Unraveling the Senses of *Phytophthora*; Leads to Novel Control Strategies?

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Abstract

Oomycetes cause devastating diseases on plants and animals. They cause major yield losses in many crop plants and their control heavily depends on agrochemicals. This is certainly true for the potato late blight pathogen *Phytophthora infestans*. Strong concerns about adverse effects of agrochemicals on food safety and environment are incentives for the development of novel, environmental friendly control strategies preferably based on natural products. Cyclic lipopeptides (CLPs) were recently discovered as a new class of natural compounds with strong activities against oomycetes including *Phytophthora*. CLPs lyse zoospores, inhibit mycelial growth and effectively reduce late blight disease. In order to unravel how *Phytophthora* senses CLPs and other environmental signals we follow two approaches. On the one hand, we monitor genome wide changes in gene expression induced by CLPs with the aim to identify the cellular pathways targeted by CLPs. On the other hand, we analyse components of ubiquitous signal transduction pathways with the aim to identify features that are unique for *Phytophthora* or oomycetes and, hence, could be suitable targets for novel anti-oomycete agents. Mining and comparing whole genome sequences have revealed that *Phytophthora* harbours many novel phospholipid modifying enzymes, unique for oomycetes. They have aberrant combinations of catalytic and regulatory domains occasionally combined with transmembrane domains. The latter resemble receptors that might be activated by extracellular ligands. Phospholipids, the substrates of these enzymes, are structural membrane components that also function in signalling. Together these findings open new avenues of research aimed at target-discovery in oomycetes.

PLANT PATHOGENIC OOMYCETES

Plant diseases cause severe crop losses and make agriculture highly dependent on adequate disease control. The most devastating plant pathogens are fungi and oomycetes (Agrios, 2005). They are look-a-likes that grow as mycelium and propagate via spores but evolved independently (Latijnhouwers et al., 2003). Fungi, together with animals, amoebozoa and dictyostelids are classified in the supergroup Unikonts (Latijnhouwers et al., 2003; Keeling et al., 2005). Oomycetes are Stramenopiles and belong to the supergroup Chromalveolates that also comprises brown algae, diatoms and apicomplexa (a.o., human pathogenic *Plasmodium*). The majority of the oomycetes are aggressive plant pathogens although some cause diseases in other higher eukaryotes including insects, fish, mammals and man. Among the plant pathogens are over eighty *Phytophthora* species, each having specific hosts (Erwin and Ribeiro, 1996), many *Pythium* species, and several obligate biotrophs, such as downy mildews and white rusts (Agrios, 2005). They infect numerous crops, ornamentals and native plants and have an enormous impact on agriculture and society. Well-known are *Plasmopara viticola* that causes downy mildew on grape, and *Phytophthora infestans,* the causal agent of late blight in potato and tomato and renowned for the Irish potato famine in 1843-45 (Erwin and Ribeiro, 1996; Govers and Latijnhouwers, 2004).

DISEASE CONTROL

It is estimated that potato late blight causes annual losses of over € 3 billion worldwide. Control mainly relies on fungicides that contain copper-, tin-, phenylamide- or cyanocetamide-oximes as active ingredients. These fungicides can prevent infection but have no curative activity. Therefore the interval between sprayings is usually short (5-7 days) and, hence, the burden on the environment is high. The term fungicide is used for a biocide that kills or reduces growth of fungi. Because oomycetes were traditionally classified in the kingdom Fungi, compounds that are used to control *Phytophthora* diseases are generally referred to as fungicides. However, fungi and oomycetes have little in common and their physiology, biochemistry and genetics differs considerably. Hence, chemicals with biocide activity on fungi are not necessarily suitable to control oomycetes (Russell, 2005). For example, SBIs (Sterol Biosynthesis Inhibitors), which represent an important class of fungicides, are not effective on oomycetes. *Phytophthora* is a sterol auxotroph and lacks genes encoding enzymes necessary for sterol biosynthesis (Govers and Gijzen, 2006). Also fungicides that target chitin biosynthesis have no effect. Cell walls of oomycetes are almost exclusively composed of glucan and cellulose with only very low levels of chitin (Bartnicki-Garcia and Wang, 1983; Meijer et al., 2006). Several compounds active against oomycetes rely on the phenylamide metalaxyl which inhibits RNA polymerase-I. However, shortly after the introduction of this oomicide the first metalaxyl-resistant *P. infestans* strains appeared and these are now widely distributed in field populations (Sukul and Spiteller, 2000). Taking into consideration that many important plant diseases are caused by oomycetes, there is a high demand for novel agents that specifically target oomycetes.

