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Evolution of the cutinase gene family: Evidence for lateral gene transfer of a candidate *Phytophthora* virulence factor

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Abstract

Lateral gene transfer (LGT) can facilitate the acquisition of new functions in recipient lineages, which may enable them to colonize new environments. Several recent publications have shown that gene transfer between prokaryotes and eukaryotes occurs with appreciable frequency. Here we present a study of interdomain gene transfer of cutinases – well documented virulence factors in fungi – between eukaryotic plant pathogens *Phytophthora* species and prokaryotic bacterial lineages. Two putative cutinase genes were cloned from *Phytophthora brassicae* and Northern blotting experiments showed that these genes are expressed early during the infection of the host *Arabidopsis thaliana* and induced during cyst germination of the pathogen. Analysis of the gene organisation of this gene family in *Phytophthora ramorum* and *P. sojae* showed three and ten copies in tight succession within a region of 5 and 25 kb, respectively, probably indicating a recent expansion in *Phytophthora* lineages by gene duplications. Bioinformatic analyses identified orthologues only in three genera of Actinobacteria, and in two distantly related eukaryotic groups: oomycetes and fungi. Together with phylogenetic analyses this limited distribution of the gene in the tree of life strongly support a scenario where cutinase genes originated after the origin of land plants in a microbial lineage living in proximity of plants and subsequently were transferred between distantly related plant-degrading microbes. More precisely, a cutinase gene was likely acquired by an ancestor of *P. brassicae*, *P. sojae, P. infestans* and *P. ramorum*, possibly from an actinobacterial source, suggesting that gene transfer might be an important mechanism in the evolution of their virulence. These findings could indeed provide an interesting model system to study acquisition of virulence factors in these important plant pathogens.

Keywords: *Phytophthora* spp.; Cutinase; *Arabidopsis*; Lateral gene transfer

1. Introduction

Lateral (or horizontal) gene transfer (LGT) is well documented in prokaryotes and can lead to the acquisition of genes conferring virulence such as antibiotic resistance and pathogenicity islands (De la Cruz and Davies, 2000; Lawrence 2005). It is unclear how important gene acquisition is in the evolution of virulence in eukaryotes. However, a recent study indicates that a recent gene transfer between two species of fungi has enabled the recipient to rapidly acquire high virulence on the host species (Friesen et al., 2006). This study highlights how a pathogen population with significantly enhanced virulence can emerge, leading to the emergence of a new damaging disease of host plants. It also brings up the controversial issues of how LGT occurs and whether recipient incompatibility barriers to gene flow are more "leaky than was previously thought" (Sanders, 2006). Another study focused on filamentous fungi and oomycetes showed that fungi-

Abbreviations: Asp, Aspartic acid; Cut, Cutinase; EST, expressed sequence tag; Gln, Glutamic acid; Gly, Glycine; His, Histidine; Ler, Landsberg erecta; LGT, lateral gene transfer; ML, maximum likelihood; NCBI, National Center for Biotechnology Information; *P.*, *Phytophthora*; rCut, *Phytophthora ramorum* cutinase; sCut, *Phytophthora sojae* cutinase; Ser, Serine; Tyr, Tyrosine.

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to-oomycete LGT has played a significant role in the evolution of the osmotrophic, filamentous lifestyle on these two separate branches of the eukaryotic tree (Richards et al., 2006). The acquisition of these genes by oomycetes likely provided a metabolic advantage which helped them to adapt to an osmotrophic lifestyle which might have facilitated a lifestyle allowing colonization of plants; the evolution of pathogenicity is usually coupled with changes in the gene inventory (De la Cruz and Davies, 2000; Lawrence 2005; Andersson, 2006a, b; Boucher et al., 2003).

Metabolic adaptation via gene acquisition indeed appears to be a common genome evolution mechanism in microbial eukaryotes. Anaerobic protists have likely adapted to a life at low oxygen concentrations at least partly via multiple gene transfers from anaerobic prokaryotes (Andersson 2006a; Ricard et al., 2006). Likewise, a prokaryote-to-eukaryote transfer of dihydroorotate dehydrogenase *Trypanosoma cruzi* likely enabled the kinetoplastid to adapt to anaerobic conditions (Annoura et al., 2005), and a gene for the same enzyme was acquired by an ancestor of *Saccharomyces cerevisiae* and made the yeast facultative anaerobic (Hall et al., 2005). These studies with many others undoubtedly confirm that there is a misconception that eukaryotes are immune to lateral gene transfer; recent phylogenomic analyses indeed indicate significant prokaryote-to-eukaryote gene transfer affecting microbial eukaryotes (Huang et al., 2004; Loftus et al., 2005; Ricard et al., 2006; Andersson et al., 2007; Carlton et al., 2007).

Economically important eukaryotic plant pathogens are found among the oomycetes. *Phytophthora* plant pathogens (oomycetes) attack a wide range of agriculturally and ornamentally important plants (Erwin and Ribeiro, 1996). Late blight of potato caused by *Phytophthora infestans* resulted in the Irish potato famine in the 19th century. A newly emerged *Phytophthora* species, *Phytophthora ramorum*, is responsible for a disease called sudden oak death that affects not only the live oaks that are the keystone species of the ecosystem but also a large variety of woody shrubs that inhabit the oak ecosystems, such as bay laurel and viburnum (Rizzo et al., 2005). *Phytophthora brassicae* is a pathogen of *Brassica* plants, it have been reported from *Brassica oleracea* and *Brassica chinensis* (Man in't Veld et al., 2002).

