Universitat Autònoma de Barcelona

Biotechnological production of antifungal proteins for crop protection

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PhD thesis

Biotechnological production of antifungal proteins for crop protection

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Barcelona, November 20

致我最爱的爸爸妈妈

Summary

Plant diseases caused by pathogenic fungi are responsible of important crop losses endangering food security and safety. Antimicrobial peptides (AMPs), exhibiting potent and durable lytic activity specifically against microorganisms, have a great potential as novel natural fungicides for the control of pathogenic fungi. However, viable exploitation of AMPs requires fast, cost-efficient, and safe production systems. The main goal of this work was to develop a sustainable platform for the production of bioactive AMPs, and to characterize them in the control of fungal infections in plants to advance in their application in agriculture.

Antifungal proteins (AFPs) secreted by filamentous fungi are a group of highly stable cysteine-rich AMPs that specifically target fungal cells. In this study, we demonstrate that *Nicotiana benthamiana* plants are an excellent biofactory for producing AFPs through transient expression using a new vector derived from the tobacco mosaic virus. Using this plant-based production system we efficiently produced two different bioactive AFPs, the *Penicillium expansum* AfpA and *Penicillium digitatum* AfpB. We found that the subcellular compartment where AFPs are accumulated has an important impact on protein yield, probably avoiding toxicity towards plant cells. The highest yields were achieved when targeting AFPs to vacuoles, reaching up to 0.170 mg/g of fresh leaves of the highly active AfpA and eight times more of AfpB (1.2 mg/g of leaf). We also show that plant crude extracts containing AFPs are fully active against plant pathogens without requiring further protein purification, thus reducing significantly downstream processing. Therefore, the developed system is efficient for the production of AFPs, and also it is economic and safe since it is based on plants.

We also developed an alternative system for the production of the linear PAF102 antifungal peptide that was recalcitrant to be produced in biological systems. This system is based on targeting the peptide to lipid droplets (LDs) through the fusion to a plant oleosin protein. Using this oleosin fusion technology, we produced PAF102 in rice seed LDs, reaching moderate yields of about 20 µg of peptide per gram of grain. Production on rice seeds is long process in order to speed the process, we successfully transferred the plant oleosin fusion technology to the *Pichia pastoris* system. We produced commercially relevant yields of PAF102 in these yeast LDs, reaching values of 180 mg/l of culture in only 4 days. The accumulation of PAF102 in the LDs of rice seeds and yeast facilitated its downstream extraction and recovery by simple flotation on dense solutions, with the recovered PAF102 being biologically active against pathogenic fungi.

Finally, we demonstrate that *in planta* produced AfpA and AfpB, either purified protein or protein extracts enriched with these two proteins, are efficient in controlling important fungal diseases on economically relevant crops, including Botrytis gray mold disease in tomato leaves and fruits, blast disease in rice plants and *Fusarium proliferatum* infection in rice seeds. Our results provide a sustainable production system of AFPs, and evidence their efficacy on protecting plants from fungal infection, strongly supporting the use of AFPs as environmental friendly and effective “green fungicides” in crop and postharvest protection.

Summary

Resumen

Los hongos patógenos de plantas causan importantes pérdidas en las cosechas, poniendo en peligro la seguridad y calidad alimentaria. Los péptidos antimicrobianos (AMPs) muestran una actividad lítica potente y duradera específicamente frente a microorganismos, por lo que tienen un gran potencial como nuevos fungicidas naturales para el control de los hongos patógenos. Su explotación requiere de sistemas de producción rápidos, eficaces, económicos y seguros. El principal objetivo de este trabajo era desarrollar sistemas de producción sostenibles de AMPs, y su caracterización en el control de infecciones fúngicas para avanzar en su aplicación en la agricultura.

Las proteínas antifúngicas (AFPs) secretadas por hongos filamentosos son un grupo de AMPs ricos en cisteínas, muy estables, activos específicamente frente a hongos. En este estudio demostramos que las plantas de *Nicotiana bentamiana* son una excelente biofactoría de AFPs mediante expresión transitoria usando un nuevo vector derivado del virus de mosaico del tabaco. Utilizando este sistema de producción en plantas, hemos producido eficientemente dos AFPs muy activas frente a hongos fitopatógenos, la AfpA de *Penicillium expansum* y la AfpB de *Penicillium digitatum*. Hemos descubierto que el compartimento subcelular donde se acumulan las AFPs tiene un impacto importante en la producción obtenida, probablemente porque su compartimentalización evita la toxicidad hacia las células vegetales. Los valores más altos se obtuvieron cuando las proteínas se acumularon en las vacuolas, alcanzando hasta 0,170 mg/g de hoja en el caso de la proteína más activa AfpA y hasta ocho veces más para la AfpB (1,2 mg/g de hoja). También demostramos que los extractos crudos de plantas que contienen AFP son activos frente a hongos, sin necesidad de purificar las proteínas reduciendo considerablemente el procesamiento del material vegetal y los costes de producción. Por lo tanto, el sistema desarrollado es eficiente para la producción de AFPs, y también es económico y seguro ya que se basa en plantas.

Además, hemos desarrollado un sistema alternativo para la producción del péptido antifúngico PAF102 que previament no había podido producirse biotecnológicamente. Este sistema se basa en acumular el péptido en las gotas lipídicas (LDs) mediante la fusión a una proteína oleosina de plantas. Mediante esta estrategia, hemos producido PAF102 en semillas de arroz en cantidades de 20 μg por gramo de semilla. Sin embargo, la producción en semillas es lenta y para acelerar el proceso hemos transferido la tecnología de la fusión a oleosinas de plantas al sistema de *Pichia pastoris*. Usando este nuevo sistema hemos obtenido rendimientos comercialmente relevantes con producciones de 180 mg/l de cultivo en sólo 4 días. La acumulación de PAF102 en las LDs de las semillas de arroz y de la levadura facilita enormemente su extracción por simple flotación en soluciones densas, permitiendo la recuperación de péptido activo frente a hongos patógenos.

Finalmente, hemos demostrado que tanto AfpA y AfpB producidas en plantas, como los extractos de plantas enriquecidos estas proteinas, son eficaces en la prevención de infecciones fúngicas en cultivos económicamente relevantes, tales como la podredumbre gris causada por *Botrytis cinerea* en hojas y frutos de tomate, el quemado del arroz causado por *Magnaporthe oryzae*, o las infecciones de las semillas de arroz por *Fusarium proliferatum*. Nuestros resultados proporcionan un sistema de producción sostenible de AFPs y demuestran su eficacia en la protección de las plantas contra las infecciones fúngicas, apoyando firmemente su uso como "fungicidas verdes" eficaces y respetuosos con el medio ambiente en la protección de cultivos y postcosecha.

Resum

Els fongs patògens de plantes causen importants pèrdues en les collites, posant en perill la seguretat i qualitat alimentària. Els pèptids antimicrobians (AMPs) mostren una activitat lítica potent i duradora específicament enfront de microorganismes, de manera que tenen un gran potencial com a nous fungicides naturals per al control dels fongs patògens. La seva explotació requereix de sistemes de producció ràpids, eficaços, econòmics i segurs. El principal objectiu d'aquest treball era desenvolupar sistemes de producció sostenibles de AMPs, i la seva caracterització en el control d'infeccions fúngiques per avançar en la seva aplicació en l'agricultura.

Les proteïnes antifúngiques (AFPs) secretades per fongs filamentosos són un grup de AMPs rics en cisteïnes, molt estables, actius específicament enfront de fongs. En aquest estudi vam demostrar que les plantes de *Nicotiana bentamiana* són una excel·lent biofactoria de AFPs mitjançant expressió transitòria usant un nou vector derivat del virus de mosaic del tabac. Utilitzant aquest sistema de producció en plantes, hem produït eficientment dues AFPs molt actives enfront de fongs fitopatògens, la AfpA de *Penicillium expansum* i la AfpB de *Penicillium digitatum*. Hem descobert que el compartiment subcel·lular on s'acumulen les AFPs té un impacte important en la producció obtinguda, probablement perquè la seva compartimentació evita la toxicitat cap a les cèl·lules vegetals. Els valors més alts es van obtenir quan les proteïnes es van acumular en els vacúols, aconseguint fins 0,170 mg / g de fulla en el cas de la proteïna més activa AfpA i fins a vuit vegades més per a la AfpB (1,2 mg / g de fulla). També vam demostrar que els extractes crus de plantes que contenen AFP són actius enfront a fongs, sense necessitat de purificar les proteïnes reduint considerablement el processament del material vegetal i els costos de producció. Per tant, el sistema desenvolupat és eficient per a la producció de AFPs, i també és econòmic i segur ja que es basa en plantes.

A més, hem desenvolupat un sistema alternatiu per a la producció del pèptid antifúngic PAF102 que prèviament no s'havia pogut produir biotecnològicament. Aquest sistema es basa en acumular el pèptid en les gotes lipídiques (LDs) mitjançant la fusió a una proteïna oleosina de plantes. Mitjançant aquesta estratègia, hem produït PAF102 en llavors d'arròs en quantitats de 20 mg per gram de llavor. No obstant això, la producció en llavors és lenta i per accelerar el procés hem transferit la tecnologia de la fusió a oleosinas de plantes al sistema de *Pichia pastoris*. Usant aquest nou sistema hem obtingut rendiments comercialment rellevants amb produccions de 180 mg / l de cultiu en només 4 dies. L'acumulació de PAF102 a les LDs de les llavors d'arròs i del llevat facilita enormement la seva extracció per simple flotació en solucions denses, permetent la recuperació de pèptid biològicament actiu.

Finalment, hem demostrat que tant AfpA i AfpB produïdes en plantes, com els extractes de plantes enriquits en aquestes proteines, són eficaços en la prevenció d'infeccions fúngiques en cultius econòmicament rellevants, com ara la podridura grisa causada per *Botrytis cinerea* en fulles i fruits de tomàquet, la piricul ariosis causada per *Magnaporthe oryzae*, o les infeccions de les llavors d'arròs per *Fusarium proliferatum*. Els nostres resultats proporcionen un sistema de producció sostenible de AFPs i demostren la seva eficàcia en la protecció de les plantes contra les infeccions fúngiques, donant ferm suport per al seu ús com a "fungicides verds" eficaços i respectuosos amb el medi ambient en la protecció de cultius, durant el seu periode al camp o postcollita.

Abbreviations

Aa: Amino acid
AFP: Antifungal Peptide
AMP: Antimicrobial Peptide
bp: base pair
cDNA: Complementary DNA
CecA: Cecropin A
CP: Coat Protein
Da: Daltons
DGAT1: Diacylglycerol O-acyltransferase 1
DNA: Deoxyribonucleic acid
Dpi: days post-infection/inoculation
dsRNA: Double stranded RNA
ECF: extracellular fluid
EDTA: Ethylenediaminetetraacetic acid
ER: Endoplasmic Reticulum
EV: Empty vector
FDA: fluorescein diacetate
FPLC: Fast Performance Liquid Chromatography
FT: Flow Through
Fwd: Forward sequence
GAP: Glyceraldehyde- 3-Phosphate Dehydrogenase
GFP: Green Fluorescent Protein
Glb1: Globulin
GluB1: Glutelin B1
GluB4: Glutelin B4
GoI: Gene of Interest
HRP: Horseradish peroxidase
KDa: Kilo Daltons
KDEL: ER retention signal sequence
LB: Left border
LD: Lipid Droplet
MAPK: Mitogen Activated Protein Kinase
MES: 2-Morpholino Ethanesulfonic Acid
MIC: Minimal Inhibitory Concentration
MP: Movement Protein
MS: Mass Spectrometry
MW: Molecular Weight
NCBI: National Center for Biotechnology
NE: No Effect
Nos: Nopaline Synthase
NT: No Tested

OB: Oil Body
OD: Optical Density
Ole18: Oleosin 18 kDa protein
ORF: Open Reading Frame
P35S: Cauliflower mosaic virus (CaMV) 35S promoter
PAGE: Polyacrylamide Gel Electrophoresis
PB: Protein Body
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PDA: Potato Dextrose Agar
PDB: Potato Dextrose Broth
PK: Protein Kinase
Plb: Protein P1b
PRS: protease recognition site
qPCR: Quantitative PCR
RB: Right border
RdRp: RNA-dependent RNA polymerase
Rev: Reverse sequence
RNA: Ribonucleic acid
ROS: Reactive Oxygen Species
SD: Standard Deviation
SDS: Sodium Dodecyl Sulphate
SE: Standard Error
SP: Signal Peptide
T35S: Cauliflower mosaic virus (CaMV) 35S terminator
TAG: Triacylglycerol
TEV: Tobacco Etch Virus
TMV: Tobacco mosaic virus
ToMV: Tomato mosaic virus
TRBO: TMV RNA-based Overexpression
UTR: Untranslated Region
UV: Ultraviolet
VS: vacuolar sorting signal
WT: Wild Type
YPD: Yeast extract, Peptone, Dextrose
δ: Ribozyme

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General introduction

1. Fungal pathogens

Fungi are an extraordinary ubiquitous and diverse group of microorganisms. Currently, there are approximately 1.5 million species of fungi, from which only about 5% of fungi are officially classified. As heterotrophic microorganisms that get nutrients by absorption, many fungi are parasites. Some parasitic fungi are pathogenic, causing diseases in host organisms. Disease-causing fungi that infect plants, animals or humans are an increasing threat to human and animal health, and to food safety and security (Fisher *et al*., 2018; Friedman and Schwartz, 2019).

The impact of fungal infections in human health is not as widely recognized as other diseases. However, they should not be ignored because they have an impact on over a billion of people, and more than 150 million suffer severe fungal diseases with fatal consequences in immunocompromised or immunosuppressed patients. Fungal infections kill more than 1.6 million people, which outreach the number of people dying from tuberculosis and malaria (Bongomin *et al*., 2017). Fungal species of *Aspergillus*, *Candida*, *Cryptococcus* and *Pneumocystis* genus are the causative agents of major invasive mycoses in human that result in more than 90% of reported fungal-related deaths (Almeida *et al*., 2019).

Infections caused by phytopathogenic fungi are also a widespread problem in agriculture (Bebber and Gurr, 2015). Microbial diseases cause severe crop losses, and 65-80% of these losses are caused by fungi around the world (Fisher *et al*., 2012; Moore *et al*., 2011). Fungi cause huge losses in the five most important crops: rice, wheat, corn, soybean and potato. If these losses were mitigated, these crops would be sufficient to feed 8.5% of the 7 billion people in 2011 (Fisher *et al*., 2012). In addition to causing crop losses directly, some plant pathogens spoil crops by producing harmful toxins. Mycotoxins are secondary metabolites produced by fungi that contaminate crops, vegetables, fruits, and animal feeds, and pose critical challenges in food and feed safety because they represent one of the main concerns in chronic toxicity (Dellafiora and Dall'Asta, 2017).

Fungal pathogens of relevance in agriculture

There are a great number of plant pathogenic fungi that cause crop diseases. This PhD Thesis is focused mainly on the following economically relevant phytopathogens: *Magnaporthe oryzae*, *Botrytis cinerea*, *Penicillium digitatum,* and *Fusarium proliferatum*.

1.1. *Magnaporthe oryzae*

M. oryzae is a filamentous ascomycete that causes the blast disease of rice crops. Rice is the most widely consumed food crop and the staple food for half of the world's population, especially in Asia, and increasingly important in Africa and South America. The blast disease is the most serious threat to global rice production, causing 10-35% losses of rice harvest in the world, and there are many efforts to control it given its economic importance (Dean *et al*., 2012).

The fungus infects almost all parts of rice plants like leaves, leaf sheath, collar, node, neck and panicles. The infection is initiated when a three-celled conidium of *M. oryzae* attaches on the surface cuticle arriving either through wind or splash dispersion (Wilson and Talbot, 2009). The teardrop-shaped conidium releases an adhesive at the tip, sticking it tightly to the plant surface. After approximately 6 h, it quickly germinates forming a single short germ tube that differentiates at its tip a highly specialized infection structure termed appressorium. Usually within 12-24 h, turgor pressure increases within the appressorium emerging a

penetration peg into leaf tissues. Then, the penetration peg differentiates into infection hyphae that move into adjacent cells in 48 h. Four to five days after infection, eye-shaped necrotic lesions form on the surface of infected rice leaves. Then, the fungus emerges bearing conidia from the lesions, and conidia are dispersed to begin the infection cycle again (Figure 1) (Wilson & Talbot, 2009).

Other than rice, *M. oryzae* can also infect more than other 50 grass species, including economically important crops, such as millet, barley, lolium and wheat causing blast diseases on them (Cruz and Valent, 2017; Inoue *et al*., 2017; Ou, 1980; Talbot, 2003). For example, wheat blast has been reported to be a fast-acting and devastating fungal disease threating food safety and security in Brazil, where it was first identified in 1985 (Inoue *et al*., 2017). Currently, the disease is widespread in South America, and recent outbreaks have been reported in Bangladesh and India, affecting as much as 15.000 hectares of land, reducing wheat yields around 51% in the affected regions. Wheat blast has become one of the most important diseases affecting wheat crops (Ceresini *et al*., 2019; Islam *et al*., 2016).

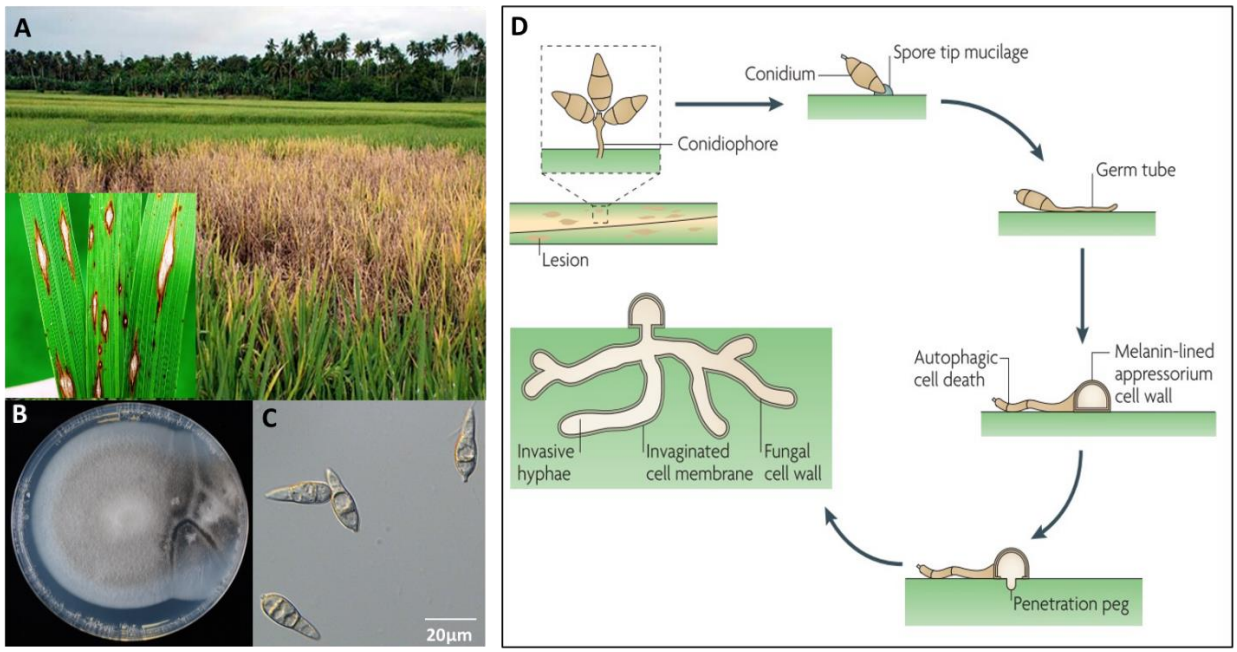

Figure 1. Rice blast disease and *Magnaporthe oryzae*. (A) images of rice fields affected by blast disease. Details of leaf symptoms are shown in the inset box. Picture taken from International Rice Research Institute (IRRI) via Flickr (CC BY-NC-SA 2.0). (B) Mycelium of *M. oryzae* after growth *in vitro* plates at 25°C for 15 days. (C) *M. oryzae* conidia. (D) Life cycle of the rice blast fungus *M. oryzae*. Scheme from Wilson and Talbot, 2009.

Blast disease caused by the fungus *M. oryzae* on cereal crops is a serious problem over the world. Resistant varieties and the application of chemical fungicides have been used for blast control. However, the useful lifespan of many resistant varieties is only 2 or 3 years after planting, due to the breakdown of the resistance in the face of the highly variable fungus population. The application of chemical fungicides is costly and environmentally detrimental, as well as long-term use also leads to resistance emergence (Miah *et al*., 2017; Skamnioti and Gurr, 2009; Wang and Valent, 2017). Hence, there is an urgent need to develop new strategies to provide durable resistance that are environmentally friendly and cost-effective. Among such new strategies, the development and use of transgenic resistant plants, as well as biocontrol agents, might represent good alternatives for blast management in the future (Amruta *et al*., 2016; Miah *et al*., 2017). The purpose of this thesis is to develop novel antifungal agents based on natural antimicrobial peptides to be

applied for the control *M. oryzae* fungal infections. These novel biofungicides with potent activities will be ecofriendly and effective strategies for crop protection.

1.2. *Botrytis cinerea*

B. cinerea is a necrotrophic plant pathogen that causes gray mold in more than 200 vegetable crops worldwide. It usually infects tissues at early stage of crop development, causing observable disease symptoms or remaining quiescent until post-harvest period. The sources of primary inoculum of *B. cinerea* range from overwintering sclerotia to conidia, or directly mycelia from infected neighbored plants. Under optimal conditions, sclerotia and mycelia produce conidia. Then, conidia are moved by the wind or splashing water onto flowers, fruits, or leaves, where they germinate and enter the plant. Germinating conidia rarely penetrate healthy tissue directly, they usually enter through wounds. Infected symptoms appear as light grayish, mushy spots on leaves, flowers and fruits. Sometimes, spots are covered by a coating of gray fungus spores, especially when humidity is high. Fruits or plants became rusty, and subsequently often develop black, stone-like sclerotia under rotted parts (Figure 2) (Petrasch *et al*., 2019).

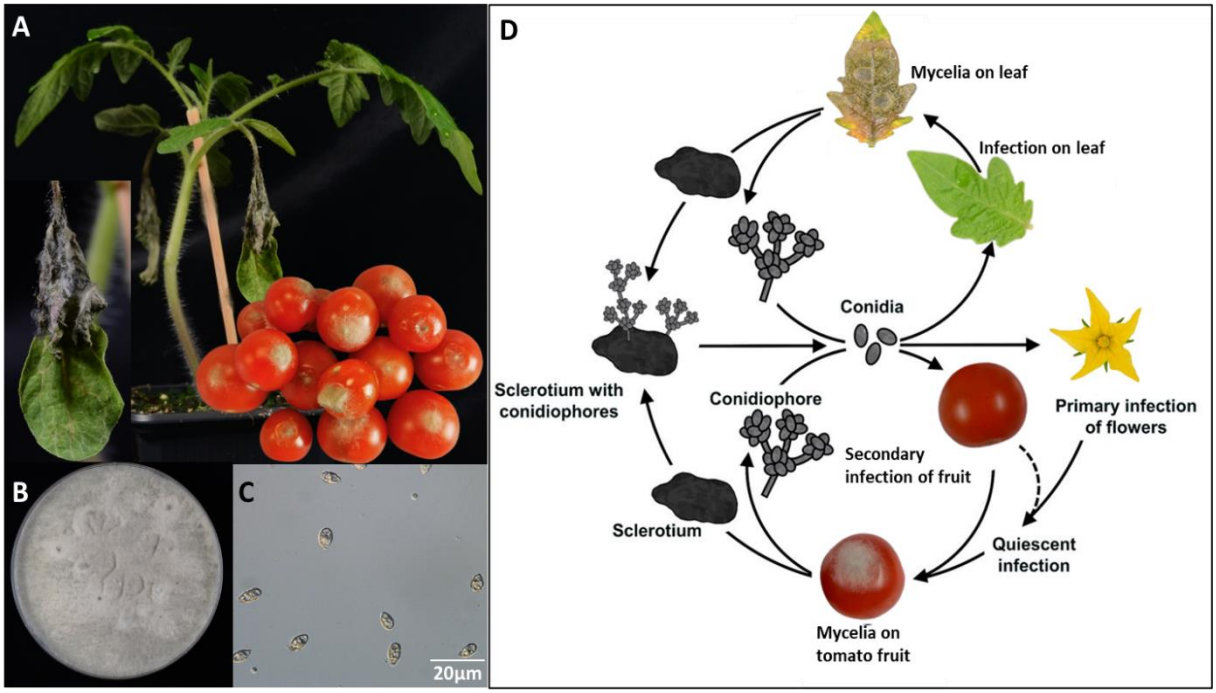

Figure 2. *Botrytis cinerea* and grey mold disease of tomato. (A) Grey mold symptoms of infected tomato leaves and fruits. (B) *B. cinerea* mycelia grown in potato dextrose agar (PDA) medium. (C) Conidia of *B. cinerea*. (D) Life cycle of *B. cinerea*. Image modified from Petrasch *et al*., 2019

Gray mold disease has a serious impact on different economically important crops, including tomato (Dean *et al*., 2012). Tomato is the world's largest vegetable crop that has achieved tremendous popularity over the last century. It is also known as protective food because tomatoes are a good source of vitamins A and C. The disease can occur in outdoor fields, greenhouses and during postharvest stages (transportation and storage). Gray mold also affects many other vegetables including chickpea, lettuce, broccoli, lima beans, cabbage, muskmelon, pepper and potato; and small fruit crops including grape, strawberry, and raspberry (Williamson *et al*., 2007). The annual economic losses and costs of *B. cinerea* disease are very difficult to estimate due to the broad host ranges. It is difficult to control this pathogen because of the diverse hosts,

variety of attack modes, and its ability to survive as sclerotia and conidia for a long time in favorable or unfavorable environments. Until now, the application of botryticides is the principal method for controlling *B. cinerea*. Botryticides represents roughly 10% of the global fungicide market (Dean *et al*., 2012). However, the increasing public concern about the synthetic fungicide residues on fruits and vegetables, and the growing problem of resistance development in pathogen populations, make necessary to find alternative strategies for *B. cinerea* control based on natural compounds to replace synthetic fungicides (Abbey *et al*., 2018).

1.3. *Penicillium digitatum*

P. digitatum is a necrotrophic filamentous fungus that causes the widespread postharvest disease of citrus fruits known as green mold, which is a very devastating disease responsible for more than 90% of citrus fruit economic losses (Julca *et al*., 2016; Palou, 2014).

The fungus is a strict wound pathogen that penetrates the fruit through surface injuries during harvest in the field or after harvest handling during transportation and marketing. Infections are initiated when conidia germinate on peel injuries on the fruit surface. At 24°C, conidia grow rapidly and invasion take place within 24-36 hours, and symptoms develop subsequently. Early symptoms appear as a moist depression on the peel that gradually expands to a circular colony of white mycelium that becomes green at the center when fungal sporulation occurs. The lesions spread rapidly, and finally the fruit decreases in size, spoils and rots (Figure 3) (Holmes and Eckert, 1999; Palou *et al*., 2016).

Traditionally, the application of synthetic fungicides is the conventional method to control *P. digitatum*. The most frequently applied are the benomyl, thiabendazole and imazalil fungicides (Palou, 2014). However, the proliferation of resistant fungal strains, as well as the public pressure to obtain healthy and safe organic fruits has driven research to find novel and durable strategies to control the disease.

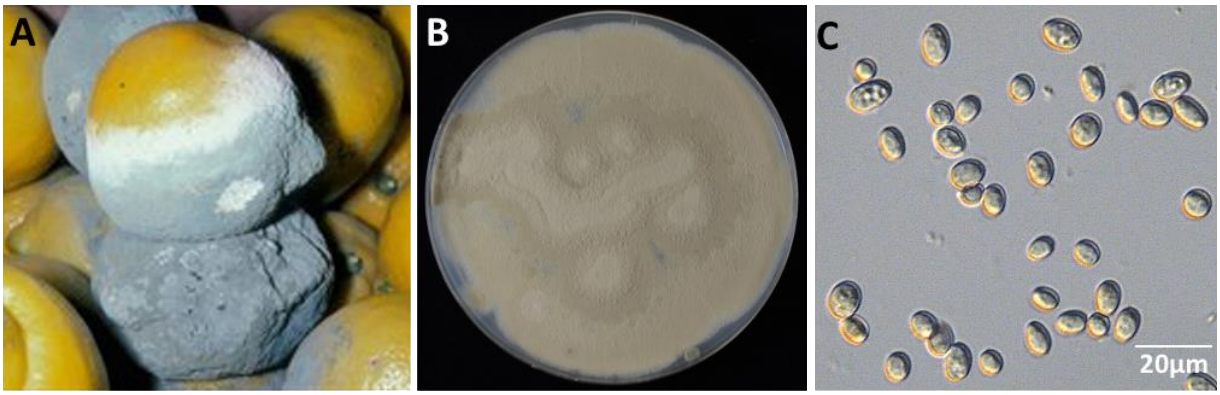

Figure 3. *Penicillium digitatum* and orange green mold (A) Orange fruit infected with *P. digitatum*; (B) Colonies of *P. digitatum* in PDA medium; (C) Conidiophores of *P. digitatum*.

1.4. *Fusarium proliferatum*

Fusarium sps are soil and seed borne fungal pathogens. They cause important losses in the productivity of economically important cereals, such as rice, maize, sorghum, bean and soybean, but also reduce the quality of grains by contamination with mycotoxins, such as fumonisins, which are a serious concern for food safety globally (Kushiro *et al*., 2012). *F. proliferatum* has been shown also to occur regularly on fruits and

vegetable roots, such as banana, tomato and watermelon, and ornamental plants, such as carnation, orchid and palms (Zhang *et al*., 2013). The fungus is taxonomically close to *Fusarium fujikuroi*, belonging to *Gibberella fujikuroi* species complex (previously known as *F. moniliforme*), and together with *F. verticillioides* and *F. andiyazi* are commonly found associated with Bakanae disease of rice (Kushiro *et al*., 2012; Quazi *et al*., 2013; Wulff *et al*., 2010). Bakanae disease occurs throughout world rice growing regions causing empty panicles and important grain losses (Ou, 1985; Wulff *et al*., 2010) (Figure 4 A-D) (Bashyal *et al*., 2016). ***F. proliferatum*** damages pollen cells and anthers of rice causing the spikelet rot disease (Lei *et al*., 2019). It also causes ear rot of maize and stalk rot of sorghum (Mesterházy *et al*., 2012).

F. proliferatum grows rapidly and produce ***woolly*** to ***cottony***, flat spreading colonies, which are initially white, cream or tan, subsequently becoming tinged with purple (Stępień *et al*., 2011) (Figure 4E). The fungus produces many spores on the surface of infected plants that can survive for several years in soil. Infection usually occurs through the plant root but also when spores blown or splashed from nearby plants fall into wounded tissues (Proctor *et al*., 2009).

Currently, the most common method to control the fungus is the use of chemical fungicides, such as natamycin and vorionazole (Sun *et al*., 2018). However, chemical fungicides are expensive, pollute ecosystems and promote the development of resistance in fungal populations, including fungal pathogens of humans.

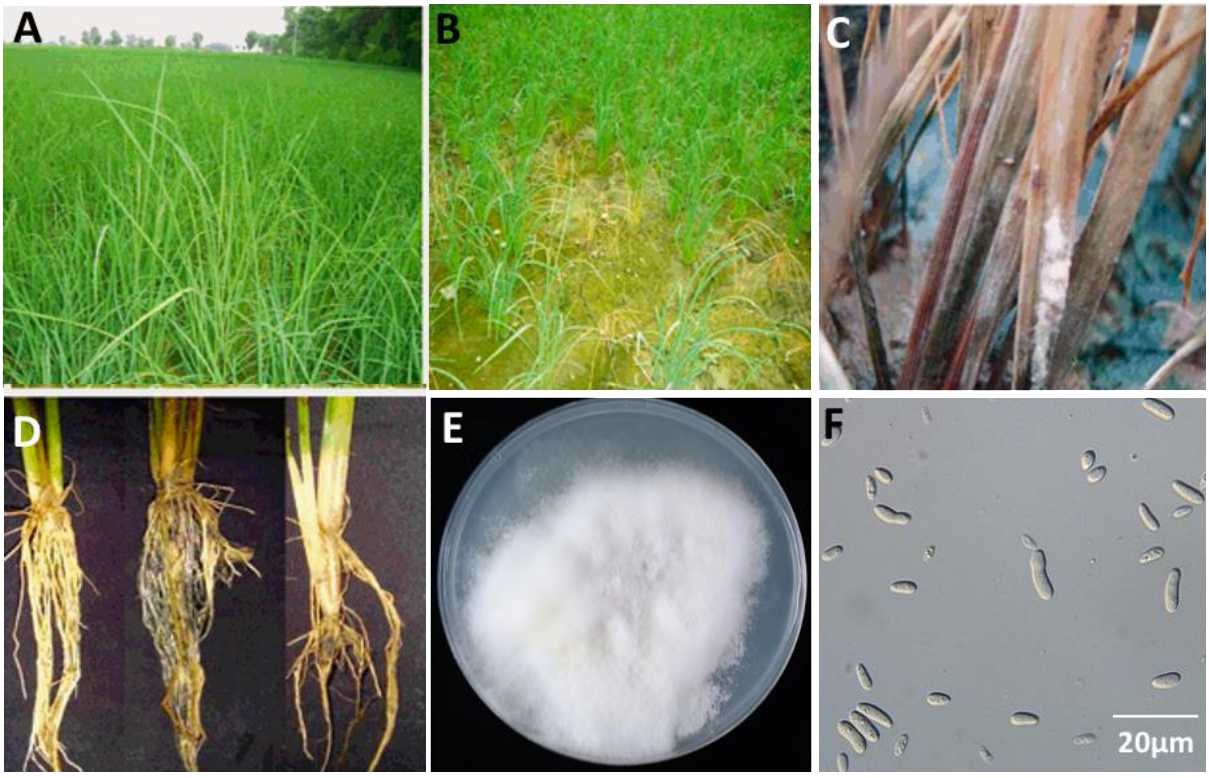

Figure 4: *Fusarium* spp. and rice Bakanae disease. Images of rice field with Bakanae disease showing seedling elongation (A) and rotting (B-D). Images are from Bashyal *et al*., 2016. Pictures of *F. proliferatum* mycelia in PDA plates (E) and conidia (F).

2. Fungicides

Fungicides are used to kill, mitigate or inhibit the growth of parasitic fungi. The number of effective fungicides is modest due to the high similarity between fungal pathogens and their hosts, in terms of cell morphology, physiology and metabolism. This similarity hampers the identification and development of compounds that target specifically fungi without affecting their hosts. Currently, only three antifungal drug classes are licensed for use in clinic, veterinaria or in crop and postharvest protection. These classes target different parts of fungal cells and are the following:

2.1. Antifungal agents acting on the fungal plasma membrane

The composition of the fungal plasma membrane is different from other eukaryotic cells, including mammalian and plant cells. Most of the fungal membranes contain ergosterol to maintain their integrity, in a similar manner that mammalian cells contain cholesterol or plant cells β-sitosterol. This difference on sterol composition at the plasma membrane has been long recognized as a target for antifungal molecules. The major groups of antifungal agents in use acting on plasma membrane include azoles and polyenes. They inhibit the fungal growth by inhibiting the synthesis of ergosterol or by interacting directly with ergosterol, respectively.

2.1.1. Inhibiting the synthesis of ergosterol

Azole molecules inhibit the fungal growth by inhibiting the synthesis of ergosterol. They are unsaturated aromatic molecules containing at least one nitrogen atom that inhibit the biosynthesis of ergosterol by targeting the lanosterol 14 α-demethylase (also known as Cyp51). Inhibition of ergosterol biosynthesis leads to the accumulation of toxic sterol precursors, reducing integrity of the fungal cell membrane and arresting fungal growth. Azoles were first introduced in the 1970s and used in different fields. The fluconazole is used in medical therapies to inhibit animal fungal pathogens. The triadimefon, flusilazole, tebuconazole, imazalil and cyproconazole are used in crop and postharvest protection against phytopathogens (Brauer *et al*., 2019). They are common antifungal agents in fungal control because of their high efficiency and broad-spectrum activity. However, nowadays, fungal resistance to azole is becoming a major public health problem. Moreover, the widespread use of azole in agriculture has serious environmental problems due to the contamination of air, soil, and plants (Azevedo *et al*., 2015).

2.1.2. Interacting directly with ergosterol

Polyenes are poly-unsaturated organic compounds generally containing 12-37 carbons and 4-7 conjugated double bonds (Ghannoum and Rice, 1999). They interact with ergosterol physiochemically and affect the stability of fungal plasma membrane. The mechanism of action is that the unsaturated part of the polyenes is directly combined with the ergosterol molecules in the plasma membrane resulting in the production of sterol complexes. The complexes provoke the membrane permeabilization, and eventually cause the leakage of cell content.

The polyene antifungal drugs include: natamycin, amphotericin B, nystatin, candicidin, pimaricin, methyl partricin, and trichomycin. Amphotericin B, from Streptococcus nodules, is the most effective and broad-spectrum therapeutic drug available for the treatment of fungal systemic infections. However, its untoward effects limit its clinical utility, such as the toxic side effects on the urinary system, especially kidney damage, and the irritation response to the digestive tract, which can cause anemia and reduce red blood cell

production (Fuentefria *et al*., 2018; Hamill, 2013). Natamycin are used to prevent *Fusarium cuneiro* infection in plants, and also can be used in the food industry as a preservative of mold growth (Dalhoff and Levy, 2015).