CYCLIC LIPOPEPTIDES

In recent years, the destructive effect of cyclic lipopeptides (CLPs) on oomycete plant pathogens has attracted considerable attention (Raaijmakers et al., 2006; De Bruijn et al., 2007). CLPs are produced by a variety of bacterial genera, including *Bacillus* and *Pseudomonas* species. Many CLPs possess growth-inhibitory activities against a range of plant and human pathogens, including viruses, mycoplasmas, trypanosomes, gram-positive bacteria, fungi and oomycetes (Finking and Marahiel, 2004; Nybroe and Sørensen, 2004; Raaijmakers et al., 2006). They are composed of a fatty acid tail linked to a short cyclic oligopeptide (Fig. 1). One of the main modes of action of CLPs described so far, involves the formation of ion channels in the plasma membrane of the target organism leading to cytolysis (Rainey et al., 1991; Hutchison and Gross, 1997; Dalla Serra et al., 1999). CLPs can be chemically produced and via structural or genetic modifications their physicochemical properties and antimicrobial activities can be altered (Baltz et al., 2005).

The Role of CLPs in Late Blight Control

Phytophthora species produce biflagellate zoospores that lack a cell wall and hence their membranes are directly exposed to the environment. Several CLPs, including massetolide A and viscosin, have zoosporicidal activities and eliminate entire zoospore populations of *P. infestans* and other oomycetes within minutes (De Souza et al., 2003; De Bruijn et al., 2007) (Fig. 2). Interestingly, CLPs also inhibit the growth of *P. infestans* in planta. In a recent study (Tran et al., 2007), we found that *Pseudomonas fluorescens* SS101, the strain that produces the CLP massetolide A, not only prevented infection of tomato leaves by *P. infestans*, but also significantly reduced expansion of existing late blight infections and sporangia formation. Given that sporangia constitute an important primary and secondary inoculum source for *P. infestans*, the adverse effects of *P. fluorescens* strain SS101 on both lesion area and sporangia formation may lead to a reduction in the epidemic progress of late blight disease of tomato. The evidence that massetolide A is an important component of the biocontrol activity of *P. fluorescens* strain SS101 against late blight of tomato was based on the observations that a massetolide A-deficient mutant was significantly less effective in biocontrol than the

wild-type strain SS101, and that application of purified massetolide A to tomato leaves and roots provided significant control of *P. infestans* (Fig. 3). The zoosporicidal activity of massetolide A, produced by *P. fluorescens* strain SS101, may explain, at least in part, the direct protection of tomato leaves against infection by zoospores of *P. infestans*. Additional experiments further indicated that strain SS101 and massetolide A also induced systemic resistance in tomato against late blight. These results showed, for the first time, that the CLP massetolide A is a bacterial determinant of induced systemic resistance (ISR) in tomato by an antagonistic *P. fluorescens* strain. In many cases, signal transduction in rhizobacteria-mediated ISR has been shown to be independent of salicylic acid (SA) and dependent on ethylene (ET) and jasmonic acid (JA) (Pieterse et al., 1998; Ton et al., 2001; Yan et al., 2002). Infection assays on *nahG* tomato plants, the transgenic derivative of cultivar 'Moneymaker' that lacks SA, suggest that also the systemic resistance induced by *P. fluorescens* SS101 or massetolide A is independent of SA signalling.

CLP-Induced Responses in *Phytophthora*

The CLP massetolide A inhibits mycelial growth of *P. infestans*, causes increased branching and hyphal swellings, reduces sporangia formation and germination of encysted zoospores, and adversely affects auto-aggregation of swimming zoospores at concentrations well below the critical micelle concentration (Tran et al., 2007). Auto-aggregation of zoospores of a *P. infestans* transformant, that constitutively produces an active α subunit of the heterotrimeric G-protein was less affected by massetolide A (Tran, 2007) suggesting that G-protein signalling plays a role in the perception of CLPs by *P. infestans* (Latijnhouwers et al., 2004; Tran, 2007). Also in mammalian cells, CLPs can interfere with canonical signalling pathways by inhibiting the activity of a $G\alpha_{q/11}$ subunit (Taniguchi et al., 2004) or a specific subclass of phospholipase C (Ui et al., 1997). In yeast, CLP treatment was found to affect phospholipid levels (Cliften et al., 1996).