Phytophthora plant pathogens deploy an array of host cell wall modifying enzymes during pathogenesis (Gotesson et al., 2002). This is largely illustrated by secreted cutinases required for hydrolyzing plant cuticular material. Cutinases are extracellular enzymes found in fungi, oomycetes and several bacterial lineages that hydrolyze cutin, an insoluble lipid-polyester that forms a major component of the plant cuticle (Kolattukudy et al., 1985; Ettinger et al., 1987). Cutinases are serine esterases and contain the classical catalytic triad of Asp, Ser, and His, with the active serine in the consensus sequence Gly–His/Tyr–Ser–X–Gly, and an oxyanion hole formed in part by Gln and Ser residues (Martinez et al., 1994). The sequence around the catalytic domain is highly conserved and provides a signature pattern for cutinases (Kolattukudy et al., 1985). A critical role for fungal cutinases in the penetration of unwounded host tissues was demonstrated for some fungi by the use of antibodies, inhibitors and by using cutinase deficient fungal mutants (Dickman and Patil, 1986; Dickman et al., 1983; Maiti and Kolattukudy, 1979; Shaykh et al., 1977). In addition, recent gene disruption studies on the cutinase gene of *Pyrenopeziza brassicae*, an ascomycete, showed molecular evidence that cutinase activity is required for pathogenicity (Li et al., 2003). Besides fungal penetration of the cuticular layer during initial stages of infection, cutinases have been suggested to have a role in spore attachment (Deising et al., 1992) and in carbon acquisition for saprophytic growth (Köller and Parker, 1989). Cutinases secreted by the fungus may partially break down the plant cuticle to produce cutin monomers, which have been shown not only to induce the expression of cutinase (Lin and Kolattukudy, 1978) but to trigger conidial germination and differentiation of appressoria (Gilbert et al., 1996; Hegde and Kolattukudy, 1997). Thus, cutinases may have a role in surface signalling that is crucial for the differentiation of essential infection structure and expression of pathogenicity factors.

Here we identify *Phytophthora* genes with sequence homology to functionally identified fungal cutinases, as well as putative bacterial homologs. The *P. brassicae* genes are shown to have an expression pattern suggestive of a role in virulence. Bioinformatic analyses of the cutinase gene family identified LGT as an important mechanism for the distribution of the gene between distantly related organismal groups with a putative bacterial origin of the *Phytophthora* homologs.

2. Materials and methods

2.1. Cultures of Arabidopsis and Phytophthora spp.

All *Phytophthora* cultures were grown and maintained as previously described (Roetschi et al., 2001). *Arabidopsis* accession Landsberg erecta (Ler) was grown as previously described (Roetschi et al., 2001).

2.2. DNA and RNA extraction

DNA and RNA were purified from mycelia with the use of the DNA-Easy Plant Mini kit and the RNeasy plant minikit, respectively (Qiagen, Basel, Switzerland), according to manufacturer's specifications. RNA from Arabidopsis has been also obtained using the RNeasy plant minikit (Qiagen, Basel, Switzerland). Quality was checked by visualization under UV light following electrophoretic separation with a molecular mass standard (1Kb DNA Ladder, Invitrogen, Switzerland) in 1% agarose (Sigma-Aldrich, Buchs, Switzerland) gel in 1x TBE, subjected to 100 V for 1 h and stained with ethidium bromide (0.5 mg/ml^{-1}). Concentrations were assayed in a S2100 Diode Array spectrophotometer (WPA Biowave, Cambridge, UK). Blotting and hybridization were done according to standard protocols (Ausubel et al., 1987).

2.3. Cloning and sequencing of P. brassicae cutinase genes

A search of EST collection from *P. brassicae* (Belbahri and Mauch, Unpublished data) identified several ESTs with strong

homology to bacterial cutinases. A *P. brassicae HH*-strain (Roetschi et al., 2001) genomic library in the vector Lambda-EMBL3 (Promega) was used to clone the cutinase genes CutA (*AY244552*) and CutB (*AY244553*) using the cutinase ESTs as probes.

2.4. Assembly of cutinase datasets

We used the PhyloGenie package (Frickey and Lupas, 2004) to assemble an initial dataset using the *P. brassicae* CutA as a probe [gi: 29838408]. The program performs similarity searches (BLAST) against the non-redundant protein database downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and then builds a Hidden Markov Model profile using HMMER (http://hmmer.wustl.edu) from the alignment of the BLAST hits with *E* values below e^{-5}. The profile is then used to search all full-length BLAST hits; sequences scoring better than the threshold (e^{-5}) are included in the dataset.

Although PHYLOGENIE previously has successfully identified gene families (Andersson et al., 2007), we complemented this automatic approach with additional similarity searches. Putative additional cutinase sequences were identified using BLAST searches against a variety of databases with three putative cutinase sequences as query sequences (*P. brassicae* [gi: 29838408], *Coprinopsis cinerea* [gi: 116503541], *Kineococcus radiotolerans* [gi: 152968054]). A dozen actinobacterial sequences with weak similarity were identified for which the consensus sequence around the cutinase catalytic active serine (Gly–His/Tyr–Ser–X–Gly) (Martinez et al., 1994) were present; the automatic approach excluded these. Although these potentially encode divergent actinobacterial cutinases, they were excluded from further analysis since their large divergence made them meaningless for phylogenetic analysis. Very weak homologies to a few other esterase sequences were also found; these were excluded since the consensus cutinase sequence was absent.