2.2. Antifungal agents acting on the fungal cell wall

The fungal cell wall contains mostly mannoproteins in the outer region, a thick intermediate layer of β-glucans, and a thin layer of chitin in the inner region. Mammalian cells lack cell walls and plant cells have a highly different cell wall to fungi. Hence, the specific elements of fungal cell walls have been long identified as targets for antifungal agents (Brogden, 2005; Georgopapadakou and Tkacz, 1995). Fungicides that have the ability to affect the cell wall of fungi have been discovered and described over the past forty years (Bowman and Free, 2006; Fuentefria *et al*., 2018). Most of these drugs act by inhibiting β-glucan synthase, such as echinocandins, rezafungin, triterpenoids (Aguilar-Zapata *et al*., 2015; Denning, 2003; Sandison *et al*., 2017; Walker *et al*., 2011), and some drugs are chitin synthase inhibitors, such as nikkomycins, polyoxins, and plagiochin (Lima *et al*., 2019; Chaudhary *et al*., 2013).

2.3. Antifungals disrupting DNA or RNA synthesis in a fungal-specific manner

As a consequence of the similarities of nucleic acid synthesis between fungi, plants and mammalian cells, it is difficult to inhibit specifically fungal nucleic acid synthesis. However, specific differences between cell types do exist and have been exploited for the development of antifungal agents. An example of this class of antifungals is the 5-fluorocytosine that interferes with fungal DNA and RNA synthesis. This compound itself is not toxic, but upon uptake into fungal cells by the cytosine permease, it is converted into the toxic 5-fluorouracil compound by the cytosine deaminase (Morris and Blaese, 2002). The 5-fluorouracil is incorporated into RNAs causing premature termination of RNA chain, and inhibits DNA synthesis by affecting thymidylate synthase (Vermes *et al*., 2000). The 5-fluorocytosine is nontoxic to mammalian cells because they lack the cytosine deaminase enzyme. Despite its numerous pharmacological advantages, the use of 5-fluorocytosine in clinical practice is decreasing because of the frequent occurrence of innate or acquired resistance in fungal pathogens, and the observed side effects to human cells since 5-fluorocytosine could be converted into 5-fluorouracil by human intestinal microflora (Vandeputte *et al*., 2012). However, the 5-fluorocytosine is being used in synergistic combinations with amphotericin B or azoles to get better fungicide activities. It is also frequently used for seed protection against fungal infection and in synergistic fungicidal compositions against various ascomycetes fungi in cereal crops. Other agricultural fungicides that exert their effect by interfering with DNA/RNA synthesis are hydroxypyrimidines that cause overexpression of the adenine phosphoribosyl transferase affecting the nucleotide pool (Yang *et al*., 2011). Also the hymexazol is a systemic soil and seed fungicide that inhibit DNA/RNA synthesis and it is used for the control of plant diseases caused by *Aphanomyces*, *Pythium*, *Fusarium* and *Corticium* species (Jampilek, 2016).

3. Antimicrobial peptides

Antimicrobial peptides (AMPs) are small peptides that exhibit lytic or inhibitory activity against bacteria, fungi and viruses. They have been found virtually in all living organisms from microorganisms to humans. AMP discovery likely started in plants, followed by the description in the 60's of brombinin in frogs and lactoferrin from milk. In the 80's, the cecropins were discovered and identified in the hemolymph of the

Hyalophora cecropia (Hultmark *et al*., 1980; Steiner *et al*., 1981). Since then, more than 1400 AMPs have been discovered and isolated (Pasupuleti *et al*., 2012).

AMPs are a diverse group of peptides with common features: (a) they are relatively short peptides (6~100 amino acids); (b) most AMPs carry a positive net charge of +2 to +11 due to the overrepresentation of lysine and arginine residues; (c) they have an amphiphilic design in which hydrophilic, cationic residues clustered together and separated from hydrophobic residues (Zasloff, 2002).

AMPs can be categorized according to their secondary structure into different groups： (a) linear peptides that adopt an α-helical structure, such as cecropin A from silk moth or the magainin from frogs; (b) β-sheet structure stabilized by one or more disulfide bridges that include defensins or defensin-like proteins found in fungi, insects, plants and mammalians; and (c) linear peptides with extended structures that do not possess a specific structural motif but rather are defined by a high content of specific residues, such as histidine, arginine, glycine or tryptophan (Zasloff, 2002). Three-dimensional structures of representative AMPs are shown in Figure 5, sequences and sources of selected AMPs are shown in Table 1.

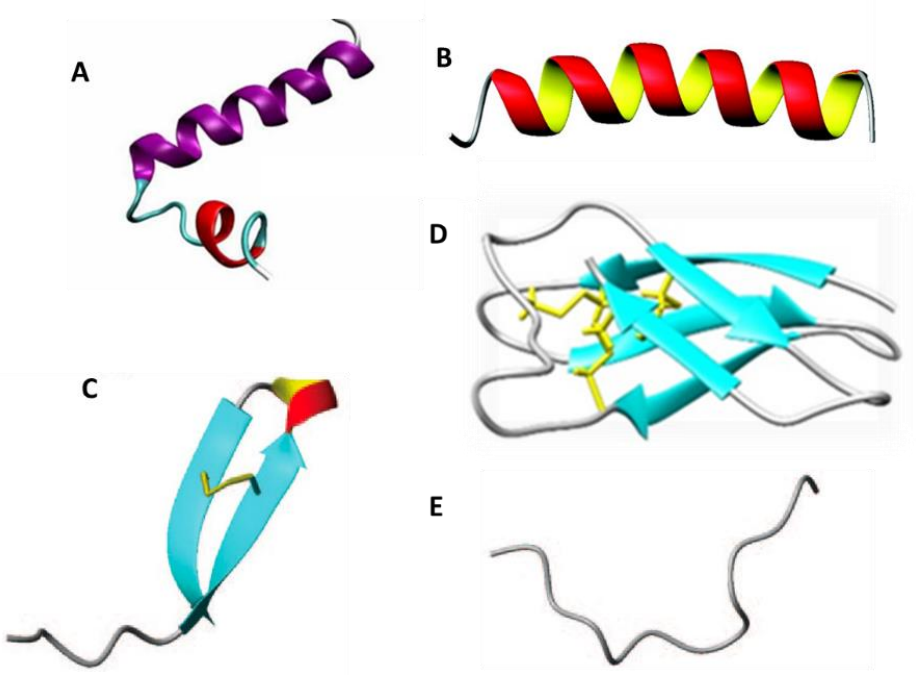

Figure 5: Three-dimensional structures of representative AMPs. (A, B) α-helical peptides corresponding to the insect cecropin A (A) and the frog magainin-2 (B); (C, D) β-sheet peptides corresponding to the crab polyphemusin (C) and the fungal defensin-like protein AFP (D); (E) extended peptide corresponding to the tryptophan-rich peptide indolicidin. Images adapted from Jenssen *et al*., 2006.

During the last years, due to the rapid increase of drug-resistant microorganisms to conventional antibiotics, together with the lack of new effective compounds, AMPs gained relevance as promising substitutes or alternatives to chemical antibiotics. Major obstacles for the exploitation of AMPs include their potential toxicity to the host cells, their sensitivity to proteases, and the high cost of their chemical synthesis. In this thesis, we work with different AMPs representative of the different structural classes: the α-helical peptide cecropin A, the extended synthetic PAF102 peptide, and ß-sheet antifungal peptides (AFPs) from fungal origin.

Table 1. Representative examples of antimicrobial peptides categorized according to their secondary structure

Group	Peptide	Mature amino acid sequence	Source	Reference
α-helical	Cecropin A	KWKLFKKIEKVGQNIRDGIKAGPAVAVVGQATQIAK	Silk moth	(Steiner *et al*., 1981)
	Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	Frog	(Zasloff, 1987)
	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human	(Gudmundsson *et al*., 1996)
	Temporin L	FVQWFSKFLGRIL	Frog	(Slmmaco *et al*., 1996)
	Melittin	GIGAVLKVLTTGLPALISSWIKRKRQQ	Insect	(Haux *et al*., 1967)
	Pexiganan	GIGKFLKKAKKFGKAFVKILKKa	Frog	(Ge *et al*., 1999)
β-sheeted	AFP	ATYNGKCYKKDNICKYKAQSGKTAICKCYVKKCPRD GAKCEFDSYKGKCYC	*A. giganteus*	(Lacadena *et al*., 1995)
	Tachyplesin-I	$KWC_1FRVC_2YRGIC_2YRRC_1R$	Horseshoe crab	(Nakamura *et al*., 1988)
	Protegrin I	$RGGRLC_1YC_2RRRFC_1VC_2VGR$	Pig	(Qu *et al*., 1997)
	HBD-1	$DHYNC_1VSSGGQC_2LYSAC_3PIFTKIQGTC_2YRGKAKC_1C_3K$	Human	(Bensch *et al*., 1995)
Extended	Indolicidin	ILPWKWPWWPWRR	Bovine	(Rozek *et al*., 2000)
	Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	Human	(Smet and Contreras, 2005)
	Apidaecin	GNNRPVYIPQPRPPHPRI	Honeybee	(Li *et al*., 2006)
	PAF26	RKKWFW	Combinatorial library	(López-García *et al*., 2002)
	PAF102	GHRKKWFWAGPARRKKWFWAGPAWRKKWFW	Rational design	(López-García *et al*., 2015)

Subscript numbers represent cysteine residues paired in disulfide linkage

3.1. Cecropins

Cecropins are renowned cationic antimicrobial peptides that were first identified from the hemolymph of *Hyalophora cecropia*. They are short peptides (typically 31-39 amino acids), and contain a strong basic N-terminal amphipathic α-helical domain linked by an intermediate hinge region to a hydrophobic C-terminal α-helical domain (Steiner *et al*., 1981).

There are several cecropin peptides including A, B, C, D and P1. Their main difference lies in the amino acid sequence, which affects their structure and functions (Steiner *et al*., 1981; Wu *et al*., 2018). Cecropin A (CecA) possess *in vitro* antibacterial and antifungal activity against several important phytopathogenic pathogens. Among them are included important plant pathogenic bacteria, such as *Erwinia amylovora*, *Pseudomonas syringae*, *Pleomorphomonas oryzae*, *Xanthomonas vesicatoria*, *Bacillus megaterium* (Ferre *et al*., 2006)*;* and important plant pathogenic fungi, such as *M. oryzae, Penicillium crustosum, B. cinerea, Fusarium* spp., *Aspergillus* spp. (Cavallarin *et al*., 1998; DeLucca *et al*., 1997). The expression of CecA in plants conferred protection against pathogens. For instance, the expression of *CecA* gene in rice confers resistance to the rice blast fungus *M. oryzae* and *Fusarium verticillioides,* and against the bacterial pathogen *Dickeya dadantii* (Bundó *et al*., 2014; Coca *et al*., 2006). CecA also has activity against other animal and human fungi, like *Beauveria bassiana*, *Candida albicans* (Lu *et al*., 2016; Yun and Lee, 2016). Cecropin B also exhibits *in vitro* activity against an important number of plant pathogenic bacteria (Jan *et al*., 2010; Mills and Hammerschlag, 1993), and its expression in rice confers resistance against *Xanthomonas oryzae* (Sharma *et al*., 2000) or in tomato against *Ralstonia solanacearum* and *Xanthomonas campestris* (Jan *et al*., 2010). In addition, Cecropin A and B exert selective cytotoxic and antiproliferative efficacy in bladder cancer cells with limited cytotoxic effects on benign cells (Suttmann *et al*., 2008). Cecropin D are less cationic and more hydrophobic than A and B, but shows also antibacterial activity against both Gram-positive and Gram-negative bacteria (Guo *et al*., 2012). Cecropin P1 was isolated from *Ascaris suum*, a parasitic nematode that resides in the pig intestine causing pig ascariasis, and shows a significant inhibitory effect on the human fungal pathogen *Candida albicans* (Zakharchenko *et al*., 2017).

3.2. PAFs

PAFs are Peptides with AntiFungal activity that are synthetic designs with improved properties as compared to natural antimicrobial peptides, such as high stability and high specificity against different plant and human pathogens. They are derivatives from the model PAF26 (RKKWFW) identified from a library of hexapeptides using a combinatorial chemistry approach. The screening was selective for peptides having high activity against filamentous fungi, but not against yeast, bacteria or human red blood cells (López-García *et al*., 2002). PAF26 showed important activity against the phytopathogens *P. digitatum, P. italicum*, *P. expansum*, *B. cinerea* and *F. oxysporum*, the human pathogens *Candida albicans*, *Aspergillus fumigatus* and several dermatophytes (López-García *et al*., 2007; Muñoz *et al*., 2012), and the model fungus *Neurospora crassa* (Muñoz *et al*., 2012). PAF26 is a cationic tryptophan-rich peptide that shows cell penetrating activity (Muñoz *et al*., 2013). Later, PAF102 was designed as a modified concatemer of the PAF26 for improved properties to be biotechnologically produced. PAF102 maintained cell penetrating properties, and high stability against proteases (López-García *et al*., 2015). It shows higher potent and specific antifungal activity against economically relevant phytopathogens, such as *F. proliferatum*, *P. digitatum*, *M. oryzae, F. oxysporum*, and *B. cinerea*, and very low toxicity to plant and mammalian cells.

3.3. Fungal antifungal proteins (AFPs)

Antifungal proteins (AFPs) secreted by filamentous fungi are small (45-64 amino acid residues), usually basic, and secreted peptides (Galgóczy *et al*., 2010). They are structurally related to defensins, a type of antifungal proteins found in insects, plants, and mammalian species, which are integral components of innate immunity (Hegedüs and Marx, 2013). AFPs contain the conserved ɣ-core motif, and have six /eight cysteine residues forming three/four disulfide bridges (Batta *et al*., 2009; Campos-Olivas *et al*., 1995). They fold into compact structures, providing them with high stability to extreme pH, high temperature and proteases. AFPs exhibit antifungal activity at very low concentrations against important human and plant fungal pathogens, and do not have toxic effects on mammalian cells or plants (Delgado *et al*., 2015; Marx *et al*., 2008; Moreno *et al*., 2003; Vila *et al*., 2001; Virágh *et al*., 2014). These characteristics make AFPs as promising candidates to be exploited as novel fungicides not only against human, but also in plant pathogens (Galgóczy *et al*., 2017; Hegedüs and Marx, 2013; López-García *et al*., 2010; Meyer, 2008).

AFPs have been classified according to phylogenetic analyses. Early classification indicated two different groups: PAF-cluster and the BP-cluster (Galgóczy *et al*., 2013; Seibold *et al*., 2011). Advances in fungal genomes sequencing have propelled the identification of new AFP-like sequences and their phylogenetic reconstruction proposed a new classification in three distinct classes: A, B and C (Garrigues *et al*., 2016, 2018). More recently a new AFP has been characterized, which seems to be the first member of a fourth class (Tóth *et al*., 2016).

Classification of AFPs

Class A contains the two most studied AFPs: the AFP from *Aspergillus giganteus,* founder member of fungal AFP family, and the second identified AFP, the PAF from *Penicillium chrysogenum*.

AFP is secreted by the mould *A. giganteus* as a 94 amino acid long inactive pro-protein that is further processed to the active mature protein of 51 amino acids. The mature AFP has a high content of cysteine, tyrosine and lysine residues (Figure 6A). The protein is positively charged under neutral condition with an isoelectric point of 8.8. AFP contains five highly twisted anti-parallel strands forming two compacted β-sheet stabilized with four disulfide bonds (Figure 6B) (Campos-Olivas *et al*., 1995). PAF is secreted by the fungus *P. chrysogenum*, has an apparent molecular mass of 6.2 kDa, and show a 42.6% amino acid identity to AFP (Marx *et al*., 1995)(Figure 6A).

PAF and AFP are abundantly secreted proteins with yields above 50 mg/L of culture medium. They show growth inhibitory activity on numerous filamentous fungi at μM concentrations without affecting plant and mammalian cells (Marx *et al.,* 2008; Meyer, 2008). The recently identified PeAfpA protein secreted by *P. expansum* also belongs to class A (Figure 6) and shows the highest antifungal activity among all identified AFPs against all tested fungi, including plant and human pathogens (Garrigues *et al*., 2018). A summary of minimum inhibitory concentration (MIC) values of AFPs against representative fungi is shown in Table 2. Class A also includes other antifungal proteins, such as AcAFP from *Aspergillus clavatus* (Skouri-Gargouri and Gargouri, 2008), and NFAP from *Neosartoria fischeri* (Kovács *et al*., 2011).

Class B are phylogenetically related to class A, and its main representative member is the AfpB protein from *P. digitatum*. *AfpB* gene was identified in the genome of the citrus postharvest pathogenic fungus *P. digitatum*, but unlike other AFPs, AfpB has not been found naturally produced by the fungus (Garrigues *et al*., 2016). In a recent study of our group and collaborators, AfpB was biotechnologically produced in *Pichia*

pastoris and in *P. digitatum*. The recombinant protein resulted to be highly active against important phytopathogenic fungi, with inhibitory concentrations much lower than other AFPs (Garrigues *et al*., 2017). Class B also included AnAFP from *A. niger* (Lee *et al*., 1999), PgAFP from *P. chrysogenum* (Rodríguez-Martín *et al*., 2010), MAFP1 from *Monascus pilosus (*Chen *et al*., 2008*)* , PAFB from *P. chrysogenum* and PeAfpB from *P. expansum*.

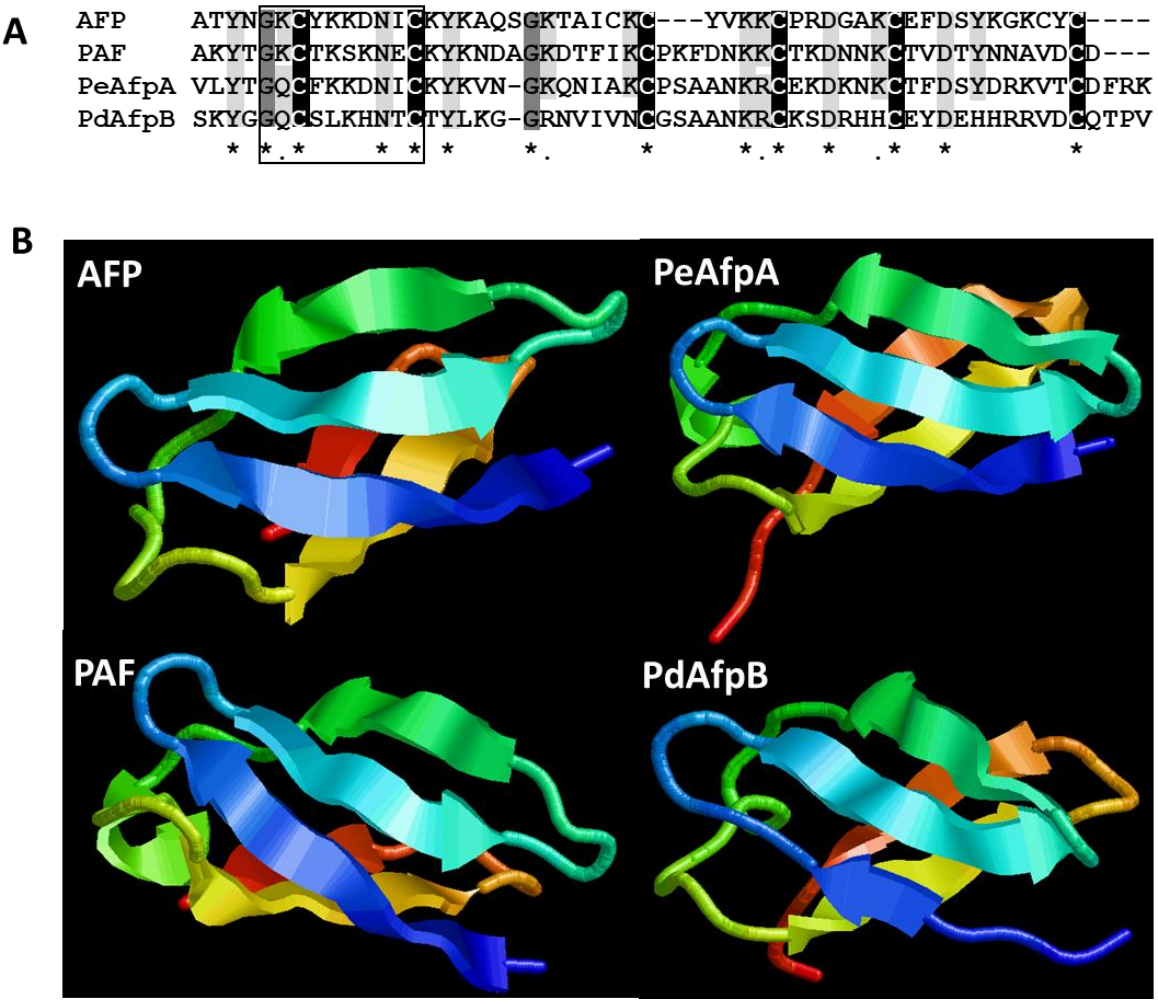

Figure 6. Amino acid sequence and structure of AFP, PAF, PeAfpA and AfpB. (A) Sequence alignment of indicated AFPs. The γ-core motif is boxed. Highly conserved cysteine and glycine patterns are shadowed in black and dark grey, respectively. Other less conserved amino acids are highlighted in light grey. (B) Comparison of the tertiary structure of indicated AFPs. The I-TASSER software was used to predict the structure of AFPs (Yang *et al*., 2014).

Class C is the most divergent class and it is represented by the BP protein from *P. brevicompactum* (Seibold *et al*., 2011). BP contains five antiparallel β-strands forming two compacted β-sheet with four disulphide bonds, and also contains α-helix structure which is absent in the other AFPs. The protein has growth inhibitory activity against the yeast ***Saccharomyces cerevisiae*** in a dose-dependent manner (Seibold *et al*., 2011). PeAfpC from *P. expansum* belongs also to class C, which contains three β-strands forming one compacted β-sheet, and the second β-sheet and α-helix structures are absent. The biotechnological production of PeAfpC was achieved in *P. chrysogenum* using the PAF-based expression cassette, but the produced protein did not exhibit antifungal activity under tested conditions (Garrigues *et al*., 2018). The

Pc-Arctin from *P. chrysogenum* also belongs to class C, this protein is active against some plant pathogenic fungi (Chen *et al*., 2013).

Table 2: Minimal inhibitory concentration (MIC) values (μg/mL) of AFP (Moreno *et al*., 2003; Vila *et al*., 2001), PAF (Garrigues *et al*., 2017), AfpB (Garrigues *et al*., 2017), PeAfpA (Garrigues *et al*., 2018) against the selected fungi.

Fungi	AFP	PAF	PeAfpA	AfpB
P. digitatum	NT*	50	1	3.2
B. cinerea	58	200	4	12.5
M. oryzae	23	>200§	16	>200§
F. proliferatum	1	NT*	NT*	NT*
P. expansum	NT*	>200§	2	3.2
P. chrysogenum	NE#	>200§	2	6.2
A. niger	1	1.6	2	3.2
A. giganteous	> 400§	NT*	NT*	NT*
P. italicum	NT*	>200§	2	1.6
Fusarium spp.	1	>200§	4	100

*NT, No Tested. #NE, No Effect. §No complete growth inhibition was detected at concentrations of 200 or 400μg/ml.

4. Mechanism of antifungal action

Knowledge on the mechanism of action of AMPs is crucial for their future development and application as novel biofungicides. Studies aimed at understanding the mechanism of AMP action have been reported extensively in recent years (Binder *et al*., 2011, 2015; Hagen *et al*., 2007; Harries *et al*., 2015; López-García *et al*., 2015; Moreno *et al*., 2006; Muñoz *et al*., 2013; Yun and Lee, 2016). Here, we will present the available knowledge on the mechanistic function of the antifungal peptides relevant for this Thesis: CecA, PAF102, AFP, AfpB and PAF.

CecA is a membrane active peptide that provokes membrane disruption following the "carpet" model mechanism of action (Oren and Shai, 1998). According to this model the integrity of the membranes is destroyed in a detergent-like manner. CecA interacts with negatively charged membrane lipids, and accumulate lining up parallel on the surface of the membrane in a carpet-like manner, affecting the fluidity and decreasing the membrane barrier properties. At low concentrations, CecA will form ion channels in the plasma membrane of target cells. When CecA reaches a threshold concentration, it will form transmembrane pores that are large enough for various molecules to penetrate (Jenssen *et al*., 2006; Silvestro *et al*., 1997) (Figure 7). It has been also reported that CecA triggers apoptosis processes at low concentrations including calcium accumulation, ROS generation, cytochrome c release to cytoplasm, and DNA fragmentation (Yun and Lee, 2016).

PAF102 has a dynamic antifungal mode of action similar to the reported for PAF26 that involves at least three stages: initial interaction of the peptide with the fungal cell envelope, internalization into target cells, and a series of complex and specific intracellular effects leading to cell death (López-García *et al*., 2015;

Muñoz *et al*., 2006, 2013) (Figure 7). At first, PAF102 locates at the surface of sensitive fungi like *F. proliferatum*. Then, it internalizes into fungal cells, where it begins to accumulate into the vacuoles. Subsequently, the peptide is transported from the vacuoles to the cytoplasm, causing the cell collapse. The internalization of the peptide into the target cell is necessary for antifungal activity, which is an energy-dependent process (López-García *et al*., 2015).

AFP disturbs the integrity of plasma membranes and inhibits the chitin biosynthesis in sensitive filamentous fungi (Hagen *et al*., 2007; Moreno *et al*., 2003, 2006; Theis *et al*., 2003). The peptide also disturbs the intracellular Ca^{2+} levels by inducing Ca^{2+} influx, which subsequently disrupts polarized cell growth. Once the integrity of the plasma membrane is broken, AFP enters the cells of sensitive filamentous fungi, and binds to anionic molecules to affect DNA and RNA synthesis (Meyer and Jung, 2018; Moreno *et al*., 2006). Interestingly, there are some reports that indicate that AFP not only binds to the cell wall or plasma membrane molecules of the sensitive fungi, but also enters the cells of resistant fungi like *P. chrysogenum* (Theis *et al*., 2003; Theis *et al*., 2005). This observation suggests that a specific interacting partner of AFP should be absent in resistant species, which are present in sensitive fungi, or alternatively, there are enzymes in resistant fungi that cause degradation of AFP intracellularly.

PAF shows a different mode of action to AFP besides their similarities in structure and antifungal activities. PAF interferes cell wall integrity pathway, as well as protein kinase A and mitogen-activated protein kinase signaling cascade pathway (Binder *et al*., 2010), and triggers apoptosis processes including calcium accumulation and ROS generation in PAF-sensitive fungi (Leiter *et al*., 2005; Marx *et al*., 2008). Interestingly, the addition of calcium to the growth medium enhances the resistance of filamentous fungi to PAF and AFP and neutralized the disturbance of Ca^{2+} uptake level. Recently, it was proven that the protein kinase signaling pathway is interconnected with intracellular calcium levels in response to PAF in *A. niger* (Binder *et al*., 2015; Meyer and Jung, 2018).

AfpB has a similar mode of action to PAF triggering regulated cell death in target fungi (Bugeda *et al*., 2020). AfpB acts through a three-stage process: i) interaction with the cell wall that requires mannoproteins; ii) internalization inside fungal cells through an energy dependent process; and iii) activation of intracellular events mediated by reactive oxygen species (ROS) production, MAP kinases and G-protein signaling pathways that lead to a transcriptional reprogramming ending in cellular collapse, and eventually in cell permeabilization (Figure 7).

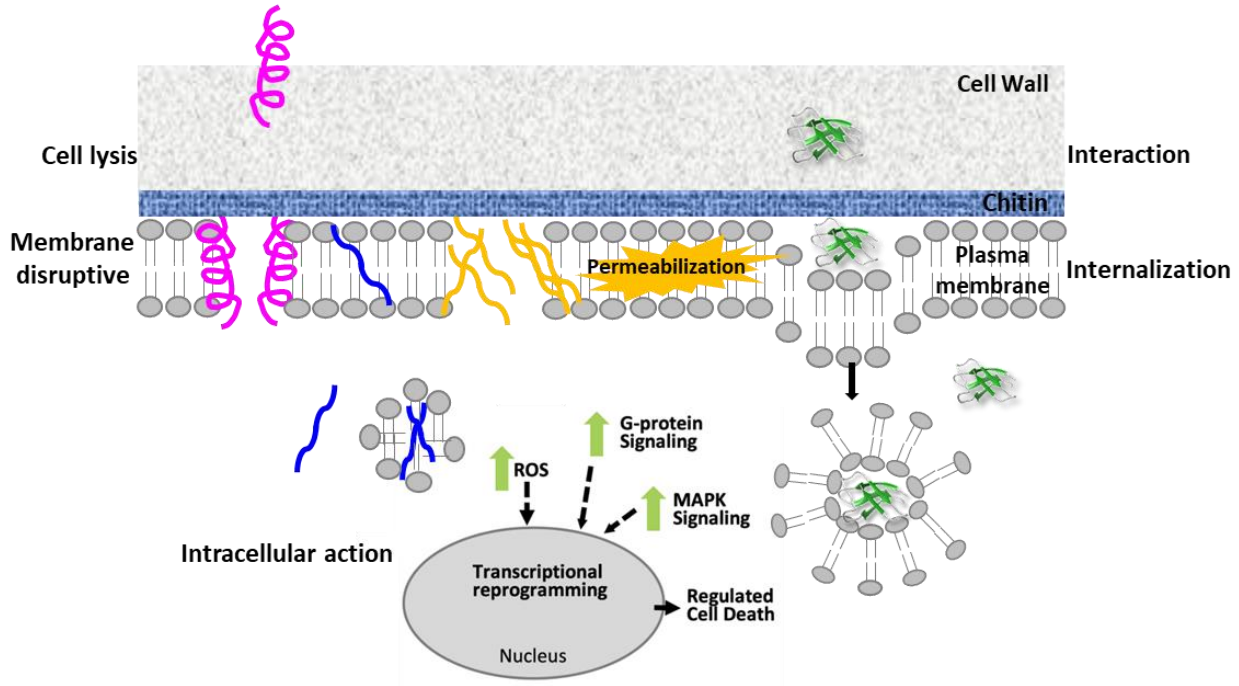

Figure7. Schematic mode of action of antimicrobial peptides. The figure represents the different modes of action: cell disrupting peptides (α-helical peptides like CecA shown in pink or extended peptides shown in orange colors) or cell penetrating peptides (extended peptides like PAF102 shown in blue or ß-sheet structured peptides like AFPs shown in green color). The mechanism of cell disrupting peptides includes the physical interaction with the cell wall, disruption of cell membranes causing their rupture and loss of biophysical properties, finally the cell killing through a lytic mechanism. The mechanism of cell non-disruptive peptides is divided in three steps: (i) the physical interaction with the cell wall, (ii) the internalization inside the cell by crossing the plasma membrane, (iii) the intracellular regulated events leading to cell death, including the production of ROS, mitogen- activated protein kinase (MAPK), and G-protein signaling, transcriptional reprogramming, and, eventually, plasma membrane permeabilization. Picture modified from Bugeda *et al*., 2020.

5. AMPs as a promising strategy for crop and postharvest protection

AMPs have been shown to be efficient in the protection of model and crop plants against fungal infections (López-García *et al*., 2012; Marcos *et al*., 2020). Strategies based in the *in-situ* AMP accumulation through transgenic expression, as well as based on AMP topical applications showed efficiency in plant protection against fungal pathogens.

Referring to the AMPs under study in this thesis, it has been reported that the expression of CecA in rice plants conferred protection to fungal pathogens, namely *M. oryzae* (Coca *et al*., 2006) or *F. verticillioides* (Bundó *et al*., 2014). Examples of AFP expression in transgenic plants conferring enhanced resistance to fungal infection can be also found in the literature (Coca *et al*., 2004; Girgi *et al*., 2006; Moreno *et al*., 2005; Oldach *et al*., 2001). For instance, the AFP from *A. giganteus* conferred protection against *M. oryzae* when expressed in rice (Coca *et al*., 2004; Moreno *et al*., 2005), to the powdery mildew fungus *Erysiphe graminis* and to the leaf rust fungus *Puccinia recondite* in wheat (Oldach *et al*., 2001), to the rust fungus *Puccinia substriata* and downy mildew *Sclerospora graminicola* in pearl millet (Girgi *et al*., 2006). In addition, AFP

conferred also resistance against Fusarium head blight when expressed in wheat as a fusion protein to a Fusarium-specific antibody (Li *et al*., 2008).

Direct application of AMPs in plant protection has been also widely studied. Topical applications of AFP onto rice leaves prevented *M. oryzae* infection and the development of blast disease lesions (Vila *et al*., 2001). Similarly, AFP application in geranium protected leaves from gray mold infections (Moreno *et al*., 2003). Applications of AFPs on tomato seedlings conferred protection against the root pathogen *F. oxysporum* without negative effects on plants (Theis *et al*., 2005). Barley and wheat leaves showed resistance against *Blumeria graminis* and *Puccinia recondite* infections, respectively, when treated with PAF protein (Barna *et al*., 2008). More recently, our group has shown that the AfpB from *P. digitatum* and the AfpA from *P. expansum* conferred protection in tomato plants against *B. cinerea* (Garrigues *et al*., 2018).

Some researchers have proved that several natural AMPs have equal or better efficacy than chemical fungicides in the control of fungal postharvest decays under controlled experimental conditions. As an example, application of the AFP on raw barley grains used in malt production can completely inhibit the growth of *Fusarium spp*., and the observed antifungal effect exceeded those of the traditionally chemical fungicides (Barakat *et al*. 2010). The spray of AFP on harvested bananas, tomato and mango fruits, significantly reduced the incidence of *Alternaria alternata* infections (Barakat *et al*., 2012; Barakat, 2014). Equally, the direct application of AfpA from *P. expansum* reduced the infection incidence of *P. digitatum* on orange fruits at very low protein concentrations (Garrigues *et al*., 2018). Topical applications of CecA also conferred protection on harvested tomatoes, in this case against *Colleotrichum coccodes* (Jones and Prusky, 2002). The synthetic PAF peptides has been shown to be efficient in the protection of citrus fruits against *P. digitatum* and *P. italicum* fungal pathogens (López-García *et al*., 2002, 2003; Muñoz *et al*., 2007).

6. Biotechnological production of AMPs

The often-low amount of AMPs obtained by purification from natural sources and the high cost of their chemical synthesis have restricted their development and application. Biotechnological production of AMPs could provide a reliable option to obtain these peptides at high yield and low cost to facilitate their exploitation. However, AMPs are bioactive peptides which are difficult to be produced in biological systems. For instance, the AMP production in microorganism-based systems is limited by host toxicity as they possess antimicrobial activity. Moreover, production platforms based in *Escherichia coli* are not very appropriate for proteins requiring posttranslational modifications, including disulphide bond formation as required AFPs for activity. Better results have been reported for the production of such peptides in fungal-based production systems, such as yeasts and filamentous fungi (Garrigues *et al*., 2017; López-García *et al*., 2010; Sonderegger *et al*., 2016; Virágh *et al*., 2014). In this work we explore different systems based in either the yeast *Pichia pastoris* or the plants *Nicotiana benthamiana* or *Oryza sativa* for the production of AMPs. Each system has advantages and drawbacks depending on the characteristics and the intended use of the AMP to be produced.

6.1. Plants as biofactories of AMPs

Plants are excellent biofactories for producing proteins of interest for research, pharma and industry. Main advantages of plant-systems are: low-cost production as they are fueled by sun light, water and soil, the high biomass that can be obtained in short time periods, the easy scale-up, and the safety of the system being free of animal and human pathogens. Additionally, as eukaryotic organisms, plant-based systems have the capability to perform appropriate posttranslational modifications, including glycosylation, folding, and disulfide bonds formation. There are different approaches to produce AMPs in plants: stable transformation and transient expression.

6.1.1. Production of AMPs in stable transformation of rice plants

Recombinant proteins can be produced in plants by stable integration of the gene encoding the protein of interest into their genome. These plants are transgenic plants that inherit the transgene stably to the next generation and stably produce the recombinant protein. The production of the recombinant protein can be constitutive in all plant tissues, inducible in response to certain stimulus or tissue-specific depending on gene regulatory sequences. Constitutive strong promoters usually enable high expression levels in the whole plant, leading to high protein accumulation levels. However, the AMPs expressed in all tissues might negatively affect growth and development of host plants, as well as to lead to the development of resistant pathogen strains due to permanent exposure to high levels of AMPs. Examples on deleterious AMP effects on the host can be found in the literature, such as the constitutive expression of a synthetic linear peptide BP100 in *Arabidopsis thaliana* and rice plants that showed toxicity towards the host plant (Company *et al*., 2014; Nadal *et al*., 2012). The constitutive CecA expression when accumulated in cell apoplasts of rice plants also caused negative effects on the host plants (Coca *et al*., 2006). Another inconvenience for constitutive expression relates to purification costs, various plant tissues have complex composition that difficult the separation and purification of target AMPs from plant material. The restriction of the peptides to certain tissues using organ-specific or inducible promoters offer clear advantages, being a more desirable strategy.

The cereal seeds as storage organs are suitable for packing large amount of recombinant proteins in a low volume, and in a stable environment with low protease activity and low water content without loss of quality for long periods of time (Lau and Sun, 2009). Moreover, the accumulation of the recombinant proteins in seeds also avoids the interfering with the vegetative growth of the plant. Thus, cereal seeds provide an attractive platform for the production of AMPs. Particularly, rice seeds are considered as a good biofactory since the rice gene transfer technology is well developed, cropping conditions are easy and well-established worldwide, and high grain yield can be obtained (Lau and Sun, 2009; Stoger *et al*., 2005; Takaiwa *et al*., 2017; Takaiwa and Yang, 2014). In addition, rice can be grown under containment conditions, and the risk of unintended gene flow is minimal compared with other crops (Lau and Sun, 2009; Stoger *et al*., 2005). Furthermore, rice is one of the important food crops, which seeds are largely consumed by humans, and the accumulation of AMPs in these storage organs can be a promising approach for the development of plant-derived edible biopharmaceuticals. Our research group has reported the efficient production of AMPs in rice seeds following two different strategies: accumulation into protein bodies (PBs) using endosperm-specific promoters or in lipid droplets (LDs) using embryo-specific promoters (Bundó *et al*., 2014; Montesinos *et al*., 2016, 2017). Restricting AMPs to subcellular organelles confers them stability and reduces their toxicity toward the host cells leading to high AMP accumulation levels. Additionally, the accumulation of AMPs into PBs or LDs have the advantage of facilitating their extraction from plant tissues

by simple centrifugation procedures on density gradients. PBs are dense organelles that easily precipitated, whereas LDs floated on dense solutions. Rice endosperm-specific promoters from the storage proteins glutelin B1, B4, or globulin1, were used for the endosperm specific expression of AMPs genes, including natural or rational designed peptides (Bundó *et al*., 2014; Montesinos *et al*., 2017). During this thesis, I collaborated with the group in the production of PAF102 antifungal peptide in the endosperm of rice seeds targeting its accumulation to PBs. Results are presented in chapter II.

