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RNAseq and Proteomics for Analysing Complex Oomycete Plant Interactions

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Abstract
The oomycetes include some of the most devastating plant pathogens. In this review we discuss the latest results from oomycete and plant studies with emphasis on interaction studies. We focus on the outcomes of RNAseq and proteomics studies and some pitfalls of these approaches. Both pathogenic interactions and biological control are discussed. We underline the usefulness of studies at several levels of complexity from studies of one organism, up to two or more and within agricultural fields (managed settings) up to wild ecosystems. Finally we identify areas of future interest such as detailed interactome studies, dual RNAseq studies, peptide modification studies and population/meta omics with or without biological control agents.

Introduction
The recent flurry of technological advancements by next-generation sequencing (NGS) and mass spectrometry (MS) have enabled genome-scale capturing of biological processes at the molecular level. Rather than being limited to a handful of measured compounds, it is now possible to capture thousands of molecules in a single study. However, these techniques have mostly been applied in the laboratory and during controlled growing conditions. The generation of such large datasets—often referred to as "-omics" data—depends partly on new pipelines for experimental set-ups, sampling, data analysis and visualization. The integration of different types of data generated in such experiments and correct biological interpretation of large datasets remain major challenges. Despite these drawbacks, the power of omics based analysis lies in its ability to facilitate a broader understanding of biological systems.

The oomycetes are a lineage of filamentous Eukaryotes that resemble fungi in their hyphal growth and heterotrophic absorptive nutrition, but are in fact more closely related to the heterokont (brown) algae and are therefore members of the Kingdom Stramenophila. Members of this group may be saprotrophic or pathogenic on a wide range of hosts, including plants, algae and animals. The most notorious and well-studied, are the plant pathogens of the genus *Phytophthora,* and include the potato late blight pathogen, *Phytophthora infestans* (responsible for the Irish potato famine in the 1840s and still a major pesticide target) and the agent of sudden oak death, *Phytophthora ramorum* responsible for global losses in forests and in horticulture. The oomycetes exhibit a high level of diversity in the nature of their pathogenic relationships. At one end of the scale, are the biotrophs, such as the downy mildews, which typically only infect a single host plant species and obtain nutrients from living cells. Members of the Phytophthora genus may have broader host ranges, for example *Phytophthora cinnamomi* can infect more than 1000 species of tree, whereas *P. infestans* is restricted to Solanaceae primarily infecting potato, tomato and tobacco plants, and infecting a wide range of organs ranging from leaves, stems to tubers. Most species of Pythium also have a broad host range; some are destructive necrotrophs such as *Pythium ultimum*, which destroy plant tissue to obtain nutrients, whilst others, e.g. *Pythium oligandrum* are mycoparasitic against a wide range of fungi and oomycetes and may have potential to be used as biological control agents (BCAs) against numerous plant diseases. Phytophthora species colonise plants in a hemibiotrophic manner, entering and evading defence responses in a similar manner to biotrophic pathogens, but later shifting to necrotrophic growth at the end of the disease cycle.

The plant immune system is complex and includes functions like specificity (specific targeting of pathogen molecules), memory (memory of prior attacks) and self-tolerance (ability to distinguish self from non-self) similar to the innate immune system of animals (Spoel and Dong, 2012). All these functions are mediated by multiple molecules, therefore rendering this system ideal for omics based investigations. The plant immune system is unique in its bi-layered organization. The first layer termed as PTI (PAMP triggered immunity) involves defense to surface-exposed conserved pathogen associated molecules termed PAMPs (Pathogen associated molecular patterns). This induces rapid responses including a Ca^{2+} burst and MAP kinase activation that in turn regulates transcription factor activity, affecting the expression of genes involved in defense compound production (Bigeard et al., 2015). In response to this, pathogens have evolved a way to circumvent PTI responses via production of specialized molecules called effectors. To counter these effectors, plants produce molecules called resistance proteins (coded by R genes) that can specifically recognize these effectors; this forms the second layer of plant immunity termed effector triggered immunity (ETI). ETI responses generally lead to a specialized form of programmed cell death (PCD) called the hypersensitive response (HR). The observation of a tussle at the molecular level between the plant and the pathogen led to the development of the "Zig-Zag" model of plant immunity (Jones and Dangl, 2006). However, this simplistic bi-layered view is being challenged lately by

discoveries that point towards a continuum wherein each layer feeds into the other (Thomma et al., 2011). Observations of the overwhelming effects of other variables (e.g. the presence of multiple pathogens with varying infection strategies) in this interaction has led to the call for additional models to better describe plant-pathogen interactions quantitatively and over correct time scales (Pritchard and Birch, 2014). Recent discoveries have also identified that various pathogens have evolved effectors that hijack a suite of "master-regulators" in the host allowing the pathogen the opportunity to perturb numerous other functions (Weßling et al., 2014). Similarly plants have also evolved immune signaling networks that can efficiently fine tune responses depending on the pathogen, or effector presented (Kim et al., 2014), indicating the highly complex and dynamic interplay between pathogen(s) and host plants.

This interplay is very well exemplified by oomycete-host plant patho-systems. For example, Steinbrenner et al. (2015) show data that indicate that two resistance protein domains from AtRPP1, are involved in direct interaction with *Hyaloperenospora arabidopsidis* effector ATR1; one to associate with the effector, and the other to sensitize the receptor and facilitate activation. This is in contrast to the interaction between P. *infestans* effector Avr2 and host resistance protein R2 wherein recognition of Avr2 by R2 is indirect, mediated via another host protein BSL1 (Saunders et al., 2012). The observed "plasticity" in several oomycete genomes (Raffaele and Kamoun, 2012) that enables them to quickly overcome host resistance mechanisms and further counter adaptation by host plants continuously drives this race for superiority between oomycete pathogens and their hosts.

In recent years, omics based investigations on oomycete-host plant pathosystems have been largely driven by genomics analysis (Dong et al., 2015; Judelson, 2012). A concerted effort by the research community has resulted in the genomes of more than sixteen different oomycetes, with a major focus on plant pathogens that exhibit biotrophic, hemibiotrophic or necrotrophic lifestyles on a variety of host plants. These genomics approaches have led to the identification of many large virulence gene families that have been predicted to encode numerous hydrolytic enzymes for the degradation of host carbohydrates, extracellular toxins (e.g. NLP toxins) and at least three families of effector proteins that enter host cells, the RXLR effectors, CHXC effectors and Crinkler (CRN) proteins (Bozkurt et al., 2012; Fawke et al., 2015).Comparative genomic analysis of pathogens with diverse host ranges, reveal striking differences in pathogenicity mechanisms. E.g. the broad host range nectrophic plant pathogen *Pythium ultimum* contains expanded families of enzymes responsible for degradation of easily accessible plant carbohydrates such as pectins, starch and sucrose and an apparent absence of RXLR effectors (Lévesque et al., 2010). On the contrary, the obligate biotrophic downy mildew *Hyaloperonospora arabidopsidis* contains reduced numbers of genes encoding such hydrolytic enzymes (Baxter et al., 2010), whilst a loss of heterozygosity may have driven the rapid adaptation of *Phytophthora capsici* to new hosts and a swift ability to overcome fungicides (Lamour et al., 2012).