In order to make the phylogenetic representation in the dataset as broad as possible also unpublished eukaryotic and prokaryotic sequences were searched at NCBI using the "other eukaryotes" and "microbial genomes" BLAST services in their genomic BLAST pages. However, only cutinase sequences from phylogenetic groups already represented in the dataset were found, and therefore no additional sequences were retrieved from these databases. Similarly, the available genome sequence data from ongoing genome projects on the microbial eukaryotes *Monosiga brevicollis* (choanoflagellate), *P. ramorum* (oomycete), *Phytophthora sojae* (oomycete), *Thalassiosira pseudonana* (diatom), *Phaeodactylum tricornutum* (diatom), *Chlamydomonas reinhardtii* (green algae), *Naegleria gruberi* (excavata) were searched using the BLAST service at the DOE-Joint Genome Institute (http://www.jgi.doe.gov/). Four and sixteen homologs were retrieved from *P. ramorum*, *Phytophthora sojae* genomes, respectively; the other genomes did not produce any significant matches. This procedure, which were performed in December 2006, resulted in a dataset with 153 putative cutinase homologs, which were aligned using CLUSTALW, version 1.83 (Thompson et al., 1994). 134 unambiguously aligned positions were identified and used in the phylogenetic analyses. Accession numbers for all included sequences and alignments are available from the authors on request.

2.5. Phylogenetic analyses

The optimal amino acid or nucleotide substitution model for each dataset was selected using the program MODELGENERATOR (Keane et al., 2006). A preliminary protein maximum likelihood (ML) phylogeny was inferred for the 153 taxa dataset with the program PHYML, version 2.4.4 (Guindon and Gascuel, 2003) with the optimal substitution model (WAG+I+G). Based on this phylogeny, a second dataset were constructed where 14 oomycete, 26 mycobacterial, and 45 fungal sequences were removed to reduce the computational burden of the phylogenetic reconstructions, while a broad taxonomic sampling was retained. A ML phylogeny was inferred together with a bootstrap analysis with 500 replicates from this 68-taxa dataset using PHYML with the optimal substitution model (WAG+I+G).

A separate dataset was constructed including the 20 unique *Phytophthora* cutinase sequences. 176 unambiguously aligned positions were identified in this dataset and the protein ML phylogenetic tree together with a bootstrap analysis with 500 replicates was inferred using PHYML with the optimal substitution model (WAG+G). For comparison, the corresponding nucleotide sequences were retrieved and a nucleotide alignment was constructed based on the amino acid alignment using MEGA, version 3.1 (Kumar et al., 2004). A nucleotide ML phylogenetic tree based on all three codon positions was inferred using PHYML, version 2.4.4. (Guindon and Gascuel, 2003) with the optimal nucleotide substitution models (TN+G+I), and a bootstrap analysis with 500 replicates with the same parameters was performed.

3. Results

3.1. P. brassicae encodes and expresses putative cutinase genes

Putative sequence homologs of cutinases were identified from an EST sequence collection of the oomycete *P. brassicae* (Belbahri, unpublished data). Two genes (CutA and CutB) putatively encoding cutinases were cloned from a genomic library constructed from genomic DNA of *P. brassicae* using the identified EST sequences with homology to bacterial and fungal cutinases as probes. To determine whether these putative *P. brassicae* cutinase genes, which were found to have identical nucleotide sequences, are expressed during the life cycle of *P. brassicae*, we conducted experiments using Northern blotting with a cutinase EST probe *in vitro*, and in presence of *Arabidopsis* seedlings. Expression of the genes was observed on total RNA extracted from germinating cysts (Fig. 1A) and 4 h after mycelia incubation with *Arabidopsis* seedlings (Fig. 1B).

3.2. A large family of putative cutinase genes in Phytophthora

Putative cutinase genes are not unique to *P. brassicae* within the genus; sequence analysis of *P. sojae* and *P. ramorum*

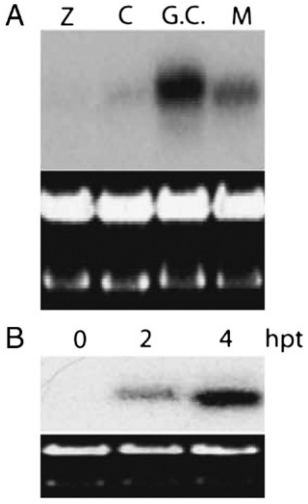

Fig. 1. Expression study of the cutinase genes from *P. brassicae*. A. Cutinase gene expression during in vitro growth of *P. brassicae*. Left side: Northern blotting study of the expression of cutinases in zoospores, cysts, germinating cysts and mycelia (Z, C, GC and M). B. CutA gene expression during interaction with *Arabidopsis thaliana*: Northern blotting study of the expression of cutinases during incubation with seedlings of the host *Arabidopsis thaliana* at 0, 2 and 4 h post-incubation.