Rice seeds have been used also to develop a novel oleosin-fusion bioproduction platform, in which AMPs are expressed as a fusion to oleosins (Montesinos *et al*., 2016). Oleosins are plant proteins with a structural role in LD formation and stabilization that are used as carriers for recombinant proteins to LDs (Van Rooijen and Moloney, 1995). LDs are specialized structures composed mainly of a core of neutral lipids (triacylglycerols and steryl esters) surrounded by a monolayer of phospholipids containing a number of different proteins, including oleosins, caleosins and sterolosins (Tzen, 2012). Oleosin fusion proteins insert spontaneously into the LDs where the oleosin embeds into the phospholipid layer and the fused AMP exposes to the surface. The production of oleosin fusions is driven by oleosin own promoters which are seed specific promoters. The fusion to oleosin not only offers stability to AMPs in the host plant, it also reduces the toxicity of AMPs towards them, and as a consequence high accumulation levels of AMPs can be reached. The accumulation of AMPs in LDs has additional advantages, such as the ability to be easily obtained by flotation on a density gradient, facilitating the recovery of AMPs from plant material. Using this strategy, the lineal antimicrobial peptide CecA was efficiently produced in rice seeds in our research group reaching high yield (40 μg CecA/g grain) (Montesinos *et al*., 2016). The technology of oleosin fusion was explored in this thesis for the production of the antifungal peptide PAF102 in rice seeds, as well as developed in *Pichia pastoris*.

6.1.2 Transient expression of AMPs in plants using viral vectors

Stable transformation of plants has the limitation of the long time required for the development. As an alternative, transient expression strategies in plant-based platforms have flourished in recent years, on the grounds that high yields of recombinant proteins can be reached in very short period of time and in a cost-efficient manner, with the additional advantage that can be escalated easily upon market demand. Moreover, transient expression also addresses regulatory issues and public concerns for genetically modified plants, since transgenic plants are not generated. The most widely used host plant for transient expression strategies is *Nicotiana benthamiana* due to certain goodness, such as its amenability for agroinfiltration, its natural ability to express heterologous genes and its rapidity to produce large amounts of biomass for easy scale-up production (Leuzinger *et al*., 2013).

Plant viral-vectors are easy to manipulate and have become a powerful tool to transiently express foreign genes in plant cells (Hefferon, 2017; Marillonnet *et al*., 2004). They are usually derived from wild type viruses in which heterologous genes are inserted in the viral genome, normally in substitution of some viral genes, like coat protein or movement protein genes. A pioneering example are the viral vectors derived from *Tobacco mosaic virus* (TMV), a single-stranded positive-sense RNA virus from *Tobamovirus* genus (Ishibashi and Ishikawa, 2016; Knapp and Lewandowski, 2001; Scholthof, 2004). A key property of TMV is the rapid accumulation of large amounts of the coat protein in infected plant tissues. Thus, TMV vectors in which the gene of interest replaces the coat protein gene result in large accumulation of the recombinant protein in plant tissues and in a short time. A major breakthrough in viral expression strategies occurred when found that virus-based vectors can be efficiently delivered into plants via *Agrobacterium tumefaciens*,

i.e. agroinfection. This fact permitted the development of the magnICON expression system by ICON Genetics GmbH (Halle/Saale, Germany) based on a deconstructed TMV (Marillonnet *et al*., 2004). The system is based on *in planta* assembly of three different modules to reconstitute a functional viral particle with the help of a recombinase, making it a highly efficient and versatile system. One limitation of the magnICON expression system is that the components need to be co-infiltrated into the same plant cell from a mix of three Agrobacterium cultures. So relatively high density of *A. tumefaciens* cell suspensions must be infiltrated into leaves in order to get the highest expression levels. Infiltration of such high density of *A. tumefaciens* may result in negative responses in the host plant (Wroblewski *et al*., 2005). Subsequently, a single-module TMV vector was developed, the TMV RNA-based overexpression (TRBO) vector with much higher agroinfection efficiency, in which most of the CP is replaced by the ORF of interest to produce large amounts of recombinant proteins (Lindbo, 2007).

In this thesis, we will explore the use of a new TMV-derived vector developed in the group of Dr. José A. Daròs at IBMCP (Valencia) that allows quick and easy cloning of the ORFs of interest using the Gibson assembly reaction for the transient expression of AMPs in *N. benthamiana* leaves.

6.2. *Pichia pastoris* as biofactory for antimicrobial peptides production

P. pastoris is a well-established system for the production of recombinant proteins, mainly applied to biopharmaceuticals and industrial enzymes. This yeast is a good production system because it is easy to genetically manipulate and grows fast in short times to high cell densities, allowing the production of grams of recombinant protein per liter of culture (Ahmad *et al*., 2014). Moreover, it is capable to perform correct protein processing and post-translational modifications. There are available strong and tightly regulated promoters for the expression of the gene encoding the protein of interest, which can be produced either intracellularly or extracellularly. Among these promoters, the alcohol oxidase 1 (*AOX1*) and the glyceraldehyde-3-P dehydrogenase (*GAP*) promoters are the most frequently used. *AOX1* is a tightly regulated promoter inducible by methanol, which holds advantages for foreign protein expression since it can drive high expression levels specifically when high cell densities are reached avoiding the toxicity due to the constitutive accumulation of heterologous proteins. However, *AOX1* promoter needs to be activated by methanol, which is a toxic and hazardous substance that makes downstream protein purification difficult. The GAP promoter is also a strong promoter that drives similar gene expression levels to *AOX1* promoter in a constitutive manner, but with the advantages of avoiding the use of toxic methanol as the carbon source (Várnai *et al*., 2014; Waterham *et al*., 1997; Zhang *et al*., 2009)

Several reports showing the successful production of AMPs in *P. pastoris* can be found in the literature (Galgóczy *et al*., 2017; Garrigues *et al*., 2017; Huynh *et al*., 2018; Landim *et al*., 2017; López-García *et al*., 2010). Some AFPs have been successfully expressed in *P. pastoris* under the control of *AOX1* promoter and by targeting the secretory pathway, resulting in the accumulation of active proteins properly folded at modest levels, far from commercial significance, but enough for research analyses. Among them could be mentioned the production of *A. giganteus* AFP reaching yields of 2.5 mg/L (López-García *et al*., 2010), the *N. fischeri* NFAP at 6 mg/L (Virágh *et al*., 2014), the *P. digitatum* AfpB at 1.4 mg/L (Garrigues *et al*., 2017) or the *Fusarium graminearum* AFP at 20 mg/L (Patiño *et al*., 2018). In the case of linear AMPs, results are even worse mainly due to the lack of stability of these peptides in yeasts and/or to the host toxicity. A successful example is the production of cecropin from the housefly *Musca domestica* at 12 mg/L or reaching up to 20 mg/L in the case of His-tagged peptide (Jin *et al*., 2006). Higher yields were obtained for the cecropin B with values around 50 mg/L of active peptide (Wang *et al*., 2011). Another example is

the expression of CecA in *P. pastoris* to be used as a biocontrol microorganism (Ren *et al*., 2012). Although the peptide *per se* was not detected, its antifungal activity was detected since the yeast strain conferred protection against apple blue mold *P. expansum*. This interesting strategy has not been widely explored and only a couple of examples can be found in the literature. One based on the production of CecA and other based on the production of the plant defensing PsD1 (Janisiewicz *et al*., 2008; Kong *et al*., 2016).

In this thesis we explore the use of *P. pastoris* for the production of linear AMPs to take advantage from the short production times required and the high yields that can be obtained. The strategy to be evaluated is the production of AMPs as oleosin fusion proteins to avoid the toxicity of AMPs towards the yeast host cells and to confer stability to the AMPs. This also allow us to evaluate whether the plant oleosin technology can be transferred to the yeast system for the production of any recombinant proteins of interest and not only AMPs.

Objectives

Objectives

The general objective of the present Ph.D thesis is to advance in the development of antimicrobial peptides as novel biofungicides for applications in crop and postharvest protection. In particular, the Thesis is focused on antifungal proteins (AFPs) from fungal origin due to their potent, specific activity against important plant pathogens, as well as their high stability that will facilitate their applications. We also investigate the rationally designed antifungal peptide PAF102 and the natural antimicrobial peptide cecropin A, both of them displaying higher antifungal activity than AFPs but suffering from less stability.

To achieve this goal, three specific objectives are addressed:

1. To develop a sustainable platform for the production of AFPs in *N. benthamiana* plants through transient expression using a new tobacco mosaic virus -derived vector.
2. To develop alternative production systems for the linear AMPs, PAF102 and cecropin A, using the oleosin technology in rice seeds and *P. pastoris* since were not efficiently produced in *N. benthamiana* plants.
3. To characterize the efficacy of *in planta*-produced AFPs in the control of plant diseases caused by fungal pathogens.

Chapter I

Efficient Production of Antifungal Proteins in Plants Using a New Transient Expression Vector Derived from Tobacco Mosaic Virus

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Efficient production of antifungal proteins in plants using a new transient expression vector derived from tobacco mosaic virus

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Xiaoqing Shi and Teresa Cordero contributed equally to this work.

Keywords: antifungal proteins, gibson assembly, *Nicotiana benthamiana*, plant biofactory, tobacco mosaic virus, viral vector.

Summary

Fungi that infect plants, animals or humans pose a serious threat to human health and food security. Antifungal proteins (AFPs) secreted by filamentous fungi are promising biomolecules that could be used to develop new antifungal therapies in medicine and agriculture. They are small highly stable proteins with specific potent activity against fungal pathogens. However, their exploitation requires efficient, sustainable and safe production systems. Here, we report the development of an easy-to-use, open access viral vector based on *Tobacco mosaic virus* (TMV). This new system allows the fast and efficient assembly of the open reading frames of interest in small intermediate entry plasmids using the Gibson reaction. The manipulated TMV fragments are then transferred to the infectious clone by a second Gibson assembly reaction. Recombinant proteins are produced by agroinoculating plant leaves with the resulting infectious clones. Using this simple viral vector, we have efficiently produced two different AFPs in *Nicotiana benthamiana* leaves, namely the *Aspergillus giganteus* AFP and the *Penicillium digitatum* AfpB. We obtained high protein yields by targeting these bioactive small proteins to the apoplastic space of plant cells. However, when AFPs were targeted to intracellular compartments, we observed toxic effects in the host plants and undetectable levels of protein. We also demonstrate that this production system renders AFPs fully active against target pathogens, and that crude plant extracellular fluids containing the AfpB can protect tomato plants from *Botrytis cinerea* infection, thus supporting the idea that plants are suitable biofactories to bring these antifungal proteins to the market.

Introduction

Disease-causing fungi that infect plants, animals and humans pose a serious threat to human and animal health, food security and ecosystem resilience (Fisher *et al.*, 2016, 2012). More people die every year from fungal infections than from malaria (Bongomin *et al.*, 2017). Fungal infections can have fatal consequences for at-risk immunocompromised patients with HIV/AIDS, anti-cancer chemotherapies, corticosteroid therapies, and organ transplantation, among others (Campoy and Adrio, 2017). In addition, fungi are a challenge to food security because they destroy major crops globally and contaminate food and feed with mycotoxins that are detrimental to animal and human health (Bebber and Gurr, 2015). Only a few classes of antifungal agents are available today, and even these are not fully effective due to the development of resistance, host toxicity, and undesirable side effects (Perfect, 2016). There is thus an urgent need to develop novel antifungals, whose properties and mechanisms of action represent improvements on the existing ones, and which can be applied in diverse fields, including crop and postharvest protection, preservation in cosmetics, materials and food, and animal and human health.

Antifungal proteins (AFPs) produced by filamentous fungi are a specific class of antimicrobial peptides (AMPs). Antifungal proteins are promising biomolecules that could be used to develop new antifungal therapies in medicine and agriculture (Garrigues *et al.*, 2017; Huber *et al.*, 2018; López-García *et al.*, 2012; Meyer, 2008). AFPs are small proteins, usually cationic, that are rich in cysteine residues, and are folded in compact structures supported by disulphide bridges, which make them highly stable and resistant to heat, proteases and extreme pH (Batta *et al.*, 2009; Campos-Olivas *et al.*, 1995; Garrigues *et al.*, 2017; Hegedüs and Marx, 2013). They exhibit potent specific antifungal activity at very low concentrations against important human and plant fungal pathogens (Garrigues *et al.*, 2017; Huber *et al.*, 2018; Marx *et al.*, 2008; Tóth *et al.*, 2016; Vila *et al.*, 2001; Virágh *et al.*, 2014), and do not have toxic effects on plant or mammalian cells (Moreno *et al.*, 2006; Szappanos *et al.*, 2006). However, the exploitation of AFPs requires efficient, sustainable and safe production systems. Antifungal proteins have been biotechnologically produced in *Pichia pastori* at relatively low yield, and more efficiently in filamentous fungi using a *Penicillium chrysogenum*-based expression cassette (Garrigues *et al.*, 2018; Huber *et al.*, 2018; López-García *et al.*, 2010; Sonderegger *et al.*, 2016; Tóth *et al.*, 2018; Virágh *et al.*, 2014). Plants represent also a good option for producing AFPs that are cysteine-rich proteins that require disulphide bridges formation and proper folding for activity. Moreover, previous reports indicate that plants can sustain AFP production (Coca *et al.*, 2004; Girgi *et al.*, 2006; Moreno *et al.*, 2005; Oldach *et al.*, 2001). These reports show that AFPs can be heterologously produced in plants, leading to improve resistance to fungal pathogens.

Plants are excellent biofactories for producing proteins and other metabolites of interest for research, pharma and industry. They are fueled by sunlight, are free from human pathogens, and compared to other systems, production can be scaled up easily. Many biotechnological tools have been developed for molecular farming, and those derived from plant viruses are prominent among them (Hefferon, 2017). Higher plants host a remarkable diversity of viruses with different genomes and strategies for genome replication and expression. However, they all have small genomes, and thus have limited capacity to harbor genetic information. Despite this limitation, plant viruses can complete complex and tightly regulated infectious processes in their host plants. Definitively, evolution has favored small and simple, but still powerful, genetic elements in plant virus genomes, a wealthy source of parts for plant biotechnology and synthetic biology.

A pioneering example of a plant virus transformed into a biotechnological tool is *Tobacco mosaic virus* (TMV). This is a plus strand RNA virus of genus *Tobamovirus,* within the family *Virgaviridae* (Ishibashi and Ishikawa, 2016; Knapp and Lewandowski, 2001; Scholthof, 2004). The genomic RNA is encapsidated by 2130 units of the viral coat protein (CP) into rigid, helical rod-shape virions of about 18 nm diameter, and 300-310 nm length. TMV RNA encodes four proteins: two 5′-end proximal overlapping 126 and 183 kDa replication proteins that are alternatively produced through a ribosomal readthrough mechanism, the 30 kDa movement protein (MP), and the 3′-end proximal 17.5 kDa CP. The two replication proteins are expressed from the genomic RNA, while the MP and CP are expressed from 3′ co-terminal subgenomic RNAs. The TMV genome also contains 5′- and 3′-untranslated regions that are important for virus translation and replication (Chujo *et al.*, 2015).

A key property of TMV is the rapid accumulation of large amounts of the viral CP in infected plant tissues. A combination of knowledge and a trial-and-error approach led to the construction of TMV-derived vectors (Dawson, 2014) such as TB-2 (Donson *et al.*, 1991; Kearney *et al.*, 1993), or 30B (Shivprasad *et al.*, 1999), which allow to express a foreign open reading frame (ORF) under the control of the viral CP promoter; in these cases, encapsidation is achieved by inserting into the recombinant virus an ORF encoding the CP of a different tobamovirus. Other vectors such as the TMV RNA-based overexpression (TRBO) vector, in which most of the viral CP is replaced by the ORF of interest, produce large amounts of recombinant proteins but are incapable of spreading systemically (Lindbo, 2007). Improvements in TMV-based vectors have also focused on increasing the efficiency of establishing the infection foci and of cloning the genes of interest. One of the most popular systems uses DNA modules delivered by *Agrobacterium tumefaciens* and assembled *in vivo* by a site-specific recombinase to produce the TMV-derived vector (Marillonnet *et al.*, 2004). This de-constructed system was further improved by introducing silent nucleotide substitutions and multiple introns into the TMV coding sequences, in order to increase infectivity (Marillonnet *et al.*, 2005). Another improved series of TMV-derived vectors uses Gateway cloning to facilitate insertion of the recombinant ORFs, which can also be fused to a broad series of epitope tags and fluorescent proteins (Kagale *et al.*, 2012).

Here, we report the development of a new TMV-derived vector system that allows quick and easy cloning of the ORFs of interest using the Gibson assembly reaction (Gibson *et al.*, 2009). We describe how we have used this system to produce large amounts of AFPs in *Nicotiana benthamiana* plants. We show that it is important to target these bioactive proteins to the extracellular space, since their toxic effects prevent them from accumulating in intracellular compartments. Moreover, by accumulating the AFPs in the extracellular fluids, downstream purification is simpler. We show also that the recombinant AFPs produced by the new system have exactly the same activity as their native counterparts of fungal origin. Finally, we demonstrate that plant extracellular fluids containing the *Penicillium digitatum* AfpB can protect tomato plants from the grey mold disease caused by *Botrytis cinerea.*

Results

A new TMV-based vector system in which ORFs of interest are inserted using the Gibson assembly reaction

As a first step toward a new TMV-derived vector system for plant biotechnology that uses the Gibson assembly reaction to insert the ORFs of interest, we constructed a TMV infectious clone that efficiently infects plants when delivered by agroinoculation (pGTMV; Figures S1 and S2; Addgene plasmid # 118755). We then transferred a fragment of the TMV cDNA containing the entire CP ORF (from A5431 to T6278) to a minimal cloning vector. In the resulting plasmid, the TMV cDNA was flanked by recognition sites for the type-IIS restriction enzyme *Bsa*I. The plasmid was manipulated to mutagenize the ATG initiation codon of the TMV CP into AGA (positions 5712-5714), and to replace most of the CP ORF (from T5757 to T6176) by a linker consisting of the recognition sites for two unique restriction enzymes (*Age*I and *Xho*I) and the LacZ blue-white selection marker. The resulting minimal intermediate plasmid (pMTMVi-N; Addgene plasmid # 118756) is represented in Figure 1 (upper part) and its exact sequence in Figure S2. This small plasmid (2376 bp) allows us to easily and efficiently insert the ORFs of interest using the Gibson assembly reaction (Gibson *et al.*, 2009). The option of assembling two or more DNA fragments in a single reaction allows us to fuse any desired peptide tag or protein moiety to the recombinant protein of interest. Once the cDNA for the recombinant protein is inserted into pMTMVi-N, the manipulated TMV genome fragment can be transferred into the TMV infectious clone (pGTMV) using a second Gibson assembly reaction (Figure 1, upper part). This requires that pGTMV be digested with restriction enzymes *Nco*I and *Pfl*23II, which have unique recognition sites in this plasmid. There are two options for generating the insert from the pMTMVi-N derivatives, the easiest being to digest the plasmid with *Bsa*I. However, if the recombinant cDNA contains *Bsa*I sites, the manipulated TMV DNA fragment can be produced by polymerase chain reaction (PCR) using primers PI and PII (Table S1), which flank the recognition sites of *Nco*I and *Pfl*23II.

In pMTMVi-N derivatives, most of the TMV CP is replaced by the ORF for the recombinant protein, such that the recombinant viruses will be unable to move systemically in infected plants. To obtain recombinant TMV clones that can move within the plant, we constructed a new minimal intermediate plasmid by transferring the fragment in the pGTMV from A5431 in TMV cDNA to G62 in the delta ribozyme cDNA. We again mutagenized the TMV CP ATG to AGA and replaced most of the TMV CP with the *Age*I-LacZ-*Xho*I linker, as above. In this case, we replaced the final 30 nt of the TMV genome (from A6366 to the end) with the 3′ end of a *Tomato mosaic virus* (ToMV) infectious clone (from G5537 to the end). This fragment includes the ToMV CP promoter, CP ORF and 3′ UTR, and provides an alternative

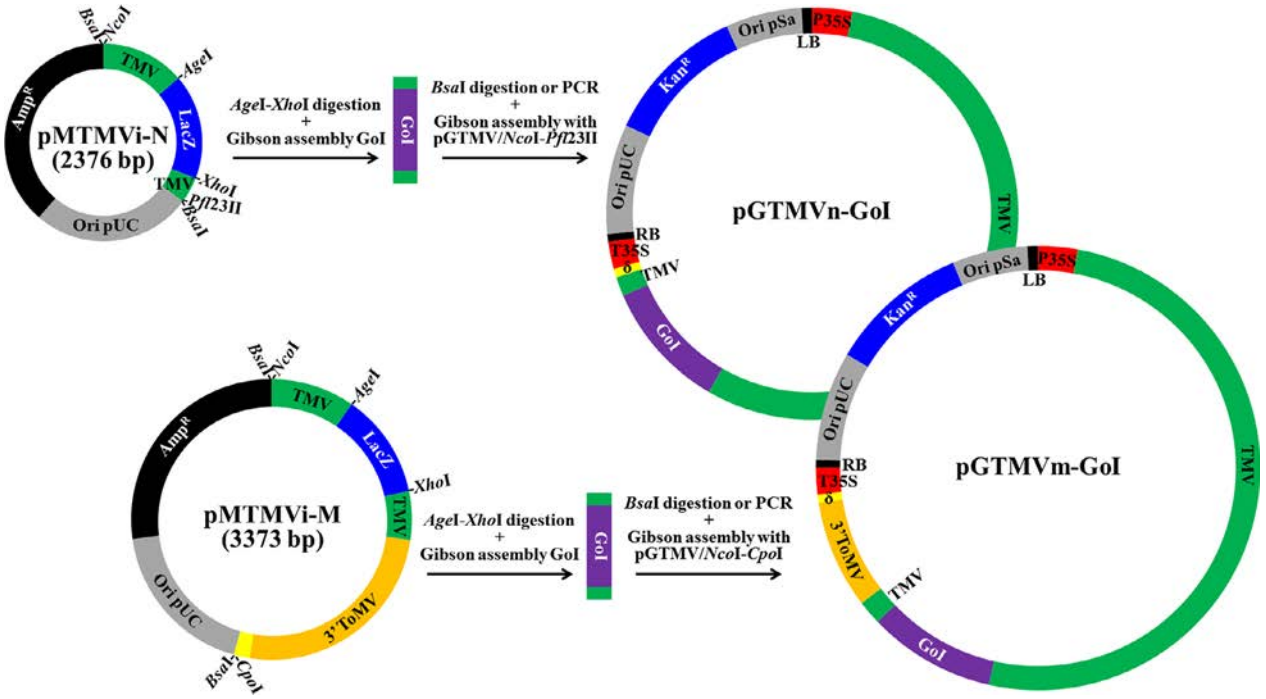

Figure 1 The TMV vector system, which allows Gibson assembly of the ORF of interest. Schematic representation of intermediate entry plasmids pMTMVi-N and pMTMVi-M, and the Gibson assembly reactions that allow insertion of the gene of interest (GoI) into pGTMV to produce the binary plasmids that will express the recombinant viruses (pGTMVn-GoI and pGTMVm-GoI). Ori pUC and Ori pSa, replication origins; Amp^R and Kan^R, ampicillin and kanamycin resistance selection markers; LacZ, fragment of *E. coli* b-galactosidase, which allows blue–white screening in the presence of X-gal; TMV and 3′ ToMV, viral cDNAs; LB and RB, left and right borders of the *Agrobacterium. tumefaciens* transfer DNA; P35S and T35S, *Cauliflower mosaic virus* (CaMV) 35S promoter and terminator; δ, ribozyme.

tobamovirus CP to encapsidate the recombinant virus, which allows it to move systemically (Shivprasad *et al.*, 1999). This second minimal TMV intermediate plasmid, called pMTMVi-M (Addgene plasmid # 118757), is shown in Figure 1 (lower part, full sequence in Figure S2). In pMTMVi-M, the M indicates movement, whereas the N in pMTMVi-N indicates nonmovement. The cDNA corresponding to the recombinant protein can be inserted into the *Age*I-*Xho*I digested plasmid using the Gibson assembly reaction, as previously described. The manipulated TMV fragment can also be reintegrated into pGTMV, using the Gibson assembly reaction with a *Bsa*I-digested insert, although in this case the destination plasmid (pGTMV) must be digested with *Nco*I and *Cpo*I. If the cDNA corresponding to the recombinant protein contains *Bsa*I sites, the insert can also be produced by PCR using primers PI and PIII (Table S1), which flank the *Nco*I and *Cpo*I recognition sites.

We confirmed that the new TMV vector system performed correctly by cloning the cDNA of reporter green fluorescent protein (GFP) into both the pMTMVi-N and pMTMVi-M intermediate plasmids, transferring the manipulated TMV fragments to pGTMV, and infiltrating *N. benthamiana* plants (Figure S3, sequences in Figure S4).

Production of the antifungal protein AfpB in leaf apoplasts of *N. benthamiana* plants

AfpB is an AFP whose gene was identified in the genome of the postharvest phytopathogenic fungus *P. digitatum* but that, unlike other AFPs, is not naturally produced by the fungus (Garrigues *et al.*, 2016). In a recent study, *P. digitatum* was genetically modified to produce AfpB and was found to be a highly active antifungal protein, with inhibitory concentrations up to one order of magnitude lower than other AFPs (Garrigues *et al.*, 2017). Therefore, there is a great interest in obtaining large amounts of AfpB, so we assessed this possibility using our new TMV vector system. We designed two different constructs to target this protein to two different subcellular compartments, namely apoplast and endoplasmic reticulum (ER). Compartmentalizing antimicrobial peptides in plant cells is known to favor their accumulation by protecting them from host proteases and avoiding their toxic effects on the host cells (Bundó *et al.*, 2014; Coca *et al.*, 2006; Montesinos *et al.*, 2016). To target AfpB to the apoplast or ER, we added the signal peptide of the tobacco AP24 protein (XP_009782398.1) to the amino terminus of the mature AfpB protein (Figure 2a). This signal peptide allows the protein to enter the secretory pathway toward the extracellular space. In the second construct, we added an additional KDEL sequence to the carboxyl terminus of the protein, facilitating retention inside the ER. We then expressed the corresponding recombinant viruses (TMVΔCP-AfpB and TMVΔCP-AfpBKDEL; Figure S4) in *N. benthamiana* leaves via infiltration with *A. tumefaciens* cultures. We observed that leaves agroinfiltrated with AfpB constructs were damaged, particularly those with the construct designed for AfpB accumulation in ER (Figure 2b). In contrast, leaves agroinfiltrated with a GFP control (TMVΔCP-GFP) showed a healthy green appearance, similar to that of the mock-agroinfiltrated leaves (Figure 2b). This suggests that the negative effects might be due to *afpB* expression rather than to TMVΔCP infection.

To confirm that AfpB was produced in *N. benthamiana* leaves, we extracted the apoplastic and total protein content, and

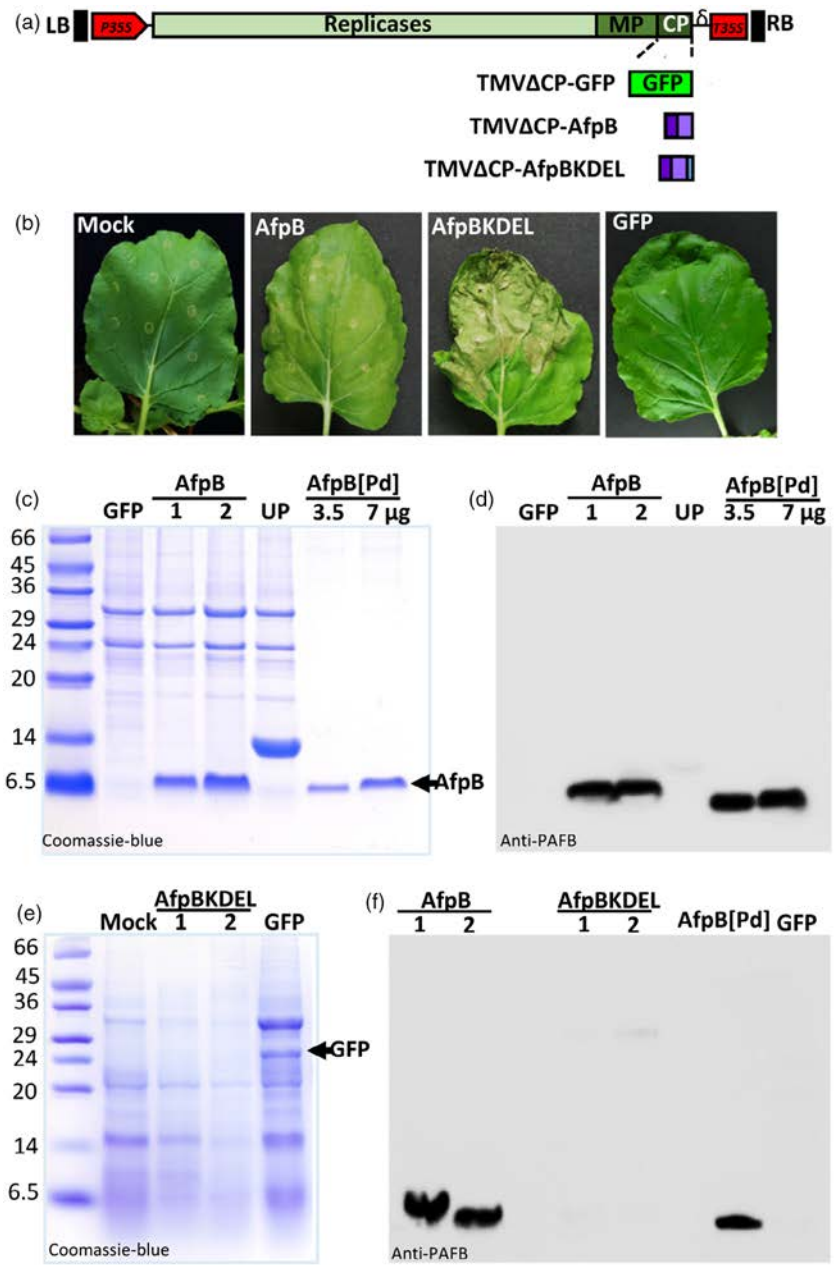

Figure 2 Production of AfpB in the apoplast of *Nicotiana benthamiana* leaves. (a) Schematic representation of the two constructs prepared and designed to accumulate AfpB in the apoplast or endoplasmic reticulum (ER) of plant cells. Both incorporate the signal peptide of tobacco AP24 protein at the N-terminus of the mature AfpB protein, and the one designed for ER accumulation also contains the ER-retention signal KDEL at its C-terminus. RB and LB, right and left border T-DNA; P35S and T35S, CaMV 35S promoter and terminator; δ, ribozyme; MP, movement protein; CP, coat protein. (b) Appearance of *N. benthamiana* leaves 7 days after agroinfiltration with the indicated constructs or with the agrobacterium induction media (mock). (c-f) Analysis of AfpB accumulation in agroinfiltrated leaves from two independent plants (1 and 2). Proteins from extracellular fluids (c, d) or acid extraction (e, f) were separated by tricine-SDS-PAGE and Coomassie-blue stained (c, e) or immunodetected using antibodies anti-PAFB (d, f). Purified AfpB (3.5 or 7 µg) from *Penicillium digitatum* cultures (AfpB[Pd]) was run in parallel as a control. ECFs from plants accumulating a cysteine-rich protein of unknown function (UP, see Figure 3) was also run in parallel for comparative purposes. Molecular weight markers are shown in kDa on the left.

analysed them by SDS-PAGE. Coomassie blue staining showed the accumulation of a small protein with same apparent molecular weight to that of the *P. digitatum* AfpB (6.46 kDa) in the extracellular fluids (ECFs) of TMVΔCP-AfpB agroinfiltrated leaves, but which was absent in leaves that had been agroinfiltrated with TMVΔCP-GFP (Figure 2c). This protein was immunodetected with specific anti-PAFB antibodies by Western blot analysis (Huber *et al.*, 2018) (Figure 2d), thus demonstrating that AfpB was produced in *N. benthamiana* leaves when targeted to the apoplastic space.

We observed no differential bands in total acid protein extracts from leaves agroinfiltrated with TMVΔCP-AfpBKDEL and mock inoculated leaves (Figure 2e). In contrast, GFP was observed clearly in the total protein extracts from TMVΔCP-GFP leaves (Figure 2e). Moreover, Western blot analysis of total acid protein extracts did not detect AfpB in leaves inoculated with TMVΔCP-AfpBKDEL, although the protein was detected in TMVΔCP-AfpB leaves (Figure 2e). These observations indicate that AfpB did not accumulate in the ER vesicles of *N. benthamiana* leaf cells, probably due to toxic effects. By comparing with the known amounts of AfpB produced by *P. digitatum*, we estimated that the accumulation of AfpB in the ECFs was ~225 ± 37 µg per gram of *N. benthamiana* leaves. Thus, the recombinant protein accounts for more than 60% of the total ECF protein.

Efficient production of proteins of fungal origin targeted to different subcellular compartments

Since AfpB was not produced when targeted to the ER, we assessed the efficiency of the TMV-based expression system using

a different fungal protein and different subcellular localizations. We selected a protein of unknown function (UP, unknown protein) encoded by the genome of *P. digitatum* (PDIG_23520); UP is a small, cysteine-rich secreted protein that has no predicted antifungal activity (Garrigues and Marcos, unpublished observations). Preliminary experiments showed that this protein was readily produced when secreted to the apoplast space (Figure 2c). We prepared three different constructs to target UP to the apoplast, the ER, or the vacuole (recombinant viruses TMVΔCP-UP, TMVΔCP-UPKDEL and TMVΔCP-UPVS, respectively; Figures 3a and S4). The constructs to target UP to the apoplast and ER were prepared as described for AfpB. The vacuolar construct carries the same N-terminal signal peptide, which allows UP to enter the secretory pathway, and also a vacuolar signal peptide at the carboxyl terminus of the protein (Neuhaus *et al.*, 1991). *Nicotiana benthamiana* leaves agroinfiltrated with these recombinant viruses appeared normal with respect to the TMVΔCP-GFP control (Figure 3b). We observed a protein with the expected mobility of *P. digitatum* UP in the ECFs obtained from TMVΔCP-UP-infiltrated leaves (Figure 3c, left panel). As judged from the intensity of bands stained with Coomassie blue, this protein achieved a higher level of accumulation than those reached by AfpB. Moreover, we obtained large amounts of this unknown protein when using TMVΔCP-UPKDEL and TMVΔCP-UPVS, the constructs designed for intracellular accumulation in ER vesicles or vacuoles (Figure 3c, right panel). These differences were striking, considering that AfpB was not detected when targeted to the ER. We estimated that the recombinant protein accumulated in the ECFs to a concentration of 4.3 ± 1.1 mg per gram of leaf fresh weight. These results demonstrate that the new TMV-based protein production system is highly efficient, and allows one to easily target recombinant proteins to different subcellular compartments.

The AfpB produced in plants is biologically active and indistinguishable from the produced by *P. digitatum*

Having established that AfpB can be produced in plants when targeted to the extracellular space, we then proceeded to its purification from plant ECFs and to determine its biological activity. For purification, we subjected ECFs from agroinfiltrated *N. benthamiana* leaves to one-step cation-exchange chromatography. We found that most proteins in the extract, except for AfpB, were not retained by the cationic column, and were found in the flow-through (Figure 4a). We then eluted the retained AfpB from the column as a single peak in fractions 14 and 15 at 0.25 M NaCl concentration, and detected a single protein band by SDS-PAGE analysis. Next we compared the antifungal activity of the purified recombinant AfpB (AfpB[Nb]) to that of the AfpB produced by *P. digitatum* (AfpB[Pd]). The activity was assayed against the producer fungus *P. digitatum*, an economically relevant pathogen of citrus fruits against which the fungal protein showed potent activity (Garrigues *et al.*, 2017). Growth inhibitory assays at different protein concentrations showed that the recombinant protein produced in biofactory plants had equivalent activity to the fungal version (Figure 4b). Importantly, the crude ECFs obtained from infiltrated plant tissues showed antifungal activity as result of AfpB production, and this activity was similar to that of the purified AfpB from fungal origin (Figure 4c). These results indicate that purification to homogeneity is not required in order to obtain a good AfpB activity.

