Plant Pathology in the 21st Century

Davide Spadaro
Samir Droby
Maria Lodovica Gullino *Editors*

Postharvest Pathology

Next Generation Solutions to Reducing Losses and Enhancing Safety

Springer

Plant Pathology in the 21st Century

Volume 11

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Preface

The 11th edition of the International Congress of Plant Pathology (ICPP-11) was held in Boston, USA, in 2018. Postharvest pathology is at the crossroad between food security and food safety, and it represents an essential element of plant pathology. This book consists of a collection of papers of the presentations given during two concurrent sessions dedicated to "*Pathogenicity and Resistance in Post-Harvest Diseases*" and "*Novel and Integrated Approaches to Control Post-Harvest Di*seases" and the workshop "*Current Issues in Food Safety and Post-Harvest Pathology*."

Losses caused by pests and diseases on fresh fruits, nuts, and grains along the supply chain (at harvest, storage, transit, and subsequent commercialization steps, before reaching the consumer) are not easily assessed. It is estimated to reach 25% of the total production in countries where postharvest facilities as well as handling and storage facilities are well developed. In developing countries, losses are often higher, exceeding 50%, because of the lack of adequate storage facilities and appropriate postharvest handling chains.

Microbial decay, caused by fungi and bacteria, is one of the main factors that determine losses compromising both the quality and safety of fruits, vegetables, nuts and grains. The development of an integrated approach for decay management, preharvest, harvest, and postharvest practices should be considered as essential components that influence the complex interaction between host, pathogen, and environmental conditions. Among postharvest practices, fruit treatments with fungicides are the most effective means to reduce decay. However, the wide consumption of high-quality fresh fruits and vegetables and the increased concerns over the possible toxicity of fungicide residues have led to the development of new alternative approaches for disease control, such as physical means and use of biocontrol agents and natural compounds. The implementation of these alternatives techniques often requires modifying the currently used postharvest practices and the development of new technologies or formulations for their applications.

Four hot topics, which have been identified in the area of postharvest diseases management, also represent the four parts of this book: i) Elucidation of the Complex Fruit-Pathogen Interactions, ii) Study of the Role of the Fruit Microbiome on the Development of Postharvest Diseases, iii) Interaction Between Postharvest

Losses and Production of Mycotoxins by Postharvest Pathogens, and iv) Development of Sustainable Strategies for the control of Postharvest Diseases.

Three chapters are dedicated to the investigation of fruit–pathogen interactions. In Chap. 1, Levin et al. present an extensive analysis of the potential effectors of *P.expansum* that may be involved in its pathogenicity on apple fruit and examine their role in suppressing fruit resistance mechanisms in the initial stages of infection. Torres et al. (Chap. 2) provide insights into the fruit–pathogen interactions, specifically in orange and apple fruit defense responses against compatible and non-host pathogens, such as *Penicillium digitatum* and *P. expansum*. Plants possess an innate immune system with disease resistance genes encoding for proteins that recognize pathogen effectors during infection. Parada-Rojas and Quesada-Ocampo (Chap. 3) review the state of the art of an important class of resistance genes—nucleotide-binding and leucine-rich repeat domains (NLRs)—in sweet potato as well as novel methods to predict NLRs in plant genomes.

Two chapters deal with the role of the fruit microbiome on the development of postharvest diseases.

Accumulating evidence indicates that the composition of the microbiota inhabiting an organism (both endo- and epiphytically) can have a profound effect on host physiology and defense responses. The global effort to characterize the endophytic and epiphytic microbiome of apple fruit is described by Wisniewski et al. (Chap. 4). The host and microbiome have co-evolved to some extent, as suggested in the holobiont concept. The implications of greater knowledge of the apple microbiome that would facilitate better disease and cultural management strategies, cultivar breeding, and abiotic stress resistance are discussed. The distribution pattern, as well as the potential utilization of endophytic microbiota that are associated with internal tissues for managing pre and postharvest pathogens, is presented in Chap. 5 by Kumar et al.

Geisen and Schmidt-Heydt (Chap. 6) develop the biological role of mycotoxin synthesis in fungi. Mycotoxins are described as pathogenicity factors of plants, colonization factors in the postharvest environment, or as specific adaptation factors for challenging and stressful environments. The overview of the interplay of the postharvest environment and mycotoxin biosynthesis opens the door to the other two chapters of the part Mycotoxins in Postharvest. Several species of *Aspergillus* and *Penicillium* contaminate nuts during the supply chain. It is, therefore, necessary to develop harvesting methods, storage conditions, processing techniques, and detoxification protocols for the management of fungal growth and mycotoxin contamination. The efficacy, the benefits, and the drawbacks of drying technologies, roasting, and cold plasma in reducing mycotoxin contamination are described by Spadaro et al. (Chap. 7). Kumar Solanki et al. (Chap. 8) deal with the role of the wheat microbiome in relation to the mycotoxin occurrence in stored grain. A better knowledge of the composition and dynamics of wheat-grain-associated microbiota is needed to identify novel beneficial microorganisms that may improve crop health and suppress the growth of potential pathogens in a sustainable manner.