genomes revealed the presence of genes with strong similarity to *P. brassicae* CutA in high copy number; 16 predicted cutinase copies (sCutA–sCutP) were identified in *P. sojae*, *P. ramorum* had four (rCutA–rCutD) copies, while two copies each could be identified from the partial genomes of *P. brassicae* and *P. infestans* (Fig. 2). In *P. sojae*, ten copies were located in a large cluster spanning a region of approximately 25 kb, and three genes are found in the same region of the *P. ramorum* genome (Figs. 2A and 3B). Thus, *P. sojae* has seven additional copies in this genomic region relative to *P. ramorum*, probably due to gene duplication in the lineage leading to *P. sojae*, or gene loss in the *P. ramorum* lineage (Figs. 2A and 3B). Three of the remaining six *P. sojae* genes are found in the proximity of a pectate lyase gene, and, again, a single putative cutinase gene is found in the same region in *P. ramorum*, suggesting gene losses or duplication in the two lineages (Figs. 2B and 3B). The remaining three *P. sojae* genes are distributed on two different scaffolds (Fig. 2C). A pattern of clustering of cell wall degrading enzyme-encoding genes was previously reported for a poly-galacturonase gene family from *Phytophthora cinnamoni* (Gotesson et al., 2002) and endoglucanases of *Phytophthora* spp. (Costanzo et al., 2006), suggesting a general pattern of clustering of plant cell wall modifying enzymes in *Phytophthora* genomes. The phylogenetic analyses of the putative oomycete cutinases indicate that the large cutinase gene cluster on scaffold 3 of *P. sojae* has a mixed origin, some are likely recent gene duplications (or possibly gene conversions) as exemplified by 100% identity between some of the paralogues (Fig. 3B). Similarly, the identity at the nucleic acid sequence level of the two putative cutinase sequences of *P. brassicae* is nearly 100% in the coding, promoter and terminator regions of the genes, suggesting a recent duplication event. Thus, analyses of four *Phytophthora* species indicate a large gene family which most likely was present in their common ancestor.

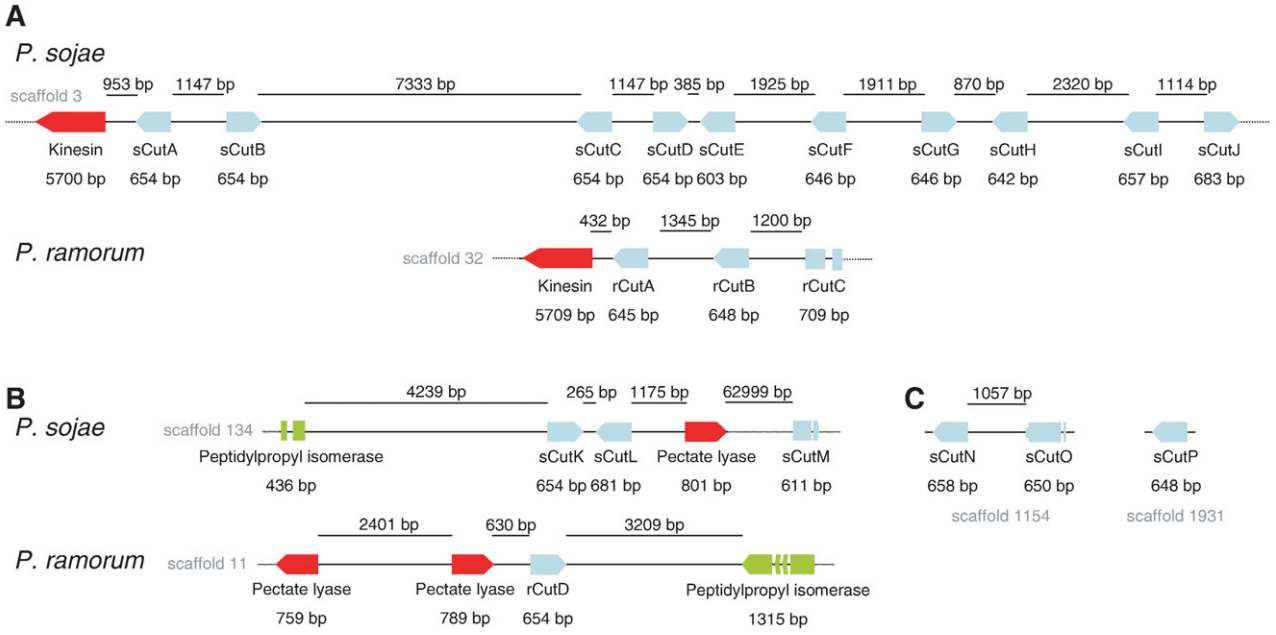

Fig. 2. Map of *Phytophthora sojae* and *Phytophthora ramorum* cutinase gene clusters. Conserved scaffold regions containing cutinase genes (blue) around the kinesin (red) (A) and pectate lyase (red) and peptidylpropyl isomerase (green) (B) genes between the two *Phytophthora* spp. are shown. Three additional cutinase genes in *P. sojae* are also shown (C). Relative distances of adjacent cutinase gene copies are indicated above the solid line representing a scaffold section. The ORF lengths are reported below the individual cutinase copy name. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