TMV-based production of alternative antifungal proteins in biofactory plants

Finally, we wanted to evaluate the new TMV-based system with other antifungal proteins. For this, we chose the well-

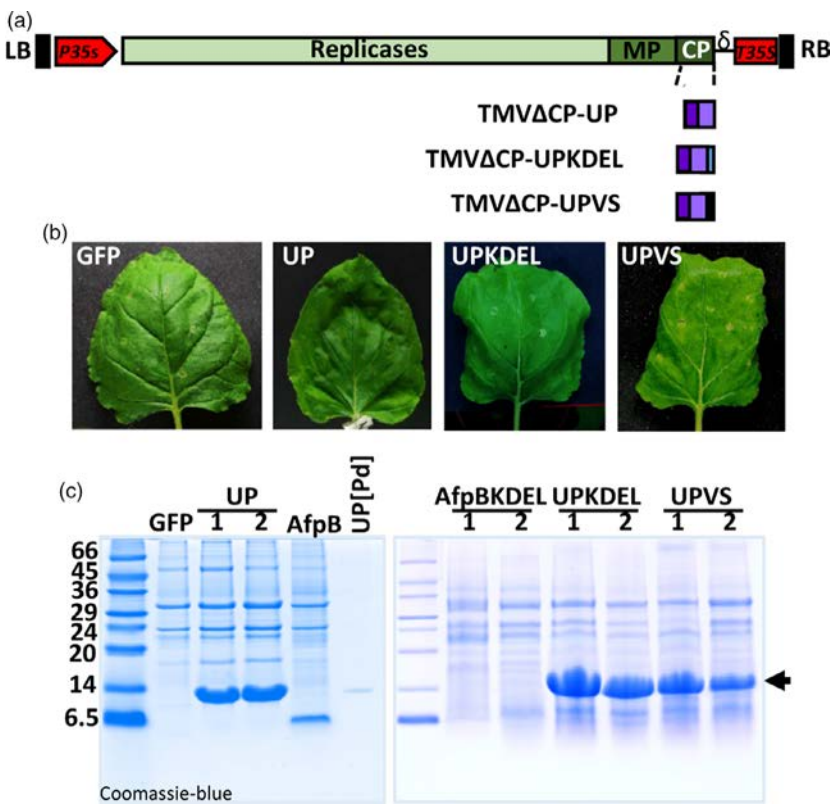

Figure 3 Production of large amounts of a *Penicillium digitatum* unknown protein in *Nicotiana benthamiana* leaves. (a) Schematic representation of the constructs generated to accumulate the protein of unknown function (UP, PDIG_23520) in the apoplast, ER, or vacuole of plant cells. The three constructs include the signal peptide of the tobacco AP24 protein at the N-terminus. A KDEL signal was added at the C-terminus in the construct designed for ER-retention, and the vacuolar signal (VS) was fused at the C-end for vacuolar accumulation. (b) Appearance of *N. benthamiana* leaves agroinfiltrated with the indicated constructs after 7 days. (c) Analysis of UP accumulation in agroinfiltrated leaves. Proteins from extracellular fluids (ECFs, left panel) or acid extraction (right panel) were separated by tricine-SDS-PAGE and Coomassie-blue stained. Purified UP from *P. digitatum* cultures was run in parallel as a control. Molecular weight markers are shown in kDa on the left.

characterized AFP from *Aspergillus giganteus* (Campos-Olivas *et al.*, 1995; Meyer, 2008; Vila *et al.*, 2001). Based on the results obtained with the AfpB, we decided to directly target AgAFP accumulation to the apoplastic space. We generated the TMVΔCP-AgAFP construct containing the sequence encoding the AP24 secretion signal peptide and the mature AgAFP (Figure S4). *Nicotiana benthamiana* leaves infiltrated with this construct had a similar appearance to the mock infiltrated leaves and leaves inoculated with the TMVΔCP-GFP control (Figure 5a). We analysed ECFs from *N. benthamiana* leaves by Western blot using the specific antibodies against AgAFP, previously described (Coca *et al.*, 2004), and found that AgAFP accumulated in the apoplasts of infiltrated leaves (Figure 5b). By comparison to known amounts of the fungal protein, we estimated the amount of protein produced to be around 196 ± 72 μg per gram of leaf fresh weight. Moreover, ECFs enriched in AgAFP showed antifungal activity against *P. digitatum* (Figure 5c). This activity seems to be at least one order of magnitude lower than that of the ECFs containing AfpB (Figure 4c), in agreement with the high activity reported for AfpB (Garrigues *et al.*, 2017). These results, obtained with a different AFP, indicate that our TMV-based system can be considered a general platform for producing antifungal proteins in biofactory plants.

Optimization of the AfpB production method for scaling the process

Syringe inoculation of plants is a time-consuming and labor intense process, and thus not suitable for large-scale production of AFPs.

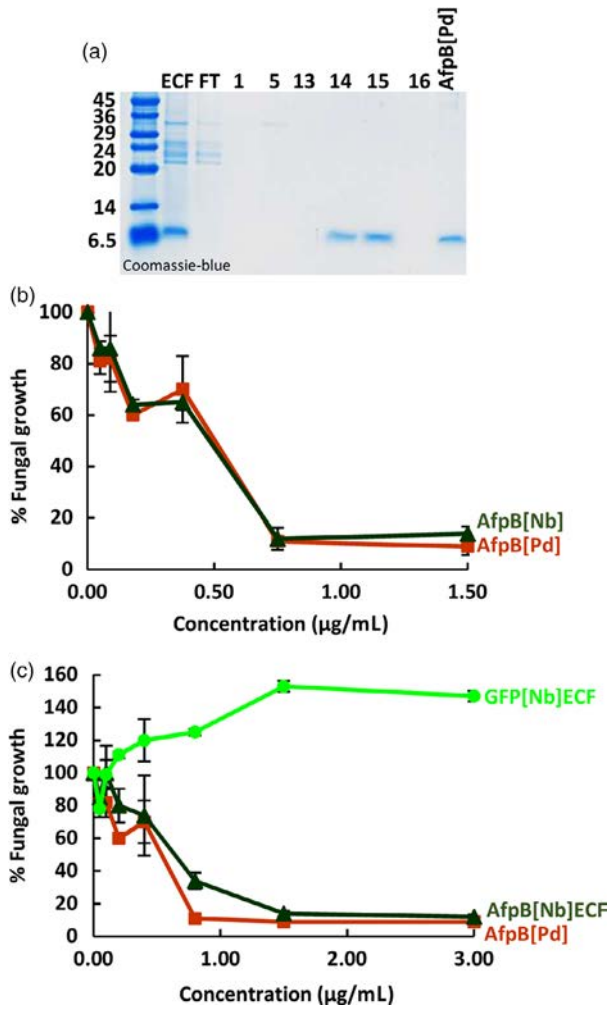

Figure 4 Equivalence of antifungal activity for the *in planta*-and *in fungus*-produced AfpB. (a) Purification of AfpB from *Nicotiana benthamiana* leaves by cationic exchange chromatography. Coomassie-blue stained tricine-SDS-PAGE gel loaded with equivalent volumes of extracelular fluid (ECF), flow-through (FT) and different eluted chromatographic fractions (1, 5, 13-16), and 3.5 μg of *Penicillium digitatum* purified protein (AfpB[Pd]). (b-c) *In vitro* inhibitory activity of AfpB against *P. digitatum* fungus. Dose–response curves comparing the fungal growth inhibitory activity of the purified AfpB[Pd] and *N. benthamiana* AfpB (AfpB[Nb]) (b), or with ECFs from leaves of *N. benthamiana* producing AfpB (AfpB[Nb] ECF) or GFP (GFP[Nb] ECF) (c). Data shown represent the mean ± SD of three biological replicates after 72 h of incubation with the indicated amounts of AfpB.

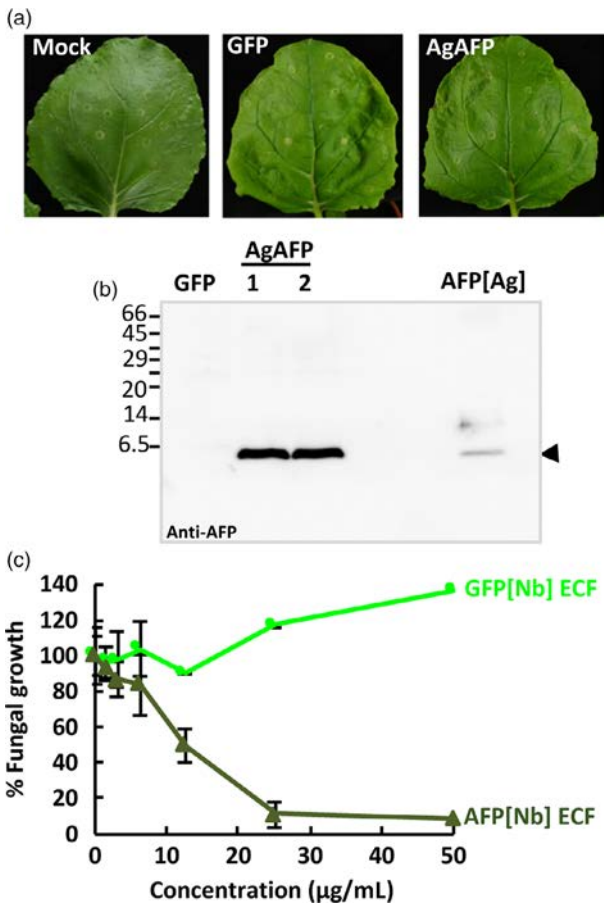

Figure 5 Production of *Aspergillus giganteus* AFP (AgAFP) in the apoplast of *Nicotiana benthamiana* leaves. (a) Appearance of *N. benthamiana* leaves 7 days after agroinoculation with the indicated constructs. (b) Analysis of AgAFP accumulation in the extracellular fluid (ECF) from two independent plants (1 and 2) by Western blot immunodetection using anti-AFP-specific antibodies. Purified AgAFP (250 ng) from *A. giganteus* cultures was run in parallel as a control (AFP [Ag]). Molecular weight markers are shown in kDa on the left. (c) Antifungal activity of ECFs containing AFP[Nb] against *Penicillium digitatum*. Dose–response curves of the fungal growth inhibitory activity of AFP[Nb] ECF in comparison to GFP[Nb] ECF. Data shown represent the mean ± SD of three biological replicates after 72 h of incubation with the indicated amounts of AFP.

We evaluated simple methods for agroinoculation that could facilitate and speed up the AFP production process. These methods included vacuum infiltration, which can be done on a large scale with robotics, and simpler spray applications, as reported previously (Hahn *et al.*, 2015). To monitor transfection efficiency easily, we used the construct TMVΔCP-GFP to produce GFP that can be visualized under UV light. In syringe- or vacuum-agroinfiltrated leaves, we observed a strong and homogeneous distribution of GFP fluorescence in the leaves at 7 days, while in sprayed leaves we could distinguish separate GFP foci (Figure 6a, upper panels). However, these GFP foci enlarged over time and merged at 12 days (Figure 6a, lower panels). These observations indicate that all three methods can efficiently produce GFP, although the spray application method requires a longer timeframe. We also tested these methods for AfpB production, and found that ECFs from agroinoculated leaves showed similar AfpB accumulation at 12 days for all three methods (Figure 6b). These results indicate that the much simpler spray application can be implemented for easy and inexpensive large-scale AfpB production.

Finally, we evaluated the stability of the *in planta* accumulated AfpB during storage as dried material, which could allow producers to uncouple biomass production from processing. Comparing AfpB accumulation in dried leaves with that in immediately frozen leaves, we observed that AfpB remains stable at least for 2 months when stored in dried leaves (Figure 6c). Therefore, AfpB can be manufactured in *N. benthamiana* without the need for immediate processing, and can be stored in dried leaves.

Protection of tomato plants against Botrytis grey mold by *in planta*-produced AfpB

Finally, we assessed the effectiveness of *in planta*-produced AfpB in plant protection assays. For that, plant protein extracts containing AfpB were evaluated against the broad-spectrum fungal pathogen *B. cinerea*. This fungus causes gray mold in plants and fresh fruits and vegetables, and is responsible for important economic losses (Dean *et al.*, 2012). AfpB produced by *P. digitatum* has *in vitro* inhibitory activity against *B. cinerea* with a MIC (minimal inhibitory concentration) value of 12.5 μM (Garrigues *et al.*, 2017). We deposited drops containing fungal conidia on tomato leaves along with *N. benthamiana* ECF containing 10 μM AfpB, as well as, drops with conidia along with the same concentration of pure AfpB produced by *P. digitatum,* and control drops with conidia along with water or ECF from GFP producing plants. Where control drops were deposited, infection symptoms were clearly visible at 4 days postinoculation (dpi); whereas where drops containing AfpB were deposited lesions were clearly smaller (Figure 7a). By quantifying lesion size using image analysis, we observed a statistically significant decrease in the damaged area in the presence of AfpB (Figure 7b). These results demonstrate that AfpB protects against *B. cinerea* infection, both for crude ECFs containing AfpB (AfpB[Nb]ECF), and for AfpB purified from fungal cultures (AfpB[Pd]). Even more interesting, the ECF extracts containing AfpB showed the same protective efficacy. Therefore, depending on the intended use for the AfpB protein, it may not be necessary to include a downstream purification process. This would reduce considerably the costs of production and biotechnological application of this antifungal biomolecule.

Discussion

In this study, we show that *N. benthamiana* plants are an excellent biofactory for producing antifungal proteins of fungal origin when transiently expressed using a TMV-derived vector. The process we have developed is fast and produces high yields of antifungal proteins, which, in addition, are bioactive in the crude ECF, or can be easily purified from crude extracts. Importantly, the recombinant proteins produced in plants have exactly the same antifungal activity as their native counterparts purified from

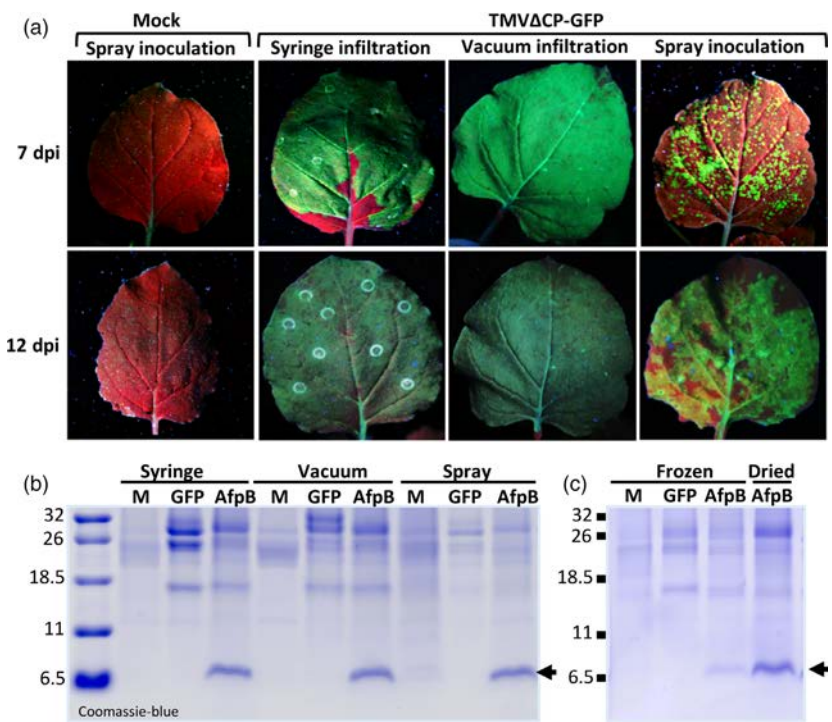

Figure 6 Optimization of AfpB production method for scaling the process. (a) Efficacy of different agroinoculation methods using TMVΔCP-GFP. Representative *Nicotiana benthamiana* leaves visualized under UV light at 7 or 12 days postinoculation (dpi), as indicated. (b) Comparative production of AfpB by agroinoculation with TMVΔCP-AfpB using the indicated methods. Coomassie-blue staining of ECF proteins from leaves at 12 dpi (c) Stability of AfpB upon dried storage of agroinoculated leaves. Coomassie-blue staining of total protein extracts obtained from leaves that were immediately frozen or dry-stored for 2 months.

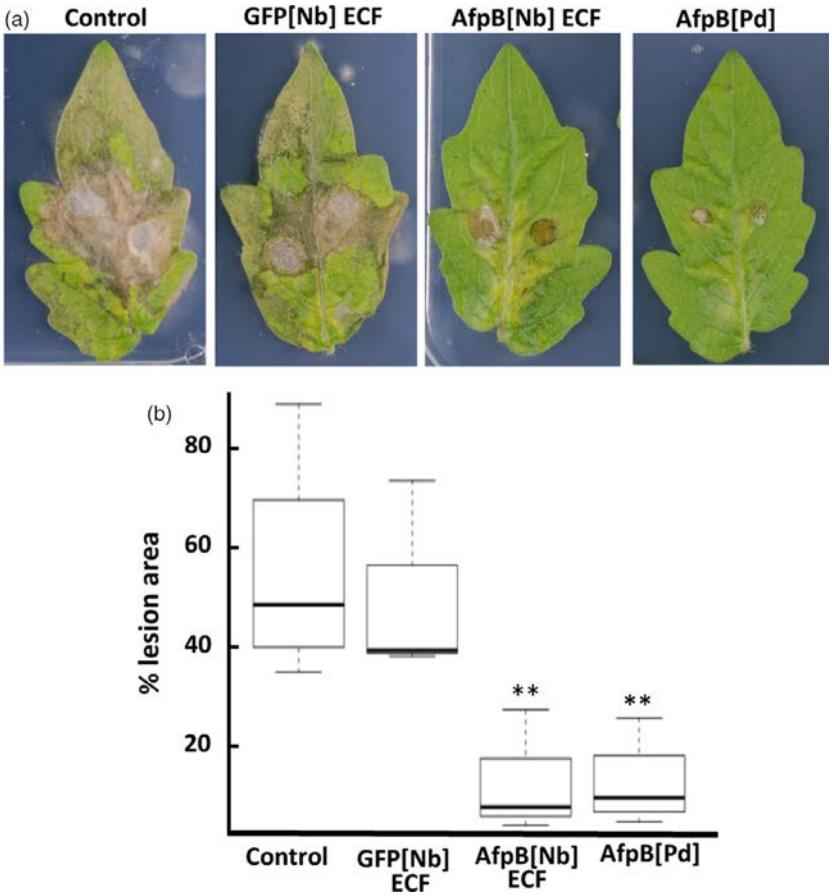

Figure 7 Protection against *Botrytis cinerea* infection on tomato leaves by *in planta*-produced AfpB produced in *Nicotiana benthamiana*. Representative leaves at 4 days after drop-inoculation with *B*. conidia suspensions (0.5×10^6 conidia/mL) along with *N. benthamiana* ECFs containing AfpB (10 μM AfpB[Nb]), GFP ECFs (GFP[Nb]) or fungal protein (10 μM AfpB[Pd]). (b) Box plot of the percentage of leaf damage area from the indicated treatments. Data represent three independent experiments. Asterisks denote statistical significance (Tukey test, $^{**}P < 0.05$).

fungi. This is a remarkable result because these small cysteine-rich proteins have been shown to be difficult to produce in a soluble, correctly folded conformation in bacterial systems (Kiedzierska *et al.*, 2008; Rosano and Ceccarelli, 2014). They only had been successfully produced as active proteins in *Pichia pastori* at relatively low yields (Garrigues *et al.*, 2017; López-García *et al.*, 2010; Virágh *et al.*, 2014), and at better yields in filamentous fungi (Garrigues *et al.*, 2017; Huber *et al.*, 2018; Sonderegger *et al.*, 2016; Tóth *et al.*, 2018). Although plant and fungal systems are similar in terms of yield and activity, the transient production of AFPs in plants represents an attractive alternative as sustainable bioproduction factories fueled by sunlight and easily scalable. The developments reported here expand the available alternatives for the exploitation of antifungal proteins and other cysteine-rich proteins such as UP, which was produced to extremely high yields.

To produce these antifungal proteins in plants, we first developed a new TMV-based vector system. The main reason for developing another TMV vector was to exploit the Gibson assembly reaction to insert the ORFs of interest into the viral genome. The Gibson assembly reaction, which is extremely efficient for inserting DNA fragments into plasmids, provides unprecedented yields when assembling several DNA fragments into a plasmid in a single step, provided these fragments contain the appropriate overlapping ends (Gibson *et al.*, 2009). This reaction facilitates the insertion of tags and the fusion of protein moieties. Moreover, we feel that modular TMV-derived systems that re-assemble *in vivo* (Marillonnet *et al.*, 2004, 2005) are unnecessarily complex, and moreover they are not freely available to the scientific community. Here, starting with an infectious clone of a TMV vulgare strain (GenBank accession number MK087763) and a small, optimized binary vector (Thole *et al.*, 2007), we constructed the plasmid pGTMV, which mediates the fast and efficient infection of *N. benthamiana* plants after infiltration with transformed *A. tumefaciens*. Marillonnet *et al.* (2005) suggested that plant agroinoculation with TMV is an inefficient process, attributed to abnormal RNA processing of the viral genome after transcription in the cellular nucleus, a step that is certainly alien to the natural TMV replication cycle. However, our experimental results demonstrate high infectivity, even at low densities of *A. tumefaciens* (Figure S5). The low infectivity previously reported in agroinoculation experiments may have been due to the use of inefficient genetic elements such as promoters and ribozymes, or the use of large, unstable binary plasmids.

Several plant-based systems have been used to produce small proteins and peptides with antimicrobial activity. While challenging due to the physicochemical properties and toxicity of many of these peptides, some of these platforms successfully produce AMPs. These platforms include leaf chloroplasts (Lee *et al.*, 2011), rice seeds (Bundó *et al.*, 2014; Montesinos *et al.*, 2016, 2017), barley seeds (Holásková *et al.*, 2018) and hairy roots (Chahardoli *et al.*, 2018). However, the yields reported when using these systems were much lower than the AFP yield achieved in *N. benthamiana* using the TMV-derived system reported here. While our estimated yield of AfpB was approximately 250 μg per gram of fresh *N. benthamiana* leaf tissue, the reported yields for Retrocyclin-101 and Protegrin-1 were ~5 and 8 μg/g of tobacco

respectively (Lee *et al.*, 2011), those for Cecropin A were ~40 μg/g of rice seeds (Montesinos *et al.*, 2016), those for LL37 were ~0.55 μg/g of barley seeds (Holásková *et al.*, 2018), and those for Lactoferrin chimera were ~4.8 μg/g of tobacco hairy roots (Chahardoli *et al.*, 2018). In addition, all of these plant-based production systems are based on stable transformation, which makes the process more complex and time consuming. Other transient expression systems have also been used for AMP production, although with lower yields than those we achieved for AFPs (Patiño-Rodríguez *et al.*, 2013; Zeitler *et al.*, 2013). Zeitler *et al.* (2013) used a TMV full-length virus strategy, to produce recombinant linear AMP SP1-1 at a yield of ~25 μg/g of *N. benthamiana* leaf tissue, while Patiño-Rodríguez *et al.* (2013) used the MagnICON system to produce Protegrin-1 at lower amounts than for the AFPs, and without providing quantitative data. In any case, even our yield of 250 μg/g for AfpB is far from the 4 mg/g achieved for other proteins, such as GFP or the *P. digitatum* unknown protein UP, which has no predicted antimicrobial activity. This highlights the difficulties associated with producing bioactive peptides in biofactory plants.

Importantly, our results with AfpB show that the subcellular localization is relevant to achieve high accumulations of bioactive peptides in plant tissues. AfpB accumulates at high levels when targeted to the extracellular space, whereas intracellular accumulation is hindered, probably by toxic effects. This observation was somehow unexpected because AFP has not previously been found to be toxic in plant cells (Coca *et al.*, 2004; Girgi *et al.*, 2006; Moreno *et al.*, 2006, 2005; Oldach *et al.*, 2001; Vila *et al.*, 2001). However, these previous studies used external application or extracellular localization of AFPs in plant tissues, so it is possible that AFPs interfere with plant cell functions once inside the cells. In contrast, the other fungal protein produced in our experiments, UP, which has no predicted antifungal activity, accumulated to similarly high intracellular and extracellular levels. These observations reveal the importance of targeting protein accumulation depending on the characteristics and activity of the recombinant protein.

Additionally, apoplastic targeting of AFPs in plant tissues greatly facilitates their purification. As observed in our work, an extremely simple purification scheme is required to purify AFPs from *N. benthamiana* tissue. ECFs can be obtained easily by vacuum infiltration of harvested leaves and sieving, yielding highly AFP-rich solutions, in which more than half of the protein content is AFPs. Starting with these ECFs, a single chromatographic step is required to purify these small proteins to homogeneity. Even better, our results show that ECFs can be used directly as antifungals with no further purification. Thus, the downstream processing of recombinant AFPs can be greatly reduced and, consequently, the overall manufacturing costs.

In addition to leaf infiltration, we have also shown that the TMV-derived system can be used to produce AFPs by spraying *N. benthamiana* tissues with *A. tumefaciens* suspensions. This simplification of the inoculation process was previously reported for the MagnICON system (Hahn *et al.*, 2015), and we have successfully implemented it for our new TMV-derived system. We demonstrate that the AFP yields achieved after 12 days by spraying leaves are similar to those achieved after 7 days by vacuum or syringe infiltration, although spraying is a much simpler inoculation strategy. Moreover, we observed that AFPs remain stable in stored *N. benthamiana* leaves for at least 2 months at room temperature. This observation suggests that the AFP production process can be decoupled from the purification process, and leaves can be conveniently stored before purification. There are clear advantages to decoupling these two processes, such as avoiding immediate processing after harvesting or otherwise freezing the plant material, with their associated production costs. These developments facilitate large-scale production of these proteins in an easy, fast cost-efficient and safe manner. In our TMV vector, the viral CP is replaced with the AFP genes, so no virus particles are created, and therefore there is no risk of viral dissemination in the environment. Also, the *afp* genes are not incorporated into the plant genome so they did not become heritable traits. Nonetheless, industrial production would require contained greenhouse conditions to avoid the release of the genetically modified *A. tumefaciens* strains and TMV clones. The green AFP manufacturing process developed here could contribute to bring these proteins into the market for practical use as antifungals. To support these applications, we also demonstrated the effectiveness of plant ECF containing AfpB in protecting tomato plants against Botrytis grey mold. *Botrytis cinerea* is one of the top ten fungal phytopathogens, causing important economic losses due to the broad range of hosts, and that damage that can occur during the production and postharvesting of vegetables, fruits and flowers (Dean *et al.*, 2012). Fungicides are the commonly used method for controlling *B. cinerea*, and botryticides represent more than 10% of the world's fungicide market. This work supports the use of green AfpB as an environment friendly and sustainable alternative to chemical fungicides.

Experimental procedures

Plasmid construction

To build pGTMV, pMTMVi-N and pMTMVi-M (Addgene plasmids # 118755, 118756 and 118757, respectively), PCR amplification reactions were performed using the Phusion high-fidelity DNA polymerase (Thermo Scientific) in HF buffer. The Gibson assembly reactions were performed using the NEBuilder HiFi DNA assembly master mix (New England Biolabs). The NEBuilder assembly tool (https://nebuilder.neb.com/; New England Biolabs) was used to design the primers. *Escherichia coli* DH5α were electroporated with the products of the Gibson assembly reaction. The full sequence of these plasmids, which was confirmed experimentally by nucleotide sequencing, is shown in Figure S2. To build plasmids expressing the various recombinant TMV clones used in this work, the intermediate plasmids pMTMVi-N or pMTMVi-M were digested with *Age*I and *Xho*I (Thermo Scientific), and the DNA fragments corresponding to the ORFs of interest were obtained by PCR and assembled using the Gibson reaction. Next, the manipulated fragment of the TMV cDNA was excised from the resulting plasmids by digestion with *Bsa*I (New England Biolabs), and assembled with the large fragment of pGTMV digested with *Nco*I-*Pfl*23II (pMTMVi-N derivatives) or *Nco*I-*Cpo*I (pMTMVi-M derivatives) (Thermo Scientific). The full sequence of all recombinant viruses is shown in Figure S4.

Agroinoculation of *N. benthamiana* leaves

Agrobacterium tumefaciens GV3101 carrying the helper plasmid pSoup (Hellens *et al.*, 2000) was transformed with plasmids to express the different TMV recombinant clones. For plant agroinoculation, overnight cultures of *A. tumefaciens* were diluted in induction medium (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone) at the appropriate optical density at 600 nm (OD_{600}), incubated for 3 h at room temperature, and infiltrated using a needle-less syringe (0.5 OD_{600}) or sprayed on

the underside of leaves using an aerograph at 2 atmospheres of pressure (1.0 OD_{600}). Importantly, the surfactant Silwet L-77 (0.1% v/v) needs to be added to the induction medium in the spray inoculation experiments for the effective agroinoculation. *N. benthamiana* plants were grown in the greenhouse at 24 °C with a 14 h light-10 h dark photoperiod. For the inoculation experiments, we used plants at the 4- to 5-leaf stage, without visible flower buds. Unless indicated in the results, leaves were harvested at 7 dpi or 12 dpi and examined for protein production. In storage experiments, leaves were dried in an incubator at 37 °C for 2 months. Plants accumulating the GFP were photographed at different time points with a Nikon D7000 digital camera under illumination with a hand-held UV lamp.

Protein analysis and purification

Protein samples were prepared by freezing agroinfiltrated leaves in liquid nitrogen and then grinding to a fine power with a mortar and pestle. Total soluble proteins were obtained in two volumes of 50 mM sodium phosphate buffer pH 7.2, 10 mM EDTA, 10 mM DTT, 0.1% (w/v) SDS and 0.1% (v/v) Triton X-100. Extracts were clarified by centrifugation at 16000 g for 15 min at 4 °C. Acidic extraction was used with AFP samples, as these proteins are pH-stable, and can be selectively extracted at low pH. The acidic buffer (pH 2.8) contained 84 mM citric acid, 30 mM Na_2HPO_4, 6 mM ascorbic acid, 0.1% (v/v) 2-mercaptoethanol. For dried leaves, total proteins were extracted in 50 mM sodium acetate (pH 5.5), 100 mM NaCl, and 10% (v/v) glycerol. Apoplastic fluids were extracted from fresh leaves by vacuum infiltration using phosphate buffered saline (PBS) buffer supplemented with 0.02% (v/v) Silwet L-77. Protein concentrations were determined using the Bio-Rad protein assay and bovine serum albumin (BSA) as standard.

Protein preparations were separated in tricine-SDS-PAGE (16.5%). Gels were stained with Coomassie blue to detect proteins, or transferred to nitrocellulose membranes (Protran 0.2 μm) to immunodetect proteins, as described previously (Coca *et al.*, 2004; Garrigues *et al.*, 2017). To detect AfpB, we used antiserum against *P. chrysogenum* PAFB, which was kindly provided by Dr. Florentine Marx (Medical University of Innsbruck, Austria) (Huber *et al.*, 2018). AFPs were purified to homogeneity by cationic chromatography using an AKTA Purifier system equipped with a 5 mL HiTrap SP HP column (GE Healthcare), as described previously (Garrigues *et al.*, 2017). AfpB from *P. digitatum* (AfpB[Pd]) was obtained from the genetically modified strain PDSG3543 and purified from culture supernatants using the same cationic chromatography (Garrigues *et al.*, 2017). Protein concentrations were determined spectrophotometrically at 280 nm in purified fractions, or by comparing the band intensities to known amounts of purified proteins in complex protein extracts, such as extracellular fluids. Signal intensities were quantified using the Quantity Tools Image Lab™ Software (Version 5.2.1) included in the ChemiDoc™ Touch Imaging System (Bio-Rad). The percentage of the recombinant protein on the total ECF protein concentration was then calculated. GFP fluorescence in protein extracts was determined as described previously (Lindbo, 2007).

Antifungal assays

Growth inhibition assays of the *P. digitatum* CECT20796 (PHI26) strain were performed in 96-well microtiter plates, as described previously (Garrigues *et al.*, 2017). Purified proteins and apoplastic fluids were dialysed against water and used at the concentrations indicated in antifungal assays.

Plant protection assays

Botrytis cinerea was kindly provided by Prof. A. Molina (CBGP collection, Madrid). Tomato plants (*Solanum lycopersicum* cultivar Marmande, known as the Mediterranean tomato) were cultivated in growth chambers at 22 °C with a 16 h light-8 h dark photoperiod. AfpB protection assays against *Botrytis cinerea* infection were performed on detached tomato leaves of 3-week-old plants on 1% (w/v) agar in water containing 2 μg/mL kinetin. Leaves were locally infected at two points with conidial suspensions (10^6 conidia/mL) by applying 20 μL drops containing 10 μM AfpB of purified protein or extracellular fluids. The progression of symptoms was followed visually. Lesion area was measured by image analysis using the Fiji ImageJ2 package. We analysed two infection points on three leaves from three independent plants in three independent experiments.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 TMV infectious clone.
Figure S2 Full sequences of pGTMV, pMTMVi-N and pMTMVi-M plasmids.
Figure S3 Analysis of GFP produced in *Nicotiana benthamiana* plants using the TMV-derived system.
Figure S4 Sequence of TMV-derived recombinant viruses TMVΔCP-GFP, TMV(ToCP)-fGFP, TMVΔCP-AfpB, TMVΔCP-AfpBKDEL, TMVΔCP-UP, TMVΔCP-UPKDEL, TMVΔCP-UPVS, and TMVΔCP-AgAFP.
Figure S5 Dilution analysis of TMVΔCP-GFP infectivity in *Nicotiana benthamiana*.
Table S1 Primers used in this work.

Chapte II

Rice Seeds as Biofactories of Rationally Designed and Cell-Penetrating Antifungal PAF Peptides

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Rice Seeds as Biofactories of Rationally Designed and Cell-Penetrating Antifungal PAF Peptides

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PAFs are short cationic and tryptophan-rich synthetic peptides with cell-penetrating antifungal activity. They show potent and selective killing activity against major fungal pathogens and low toxicity to other eukaryotic and bacterial cells. These properties make them a promising alternative to fulfill the need of novel antifungals with potential applications in crop protection, food preservation, and medical therapies. However, the difficulties of cost-effective manufacturing of PAFs by chemical synthesis or biotechnological production in microorganisms have hampered their development for practical use. This work explores the feasibility of using rice seeds as an economical and safe production system of PAFs. The rationally designed PAF102 peptide with improved antifungal properties was selected for assessing PAF biotechnological production. Two different strategies are evaluated: (1) the production as a single peptide targeted to protein bodies and (2) the production as an oleosin fusion protein targeted to oil bodies. Both strategies are designed to offer stability to the PAF peptide in the host plant and to facilitate its downstream purification. Our results demonstrate that PAF does not accumulate to detectable levels in rice seeds when produced as a single peptide, whereas it is successfully produced as fusion protein to the Oleosin18, up to 20 μg of peptide per gram of grain. We show that the expression of the chimeric *Ole18-PAF102* gene driven by the *Ole18* promoter results in the specific accumulation of the fusion protein in the embryo and aleurone layer of the rice seed. Ole18-PAF102 accumulation has no deleterious effects on seed yield, germination capacity, or seedling growth. We also show that the Oleosin18 protein serves as carrier to target the fusion protein to oil bodies facilitating PAF102 recovery. Importantly, the recovered PAF102 is active against the fungal phytopathogen *Fusarium proliferatum*. Altogether, our results prove that the oleosin fusion technology allows the production of PAF bioactive peptides to assist the exploitation of these antifungal compounds.

Keywords: PAF, antifungal, pathogens, fungi, rice, seed, oil bodies, protein bodies

INTRODUCTION

Infections caused by fungi pose a serious threat to human and animal health and to food security and safety (Fisher et al., 2012). Invasive fungal diseases have significantly increased in recent decades and are important causes of mortality, particularly in immunocompromised patients, killing about one and a half million people every year. This value exceeds the death rate for malaria or breast cancer (Brown et al., 2012). Plant disease epidemics caused by fungi and fungal-like oomycetes are an old problem that have been further exacerbated by intensive agricultural practices, globalization, and climate change (Bebber and Gurr, 2015). Today, crop-destroying fungi account globally for yield losses of ~20%, with further 10% postharvest losses (Fisher et al., 2018). In addition, food safety is challenged by mycotoxigenic fungi that contaminate food and feed with detrimental toxins for human health. The limited number of licensed antifungals currently available, together with the unprecedented rise of multidrug-resistant pathogenic fungi, makes crucial the development of novel antifungal compounds to combat fungal infections (Perfect, 2016; Fisher et al., 2018). Antimicrobial peptides (AMPs) are being actively explored to fulfill the need of novel antifungals with potential applications in crop protection, food preservation, and medical therapies.

AMPs are peptides and small proteins produced by most living organisms that exhibit lytic or inhibitory activity against microorganisms (Zasloff, 2002; Zhang and Gallo, 2016). However, most AMPs are obtained at low yields from natural sources, and some of them show properties, such as low stability or low specificity, that might compromise their applications. Their peptidic nature enables the rational design of novel molecules with improved properties to be produced at high yields through biotechnological systems. PAF102 was designed as a novel antifungal peptide with improved properties and optimized to be produced in biofactories (López-García et al., 2015). PAF102 is a modified concatemer of the hexapeptide PAF26 (RKKWFW), which was identified through a combinatorial approach as a Peptide with specific AntiFungal activity (PAF) (López-García et al., 2002, 2015; Muñoz et al., 2013). PAF102 shows potent antifungal activity against economically relevant phytopathogens and very low toxicity to other eukaryotic cells, including human erythrocytes. The antifungal mechanism of PAF102 is similar to that of the parental PAF26, and it involves the interaction with the fungal cell envelope, followed by cell penetration and intracellular effects that cause cell death (Muñoz et al., 2013; López-García et al., 2015). This mode-of-action is different to the one of licensed antifungal drugs; thus, PAF peptides might be an alternative to combat fungal pathogens. However, the difficulties of cost-effective manufacturing of PAFs by chemical synthesis, or by conventional microbial-based production systems associated to host toxicity, have hampered their development for practical use.

