sequenced to date. Unexpectedly, however, the oomycetes were found to have both active and probably-inactive forms of an AP similar to vertebrate BACE, the enzyme responsible for initiating the processing cascade that generates the β-amyloid peptide central to Alzheimer’s Disease. The oomycetes also encode enzymes similar to plasmepsin V, a membrane-bound AP that cleaves effector proteins of the malaria parasite Plasmodium falciparum during their translocation into the host red blood cell. Since the translocation of Phytophthora effector proteins is currently a topic of intense research activity, the identification in Phytophthora of potential functional homologues of plasmepsin V would appear worthy of investigation. Indeed, elucidation of the physiological roles of the APs identified here offers areas for future study. The significant revision of gene models and detailed annotation presented here should significantly facilitate experimental design.
drastically different and there is considerable co-linearity between them. Genome size expansion in *P. infestans* has mainly resulted from escalation in the number of repeat regions and transposons [4]. Analyses of *Phytophthora* genomes have identified genes encoding proteins containing novel combinations of previously identified domains [4,6-9].

In common with other organisms for which genome sequences have been elucidated, one protein category which is highly represented in the three *Phytophthora* genomes is that of proteolytic enzymes [4]. These protein hydrolases have a wide variety of roles in all organisms including nutrient provision, stress responses and cell death processes. Proteinases are categorised into a number of catalytic types, dependent on the nature of the nucleophile that participates in the catalytic reaction. The MEROPS database (http://merops.sanger.ac.uk) classifies proteinases into clans which contain all the proteins that have arisen from a single evolutionary origin [10]. A clan represents one or more families of proteinases that reveal their evolutionary relationship through similarities at the primary and 3D-structural levels. Our interests over many years have focussed on aspartic proteinases, which belong to the AA clan. Within this clan, most members reside in the A01 family and are related to the archetypal patriarch, pepsin, or in family A02 which encompasses proteinases from retroviruses including HIV [11]. APs participate in a variety of physiological and pathological processes [11] in vertebrates (e.g. renin in hypertension and the β-secretase or BACE in Alzheimer’s Disease [12]) while in plants APs have roles in senescence, stress responses and fertilization [13] as well as in defence against pathogens [14]. Reciprocally, APs are deployed by pathogens to facilitate infection e.g. HIV/AIDS [15], thrush (candidiasis) and malaria [16]. Inhibitors targeted against HIV-proteinase are now in widespread use in therapy for treatment of HIV-infected individuals [17]. Recently it has been shown that one AP of *Plasmodium falciparum* is an ideal target for development of therapeutic inhibitors to treat malaria [18,19]. This AP is responsible for processing *Plasmodium* effector proteins in a step that is crucial for their export into the host red blood cell, thereby reprogramming the host cell to meet the demands of the parasite.

With these examples serving to illustrate the reliance of both host and pathogens on AP activities, it was of considerable interest to establish and annotate the complement of APs encoded within the three *Phytophthora* genomes, particularly since one of the major current subjects of oomycete research is on effectors that modulate host cell physiology and immune responses. *Phytophthora* effectors are small, secreted proteins that undergo translocation into host cells [20] in a manner analogous to that for the *Plasmodium* effectors [18,19]. However, the components of the *Phytophthora* translocation machinery remain to be identified. Here we describe the complete AP family of three *Phytophthora* species and identify some of its members which may participate in effector translocation.

**Results and Discussion**

Genome sequences of *P. infestans* (available through the Broad Institute website http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html), *P. sojae* and *P. ramorum* (from JGI at http://genome.jgi-psf.org/) were analysed for the presence of genes encoding putative pepsin-like aspartic proteinases as described in the Methods. Pepsin-like APs from family A01 are typically produced in the form of a precursor, consisting of a signal peptide followed by a propeptide region that has to be excised from thezymogen polypeptide chain in order to generate the mature enzyme. The mature enzyme region consists of two homologous catalytic domains, each of which provides a catalytic Asp residue to the active site [11,21]. Each Asp residue is located within the hallmark motif Asp-Thr/Ser-Gly, which is followed by a hydrophobic-hydrophobic-Gly sequence, as exemplified by the family patriarch, pig pepsin (Additional File 1). Together, these motifs form a structural feature known as a psi loop [22]. A Tyr residue is located at position 75 (pepsin numbering) in a β-hairpin loop which overlies the active site. This residue is strictly conserved in all active pepsin-like APs and serves as a landmark residue that can be anticipated to be present in newly-identified AP sequences. A number of other motifs and landmark residues (including Trp 39 and several intramolecular disulphide bonds - Additional File 1) are present at characteristic locations along A01 family member polypeptides.

A total of 11 predicted protein sequences exhibiting some or all of these hallmark features and landmark residues were identified in *P. infestans*, with 12 and 14 entries detected in *P. sojae* and *P. ramorum*, respectively. Inspection of the sequences revealed that, in *P. ramorum*, gene model Pr_73565 was erroneous due to incorrect assembly of the DNA sequence in this region and gene model Pr_84645, present in a correctly assembled sequence, was a pseudogene lacking at least 250 residues of the N-terminus of a functional AP. In *P. sojae*, the sequence encoded by gene Ps_129567 was highly similar to APs but the two hallmark Asp-Thr/Ser-Gly motifs were replaced by Asp-Val-Met and Phe-Thr-Met respectively, such that the encoded protein could not possibly be active. This entry, which lacked an orthologue in both *P. infestans* and *P. ramorum*, was thus considered also to be a pseudogene. The above three gene models were thus excluded from further analyses.
The remaining 34 genes were the only ones consistently identified by repeated search using PSI-Blast [23] or HMMer [24], so that it would appear no further AP homologues are present within these three genomes. These 34 gene models were analysed (Table 1) and are further discussed in groups on the basis of orthology. Comparison of the orthologues from the three species revealed in a number of cases that the gene models, as originally annotated (Table 1), were likely to be incorrect. The wealth of information available on pepsin-like AP sequences (http://merops.sanger.ac.uk) including the hallmark motifs/landmark residues described above, was applied together with available EST and trace file data, to guide the final gene predictions for putative APs encoded in the three Phytophthora genomes. Additional File 2 lists the gene models that required correction. More than half of the original gene models annotated in the P. sojae and P. ramorum databases were correct, whilst almost all of the original entries for P. infestans were erroneous. The annotation errors that were identified (Additional File 2) included mis-identification (i) of the initiator Met residue, (ii) of intron presence/absence, (iii) of the 5’ and/or 3’ splice junctions of introns as well as (iv) sequence trace mis-reads or (v) trace mis-usage in assembly of the P. infestans sequence due to heterozygosity. Annotation errors in two P. infestans genes due to sequencing related issues were corrected by re-examination of original sequence trace files at NCBI and by re-sequencing relevant regions. The corrected nucleotide sequences of these genes (PITG_08190 and PITG_09387) are deposited in NCBI under accession numbers HM588685 and HM588686, respectively. For all gene models, the intron positions were verified by inspection of EST information, if available, and by comparison with DNA sequences of the orthologous gene in the other two species for the presence or absence of possible splice junctions at the equivalent nucleotide position(s). The corrected gene models and their encoded polypeptides are summarised in Table 2. All corrections proposed for the P. infestans gene models were confirmed by available EST data or by cloning and sequencing the cDNA. Two of the three proposed corrected gene models for P. sojae (Ps_157552, Ps_158877) were confirmed by cloning and sequencing the cDNA, while for the third (Ps_135764), correction could not be confirmed as cDNA amplification was unsuccessful. Corrections in P. ramorum gene models were not experimentally validated as this species has a quarantine status. An initial insight into the expression patterns of these APs was obtained from microarray analysis of P. infestans genes using the genome-wide NimbleGen array [4]. Consistent levels of expression were observed for each of the eleven PiAP genes during culture on different agar media and during infection on potato (Additional File 3). The final protein sequences predicted for each of the 34 APs from the three Phytophthora species are listed individually in Additional File 4. Each of these sets of orthologous polypeptides was examined for the presence/absence of a signal peptide and a propart segment preceding the mature enzyme region. These features are summarised in Table 3. The individual signal peptide/propart segment sequences for each of the 12 sets of orthologues are aligned in Additional File 5. In order to