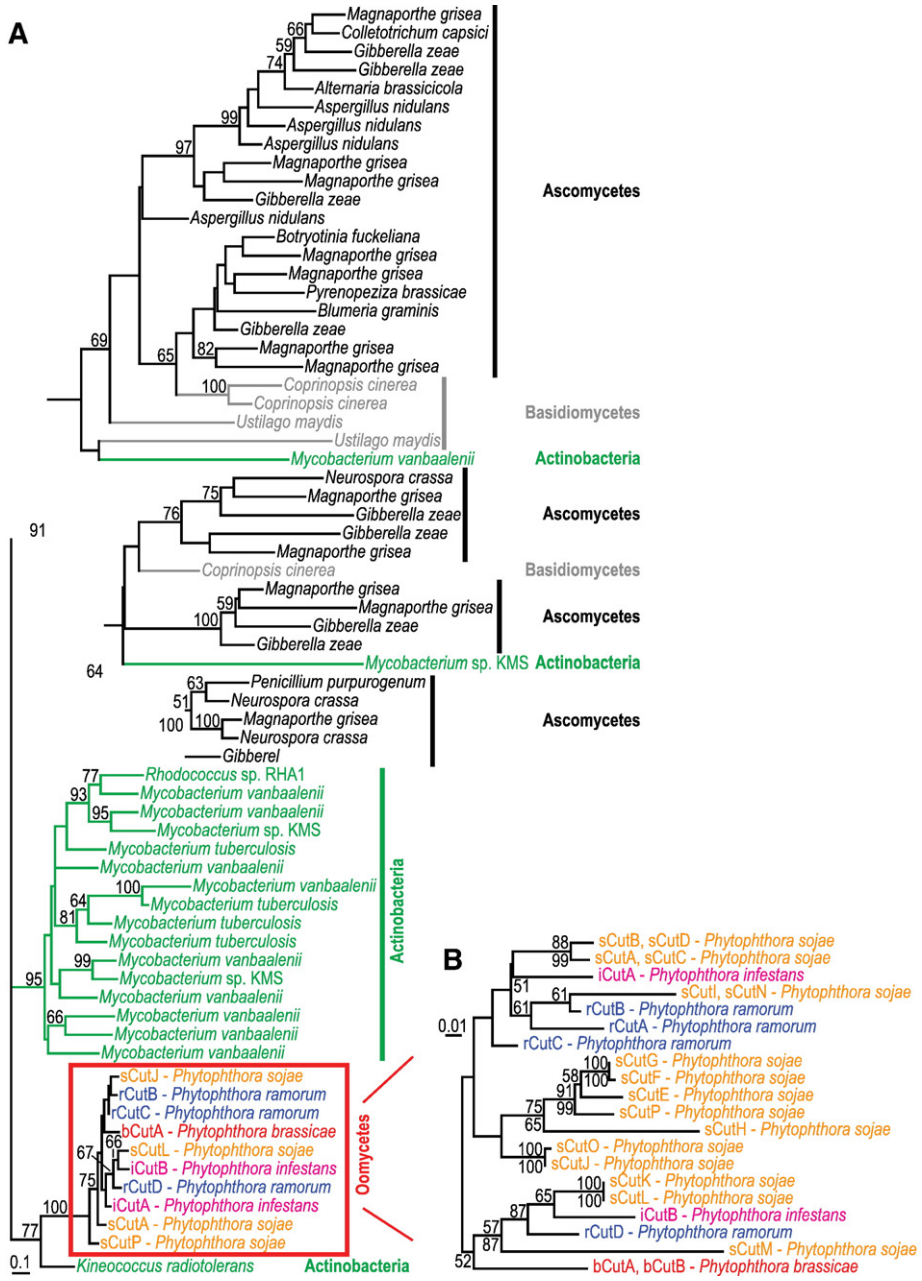

Fig. 3. Protein maximum likelihood trees of cutinase. A. Phylogenetic tree based on 134 unambiguously aligned positions of cutinase. The Γ shape parameter, α, and the fraction of invariable sites were estimated to 1.719 and 0.034, respectively. B. Phylogenetic tree based on 176 unambiguously aligned positions regions of 20 unique oomycete cutinases. Only one of pair-wise identical sequences are included as indicated by two different protein names on the same terminal node. The Γ shape parameter, α, was estimated to 0.380. Protein maximum likelihood bootstrap values are shown above the branches. Only values >50% for bipartitions are shown. Nucleotide maximum likelihood bootstrap support values are shown below the branches in B. The trees are arbitrarily rooted.

3.3. The gene family most likely encode functional and secreted cutinases

All members within the gene family putatively encoding cutinases could be conceptually translated into proteins using BioEdit (Hall, 1999). These varied in length from 210 to 294 amino acid residues (Fig. 2). The level of pair-wise amino acid identity between these 24 oomycete cutinase sequences ranged from 66 to 100% (Fig. 3B). Based on a combination of prediction models implemented by the SignalP 3.0 server (Bendtsen et al., 2004; Nielsen et al., 1997), a signal peptide was predicted in all 24 cutinases amino acid sequences with a cleavage site located between the 18th and 28th amino acid residue, indicating that they are likely secreted from the cell, as expected from a protein with cutinase activity. All sequences, except *P. sojae* CutM protein, contain the classical catalytic triad of Asp, Ser, and His; with the active serine in the consensus sequence Gly–His/Tyr–Ser–X–Gly (Martinez et al., 1994) providing the signature pattern for cutinases (Kolattukudy et al., 1985). These observations suggest that all genes in the identified gene family, with the possible exception of sCutM, are functional cutinases and secreted. Intriguingly, these

observations are in agreement with a role as a virulence factor within these four plant pathogens.