With regards to plants, genome sequences of only two, namely Arabidopsis and Rice were complete and available between the years 2000 and 2006. However in the last 7 years, the number of plant genomes sequenced has rapidly increased (Bolger et al., 2014). The majority of economically important plants (including several "true hosts" of oomycetes) that have been sequenced are polyploid and have large genomes. Earlier sequencing methods were therefore unable to sequence these complex genomes. New developments in sequencing methodologies and reduced costs have enabled sequencing of these genomes (Bolger et al., 2014). However for some plants, such as potato, the heterozygosity and tetraploid nature of the genome still makes it difficult to sequence. Therefore to circumvent this problem in potato a doubled monoploid was sequenced (Consortium, 2011). Further characterization of polyploid genomes is complicated. For example, resistance gene (R gene) analysis in the genomes of tomato and potato revealed that most R genes were present as tandem duplications (Andolfo et al., 2013; Jupe et al., 2012) and many of them seem to be non-functional (Mace et al., 2014) ; Lenman et al., 2015). Therefore just through *in silico* identification of the gene it becomes difficult to predict its functionality, since information regarding, for example, which copy of the R gene is expressed and activated is required, just as it is for pathogen-derived effectors.

Incorporation of RNAseq and proteomic methodologies are facilitating improved analysis of gene functions in economically important plants and both plant pathogenic and mycoparasitic oomycetes. Some progress towards this end has been made by linking proteomics data with gene information in the model plant Arabidopsis (Usadel et al., 2012). We foresee integration of RNAseq and proteomic data sources into existing comparative analysis platforms such as "Phytozome" (http://phytozome.jgi.doe.gov/pz/portal.html) and "PLAZA" (http://plaza.psb.ugent.be/). This will enable seamless knowledge transfer between model and true host plants and will drive future research associated with understanding the role of genome complexity in pathogen responses.

Insights into oomycete effector biology using RNAseq

Sanger sequencing of ESTs and differential expression analysis using custom made Affymetrix GeneChips gave early clues as to the dynamics of pre-infection development and effector gene expression in *P. infestans* (Judelson et al., 2008; Randall et al., 2005). In the post-genomics era of oomycete research, and with possibilities for large-scale deep sequencing of transcriptomes, it is now possible to document the dynamics of *P. infestans* development throughout the disease cycle, from biotrophy to necrotrophy. One such recent study showed that an induction of apoplastic and cytoplasmic effectors and a tight temporal regulation of pathways involved in suppression of host defence responses characterized the biotrophic phase of infection (Zuluaga et al., 2015a). Four effectors that suppress necrosis were identified and such

proteins may serve to prolong the biotrophic stage. 818 genes that had not been previously reported in the *P. infestans* genome were also identified. Such "orphan genes" may represent errors in annotation, non-coding RNAs, expressed pseudogenes or alternate splice variants (Zuluaga et al., 2015a), and thereby illustrate the power of deep sequencing approaches using RNAseq technology.

Next-generation sequencing and RNAseq techniques have been, and continue to be, very useful for the study of the infection biology of recalcitrant oomycetes such as the downy mildews, which as obligate biotrophs are hard to work with in a laboratory setting. Large-scale EST sequencing of the downy mildew pathogen *Hyaloperonospora arabidopsidis* during infection of Arabidopsis revealed the presence of isolate-specific effectors (Cabral et al., 2011). Recently, the Jones laboratory has developed a high-throughput mRNA expression profiling method (Expression Profiling through Random Sheared cDNA tag Sequencing [EXPRSS]) which detects the differential expression of more genes with a higher sensitivity than microarray or traditional RNAseq methods (Rallapalli et al., 2014). Expression analysis of both host and pathogen using this method has identified that *H. arabidopsidis* suppresses responsiveness to salicylic acid (SA) in host cells containing pathogen haustoria through which host-translocated effectors are delivered. An *H. arabidopsidis* RXLR effector, HaRxL62, which enhances host susceptibility was highly expressed during this assay and was shown to suppress host responsiveness to SA (Asai et al., 2014).

Massive parallel transcriptome sequencing of lettuce infected with the downy mildew *Bremia lactucae* revealed expression of approximately 78 effectors with RXLR-like features, as well as elicitins, necrosis inducing peptides, glucanase inhibitors and lectins and an enrichment of cysteine-rich proteins (Stassen et al., 2012). RXLR and CRN-type effectors were also identified in a sunflower (*Helianthus annuus)* - downy mildew (*Plasmopara halstedii)* interaction trancriptome sequenced by 454 FLX pyrosequencing (As-Sadi et al., 2011). An extensive mRNAseq analysis of expression of pathogen genes over a time course of infection of cucumber (*cucumis stivus)* by the downy mildew *Pseudoperonospora cubensis* identified expression of RXLR-like effectors and other pathogenicity genes. Interestingly, comparison of these expression profiles with expression data from a timecourse of *P. infestans* infection of potato (*Solanum tuberosum)* revealed a substantial overlap in molecular events and gene expression in virulence of the biotrophic *Ps. cubensis* and the hemibiotrophic *P. infestans* (Savory et al., 2012). Similarly, Kunjeti et al. (2012) found that many of the transcripts identified from *Phytophthora phaseoli* infection of lima bean by RNAseq were homologous to known or putative virulence genes in *P. infestans.* Surprisingly, although RXLR and elicitin type effectors were upregulated during host plant infection, CRN effectors were not. *Phytophthora capsici* is a soilborne necrotroph with a broad host range. RNAseq of pre-infection spore development in this pathogen identified major differences between the expressed gene content of the mycelium, zoospores and germinating cysts, and a total of 98 predicted effector genes were identified, including members of the RXLR and CRN classes. Nineteen of these effector genes were shown to also be expressed during infection and were able to suppress host cell death (Chen et al., 2013). RXLR and CRN effectors are therefore expressed by a wide range of oomycete pathogens during colonization and infection of host plants, and may be ancient and conserved pathogenicity mechanisms in the oomycetes. However, pyrosequencing of the transcriptome of the interaction between the brown alga *Ectocarpus siliculosus* by the obligate biotroph and basal oomycete *Eurychasma dicksonii* failed to positively identify putative RXLR and CRN effector transcripts, suggesting either that they are only present in low abundance in this interaction or that they arose later on in the oomycete lineage after the oomycetes left their marine environment (Grenville-Briggs et al., 2011).