| Table 1 Aspartic Proteinase Genes in Phytophthora spp |
|----------------------------------------------------|
| Phytophthora infestans | Phytophthora sojae | Phytophthora ramorum |
|------------------------|--------------------|---------------------|
| PITG accession number  | Predicted intron number | Predicted length (residues) |
| Ps accession number    | Predicted intron number | Predicted length (residues) |
| Pr accession number    | Predicted intron number | Predicted length (residues) |
| 09387 2 815 109366 0 393 72239 0 389 |
| 12357 0 469 144332 0 472 84847 0 469 |
| 05114 2 545 132274 5 379 77872 8 698 |
| 0525 0 737 138576 0 751 84399 0 753 |
| 0975 1 512 136075 0 587 73566 0 582 |
| 06004 3 307 - - - 73546 0 432 |
| 08190 1 556 135399 0 585 78678 0 611 |
| 06900 5 479 135764 3 627 79884 4 644 |
| 02623 2 681 144093 2 719 77495 2 636 |
| 02624 2 350 144091 11 806 77493 3 360 |
| 11522 1 446 141367 0 531 74839 0 525 |

The number of introns and length of each polypeptide predicted by the database-annotated gene models (January 2010) are depicted for the orthologues from P. infestans, P. sojae and P. ramorum set out in each row.
simplify the descriptions that follow, the identifiers PxAP1-12 were assigned to each of these sets of orthologues.

Except for PxAP1, the polypeptides continued beyond the conventional location of the C-terminus of an archetypal AP, as defined by the reference standard, pig pepsin (Additional File 1). Each of the PxAP2 - PxAP12 polypeptides thus contained a C-terminal extension (Table 3). These extensions differed markedly in length and sequence (Additional File 6) and could only be aligned with the sequences of their orthologue(s) from the other *Phytophthora* species, and not with any other sequence in the set of entries or with any archetypal AP. Only short extensions were present in the PsAP3 and PrAP3 polypeptides (Additional File 6). The C-terminal extensions for PxAP2 and PxAP4 - PxAP12 were considerably longer and all contained at least one stretch of approximately 20 consecutive hydrophobic residues (marked in Additional File 6 by a box) which are predicted to provide a membrane-spanning segment. Thus, almost all the *Phytophthora* APs (PxAP2 and PxAP4 - PxAP12) are predicted to be membrane-bound enzymes. While it is not uncommon for individual APs from other species to be membrane-anchored in such a fashion (e.g. human BACE, plasmepsin V from *Plasmodium falciparum*), it is unprecedented for almost the entire AP complement of an organism to appear to be membrane-bound enzymes. Considerable experimental effort will need to be invested to establish whether all of these predicted polypeptides are indeed membrane-associated but in previous investigations, APs that have been predicted to contain a membrane-associated segment have always been proven by subsequent experimental investigations to be membrane-attached. Further consideration will be given to this aspect for the individual cases described below.

The sequences of the mature enzyme regions of each of the 12 sets of orthologues from the three *Phytophthora* species were aligned and a phylogenetic tree was generated (Figure 1). This resolved the PxAP1-12 sequences into five clades that are related to well-documented APs from other species. Each *Phytophthora* species is thus equipped with an AP gene family, the members of which have distinct gene organisations (Table 3) and encode polypeptides with considerably different sequences (Figures 2, 3, 4, 5) and, potentially, distinct activities. Each of the clades will be considered in turn.

| Table 3 Component segments flanking the N and C-terminal ends of the mature enzyme regions of the *Phytophthora* Aspartic Proteinases |
| --- |
| **Signal Peptide** | **Pro-** | **C-terminal extension** | **Number of Trans-membrane domains** |
| PxAP1 | + | + | - | 0 |
| PxAP2 | - | + | + | 1 |
| PxAP3 | + | + | + | 0 |
| PxAP4 | + | + | + | 1 |
| PxAP5 | + | + | + | 7 |
| PxAP6 | + | - | + | 1 |
| PxAP7 | + | - | + | 1 |
| PxAP8 | + | - | + | 1 |
| PxAP9 | + | - | + | 1 |
| PxAP10 | + | + | + | 1 |
| PxAP11 | + | + | + | 1 |
| PxAP12 | + | + | + | 1 |

Table 2 Corrected Aspartic Proteinase gene models in *Phytophthora* spp

| Phytophthora infestans | Phytophthora sojae | Phytophthora ramorum |
| --- | --- | --- |
| **Gene model** | **introns** | **Length** | **Gene model** | **introns** | **Length** | **Gene model** | **introns** | **Length** |
| PxAP1 | PITG_09387 | 0 | 390 | Ps_109366 | 0 | 393 | Pr_77239 | 0 | 389 |
| PxAP2 | PITG_12357 | 0 | 472 | Ps_144332 | 0 | 472 | Pr_84847 | 0 | 469 |
| PxAP3 | none | 0 | 157552 | 4 | 425 | Pr_77872 | 4 | 414 |
| PxAP4 | PITG_05114 | 1 | 559 | Ps_132274 | 1 | 573 | Pr_82845 | 1 | 548 |
| PxAP5 | PITG_10524 | 0 | 737 | Ps_138576 | 0 | 751 | Pr_84399 | 0 | 753 |
| PxAP6 | PITG_04975 | 0 | 566 | Ps_136075 | 0 | 587 | Pr_73566 | 0 | 582 |
| PxAP7 | PITG_05004 | 0 | 444 | none | 0 | 587 | Pr_73546 | 0 | 432 |
| PxAP8 | PITG_08190 | 0 | 574 | Ps_135399 | 0 | 585 | Pr_78678 | 0 | 586 |
| PxAP9 | PITG_06900 | 4 | 647 | Ps_135764 | 4 | 654 | Pr_79884 | 4 | 644 |
| PxAP10 | PITG_02623 | 2 | 681 | Ps_144093 | 2 | 719 | Pr_77495 | 2 | 650 |
| PxAP11 | PITG_02624 | 4 | 638 | Ps_144091 | 4 | 642 | Pr_77493 | 4 | 640 |
| PxAP12 | PITG_11522 | 0 | 522 | Ps_141367 | 0 | 531 | Pr_74839 | 0 | 525 |