3.4. Patchy phylogenetic distribution suggest a recent origin in oomycetes within heterokonts

In order to study the evolution of cutinases in more detail, we have performed bioinformatic and phylogenetic analyses on available cutinase homologs. Our extensive database mining only identified additional cutinase homologues within Actinobacteria and fungi, two phylogenetic groups distantly related to oomycetes. According to the current view of eukaryotic phylogeny oomycetes belong to heterokonts within the supergroup Chromalveolates, while fungi belong to Opisthokonts (Baldauf 2003; Simpson and Roger 2004; Adl et al., 2005). The absence of cutinases in finished genomes of other heterokonts (i. e. diatoms), as well as in ciliates and apicomplexa suggests a recent origin of cutinase in oomycetes, rather than an ancient presence in the common ancestor of chromalveolates. Similarly, our searches failed to identify cutinase genes in any of the metazoan or choanoflagellate genomes suggesting absence of cutinase in the ancestral opisthokonts genome. Cutinase homologues were found only in three actinobacterial genera and no archaea, indicating a very narrow phylogenetic distribution within prokaryotes and, again, suggesting a recent origin in Actinobacteria. Thus, it appears unlikely that the common ancestor of bacteria and eukaryotes encoded cutinase genes; a recent origin followed by distribution via gene transfer seems like a more likely evolutionary scenario to explain the presence of cutinase genes only in these three distantly related phylogenetic groups.

3.5. Cutinase has been distributed via inter- and intra-domain gene transfer

To further study the origin of the cutinase genes we performed phylogenetic analyses on the cutinase homologues (Fig. 3A). Two major groups were observed, one containing all fungal sequences with two mycobacterial sequences, and another with the rest of the actinobacterial sequences and the oomycete sequences separated with a bootstrap support value of 91% for the bipartition. Thus, the phylogenetic analyses do not support a common ancestry of eukaryotic cutinase, in agreement with the narrow phylogenetic distribution of the enzyme. However, the oomycete sequences are split from all other sequences, strongly suggesting a common ancestry of cutinase in these plant pathogens. These sequences are forming an adjacent group with a *Kineococcus* sequence, which in turn are adjacent to the large actinobacterial group consisting of mycobacterial and a single *Rhodococcus* sequence (Fig. 3A). Thus, the oomycete sequences are found nested with actinobacterial sequences, weakly suggesting an actinobacterial-to-heterokont gene transfer event for which *Kineococcus* would be the closest sequenced present-day relative to the putative donor lineage. However, the absence of cutinase genes in relatives to *Kineococcus* and *Mycobacterium* indicate that the genes could have different origins in these lineages. Obviously, further taxon sampling is needed to determine the donor and recipient lineages of the gene transfer in more detail. Interestingly, the phylogenetic analyses indicate additional transfer of cutinase genes. The *Rhodococcus* sequence is nested within *Mycobacterium* sequences suggesting a recent acquisition of cutinase in the *Rhodococcus* lineage from a mycobacterial source (Fig. 3A). More surprisingly, mycobacterial sequences are found in two positions within fungi. Unfortunately, the bootstrap support for the exact positions within fungi is poor, making it impossible to identify putative donor lineages, or to determine the direction of transfer.

4. Discussion

Cutin plays a key role in protection against the entry of pathogens into plants, and its enzymatic degradation has proved to be one of the first steps in the infection process for plant pathogens. For example the invasion of plants by phytopathogenic fungi is based on the secretion of an extracellular cutinase (Li et al., 2003). In this study we demonstrate the presence of multiple copies of putative cutinases in the phytopathogen oomycete genus *Phytophthora*, with as much as 16 copies in the genome of *P. sojae* (Figs. 2 and 3B). We show that putative *P. brassicae* cutinases are expressed during early stages of infection, induced by contact with the host, *Arabidopsis thaliana* (Fig. 1). In addition, the signature pattern for cutinases is present in the *Phytophthora* proteins, and they do encode signal peptides for secretion from the oomycete cell. These observations are in agreement with cutinase activity expressed in response to contact with host cells. Furthermore, the gene family is absent from sequenced non-pathogenic heterokonts (i. e. diatoms), in agreement with a role as virulence factors for the *Phytophthora* cutinases.

The oomycete cutinase genes most likely originated via gene transfer, possibly from a bacterial lineage (Fig. 3A). Inter-domain transfer of cutinase has indeed been suggested previously based on similarity searches of a smaller dataset (Gamieldien et al., 2002). An alternative scenario could be that the gene was present in the last common ancestor of bacteria and eukaryotes and subsequently was lost in the lineages that do not encode the enzyme, as has been argued in the past (Kinsella and McInerney, 2003). However, this possibility is dismissed for two major reasons. Firstly, the absence of the gene in close relatives of oomycetes and fungi and the polyphyly of the eukaryotic sequences in the phylogenetic analyses makes a differential gene loss scenario unlikely since a large number of loss events have to be inferred to explain these observations. Gene families with patchy distribution are indeed likely to be spread between distantly related organisms with similar lifestyles via gene transfer events (Andersson et al., 2006). Secondly, to our knowledge, cutin is only made by plants and it seems very unlikely that a protein with cutinase activity was present in the common ancestor of eukaryotes since the substrate for the enzyme almost certainly was unavailable at the time. Therefore, a more recent origin of cutinase – after the emergence of plants – followed by gene transfers appears like a more reasonable evolutionary scenario.