Small RNA (sRNA) studies on oomycete pathogens have demonstrated that some effectors are regulated by sRNAs. This regulation allows for the development of variation in pathogenicity and virulence toward plant resistance genes (Qutob et al., 2013; Vetukuri et al., 2012). Silencing of the *Phytophthora sojae*, effector *Avr3a*, is reported to be controlled by 25 nt *cis*-acting sRNAs, and the silencing signal appears to be heritable and confers virulence on plants carrying the Avr3a-recognizing *R* gene *Rps3a* (Qutob et al., 2013). Studies in both fungi and oomycetes have suggested the existence of bidirectional transport of sRNAs. For instance 21-22nt *Botrytis cinerea* sRNAs induced during the infection process target host genes in *Arabidopsis* and tomato by hijacking host sRNA machinery (Weiberg et al., 2013), whereas sRNAs from the potato targeting oomycete *P.infestans* are reported from a host induced gene silencing sRNA screen (Jahan et al., 2015), suggesting that RNA has a multifaceted role in host-pathogen interactions.

RNAseq for improved understanding of plant-oomycete interactions

Over the past few years, RNAseq technology has fostered the move from studying model plant patho-systems to true host-pathogen interactions. RNAseq has been rapidly outcompeting traditional microarrays because unlike microarrays, which are limited to the availability of sequenced genome/prior information, RNAseq is independent of the same (Wang et al., 2009). Since the procedure is not limited to sequenced genomes, the possibilities of identifying new components regulating plant-pathogen interactions are dramatically improved. Recently Zuluaga et al. (2015b) used 454 pyrosequencing to identify 94 000 different tomato transcripts that altered in response to *Phytophthora infestans* inoculation of leaves. Microarray analysis using the tomato genome (Jupe et al., 2013b) would not have detected the novel components such as alternative spliced transcripts or fusion transcripts that constitute the large number of transcripts found by Zuluaga et al. (2015b). Oomycetes like *P. infestans* cause disease in different tissues or organs; a strategy that uses specific RNAseq analyses can be helpful in elucidating novel components involved in tissue or organ specific oomycete

interactions. Similarly, RNAseq has also enabled researchers to perform detailed exploration of alternative splicing/miRNA processes involved in plant defense (Wang et al., 2010). For instance, RNAseq has been used to detect novel miRNAs involved in plant-oomycete interactions. Zhao et al. (2015) assayed miRNA profiles of 9 near isogenic lines (NILs) of soybean, each carrying a unique resistance gene (Rps; resistance to *Phytophthora sojae*) in a susceptible background, in response to *Phytophthora sojae* using RNAseq. The study identified 369 putative miRNAs to be differentially expressed in the NILs and the background, 63 of these could not be classified into known miRNA families and hence were regarded as unique to this patho-system. The study also found that miRNAs were repressed in the resistant NILs. RNAseq enabled transcriptomic analysis of the same material was also performed (Lin et al., 2014). Based on target identification analysis of miRNA and corresponding RNA data, the authors were able to show that general down regulation of miRNAs after pathogen inoculation results in the up regulation of corresponding defense genes leading to observed resistance. Alternative splicing is another facet of RNA mediated regulation of immunity that can be uncovered using RNAseq (Mandadi and Scholthof, 2015), although there has been no report on the use of RNAseq to specifically uncover host alternative splicing events in response to oomycetes, RNAseq has revealed evidence of alternative splicing in effector coding transcripts in the oomycete cucurbit pathogen *Pseudoperonospora cubensis* (Burkhardt et al., 2015).

Plant disease resistance is mediated by highly specific resistance genes (R genes); currently genome dependent map based cloning methods that are laborious and time consuming are used to identify these R genes. RNAseq has been used to circumvent these traditional approaches. For instance, Strauß et al. (2012) used an innovative RNAseq based approach wherein transcription activator-like effector (TALE) protein AvrBS4 was used as a probe to identify the corresponding *Bs4C* resistance gene in pepper. Analysis of oomycete genomes has revealed the presence of vast repertoires of effectors with probably different pathogenicity strategies, however there has been slow progress in identifying their targets in host. Therefore RNAseq combined with strategies such as effector screens (Du et al., 2015), that facilitate isolated response of novel oomycete effectors, could be used to identify R genes or effector targets involved in plant-oomycete interactions. With regards to R gene mapping, advancements to traditional methods through the use of high throughput genomic data are already underway, for example with the use of RenSeq it is possible to specifically enrich for R genes from genomic libraries as shown by Jupe et al. (2013a) in potato. Using this method, the authors were able to identify many more NB-LRR R genes in comparison to the published potato genome sequence. Although RenSeq is based on genomic libraries, the possibility to apply this technique to long-read based PAC-bio sequencing that reduces assembly issues as well as the use of expression datasets or exomes could simplify the identification of R genes involved in oomycete resistance.

Use of dual RNAseq methodology that allows for simultaneous quantification of gene expression in the host and the pathogen during the infection process can enable the discovery of multiple effectors produced by an oomycete during infection. Furthermore it may assist in the identification of candidates for corresponding host targets as negative correlation between repressed transcripts and effector transcript up regulation is expected. Simple microarrays from which much information about general defense responses in arabidopsis, potato and tomato to various biotic interactions have been gained (Ali et al., 2014; Lodha and Basak, 2012) are not particularly amenable for studies aimed towards effector target identification (Westermann et al., 2012). Some progress however has been made with the use of composite microarrays. In a recent study, custom-made composite microarrays were employed to study the *Phytophthora capsici* -tomato patho-system (Jupe et al., 2013b). Although it is relatively easy without a trained bioinformatician to analyze data obtained from microarrays, the use of microarrays in fact is not ideal if the focus is on identification of lowly expressed effector targets or elucidation of various other factors that enable transcriptional plasticity (e.g. miRNAs). Hayden et al. (2014) used a dual RNAseq approach to investigate the *Phytophthora ramorum- Notholithocarpus densiflorus* patho-system, and although they did find induction of effector proteins 5 days after inoculation (dai), they were unable to detect pathogen associated transcripts 1 dai, and the authors cite poor sequencing depth as a reason for this observation. Higher sequencing depth is an even more important pre-requisite in this regard as unlike other host-pathogen patho-systems, oomycete infections might be characterized by the low relative abundance of pathogen associated transcripts (Derevnina and Michelmore, 2015). Inclusion of crossing populations with differing resistance in the experimental set up and the use of dual RNAseq could help in elucidating novel facets of effector interactions with host targets in plant-oomycete interactions.