Genes are presented as groups of orthologues. The generic descriptors PxAP1-12 are used to denote the respective encoded polypeptides. The number of residues in each polypeptide is indicated.
by orthologous single exon genes (Table 2). The PxAP1 polypeptides (Table 3) display all of the essential components i.e. signal peptide, propeptide segment (Additional File 4) and two-domain mature enzyme region (Figure 2), including all the necessary hallmark motifs and residues of archetypal pepsin-like APs described earlier (Additional File 1). These polypeptides are not extended at their C-terminus. The phylogenetic analysis revealed that the three PxAP1 polypeptides cluster closely with fungal APs (Figure 1) that are known to be located in the vacuole of the cell. This subfamily, with saccharopepsin from *S. cerevisiae* as the parent member, is assigned the identifier A01.018 in the MEROPS database. Analyses of all sequenced fungal genomes has revealed that the only AP that is omnipresent is the vacuolar enzyme [25]. Thus, it seems likely that PiAP1, PsAP1 and PrAP1 are the respective vacuolar APs from each *Phytophthora* species. PiAP1 has been produced in recombinant form and shown to have AP activity on haemoglobin (F. Govers, Wageningen University, personal communication).

The polypeptides of the PxAP2 in clade 2 (Figure 1) are atypical in that they do not contain a signal peptide or a non-classical secretion signal (Table 3 and Additional File 5). This is highly unusual in APs although precedents have been observed in fungi including *Botrytis cinerea* [25] and in bacterial species of marine origin [26]. It is not obvious how these polypeptides are able to become incorporated into a membrane through the (single) hydrophobic segment in their C-terminal extensions (Additional File 6) when there is no signal peptide to mediate transfer into the endoplasmic reticulum. Experiments using fusion constructs with reporter proteins will be necessary to resolve this conundrum.

Within clade 3, five clusters (PxAP3 - PxAP7) were resolved from one another (Figure 1). More profound phylogenetic analysis (not shown) indicates that this clade forms a monophyletic clade with three entries from two filamentous fungi, i.e. the zygomycetes *Rhizopus oryzae* and *Phycomyces blakesleeanus*. PxAP3 encoding genes were only detected in *P. sojae* and *P. ramorum* (gene models Ps_157552 and Pr_77872; Table 1, Table 2). Despite repeated searching, an orthologue of these two in *P. infestans* could not be detected. The PxAP3 genes are located in regions of co-linearity. Scrupulous search of the five flanking genes (upstream and downstream) in the *P. sojae* and *P. ramorum* genomes revealed that orthologues are present in similar arrangement in *P. infestans* DNA, however, on separate contigs. The gap between the contigs corresponds to the likely location of the “missing” AP gene. Moreover, no un-assembled reads were identified in *P. infestans* that matched PsAP3 or PrAP3 sequences.

The originally-annotated gene models for Ps_157552 and Pr_77872 both required considerable correction (Additional Files 2 and 4). The 5'-splice donor site of the third intron in Ps_157552 was identified to be a GC dinucleotide sequence. This dinucleotide has been found to be used as the 5'-donor junction at low frequency (~1%) in a number of fungi [27] but no information is available as yet on its use in oomycetes. The amendments to the Ps_157552 and Pr_77872 gene models (Additional Files 2 and 4) resulted in predicted polypeptide sequences that contained both signal peptide and propeptide segments (Table 3; Additional File 5). The sequences of the mature enzyme regions (Figure 3) showed a pairwise identity of 71%. Both polypeptides contained all three of the disulphide bonds that are common in vertebrate APs and they were only slightly extended at their C-terminal end relative to pepsin (Additional File 6). PsAP3 and PrAP3 can be predicted to display different substrate specificities. The region between residues 292 and 298 is known conventionally as the “polyproline loop” and has been demonstrated to
be a major determinant for substrate specificity [22]. In PrAP3, the sequence LGDDLYW of this region has two hydrophobic residues is likely to influence specificity. In these two hydrophobic residues is likely to influence specificity.

Like PxAP3, the PxAP4 and PxAP5 polypeptides from Phytophthora contained the conventional AP segments of a signal peptide and propart (Table 3; Additional File 6). Indeed, the C-terminal extensions in PxAP4 (138 residues with one putative membrane-spanning stretch, the PxAP5 extensions were substantially longer but, whereas the extensions in PxAP4 consisted of 136-151 residues, the corresponding extensions in PxAP5 consisted of 152-165 residues). Each of the C-terminal extensions in P. sojae PIAP5 (322 residues) and its orthologues encompassed no less than seven potential transmembrane segments (Additional File 6), an architecture highly reminiscent of the seven transmembrane stretches that constitute the super-family of G-protein coupled receptors (GPCR) [28]. Blast search using the sequence of the C-terminal extension of PIAP5 as query, identified the first non-self hit (with an E-value of 0.001) as RpkA, a G-protein coupled receptor family protein from Dictyostelium discoideum [29]. A 55.5% similarity in a 265 amino acid overlap was calculated between the two sequences using Smith-Waterman analysis [30]. The intriguing possibility arises that, in Phytophthora species, a fusion protein is produced in which the N-terminal partner is an AP connected to a putative GPCR as the C-terminal component. Self-processing to release the AP component would generate the mature GPCR. Such a self-processing phenomenon is unprecedented, although receptors activated by extrinsic proteinases are well-

Figure 2 Alignment of the mature enzyme regions of PxAP1 and PxAP2 predicted polypeptides with pig peptain. The numbering system is that of pig peptain (PepA_Pig), and the vacuolar enzymes from S. cerevisiae (ScPep4p) and N. crassa (NcPep4) are included for comparison. The DT/G and hydrophobic-hydrophobic-Gly motifs forming the psi loops in each domain are boxed, as are the landmark residues Y75 and W39.
Figure 3 Alignment of the mature enzyme regions of the PxA3 - 7 predicted polypeptides with pig peptin. The numbering system is that of pig peptin (PepA_Pig) and is highlighted by a dotted box (■).
The final two sub-groups that were resolved within clade 3 consisted of PxAP6 and PxAP7 (Figure 1). No orthologue in P. sojae or PrAP7 sequences. The simplest interpretation is that an ancestor of the PxAP7-encoding gene was present in the orthologue in P. sojae, although it cannot be excluded that substitutions have accumulated to an extent that prevented recognition of the gene.