The phylogenetic tree of the cutinases is unrooted (Fig. 3A) and the present taxonomic sampling of cutinases almost certainly only represent a small fraction of the true sequence diversity; more taxonomic groups will likely be shown to encode cutinase homologs as the size of the sequence databases increases. Accordingly, the directions of transfers between the three represented groups are difficult to identify and the analysis is inconclusive regarding which group the cutinase gene originated. However, the sequence analysis identified a relatively large diversity of cutinases within *Phytophthora* (Fig. 3B) indicating an evolutionary history in the genera long enough to expect native sequence patterns in the gene sequences.

The presence of genes encoding cutinases in the Actinobacterial genera, such as *Mycobacterium*, not known to be plant pathogens might be surprising. However, the presence of a cutinase in a genome confers the ability to hydrolyze plant cuticular material to the organism. This substrate could indeed be used as carbon sources for non-pathogenic organism; mycobacterial species – including *M. vanbaalenii* and *Mycobacterium* sp. KMS – are frequently isolated from soil environments where dead plant matter should be present (Miller et al., 2004). The mechanism by which the eukaryotes acquired the genes is not the object of this study. Nevertheless, symbiotic relationships between prokaryotes and eukaryotes and transfection by viruses, are two mechanisms that could explain such transfers (Gogarten, 2003). Interestingly, recent data indicate that bacterial-type genes are abundant in some viruses that infect eukaryotes suggesting that they indeed could be vectors for inter-domain gene transfers (Filée et al., 2006).

5. Conclusions

This work clearly demonstrates that a gene family most likely encoding enzymes with cutinase activities, which are strong candidate to be virulence factors, has been acquired by *Phytophthora* spp. during evolution via LGT. Our findings suggest that the acquisition of this gene likely was important for oomycetes to be able to colonize host plants, presumably a new ecological niche for these organisms. At any rate, our study provides another example of metabolic adaptation via gene acquisition in microbial eukaryotes. We predict that systematic analyses of a much wider array of gene families will show that LGT from various sources is an important evolutionary mechanism in genome evolution among oomycetes.

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References

Adl, S.M., et al., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J. Eukaryot. Microbiol. 52, 399–451.

Andersson, J.O., 2006a. Genome evolution of anaerobic protists: metabolic adaptation via gene acquisition. In: Katz, L.A., Bhattacharya, D. (Eds.), Genomics and Evolution of Microbial Eukaryotes. Oxford University Press, Oxford, pp. 109–122.

Andersson, J.O., 2006b. Convergent evolution: gene sharing by eukaryotic plant pathogens. Curr. Biol. 16, R804–R806.

Andersson, J.O., Hirt, R.P., Foster, P.G., Roger, A.J., 2006. Evolution of four gene families with patchy phylogenetic distribution: influx of genes into protest genomes. BMC Evol. Biol. 6, 27.

Andersson, J.O., et al., 2007. A genomic survey of the fish parasite *Spironucleus salmonicida* indicates genomic plasticity among diplomonads and significant lateral gene transfer in eukaryote genome evolution. BMC Genomics 8, 51.

Annoura, T., Nara, T., Makiuchi, T., Hashimoto, T., Aoki, T., 2005. The origin of dihydroorotate dehydrogenase genes of kinetoplastids, with special reference to their biological significance and adaptation to anaerobic, parasitic conditions. J. Mol. Evol. 60, 113–127.

Ausubel, F.M., et al., 1987. Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Interscience, New York.

Baldauf, S.L., 2003. The deep roots of eukaryotes. Science 300, 1703–1706.

Bendtsen, J.D., Nielson, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Boucher, Y., Douady, C.J., Papke, R.T., Walsh, D.A., Boudreau, M.E.R., Nesbø, C.L., Case, R.J., Doolittle, W.F., 2003. Lateral gene transfer and the origins of prokaryotic groups. Annu. Rev. Genet. 37, 283–328.

Carlton, J.M., et al., 2007. Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis. Science 315, 207–212.

Costanzo, S., Ospina-Giraldo, M.D., Deahl, K.L., Baker, C.J., Jones, R.W., 2006. Gene duplication event in family 12 glycosyl hydrolase from *Phytophthora* spp. Fungal Genet. Biol. 43, 707–714.

Deising, H., Nicholson, R.L., Haugh, M., Howard, R.J., Mendgen, K., 1992. Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. Plant Cell 4, 1101–1111.

De la Cruz, F., Davies, J., 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. Trends Microbiol. 8, 128–133.

Dickman, M.B., Patil, S.S., Kolattukudy, P.E., 1983. Effects of organophosphorous pesticides on cutinase activity and infection of papayas by *Colletotrichum gloeosporioides*. Physiol. Biochem. 73, 1209–1214.

Dickman, M.B., Patil, S.S., 1986. Cutinase deficient mutants of *Colletotrichum gloeosporioides* are non-pathogenic to papaya fruit. Physiol. Mol. Plant Pathol. 28, 235–242.

Erwin, D.C., Ribeiro, O.K., 1996. Phytophthora diseases worldwide. APS Press. American Phytopathological Society, St. Paul, MN.

Ettinger, W.F., Thukral, S.K., Kolattukudy, P.E., 1987. Structure of cutinase gene, cDNA and the derived amino acid sequence from phytopathogenic fungi. Biochemistry 26, 7883–7892.

Filée, J., Siguier, P., Chandler, M., 2006. I am what I eat and I eat what I am: acquisition of bacterial genes by giant viruses. Trends Genet. 23, 10–15.

Frickey, T., Lupas, A.N., 2004. PhyloGenie: automated phylome generation and analysis. Nucleic Acids Res. 32, 5231–5238.