Because of their zoosporicidal activity and their growth-inhibitory effects, CLPs are promising as a substitute or supplement in the protection of crops against oomycete pathogens (Raaijmakers et al., 2006). However, the molecular responses of oomycetes to treatment with CLPs are not characterized. To analyse the effects of CLPs on gene expression in *P. infestans*, a pilot experiment was performed with a limited edition of an Affymetrix® GeneChip containing over 15,000 *P. infestans* unigenes (Judelson et al., 2008). Preliminary data showed that approximately 300 genes in *P. infestans* had a higher expression after exposure to the CLP massetolide A. At least twelve of these encode membrane transporters, including MFS- and ABC-transporters, which may act as a first line of defence against the deleterious effects of CLPs.

PHOSPHOLIPID SIGNALLING AND METABOLISM

Phospholipids are structural components of cell membranes and act as second messengers in a variety of signal transduction networks. In eukaryotes, signalling cascades are often initiated when membrane bound G-protein coupled receptors (GPCRs) are triggered by extracellular stimuli. Upon ligand binding, GPCRs feed into downstream pathways via G-proteins, thereby activating or repressing various components, including phospholipid modifying enzymes. The finding that treatment of zoospores of *P. infestans* and *Phytophthora palmivora* with phosphatidic acid (PA) or the G-protein activator mastoparan results in encystment suggests that G-protein and phospholipid signalling play a key role in zoospore differentiation in these organisms (Latijnhouwers et al., 2002; Zhang et al., 1992).

Phytophthora Has Several Novel PLDs but Lacks PLC

The production of PA is catalyzed by phospholipase D (PLD). Mastoparan is known to activate PLD (Latijnhouwers et al., 2002) and therefore PLD could be an important signalling component in *Phytophthora*. Until recently, two major classes of PLDs were known: PXPH-PLDs that are ubiquitous in eukaryotes, and C2-PLDs that are

only found in plants (Eliáš et al., 2002; Meijer and Munnik, 2003). In recent years we mined the genome sequences of three *Phytophthora* species (*P. sojae*, *P. ramorum* and *P. infestans*) and one downy mildew (*Hyaloperonospora parasitica*) and found an astonishing wide repertoire of novel phospholipid modifying enzymes proteins that are conserved in *Phytophthora* and *Hyaloperonospora*, but absent in other eukaryotes (Table 1). They often have a characteristic catalytic domain combined with a variety of structural or regulatory domains (Bakthavatsalam et al., 2006; Meijer and Govers, 2006; Tyler et al., 2006; H.M.J. Meijer and F. Govers, unpublished). A striking feature was the absence of a gene encoding a canonical phospholipase C (PLC), a universal signalling component that is conserved throughout the eukaryotic kingdom (Meijer and Munnik, 2003). Similar to PLD, PLC is one of the enzymes involved in the production of PA.

In *Phytophthora* the absence of PLC seems to be compensated by the presence of a variety of different PLDs. *Phytophthora* has a conventional PXPH-PLD but, in addition, it has two PLDs (PXTM-PLD and TM-PLD, respectively) in which the catalytic PLD domain is preceded by transmembrane domains and a novel family of small PLDs, the majority of which has a secretion signal (sPLD-likes) (Fig.4) (Meijer and Govers, 2006). The receptor-like structure of PXTM-PLD and TM-PLD suggests that these proteins can perceive and transmit extracellular signals, resulting directly in the activation of PLD. In collaboration with the laboratory of Kurt Lamour (University of Tennessee) we obtained a TILLING mutant of *P. sojae* that has a premature stop codon in the *PsPXTM-PLD* gene and, hence, lacks a functional PXTM-PLD protein (Lamour et al., 2006). This mutant has a severely reduced growth rate and aberrant colony morphology (Fig. 5) and, apparently, PXTM-PLD plays a role in growth and development. Such a *Phytophthora* specific enzyme could be a suitable drug target. The existence of secreted PLDs suggests that *Phytophthora* secretes PLDs that could play a role in pathogenesis. We recently demonstrated that PLD activity is present in the exudate of *P. infestans* when it is cultured in liquid medium. We are currently characterizing the biochemical features of these secreted PLDs and their putative function in the interaction with host plants.