The numbering system is that of pig pepsin. The DT/SG and hydrophobic-hydrophobic-Gly motifs forming the psi loops in the mature enzyme regions of PxAP8 and PxAP9 predicted polypeptides with human BACE (Hs_BACE1) and BACE2 (Hs_BACE2).

The Cys residues contributing the three disulphide bonds in BACE are shaded and their known pairing is indicated by thick (—) and light (—). Residue 80 is also boxed to indicate the presence of a W residue at this location in PxAP8 and BACE/BACE2.

PrAP7- and PrAP9-encoding genes are located in co-linear orthologous regions of the P. infestans and P. ramorum genomes, as confirmed by the organization of flanking genes. In P. sojae, the corresponding genomic region had a similar co-linearity arrangement with the exception of two missing genes, one of them in the region where an orthologous AP-encoding gene should have been located. Moreover, no unassembled reads were identified in P. sojae that matched the PrAP7 or PrAP9 sequences. The simplest interpretation is that an ancestor of the PxAP7-encoding gene was present in Phytophthora, and deletion of the region resulted in loss of function or PrAP7 sequences. The simplest interpretation is that an ancestor of the PxAP7-encoding gene was present in P. sojae, although it cannot be excluded that substitutions have accumulated to an extent that prevents recognition of the gene.

The final two sub-groups that were resolved within clade 3 consisted of PxAP6 and PxAP7 (Figure 1). No orthologue was detected in P. sojae [31]. Further investigations into the Phytophthora AP-GPCR fusion-proteins would appear to be well-merited.

documented [31]. Further investigations into the Phytophthora AP-GPCR fusion-proteins would appear to be well-merited.
Alignment of the mature enzyme regions of PxAP10 - 12 predicted polypeptides with representative plant aspartic proteinases and plasmepsin V.

Figure 5 Alignment of the mature enzyme regions of PxAP10 - 12 predicted polypeptides with representative plant aspartic proteinases and plasmepsin V. The numbering system is that of pig pepsin. Boxes identifying the hallmark features/landmark residues are as described in the legend to Figure 2. Cys residues and their likely pairing in intramolecular disulphide bonds are indicated by shading with black (●) and dotted (○) lines, respectively. The two Cys residues which are reported to participate in intermolecular disulphide bonds in Ath_CDR1 are marked with a star (★)
The PxAP6 and PxAP7 polypeptides showed an unusual variation in the component segments that constitute an archetypal AP in that a propart was absent (Table 3). In each case, the signal peptide (Additional File 5) immediately precedes the mature enzyme region (Figure 3) which, in turn, is followed by a C-terminal extension (Additional File 6). As stated above, almost all pepsin-like APs are synthesised in the form of a precursor. The propart segment makes a number of essential contributions, such as ensuring proper folding and intracellular sorting of the zymogen polypeptide, and facilitation of its activation into the mature enzyme form upon encountering the appropriate conditions [11,32]. Very recently, however, APs lacking a propart segment have been described from marine bacteria [26] and fungi [25], hence this unusual architecture of the PxAP6 and PxAP7 polypeptides in Phytophthora species is not without precedent.

BACE-like Aspartic Proteinasises

The PxAP8 and PxAP9 polypeptides were resolved by the phylogenetic analysis into clade 4 (Figure 1). These polypeptides also contained a signal peptide (Additional File 5), the mature enzyme region (Figure 4) and a C-terminal extension (Additional File 6) but they too lacked a propart (Table 3 and Additional File 5), just like PxAP6 and PxAP7.

The phylogenetic analysis (Figure 1) revealed that these proteins cluster within clade 4 with human BACE and its parologue, BACE2 (sub-families A01.004/041 in the MEROPS database). The mature enzyme forms of BACE/BACE2 are initially synthesised in precursor forms that do contain propart segments. While the presence of the propart has little effect on the intrinsic proteolytic activity, its inclusion at the N-terminus of proBACE/proBACE2 ensures much more rapid folding of the polypeptide(s) than is observed when only the mature forms of each enzyme are produced [[33,34] and references therein].

BACE and BACE2 were among the first APs to be identified as having a C-terminal extension which ensured they were membrane-bound. The mature enzyme regions (Figure 4) have hallmark features which distinguish A01.004/041 subfamily members from classical APs such as pepsin. Among these are: (i) the highly-conserved Trp39 (see Figures 2 and 3) is replaced by Ala and is itself relocated to a position (residue 80) just downstream from the essential Tyr75 residue on the β-hairpin loop that overhangs the active site cleft (see Background), (ii) disulphide bonds in characteristic locations that increase stability of the polypeptide. These additional Cys residues and the Trp39Ala replacement are all present in PxAP8 and PxAP9 proteins (Figure 4).

BACE has been identified as the β-site Alzheimer’s Precursor Protein (APP) Cleaving Enzyme (or β-secretase) responsible for the cleavage that generates the free N-terminal end of the Aβ peptide which plays a central role in Alzheimer’s disease [35]. For almost a decade, BACE has been the target of intense efforts to develop inhibitors to alleviate the dementia of Alzheimer’s disease. Genes encoding BACE and its parologue, BACE2, have been identified in species ranging from humans through elephant, opossum, armadillo, hedgehog, shrew, and fish, to platypus (http://www.merops.ac.uk). To the best of the authors’ knowledge, the identification of PxAP8-encoding genes from three Phytophthora species provides the first indication that BACE-like, membrane-bound APs may not be limited to the vertebrates. Intriguingly, the Phytophthora genomes also contain genes encoding homologues of presenilin, nicastrin, anterior pharynx defective-1(APH-1) and presenilin enhancer-2 (PEN-2) (although some gene models require substantial correction; not shown). These are all components of the human γ-secretase complex which completes the proteolytic processing by releasing the Aβ peptide at its C-terminal end from the Alzheimer’s Precursor polypeptide [36]. The presence of both β- and γ-secretase gene products in microbes such as Phytophthora suggests that their function is not limited to the processing of a brain polypeptide related to Alzheimer’s disease. These proteins therefore seem to be very ancient features that are conserved between oomycetes and vertebrates, and they have been coopted in vertebrate species to cleave the brain APP polypeptide.