Although RNAseq offers the benefit of generating large amounts of data for analysis, the challenge has been to extract and generate knowledge from the data produced (Sboner et al., 2011). New developments like use of RNAseq in bulked segregant analysis (BSA) that facilitates fine mapping of genes is being done (Martin et al., 2013). Much needed focus has also been put onto improving experimental designs that can yield targeted results (Yang and Wei, 2015). Technical aspects that need redress are issues related to inherent biases in library construction and data analyses methods/storage. These have been reviewed widely (Kaufmann and Busch, 2013; Martin et al., 2013; McGettigan, 2013). There is also a need for simultaneous improvements in statistical tools, since the current tools for the analysis of RNAseq datasets have been shown to have lower power in identifying differentially regulated transcripts in comparison to statistical tools that analyze microarrays (Kaufmann and Busch, 2013). In the recent past, improvements have been made to traditional probe dependent methodologies, and some of these could assist in the development of standardized data analysis

methods for RNAseq data processing, analysis, integration, visualization and storage.

Proteomics of Oomycetes

Proteomics can be defined as the study of the whole protein complement of an organism, tissue, cell or subcellular compartment. The intracellular levels of mRNA and protein correlate, but with a broad range of variation (Schwanhausser et al., 2013; Schwanhäusser et al., 2011). In addition, proteins in an intracellular compartment may be synthesized in advance and transported there (e.g. secreted proteins). This makes it difficult to predict the level of a protein from mRNA expression data alone. Proteomics offers the opportunity to obtain direct information about protein abundance, as well as information about post translational modifications.

Early proteomic studies of oomycetes were conducted by two-dimensional gel electrophoresis (2DGE). Krämer et al. (1997) showed for the first time, that the asexual life stages and infection structures of *P. infestans* could be produced *in vitro* in a synchronised fashion, and that the switch between spore types and germination was accompanied by the synthesis of stage-specific proteins. Ebstrup et al. (2005) built upon this work, by further identifying differentially expressed proteins during cyst germination and appressorium formation. A major limitation of the early studies was that very few sequences were available. For this reason, many of the early research articles are 2D gel based studies comparing life stages or organisms, but not identifying protein spots that change in abundance. Another limitation was that 2D-gel based studies are restricted to the subset of a proteome that can be resolved by 2DGE such as non-basic, non-membrane and average sized proteins. However, with extensive new sequence data, new instrumentation for HPLC-MSMS based proteomics as well as new methods for quantitative analysis, both label free and chemical label based methods, opens up new possibilities for a coming-of-age of proteomics in oomycete and plant research.

A number of authors have used proteomics to compare different oomycete life stages. In a study of *Phytophthora palmivora* (the causal agent of blackpod of cocoa), 1 % of proteins resolved by 2DGE were specific for each of the pre-infection stages of the asexual lifecycle and 30% co-migrated with *P. infestans* proteins (Shepherd et al., 2003), giving a glimpse of the comparative power to come from such studies. Some of the first evidence for the presence and secretion of extracellular effectors from *P. infestans* was provided by a combined *in silico* EST data mining approach and 2DGE of secreted proteins (Torto et al., 2003). Grenville-Briggs et al. (2005) used a combination of 2DGE and Suppressive Subtractive Hybridisation (SSH) to assess the nutritional requirements of *P. infestans* asexual development, revealing the importance of amino acid biosynthesis in *P. infestans* appressorium production. Savidor et al. (2008) were the first to use large scale quantitative HPLC-MSMS proteomics methods in oomycete research when they compared mycelia and germinating cysts of *P. sojae* and *P. ramorum*. In this project they identified 3897 *P. ramorum* and 2970 *P. sojae* proteins. The quantitative analysis yielded candidates for early infection, vegetative growth and other proteins important for determining host specificity.Grenville-Briggs et al. (2010) isolated cell wall proteins from *P. infestans* vegetative mycelium, sporulating mycelium, and germinating cysts with appressoria. 31 cell wall associated proteins were identified. All proteins identified in germinating cysts with appressoria were either putative effector or pathogen-associated molecular pattern (PAMP) molecules, indicating that the cell wall is an important reservoir for this type of molecules. Hosseini et al. (2015) compared hyphae and germinating cysts of *P. sojae* and the recently discovered species *P. pisi*. Using HPLC-MSMS and quantitative analyses, 58 candidates for infection and 23 candidates for vegetative growth were identified. The secretome of *P. infestans* was recently mapped by Meijer et al. (2014) wherein over 200 secreted proteins from *P. infestans* were identified.

Resjö et al. (2014) performed the first phosphoproteomics study of any oomycete. Six life stages of *P. infestans* were compared and more than 2000 phosphosites were identified. The Crinkler (CRN) group of effectors was extensively phosphorylated and a number of phosphorylations characteristic for appressoria, such as transport and signal transduction proteins were demonstrated. In the future, the study of other post-translational modifications, such as methylation and acelylation might prove useful in order to increase our understanding of the mechanisms behind host resistance and immunity as has been shown in other plant-pathogen systems.

Proteomics of plant-oomycete interactions

The first proteomics study that investigated the effect of *P. sojae* infection on soybean root cell wall proteins was performed by Mithöfer et al. (2002). The proteins were analysed using one-dimensional SDS-PAGE, and protein bands of interest were excised and analysed using ESI-MS/MS analysis. Like many early proteomics studies, this one was hampered by a lack of sequence data. Since the soybean genome had not been sequenced at that time, the authors had to rely on the presence of proteins with sufficiently high homology in other databases in order to identify the analysed protein. For this reason, only four out of eight excised bands could be identified.

Subsequent studies have frequently used a two-dimensional SDS-PAGE based approach. This technique has the advantage that the quantitative analysis is independent of protein identification. Hence, different conditions (such as infected/healthy) can be compared and the number of (detectable) proteins that are differently abundant in the different conditions can be determined without any protein identification. Proteins can then be identified using techniques and sequence data are available. In the cases where the genome of the organism in question has not been sequenced, the most common approaches are to use ESTs or sequences from species with a high degree of sequence similarity. When 2D-gel based experiments designed to study the effect of oomycete infection are compared, some common themes

emerge. Typically, 20-80 proteins that display significant changes in abundance in response to infection are identified and these proteins are often metabolic enzymes, pathogenesis proteins (PR) proteins and proteins involved in the production of reactive oxygen species during the oxidative burst.