Plants provide a platform for the production of AMPs that offer advantages in terms of cost-effectiveness and scalability as they are economical and easy to grow, as well as of safety because of the low risk of contamination with human and animal pathogens (Twyman et al., 2003). Particularly, rice seeds have been reported as efficient bioreactors of AMPs, including natural or rational designed peptides (Bundó et al., 2014; Montesinos et al., 2016, 2017). AMP production is favored by limiting their accumulation to seeds that avoids the negative impacts on plant performance reported in some cases when accumulated in vegetative tissues (Coca et al., 2006; Nadal et al., 2012; Company et al., 2014). Several seed-specific promoters are now available to drive strong expression of *AMP* genes either in the rice endosperm or embryo (Qu and Takaiwa, 2004). Among endosperm-specific promoters are the ones from the seed storage proteins glutelins and globulins, including the *GluB1*, *GluB4*, and *Glb1* promoters; and among embryo-specific promoters are the ones from the oleosin proteins, such as the *Ole18* promoter. Another factor that favors the production of AMPs in seeds is their confinement into storage organelles, such as protein bodies (PBs) or oil bodies (OBs) (Bundó et al., 2014; Montesinos et al., 2016, 2017). Storage organelles offer a stable environment for packing a large amount of AMPs, together with host cell protection from AMP exposure. Proteins can be targeted to PBs through signal peptides linked at their N-terminus and/or KDEL sequence at their C-terminus, together through intrinsic physicochemical properties in certain storage proteins (Khan et al., 2012; Takaiwa et al., 2017). OB targeting is achieved using oleosin proteins as carriers (van Rooijen and Moloney, 1995; Montesinos et al., 2016). Oleosins are the most abundant structural proteins of plant seed OBs, whose lipophilic character and secondary structure determine their association to OBs (Abell et al., 1997). Both PBs and OBs have served to stabilize AMPs in rice seeds and to reach high yields (Bundó et al., 2014; Montesinos et al., 2016).

This work explores the feasibility of using rice seeds as a platform for the production of cell-penetrating antifungal PAF peptides, exemplified as PAF102. Two different strategies are evaluated: (1) the production as a single peptide targeted to PBs or (2) the production as an oleosin fusion protein targeted to OBs. Here, we report that PAF103, a His-tagged and KDEL-extended PAF102, was not accumulated to detectable levels in rice seeds, whereas PAF102 was successfully produced as fusion to the Ole18 protein in rice seeds. We demonstrate that the Ole18-PAF102 fusion protein was accumulated in OBs without affecting seed yield or germination capacity. We also show that biologically active PAF102 can be recovered from rice OBs. Our results demonstrate that the oleosin fusion technology is a good strategy for the production of PAF antifungal peptides.

MATERIALS AND METHODS

Preparation of Plant Expression Vectors

Four different constructs were prepared for the expression of the synthetic *PAF* genes in rice seeds (**Figure 1A**). Three of them were designed for the production of a PAF as an individual peptide, and the last one as a fusion to the rice Oleosin 18 kDa

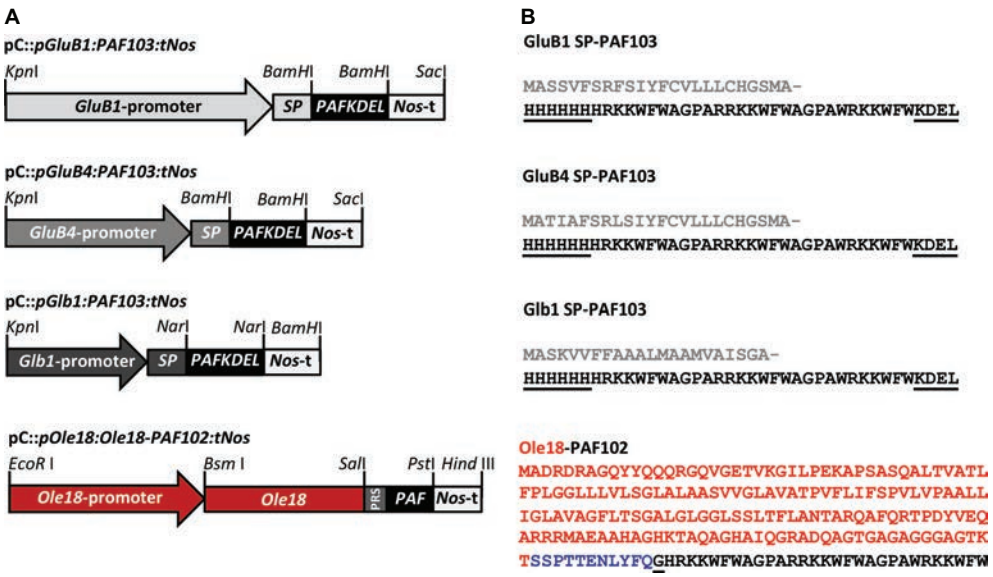

FIGURE 1 | Gene constructs for PAF production in rice seeds. **(A)** Schematic representation of the constructs in which the expression of the synthetic *PAF103* gene was controlled by the 2.3 kb *GluB1*, or the 1.4 kb *GluB4,* or 0.9 kb *Glb1* promoters, and the expression of the chimeric *Ole18-PAF102* fusion gene by the *Ole18* promoter. The corresponding signal peptides (SP) were included in the PAF103 constructs. The *Nos* terminator (Nos-t) was present in all the constructs. PRS corresponds to the TEV protease recognition sequence that links the Ole18 protein to PAF102. Relevant restriction enzyme sites for cloning purposes are indicated. **(B)** Amino acid sequences of PAF103 peptide and Ole18-PAF102 fusion protein. Gray sequences correspond to SPs of the corresponding storage proteins, which are post-translationally cleaved; black sequences to the mature PAF103 or PAF102 peptides; red sequence to the 18 kDa oleosin (Ole18) protein; and blue sequence to PRS. Underlined sequences correspond to the differential residues between PAF peptides.

protein (Ole18). The individual peptide was His-tagged in N-terminal and KDEL-extended in C-terminal resulting in a new PAF that was named PAF103 (**Figure 1B**). The *PAF103* gene was synthesized by GenScript based on the codon usage bias in *Oryza sativa* and flanked in both ends by *BamH*I restriction sites (**Supplementary Figure S1**). To drive the expression of *PAF103*, three different endosperm-specific promoters were used, namely *Glutelin B1* (*GluB1*), *Glutelin B4* (*GluB4*), and the 26 KDa *Globulin* (*Glb1*) (Qu and Takaiwa, 2004). Additionally, the sequence encoding the signal peptide of the corresponding seed storage proteins was fused to the N-terminus of the *PAF103* gene for internalization into the endoplasmic reticulum (ER) system (**Figure 1B**). The vectors containing the *pGluB1:PAF103:tNos* and *pGluB4: PAF103:tNos* constructs were prepared replacing the *BamH*I-*BamH*I *CecA-KDEL* DNA fragment by the *BamH*I-*BamH*I *PAF103* DNA fragment into the previously described pC::*pGluB1:CecAKDEL:tNos* and pC::*pGluB4:CecAKDEL:tNos* vectors (Bundó et al., 2014). The pC::*pGlb1:PAF103:tNos* vector was prepared by replacing the *Nar*I-*Nar*I *BP178* fragment by a *Nar*I-*Nar*I *PAF103* fragment into the previously described pC::*pGlb1:BP178:tNos* vector (Montesinos et al., 2017). The *Nar*I-*Nar*I *PAF103* fragment was obtained by PCR amplification from the GenScript clone using the oligonucleotides NarIPAF103_ fwd and PAF103NarI_rev in **Supplementary Table S1**.

An additional vector for the expression of the chimeric gene encoding an Ole18-PAF102 fusion protein was prepared (**Figure 1A**). In this case, the gene expression was driven by the embryo-specific *Ole18* own promoter (Montesinos et al., 2016). The fusion protein corresponds to the Ole18 protein linked to the PAF102 peptide through a Tobacco Etch Virus NIa protease recognition site (TEV protease) (**Figure 1B**), without His-tag or KDEL extension but with an extra glycine residue at N-terminus that remains after TEV protease digestion. The construct was prepared by PCR amplification of the *Ole18* promoter and the Ole18 protein coding sequence (*pOle18:Ole18*) from the pC::*pOle18:Ole18-CecA:tNos* vector (Montesinos et al., 2016) using the primers in **Supplementary Table S1**, which introduce an *Eco*RI and a *Sal*I site at 5′ and 3′ ends of the fragment, respectively. The PAF102 coding sequence extended in frame at N-terminus with the TEV protease recognition site (PRS), and flanked by *Sal*I and *Pst*I restriction sites, was synthesized by GenScript (**Supplementary Figure S1**). The *Nopaline Synthase* (Nos)-terminator sequence was introduced into the cloning vector containing the *PRS-PAF102* (pUC57::*PRS-PAF102*) as a *Pst*I-*Hind*III fragment, which was amplified by PCR using the primers that add these restriction sites (**Supplementary Table S1**). Into this plasmid, the *pOle18:Ole18* fragment was also introduced as an *Eco*RI-*Sal*I restriction fragment. Finally, the whole construct was mobilized to the binary vector pCAMBIA1300 as an *Eco*RI-*Hind*III fragment to generate the pC::*pOle18:Ole18-PAF102:tNos* vector for rice transformation (**Figure 1A**). All the constructs were then verified by nucleotide sequencing.

Production of Transgenic Plants

Transgenic rice plants (*Oryza sativa* cv. Ariete) were produced by *Agrobacterium*-mediated transformation of embryogenic calli

as previously described (Sallaud et al., 2003). Transgene insertion was confirmed in the regenerated plants by PCR analysis using leaf genomic DNA as template. The positive plants were selected to obtain homozygous lines in the T2 generation. The homozygous lines were identified by segregation of hygromycin resistance afforded by the *htp*II marker gene in the T-DNA region of pCAMBIA1300-derived vectors. The transgene copy number was estimated by quantitative PCR (qPCR) using the *Sucrose Phosphate Synthase* (*SPS*) reference gene as previously described (Yang et al., 2005; Bundó et al., 2014). Rice plants transformed with the empty vector (pCAMBIA 1300) were also produced as a control for this study. All rice plants were grown at 28 ± 2°C with a 14/10 h light/dark photoperiod.

Protein Extraction and Immunoblot Analysis

Protein extracts were prepared from dehulled mature seeds (10 seeds, ~200 mg) imbibed in water for 1 h. Seeds were ground and homogenized in a sucrose-containing buffer (10 mM phosphate buffer pH 7.6, 0.6 M sucrose). After filtration with miracloth, homogenates were centrifuged at low speed (200*g*) to remove cellular debris and starch. Clarified homogenates were then centrifuged at high speed (2,000*g*) to obtain PB-enriched fractions, as the precipitated dense fractions (Bundó et al., 2014), or the OB-enriched fractions, as the floating fractions (Montesinos et al., 2016). PB-enriched fractions were resuspended directly in SDS-loading buffer, separated on tricine-SDS-PAGE (16.5%), transferred to a nitrocellulose membrane (Amersham Protran 0.2 μm), and immunodetected using commercial monoclonal antibodies anti-His tag (A00186 GeneScript).

Immunoblot analysis of OB-associated proteins was done after solubilization in SDS-loading buffer, separation in SDS-PAGE, transfer to nitrocellulose membranes (Amersham Protran 0.4 μm), and immunodetection with antibodies against the PAF102 (1:1,000 dilution, this work) and the rice Ole18 [1:2,000 dilution, (Montesinos et al., 2016)]. Mouse polyclonal antibodies against synthetic PAF102 (GeneScript) were produced at the Laboratory Animal Facilities (registration number B9900083) of the Center for Research and Development (CID) from the Spanish National Research Council (CSIC), in strict accordance with the bioethical principles established by the Spanish legislation following international guidelines. The protocol was approved by the Committee on Bioethics of Animal Experimentation from CID and by the Department of Agriculture, Livestock, Fisheries, Food and Environment of the Government of Catalonia (permit number DAAM:7461). All efforts were made to minimize suffering of the animals. Four injections of synthetic PAF102 (0.5 mg each) in a three-weekly basis were applied to mice, which were bleed 1 week after the last injection to obtain the PAF102 antiserum.

The amount of PAF102 accumulation per seed was estimated on immunoblot by quantification of signal intensities of Ole18-PAF102 to known amounts of synthetic PAF102. Signal intensities were quantified using the Quantity Tools Image Lab™ Software (Version 5.2.1) included in the ChemiDoc™ Touch Imaging System (Bio-Rad, USA).

RT-PCR Analysis of Transgene Expression

Transgene expression was determined by RT-PCR analysis of total RNA isolated from a pool of 10 immature seeds (before seed desiccation, around 20–25 days after flowering). Total RNA was extracted using the method previously described (Chang et al., 1993). DNase-treated RNA (1 μg) was retrotranscribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) using the oligo(dT) primer. PCRs were carried out using specific primers (**Supplementary Table S1**) that annealed to GluB1, GluB4, and Glb1 SP encoding sequences (forward primers) and to the *PAF103* gene sequence (reverse primers). Amplified transcripts encoded by the three different transgenes were compared to the *OsEF1a* housekeeping transcripts (Os03g08060).

In situ Immunodetection of PAFs in Whole Seeds

PAF103 and PAF102 accumulation in the transgenic rice seeds was analyzed by *in situ* immunodetection using the anti-His tag (dil 1:1,000) and anti-PAF102 antibodies (dil 1:500), respectively, and the fluorescent labeled AlexaFluor448 anti-mouse secondary antibody (Molecular Probes, 1:5,000 dilution) as previously described (Bundó et al., 2014).

Fungal Infection Assays on Rice Seeds

Transgenic rice seeds were evaluated for resistance to the seed fungal pathogen *Fusarium proliferatum* as previously described (Bundó et al., 2014). Briefly, 12 surface-sterilized seeds per line and treatment were placed on MS medium without sucrose and then inoculated with 50 μl of sterile water (control) or of *F. proliferatum* spore suspension (10^3 spores/ml). Seeds were allowed to germinate for 7 days to determine the percentage of germination under control or infection conditions. Three independent assays were performed.

OBs Isolation and PAF102 Purification

OBs were isolated from the OB-enriched fractions by two consecutive cycles of flotation-centrifugation on a sucrose containing buffer. The integrity of the isolated OBs was tested by selective staining with Nile red (1 ng/ml, Sigma) and confocal fluorescent visualization. PAF102 was recovered from the OBs containing the Ole18-PAF102 protein by digestion with TEV protease (Invitrogen, 1:100). Proteolytic digestion was conducted overnight at 30°C in the TEV protease buffer supplemented with 0.25 M sucrose. TEV protease efficiency was estimated based on the disappearance of the Ole18-PAF102 signal in immunoblot analysis by quantification of signal intensities.

Antifungal Assays

Growth inhibition assays of *F. proliferatum* were performed in 96-well flat-bottom microtiter plates, as previously described (López-García et al., 2015). Basically, 70 μl of fungal conidia

(1.4×10^3 conidia/ml) in half strength of potato dextrose broth (PDB) containing 0.02% chloramphenicol were mixed in each well with 30 µl of samples in OB resuspension buffer (0.2 M sucrose; 10 mM Tris-HCl pH 7.5; 0.02% Tween-20). Samples were prepared in triplicate. Plates were incubated with agitation for 72 h at 28°C. Fungal growth was monitored every 24 h by measuring the optical density (OD) at 600 nm using a Spectramax M3 reader (Molecular Devices), and mean values and standard deviation (SD) were calculated. Experiments were repeated twice.

RESULTS

Generation and Characterization of Transgenic Rice Plants

Three different constructs were prepared for the expression of a *PAF* synthetic gene in rice seed endosperm (**Figure 1A**). Two of them contain the glutelin promoters (*pGluB1* and *pGluB4*) to drive expression in the peripheral region of the endosperm, and the other one contains the 26 kDa globulin promoter (*pGlb1*) to drive expression in the inner starchy endosperm tissue (Qu and Takaiwa, 2004). All three constructs were designed to produce an individual PAF peptide targeted to PBs by including the signal peptides (SP) of the corresponding storage protein and the KDEL signal. The N-terminal signal peptides target proteins to the secretory pathway, are co-translationally cleaved, and are indispensable for PB sorting (Takagi et al., 2005b). However, the C-terminal endoplasmic reticulum (ER) retention sequence (KDEL) remains in the mature proteins, and although it is not strictly required for PB deposition, it is reported to favor accumulation levels (Takagi et al., 2005b). In addition to the KDEL sequence, the PAF102 was His-tagged at the N-terminus to facilitate its purification from rice endosperm, resulting in a new PAF peptide that was named PAF103 (**Figure 1B**). Growth inhibitory activity against the *F. proliferatum* fungal pathogen revealed equivalent antifungal activity for both peptides, namely PAF102 and PAF103, with a minimal inhibitory concentration (MIC) value of 3.5 µM.

One additional construct was prepared to produce PAF102 as a fusion protein to the Ole18 (**Figure 1A**). In this case, the expression of the chimeric fusion gene *Ole18-PAF102* is directed by the embryo specific promoter of the *Ole18* gene (Qu and Takaiwa, 2004). This strategy intends to target PAF102 to OBs.

Transformation of embryogenic rice calli was performed *via Agrobacterium tumefaciens*. Using the hygromycin resistance for selecting transformed calli, a similar number of plants was regenerated for each transformation event (around 10 independent lines obtained from independent calli). The presence of the transgenes was confirmed in most of the regenerated plants by PCR analysis using leaf genomic DNA as template. The positive plants were grown under containment greenhouse conditions to obtain homozygous transgenic lines in T2 generation. Four to five independent homozygous lines per construct were identified based on the segregation of the hygromycin resistance marker. No apparent adverse effects on growth, flowering, or grain yield

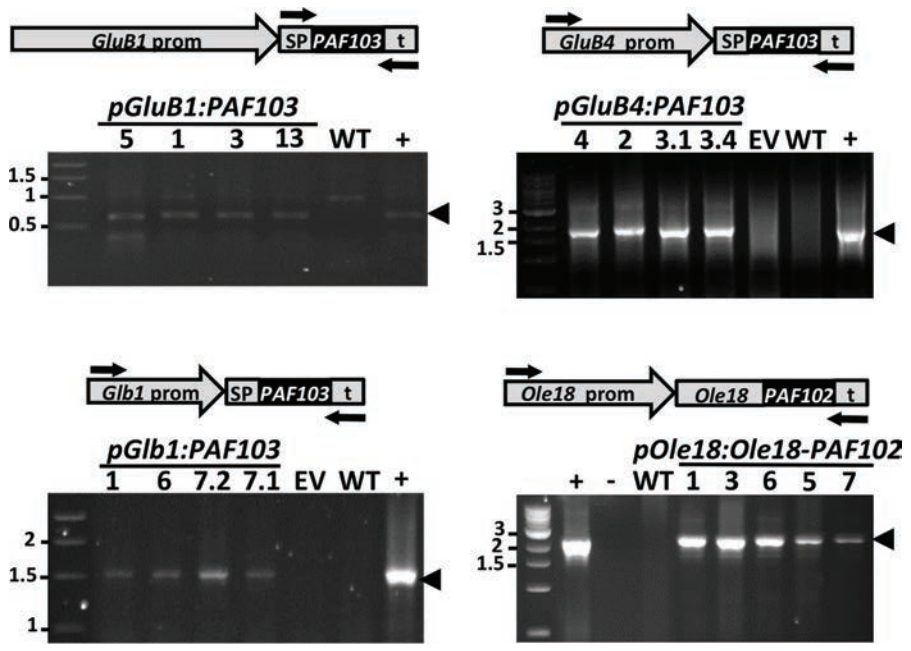

FIGURE 2 | Detection of the transgenes in the rice plant genome. PCR analysis on genomic DNA purified from leaves of wild-type (WT) or transgenic lines carrying the empty vector (EV) or the indicated transgenes. Plasmid DNA was used as a positive control (+). Arrows indicate the position of the specific oligonucleotides used for PCR amplification. The size of amplified fragments showed full length transgene insertion. Molecular markers are shown on the left in kpb.

were observed on the transgenic PAF plants across generations; all of them performed similar to the plants transformed with the empty vector grown simultaneously under the same conditions. Stability and inheritance of transgenes in T3 plants were then confirmed by PCR analysis of genomic DNA, as done in the T0 plants (**Figure 2**). The copy number of transgene insertions in the different lines was estimated by qPCR analysis in comparison to the *SPS* single copy gene in the rice genome. The results show that the transgenes were present in a single copy in all the selected homozygous lines, in agreement with the segregation in previous generations of the antibiotic resistance marker encoded in the T-DNA (**Supplementary Table S2**).

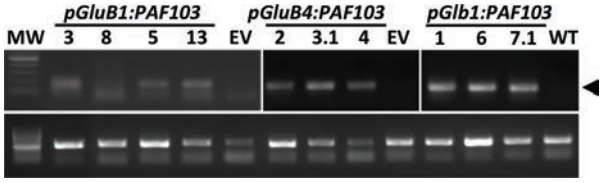

FIGURE 3 | *PAF103* transgene expression in rice seeds. RT-PCR analysis of indicated transgenes in immature rice seeds. PAF103 transcripts (upper bands) were compared to the OsEF1a housekeeping gene transcripts (lower bands).

PAF103 Does Not Accumulate in PBs of Rice Seeds

The accumulation of the single PAF103 peptide was evaluated on T2 homozygous seeds carrying the constructs *pGluB1:PAF103*, *pGluB4:PAF103*, or *pGlb1:PAF103*. Seed protein extracts enriched in dense organelles were successfully used previously for detection of cationic antimicrobial peptides deposited in PBs, such as cecropin A or BP178 (Bundó et al., 2014; Montesinos et al., 2017). Accordingly, we prepared PB-enriched extracts from mature seeds of all the homozygous lines carrying the constructs for *PAF103* expression. Western blot analysis using anti-His tag antibodies for PAF103 detection revealed no differential band on protein extracts from transgenic lines in comparison to wild type, whereas PAF103 was clearly immunodetected on a wild-type extract supplemented with the synthetic PAF103 peptide (**Supplementary Figure S2**). This result suggests that PAF103 does not accumulate in the transgenic seeds. To discard an extraction problem, we conducted an *in situ* immunodetection assay in whole seeds that equally failed to detect PAF103 on the transgenic seeds.

A different approach to assess PAF103 accumulation is through the detection of its antifungal activity. Thus, transgenic *PAF103* seeds were then evaluated for resistance to *F. proliferatum*. However, *PAF103* seeds showed similar, or even increased, susceptibility to the fungal pathogen than wild-type or empty vector seeds (**Supplementary Figure S3**). All the seeds from the different lines showed reduced germination capacity and reduced seedling growth after inoculation with fungal spores, whereas they germinated and grew normally under control conditions. Therefore, the antifungal activity of PAF103 was not detected in the transgenic rice seeds, providing additional support that the PAF103 peptide is not accumulated in these seeds.

Given that transgenes were integrated in the genome of all the independent transgenic *PAF103* lines but the transgene products were not detected, we evaluated the transgene expression in immature seeds at the developmental stage where *GluB1*, *GluB4*, and *Glb1* promoters are active (Qu and Takaiwa, 2004). We amplified the corresponding mRNAs in the tested independent lines (**Figure 3**) by RT-PCR analysis on total RNA isolated from seeds and using specific primers (**Supplementary Table S1**). These results indicate that although *pGluB1:PAF103*, *pGluB4:PAF103*, or *pGlb1:PAF103* transgene is expressed in the rice seeds, the corresponding product PAF103 does not accumulate to detectable levels.

PAF102 Accumulates as Fusion to the Ole18 Protein in Rice Seeds

The accumulation of the PAF102 when fused to the Ole18 protein was evaluated in the T2 homozygous seeds of transgenic lines carrying the *pOle18:Ole18-PAF102*. We first analyzed PAF102 accumulation by *in situ* immunodetection in whole mount seeds. As shown in **Figure 4A**, PAF102 was immunodetected in the seed embryo and aleurone layer of *pOle18:Ole18-PAF102* seeds but not in wild-type seeds. This distribution pattern corresponds to the expression pattern of the *Ole18* promoter (Qu and Takaiwa, 2004) and correlates with OB accumulation in rice seeds (Montesinos et al., 2016).

In order to confirm that PAF102 was produced as a fusion protein and retains the natural targeting of Ole18, we isolated OBs from seeds of five independent *pOle18:Ole18-PAF102* homozygous lines. After solubilization, OB-associated proteins were separated by SDS-PAGE and immunodetected using anti-PAF102 antibodies. A polypeptide of apparent molecular mass of 23 kDa (the expected mass of the fusion protein is 23.14 kDa, corresponding to 18 kDa of oleosin + 1.2 kDa of TEV protease recognition size + 3.94 kDa of PAF102) was clearly detected in the OBs of four out of five *pOle18:Ole18-PAF102* lines and was absent in the empty vector and wild-type OBs (**Figure 4B**). This protein was also immunoreacting with the anti-Ole18 antibodies as an additional and less intense band than the one corresponding to the Ole18 protein. The accumulation of the fusion protein seems not to alter the protein profile of OBs as visualized by protein Coomassie blue staining. These results demonstrate that PAF102 accumulates in rice OBs when fused to the Ole18 protein.

The amount of produced PAF102 was estimated in T3 seeds in comparison to known amounts of PAF102. The highest value was found in the line #6 producing 20 ± 3 μg/g of seed, and the mean value for the four independent lines was 15 ± 6 μg/g of seed.

In addition to be stably produced across plant generations, the fusion protein remains stable in seeds during long-time storage at room temperature. The fusion protein was still detected in seeds after 3 years of storage in the laboratory with a mean value of 16 ± 6 μg/g of seed.

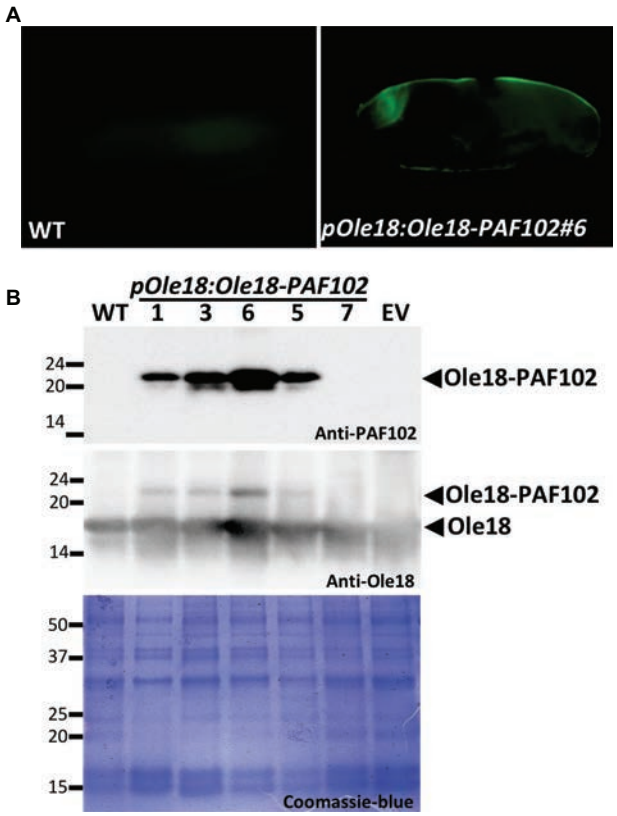

FIGURE 4 | PAF102 accumulates stably in rice seeds as an oleosin fusion protein. **(A)** *In situ* immunolocalization of PAF102 in *pOle:Ole18-PAF102* transgenic seeds (line #6) in comparison to wild-type (WT). Immunoreaction was detected using a fluorescent-labeled secondary antibody. **(B)** Immunoblot analysis of OB fractions purified from seeds of WT, empty vector (EV), and five independent transgenic lines carrying the *pOle18:Ole18-PAF102* transgene, using anti-PAF102 or anti-oleosin18 antibodies as indicated. Protein profile of OB fractions is shown by Coomassie blue staining of SDS gel. Proteins were purified from recent harvested seeds.

Ole18-PAF102 Accumulation Has No Negative Impact in Rice Plant Performance

PAF102 is a cell-penetrating peptide that kills fungal cells intracellularly (López-García et al., 2015). In order to evaluate potential toxicity of PAF102 fused to Ole18 and accumulated inside the embryonic rice cells, we characterized phenotypically the transgenic rice plants expressing the *Ole18-PAF102* under the control of the *Ole18* promoter. These plants showed normal phenotypical appearance during the vegetative phase, similar to the wild-type and empty vector plants (**Figure 5A**). Interestingly, they did not show a penalty in grain yield (**Figure 5B**), indicating that the accumulation of the Ole18-PAF102 does not affect seed production. Although differences in seed weight were observed among lines, those seeds accumulating the highest levels of Ole18-PAF102 showed similar weight on average to the control wild-type and empty vector seeds (**Figure 5C**). These data indicate that accumulation of the Ole18-PAF102 has no impact in seed filling. Additionally, seeds accumulating the recombinant fusion protein germinated at the same rate and timing as the control seeds, and their seedlings showed similar appearance (**Figures 5D,E**). Thus, the presence of Ole18-PAF102 in the OBs seems not to affect the viability of rice seeds and seedling growth. Altogether, these results suggest that the expression of *pOle18:Ole18-PAF102* does not alter the fitness of the rice plants.

Ole18-PAF102 Accumulation Does Not Protect Rice Seeds Against Fungal Infection

To investigate whether the fusion protein Ole18-PAF102 retains the antifungal activity of the single PAF102 peptide, we evaluated the *pOle18:Ole18-PAF102* seeds for resistance to *F. proliferatum*. These transgenic seeds showed similar susceptibility to the fungal pathogen than the wild-type or empty vector seeds (**Figure 6**). All the seeds from the different lines showed reduced germination capacity and reduced seedling growth after inoculation with fungal spores. These data suggest that the Ole18-PAF102 does not protect plants *in situ* against the fungal infection.

Biologically Active PAF102 Is Recovered From pOle18:Ole18-PAF102 Seeds

We next assessed the recovery of the single PAF102 peptide from rice seed OBs carrying Ole18-PAF102. For that, we digested the recombinant OBs with the TEV protease, since Ole18 and PAF102 polypeptides were linked through the protease recognition site. The immunoblot analysis of OB fractions before and after proteolytic digestion is shown in **Figure 7A**. We observed that the fusion protein nearly disappeared after protease digestion of the OBs from two *pOle18:Ole18-PAF102* transgenic lines (#3, #6). Subtle differences were detected among lines and experiments, and TEV protease efficiency was calculated at 87.5 ± 6.5% on average. These data indicate a high efficiency of proteolytic processing of the fusion protein Ole18-PAF102 on intact OBs. Next, we investigated the presence of the PAF102 single peptide in the protease-digested fractions. We immunodetected a polypeptide in the fractions of lines #3 and #6 with a higher electrophoretic mobility to the synthetic PAF102 peptide, but that was absent in the WT fractions (**Figure 7B**). Equally, the polypeptide immunodetected in the EV fractions supplemented with synthetic PAF102 peptide showed a different mobility than the synthetic PAF102 peptide alone. These results indicate that, in the presence of plant extracts enriched with OBs, PAF102 exhibits different electrophoretic mobility, consistent with the altered electrophoretic mobility that has been previously reported for other small cationic peptides (Coca et al., 2006; Bundó et al., 2014; Montesinos et al., 2016, 2017). We also observed a couple of immunoreactive bands for the pure synthetic PAF102 indicating a tendency to form multimers (**Figure 7B**). Thus, our data suggest that PAF102 is released from the fusion protein and associates with other compounds in OB fractions or multimerizes.

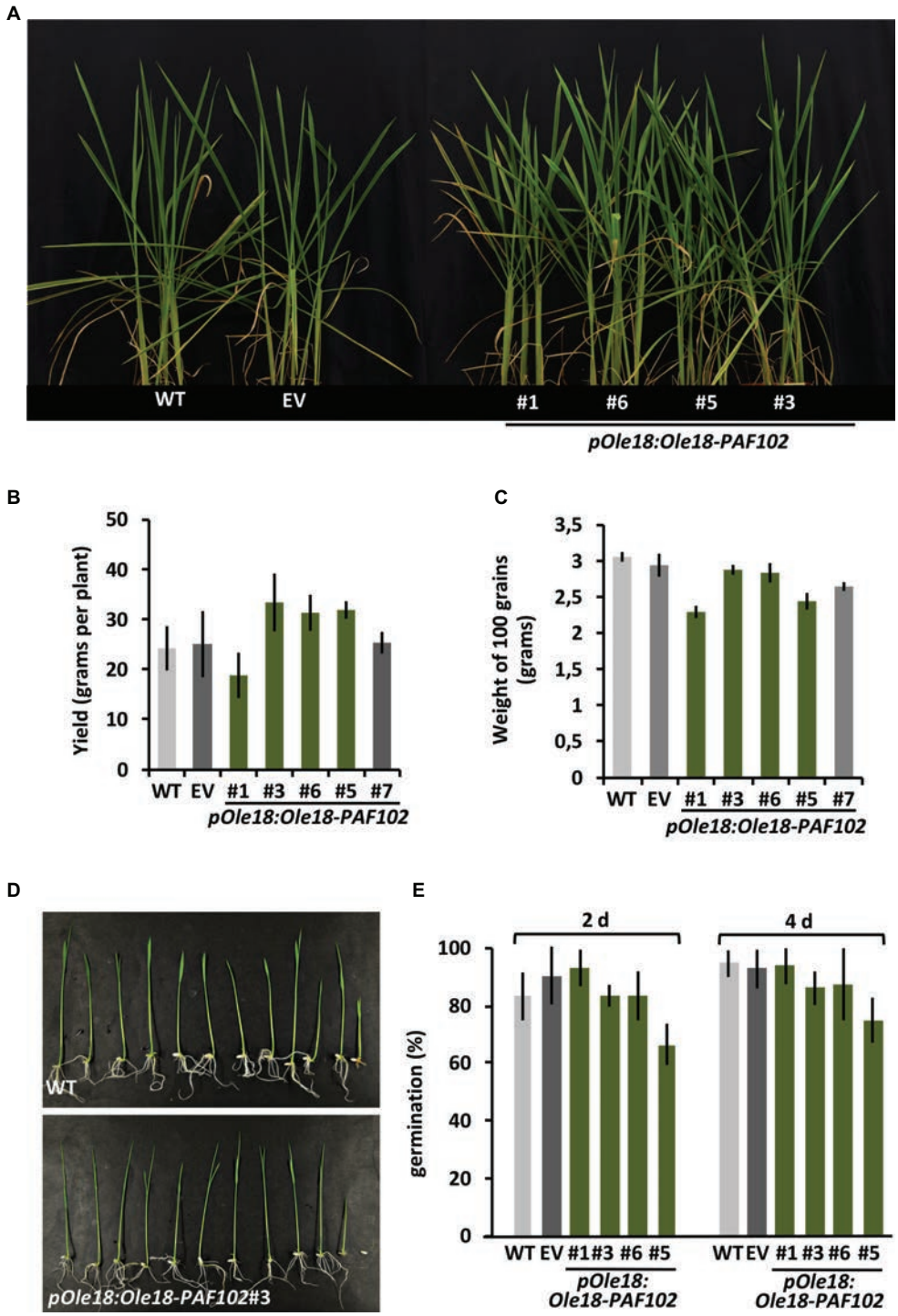

FIGURE 5 | Production of the Ole18-PAF102 fusion protein in seeds has no negative impact in rice plant performance. **(A)** Phenotypical appearance of the wild-type (WT) and the transgenic rice plants carrying the empty vector (EV) or the *pOle18:Ole18-PAF102* gene at 30 days after sowing. **(B)** Average grain yield per plant calculated from four plants per line in three independent assays (n = 12). **(C)** Average weight of 100 seeds per line (n = 12). **(D)** Phenotypical appearance of seedlings at 7 days post imbibition. **(E)** Percentage of germinated seeds at 2 and 4 days after imbibition. Values correspond to the mean value of three independent assays. Error bars represent standard deviation.

To characterize the released PAF102 peptide, we tested the antifungal activity of the different OB fractions in *in vitro* fungal growth inhibitory assays (**Figures 7C–E**). First, we checked whether the synthetic PAF102 was active against *F. proliferatum* in the OB isolation buffer (**Figure 7C**). We observed total fungal growth inhibition at the concentration of 4 μM PAF102.

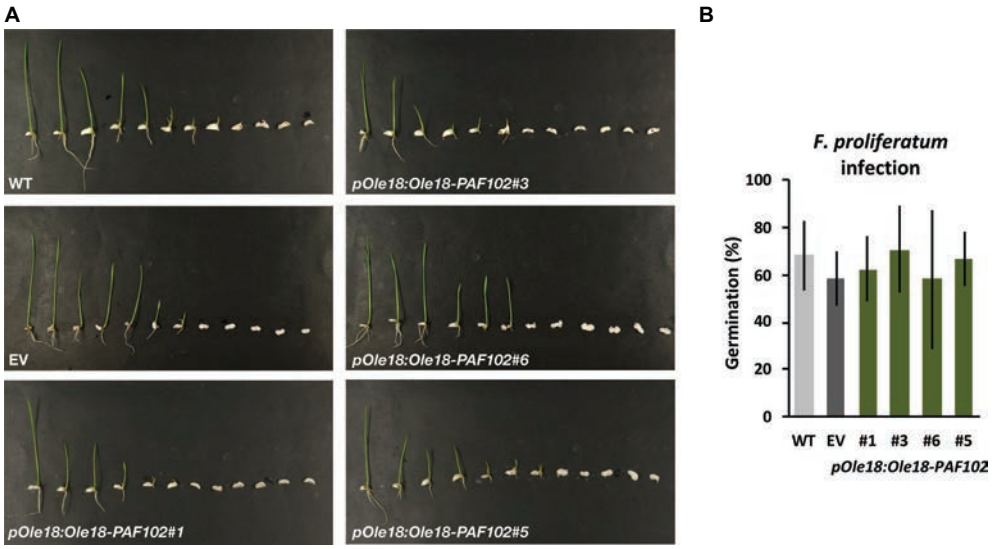

FIGURE 6 | Ole18-PAF102 accumulation does not protect rice seeds against the fungal pathogen *F. proliferatum*. **(A)** Phenotypical appearance of wild-type (WT), empty vector (EV), and *pOle18:Ole18-PAF102* transgenic seedlings (lines #1, #3, #6, and #5) at 7 days after inoculation with *F. proliferatum* spore suspensions (10^3 spores/ml). Pictures are representative of three independent experiments. **(B)** Percentage of seed germination upon infection in comparison to control conditions (see **Figure 5D**). The graph shows mean and standard deviation values of the indicated lines from three independent assays.