The fourth part, dedicated to the development of sustainable strategies for postharvest disease control, is composed of four chapters. Adaskaveg et al. (Chap. 9)

offer an overview of the chemical management of postharvest diseases of subtropical and tropical fruits, such as citrus, pomegranates, pineapples, and avocado. For subtropical crops, new fungicides have been introduced to manage major diseases, ranging from conventional (propiconazole), reduced-risk (pyrimethanil, azoxystrobin, fludioxonil) to bio-pesticide (natamycin), and also pre-mixtures. Postharvest fungicide applications with aqueous recycling drenches are one of the most effective strategies to prevent fruit decay. Adaskaveg et al. (Chap. 10) describe different sanitation practices to recycle fungicide drenches. Among alternative means to chemical fungicides to control postharvest decay of fresh horticultural products, low-toxicity chemicals, classified as food additives or Generally Recognized As Safe (GRAS) compounds, are of interest because of their low toxicological effects on mammals and minimal impact on the environment. Palou and Pérez-Gago (Chap. 11) introduce the reader to GRAS salts as a viable alternative for postharvest preservation of fresh horticultural products. Finally, Ippolito et al. (Chap. 12) focus on the advantages and drawbacks of electrolyzed water as a novel broad-spectrum sanitizer for postharvest disinfection to maintain commodities and facilities free from fungal and bacterial pathogens.

We hope this book will be helpful to researchers, extension services, and technicians working in the postharvest industry as well as students interested to this field.

Fruit–pathogen interactions, fruit microbiome, mycotoxins, and postharvest pest and disease management strategies are the keywords for this forefront book that provides interesting hints and suggestion to further develop the challenging discipline of postharvest pathology. Next Generation Solutions for reducing postharvest losses and enhancing food safety are at your fingertips!

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Contents

Chapter 1
Role of Effector Proteins in the Virulence of *Penicillium expansum* on Apple Fruit

Elena Levin, Samir Droby, Michael Wisniewski, and Christopher Dardick

Abstract *Penicillium expansum* is a ubiquitous postharvest pathogen that infects a wide range of fruits, including pome and a variety of stone fruit. Virulence factors that mediate pathogenicity in postharvest-decay fungi have not been fully defined, especially in regard to if and how the pathogen is able to modulate fruit defense mechanisms, particularly at the initial stages of infection. In this chapter, we present an extensive analysis of the potential effectors of *P.expansum* that may be involved in its pathogenicity on apple fruit and examine their role in suppressing fruit resistance mechanisms in the initial stages of infection. The ability of *P. expansum* to secrete factors that are able to down-regulate ROS production was initially assessed. Results demonstrated that there are proteins secreted by *P. expansum* that are able to down-regulate host-response to presence of the pathogen. A combination of different bioinformatic and genetic approaches were used to identify and characterize potential effectors in *P. expansum*. In particular, the small cysteine-rich proteins, *Pescr1* and *Pescr2,* were investigated. Additionally, the role of LysM proteins, NLPs, and one proteolytic enzyme, peptidase S8, in the virulence of *P. expansum* was also examined.

Keywords NLP effectors · LysM effectors · Pathogenicity · Necrotrophic fungi · Small · Cysteine-rich proteins · PePRT protein