The work of the group of Frank Colditz, on the *Medicago truncatula* - *Aphanomyces euteiches* pathosystem provides a number of interesting examples of different experimental approaches. In one of their earlier studies, a healthy *M. truncatula* was compared with plants infected with *A. euteiches* (Colditz et al., 2004). Twelve proteins with differential abundance were identified. Among these were heat shock proteins and proteins belonging to the PR 10 family. In a subsequent experiment, the PR10-1 gene of *M. truncatula* was silenced and the effect of *A. euteiches* infection of the transgenic plants was compared with the effect of infection of wild type plants (Colditz et al., 2007). The transgenic plants were shown to be more resistant to *A. euteiches*. The proteomic analyses demonstrated increased abundance of other families of PR proteins in the PR10-1-silenced line. In another paper published in 2009, *M. truncatula* cells grown in suspension culture were used to compare the effect of infection with *A. euteiches* zoospores with elicitation using filtered medium from *A. euteiches* culture (Trapphoff et al., 2009). The filtered medium contained a mixture of secreted *A. euteiches* proteins and cell wall proteins released from dead cells. The purpose was to compare the effect of infection with the earliest reaction of the *M. truncatula* cells, triggered by PAMPs from *A. euteiches*. 44 proteins were identified. Infection resulted in increased abundance of 38 proteins, while elicitation resulted in the increased abundance of 16 proteins. Of these, 10 where induced by both treatments. The proteins induced by infection were involved in production of ROS, redox reactions, signal transduction, cell wall restructuring, PR proteins, carbohydrate metabolism and amino acid biosynthesis. The proteins induced by elicitation were mainly involved in the production of ROS and redox reactions. Interestingly, PR proteins and proteins involved in carbohydrate metabolism were not induced by elicitation. Another paper published later included proteomics to compare the effects on *M. truncatula* infection by *A. euteiches* with co inoculation with two symbiotic soil microorganisms (Schenkluhn et al., 2010). The proteins induced by pathogenic infection were similar to those described previously, whilst co-infection of pathogenic and symbiotic microorganisms did not induce any PR-proteins and induced less of other pathogen-induced protein patterns.

The first proteomics study of oomycete-plant infection that used a technique other than 2D SDS-PAGE was performed by Palmieri et al. (2012). This study was also the first to benefit from a complete genome sequence. They used gel-free proteomics to study the mechanism by which the beneficial microorganism *Trichoderma harzianum* T39 reduces infection of *Plasmopara viticola* in grapevine. 800 proteins were identified and quantified, 246 of which displayed changes in abundance between some of the different conditions. The combination of gel free methods and a more comprehensive database resulted in a dramatical increase in the number of identified and quantified proteins, compared to 2D-gel based approaches. This is a general trend and one of the major reasons that gel free methods have become much more commonly used in recent years. As a rule, gel-free methods (i.e. HPLC-MSMS based) result in hundreds to thousands of identified proteins. While identification is straightforward, quantification can be more challenging. Palmieri et al. (2012) used iTRAQ, a quantification method based on chemical labeling of the samples, but various other label free methods also exist.

Apoplastic effectors will often degrade secreted proteins from the infected plant. Ali et al. (2014) used this fact in a study where they combined RNA measurements and proteomics in order to get a more global understanding of these interactions. They isolated apoplastic molecules from one susceptible and two resistant potato cultivars during *P. infestans* infection. A total of 1639 proteins were included in the quantitative data analysis. Of these, 1075 were found to vary in at least one comparison between the different plants and stages of infection. To identify potential apoplastic effector targets, proteins with reduced abundance and increased RNA during infection were selected. The idea behind this strategy was that the plant will increase transcription of defense related proteins in response to infection (which will lead to increased mRNA), but the pathogen will use apoplastic effectors to hydrolyze the corresponding protein (which will lead to decreased abundance of the protein). Another strategy was to search for proteins that displayed decreased abundance in the susceptible cultivar but not in the resistant cultivars. Among the effector target candidates identified by that analysis were the protease C14, which has previously been identified as an effector target. Ali et al. (2014) also demonstrated the usefulness of combining protein sequences generated de novo from RNAseq data with published databases. Using this strategy, they were able to increase the number of identified proteins by 17 %, as compared to using only the published protein database.

It has so far been very difficult to identify pathogen proteins in samples from infected plants. The reason for this is probably that the plant proteins are so abundant compared to the proteins of the pathogen that it becomes impossible to detect them. True interactome studies are an important goal for the future, but will probably require the use of techniques such as laser microdissection for specific isolation of infected tissue. However, even so it may be problematic to design proper controls. Another option is to use targeted proteomic methods such as selected reaction monitoring (SRM). In this type of HPLC-MSMS analysis, pre-selected peptides are monitored throughout an entire HPLC gradient (Picotti et al., 2009). This makes it possible to detect the peptides of interest with very high sensitivity, even in a complex mixture. By monitoring several peptides from a single protein, pre-specified proteins can be detected with high sensitivity. Hopefully, these methods, combined with the ever-increasing sensitivity of new mass spectrometers will make such studies possible in the future. Another technique that may be useful for future

analysis is SWATH acquisition. In conventional MSMS analysis, only selected peptides are subjected to fragmentation and identification in the mass spectrometer. In SWATH acquisition, all peptides are fragmented, and the resulting masses are assigned to their respective parents based on elution time and other properties. The result is a dataset that can be re-analysed retrospectively, to look for certain proteins or post-translational modifications (Gillet et al., 2012).

Oomycete biological control and omics

One promising method for increasing disease resistance in crops and controlling oomycete pathogens is by addition of microbial antagonists that suppress pathogens also called biological control agents (BCA). There are a number of microorganisms that belong to bacterial, fungal and oomycetes lineages that mycoparatize oomycete pathogens such as *Burkholderia* spp.(Heungens and Parke, 2000), *Bacillus* spp.(Baker et al., 1983), *Streptomyces* spp. (Xiao et al., 2002), *Candida* spp. (Saligkarias et al., 2002), *Trichoderma* spp.(Anandaraj and Sarma, 1995; Sawant et al., 1995) and *Pythium* spp. (Gerbore et al., 2014; Horner et al., 2012). Despite the presence of BCAs in the market for over two decades, the usage of BCAs compared to conventional pesticides is very limited due to low success rate in controlling plant diseases (Alabouvette et al., 2006). The commercialization of BCA has been constrained by lack of understanding of the complex interactions between BCA- host plant - surrounding microbial flora, and pathogens in the field. Genomics, transcriptomics and proteomics can aid in successful commercialization of BCAs by identification of key virulence genes, selection of virulent microbial isolates, identification of genes involved in the production and secretion of antimicrobial compounds and fitness, as well as other characteristics that affect cultural variability and stability of a BCA. More specifically, laboratory conditions which affect sporulation and germination of spores should be carefully addressed since these factors affect maintenance, preservation and the selection of isolates for commercialization.

Recent genome and transcriptome studies on BCAs identifed virulence factors, other important genes and pathways involved in mycoparasitism (Baroncelli et al., 2015; Gao et al., 2011; Horner et al., 2012; Velasquez et al., 2014). Interestingly, in a recent pilot transcriptome sequencing study of the mycoparasitic oomycete, *Pythium oligandrum*, several transcripts with similarity to effectors expressed by plant pathogenic oomycetes were identified, such as glucanases, proteases, protease inhibitors, Crinkler effectors, and elicitors (Horner et al., 2012). This suggests that oomycetes with different hosts and different modes of growth have nevertheless evolved similar infection strategies (Grenville-Briggs et al., 2013; Horner et al., 2012). We foresee more detailed comparative analysis of effector biology of mycoparasitic and plant pathogenic oomycetes, also including the host in the future (Figure 1).