Friesen, T.L., et al., 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. Nat. Genet. 38, 953–936.

Gamieldien, J., Ptitsyn, A., Hide, W., 2002. Eukaryotic genes in *Mycobacterium tuberculosis* could have a role in pathogenesis and immunomodulation. Trends Genet. 18, 5–8.

Gilbert, R.D., Johnson, A.M., Dean, R.A., 1996. Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. Physiol. Mol. Plant Pathol. 48, 335–346.

Gogarten, J.P., 2003. Gene transfer: gene swapping craze reaches eukaryotes. Curr. Biol. 13, R53–R54.

Gotesson, A., Marshall, J.S., Jones, D.A., Hardham, A.R., 2002. Characterization and evolutionary analysis of a large polygalacturonase gene family in the oomycete plant pathogen *Phytophthora cinnamomi*. Mol. Plant Microbe Interact. 15, 907–921.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hall, C., Brachat, S., Dietrich, F.S., 2005. Contribution of horizontal gene transfer to the evolution of *Saccharomyces cerevisiae*. Eukaryot. Cell 4, 1102–1115.

Hegde, Y., Kolattukudy, P.E., 1997. Cuticular waxes relieve self-inhibition of germination and appressorium formation by the conidia of *Magnaporthe grisea*. Physiol. Mol. Plant Pathol. 51, 75–84.

Huang, J., Mullapudi, N., Lancto, C.A., Scott, M., Abrahamsen, M.S., Kissinger, J.C., 2004. Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in *Cryptosporidium parvum*. Genome Biol. 5, R88.

Keane, T.M., Creevey, C.J., Pentony, M.M., Naughton, T.J., McLnerney, J.O., 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 6, 29.

Kinsella, R.J., McInerney, J.O., 2003. Eukaryotic genes in *Mycobacterium tuberculosis*? Possible alternative explanations. Trends Genet. 19, 687–689.

Kolattukudy, P.E., Sollday, C.L., Woloshuk, C.P., Crawford, M., 1985. Molecular biology of the early events in the fungal penetration into plants. In: Timberlake, W.E. (Ed.), Molecular Genetics of Filamentous Fungi. Alan R. Liss, Inc., New York, pp. 421–438.

Köller, W., Parker, D.M., 1989. Purification and characterisation of cutinase from *Venturia inequalis*. Phytopathology 79, 278–283.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5, 150–163.

Lawrence, J.G., 2005. Common themes in the genome strategies of pathogens. Curr. Opin. Genet. Dev. 15, 584–588.

Li, D., Ashby, A.M., Johnstone, K., 2003. Molecular evidence that the extracellular cutinase Pbc1 is required for pathogenicity of *Pyrenopeziza brassicae* on oilseed rape. Mol. Plant-microb. Interact. 16, 545–552.

Lin, T.S., Kolattukudy, P.E., 1978. Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani f. sp. pisi.* J. Bacteriol. 133, 942–951.

Loftus, B., et al., 2005. The genome of the protist parasite *Entamoeba histolytica*. Nature 433, 865–868.

Maiti, I.B., Kolattukudy, P.E., 1979. Prevention of fungal infection of plants by specific inhibition of cutinase. Science 205, 507–508.

Man in't Veld, W.A., de Cock, A.W.A.M., Ilieva, E., Lévesque, C.A., 2002. Gene flow analysis of *Phytophthora porri* reveals a new species: *Phytophthora brassicae* sp. nov. Eur. J. Plant Pathol. 108, 51–62.

Martinez, C., Nicolas, A., van Tilbeurgh, H., Egloff, M.P., Cudrey, C., Verger, R., Cambillau, C., 1994. Cutinase, a lipolytic enzyme with a preformed oxyanion hole. Biochemistry 33, 83–89.

Miller, C.D., Hall, K., Liang, Y.N., Nieman, K., Sorensen, D., Issa, B., Anderson, A.J., Sims, R.C., 2004. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading Mycobacterium isolates from soil. Microb. Ecol. 48, 230–238.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Ricard, G., et al., 2006. Horizontal gene transfer from bacteria to rumen ciliates indicates adaptation to their anaerobic carbohydrates rich environment. BMC Genomics 7, 22.

Richards, T.A., Dacks, J.B., Jenkinson, J.M., Thornton, C.R., Talbot, N.J., 2006. Evolution of filamentous plant pathogens: gene exchange across eukaryotic kingdoms. Curr. Biol. 16, 1857–1864.

Rizzo, D.M., Garbelotto, M., Hansen, E.M., 2005. *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. Annu. Rev. Phytopathol. 43, 309–335.

Roetschi, A., Si-Ammour, A., Belbahri, L., Mauch, F., Mauch-Mani, B., 2001. Characterization of an *Arabidopsis-Phytophthora* pathosystem: resistance requires a functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J. 28, 293–305.

Sanders, I.R., 2006. Rapid disease emergence through horizontal gene transfer between eukaryotes. Trends Ecol. Evol. 21, 656–658.

Shaykh, M., Soliday, C., Kolattukudy, P.E., 1977. Proof for the production of cutinase by *Fusarium solani* f. sp. Pisi during penetration into its host, *Pisum sativum*. Plant Physiol. 60, 170–177.

Simpson, A.G.B., Roger, A.J., 2004. The real 'kingdoms' of eukaryotes. Curr. Biol. 14, R693–R696.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.