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Introduction

Penicillium is a fungal genus containing 354 recognized species (Visagie et al. 2014) that are distributed globally, where they inhabit a large variety of substrates, from soil to foods. *Penicillium expansum* is a postharvest, necrotrophic pathogen that infects a wide range of fruits, including pome and a variety of stone fruit (e.g. peaches, nectarines, plums, and apricots) (Cappellini et al. 1987; Jurick et al. 2011). *P. expansum* is regarded as the most important postharvest pathogen of apple fruit and is of great concern to the fruit processing industry due to its ability to synthesize toxic metabolites such as patulin (Wouters and Speijers 1996; McCallum et al. 2002). Direct fruit losses due to the development of decay during long-term storage of apple can be considerable and dependent on the cultivar, postharvest handling processes and storage conditions. Infection of apples by *P. expansum* usually takes place through wounds inflicted during fruit harvesting and subsequent postharvest handling. Spores germinate in the wounds and colonize the surrounding tissue within 24 h. A visible breakdown of the tissue surrounding the infection site occurs within 48 h. A unique feature of *P. expansum* compared to other postharvest pathogens is its ability to grow at low temperatures. This ability allows it to survive and grow during low–temperature storage of fruit. *P. expansum,* commonly referred to as blue mold, is a significant problem in production systems that do not use pre- or postharvest fungicides (Tahir et al. 2015).While apple producers rely on the use of synthetic fungicides for decay control, future use of postharvest fungicides will be restricted or banned by many countries (Droby et al. 2009). Alternative postharvest strategies, based on biological approaches, are being developed but are currently very limited due to their reduced efficacy under commercial conditions. Although one of the best means to control plant diseases is the use of resistant cultivars, little attention has been devoted to postharvest disease resistance in apple breeding programs (Janick et al. 1996). This is due both to a lack of sources of genetic resistance in domesticated apples and the current prohibitive cost of maintaining trees in trial plots for several years. Hence, the identification of resistance as a heritable trait would represent a significant accomplishment. To this purpose, the identification of DNA markers for resistance to post-harvest decay would increase the feasibility of breeding resistant cultivars through marker-assisted selection (MAS) of seedlings prior to field planting. In this regard, Norelli et al. (2017) tested the hypothesis that the observed resistance of wild *Malus sieversii* accessions PI613981 to infection by *P. expansum* is due to the effect of specific genetic loci. The analysis of a *M.* × *domestica* 'Royal Gala' X *M. sieversii* PI613981 mapping population (GMAL4593) identified two QTLs located on linkage groups (LG) 3 and LG10 that were associated with resistance to *P. expansum* and were designated qM-Pe3.1 and qM-Pe10.1, respectively. A DNA test for qM-Pe3.1, which will greatly improve the efficiency of breeding apple for blue mold resistance, has been developed and is currently being used to identify and introgress predicted blue mold resistance in progeny segregating for qM-Pe3.1 (Norelli et al. 2017). A better understanding of the mechanism of pathogenicity of *P. expansum* on apple and how the pathogen is able to overcome

innate immunity on fruit would provide new insights that could be used for developing innovative management strategies to control this postharvest pathogen.

Plant Defense Mechanisms

Plants respond to pathogen attacks using different defense mechanisms. Innate or preformed defense mechanisms involve structural barriers such as a waxy cuticle and the cell wall or preformed antimicrobial compounds that function to prevent the colonization of the tissue (Jackson and Taylor 1996; Osbourn 1996). Once the structural barriers of a host have been breached, plants respond to infection using a two-branched innate immunity system (Jones and Dangal 2006). The first branch recognizes and responds to pathogen-associated-molecular-patterns (PAMPs), molecules that are common to many classes of microbes, including non-pathogens. This type of immune response is referred to as PAMP-triggered immunity (Doehlemann et al. 2009; Nürnberger and Kemmerling 2009). Pathogens, however, have evolved the use of effectors that interfere with PAMP-triggered immunity and results in effector-triggered susceptibility (Boller and He 2009). In the second branch of innate immunity systems, a given pathogen effector is recognized by a resistance protein resulting in effector-triggered immunity (ETI). This second level of immunity can itself be suppressed by other pathogen effectors (Houterman et al. 2008), resulting in the evolution of complex host-microbe interactions (Chisholm et al. 2006).

Pathogenicity of *P. expansum* on Fruit

Fungal-plant pathogens have developed several mechanisms of pathogenicity that enhance their virulence (Perez-Nadales et al. 2014). In regard to the *Penicillium* spp. host-pathogen interaction, wounding of the fruit surface, secreted nutrients, and volatiles stimulate spore germination (Droby et al. 2008). Spore germination is followed by penetration and colonization of the fruit tissue. *P. expansum* is able to cause decay at the low temperatures used for fruit storage, and over-mature or long-stored fruits are especially susceptible to infection. In most cases, necrotrophic fungal plant pathogens promote host cell death by secreting toxic extracellular proteins and secondary metabolites and then utilize the nutrients released from dead host cells for growth (Van Kan 2006). In the early stages of infection and disease development, a wide range of virulence mechanisms are employed by these pathogens to overcome the innate and induced resistance responses of their hosts. These include the secretion of diverse phytotoxic compounds, cell-degrading enzymes, and proteinaceous effectors that function in manipulating host defense mechanisms (Wang et al. 2014). Only a relatively few protein effectors have been identified in

necrotrophic pathogens, however, and the molecular interactions between these effectors and host plants, in most cases, remain to be elucidated.

While the availability of the genome sequences of several of *P. expansum* strains can be used in the identification of virulence genes (Ballester et al. 2015), there is general lack of knowledge about virulence elements that determine pathogen aggressiveness in *P. expansum*, including information on effectors that can subvert fruit defenses.