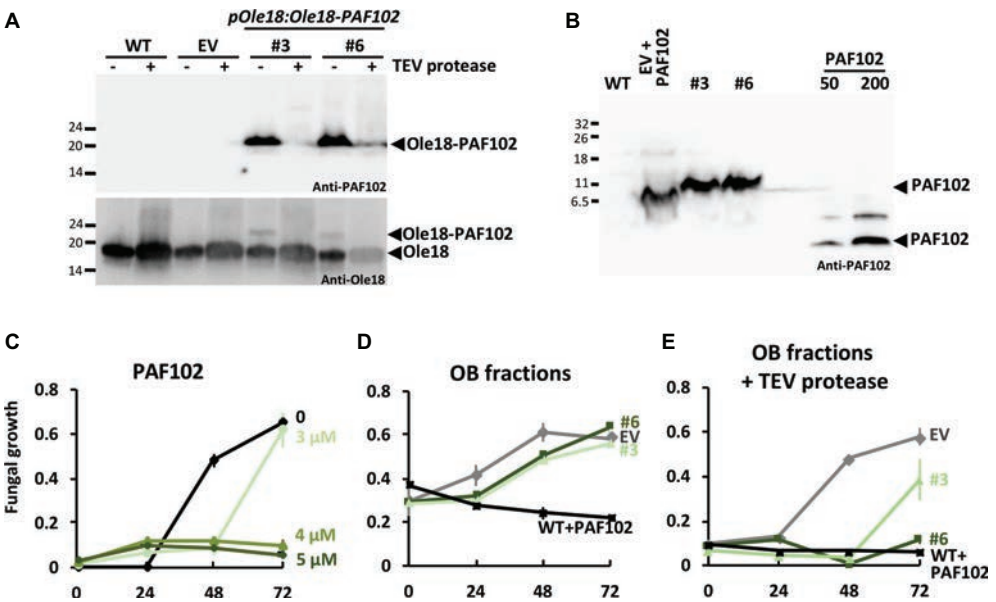

FIGURE 7 | Recovery of active PAF102 from rice seed OBs. **(A,B)** Immunoblot analysis of equivalent amount of OB fractions from wild-type (WT), empty vector (EV), and *pOle18:Ole18-PAF102* lines #3 and #6 seeds, before (–) or after (+) TEV protease digestion, and using anti-PAF102 or anti-Ole18 antibodies. Digested fractions were run in a tricine-SDS gel in parallel to synthetic PAF102 (50 and 200 ng) or to synthetic PAF102 (200 ng) added to EV fractions **(B)**. **(C–E)** *In vitro* growth inhibitory activity of *F. proliferatum* by synthetic PAF102 peptide at indicated concentrations **(C)**, by OB fractions **(D)**, or by OB fractions digested with TEV protease **(E)** from indicated lines. WT fractions were supplemented with 5 µM of synthetic PAF102. Graphs show the mean of OD_{600nm} of triplicate samples ± SD.

This inhibitory concentration agrees with reported values (López-García et al., 2015). Then, we tested intact OBs carrying the Ole18-PAF102 fusion protein from two independent lines in comparison to OBs from empty vector lines, and we did not detect fungal growth inhibitory activity (**Figure 7D**). This is an additional evidence that the fusion protein Ole18-PAF102 has no antifungal activity, as suggested by the fungal infection assays of the seeds accumulating the fusion protein (**Figure 6**). Finally, OB fractions digested with TEV protease containing the released PAF102 showed clear growth inhibitory activity against *F. proliferatum*, whereas the empty vector fractions did not possess antifungal activity (**Figure 7E**). OB fractions

from line *pOle18:Ole18-PAF102* #6 showed higher inhibitory capacity than those from line #3, in agreement with the protein accumulation levels. The inhibitory activity depicted by fractions from line #6 was similar to wild-type fractions supplemented with the synthetic PAF102. According to the activity, the amount of PAF102 in OB fractions was around 3–4 μM (30 μl from a total of 100 μl obtained from 10 seeds), which represents 13.3–17.6 μg/g of seed. These values agree with the estimation for the fusion protein Ole18-PAF102, which was 15 ± 6 μg/g of seed. Therefore, the results indicate that most of the PAF102 was released after the proteolytic digestion of the Ole18-PAF102 fusion protein as an active antifungal peptide.

DISCUSSION

Our study demonstrates that rice seeds can be used as biofactories of rationally designed antifungal peptides, exemplified in the PAF102. The production of this small bioactive peptide was only feasible when fused to the Ole18 protein, and its accumulation was not detected when expressed as a single peptide. Since the identification of PAF26 (López-García et al., 2002), this and other PAF-derived peptides have been recalcitrant to be produced through biotechnology (either in bacteria, yeast, or higher eukaryotes). Therefore, one major point of novelty of the current study is to manage the biotechnological production of PAF peptides through fusion to oleosin. We have defined a model for the mode of action of fungal-specific and PAF-derived peptides in three steps: interaction with fungal cells, internalization, and intracellular killing (Muñoz et al., 2013; López-García et al., 2015). This implies that the specificity of these peptides relies in the interaction with the fungal cell envelope and the subsequent internalization. Once the peptide is inside the cell, it might be active and killing other cells different than fungal cells, such as bacteria cells. We speculate that the peptide produced in any cell factory could be toxic when it accumulates intracellularly, unless fused to a carrier such as the oleosin, as demonstrated in this study. The fusion of PAF102 to the Ole18 protein targeted the peptide to the OBs, where it remains stable during long-time storage and accumulates to high amounts of up to 20 μg/g of seeds for such small peptides (3.9 kDa).

The production of PAFs as single peptides was approached using three different promoters, namely *pGluB1*, *pGluB4*, and *pGlb1*, which drive strong endosperm-specific expression. Our approach was based on targeting the peptide to PBs with the three corresponding seed storage protein signal peptides and the KDEL extension to produce the derivative PAF103. These SPs are known to guide protein sorting into PBs, including antimicrobial peptides (Müntz, 1998; Yang et al., 2003; Ibl and Stoger, 2012; Bundó et al., 2014; Montesinos et al., 2017; Takaiwa et al., 2017). The KDEL extension was introduced because it normally increases protein accumulation levels in PBs (Takagi et al., 2005b; Takaiwa et al., 2017). The two glutelin promoters, namely *pGluB1* and *pGluB4*, have been reported to direct expression to the outer endosperm and have been successfully used to express the *Cecropin A* gene, encoding a small, linear, and cationic antimicrobial peptide (Bundó et al., 2014). The globulin promoter directs the expression to the inner endosperm and worked better than *pGluB1* and *pGluB4* for the expression of the *BP178* gene, encoding also a synthetic small, linear, and cationic antimicrobial peptide (Montesinos et al., 2017). Although we showed that all the three promoters directed the expression of the gene to the rice seeds, we could not detect the product using different methods. Failed detection supports that the peptide did not accumulate in the rice seed. Given that all the rice plants expressing the *PAF* transgenes showed normal growth and development, and no altered seed filling and yield, cytotoxic effects seem not to be responsible for the lack of PAF accumulation. The most plausible reason is peptide instability in plant tissues. Further experiments are needed to understand why these peptides are not accumulated in rice seeds. Whatever was the reason, our results clearly show difficulties to produce PAFs as single peptides in rice seeds.

A better strategy for the production of this type of peptides is the fusion to the Ole18 protein. The fusion protein guided by the oleosin is embedded in OBs where peptides are immobilized and inactivated. Our assays clearly show that Ole18-PAF102 does not exhibit *in vivo* or *in vitro* activity against fungi, but as soon as it is released from the Ole18 it becomes active. The immobilization in OBs confers protection and offers stability to PAF102, allowing its accumulation. In our experiments, the amount of PAF102 reached up to 20 μg/g of seed, which taking into account the low molecular weight of the peptide (3.9 kDa), corresponds to 5.1 nmoles/g of seed. This yield is a little lower than the one obtained with cecropin A using the same production strategy (8 nmoles/g) (Montesinos et al., 2016), but still on the average of reported yields for small peptides in rice seeds (0.03–10 nmoles/g of seed) (Yasuda et al., 2005; Takagi et al., 2005a,b, 2008, 2010; Suzuki et al., 2011; Wakasa et al., 2011; Wang et al., 2013), or even proteins, such as lysozyme yielding up to 80 μg/g (5.6 nmoles/g of seed) when produced from a single expression cassette or 150 μg/g (10.5 nmoles/g of seed) from two independent expression cassettes (Hennegan et al., 2005). Most of these recombinant proteins were produced in the rice endosperm, which accounts for most of the grain volume (90%), whereas our strategy of protein accumulation in OBs is restricted to the rice embryo and the aleurone layer that represents only 10% of the rice grain volume. It would be interesting to explore the production of PAF102 in OB-rich seeds such as safflower, sesame, rapeseed, soybean, or sunflower. These oily seeds have been used to produce recombinant proteins using the oleosin fusion technology (Parmenter et al., 1995; Boothe et al., 2010; Nykiforuk et al., 2011). Reported yield ranges from 0.13% of total protein for insulin in *Arabidopsis thaliana* seeds (Nykiforuk et al., 2006), 0.27% for hirudin in *Brassica napus* seeds (Parmenter et al., 1995), to 0.55% for human growth factor protein in safflower (Boothe et al., 2010). Taking into account that rice grain is

not particularly rich in OBs, and our PAF yield in rice is 0.025% of seed proteins, we predict that commercial relevant values might be reached in oily seeds. Therefore, our proof-of-concept study indicates that the technology of oleosin fusion might be the best strategy to produce PAF peptides with the projection to improve yields in oily crops.

In addition to offer stability, OB accumulation facilitates the purification of PAFs from plant material by simple flotation in dense sucrose solutions. However, the PAF102 immobilized in the OBs was not active and required to be released from the Ole18 for activity. Equally, the antimicrobial peptide CecA did not exhibit activity while immobilized on the OBs (Montesinos et al., 2016), whereas other proteins have been reported to be active while associated to OBs, such as the ß-glucuronidase (van Rooijen and Moloney, 1995), a xylanase (Liu et al., 1997; Hung et al., 2008), the D-hydantoinase (Chiang et al., 2006), or the D-psicose-3-epimerase (Tseng et al., 2014), among others. The lack of activity shown by the antimicrobial peptide PAF102 while immobilized in OBs might be related to its mode of action. Being attached to OBs might prevent the PAF102 internalization into fungal cells, a process that is required for its antifungal action (Muñoz et al., 2013; López-García et al., 2015). Appropriately, antifungal activity was recovered upon release from OBs by TEV protease digestion, exhibiting equivalent activity against *F. proliferatum* to the synthetic peptide. Therefore, our results show that biologically active PAFs can be produced *in planta*.

Although rice seeds are starchy more than oily, and they might not be the best host for production of oleosin fusion proteins, they offer unique opportunities as bioreactors since the rice gene transfer technology is well developed, cropping conditions are easy and well-established worldwide, and high grain yield can be obtained (Stoger et al., 2005; Takaiwa et al., 2017). Moreover, the accumulation in seeds provides long-term stability during storage at room temperature, up to 3 years in the case of PAF102. Seeds can be stockpiled without the need to synchronize production with product demand. Additionally, the OBs are restricted to the embryo cells and aleurone layer in the rice grain. Thus, the production of the Ole18-PAF fusion protein driven by the *Ole18* promoter was only found in these specific tissues. Along with the embryo, the seed coats, including aleurone and pericarp, are separated during rice milling to obtain the white refined grain and remain as the rice bran by-product. Consequently, downstream purification is facilitated using the PAF102-enriched rice bran as the starting plant material. The use of rice bran for the production of PAFs could add an extra value to this by-product assisting their exploitation to bring them to market.

ETHICS STATEMENT

Mouse polyclonal antibodies were produced at the Laboratory Animal Facilities (registration number B9900083) of the Center for Research and Development (CID) from Spanish National Research Council (CSIC), in strict accordance with the bioethical principles established by the Spanish legislation following international guidelines. The protocol was approved by the Committee on Bioethics of Animal Experimentation from CID and by the Department of Agriculture, Livestock, Fisheries, Food and Environment of the Government of Catalonia (permit number DAAM:7461). All efforts were made to minimize suffering of the animals.

AUTHOR CONTRIBUTIONS

JM, BL-G, and MC conceived and designed the study. BL-G and MB prepared the gene constructs to be introduced in rice. MB carried out all rice transformation experiments and the molecular characterization of transgenic plants. MB, MV, and MC characterized phenotypically the generated transgenic rice plants. XS, MV, and MC characterized PAF production in rice seeds. MC coordinated the study and prepared the manuscript. All the authors read and approved the final manuscript.

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Chapte III

Biotechnological Production of the Cell Penetrating Antifungal PAF102 Peptide in *Pichia pastoris*

frontiers in Microbiology

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Biotechnological Production of the Cell Penetrating Antifungal PAF102 Peptide in *Pichia pastoris*

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Antimicrobial peptides (AMPs) have potent and durable antimicrobial activity to a wide range of fungi and bacteria. The growing problem of drug-resistant pathogenic microorganisms, together with the lack of new effective compounds, has stimulated interest in developing AMPs as anti-infective molecules. PAF102 is an AMP that was rationally designed for improved antifungal properties. This cell penetrating peptide has potent and specific activity against major fungal pathogens. Cecropin A is a natural AMP with strong and fast lytic activity against bacterial and fungal pathogens, including multidrug resistant pathogens. Both peptides, PAF102 and Cecropin A, are alternative antibiotic compounds. However, their exploitation requires fast, cost-efficient production systems. Here, we developed an innovative system to produce AMPs in *Pichia pastoris* using the oleosin fusion technology. Oleosins are plant-specific proteins with a structural role in lipid droplet formation and stabilization, which are used as carriers for recombinant proteins to lipid droplets in plant-based production systems. This study reports the efficient production of PAF102 in *P. pastoris* when fused to the rice plant Oleosin 18, whereas no accumulation of Cecropin A was detected. The Ole18-PAF102 fusion protein targets the lipid droplets of the heterologous system where it accumulates to high levels. Interestingly, the production of this fusion protein induces the formation of lipid droplets in yeast cells, which can be additionally enhanced by the coexpression of a diacylglycerol transferase gene that allows a three-fold increase in the production of the fusion protein. Using this high producer strain, PAF102 reaches commercially relevant yields of up to 180 mg/l of yeast culture. Moreover, the accumulation of PAF102 in the yeast lipid droplets facilitates its downstream extraction and recovery by flotation on density gradients, with the recovered PAF102 being biologically active against pathogenic fungi. Our results demonstrate that plant oleosin fusion technology can be transferred to the well-established *P. pastoris* cell factory to produce the PAF102 antifungal peptide, and potentially other AMPs, for multiple applications in crop protection, food preservation and animal and human therapies.

Keywords: antimicrobial peptides, antifungal peptides, *Pichia pastoris*, plant oleosin, cecropin A, PAF peptides, lipid droplets

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INTRODUCTION

Antimicrobial peptides (AMPs) are natural compounds with antimicrobial activity toward a wide range of fungi and bacteria. They are a diverse group of peptides with no consensus sequence associated to their biological activity but sharing common features: most of them are cationic, relatively hydrophobic and amphipathic molecules (Zasloff, 2002). Being amphipathic facilitates their selective interaction with negatively charged microbial membranes, so are capable of functioning as membrane-disruption peptides or cell-penetrating peptides interfering with key intracellular processes (Zasloff, 2002; Nicolas, 2009). In general, AMPs seem to act on low-affinity targets, so with little propensity to develop resistance in microbial targets (Zasloff, 2002; Hancock and Sahl, 2006; Peschel and Sahl, 2006; Sierra et al., 2017; Yu et al., 2018; Ghosh et al., 2019). The growing problem of resistance to conventional antibiotics together with the lack of new effective molecules has stimulated interest in developing AMPs as an alternative (Hancock and Sahl, 2006; Zhang and Gallo, 2016; Ghosh et al., 2019). In addition, AMPs have different mechanisms of action to other antimicrobials, reinforcing their enormous potential as a complement to conventional antibiotics. They have multiple applications in medical therapies, food preservation and crop protection (Marcos et al., 2008; López-García et al., 2012; Montesinos et al., 2012; Kang et al., 2017).

Although research on AMP development has been highly active recently, few molecules have entered the market. Major challenges to be solved before natural AMPs can be fully exploited relate to their target specificity and stability, as well as production costs. The advances in peptide synthesis have enabled the *de novo* synthesis and rational design of AMPs with improved properties (Marcos et al., 2008), with the hexapeptide PAF26 (RKKWFW) identified through a combinatorial approach as a novel peptide with specific antifungal activity and cell penetrating properties (López-García et al., 2002), and PAF102 rationally designed as a modified concatemer of PAF26 of 29 amino acid residues with high potency and specificity toward fungal cells, low toxicity to mammalian cells, high stability to proteolysis and for optimal production in plants (López-García et al., 2015). Other strategies are based on the modification of natural AMPs, such as the cecropin::melittin hybrids that are designed to decrease the toxicity of the high hemolytic melittin peptide (Cavallarin et al., 1998; Badosa et al., 2007, 2013). However, some naturally occurring peptides, such as the 37 residues peptide cecropin A (CecA) isolated from insects, have potent lytic activity against major bacterial and fungal pathogens without additional modifications (Steiner et al., 1981; Cavallarin et al., 1998; Moreno et al., 2003). More importantly, CecA has no lytic activity against mammalian erythrocytes or lymphocytes (Steiner et al., 1981; Suttmann et al., 2008). These properties make this natural peptide a good candidate to be developed as an antimicrobial in multiple fields of applications.

The other major limitation for most AMPs is their production costs. The amount of AMPs produced in living organisms is very low, and their extraction and purification requires complex and costly procedures. Chemical synthesis is only economically viable for short peptides and high value applications. Their peptidic nature enables production through biotechnological systems, but, depending on the nature of the AMPs, their production in conventional microbial systems is frequently not very efficient due to toxicity toward host cells or proteolysis of the products (Valore and Ganz, 1997; Bryksa et al., 2006; Jin et al., 2006; Wang et al., 2018). Recently, our group has demonstrated that rice seeds are good biofactories for linear AMPs that, otherwise, are difficult to produce in biological systems (Bundó et al., 2014, 2019; Montesinos et al., 2016, 2017). We found that, interestingly, PAF102 and CecA could be efficiently produced in rice seeds as oleosin fusion proteins targeted to oil bodies (OBs) (Montesinos et al., 2016; Bundó et al., 2019). OBs, also known as lipid droplets (LDs), are specialized structures composed mainly of a core of neutral lipids (triacylglycerols and steryl esters) surrounded by a monolayer of phospholipids containing a number of proteins that differ considerably between species (Chapman et al., 2012). Oleosins are plant specific proteins with a structural role in OB formation and stabilization (Tzen, 2012). They have been used as a carrier of recombinant proteins to OBs, facilitating their subsequent purification from plant material (van Rooijen and Moloney, 1995). Consequently, plant OBs have emerged as a target for biotechnological production of recombinant proteins (Parmenter et al., 1995; Nykiforuk et al., 2006, 2011; Boothe et al., 2010).

The purpose of the present study was to investigate whether oleosin fusion technology could be transferred to the well-established production system based on *Pichia pastoris* to efficiently produce AMPs. *P. pastoris* has been developed as an excellent host for the production of biopharmaceuticals and industrial enzymes. The growth of yeast is fast, with very high cell densities producing grams of recombinant protein per liter of culture (Ahmad et al., 2014). Here, PAF102 was efficiently produced in *P. pastoris* when fused to the rice plant Oleosin 18, whereas no accumulation of CecA was detected. The plant oleosin was correctly targeted to the LDs in the heterologous system and carries the PAF102 peptide, leading to high accumulation of the fusion protein in these vesicles. Moreover, the recombinant LDs were easily isolated by flotation on density gradients for PAF102 purification. We also show that the recovered PAF102 was biologically active against pathogenic fungi. Our results demonstrate that *P. pastoris* allows fast and efficient production of AMPs through oleosin fusion technology.

MATERIALS AND METHODS

Plasmid Constructs, Yeast Strains and Growth Conditions

A construct was prepared to produce the fusion protein of Oleosin 18 to the Green Fluorescent Protein (Ole18-GFP) in *P. pastoris* (**Figure 1**). The open reading frame of the rice *Ole18* (LOC_Os03g49190) without the stop codon was amplified by PCR from the plant vector pC::pOle18:Ole18-CecA (Montesinos et al., 2016) using the primers indicated in **Supplementary Table 1** (EcoRI_Ole18fwd and Ole18rev) and the Phusion high-fidelity DNA polymerase (Thermo Scientific, Spain). The *GFP*

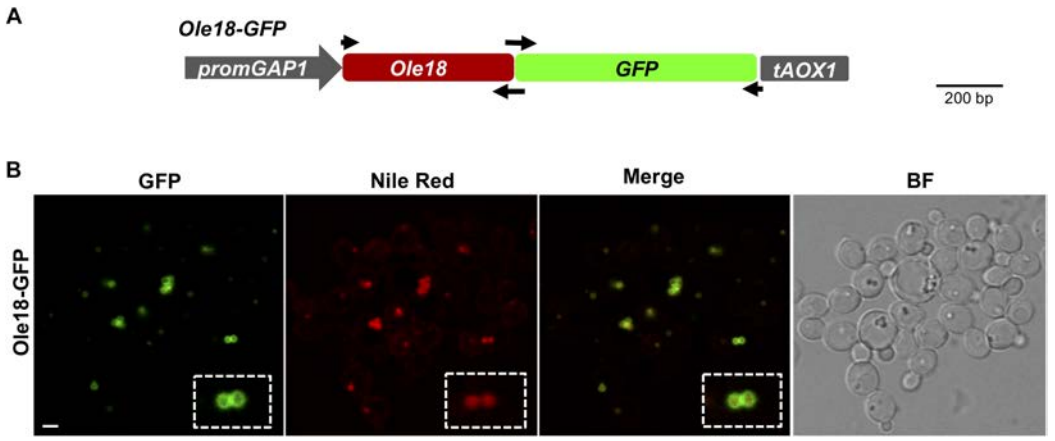

FIGURE 1 | Ole18-GFP is targeted to *P. pastoris* LDs **(A)** Gene construct used to produce the Ole18-GFP fusion protein in *P. pastoris* cells. Expression of the fusion gene encoding the rice Ole18 protein tagged with the Green Fluorescent Protein (Ole18-GFP) was under the control of the constitutive promoter *GAP1* (promGAP1) and the *AOX1* terminator (tAOX1). Arrows indicate the position of primers used for preparing the construct **(B)** Confocal laser microscopy images of cells accumulating the Ole18-GFP protein visualized under fluorescence for GFP, Nile red, and the merged GFP and Nile red image. The corresponding bright field image (BF) is also shown. Scale bar = 2 μm.

open reading frame was amplified from the pYGWY yeast vector containing the GFP tag (Popa et al., 2016) using the GFPfwd and XbaI_GFPrev primers (**Supplementary Table 1**). Amplified DNA fragments were assembled by the Gibson reaction using the NEbuilder HiFi DNA assembly master mix (New England Biolabs, United States). The assembled *Ole18-GFP* fragment was then digested with the *Eco*RI and *Xba*I restriction enzymes, and cloned into these restriction sites of the integrative yeast vector pGAPHA for expression, driven by the Glyceraldehyde-3-Phosphate Dehydrogenase (*GAP*) constitutive promoter. This plasmid derives from pGAPZA (Invitrogen, Thermo Scientific, Spain), in which the Zeocin^{TM} resistance marker was replaced by the hygromycin resistance one (Adelantado, 2016).

Two more constructs were prepared to produce the Ole18-CecA and Ole18-PAF102 fusion proteins in *P. pastoris* (**Figure 2**). The *Ole18-CecA* open reading frame was amplified by PCR from the plant vector pC::pOle18:Ole18-CecA (Montesinos et al., 2016) using the EcoRI_Ole18fwd and XhoI_CecArev primers, shown in **Supplementary Table 1**. The *Ole18-PAF102* was amplified from the pC::pOle18:Ole18-PAF102 (Bundó et al., 2019) using the same forward primer and the XhoI_PAF102rev as the reverse primer (**Supplementary Table 1**). The fusion genes were introduced into the pGAPHA plasmid as *Eco*RI-*Xho*I fragments behind the *GAP* promoter. As a control, we also prepared a construct for the single Ole18 protein (**Figure 2**). For that, the *Ole18* open reading frame was amplified with EcoRI_Ole18fwd and XhoI_Ole18rev primers (**Supplementary Table 1**), and transferred to the pGAPHA plasmid as an *Eco*RI-*Xho*I fragment.

An additional construct was prepared for the production of Diacylglycerol O-AcylTransferase 1 (Dgat1) from *Arabidopsis thaliana* (At2g19450) in *P. pastoris*. In this case the open reading frame was amplified from the G16373 clone provided by the Arabidopsis Biological Resource Center (ABRC, Ohio State University, United States) using the primers Dgat1_fwd and Dgat1_rev (**Supplementary Table 1**). The amplified fragment was inserted into the pGAPZA plasmid (Invitrogen, Thermo Scientific, Spain) using the Gibson assembly reaction. This plasmid allows gene expression under the control of the *GAP* promoter and confers Zeocin^{TM} resistance. All the constructs were verified by DNA sequencing.

Gene constructs were transferred to the wild-type *P. pastoris* strain X-33 by electroporation. Recombinant colonies were isolated using the appropriate antibiotic selection at 100 μg/ml of Zeocin^{TM} or 300 μg/ml of hygromycyn B, and then confirmed by PCR. Two to three independent colonies were analyzed by transformation event with similar results in the different assays. Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30°C. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) of the culture at different time points, or by plating serial 1/10 dilutions of an exponential growing culture on YPD agar plates.

Lipid Droplets Fractionation

Lipid droplets (LDs), along with their bound proteins, were isolated by density gradient centrifugation provided by Ficoll (Sigma-Aldrich, Spain), as previously described, with some modifications (Mannik et al., 2014). Basically, the method was adapted to lyse the cells by sonication on ice. Cells grown in YPD medium to late logarithmic phase were resuspended in Ficoll buffer I (12% Ficoll, 10 mM Tris–Hcl, 200 μM EDTA) at a concentration of 2 ml/g of cells. Cells were then lysed through seven consecutive cycles of sonication of 20 s each. After clarifying the lysate from the cell debris by low speed centrifugation, the supernatant was overlaid with the same volume of Ficoll buffer I and centrifuged at high speed (100,000 *g*) for 1 h in a SW40 rotor. LDs were recovered in the floating white layer, and then, were overlaid with a volume of Ficoll buffer II (8% Ficoll, 10 mM Tris–HCl, 200 μM EDTA). After a second round of flotation-centrifugation under the same conditions, LDs were

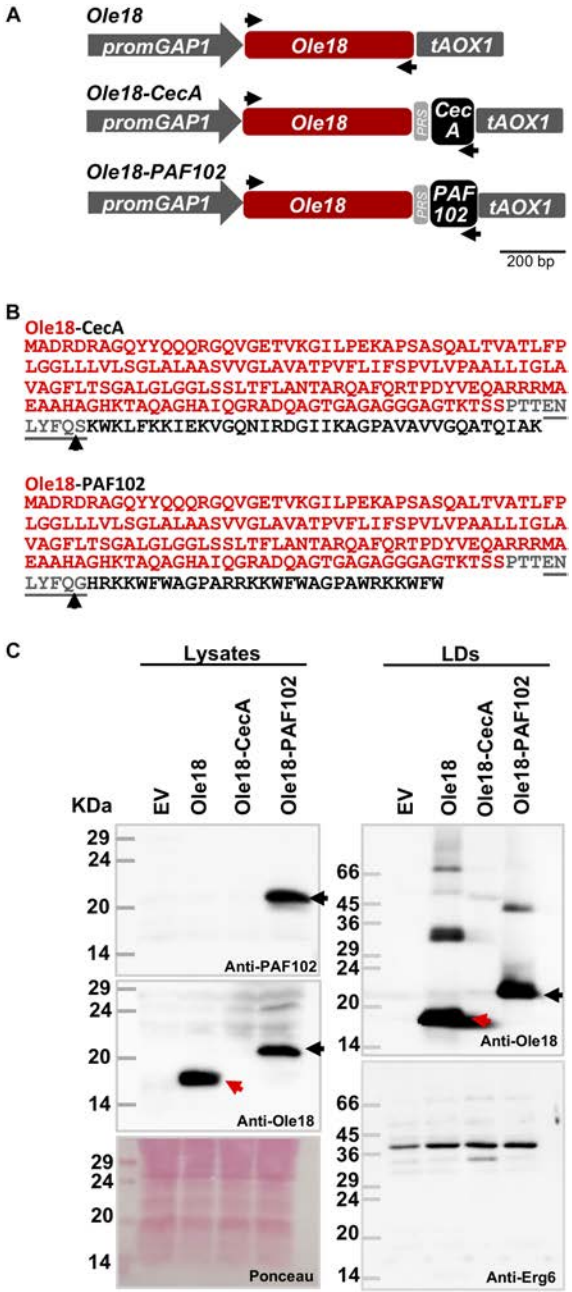

FIGURE 2 | Ole18-PAF102 accumulates in *P. pastoris* LDs. **(A)** Gene constructs used to produce the rice Ole18 and the Ole18-CecA and Ole18-PAF102 fusion proteins in *P. pastoris cells*. Ole18 protein and the CecA and PAF102 antimicrobial peptides are linked through the TEV protease recognition site (PRS). Arrows indicate the position of primers used for preparing the constructs. **(B)** Amino acid sequence of the Ole18-CecA and Ole-PAF102 fusion proteins with Ole18 protein (red) linked through PRS (gray) to CecA or PAF102 peptides (black). Arrows indicate TEV protease cleavage site. **(C)** Immunoblot analysis of equivalent total protein extracts (lysates) and LDs from *P. pastoris* cell cultures transformed with empty vector (EV) or the indicated gene constructs, using anti-PAF102, anti-Ole18 or anti-Erg6 antibodies as indicated. Ponceau staining of the membrane is shown as protein loading control (25 μg of total protein). Red arrows indicate Ole18 protein bands and black the Ole18-PAF102 fusion protein bands. Molecular weight markers are indicated in kDa on the left.

obtained at high purity in the floating fat pad. The purity and integrity of recovered LDs were confirmed by selective staining with Nile red (1 ng/ml, Sigma-Aldrich, Spain) and fluorescence microscopy visualization.

Immunoblot Analysis of LD-Associated Proteins and PAF102 Purification

LD-associated proteins were solubilized in loading buffer, separated by SDS-PAGE (15% acrylamide, Laemmli, 1970), transferred to nitrocellulose membranes and immunodetected with different antibodies: anti-Ole18 (1:2000 dilution, Montesinos et al., 2016), anti-CecA (1:500 dilution, Coca et al., 2006), anti-PAF102 (1:1000 dilution, Bundó et al., 2019), and anti-Erg6 (1:500 dilution, Paltauf et al., 1994). PAF102 accumulation was estimated on immunoblot by quantification of signal intensities of Ole18-PAF102 to known amounts of synthetic PAF102, using the Quantity Tools Image Lab™ Software (Version 5.2.1) included in the ChemiDoc™ Touch Imaging System (Bio-Rad, United States).

OBs containing Ole18-CecA or Ole18-PAF102 proteins were isolated from *pOle18:Ole18-CecA* and *pOle18:Ole18-PAF102* rice seeds as previously described (Montesinos et al., 2016; Bundó et al., 2019). OB fractions obtained from ten seeds and resuspended in 100 μl buffer (0.2 M sucrose, 10 mM phosphate buffer pH 7.6) were separated into four aliquots (25 μl each). Protease inhibitor cocktail (Sigma-Aldrich, Spain) was added to two of these, and then 25 μg of total protein extracts from the empty vector *P. pastoris* strain was added to all them. One with and one without the inhibitor were immediately frozen and the other two incubated at room temperature overnight. Proteins were then analyzed by immunoblot using anti-Ole18 specific antibodies.

PAF102 was recovered from the LDs containing the Ole18-PAF102 fusion protein by digestion with Tobacco Etch Virus (TEV) protease (Invitrogen, Thermo Fisher Scientific, Spain, 1:100), as the Ole18 protein was linked to the PAF102 peptide through a TEV protease recognition site (ENLYFQ/G). Proteolytic digestion was overnight at 30°C in TEV protease buffer. The estimated efficiency of the TEV protease was based on the disappearance of the Ole18-PAF102 signal by immunoblot analysis. The released PAF102 was immunodetected using anti-PAF102 antibodies by western-blot, after acetone precipitation, separation in Tricine-SDS-PAGE (18% acrylamide, Schägger and von Jagow, 1987) and transfer to nitrocellulose membranes (Whatman® Potran 0.2 μm).

Confocal and Fluorescence Microscopy

Cells of *P. pastoris* carrying the different gene constructs were visualized under confocal fluorescent microscopy with a Leica TCS SP5 microscope. For that, cells grown overnight in liquid medium to late logarithmic phase (1 ml of cultures) were concentrated by centrifugation for 2 min at 600 *g*, washed in water, resuspended in phosphate-buffered saline (PBS) containing 1 ng/ml of Nile red and directly visualized under a coverslip. GFP signal was detected at 500–550 nm through

excitation with an Argon ion laser emitting a 488 nm and Nile red signal at 570–670 nm using DPSS laser emitting at 561 nm.

Cell viability was evaluated by fluorescein diacetate (FDA, Sigma-Aldrich, Spain) staining (Jones and Senft, 1985). Cell cultures grown at logarithmic phase (OD_{600} = 1) were concentrated, washed and resuspended in PBS containing FDA (10 μg/ml) and stained for 20 min. After washing, the cells were visualized under fluorescent microscopy using an Axiophot Zeiss upright wide field fluorescence microscope.

The size of the intracellular LDs was estimated by image analysis using ImageJ software. The Z-projection fluorescent images were converted to binary images by applying a threshold that highlights all the structures to be measured. The area of LDs was determined using the particle analysis tool and taking into account only those droplets with spherical geometry. The 100 LDs measured per line were clustered in different size ranges and the percentage of LDs in each group was calculated. To determine the amount of LDs in the different cell lines, LDs were counted in three different replicas of the projection images of 30 cells from at least three different experiments.

Antifungal Activity Assays

Growth inhibition assays of *Fusarium proliferatum* were in 96-well flat-bottom microtiter plates as previously described, with minor changes (López-García et al., 2015). Basically, 50 μl of fungal conidia (5×10^4 conidia/ml), in 1/10 diluted potato dextrose broth (PDB) containing 0.02% (w/v) chloramphenicol, were mixed in each well with 50 μl of LD fractions. The Ficoll buffer II of LD fractions was exchanged with sterile deionized H_2O containing 0.02% Tween-20 using Zeba^{TM} Spin Desalting columns (Thermo Fisher Scientific, Spain). Samples were prepared in triplicate. Plates were incubated with agitation for 72 h at 28°C. Fungal growth was monitored every 24 h by measuring the OD_{600} using a Spectramax M3 reader (Molecular Devices, United States), and mean values and standard deviation (SD) were calculated. Experiments were repeated twice with similar results.

RESULTS

Ole18-GFP Fusion Protein Is Targeted to *P. pastoris* LDs

A gene construct was prepared to study the subcellular localization of the rice Ole18 protein in *P. pastoris* (**Figure 1A**). This construct was designed to express a *Ole18-GFP* fusion gene under the control of the constitutive promoter *GAP1* in the integrative yeast vector pGAPHA. Cell colonies carrying the gene construct were obtained by genetic transformation. Their growth rate in rich medium was similar to cells transformed with the empty vector (EV). Confocal fluorescence microscopy revealed the GFP signal surrounding spherical intracellular vesicles with diameters of 0.1–1 μm (**Figure 1B**). These vesicles co-localized with those labeled with Nile red, a fluorescent stain for neutral lipids used as an LD marker (Greenspan et al., 1985). These results indicate that the Ole18-GFP fusion protein is produced and accumulates mostly in the periphery of LDs of *P. pastoris* cells. Hence, the rice Ole18 protein was efficiently produced and correctly targeted to LDs in this heterologous system, as previously reported for other plant oleosin proteins produced in *Saccharomyces cerevisiae* yeast cells (Ting et al., 1997; Beaudoin et al., 2000; Parthibane et al., 2012; Jacquier et al., 2013; Vindigni et al., 2013). Our results also suggest that Ole18 can be used as a carrier protein to LDs in Pichia cells.

Ole18-PAF102 Accumulates in *P. pastoris* LDs

To investigate whether the oleosin fusion technology can be used to produce AMPs in *P. pastoris*, we prepared two new gene constructs (**Figure 2A**). They were designed to produce the CecA and PAF102 antimicrobial peptides as fusion to the rice Ole18 protein (**Figure 2B**). Proteins were linked through a TEV protease recognition site for the subsequent release of the AMPs from the fusion protein. We also prepared an additional construct to produce the single Ole18 protein as a control (**Figure 2A**). Transformed colonies were obtained for each construct, and integration of the transgenes was confirmed by PCR. Total protein extracts from the recombinant cultures grown in YPD media to the late logarithmic phase were analyzed by SDS-PAGE and immunoblotting (**Figure 2C**, right panels). Using specific antibodies to the rice Ole18, we detected a band of the expected size (around 18 kDa) in the lysates obtained from *Ole18* strain cultures (**Figure 2C**, red arrows). A higher band was detected in the lysates from *Ole18-PAF102* cultures (**Figure 2C**, black arrows) with an apparent size around 23 kDa, the expected size for the Ole18-PAF102 fusion protein (the theoretical molecular weight is 22.17 kDa). This band was also immunodetected using the anti-PAF102 specific antibodies. Our results indicate that the Ole18-PAF102 fusion protein was produced and stably accumulated in *P. pastoris* cells.