Novel Phosphatidylinositolphosphate Kinases with a GPCR Signature

Another peculiar class of novel proteins discovered by genome mining are the GPCR-PIPKs. These are phosphatidylinositolphosphate kinases (PIPKs) that possess a GPCR at the N-terminus. *Phytophthora* has twelve distinct GPCR-PIPKs genes (Bakthavatsalam et al., 2006; Meijer and Govers, 2006). The only other GPCR-PIPK gene described to date is *rpkA*, a single copy gene in *Dictyostelium* that is known to be involved in cell density signalling (Bakthavatsalam et al., 2007). The GPCR signature combined with a PIPK domain in one protein suggests that GPCR-PIPKs can perceive extracellular signals that result directly in the activation of PIPK. Subsequently, the PIPK activity leads to further downstream signalling (Meijer and Munnik, 2003). These findings nourish the thought that *Phytophthora* has developed sensing systems that can bypass heterotrimeric G-proteins. Heterotrimeric G-proteins are composed of three subunits, α, β and γ. Most eukaryotes have multiple Gα subunits and multiple Gβ subunits, each with their own specific activity or tissue specificity. In contrast, *Phytophthora* species have only one α subunit and one β subunit gene, suggesting that either the same heterotrimeric G protein is active all over, in every developmental stage or cell type, or that certain stages or cell types are devoid of a heterotrimeric G protein. The latter seems to be the case. In *P. infestans* the Gα and Gβ subunit genes (*Pigpa1* and *Pigpb1*, respectively) show differential expression with the highest mRNA levels in sporangia. In mycelium expression is low (for *Pigpb1*) or below detection level (for *Pigpa1*) (Laxalt et al., 2002). Silencing of the *Pigpa1* gene in *P. infestans* led to hypovirulence and severely affected the swimming behaviour of zoospores but mycelium growth, sporangia formation and oospore formation were not disturbed (Latijnhouwers et al., 2004). Silencing of *Pigpb1* resulted in transformants that did not sporulate. The mycelium grew more dense but growth rate was not reduced (Latijnhouwers and Govers, 2003). Taken together these findings not only show that ubiquitous signalling pathways

via heterotrimeric G-proteins are essential for the disease cycle of *Phytophthora*, but also emphasize the importance of alternative signalling pathways. In this respect, pathways involving the family of novel oomycete specific GPCR-PIPKs might be important (Bakthavatsalam et al., 2006). Future research aims at elucidating the PIPK activity of the GPRC-PIPKs and identifying their ligands. The PIPK-GPCRs are interesting drug targets, especially when considering that the majority of pharmaceutical drugs have GPCRs as target.

CONCLUSIONS

Current practices used to reduce yield losses caused by *Phytophthora* diseases primarily rely on chemical crop protection. The appearance of more aggressive isolates, and isolates that are no longer inhibited by chemical protectants, have led to an increased demand for novel control strategies. Moreover, the increase of public concern about adverse effects of agrochemicals on food safety and environment stimulate the search for control strategies that are more durable and preferably based on natural products. CLPs are natural products. They are produced by bacteria that exhibit biocontrol activity and have destructive effects on oomycetes. Their zoosporicidal activity and involvement in induced systemic resistance in plants are attractive properties and their potential as oomicide should be explored further. The many novel proteins that are discovered in oomycetes by genome mining and comparative genomics offer another opening towards alternative control strategies. The occurrence of enzymes with aberrant combinations of catalytic and regulatory domains suggests that oomycetes possess unique circuits in metabolic or signalling pathways. Supporting this suggestion are the novel types of receptors that may function in monitoring and transmitting extracellular stimuli. Pinpointing targets in these unique circuits or receptors, and identifying compounds that can disrupt these targets or mimic the natural ligands of the unique receptors, is yet another promising strategy that may result in novel oomicides.

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Tables

Table 1. Number of *Phytophthora* genes encoding known or novel phospholipid modifying enzymes.