Several reports on the potential role of different factors that mediate pathogenicity and virulence in *P. expansum* on apple fruit have been published in recent years. Acidification of host tissue leading to enhanced production of plant cell-wall-degrading fungal enzymes, as well as the production of patulin are among the suggested factors influencing the pathogenicity and virulence of *P. expansum* (Prusky et al. 2004; Sanzani et al. 2012; Barad et al. 2014; Vilanova et al. 2014). Patulin is a secondary metabolite and its role in the development of blue mold decay on apples remains equivocal. While some authors have implicated its involvement in pathogenicity (Sanzani et al. 2012; Barad et al. 2014), other authors have reported the lack of an association between virulence and patulin production in *P. expansum* (Ballester et al. 2015; Li et al. 2015). It appears that, although patulin could not be required to infect apples, it may act as an apple cultivar-dependent aggressiveness factor (Snini et al. 2016).

Previous studies have linked the secretion of polygalacturonases, glutamate decarboxylases, calmodulin or C-4 methylesterol oxidases with the pathogenicity of *P. expansum* in apples by characterizing genes that are differentially expressed during the infection process (Sánchez-Torres and González-Candelas 2003). Qin et al. (2007) also linked polygalacturonases to the pathogenicity of *P. expansum* by analyzing its cellular and extracellular proteomes. Moreover, Tian et al. (2013) reported that proteins involved in antioxidant metabolism, such as catalases and superoxide dismutases, are related to the pathogenicity of blue mold. The hypothesis that *P. expansum* has the ability to acidify the environment in colonized tissue by the accumulation of D-gluconic acid has also been proposed as a mechanism of *P. expansum* pathogenicity in apples (Vilanova et al. 2014).

Fruit pH has been suggested to be an important factor in postharvest diseases due to its direct effect on conidial germination (Pelser and Eckert 1977) and on the regulation of virulence factors (Alkan et al. 2013). These authors reported that when *P. expansum* colonizes apple fruit tissues, it acidifies its local surroundings causing the enhancement of its pathogenicity by activating pathogenicity factors (Alkan et al. 2013).

Virulence factors that mediate pathogenicity in postharvest-decay fungi have not been fully defined, especially in regard to if and how the pathogen is able to modulate fruit defense mechanisms, particularly at the initial stages of infection. While pathogen effectors responsible for suppressing host defense mechanisms are believed to play a major role in the pathogenicity of necrotrophic pathogens, their potential involvement in the apple-*P. expansum* system has not been fully elucidated.

Fungal Effectors and Their Role in the Pathogenicity of Necrotrophic Fungi

Fungal plant pathogens are classified into three groups based on their mechanisms of pathogenicity. The groups are: biotrophs (obtaining nutrients from the host directly, but not killing the tissue); hemi-biotrophs (fungi that have an early biotrophic stage but then become necrotrophic); and necrotrophs (fungi that kill cells and tissues to obtain nutrients). Most of these fungal pathogens utilize effectors to suppress innate and induced resistance mechanisms, which allows them to invade host tissues (Kleemann et al. 2012).

Pathogen effectors are defined as molecules that manipulate host cell structure and have dual function in a manner that in cases it facilitates infection and in others triggers defense responses (Kamoun 2006). Necrotrophic fungi have been generally considered pathogens that rely on a plethora of non-host-specific mechanisms to infect their host. Studies on effectors in necrotrophic fungi have demonstrated, however, that they are capable of selectively suppressing host defenses before an effective defense response can be initiated by the plant host. However, the mode of action of only few of these effectors have been studied. PtrToxA, a proteinaceous effector in *Pyrenophora tritici-repentis* the causal agent of tan spot disease of wheat (*Triticum aestivum L.*), was demonstrated to be present within living mesophyll cells of sensitive wheat cultivars. PtrToxA is targeted to the chloroplast and apparently disrupts photosynthetic electron transport, leading to ROS accumulation and plant cell death upon light exposure (Tan et al. 2010). Weiberg et al. (2013) demonstrated that *Botrytis cinerea,* a highly destructive plant necrotrophic pathogen, is able to suppress host immunity in *Arabidopsis* and tomato plants through small RNAs that highjack RNA interference (RNAi) machinery and selectively silence host immunity genes. In other studies, host-selective toxins were identified that function in effector-triggered susceptibility (Friesen et al. 2007, 2008; Manning et al. 2007; Abeysekara et al. 2009; Liu et al. 2009, 2012). LysM effectors have been shown to contribute to pathogenicity and virulence in several pathogen-plant systems (Bolton et al. 2008; De Jonge et al. 2010). Necrosis and ethylene-inducing peptide 1 (NEP1)-like (NLP) effectors, found in many plant pathogens, have also been shown to play a role in the pathogenicity of necrotrophic fungi (Schouten et al. 2008; Dallal Bashi et al. 2010). Other effectors, such as the PeSte12 transcription factor, were reported to affect lesion development of *P. expansum* on apple (Sánchez-Torres et al. 2018). During axenic growth, PeSte12 appears to act as a negative regulator of genes related to detoxification, ATPase activity, protein folding, and basic metabolism. Knocking out *PeSte12* gene significantly affected the rate of decay development on apple fruit stored at 0 °C. No significant effect, however, was observed when apple fruits were inoculated with knockout mutants and stored at 20 °C (Sánchez-Torres et al. 2018).