While the PxAP8 polypeptides exhibited the hallmark characteristics of BACE/BACE2 subfamily members, the PxAP9 proteins lacked one feature that is necessary for an AP to be active as an enzyme. The Tyr residue located at position 75 (pig pepsin numbering) is conserved in every pepsin-like AP that is known to be active, although it does not participate directly in the catalytic mechanism [11,21]. In PxAP9, this Tyr residue is replaced by Phe (Figure 4) so the catalytic activity can be postulated to be compromised, at best. A protein engineering study revealed that the replacement of Tyr75 in a fungal AP by Asn or Phe permitted activity, albeit at a considerably reduced level, while all other replacements abolished activity [37]. However, a number of catalytically-inactive APs have been reported previously, including the pregnancy-associated glycoproteins (PAGs) found in the placenta of ungulates (subfamily A01.971 in MEROPS) [38] and perhaps most notably, the Bla g2 protein (subfamily A01.950 in MEROPS) which is a predominant allergen from cockroach [39]. The crystal structure of Bla g2 shows that it has the fold and three-dimensional structure typical of pepsin-like APs [40]. Bla g2 and the PAGs from Ovis aries and Capra hircus all have a Phe residue at position 75. These three proteins have additional substitutions at the
two active site DT/SG motifs, cementing their catalytic inactivity. Their function has been postulated to include roles in peptide-binding and presentation, but without cleavage. If PxAP9 should also prove to be catalytically ineffective, the active site will still be accessible for the binding of peptides that cannot undergo cleavage and so a role similar to the PAGs/Blag2 in peptide binding might be feasible. This intriguing possibility is rendered all the more fascinating with the realisation that, in contrast to the PAGs/Blag2, these Phytophthora orthologues are likely to be membrane-bound through their C-terminal extensions (Additional File 6) and might function as a novel type of surface receptor. In this regard, it may well be noteworthy that the putative intracellular domain comprised of the residues downstream from the transmembrane segment in the C-terminal extension in PxAP9 is considerably longer than its PxAP8 counterpart (Additional File 6). Neither of the C-terminal extensions, however, contains a discernible Pfam domain or protein motif. Thus, all three Phytophthora species may produce one catalytically-active BACE-like enzyme (PxAP8) and one peptide-binding, membrane-bound enzymatically inactive parologue with an intracellular extension of unknown function (PxAP9).

**Nepenthesin-like Aspartic Proteinases (MEROPS subfamily A1B)**

The remaining three AP genes in each Phytophthora genome (Table 2), encoding polypeptides PxAP10, PxAP11 and PxAP12 cluster into clade 5 with representative APs from MEROPS subfamily A1B including the patriarch of plant origin, nepenthesin, and plasmepsin V from Plasmodium falciparum (Figure 1). The PxAP10 -12 polypeptides contrast with their PxAP6 - 9 counterparts in having the archetypal AP architecture of a signal peptide followed by a propeptid (Additional File 5), a mature enzyme region (Figure 5), terminating with C-terminal extensions (Additional File 6). PxAP10 -12 differed, however, from all of the other Phytophthora APs (PxAP1 - 9) in having a small extra segment inserted (dotted box in Figure 5) in the region just upstream from Tyr75 (in pig pepsin numbering). No less than seven Cys residues are present between residues 39 - 75, with a further three Cys residues (shaded in Figure 5) conserved at characteristic locations elsewhere in the mature enzyme regions of the nine Phytophthora APs in clade 5. Together, these Cys residues are likely to form five specific disulphide bridges, paired as indicated in Figure 5. This arrangement of disulphide bridges has been deduced from biochemical analyses of the APs of plant origin [41,42] that cluster with PxAP10 -12 in clade 5. Indeed, most of the APs in plants [43] contain these Cys residues as well as the insert and they are grouped into subfamily A1B in the MEROPS database.

The plant APs identified in clade 5 are readily distinguished on the basis of their functional activity from one another and from others that are not included here for the sake of brevity, such as the nucellins (subfamily A01.073), regulators of the degradation of nucellar cells after ovule fertilisation [44], and the Chloroplast Nucleoids DNA-binding (CN4D1) proteinase (subfamily A01.050) which catalyses the degradation of RUBISCO in senescent leaves [45]. Nepenthesins (A01.040 subfamily) are extracellular enzymes secreted by the carnivorous plant, Nepenthes, to digest its prey [41] while CDR1 of Arabidopsis plays a crucial role in activating resistance of Arabidopsis against microbial pathogens [14]. Overexpression of CDR1 leads to constitutive disease resistance, while gene silencing compromises resistance. The CDR1 gene product is suggested to release an as yet unidentified peptide that activates a signalling cascade to induce disease resistance in a salicylate-dependent manner [14]. Recombinant CDR1 protein has been produced in E. coli and shown to be active as an AP in a dimeric form [42]. Two intermolecular disulphide bonds were proposed to be responsible for the covalent association of the two polypeptide chains in the proteolytically-active homodimer [42]. The Cys residues involved in the homodimer formation are highlighted in Figure 5 by a star (*). Cys residues are present at identical locations within PxAP10 (Figure 5), suggesting that this enzyme may also function as a homodimer. In contrast, PxAP11 is devoid of cysteine residues in this region and so, based on this criterion, should function as a monomer. PxAP12 does contain two cysteine residues but at different positions within this region of the polypeptide (Figure 5). Whether these remain as free cysteines or are involved in disulphide bonds, intra- or inter-molecular, requires experimental investigation.

One feature does serve to distinguish between the plant APs and the nine PxAP10 -12 polypeptides clustered in clade 5 (Figure 1). Most plant APs do not have C-terminal extensions (with NP_199124 from Arabidopsis included in Figure 1 as one of few exceptions). In contrast, all nine of the oomycete PxAP10 -12 polypeptides do have C-terminal extensions, ensuring that all are membrane-bound enzymes (Additional File 6).

In addition to plant APs, plasmepsin V was resolved into clade 5 (Figure 1) as the entry most closely associated with the PxAP10 -12 polypeptides. Plasmepsin V is one of ten APs encoded within the genome of Plasmodium falciparum [16], the causative agent of malaria. This enzyme (Figure 5) has a C-terminal extension with a hydrophobic stretch (not shown) that results in its anchoring as a membrane-bound polypeptide [46]. Very recently, plasmepsin V has been demonstrated to be the (aspartic) proteinase responsible for processing the precursors of Plasmodium effector proteins at the PEXEL motif (RxLxE/Q); this step...
is essential for the translocation of these polypeptides into the host red blood cell [18,19]. The malaria parasite exports hundreds of such effector proteins across the parasitophorous vacuolar membrane into the human red blood cell, thereby reprogramming the host cell to meet the needs of the parasite. Plasmepsin V is conserved in Plasmodium species but is not produced in vertebrates susceptible to parasitic infection and thus serves as a target enzyme against which to develop inhibitors as novel therapeutic agents for treatment of malaria.