As mentioned earlier a proteomic study on priming of defenses in grapevine against the oomycete *Plasmopara viticola,* the causative agent of downy mildew with biocontrol agent *Trichoderma harzianum* T39, demonstrated accumulation of proteins involved in signal transduction activating different defense pathways, reactive oxygen species and callose formation at infection sites (Palmieri et al., 2012). Studies on interactions of BCA *Metschnikowia fructicola*, and its target fungal pathogen *Penicillium digitatum*, and plant host red grapefruit have revealed the biology and mechanism associated with bio-control activity (Hershkovitz et al., 2013). In this study over 250 genes have shown differential expression and may potentially affect the biocontrol activity of *M. fructicola.* Presently, there is no study focusing on BCA-oomycete pathogen or host multipart interactions in actual field conditions which are of utmost importance for effective control of plant diseases.

Field-omics and crop systems biology

The recent omics advances enable genome-scale capturing of complex biological processes at the molecular level in crop fields. This opens up new possibilities for understanding the relationships between genotype, environment and management interventions such as fungicide treatments. However, combining different types of data obtained from the field and understanding the biological relevance of large datasets remain challenging. We have earlier pointed out the need to create a cross-disciplinary platform for experimental design, sampling and subsequent analysis of large-scale molecular data from field trials by introducing the term “Field-omics" (Alex-andersson et al., 2014). Field-omics strives to couple information from genomes, transcriptomes, proteomes, metabolomes and metagenomes to the long-established practice in crop science of conducting field trials as well as to adapt current strategies for recording and analysing field data to facilitate integration with '-omics' data." This concept creates a basis for crop systems biology, which models complex crop traits by combining functional genomics with crop physiology and biochemistry (Yin and Struik, 2010). Future exciting models might be built on for example how plants develop over the whole season, based on several different inputs including long term weather forecasts and molecular information that may predict the severity of Phytophthora outbreaks and could be a future tool for crop selection relating to a certain field in a specific year.

Multistress conditions affects pathogen interactions, and therefore field studies are necessary, particularly since in such conditions non-dominating resistance genes may be in play. Natural light conditions also influence the infection success of pathogens (Kangasjärvi et al., 2012), which is important to consider since plant-pathogen interactions in the laboratory are mostly conducted in relatively low and constant light regimes. Field resistance, a phenomena only observed in a field situation and not in the laboratory, has been observed to pathogens such as *Magnaporthe oryzae* (Miah et al., 2013), and *Phytophthora infestans* (Rietman et al., 2012). This phenonema is assumed to be controlled by more than a single genetic locus and dependent on environmental and management cues. For example, in the late blight-resistant potato cultivar, Sarpo Mira, a discrepancy between resistance in laboratory tests and

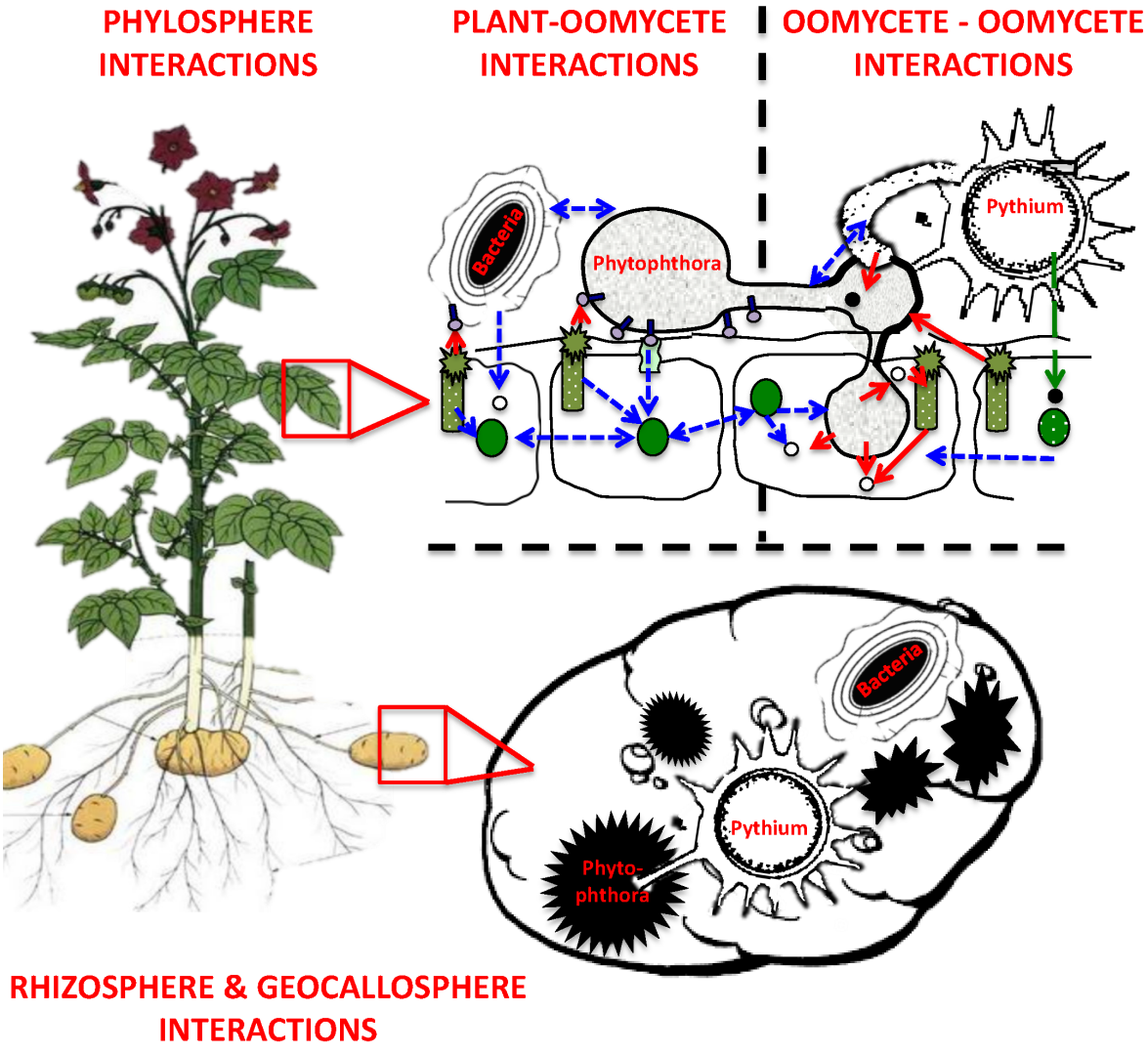

Figure1. Schematic representation of potential plant-oomycete and oomycete-oomycete interactions under field conditions as exemplified by *Phytophthora infestans* interactions with potato and with the mycoparasitic oomycete *Pythium oligandrum* and Bacteria in the phyllosphere, rhizosphere and geocallosphere.

field trials was discovered and later linked to an additional resistant factor conferring field resistance (Rietman et al., 2012). It is also clear that resistant oomycete-plant interactions can influence other biotic interactions such as insect oviposition (Abreha et al., 2015).