P. expansum Secretes Factors That Down-Regulate ROS Production in Apple During Infection

An extensive analysis of potential effectors of *P.expansum* was conducted to identify potential effectors involved in the pathogenicity of *P.expansum* on apple fruit and to examine their role in suppressing fruit resistance mechanisms during the initial stages of infection. The first stage of this study was directed at understanding the response of fruit tissue to elicitors secreted by *P. expansum* mycelia. Fragments of fungal cell walls, such as chitin oligomers, are known to act as elicitors that trigger ROS production in host tissues. The induced ROS usually serves as a signal to trigger downstream pathways leading to the activation of resistance mechanisms (Lamb and Dixon 1997; Torres et al. 2006; Torres 2010). Necrotrophic pathogens usually secrete effectors that suppress ROS production during the initial stages of the infection process to inhibit the activation of induced resistance responses by the host and thus allow it to establish infection (Wang et al. 2014). In our study, apple wounds were inoculated with 50 mg/ml of a preparation of *P. expansum* cell walls mixed with culture filtrate that was derived from the fungus growing in an apple juice medium. This was done to determine if *P. expansum* secretes factors that are able to down-regulate ROS production. ROS production was measured in wounded tissues using fluorescence staining. As illustrated in Fig. 1.1, wounds treated with the fungal cell wall preparation plus the addition of culture filtrate markedly reduced ROS production in host tissues, relative to treatments with fungal cell walls alone. This suggests that materials secreted into the culture filtrate play an active role in down-regulating ROS production in apple tissue. Subsequently, culture filtrate was first boiled and then mixed with cell walls before being administered into apple wounds to determine if the ROS-suppressive factors were proteins. In this case, results indicated that the fluorescence level in apple tissues was significantly higher relative to the fluorescence in wounds inoculated with cell walls with not boiled culture filtrate (Fig.1.1). The results of this experiment suggest that proteins secreted by *P. expansum* are able to inhibit or prevent host-response to fungal infection. This effect was also described by Buron-Moles et al. (2015) in apples inoculated with host and non-host species of *Penicillium.* In that study, production of hydrogen peroxide (H_2O_2) in immature apple fruit tissues that was induced by wounding was suppressed by *P. expansum* to much greater extent than it was by the non-host pathogen, *P. digitatum* (Buron-Moles et al. 2015).

Discovery and Functional Analysis of Effectors in Plant Pathogenic Fungi

A combination of biochemical, genetic, and bioinformatic strategies have often been used for the discovery and characterization of effector genes in plant pathogenic fungi (Kamoun 2006; Raffaele et al. 2010). Recent advances in genomics,

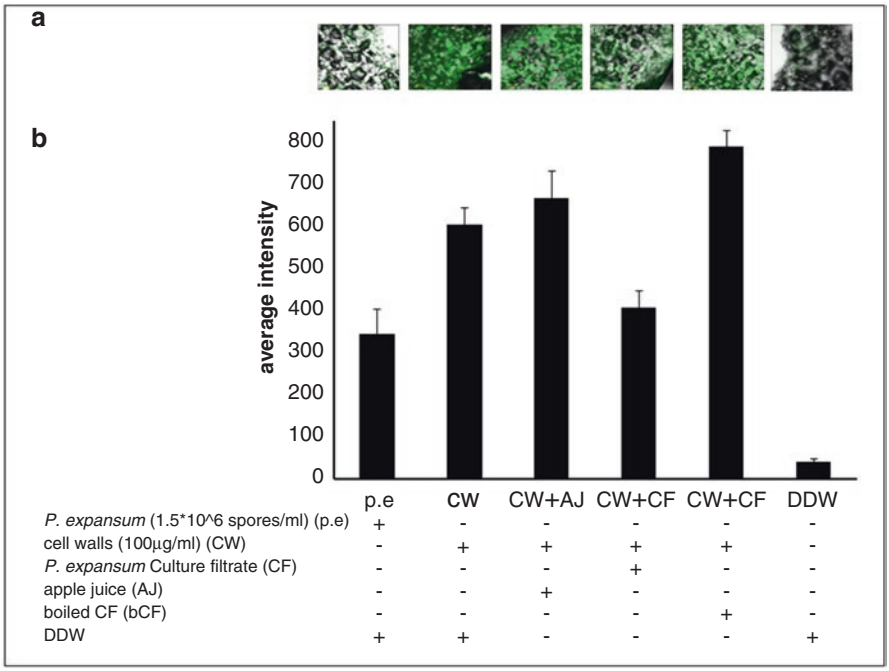

Fig. 1.1 Effect of the factors secreted by *P. expansum* on the ROS production in apple wounds**.** (**a**) confocal microscope image of ROS production in apple wounds inoculated with *P. expansum* spores (p.e), *P. expansum* cell walls fragments (CW) with or without addition of *P. expansum* culture filtrate (CF) visualized by DCF staining, (**b**) quantification of the fluorescence intensity in the images