Enzyme	known/novel	Remarks
Phosphatidylinositol synthase	1 / -	
Phosphatidylinositol kinase	10 / 13	novel domain structures
Phosphatidylinositolphosphate kinase	2 / 14	12 are GPCR-PIPKs
Phospholipase C	0 / 0	
Diacylglycerol kinase	1 / -	
Phospholipase D	1 / 17	12 have a signal peptide and 2 resemble transmembrane receptors

Figures

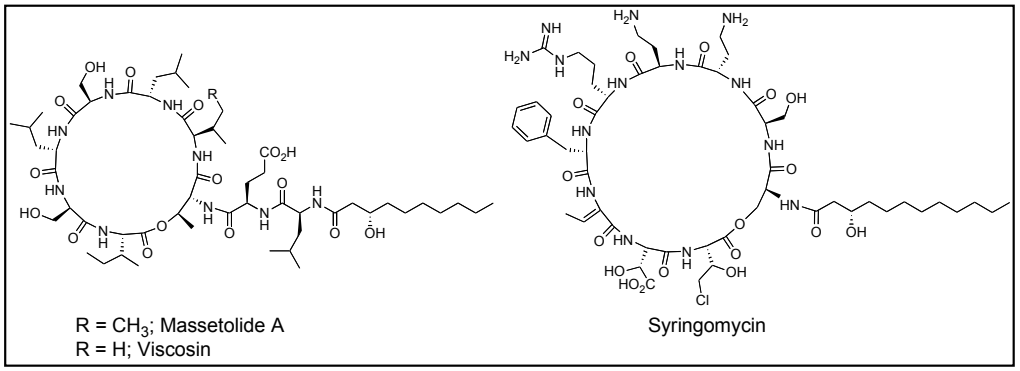

Fig. 1. Structures of the CLPs massetolide A, viscosin and syringomycin (drawings made by Dr. J. van Maarseveen, University of Amsterdam, Netherlands).

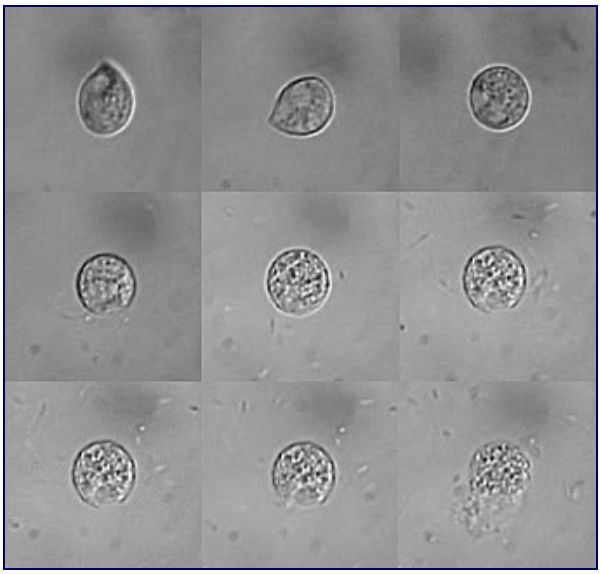

Fig. 2. Time-lapse video microscopy of the zoosporicidal activity of cyclic lipopeptides (CLPs). *P. infestans* zoospores explode (from top left to bottom right) within one minute after exposure to the CLP massetolide A (Source: De Bruijn et al., 2007).

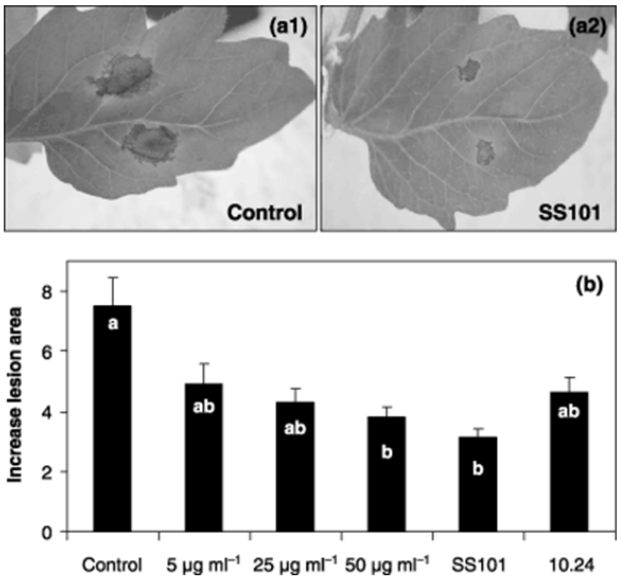

Fig. 3. Effect of *P. fluorescens* SS101 on the growth of existing late blight lesions. Tomato leaves were first inoculated with *Phytophthora infestans* zoospores. After the initial late blight lesions were formed, lesion areas were measured and leaves were subsequently treated with cell suspensions of *P. fluorescens* SS101, its massetolide A-deficient mutant 10.24 or with different concentrations of partially purified massetolide A. Five days later, lesion sizes were determined again and the increase in lesion area was calculated. (a2) Typical effect of *P. fluorescens* SS101 on the growth of existing lesions compared to the non-treated control (a1); (b) effect of SS101, 10.24 and different concentrations of massetolide A on the increase in lesion size. The means of 6 replicates are shown and error bars represent the standard error of the mean. Means with a different letter are significantly different ($p<0.05$) (Source: Tran et al., 2007).