RNA samples from different vineyards were used in a genome-wide expression study and several transcripts for pathogenesis-related (PR) proteins were found to be independent of external stress cues (Dal Santo et al., 2013). In cultivated and wild potato, large scale secretome sampling revealed surprisingly few samples with high levels of PR proteins (less than 30% in average) even with a high disease pressure (Ali et al., 2014; Åsa Lankinen personal communcation). These proteins have been used as classical biomarkers for stress-induction in the laboratory, but might be unsuitable as biomarkers in the field where they seem to be absent or present constitutively.

Obtaining global proteome samples from the field would be labor-intensive and require complicated fractionation steps impeding large-scale analysis. Consequently, targeted approaches are currently necessary in order to obtain biologically relevant results. The secretome fraction containing apoplastic proteins constitutes the interface between intracellular processes and the environment, and is of great interest for plant pathogen interaction studies, since it includes apoplastic effectors and many signaling and enzymatic components. It is fast and relatively easy to isolate from other cellular compartments, even in the field, with a low level of contamination (Alexandersson et al., 2013); Andreasson et al., 2015) but it needs processing before freezing the samples. Another way to overcome the complexity associated with protein quantification and

identification by MS is to analyse a few peptides by selected reaction monitoring (SRM). As mentioned above, SRM has a high quantitative accuracy and can discriminate between protein isoforms in contrast to shotgun proteomics (Addona et al., 2009), but requires detailed knowledge about the system. Another way of getting interesting fractions is to isolate for example the phosphoproteome, or methylated proteins, but this requires more samples as the technical variation increases. These studies will be crucial to set a baseline for molecular field studies and to learn about the differences in global molecular mechanisms compared to a laboratory setting.

To explore the general effect of spatial variance or management methods, isolation of RNA is still preferable since it is relatively easy to sample if the specimen can be flash frozen and gives a global molecular view. Proteomics is "closer to the phenotype" than RNA but more dependent on stability of different fractions or specific molecules (see above). Metabolites might currently be more suitable to study variance in plant-tissue composition for desired properties, especially if the genes affecting the metabolites of key-interest are poorly characterized.

Samples from the field also include different micro-organisms. Despite improvements in microbial cultivation techniques, culturable organisms so far identified in complex environmental samples are likely to represent only a minor fraction of the organisms present (Sørensen et al., 2009). Meta-transcriptomics, data from a community of several different organsims, sequenced directly from the field can facilitate our understanding of the significance of plant-microbe interactions in an ecological context. Coupling metagenomics and meta-transcriptomics to agricultural practices is likely to have a large impact on the understanding of biocontrol agents, plant defense induction, pathogen spread, spraying-regimes and integrated pest management. Bernard et al. (2012) detected shifts in soil microbial community structure using fatty acid methyl ester (FAME) profile analysis of soils from organic and conventional potato production systems. These results demonstrate that cultural practices such as organic amendments, rotations, and the use of biological control agents can significantly impact local microbial communities in both organic and conventional potato farming systems. The success of an oomycete as a pathogen may be influenced by the community of microbes occupying the same ecological niche, and the oomycetes themselves may also influence the colonisation of plants by other microbes. For example, the presence of downy mildew *(Bremia lactucae)* on romine lettuce promotes subsequent colonisation by human enteric pathogens *E. coli* 0157:H7 and *Salmonella enterica* (Simko et al., 2015). Previously, oomycetes in soil have been targeted with limited success (Coince et al., 2013), however the metatranscriptomics approach used by Geisen et al. (2015) revealed the lineage to be ubiquitous, abundant and active in many soil types. Espindola et al. (2015) have recently developed a new approach to utilise such data by analysis of metagenomics data with electronic-probe diagnostic nucleic acid analysis (EDNA) for rapid and sensitive detection of oomycete sequences in metasequence data.These data suggest oomycetes might play important roles in structuring natural plant communities. Their ubiquitous nature may also contribute to the rapid development of oomycete diseases in several agricultural or natural systems. One of the complicated issues in rhizosphere metatranscriptome studies is the number of different compartments to sample, such as: 1) soil close to roots, 2) root surface microbes (rhizosphere epiphytic community), 3) root colonising microbes (endophytes), 4) tuber/stolen associated community (geocaulosphere microbes) 5) leaf material (pathogens and endophytes), and 6) vascular tissues. Lundberg et al. (2012) surveyed the root microbiome of the model plant Arabidopsis using metatranscriptomic approaches and were able to identify distinct groups of microbes that were highly abundant in individual compartments. Peiffer et al. (2013) surveyed the metatranscriptome of the maize root microbiome under field conditions and identified substantial diversity in bacterial species present in the rhizosphere in relation to bulk soil analysis. Such results should facilitate expanded studies to identify robust plant-microbe interactions at the level of individual polymorphisms by genome wide association, so potential for that plant-microbiome interactions can ultimately be incorporated into plant breeding. However, the majority of species identified in similar studies are prokaryotic in nature and studies incorporating analysis of oomycetes or other Eukaryotes from other agriculturally relevant crop species are largely lacking.

Multi-stress experiments require also that spatial and temporal variance is taken into account and recorded. Micro-climate and soil composition may vary not only between fields but also over single plots. Sampling is also dependent on plant developmental stage and variance of biotic factors such as disease or symbiont load as well as management. This has profound implications on study-design and sampling strategies for field trials (Alexandersson et al., 2014). Phenotyping and remote sensing of crops in the field is a rapidly developing area. One of the main challenges is still the ability to handle, analyze and visualize large-scale data in a way that it really advances biological understanding. Interestingly, compared to ecosystems and clinical data, the agricultural field as an entity is a simpler and a more controlled structure and thus has the power to connect between the ecological and greenhouse system levels, and could serve as a model for ecosystems studies in the wild.