proteomics, and bioinformatics have facilitated the implementation of functional genomic pipelines to identify effectors from nucleotide and protein sequence data sets. As suggested by Kamoun (2006), a typical pipeline consists of two major steps: use of data mining tools to identify candidate genes that fulfill a list of specific criteria, followed by analysis and validation of these candidate genes by functional assays, such as expression *in planta* and evaluation of effector-like activity. This approach was successfully used to develop a pipeline for the discovery of *Phytophthora infestans* effectors (Torto et al. 2003; Raffaele et al. 2010). Identification of candidate secreted proteins was facilitated by the fact that in Oomycetes, as in other eukaryotes, most secreted proteins are exported through a common secretory pathway that utilizes short, N-terminal, amino-acid sequences known as signal peptides (Torto et al. 2003). Signal peptides are highly degenerate and cannot be identified using DNA hybridization or PCR-based techniques. Nonetheless, computational tools, particularly SignalP software (Nielsen 2017), can be used to assign a signal peptide annotation to unknown amino acid sequences (Menne et al. 2000; Schneider and Fechner 2004).

One of the problematic challenges facing researchers in the identification of potential effector proteins is the lack of common features in effector genes/proteins within and across species. Since there is an ever-increasing number of sequenced genomes of plant pathogenic fungi, computational tools have become a widely used approach for effector-prediction (Sperschneider et al. 2013, 2014, 2015; Syme et al. 2013). Criteria such as presence of a secretion signal, species-specificity, small size, a large number of cysteine residues, presence of specific protein domains and motifs found in known fungal effectors, signatures of positive selection, recent gene duplication, and homology to proteins experimentally proven to affect the outcome of pathogen-host interactions have all been used to identify potential effectors (Guyon et al. 2014; Levin et al. 2019a).

In the present study, nine potential effectors of *P. expansum* were examine, four of which were detected by a pipeline developed by Levin et al. (2019a). The remaining five effector candidates were members of known effector families reported to contribute to the virulence of other plant-pathogenic fungi.

Small, Cysteine-Rich Proteins in *P. expansum*

Small cysteine-rich secreted proteins with unknown function are among the most established parameters for the identification of potential effectors (Sperschneider et al. 2015). Analysis of the *P. expansum* secretome revealed 25 small (< 300 aa) cysteine-rich (>4) proteins with unknown function. Three of them exhibited a high level of expression during apple infection and one was induced during spore germination (Levin et al. 2019a). The effect of two proteins on the virulence of *P. expansum* was examined: *Pescr1* (PEX2_056460), expressed in ungerminated spores, and *Pescr2* (PEX2_002470) expressed during apple infection.

Deletion of *Pescr1* had no effect on the radial growth rate and colony morphology of mutant strains of *P. expansum* when grown on PDA medium (Fig. 1.2a, b). The *Pecsr2* null mutant, however, exhibited a slightly lower rate of radial growth compared to the wild-type (WT) strain and the ectopic mutant. 'Golden Delicious' apples were inoculated with the two null mutants to examine the effect of the deletions on pathogenicity. The rate of lesion development and the percent of infected wounds were compared between null mutants, ectopic mutants, and the WT strain. No significant differences in infection incidence were observed between either of the mutants. As illustrated in Fig. 1.2c, however, lesion diameter caused by the *Pescr2* mutant was significantly smaller than the lesion diameter induced by the WT strain and ectopic mutants. This result is similar to the results obtained for the disruption of *Ecp1* or *Ecp2* genes coding for cysteine-rich extracellular proteins that were abundantly secreted by *Cladosporium fulvum* during colonization of tomato leaves, which compromised its virulence (Laugé et al. 1997). Moreover, functional analyses of *F. oxysporum f. sp. lycopersici* cysteine-rich, secreted proteins encoded by avirulence (AVR) genes (*Avr1*, *Avr2*, and *Avr3*) revealed that Avr2 and Avr3, in addition to triggering resistance in tomato plants carrying the R genes *I-2* and *I-3*,