In contrast, no immunoreactive band was detected in the protein extracts from the *Ole18-CecA* cultures, using anti-Ole18 (**Figure 2C**) or anti-CecA antibodies (data not shown). The theoretical molecular weight of Ole18-CecA fusion proteins is 22.32 kDa. Up to five independent *Ole18-CecA* transformed colonies confirmed for transgene integration were tested, with similar results. This data suggests that the Ole18-CecA fusion protein did not accumulate at detectable levels in the yeast cells.

We then isolated the LDs from the different recombinant yeast strains by gradient density centrifugation. LD fractions were stained with Nile red and visualized under fluorescence and bright field microscopy. Samples had a high content of intact, high purity LDs. After solubilizing in SDS loading buffer, the LD proteins were separated by SDS-PAGE and immunodetected using specific anti-Ole18 antibodies (**Figure 2C**, right upper panel). We clearly detected the presence of Ole18 (red arrow) and Ole18-PAF102 (black arrow) proteins in the LD fractions, but not the Ole18-CecA protein (**Figure 2C**, right upper panel). The isolated fractions were additionally confirmed as LD fractions by immunodetection of the Erg6 protein (**Figure 2C**, right lower panel), an enzyme of the ergosterol biosynthetic pathway known to be associated with the LDs in *P. pastoris* cells

(Ivashov et al., 2013). These results show that Ole18-PAF102 accumulates in *P. pastoris* LDs.

Ole18-CecA Is Susceptible to *P. pastoris* Proteases

Given that Ole18-CecA was not detected in total or LD protein extracts from the recombinant yeast strain, we hypothesized a potential toxicity problem related to the CecA antimicrobial activity, and this would lead to growth defects. However, when the growth of *Ole18-CecA* cells was monitored, we found they grew similarly to the strains transformed with the EV, the *Ole18*, and the *Ole18-PAF102* gene constructs (**Figure 3A**). Additionally, the morphological appearance and viability of *Ole18-CecA* cells was similar to EV transformed cells when visualized under fluorescence microscopy after fluorescein diacetate viable cell staining (**Figure 3B**). We found no evidence of toxicity associated to the *Ole18-CecA* gene in our experiments.

Another possible scenario could be that Ole18-CecA does not accumulate due to instability in the yeast cells. To test the stability, we obtained the fusion protein from *promOle18:Ole18-CecA* rice seeds previously generated in our group (Montesinos et al., 2016). These rice seeds accumulate large amounts of Ole18-CecA protein in OBs (40 μg per gram of rice seeds). Isolated rice OBs containing Ole18-CecA protein were incubated *in vitro* with *P. pastoris* protein extracts from EV strain, and then analyzed by immunoblotting (**Figure 3C**). There was a clear reduction in the amount of Ole18-CecA after incubation with Pichia protein extracts unless protease inhibitors were added. As a control, we also analyzed the Ole18-PAF102 fusion protein produced in rice seeds (Bundó et al., 2019). Interestingly, Ole18-PAF102 remained stable after overnight incubation with the Pichia protein extracts. These results suggest that Ole18-CecA does not stably accumulate in *P. pastoris* cells because it is susceptible to the yeast proteases, whereas Ole18-PAF102 is more resistant to yeast proteases (**Figure 3C**) and accumulates to high levels in the yeast system (**Figure 2C**). In contrast, accumulation of Ole18-CecA is higher than Ole18-PAF102 in rice (**Figure 3C**), probably associated to a higher susceptibility of Ole18-PAF102 to rice proteases. Therefore, *P. pastoris* seems to be an efficient system for the production of Ole18-PAF102, and hereinafter we focus on this antifungal peptide.

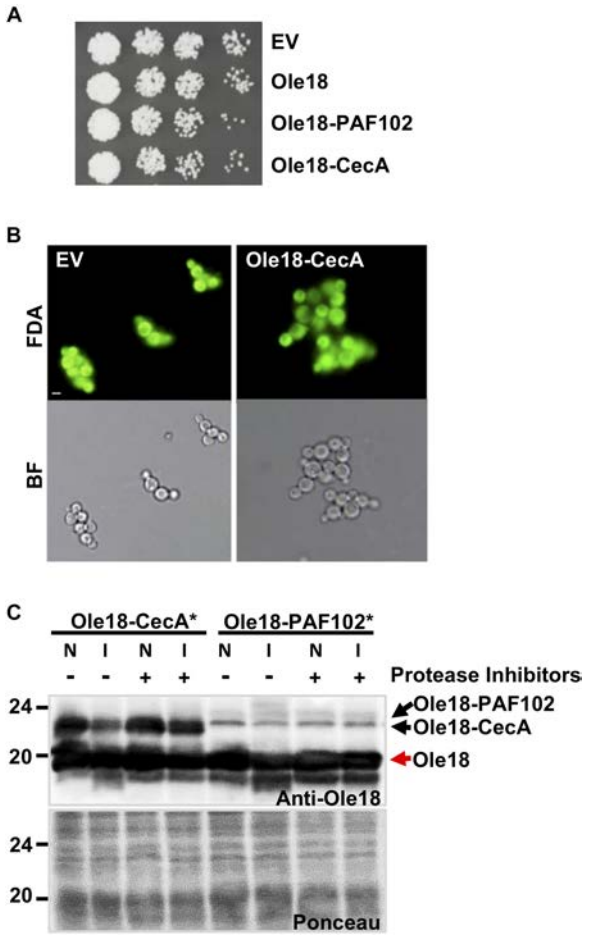

FIGURE 3 | Ole18-CecA is susceptible to *P. pastoris* proteases. **(A)** Growth of recombinant strains carrying empty vector (EV), *Olel8, Olel8-PAF102, Olel8-CecA*. Aliquots of 5 μl of ten serial dilutions of log phase cultures (OD_{600} = 1) were spotted on YPD agar media and allowed to grow for 2 days at 30°C. **(B)** Images of fluorescence and bright field microscopy of EV and *Olel8-CecA* cells after fluorescein diacetate (FDA) viability staining. **(C)** Immunoblot of OB fractions isolated from rice seeds producing Ole18-CecA* or Ole18-PAF102* fusion proteins, in the presence of total protein extracts (25 μg) from EV yeast cells, supplemented without (–) or with (+) protease inhibitors, incubated (I) or not (N) overnight at room temperature.

Ole18-PAF102 Production Induces Proliferation of LDs in *P. pastoris*

To further characterize the production of Ole18-PAF102 in the yeast cells, we closely inspected the recombinant cells under confocal microscopy (**Figure 4A**). We observed that their morphological appearance was similar to *EV* and *Ole18* cells, but they contained a higher number of LDs, with the cell-to-cell LD number and size highly variable. As the number of LDs was difficult to quantify at the cell level, we used a large population (30 cells), detecting a statistically significant increase in the cells accumulating Ole18-PAF102 (**Figure 4B**). Although there was also a tendency for a higher number of LDs in *Ole18* cells, no significant differences were quantified in comparison to EV cells. These results differ from reports in the literature showing an induction of LD proliferation mediated by other plant oleosins in the yeast *S. cerevisae* (Ting et al., 1997; Jacquier et al., 2013; Jamme et al., 2013; Vindigni et al., 2013). We also observed an increase in the size of the recombinant LDs, more pronounced in those of Ole18-PAF102 (**Figure 4C**). Our results suggest that the antifungal PAF102 peptide fused to Ole18 determines an increase in LDs in *P. pastoris* cells.

A. thaliana Dgat1 Expression Induces LD Formation in *Ole18-PAF102* Yeast Cells

With the purpose to increase the amount of LDs and presumably the accumulation of Ole18-PAF102 in the yeast cells, we expressed the *Dgat1* gene from *A. thaliana* in *P. pastoris* cells.

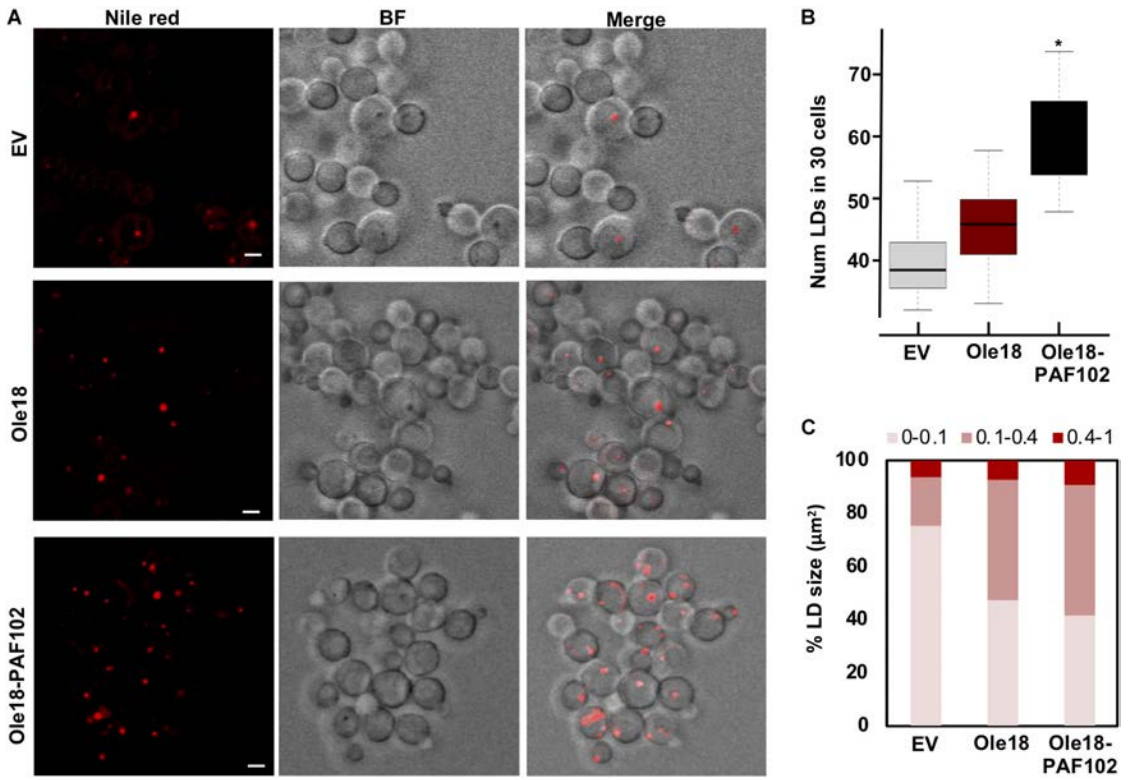

FIGURE 4 | Ole18-PAF102 production induces proliferation of LDs in *P. pastoris* cells **(A)** Confocal microscopy images of EV, Ole18, and Ole18-PAF102 recombinant cells at late log growth phase stained with Nile red. Bright field (BF) images, and fluorescence and BF merged images are also shown. Images correspond to single focal sections. Bars = 2 μm. **(B)** Total number of LDs per 30 cells of the indicated strains. LDs were counted in two replicates per assays and five independent assays (n = 10). Asterisk denotes statistically significant differences (Tukey test, $p < 0.005$) **(C)** Distribution of range sizes (area in μm^2) from 50 LDs from cells of indicated strains.

Dgat enzymes catalyze the acyl-CoA-dependent acylation of sn-1,2-diacylglycerol to produce triacylglycerol (TAG) (Liu et al., 2012). This is the rate-limiting activity for TAG biosynthesis and a target for engineering increase in oil content in microorganisms (Liu et al., 2012). The expression of the Arabidopsis *Dgat1* in *S. cerevisiae* has been reported to enhance the content in TAG and LD formation (Bouvier-Navé et al., 2000; Aymé et al., 2014). We prepared a new construct containing the coding sequence of the *AtDgat1* in the integrative pGAPZA vector, which carries another selection marker to allow double transformant selection (**Figure 5A**). To easily monitor the LD content, *Dgat1* transformation was first examined in the *Ole18-GFP* strain. Visualization of cells under confocal microscopy revealed an increase in the content of LDs in the recovered transformant cells (**Figure 5B**). Although there was a large variability among cells, most of *Ole18-GFP* possessed one or two LDs whereas the double transformant *Ole18-GFP*/*Dgat1* had three or four LDs per cell. The number of LDs was significantly higher in those cells expressing the *Dgat1* gene (**Figure 5C**), indicating that expression of the Arabidopsis *Dgat1* gene induces LD formation in *P. pastoris*, as reported for *S. cerevisiae* (Bouvier-Navé et al., 2000; Aymé et al., 2014).

We then introduced the *Dgat1* gene construct in the *Ole18-PAF102* strain. Double transformants were recovered and visualized under confocal microscopy. Nile red staining of LDs revealed accumulation of cellular LDs mediated by the expression of the *Dgat1* gene (**Figure 5D**). We found a statistically significant increase of around three times in the number of LDs of double transformant cells (**Figure 5E**), which not only had more LDs, but were also larger in size (**Figure 5F**). Consistent with our previous observations, there was high variability in the number and size of LDs in the cell population. Monitoring cell growth through optical density (OD_{600}), we also found that cells expressing *Dgat1* were slower reaching high densities (**Figure 5G**). This slow growth was not only measured in the *Ole18-PAF102* strain but also in *EV* cells, and both EV and *Ole18-PAF102* single transformants had similar growth rates. This indicates that the delay may be determined by *Dgat1* gene expression. Altogether, our results show that the LD content is further enhanced in *Ole18-PAF102* strain by expression of the Arabidopsis *Dgat1* gene, albeit requiring a longer period of culture growth.

Biologically Active PAF102 Is Recovered From Yeast Cells

Knowing that double transformant *Ole18-PAF102/Dgat1* cells contain a high number of LDs, and that Ole18-PAF102 is

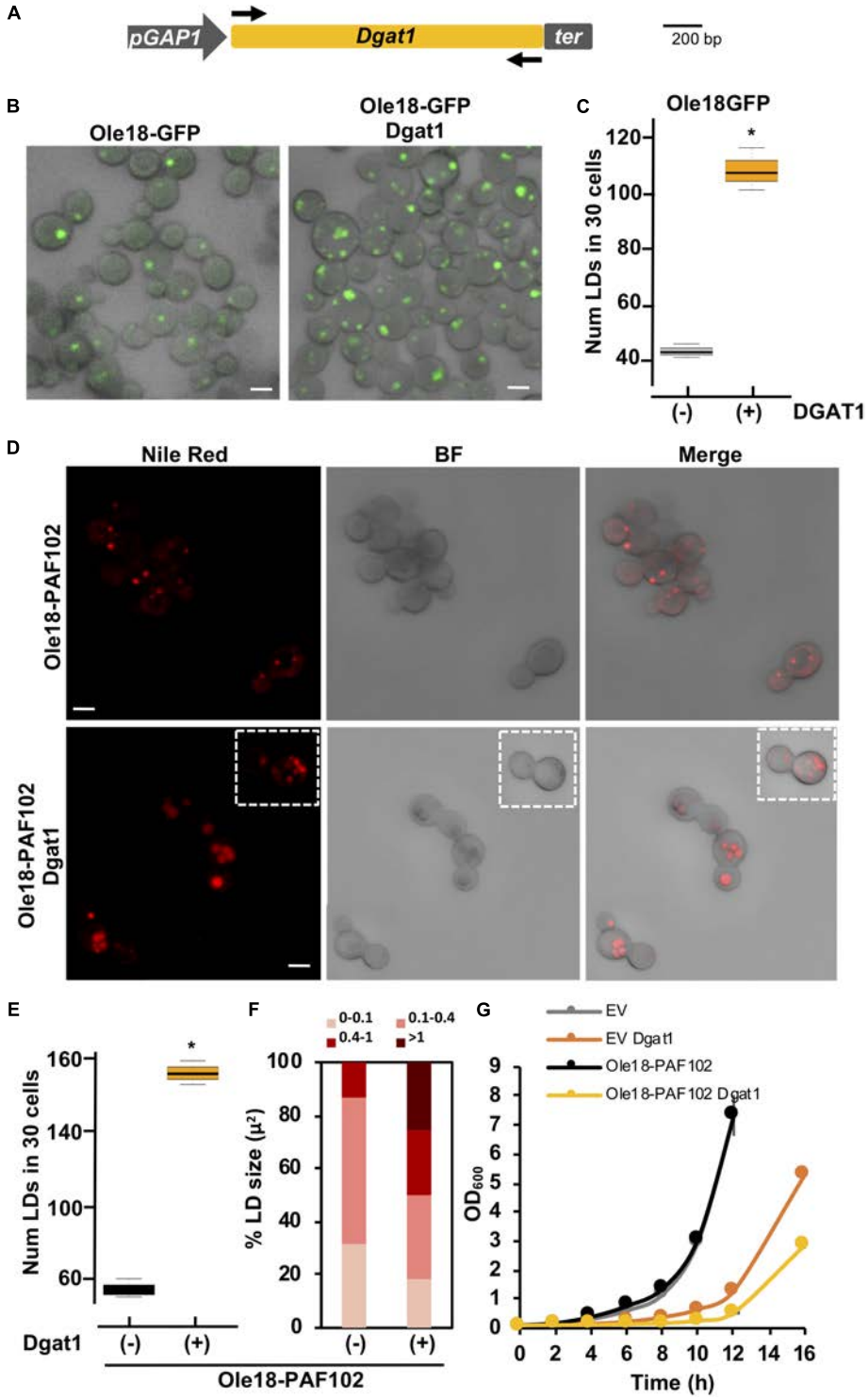

FIGURE 5 | *A. thaliana* Dgat1 expression induces LD formation in Ole18-PAF102 yeast cells **(A)** Gene construct introduced into the pGAPZA vector for producing *A. thaliana* Diacylglycerol o-acyltransferase (Dgat1) enzyme (At2g19450) in *P. pastoris*. **(B)** Confocal microscopy images of Ole18-GFP cells with or without the Dgat1 construct, as indicated. Bright field (BF) and fluorescence merged images are shown. **(C)** Total number of LDs per 30 cells of the indicated strains. LDs were counted in three replicates. Asterisk denotes statistically significant differences (Tukey test, $p < 0.005$) **(D)** Confocal microscopy images of Nile red stained Ole18-PAF102 cells with or without the Dgat1 construct as indicated. Bright field (BF), and fluorescence and BF merged images are also shown. Images correspond to single focal sections. **(E)** Total number of LDs per 30 cells of the indicated strains. LDs were counted in three replicates. Asterisk denotes statistically significant differences (Tukey test, $p < 0.005$) **(F)** Distribution of range sizes (area in μm^2) from 50 LDs from strain cells indicated. **(G)** Growth of indicated strain cultures. Graph represents the mean $OD_{600} \pm$ SD value of triplicate cultures measured every 2 h. Bars = 2 μm.

accumulated in these LDs, we evaluated the amount of PAF102 peptide produced by this strain. We isolated the LDs by density gradient centrifugation and analyzed their Ole18-PAF102 content by immunodetection. As shown in **Figure 6A**, Ole18-PAF102 accumulation was higher in the *Dgat1* expressing strain and correctly targeted to LDs. We estimated that the fusion protein accumulates at 67 ± 2 mg/l in the *Ole18-PAF102* strain, and reaches up to 180 ± 5 mg/l in the *Ole18-PAF102/Dgat1* strain. This represents a near three-fold increase in the fusion protein accumulation levels, congruently with the three-fold increase in cellular LD content (**Figure 5E**).

Using this high producer *Ole18-PAF102/Dgat1* strain, we assessed the recovery of the single PAF102 peptide from the LDs containing the Ole18-PAF102 fusion protein. TEV protease was used to digest the recombinant LD fraction since the Ole18 and PAF102 polypeptides were linked through the TEV protease recognition site (PRS). In immunoblot analysis of LD fractions after proteolytic digestion (**Figure 6B**), we observed that the fusion protein detected by anti-PAF102 antibodies was almost absent (upper panel), and a protein slightly larger than Ole18 appeared, recognized by anti-Ole18 antibodies (lower panel). After TEV protease digestion, most of the PRS remained attached to the Ole18 protein. This gives the Ole18 released from the fusion protein by TEV protease digestion a theoretical molecular weight of 18.25 kDa, whereas the single Ole18 is only 17.15 kDa. The bands detected in western-blot analysis are consistent with this difference in size and suggest that the fusion protein was processed by the TEV protease. Based on the disappearance of the fusion protein in different assays, the TEV protease efficiency was calculated at 86% ± 10.5 on average. This data indicates a high efficiency of proteolytic processing of the fusion protein Ole18-PAF102 on intact LDs. We then investigated the presence of the PAF102 single peptide in the protease digested fractions. We immunodetected a polypeptide in the Ole18PAF102 LDs with similar mobility to the synthetic PAF102 (sPAF102) added to the EV LDs, or to the sPAF102 alone (**Figure 6C**). This band was not present in Ole18 LDs or in non-digested Ole18PAF102 LDs, indicating that the fusion protein was correctly processed and the PAF102 released.

To characterize the released PAF102 peptide, we tested the antifungal activity of the LDs fractions before and after TEV protease digestion. For that, we monitored the growth of *F. proliferatum* in the presence of LDs at different concentrations. We did not detect any fungal growth inhibitory activity in the intact LD fractions, regardless of whether they were from EV, *Ole18* or *Ole18-PAF102* strains (**Figure 6D**), whereas the sPAF102 completely inhibited *F. proliferatum* growth at the very low concentration of 2 μM. This data indicates that the fusion protein Ole18-PAF102 was not active, at least when embedded into LDs. However, TEV protease digested Ole18-PAF102 LDs clearly inhibited the growth of *F. proliferatum*, and the inhibitory activity was equivalent to that of EV LDs supplemented with sPAF102 and incubated with TEV protease (**Figure 6E**). It is worth noting that the synthetic PAF102 was more active in the presence of EV LDs and TEV protease, completely inhibiting fungal growth at the even lower concentration of 0.25 μM. Given that Ole18 LDs and TEV protease did not have any inhibitory activity (**Figure 6E**), TEV protease could be assisting PAF102 activity. Our results demonstrate that when PAF102 is produced in *P. pastoris* using the oleosin fusion technology, it is biologically active and equivalent to that produced by chemical synthesis.

DISCUSSION

This study reports that *P. pastoris* is an excellent cell factory for the fast and efficient biotechnological production of the antifungal PAF102 peptide, and probably other PAF peptides. The process developed here is an innovative system based on the plant oleosin fusion technology that we successfully transfer to the heterologous yeast system. It has so far been difficult to produce rationally designed PAF antifungal peptides in conventional microbial expression systems, probably due to toxicity toward host cells. We recently demonstrated that these bioactive peptides could be produced in plant biofactories as oleosin fusion proteins (Bundó et al., 2019). The fusion of PAF102 to the rice Ole18 protein reduces its toxicity to the host cells allowing its accumulation to large amounts. We show here that the Ole18-PAF102 fusion protein is also produced in yeast cells and targeted to LDs where large amounts accumulate. By coexpressing the Arabidopsis *Dgat1* gene, *P. pastoris* cells produced up to 180 mg/l of PAF102 peptide in only 4 days. This yield was obtained from shake-flash cultures, and typically recombinant protein yield can be increased in fermenter cultures. This demonstrates that our new system is a considerable improvement on the previous rice-seed based system in terms of production times and yields of commercial relevance.

Nevertheless, the natural CecA peptide is not produced at detectable levels in this *P. pastoris* system, although it was efficiently produced in rice seeds when fused to Ole18, even to higher levels than PAF102 (Montesinos et al., 2016). Recombinant protein production is not only dependent on gene expression and protein synthesis but also on its stability in host cells to allow its accumulation to significant amounts. Therefore, the lack of Ole18-CecA accumulation to detectable levels by immunoblot analysis might be due to instability in *P. pastoris* cells. Indeed, our results show that CecA is highly susceptible to the yeast proteases. In contrast, PAF102 seems to be more resistant to Pichia proteases and accumulates to high levels. Reports have been already published on the high susceptibility to proteolytic degradation of CecA by fungal proteases (Bland and Lucca, 1998) or of CecA-derived peptides by tobacco plant proteases (Cavallarin et al., 1998). However, CecA stably accumulated in transgenic rice plants, suggesting limited proteolysis by rice proteases (Coca et al., 2006; Bundó et al., 2014; Montesinos et al., 2016). This data indicates differential degradation of CecA by host proteases, with its stability varying from one system to another: the stability of CecA to protein extracts derived from the host system should first be tested when planning to produce CecA in a new system. Still, the use of protease-deficient strains might improve production of CecA in *P. pastoris*. These strains have been used for the production

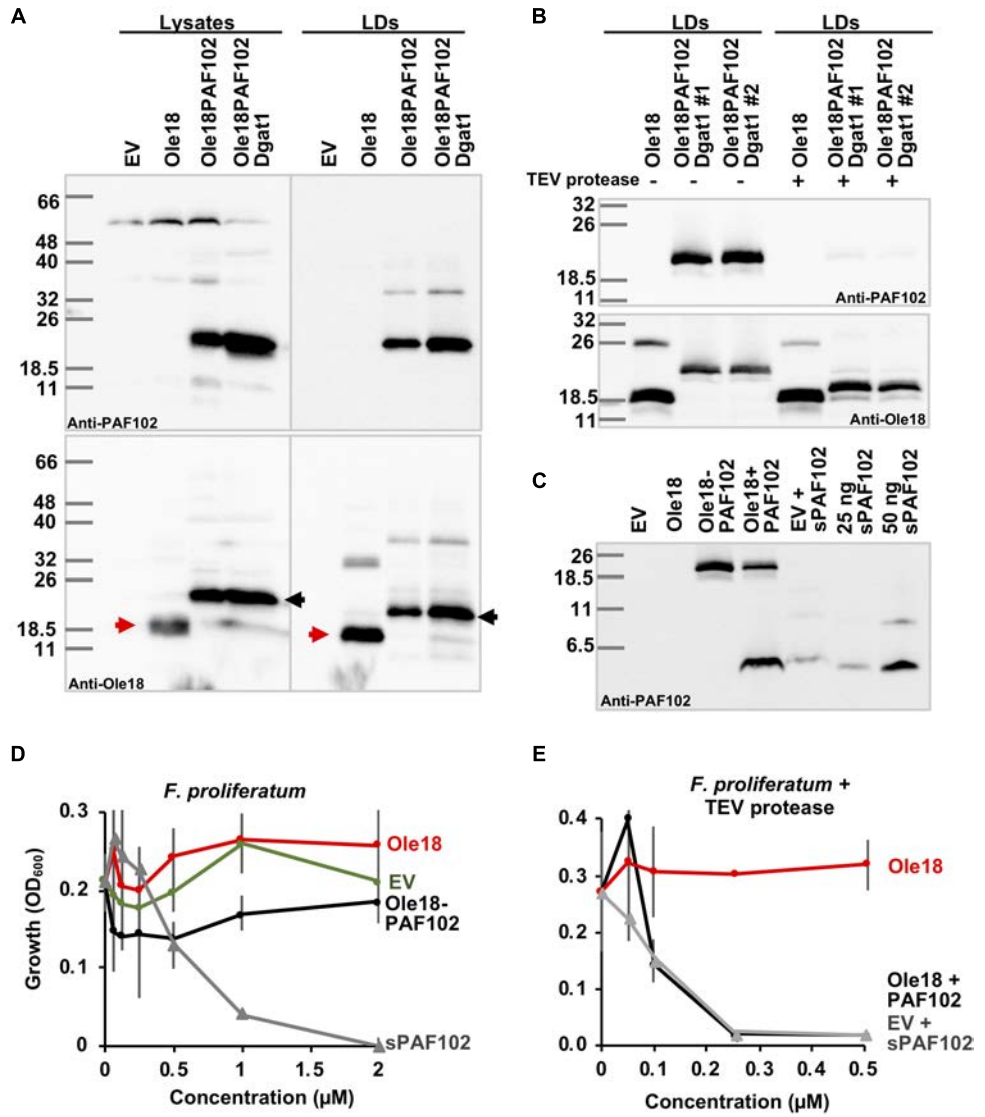

FIGURE 6 | Biologically active PAF102 is recovered from yeast cells. **(A)** Immunoblot of total protein extracts (25 μg) and LD proteins from *P. pastoris* cell cultures transformed with empty vector (EV) or the indicated gene constructs, using anti-Ole18 or anti-PAF102 antibodies as indicated. **(B)** Immunoblot analysis of LDs fractions before (–) or after (+) TEV protease digestion using anti-PAF102 or anti-Ole18 antibodies as indicated. LDs were from Ole18 and Ole18PAF102/Dgat1 (#1 and #2 independent colonies) strains. **(C)** Immunoblot analysis of PAF102 peptide released from the fusion Ole18-PAF102 protein. Proteins from LDs were precipitated with acetone after TEV protease digestion and separated in Tricine-SDS-PAGE. Synthetic PAF102 (sPAF102, 25 or 50 ng), alone or in combination with the EV proteins, was run in parallel for comparative purposes **(D,E)** *F. proliferatum* growth inhibitory assays by indicated LD fractions in comparison to synthetic PAF102 (sPAF102) at varying concentrations **(D)** or by TEV digested LDs fractions in comparison to EV LDs supplemented with sPAF102 **(E)**.

of protease-sensitive proteins with variable success depending on the proteases involved in the degradation events (Ahmad et al., 2014). Our results suggest that the efficiency of the production system here developed would be dependent on the peptide to be produced.

Oleosin proteins have an important structural role in facilitating the formation and stability of plant LDs. They are small lipophilic proteins with a conserved central hydrophobic domain, inserted within the phospholipid bilayer of LDs, and two variable amphipathic N and C terminal domains covering the LD surface (Abell et al., 1997). Oleosins have an intrinsic affinity to membranes containing neutral lipids, associating spontaneously with them and assisting their sequestration into domains and ultimately in the formation of a particle filled with neutral lipids. Yeast LDs do not have similar structural proteins, but perform their functions properly (Koch et al., 2014). However, oleosins from different plant species produced in *S. cerevisiae* cells target LDs and induce the formation of LDs (Ting et al., 1997; Jacquier et al., 2013; Jamme et al., 2013; Vindigni et al., 2013). In agreement with these reports, rice Ole18 targets and stably accumulates in LDs in *P. pastoris*. The fusion of PAF102 to the Ole18 in the C-terminal also targets LDs, with the

hydrophobic domain of Ole18 embedding into the TAG matrix and the PAF102 remaining on the surface. There were more and larger LDs in the cells accumulating the Ole18-PAF102 fusion protein than in those accumulating only the Ole18. It seems that the presence of PAF102 stabilizes the recombinant LDs and promotes their formation, probably due to the cationic and amphipathic character of PAF102 and its phospholipid affinity. This suggests that the oleosin fusion technology could be successfully applied to other AMPs with a similar cationic and amphipathic character.

A valuable addition to the production system is the coexpression of *AtDagt1* and *Ole18-PAF102* genes. *AtDag1* expression enhances the TAG content in yeast cells (Bouvier-Navé et al., 2000; Aymé et al., 2014). This high content of TAG leads to overaccumulation of the Ole18-PAF102 fusion protein, resulting in high yields of the recombinant protein. This is consistent with the requirement of TAGs for the stable accumulation of the *A. thaliana* Oleosin 1 in yeast cells (Jacquier et al., 2013). Oleosin 1 was easily degraded in mutant strains of *S. cerevisae* deficient in TAGs that were not able to produce and to accumulate neutral lipids. In contrast, cells with a high content of TAGs offer sufficient anchoring surface for the Ole18-PAF102, providing stability and allowing the accumulation of very large amounts of the fusion protein. The yield could be further improved by using modified *Dagt1* genes to enhance the storage lipid content in yeast (Greer et al., 2015).

One of the main advantages of the oleosin fusion technology is the simplicity of extraction and purification of the recombinant proteins accumulated in LDs from plant material. Similarly, *P. pastoris* LDs with the Ole18-PAF102 can be separated from other cellular components by simple flotation in density gradients. The yeast isolated LDs containing the Ole18-PAF102 had no activity against fungal targets, consistent with previous results with LDs from rice seeds (Bundó et al., 2019). PAF102 is not active while immobilized in the LDs, requiring release from the Ole18 for activity. We speculate that this cell penetrating peptide cannot enter the fungal target cell while attached to LDs, a process that is necessary for its antifungal action (Muñoz et al., 2013). Once released from Ole18, PAF102 produced in *P. pastoris* was biologically active and indistinguishable from the synthetic peptide. PAF102 has potent activity against *F. proliferatum*, a plant pathogen that contaminates cereal grain with mycotoxins, reducing grain quality and causing severe economic losses (Vismer et al., 2019). Here we demonstrate that biologically active PAF102 peptide can be produced quickly and efficiently in *P. pastoris* using the rice oleosin protein as a carrier to LDs.

Our results suggest that the oleosin fusion technology can be transferred to yeast cells for the production of recombinant proteins other than the AMPs here investigated. Particularly, we also successfully produced the GFP as an Ole18 fusion protein in *P. pastoris.* The main advantage of yeast over plant systems is the reduced production time. Oleosin technology in plants requires completion of the plant life cycle, since LDs are mainly accumulated in seeds: depending on the species, this could take several months whereas production in *P. pastoris* only requires several days. The production of other proteins should be evaluated to determine the robustness of the developed system.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

MC conceived and designed this study. PF contributed to the design. CP and TR prepared the gene constructs and carried out the yeast transformation experiments. CP and XS characterized the yeast transformants and the accumulation of AMPs in yeast cells. CP set up the LD isolation protocol. XS set up the recovery and evaluation of PAF102 from LDs. MC coordinated the study and prepared the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01472/full#supplementary-material

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Chapter IV
Improving the Biotechnological Production of AFPs in Plants for Crop Protection

Improving the biotechnological production of AFPs in plants for crop protection

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Abastract

Fungi and fungal-like oomycetes are the major pathogens of plants and pose a serious threat to food security and safety. Antifungal proteins (AFPs) secreted by filamentous fungi have a great potential to control phytopathogenic fungi. Among them, the recently characterized AfpA from *Penicillium expansum* and AfpB from *Penicillium digitatum* are particularly outstanding for their potent, specific and durable activity against economically relevant plant pathogens. Their application in agriculture requires from efficient, economic and safe production systems. Here, we improve our previously developed AFP production system, based on *Nicotiana benthamiana* leaves and a tobacco mosaic virus vector, to obtain commercially relevant yields in a cost-effective manner. The main add consists in vacuolar targeting of the highly active AFPs that increases seven times their yields. Using the improved system, we were able to biotechnologically produce AfpA and to enhance AfpB production up to 1.2 mg/g fresh leaves. Moreover, we demonstrate that AfpA- and AfpB-enriched plant extracts are equally active to the counterpart proteins from fungal origin, without requiring further purification and reducing downstream processing, and consequently production cost. Importantly, we show these plant extracts are efficient controlling tomato plants and fruits against Botrytis gray mold, rice plants against blast disease and rice seeds against *Fusarium proliferatum* infections. Thus, plant extracts can be used as safe and economic biofungicides in spray applications on plant leaves, or alternatively surface coated on seeds or fresh fruits. Our results on the development of a sustainable and efficient production system in plant biofactories, and on the demonstration of the efficacy of plant products, represent an important advance in the use of AfpA and AfpB as environmental friendly and effective "green fungicides" in crop and postharvest protection.

INTRODUCTION

Fungi and fungal-like oomycetes are the major pathogens of plants. They are the causal agents of important plant diseases, including epidemics in staple and commodity crops that feed billions of people. Crop-destroying fungi account globally for yield losses of ∼20%, with a further 10% at postharvest, posing a serious threat to food security (Fisher et al., 2018). The two most economically relevant fungi are *Magnaporthe oryzae* and Botrytis cinerea (Dean et al., 2012). The first one affects the production of rice that is the cereal crop feeding half of the global population. The most severe and widely distributed rice disease is caused by *M. oryzae* and it is responsible for 10 to 30% grain losses equivalent of feeding 60 million people (Skamnioti and Gurr, 2009). The second one, B. cinerea is responsible for the gray mold disease affecting almost all vegetable and fruit crops, including economically important crops such as grape, strawberry and tomato. This fungal pathogen causes serious losses at pre-harvest period but most importantly in postharvested fresh fruits, vegetables and flowers. Annual losses due to gray mold disease are estimated between $10 to $100 billion worldwide (Weiberg et al., 2013). Additionally, mycotoxigenic fungi also endanger food safety because they contaminate food and feed with toxic compounds for human and animals (Bennett and Klich, 2003). Fusarium species infect and colonize important cereal crops such as maize, rice, wheat and oats, and produce three of the most important mycotoxins commonly found in grains grown in America, Europe and Asia (Bennett and Klich, 2003). Currently, the control of fungal infections mainly relies on chemicals that affect human health, negatively impact ecosystems, and promote the development of resistance, not only in plant pathogens but also in human pathogens (Fisher et al., 2020). Novel, ecofriendly fungicides are in urgent need to avoid future uncontrollable outbreaks of fungal infections in agriculture.

Antifungal proteins (AFPs) produced and secreted by fila-

mentous fungi are a promising alternative, or complement, to conventional fungicides (Galgóczy and Marx, 2019; Marx et al., 2008; Meyer, 2008; López-García et al., 2012; Marcos et al., 2020). AFPs are small, cationic and cysteine-rich proteins that are folded in compact structures supported by disulphide bridges, making them highly stable against harsh environmental conditions (Batta et al., 2009; Campos-Olivas et al., 1995; Garrigues et al., 2017, 2018). They exhibit potent, specific antifungal activity in the micromolar range against important plant pathogens (Garrigues et al., 2017, 2018; Hegedüs and Marx, 2013; Huber et al., 2018; Meyer, 2008; Vila et al., 2001; Virágh et al., 2014), and there are several reports showing that the topical application of AFPs is an effective treatment for plant and fruit protection against fungal infections (Shi et al., 2019; Vila et al., 2001; Moreno et al., 2003; Garrigues et al., 2018; Theis et al., 2005). Interestingly, the mode of action of AFPs is completely different to licensed antifungal drugs. AFPs act through multiple targets on fungal cells, including components of the cell wall, the plasma membrane, and intracellular elements which would hinder the development of fungal resistance (Bugeda et al., 2020; Delgado et al., 2015; Moreno et al., 2006; Marx et al., 2008; Meyer, 2008; Paege et al., 2019; Virágh et al., 2015). All these properties make AFPs attractive molecules to be developed as biofungicides for crop and postharvest protection.