Conclusions

Omics based technologies have and are revolutionizing the approach to study complex and agriculturally important systems like host plant-oomycete patho-systems (Figure 2). Genome sequencing data is shedding light on the blue-print that causes complexities observed in plants and oomycete pathogens. Transcriptomics and proteomics are now adding layers to this by unraveling functionality that arises from this blue-print. Much of our understanding of plant-oomycete patho-systems has been driven by studying both the host and the oomycete separately. However, with improvements in transcriptomic and proteomic methodologies, we foresee an increased in

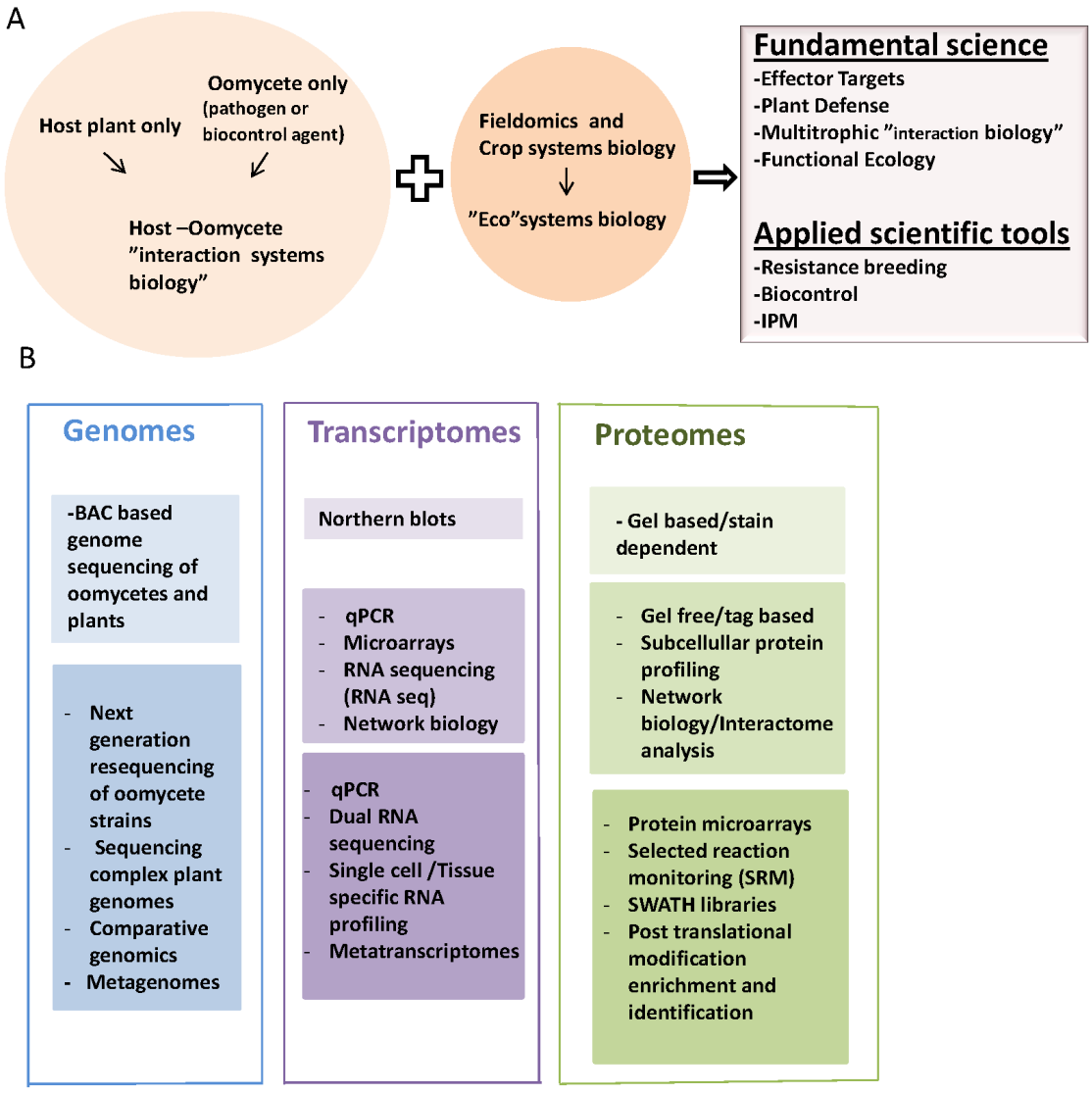

Figure 2. A. Suggested strategy and goals for the study of host-oomycete patho-systems. B. Various techniques discussed in the review that aid in deepened understanding of host-oomycete pathosystems. Techniques listed in boxes shaded in lighter gradient of color indicate older techniques, while techniques listed in boxes with increasing color gradient refer to current and future technologies respectively.

studies of these individual systems together as an "interaction system" in specific tissues. These interactions can also include biocontrol agents due to the rising prospects of their use as effective control strategies in an integrated pest management system. Another level of understanding of these multiple interaction systems in the context of crops and their ecosystems can be gained through fieldomics where the samples are taken in the field in order to grasp the multi-stress environment, currently most probably lacking mechanistic proteomic and post transcriptional analyses. Together, these studies will create a basis for crop systems biology and new understanding at the ecosystem level. Crop losses incurred due to oomycete infections are immense and current control strategies through fungicides and traditional R gene based resistance breeding programs will be strengthened, since new methods can be developed with data obtained from Omics driven research. At the same time, these studies will also improve fundamental biological and biochemical understanding of this "interaction-system".

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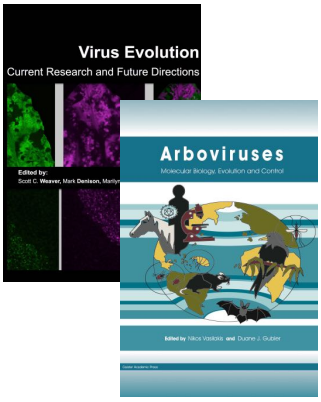

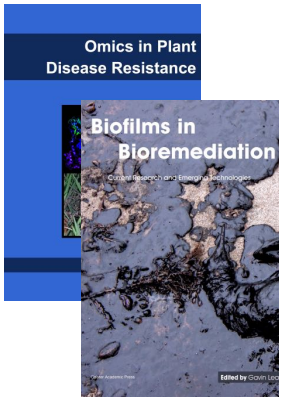

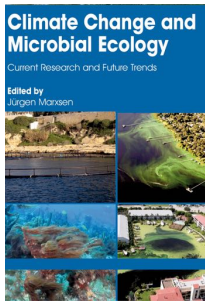

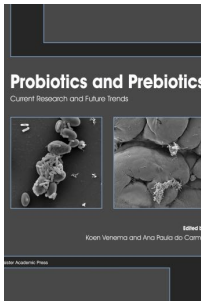

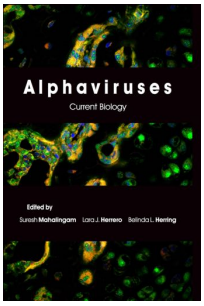

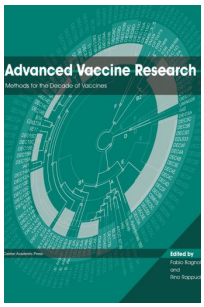

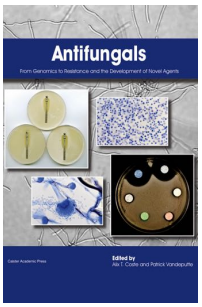

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