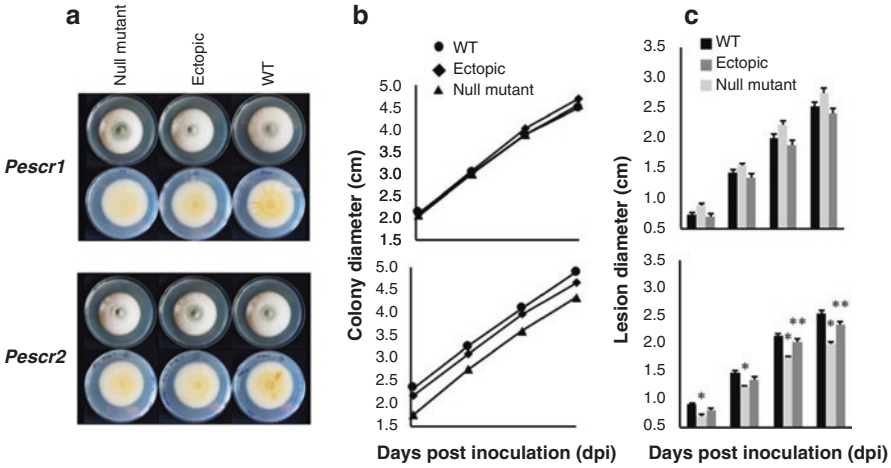

Fig. 1.2 Effect of the targeted deletion of genes coding for small cystein-rich proteins PeSCR1 and PeSCR2 on the growth rate, colony morphology and virulence. (**a**) colony morphology at 7 dpi, (**b**) radial growth on PDA medium, (**c**) lesion diameter during apple infection of the wild type (WT), extopic mutants (E), and null mutants. Radial growth and lesion diameter results are an average of three independent measurments

respectively, were both required for full pathogenicity insusceptible tomato lines (Rep et al. 2004; Rep 2005; Houterman et al. 2009). *F. oxysporum f. sp. lycopersici* effector Avr1, however, does not contribute to virulence insusceptible cultivars.

LysM Proteins in *P. expansum*

Genetic homologues of lysine motif (LysM) effectors have been identified in many fungal species, and were reported to have a major effect on virulence in several plant pathogens (Bolton et al. 2008; De Wit et al. 2009; De Jonge et al. 2010; Marshall et al. 2011; Mentlak et al. 2012; Takahara et al. 2016; Kombrink et al. 2017). Analysis of the genome of *P. expansum* revealed 18 genes possessing a LysM motif. Eleven of these LysM genes were predicted to also contain a secretion signal, while only four (*PeLysM1*, *PeLysM2, PeLysM3, PeLysM4)* were found to be actively transcribed during the early stages of the infection process of apple (Levin et al. 2017). A further analysis was conducted of the amino acid sequences of the LysM motif of these genes. An amino acid alignment of the LysM motifs in *P. expansum* with LysM domains from known fungal effectors revealed a high level of homology to the LysM domains of the *Trichoderma atroviride* TAL6 protein rather than other LysM effector proteins (Levin et al. 2017). Notably, TAL6 was demonstrated to be involved in self-signaling processes during fungal growth (Seidl-Seiboth et al. 2013).

The analysis of the knockout mutants of each of the four LysM genes included an examination of growth rates, colony morphology, sporulation, germination, and

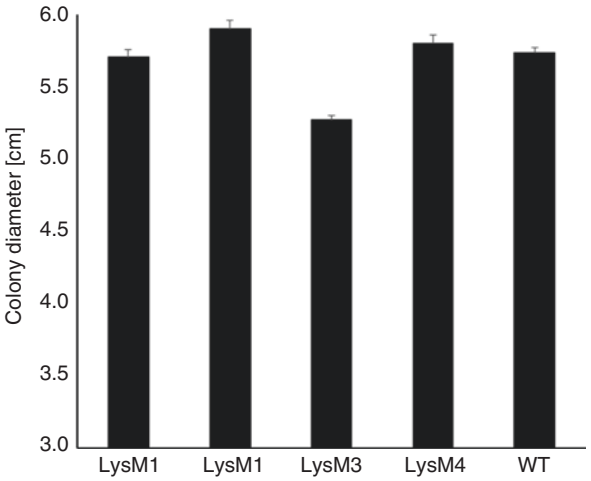

Fig. 1.3 Effect of the targeted deletion LysM genes on the growth rate. Diameter of colonies of LysM null mutants *PeLysM1, PeLysM2, PeLysM3, PeLysM4* and wild type (WT) grown on PDA medium, measured 7 days post inoculation. The results are an average of three independent measurements