Intriguingly, Phytophthora species also secrete effector proteins that are important in modulating host defence and cell death mechanisms and many such effector proteins contain the internal sequence motif RxLR, similar to the Plasmodium PEXEL motif [8,47,48]. Phytophthora effector proteins are secreted after removal of their signal peptide, giving rise to a polypeptide that contains in its N-terminal region the RxLR-containing domain which is essential for subsequent delivery into the plant cell [47-50]. It is tempting to consider that a Phytophthora effector precursor might undergo further processing to remove the RxLR domain, thereby releasing the mature, biologically active effector domain. The similarities described above between plasmepsin V and its PxAP10, PxAP11 and PxAP12 orthologues in Phytophthora suggests a conserved biological role. By analogy to the role of plasmepsin V in processing of Plasmodium effector proteins [18,19], one or more of its Phytophthora orthologues may reside in the translocon and act in the processing of Phytophthora effector precursors. There are obvious structural and genetic differences between the secretion mechanisms of the two parasites. The genome sequences of Phytophthora species lack genes homologous to the translocon complex components identified in Plasmodium [51]. Nevertheless, our hypothesis invites experimental investigation into targeting these particular APs in Phytophthora as a pathogenic Achilles heel through which the devastating losses from infection by Phytophthora might be reduced.

Conclusions

One of the twelve pepsin-like APs encoded within the genomes of the three Phytophthora species is predicted to be produced as an unprecedented fusion protein with a putative G-protein coupled receptor as the C-terminal partner. The other APs appear to be related to existing enzymes from other species that have already been documented. These include the vacuolar enzyme, and somewhat more unexpectedly, BACE/BACE2-like APs in an active and likely inactive form akin to the Bla g2/PAG proteins, and plasmepsin V-like enzymes. Whether the latter are functional homologs of their Plasmodium counterpart, operating in the processing of RxLR-type effector proteins during transit to the plant cell, would appear to be worthy of immediate experimental investigation given the high level of current interest in this topic. Elucidation of the physiological roles of the APs identified here offers highly fertile areas ripe for examination. The significant revision of the erroneous gene models and the detailed annotation presented here should be highly beneficial in facilitating future experimental design.

Methods

Data sources and analyses

Sequence data of Phytophthora infestans were retrieved from the Broad Institute website (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html). Sequence data of P. sojae and P. ramorum were retrieved from the website of the DOE Joint Genome Institute (http://genome.jgi-psf.org/). In cases where errors were suspected in P. infestans DNA sequences (gene models PITG_08190 and PITG_09837), the original trace files at http://www.ncbi.nlm.nih.gov/Traces/home were analyzed in more detail. In all cases the expected errors could be confirmed by analysing the trace representing the two alleles of the genes in P. infestans strain T30-4. The sequence error was further validated by amplification of the entire coding sequence of the genes from T30-4 genomic DNA using specific primers (underlined) linked to standard sequencing primers: 08190F1: 5′-TTTAGTGAAACCCTCAGATCGTTAAGTGCACCGTGGTTATAC-3′; 08190F2: 5′-TTTAGTGAAACCCTCAGATCGTTAAGTGCACCGTGGTTATAC-3′; 08190R1: 5′-AACAGCTCCTCGCCCTTGGGA-GATGGTACGTCCGCGG-3′; 08190R2: 5′-AACAGCTCCTCGCCCTTGGGA-GATGGTACGTCCGCGG-3′; 09387F: 5′-TTTAGTGAAACCCTCAGATCGTTAAGTGCACCGTGGTTATAC-3′; 09387R: 5′-AACAGCTCCTCGCCCTTGGGA-GATGGTACGTCCGCGG-3′.

The PCR products were purified and subsequently sequenced (Macrogen, Amsterdam, The Netherlands). The corrected nucleotide sequences were deposited in NCBI (PITG_09387 = PiAP1: HM588685; PITG_09837 = PiAP8: HM588686).

Expression of the eleven P. infestans AP genes was determined from analysis of the data derived from the NimbleGen microarray (Accession Number GSE14480), as described previously [4].

Identification of Phytophthora APs by PSI-Blast and HMMer profile alignment

A first HMMer search [24] was performed with default cut-off values of 10 using a profile based on the reference holozyme sequence set as provided by MEROPS. A second HMMer search was done with a profile that we
generated based on over 5000 AP sequences identified by NCBI Entrez and verified by MEROPS batch analysis [52]. PSI-Blast was performed, using cut-off values of 10, with the PiAP1, PiAP8 and PiAP10 as queries against the NR database restricted to the taxid Phytophthora (which includes the complete proteome sequences of P. infestans but not of P. sojae or P. ramorum) using a Position Specific Substitution Matrix obtained by converging PSI-blast against the Swissprot database. PSSM generation using NR or Refseq databases failed due to high contamination in the first Blast iteration using the Blosum62 substitution matrix.

Protein sequence analyses

Once the open reading frames of relevant gene entries were established, the resulting protein sequences were analysed in various ways. The mature enzyme region was determined by using the Pfam protein families database [53]. The SignalP server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptidase cleavage site and the absence of non-classical secretion signals in the PxAP2 sequences was verified using the SecretomeP server (http://www.cbs.dtu.dk/services/SecretomeP/). The cleavage site between pro-Imp and mature enzyme region was predicted from detailed inspection of the sequences. The prediction of transmembrane domains was performed using the web-tools ConPred II [54] or TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/) [55]. GPCR relationship was analysed by PRED-GPCR (http://athina.biol.uoa.gr/bioinformatics/PRED-GPCR/) [56].

Sequence alignments in Figures 2, 3, 4 and 5 were essentially prepared using T-Coffee [57] at the EBI server (http://www.ebi.ac.uk), and manually adjusted in a limited number of places to better align structural features that are hallmarks of APs.

Phylogeny

The alignments shown in Figures 2, 3, 4 and 5 were combined using T-Coffee [57] with manual corrections. Non-homologous N- and C-terminal sequences were removed. A maximum likelihood (ML) tree was made using PHYML [58], Bootstrap analysis was performed by combining the “seqboot” and “consense” commands from PHYML [59] and the ML algorithm from PHYML. Trees were analyzed using Dendroscope [60], Bootstrap analysis indicated poor resolution of the PxAP2 clade. In order to improve the resolution of this clade we included the sequence of human gastricin which was the first non-self hit detected in a Blast analysis using PiAP2 as a query. Similarly, in order to improve the resolution between Plasmepsin V and the PxAP10-12 clade, the sequence XP_002180775 from Phaeodactylum tricornutum (from the sister phylum of diatoms) was included in the phylogeny.