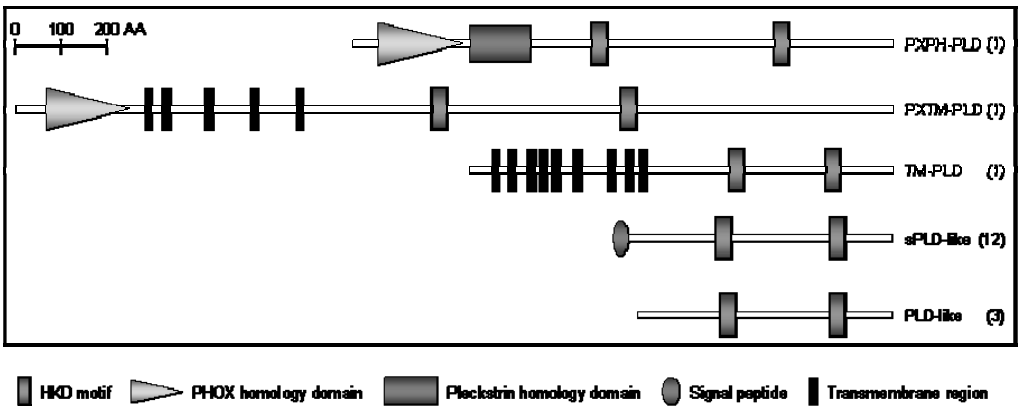

Fig. 4. The five phospholipase D (PLDs) subfamilies in *Phytophthora*. The duplicated HKD motifs represent the catalytic PLD domain. In brackets is the number of genes in each species. The size bar represents 200 amino acids. With more than one member per family the size shown is an average (Source: Meijer and Govers, 2006).

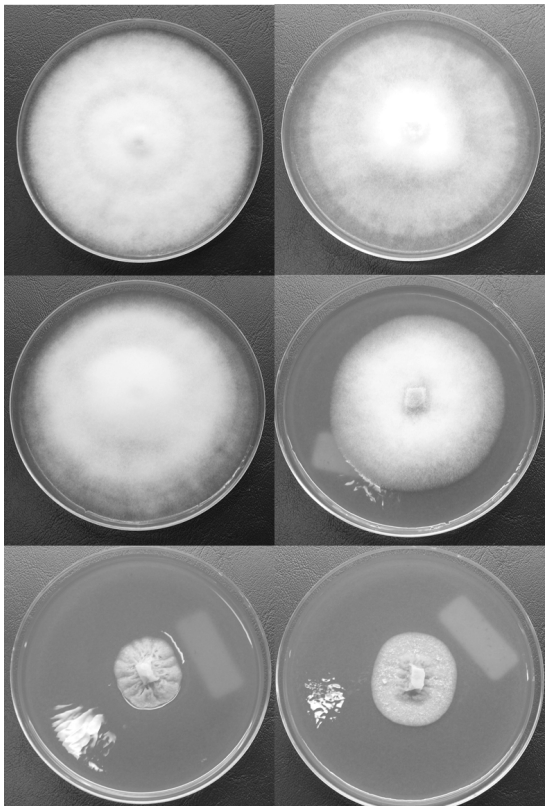

Fig. 5. Colony morphology of the wild-type *Phytophthora sojae* isolate P6497, and representatives of oospore progeny derived from the heterozygous PsPXTM-PLD TILLING mutant K2870 after 9 days of growth in the dark on full-strength V8 agar. The TILLING mutant has a premature stop codon in the *PsPXTM-PLD* gene resulting in a truncated protein that lacks the second HKD motif (see Fig. 4). Top left: P6497, wild type. Top right: LT2489, heterozygous mutant progeny (Wt/mt). Middle left and right: LT2938 and LT2662, homozygous wild-type progeny (Wt/Wt). Bottom left and right: LT2506 and LT2501, homozygous mutant progeny (mt/mt). (Source: Lamour et al., 2006).