effects on pathogenicity. No significant effect of the null mutations on infection incidence or the rate of lesion development was observed on 'Golden Delecius' apples. The *PeLysM3* null mutant, however, exhibited a lower rate of radial growth on PDA plates compared to the WT strain and the other mutants (Fig. 1.3). The *PeLysM3* null mutant also exhibited a significantly lower percent of spore germination, as well as shorter germ tubes on spores that did germinate. Collectively, these results suggest that *P. expansum* LysM proteins may play a potential role in growth processes, similar to TAL6 in *T. atroviride,* rather than acting as effectors in fungal-plant interactions. Notably, a yeast two-hybrid (Y2H) analysis, conducted to gain insight into the potential interaction of the secreted LysM proteins with apple proteins, revealed LysM binding to a wide range of apple proteins, including those involved in plant defense processes and iron sequestration (Levin et al. 2017). This suggests that LysM proteins in *P. expansum* may play a potential role in modifying host response to the pathogen during the infection process. Importantly, it is also possible that interruption of a single effector gene would not have a determinable effect on virulence due to complementation by other LysM family members. In this regard, among three potential LysM effectors (Mg1LysM, Mg3LysM, and MgxLysM) produced by *Mycosphaerella graminicola*, the null mutant of *Mg3LysM* affected pathogen infection, while the knockout strain of *Mg1LysM* had no effect on *M. graminicola* pathogenicity (Marshall et al. 2011). Moreover, an 80-lineage-specific LysM gene in *Verticillium dahlia* (strain VdLs17), was reported to contribute to the virulence of *V. dahliae* in tomato but not in *Arabidopsis* and tobacco (Kombrink et al. 2017). An elaborate study of the LysM protein family in *P. expansum* was recently conducted by Chen et al. (2020). The effect of 16 LysM deletion mutants on the growth and virulence of *P. expansum* on apples was analyzed. Growth rates of 3 out of 16 null mutants on a PDA medium were demonstrated to be slower, relative to the WT strain. Eleven of the 16 LysM null mutants, however, exhibited enhanced virulence on apples. Notably, only 2 of the 16 null mutants were less virulent on apples than the WT strain.

NLP Proteins in *P. expansum*

Effector proteins are generally species-specific and lack common features within and across species (Sperschneider et al. 2015). Some effector families, however, have been found to play a role in numerous plant-pathogen interactions. Nep1-like proteins (NLPs), produced by various microorganisms with different lifestyles, were shown to be involved in the virulence of several plant pathogens (Mattinen et al. 2004; Dallal Bashi et al. 2010; Santhanam et al. 2013).

Use of the effector-prediction pipeline developed by Levin et al. (2019a) identified 17 candidate genes coding for proteins most likely involved in the pathogenicity and virulence of *P. expansum*. An NLP encoding gene, *Penlp1* (PEX2_080220), was identified as one of the top effector-candidates, since *Penlp1* encoded a small (240 aa), cysteine rich (5 cysteines) protein and was highly induced in the initial stages of apple infection. Importantly, proteins possessing an NPP1-like domain were previously demonstrated to contribute to the pathogenicity of plant pathogens (Schouten et al. 2008; Dallal Bashi et al. 2010). Further analysis of the *P. expansum* genome, revealed an additional gene, *Penlp2* (PEX2_071150) possessing a NPP-1 domain (IPR008701). *Penlp1* and *Penlp2* encode a type 1 and type 3 NLP, respectively. While *Penlp1* expression was found to be strongly induced during apple infection, the highest level of *Penlp2* expression was found in ungerminated spores (Levin et al. 2019b). Although, both proteins were found to induce necrosis when transiently expressed in *Nicotiana Benthamiana* leaves, only the deletion of *Penlp1* had an effect on the pathogenicity of *P. expansum* (Levin et al. 2019b). Apples infected with the null mutant of *Penlp1*, developed smaller lesions than apples inoculated with the WT strain and the ectopic mutant (Fig. 1.4).

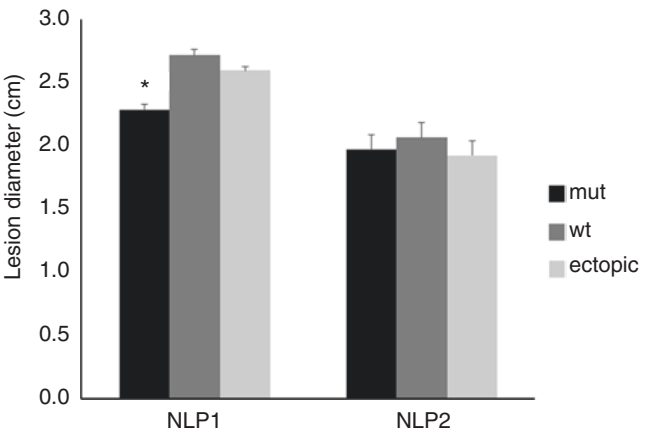

Fig. 1.4 Effect of the targeted deletion of NLP genes on *P. expansum* virulence on apple. Lesion diameter during apple infection caused by wild type (WT), ectopic mutants (E), and null mutants (mut) of *PeNLP1* and *PeNLP2* measured 6 days post inoculation. The results are an average of three independent measurements