List of Abbreviations

Aspartic proteinase (AP) genes are listed according to their identifiers (e.g. PIPG, Ps or Pr) in the respective genome databases. The encoded polypeptides are identified as PiAP, PsAP or PrAP with a number. The generic descriptor PxAP with a number is used to simplify the description of any one sub-group of AP polypeptides from all three species. BACE: β-site Alzheimer’s Precursor Protein Cleaving Enzyme; PAG: Pregnancy Associated Glycoprotein; Bla g2: Cockroach Allergen from Blatella germanica; CDR-1: Constitutive Disease Resistance gene-1 protein product.

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Additional material

Additional file 1: Amino acid sequence of pig pepsin with characteristic motifs and features of Aspartic Proteinases. The DTG and hydrophobic/hydrophobic-Gly motifs forming the psi loops in each domain are boxed, as are the landmark residues Tyr75 and Trp39. The Cys residues contributing the three disulphide bonds are shaded and their pairing is indicated by thick black lines.

Additional file 2: Corrections made to erroneous Phytophthora Aspartic Protease gene models (as represented in databases in January 2010)

Additional file 3: Gene expression analysis of P. infestans AP genes by NimbleGen microarray

Additional file 4: Corrected Phytophthora Aspartic Protease gene models

Additional file 5: Signal peptides and pro-Imp segments at the N-terminal end of the mature enzyme regions of PxAP1-12

Additional file 6: Alignment in orthologous sets of the extensions at the C-terminal ends of the mature enzyme regions of PxAP2 - 12 detailed in Figures 2, 3, 4, 5. Gaps were introduced to optimize alignment. Hydrophobic residues contributing potential membrane-spanning stretches are boxed.
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Authors’ contributions
JK conceived the study, performed analyses on gene models, generated alignments shown in Figures 2, 3, 4, 5 and drafted the manuscript. HUGM analysed gene models, performed co-linearity studies, reassembled trace files for gene model corrections, and sequenced genome fragments and cDNA clones to validate gene models. ARH generated the phylegetic tree and multiple sequence alignments. JALV analysed gene models, coordinated the entire study, and prepared the final manuscript. All authors contributed to writing the manuscript and approved the content. They declare that they have no competing financial interests.

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References
1. Brasier C. Phytophthora biodiversity: How many Phytophthora species are there? In Proceedings of the 4th ILFRO workshop on Phytophthora in forest and natural ecosystems. Edited by Goheen E. USDA Forest series; 2008 101-115.

2. Rostan JA. Tracking historic migrations of the Irish potato famine pathogen, Phytophthora infestans. Microbes and Infection 2002; 4:1369-1377.

3. Haverkort A, Boonekamp P, Hutten R, Jacobsen E, Lotz, L, Kessal G, Visser R, van der Vossen E. Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. Potato Res 2008; 51:47-57.

4. Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, Cano LM, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller L, Provart N, Duggan DJ, Zody MC, Nusbaum C, Win J, Thines M, Kamoun S. Ten things to know about oomycete effectors. Mol Plant Pathol 2009, 10:795-803.

5. Khan AR, James MJG. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Proc Natl Acad Sci 1998; 95:15-836.

6. Cooper JB, Khan G, Taylor G, Tickle IJ, Blundell TL. X-ray analyses of aspartic proteases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. J Mol Biol 1990, 214:199-222.

7. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997, 25:389-400.

8. Eddy SR. Profile hidden Markov models. Bioinformatics 1998, 14:755-763.

9. ten Have A, Espino JJ, Dekkers E, Van Sluyter SC, Brito N, Kay J, Gonzalez C, van Kan JAL. The Botrytis cinerea aspartic protease family. Fung Genet Biol 2010, 47:53-65.

10. Rawlings ND, Bateman A. Pepsin homologues in bacteria. BMC Genomics 2009, 10:432.

11. Rep M, Duyvestijn RGE, Gale L, Usgaard T, Cornelissen BJC, Ma Li, Ward TJ. The presence of GC-AG introns in Neurospora crassa and other euascomycetes determined from analyses of complete genomes: Implications for automated gene prediction. Genomics 2006, 8:338-347.

12. Boekhout T, Hippler M, Smit MNM, de Vries W, de Weger RAAM, Pennings JJM. The presence of GC-AG introns in Neurospora crassa and other euascomycetes determined from analyses of complete genomes: Implications for automated gene prediction. Genomics 2006, 8:338-347.

13. Sinosi J, Faro C. Structure and function of plant aspartic proteasines. Eur J Biochem 2004, 271:2067-2075.

14. Xia YJ, Suzuki H, Borevitz J, Blount J, Guo ZL, Patel K, Dixon RA, Lamb C. An extracellular aspartic protease functions in Arabidopsis disease resistance signaling. EMBO J 2004, 23:980-988.

15. Craig C, Race E, Sheldon J, Whittaker L, Gilbert S, Moffatt A, Rose J, Dissanyake Y, Chim GW, Duncan I, Cammack N. HIV protease genotype and viral sensitivity to HIV protease inhibitors following saquinavir therapy. AIDS 1998, 12:1611-1618.

16. Coombs GR, Goldberg DE, Klembo M, Berry C, Kay J, Mottram JC. Aspartic proteases of Plasmodium falciparum and other parasitic protozoa as drug targets. Trends Parasitol 2001, 17:532-537.

17. Wlodawer A, Vondrasek J. Inhibitors of HIV-protease: a major success of structure-assisted drug design. Ann Rev Biomol Struct 1998, 27:249-284.

18. Boddey JA, Hodder AN, Gunther S, Gilson PR, Patisours H, Kapp EA, Pearce JA, de Koning-Ward TF, Simpson RJ, Crabbe BS, Cowman AF. An aspartic protease directs malaria effector proteins to the host cell. Nature 2010, 463:627-631.

19. Russo I, Babbitt S, Muradalhvaran V, Butler T, Okman A, Goldberg DE. Plasmapesin V licenses Plasmodium proteins for export into the host erythrocyte. Nature 2010, 463:632-636.

20. Shimock JI, Suttema E, Cano LM, Bocksutz KT, Olva R, van Damme M, Schwerz S, Raffaele S, Chaparro-Garcia A, Farrer R, Segreton ME, Bos J, Haas BJ, Zody MC, Nusbaum C, Win J, Thines M, Kamoun S. Ten things to know about oomycete effectors. Mol Plant Pathol 2009, 10:795-803.

21. Khan AR, James MJG. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Proc Natl Acad Sci 1998; 95:15-836.

22. Cooper JB, Khan G, Taylor G, Tickle IJ, Blundell TL. X-ray analyses of aspartic proteases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. J Mol Biol 1990, 214:199-222.

23. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997, 25:389-400.

24. Eddy SR. Profile hidden Markov models. Bioinformatics 1998, 14:755-763.

25. ten Have A, Espino JJ, Dekkers E, Van Sluyter SC, Brito N, Kay J, Gonzalez C, van Kan JAL. The Botrytis cinerea aspartic protease family. Fung Genet Biol 2010, 47:53-65.

26. Rawlings ND, Bateman A. Pepsin homologues in bacteria. BMC Genomics 2009, 10:432.

27. Rep M, Duyvestijn RGE, Gale L, Usgaard T, Cornelissen BJC, Ma Li, Ward TJ. The presence of GC-AG introns in Neurospora crassa and other euascomycetes determined from analyses of complete genomes: Implications for automated gene prediction. Genomics 2006, 8:338-347.

28. Boekhout T, Hippler M, Smit MNM, de Vries W, de Weger RAAM, Pennings JJM. The presence of GC-AG introns in Neurospora crassa and other euascomycetes determined from analyses of complete genomes: Implications for automated gene prediction. Genomics 2006, 8:338-347.

29. Bakthavatsalam D, Brazill D, Gomer RH, Eichinger L, Rivero F, Noegel AA. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Proc Natl Acad Sci 1998; 95:15-836.

30. Smith TF, Waterman MS. Identification of common molecular subsequences. J Mol Biol 1981, 147:185-197.

31. Traynellis SF, Trejo J. Protease-activated receptor signaling: new roles and regulatory mechanisms. Curr Opin Hematol 2007, 14:230-235.

32. Horimoto Y, Dee DR, Yada Y. Multifunctional aspartic peptidease prosegments. New Biotechnol 2009, 25:318-324.

33. Shi XP, Chen E, Yin KC, Na S, Garsky VM, Li YM, Platchek M, Register RB, Sardana MK, Thiebeau J, Wood T, Shafer JA, Noegel AA. A G protein-coupled receptor with a lipid kinase domain is involved in cell-density sensing. Curr Biol 2007, 17:892-897.

34. Smith TF, Waterman MS. Identification of common molecular subsequences. J Mol Biol 1981; 147:185-197.

35. Tomasselli AG. Large scale refolding and purification of the catalytic domain of Human BACE-2 produced in E. coli. Proteins: Structure, Function, and Bioinformatics 2009, 73:29-42.
36. Tian Y, Bassit B, Chau DM, Li YM: An APP inhibitory domain containing the Flemish mutation residue modulates gamma-secretase activity for A beta production. *Nature Struct Mol Biol* 2010, 17:151-159.

37. Park YN, Akawa J, Nishiyama M, Hornouchi S, Beppu T: Involvement of a residue at position 75 in the catalytic mechanism of a fungal aspartic protease, *Rhizomucor pusillus* pepsin. Replacement of tyrosine 75 on the flap by asparagine enhances catalytic efficiency. *Protein Eng* 1996, 9:869-875.

38. Xie SC, Green J, Beckers JF, Roberts RM: The gene encoding bovine pregnancy-associated glycoprotein-1, an inactive member of the aspartic protease family. *Gene* 1995, 159:193-197.

39. Pomes A, Chapman MD, Valles LD, Blundell TL, Dhanaraj V: Cockroach allergen Bla g2: structure, function, and implications for allergic sensitization. *Am J Respir Crit Care Med* 2002, 165:391-397.

40. Gustchina A, Li M, Wünschmann S, Chapman MD, Pomes A, Wlodawer A: Crystal structure of cockroach allergen Bla g2, an unusual zinc binding aspartic protease with a novel mode of self-inhibition. *J Mol Biol* 2005, 348:413-444.

41. Ashauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, Kubomura-Yoshida N, Iwamatsu A, Shibata C, Inoue H, Takahashi K: Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteases. *Biochem J* 2004, 381:295-306.

42. Simoes I, Fato R, Bur D, Fato C: Characterization of recombinant CDR1, an Arabidopsis aspartic protease involved in disease resistance. *J Biol Chem* 2007, 282:31358-31365.

43. Takahashi K, Niwa H, Yokota N, Kubota K, Inoue H: Widespread tissue expression of nepenthesin-like aspartic protease genes in Arabidopsis thaliana. *Plant Physiol Biochem* 2008, 46:724-729.

44. Chen F, Foolad MR: Molecular organization of a gene in barley which encodes a protein similar to aspartic protease and its specific expression in nucellar cells during degeneration. *Plant Mol Biol* 1997, 35:821-831.

45. Nakano T, Murakami S, Shoji T, Yoshida S, Yamada Y, Sato F: A novel protein with DNA binding activity from tobacco chloroplast nucleoids. *Plant Cell* 1997, 9:1673-1682.

46. Klemba M, Goldberg DE: Characterization of plasmepsin V, a membrane-bound aspartic protease homolog in the endoplasmic reticulum of *Plasmodium falciparum*. *Mol Biochem Parasitol* 2005, 143:183-191.

47. Birch PRU, Boeving PK, Gilroy EM, Hein I, Pritchard L, Whisson SC: Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. *Curr Opin Cell Biol* 2008, 11:379-379.

48. Govers F, Bourmeester K: Effector trafficking: RXLR-deEER as extra gear for delivery into plant cells. *Plant Cell* 2008, 20:1728-1730.

49. Dou D, Kale SD, Wang X, Jiang RHY, Bruce NA, Arredondo FD, Zhang X, Tyler BM: RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 2008, 20:1940-1947.

50. Tyler BM: Entering and breaking: virulence effector proteins of oomycete plant pathogens. *Cell Microbiol* 2009, 11:13-20.

51. de Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, Papenfuss AT, Sanders PR, Lundie RJ, Maier AG, Cowman AF, Crabb BS: A newly discovered protein export machine in malaria parasites. *Nature* 2009, 459:949-954.

52. Rawlings ND, Morton FR: The MEROPS batch BLAST: a tool to detect peptidases their and non-peptidase homologues in a genome. *Biochimie* 2008, 90:243-259.

53. Finn RD, Mistry J, Tate J, Coggill P, Heeger A, Pollington JE, Gavin O., Gunasekaran P, Ceric G, Forslund K, Holm L, Sonnhammer EL, Eddy SR: Bateman A: The Pfam protein families database. *Nucl Acids Res* 2010, 38:D211-222.

54. Ariai M, Mitsuhashi H, Ikeda M, Xia JX, Kikuchi T, Sarate M, Shimizu T: ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. *Nucl Acids Res* 2004, 32:W300-309.

55. Moller S, Croning MDR, Apweiler R: Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* 2001, 17:464-653.

56. Papasaias PK, Bagos PG, Lithoszi ZJ, Promponas VJ, Hamodrakas SJ: PRED-GPCR: GPCR recognition and family classification server. *Nucl Acids Res* 2004, 32:W380-382.

57. Notredame C, Higgins DG, Heering J: T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000, 302:205-217.

58. Guindon S, Gascuel O: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *System Biol* 2003, 52:696-704.

59. Felsenstein J: PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 1989, 5:164-166.

60. Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R: Dendroscope: An interactive viewer for large phylogenetic trees. *BMC Bioinformatics* 2007, 8:460.

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