Currently, there are more than 50 members in the AFP family, including the founder AFP from *Aspergillus giganteus* , and the recently identified AfpB and AfpA from *Penicillium digitatum* and *Penicillium expasum*, respectively (Lacadena et al., 1995; Garrigues et al., 2018, 2016). Among all of them, AfpA is one of the most potent AFPs, exhibiting the highest antifungal activity against all tested fungi in *in vitro* assays, including economically relevant plant pathogens (Garrigues et al., 2018). AfpA is also efficient in *in vivo* protection assays against postharvest decay fungal pathogens, such as B. cinerea, *P. digitatum* and *P. expansum* (Gandía et al., 2020; Garrigues et al., 2018). Although less active than AfpA, AfpB also shows strong activity in the low micromolar range against important phytopathogens (Garrigues et al., 2017), and it is able to efficiently control the Botrytis gray mold in tomato plants (Shi et al., 2019). These two proteins represent good candidates for the development of new fungicides.

The viable exploitation of AFPs requires a safe, efficient and economic production. Recently, we have developed a cost-efficient plant-based system that allows the production of large amounts of AFPs in short periods of time (Shi et al., 2019). The system uses a modified Tobacco Mosaic Virus (TMV) in which the coat protein sequences were replaced by afp genes to trigger the transient expression of AFPs in *Nicotiana benthamiana* leaves. Using this system, we efficiently produced *P. digitatum* AfpB and *A. giganteus* AFP when targeted to the apoplastic space. The objective of the present study is to evaluate the system for the production of the highly active AfpA from *P. expansum*. We report here that the system is efficient for the production of highly bioactive proteins, such as AfpA, when targeted to vacuoles instead of the apoplastic space. Moreover, we also found that the production of AfpB can be improved by vacuolar targeting when compared to our previous results in the apoplast. Additionally, we demonstrate that the plant vacuolar AFPs tagged with a vacuolar sorting sequence are equivalent in activity to their counterparts from fungal origin. Importantly, we show that the plant protein extracts containing AfpA and AfpB are efficient protecting tomato plants and fruits against Botrytis gray mold, rice plants against blast disease and rice seeds against *Fusarium proliferatum* infections. This work represents an important advance in the development of AfpA and AfpB as biofungicides for crop and postharvest protection.

RESULTS

Production of AfpA in *N. benthamiana* plants

For the biotechnological production of AfpA, we used the production system previously developed in our group that works efficiently with other AFPs (Shi et al., 2019). The system is based on transient expression in N. bentamiana leaves using a TMV-derived vector and assisted by agroinfiltration. We prepared a first construct to target the protein to apoplasts in order to ease its downstream purification (Figure 1a). The cDNA encoding the mature AfpA extended in N-terminal with a secretory signal (Figure 1b) was chemically synthesized with a codon usage adapted to *N. benthamiana* , cloned in the intermediate vector pMTMVi-N, and then transferred into the final destination vector pGTMV for the expression of the recombinant virus TMV△CP-AfpA. The N-terminal signal peptide allows AfpA to enter the secretory pathway toward the extracellular space, it is processed during trafficking, and is absent in the mature apoplastic protein. The construct was then agroinfiltrated in *N. benthamiana* leaves for the expression of the recombinant virus, and compared to the construct for expression of the green fluorescent protein (TMV△CP-GFP), previously described (Shi et al., 2019). After 7 days, we observed that some TMV△CP-AfpA agroinfiltrated leaves showed a severe wilting appearance (Figure 1c, AfpA1), whereas another leaves showed a normal green healthy appearance, quite similar to the control TMV△CP-GFP agroinfiltrated leaves (Figure 1c, AfpA2). To confirm that AfpA was produced, leaf proteins were extracted using an acidic buffer for basic proteins such as AFPs, and were analyzed by Western-blot using specific anti-AfpA antibodies (Figure 1d). We detected

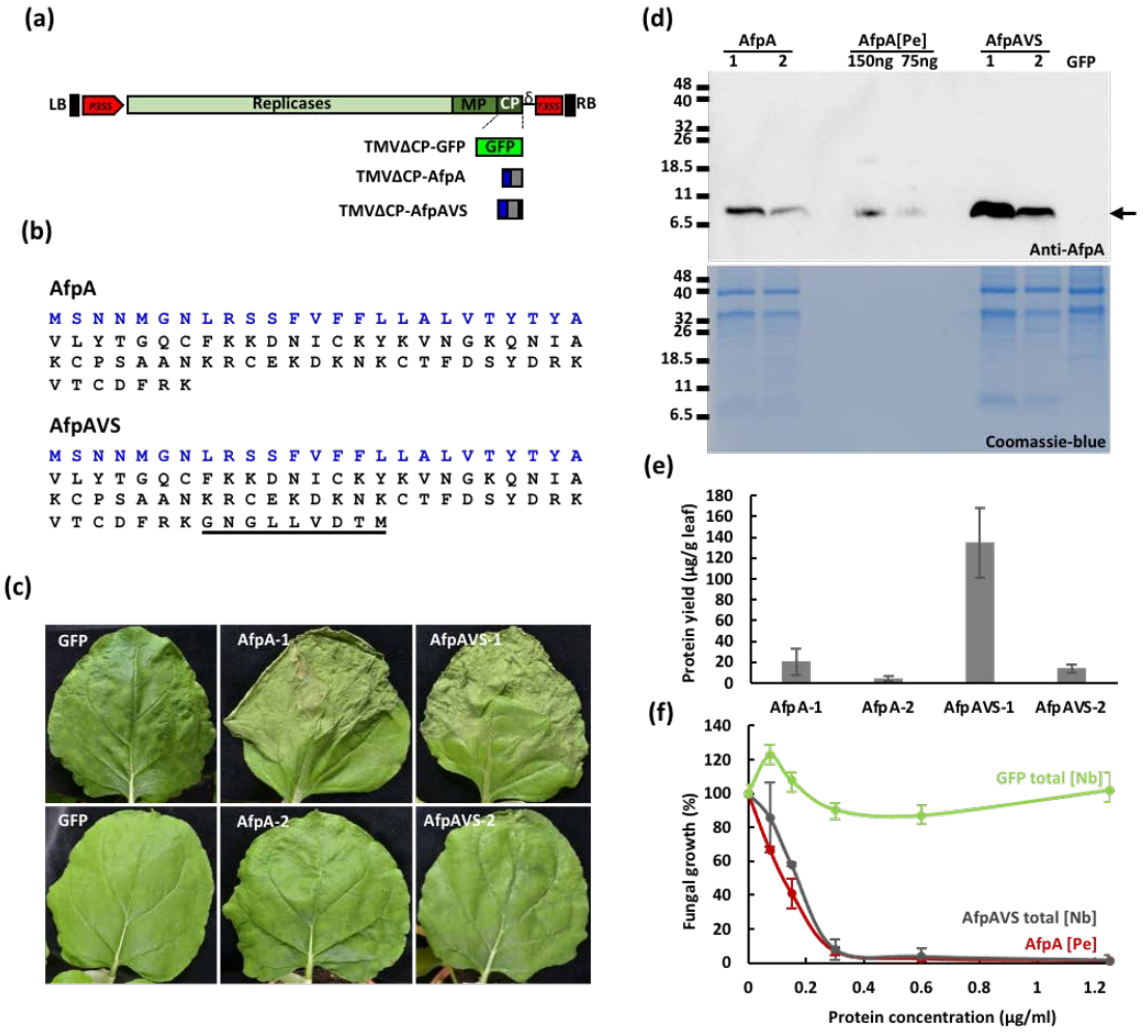

Figure 1. **Biotechnological production of AfpA**. (a) Schematic representation of the constructs to produce GFP or AfpA using the TMV-derived vector. Constructs were designed to accumulate GFP in the cytoplasm and AfpA in the apoplast or in the vacuole of plant cells. The two AfpA constructs incorporate the signal peptide of tobacco AP24 protein at the N-terminus, and in the vacuolar construct, the vacuolar signal (VS) at the C-terminus of the mature protein. RB and LB, right and left T-DNA borders; P35S and T35S, CaMV 35S promoter and terminator, respectively; δ, ribozyme; MP, movement protein; CP, coat protein. (b) Amino acid sequences of AfpA and AfpAVS proteins. The processed signal peptide is shown in blue and the retained vacuolar signal is underlined. (c) Appearance of *N. benthamiana* leaves 7 days after agroinfiltration with the constructs for the production of indicated proteins. Two different phenotypical appearances (1 and 2) were observed. (d) Analysis of AfpA accumulation in the leaves with the two different appearances. Acidic protein extracts were separated by tricine-SDS-PAGE and immunodetected using antibodies anti-AfpA (upper panel) or Coomassie-blue stained (lower panel). Two different amounts (75 and 150 ng) of AfpA purified from *P. expansum* cultures (AfpA[Pe]) were run in parallel as a control. Molecular weight markers are shown in kDa on the left. (e) AfpA accumulation in leaves as estimated by comparison with known amounts of purified AfpA[Pe]. Values correspond to the mean $\pm$ SD of at least three biological replicates from three independent assays. (f) *in vitro* inhibitory activity of plant total protein extracts containing AfpAVS against *P. digitatum* fungus. Dosis–response curves comparing the fungal growth in the presence of AfpAVS, in total plant extracts (AfpAVS total [Nb]), or purified AfpA[Pe], with the control plant extracts with GFP (GFP total [Nb]).

an immunoreactive band with the same apparent molecular weight to the fungal AfpA, which showed stronger intensity in the samples from wilting leaves than healthy leaves. The protein was barely visible by Coomassie-blue staining (Figure 1d), and estimated to accumulate only at 4.4 $\pm$ 2.4 µg per gram of healthy leaves or at 20.5 $\pm$ 12.2 µg per gram of

wilting leaves. These values were at least 10 times lower than those reached by other AFPs and around 200 times lower than those reached by non-bioactive molecules using the same production system (Shi et al., 2019). These observations demonstrate that AfpA is produced in *N. benthamiana* leaves when targeted to the apoplastic space, yet it is moderately accumulated and accompanied of cytotoxic effects.

Compartmentalizing antimicrobial peptides in plant cells is known to favor its accumulation by avoiding toxic effects on their host cells (Bundó et al., 2019; Montesinos et al., 2016; Coca et al., 2006). A subcellular compartment of particular interest in for the deposition of high amounts of proteins in plant cells is the vacuole, since it occupies most of the cell volume. However, it is considered a hostile environment for its lytic characteristics. Taking into account that AFPs are quite resistant to proteolysis, we examined AfpA production in *N. benthamiana* leaves when targeted to vacuoles. We prepared a new construct to produce AfpA with the secretory signal in N-terminal and extended in C-terminal with the vacuolar sorting signal (VS) of tobacco chitinases (Neuhaus et al., 1991) (Figure 1b). *N. benthamiana* leaves agroinfiltrated with this construct (TMV△CP-AfpAVS) also showed two different appearances, like we observed for apoplastic AfpA (Figure 1c). Western-blot analysis of acidic protein extracts also immunodetected a protein band with a slightly higher molecular weight than AfpA, and higher intensity in wilting than in healthy leaf samples (Figure 1 d). The protein band was also visible by Coomassie-blue staining in comparison to the GFP control leaves, and it always was more intense to the apoplastic AfpA (Figure 1d). The size increase agrees with that expected for AfpA extended with the vacuolar sorting signal that remained attached in the mature protein: the molecular weight of mature AfpA after processing the signal peptide is 6.644 KDa, whereas the vacuolar variant AfpAVS is 7.545 KDa. The quantification of band intensities in comparison with known amounts of fungal purified protein shows that vacuolar targeting leads to an average increase of 7 fold (Figure 1e). This data shows that AfpA yields are improved when targeted to the plant cell vacuole.

Having established that AfpA can be produced in plants, we then evaluated its antifungal activity. Total protein extracts containing the vacuolar variant (AfpAVS) were assayed against the pathogen of citrus fruits *P. digitatum* highly susceptible to the fungal AfpA (Garrigues et al., 2018). After dialyzing protein extracts containing AfpAVS against water, they were used at different AfpAVS concentrations in growth inhibitory assays, where they showed equivalent antifungal activity to pure AfpA from fungal origin (Figure 1f). As a control, we used protein extracts containing GFP at the same dilutions, which did not show growth inhibitory activity against the same fungus. These results indicate that *in planta*-produced AfpAVS is biologically active and equivalent to AfpA produced by P. expansum, and that the vacuolar sorting signal added to the vacuolar variants do not affect their antifungal activity. Moreover, they demonstrate that protein purification is not required in order to obtain good AfpA activity.

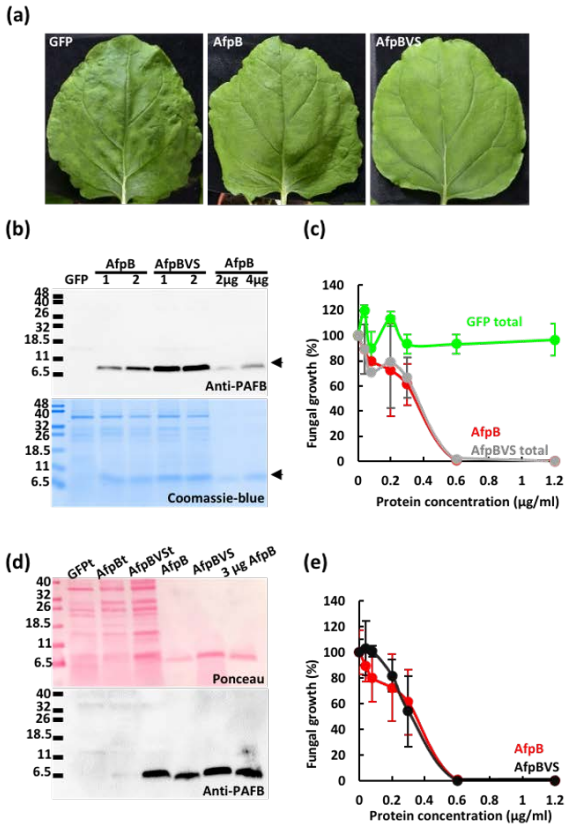

Figure 2. **Production of AfpB in vacuoles of *N. benthamiana* leaves**. (a) Leaf appearance at 7 days after agroinfiltration with the constructs for the production of indicated proteins. (b) Analysis of AfpB accumulation in vacuoles (AfpBVS) compared to apoplasts. Acidic protein extracts from two different leaves (1 and 2) were separated by tricine-SDS-PAGE and immunodetected using antibodies anti-PAFB (upper panel) or Coomassie-blue stained (lower panel). Molecular weight markers are shown in kDa on the left. (c) *in vitro* inhibitory activity of plant total protein extracts containing AfpBVS against *P. digitatum* fungus in comparison to purified AfpB and the control plant extracts with GFP. (d) Purification of AfpBVS by size filtration. Ponceau stained tricine-SDS-gel loaded with total protein extracts containing the indicated proteins (GFPt, AfpBt, AfpBVSt) and the filtered extracts to monitor purification (upper panel), or western-blot immunodetected with anti-PAFB antibodies. (e) In vitro inhibitory activity of purified AfpBVS against *P. digitatum* fungus in comparison to purified AfpB.

Production of AfpB in vacuoles of *N. benthamiana* leaves

Provided that AfpA production is improved when targeted to vacuoles, we evaluated the production of AfpB, another highly active antifungal protein. AfpB accumulated at high levels in the apoplastic space, but did not accumulate in the endoplasmic reticulum due to toxic effects (Shi et al., 2019). We prepared a similar construct for the production of AfpB extended in C-terminal with VS sequence (TMV△CP-AfpBVS). Contrastingly to AfpAVS, agroinfiltrated leaves with this construct did not show any leaf damage (Figure 2a). Leaves were green and healthy, like the control leaves producing GFP or the apoplastic AfpB. Western-blot analysis of acid protein extracts from AfpBVS leaves detected an immunoreactive band slightly higher to apoplastic AfpB (Figure 2b, upper panel), in agreement with the increment in size expected for the extended protein with the vacuolar sorting peptide (theoretical molecular weight 6.462 vs 7.363 kDa). The presence of AfpB and AfpBVS proteins in the plant extracts was also visualized by Coomassie-blue staining (Figure 2b, lower panel). The accumulation of AfpBVS was around four times higher than AfpB, reaching mean yields of 1.2 mg of protein per gram of fresh leaf. Additionally, we assayed the plant protein extracts enriched with AfpBVS against *P. digitatum* fungus in comparison to the same amounts of AfpB produced in *N. benthamiana* and purified to homogeneity. As shown in Figure 2c, the fungal growth inhibitory activity of plant extracts containing AfpBVS was equivalent to the purified AfpB by contrast to GFP plant extracts. This data demonstrates that AfpBVS shows the same biological activity as AfpB, and equal antifungal activity can be provided with total protein extracts without requiring additional purification. Even though, protein purification can be easily achieved using centrifugal filters and separation on a size-basis, as monitored by tricine-SDS-PAGE (Figure 2d). Purified AfpBVS confirmed the same growth inhibitory activity against *P. digitatum* as the purified AfpB (Figure 2e), showing again that the addition of the vacuolar sorting sequence does not affect AfpB activity.

Production of cysteine-rich proteins in vacuoles

These studies indicate that the plant vacuole is an appropriate compartment for the accumulation of highly active and stable proteins, such as AfpA and AfpB. In order to assess whether vacuoles are particularly suitable as reservoirs of bioactive proteins, we evaluated the efficiency of the system with a different cysteine-rich stable fungal protein that does not show antifungal activity, namely Sca protein (Garrigues et al., 2020). This novel protein is particularly well produced using the TMV-derived expression system reaching yields greater than 4 mg per gram of leaf when accumulated in the apoplastic space (Shi et al., 2019). Using the same vacuolar sorting signal (VS), we targeted Sca accumulation to plant vacuoles (ScaVS) and we compared it to apoplastic yields (Sca) (Figure 3). Leaves agroinfiltrated with Sca and ScaVS recombinant virus appeared normal, similar to the control GFP leaves (Figure 3a). We extracted the extracellular fluid (ECF) and total proteins with acidic buffer from independent leaves, and analyzed by SDS-PAGE. Coomassie blue staining revealed a band of stronger intensity in ECFs from leaves expressing the apoplastic Sca virus (TMV△CP-Sca) than the vacuolar Sca virus (TMV△CP-ScaVS) (Figure 3b). Whereas, Sca and ScaVS band intensities were similar in total protein extracts. These data suggest that Sca is correctly secreted to the extracellular space, whereas ScaVS mostly remained in the interior of the plant cells. Probably, the detected ScaVS in ECFs is due to cell rupture during the extraction process. Moreover, the quantification of Sca and ScaVS band intensities in total extracts showed similar accumulation levels (Figure 3c). Therefore, vacuolar targeting did not improve the accumulation of non-bioactive proteins, reaching similar yields in apoplastic space and vacuoles.

AFP-enriched plant extracts protect tomato plants and fruits from Botrytis grey mold

We next assessed the effectiveness of plant extracts containing AfpA and AfpB against B. cinerea infection in tomato plants, given that the counterpart fungal proteins are active against this fungus (Garrigues et al., 2018; Shi et al., 2019). We deposited drops containing fungal conidia on tomato leaves along pure proteins AfpA from *P. expansum* (AfpA[Pe]) or AfpBVS from *N. benthamiana* (AfpBVS[Nb]) or total protein extracts containing 10 µM of AfpAVS or AfpBVS, and compared them to drops of conidia along same amounts of GFP total protein extracts as a control. After a couple of days, infection lesions were visible where control drops (GFP protein extracts or sterile water) were deposited, whereas no damage was visible where drops containing AFPs were deposited. These lesions developed with days being clearly spread all over the leaves by 5 days on control drops, whereas they were not observed or clearly smaller where drops contained either AfpA or AfpB (Figure 4a). By quantifying lesion area using image analysis, we observed a statistically significant decrease in the damaged area when AfpA, AfpAVS or AfpBVS were present (Figure 4b). Moreover, the total extracts enriched in AfpAVS or AfpBVS showed the same protective efficacy as the AfpA or AfpBVS pure proteins. Fungal biomass was rather detected by qPCR analysis with DNA from leaves treated the antifungal proteins (Figure 4c). These results demonstrate that plant produced vacuolar variants of AfpA and AfpB are effective on the protection of tomato leaves against B. cinerea infection without the need of additional downstream purification processes.

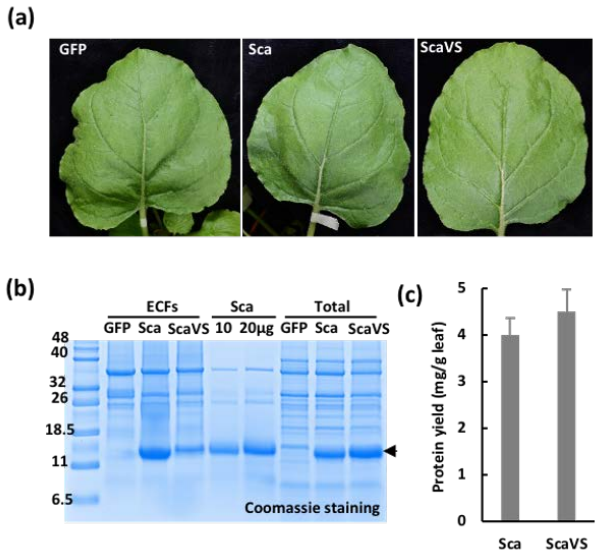

Figure 3. **Production of the fungal cysteine-rich protein Sca in vacuoles**. (a) Leaf appearance at 7 days after agroinfiltration with the constructs for the production of indicated proteins. (b) Analysis of Sca accumulation in agroinfiltrated leaves. Proteins from extracellular fluids (ECFs) and acid extraction (total) were separated by tricine-SDS-PAGE and Coomassie-blue stained. Known amount of Sca protein were run in parallel for quantification. (c) Sca yields in *N. benthamiana* leaves when accumulated in the apoplastic space (Sca) or in the vacuole (ScaVS). Values correspond to the mean ± SD of three biological replicates from three independent assays.

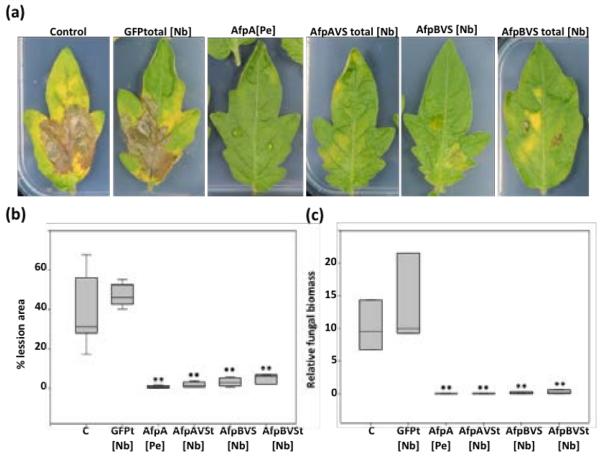

Figure 4. **Protection against Botrytis cinerea infection on tomato leaves by plant extracts enriched in AFPs**. (a) Representative leaves at 5 days after drop-inoculation with conidia suspensions (0.5 x 106 conidia/ml) along with sterile water (control) or *N. benthamiana* protein extracts containing AfpAVS (AfpAVS total [Nb]), AfpBVS (AfpBVS total [Nb]) or GFP (GFP total [Nb]), or purified protein from *P. expansum* cultures (AfpA[Pe]) or from *N. benthamiana* leaves (AfpBVS[Nb]). The antifungal protein concentration was always 10 µM. (b) Box plot of the percentage of leaf damage area from the indicated treatments. Data represent at least three independent experiments in which at least 3 leaves were assayed with two drop-inoculations. (c) Box plot of relative fungal biomass from infected leaves as determined by qPCR of *B. cinerea CutA* gene and *S. lycopersici* Act2 gene. Asterisks denote statistical significance (Tukey test, ** $p<0.05$).

We also evaluated the efficacy of the plant produced antifungal proteins on tomato fruits. Commercial tomato cherries were drop inoculated with B. cinerea conidia on needle-prick wounds and incubated at room temperature for 9 days. To test the protection effect of AfpA and AfpB, tomatoes were also inoculated with drops containing the fungal conidia along with the antifungal proteins. We observed that those tomatoes inoculated with control drops containing sterile water or GFP plant extracts were infected in most of the cases, and the wounds covered with gray mold in 80% of tomatoes (Figure 5a). However, tomatoes inoculated with drops containing AfpA, either pure protein or plant extracts enriched in AfpAVS, were mostly non-infected (Figure 5a). Similarly, the number of infected tomatoes was significantly reduced when AfpBVS was applied along with the fungal conidia (Figure 5a). The quantification of the infected tomato showed a statistically significant reduction of average of 50% of gray mold incidence when either AfpA or AfpB was applied (Figure 5b). These results demonstrate that both proteins are effective preventing B. cinerea infections in tomato plants and fruits, and that plant protein extracts enriched in either of these protein could be used for the control of grey mold in tomato.

AFP-enriched plant extracts protect rice seeds against *F. proliferatum* infection

We also tested the effect of AfpA and AfpB on rice seeds against *F. proliferatum* infection. This fungus is found in the Fusaria sps complex associated to Bakanae rice disease, responsible for important crop losses and contamination of grain with mycotoxins (Wulff et al., 2010). Because this fungus inhibited seed germination, we evaluated the germination ability of drop-inoculated seeds with conidia suspensions along with AfpA or AfpB proteins, either pure protein or plant extracts containing them at the same concentrations. Results in Figure 6 show that *F. proliferatum* was clearly affecting the germination and growth of seedlings in control conditions (drops containing conidia along with sterile water or GFP plant extracts), around the 70% of seeds were completely dead or seriously affected in growth, and only the 30% of seeds performed healthy. Whereas the presence of AfpA clearly inhibited fungal growth and the germination and growth was not affected in more than a 95% of the rice seeds (Figure 6b). AfpB also improved ger-

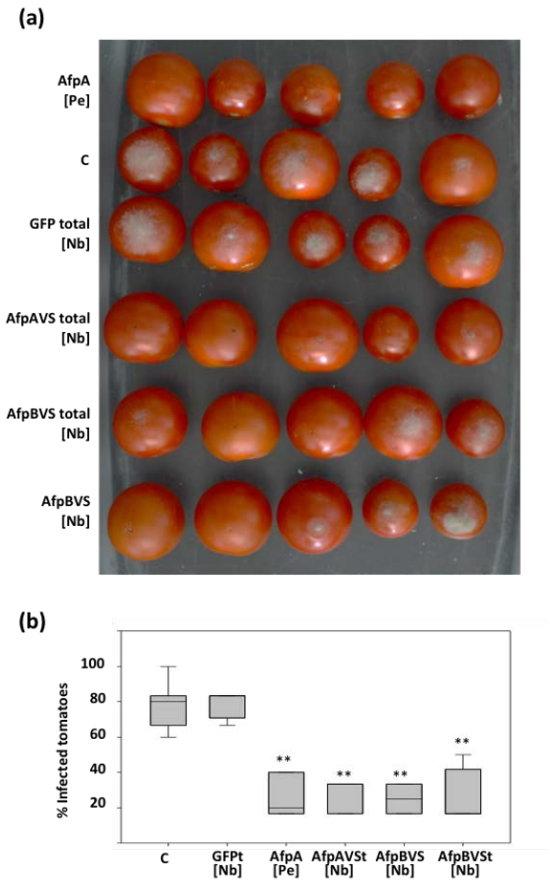

Figure 5. **Protection against gray mold infection of tomato fruits by plant extracts enriched in AFPs**. (a) Representative tomatoes at 9 days after drop-inoculation on needle-prick wounds with conidia suspensions (0.5 x 106 conidia/ml) along with sterile water (control) or *N. benthamiana* protein extracts containing AfpAVS (AfpAVS total [Nb]), AfpBVS (AfpBVS total [Nb]) or GFP (GFP total [Nb]), or purified protein from *P. expansum* cultures (AfpA[Pe]) or from *N. benthamiana* leaves (AfpBVS[Nb]). The antifungal protein concentration was always 10 µM. (b) Box plot of the percentage of infected tomatoes from indicated treatments. Data represent four independent assays in which 6 tomatoes were inoculated with each treatment (n= 24). Asterisks denote statistical significance (Tukey test, ** $p<0.05$).

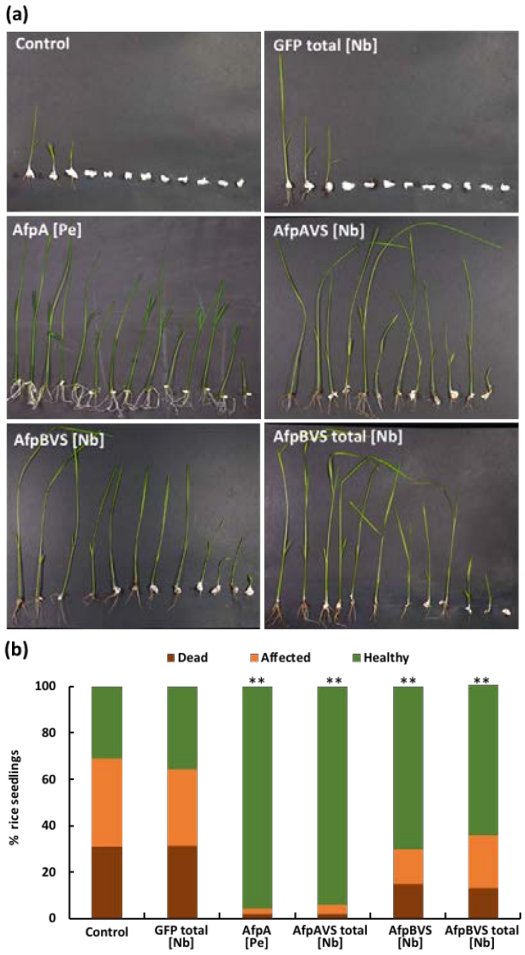

Figure 6. Rice seed protection against *Fusarium proliferatum* infection by AfpA and AfpB. (a) Appearance of rice seedlings 7 days after gemination from seeds drop-inoculated with a spore suspension (104 spores/ml) along with sterile water (control), or *N. benthamiana* protein extracts containing AfpAVS (AfpAVS total [Nb]), or AfpBVS (AfpBVS total [Nb]), or GFP (GFP total [Nb]), or purified proteins from *P. expansum* cultures (AfpA[Pe]) or from *N. benthamiana* leaves (AfpBVS[Nb]). The antifungal protein concentration was always 10 µM. (b) Graph represents the percentage of seeds that did not germinate (dead), seedlings retarded in growth (affected), and seedlings with normal growth (healthy). Values are the mean of 4 different assays using 12 seeds per treatment. Asterisks denote statistical significance (Tukey test, ** $p<0.05$).

mination and growth on seeds inoculated with *F. proliferatum* , although in a lower extent to AfpA rates. Interestingly, same protective effects were afforded by the AfpAVS- or AfpBVS-enriched plant extracts compared to the respective pure proteins. These results suggest that AfpA and AfpB can protect seeds from fungus-infecting seeds during storage and germination.

Plant produced AFPs protect rice leaves against blast disease

Finally, we assessed the effectiveness of AfpA and AfpB against blast disease of rice plants. *M. oryzae* , the blast disease fungus, is not particularly susceptible either to AfpB or AfpA in *in vitro* growth inhibitory assays (Garrigues et al.,

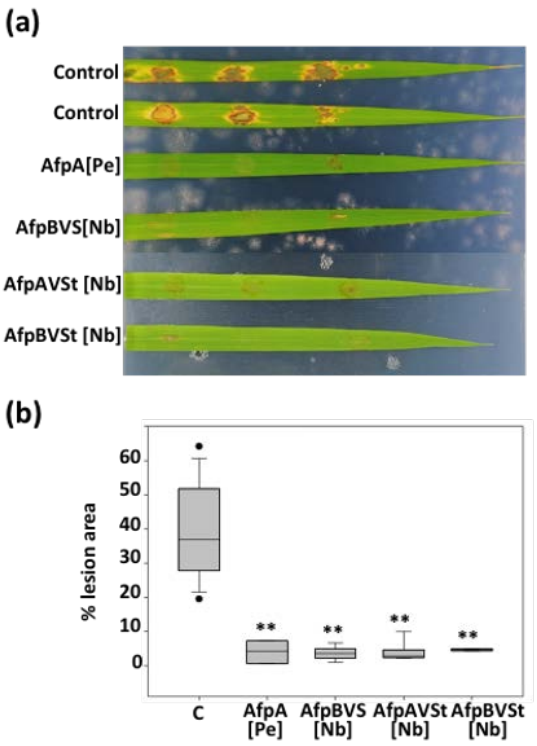

Figure 7. **Rice leaf protection against blast disease by AfpA and AfpB**. (a) Representative leaf symptoms at 5 days post inoculation with drops containing Magnaporthe oryzae spores (5x105 spores/ml) along with sterile water (control), or with AfpA[Pe] or AfpBVS[Nb] pure proteins, or plant extracts containing the proteins (AfpAVSt [Nb] or AfpBVSt[Nb]). Antifungal proteins were at 10 µM in all the treatments. (b) Box plot of the average of lesioned area (%) of three independent assays with 3 leaves per treatment and 3 inoculation points (n= 27). Asterisks denote statistical significance (Tukey test, ** $p<0.05$).

2017, 2018). The protection assays were carried out using a detached leaf infection assay. For inoculations, we deposited on three points on each leaf drops of spore suspensions along the pure proteins AfpA [Pe] or AfpBVS [Nb]. We observed the typical blast lesions with diamond-shape and necrotic borders in leaves inoculated only with *M. oryzae* spores, whereas no symptoms or small necrotic lesions were visible in those leaves inoculated with the spores along with either AfpA [Pe] or AfpBVS [Nb](Figure 7). These observations indicate that both proteins are able to inhibit the fungal infection on the leaves. And, as previously observed same protection effects were conferred by the plant protein extracts containing AfpAVS [Nb] or AfpBVS [Nb], indicating that further protein purification is not needed.

DISCUSSION

In this study, we show that transient expression in *N. benthamiana* leaves using a TMV-vector and accumulation in vacuoles is an optimum strategy for the biotechnological production of antifungal proteins at commercially relevant yields. We found that the plant vacuole is a good subcellular compartment for the deposition of bioactive compounds like AFPs, where they can accumulate in high amount without affecting host cells. Additionally, we demonstrate that crude protein extracts enriched with the AFPs are effective in protecting crop plants, and postharvest fresh fruits and seeds from fungal infection. The system developed here is fast, economic, scalable and safe. The process only takes seven days as a transient expression system, and simple without complex downstream development. It is economic and scalable since plants are cheap and easy to grow, and it is safe since plants are free of human pathogens. Hence, it is excellent for the production of AFPs for crop and postharvest protection.

Subcellular localization has a profound impact on protein yields, particularly for bioactive proteins such as AfpA. We previously reported that AfpB did not accumulate in *N. benthamiana* leaves when targeted to endoplasmatic reticulum associated to toxic effects, whereas it reached high yields when sorted to the extracellular space (Shi et al., 2019). Based on our previous results, we first approached the production of AfpA extracellularly, but we detected low protein accumulation levels accompanied by leaf wilting phenotypes. These results showed that AfpA has toxic effects in plant cells even when deposited outside the cells. This differential behavior of AfpA and AfpB might be determined by their mode of action, which is well characterized for AfpB but completely unknown for AfpA. It is known that AfpB is a cell penetrating protein that specifically targets fungal cells (Bugeda et al., 2020), thus apoplastic AfpB should not have any effect on plant cells. In the case of AfpA, it is a highly active protein against fungal cells that might be acting as a cell membrane disrupting protein as reported for other AFPs (Paege et al., 2019; Meyer, 2008; Moreno et al., 2006). In this way, AfpA might be affecting plant cell membranes when it is synthetized and it entered the cell secretory pathway during the transit to the apoplast. Once in the apoplast, AfpA might have no effect in plant cells due to the presence of the cell wall. Further studies on AfpA activity against biological cells would help to understand why AfpA fails to accumulate in the apoplastic space.

Better yields were obtained when AfpA was targeted to the vacuoles, reaching values closer to those of AfpB in the apoplasts. Vacuoles are considered hostile compartments for protein accumulation due to their proteolytic function, yet AfpA, which is particularly resistant to proteases, got accumulated to moderate levels. The only drawback is that

the deposition of AfpA in vacuoles is sometimes accompanied by cytotoxic effects leading to wilting leaves. In the case of AfpB, yields were also increased when sorted to vacuole without any detectable detrimental effect on leaves. These observations point again to differences between the mode of action of AfpA and AfpB, which are also evident from their different antifungal potency against fungal pathogens. Intriguingly, Sca protein, that does not display antifungal activity and accumulates at higher levels than AFPs, reached similar high yields in both vacuoles or apoplasts. These results indicate that AFPs increased yields when sorted to vacuoles are not only due to great vacuolar volume but also because this subcellular compartment seems to serve as an appropriate reservoir of bioactive compounds. There are only few reports on successful production of foreign proteins in vegetative vacuoles, mainly human proteins, such as recombinant antibodies (Ocampo et al., 2016), collagen (Stein et al., 2009), complement factor C5a (Nausch et al., 2012) or transglutaminase (Marín Viegas et al., 2015). Here, we demonstrate that vacuolar targeting can be an excellent strategy to improve yields of bioactive proteins reaching yields in the order of grams of protein per kg of fresh leaves.

We have successfully produced vacuolar AfpA and AfpB protein variants extended in C-terminal with an extra VSS (GNGLLVDTM) in a bioactive conformation. This extension apparently does not interfere with their antifungal activity, but helps in sorting them into vacuoles. Purified vacuolar variants or crude protein extracts enriched in them showed antifungal activities equivalent to the corresponding proteins from fungal origin. The finding that crude protein extracts are active is very relevant since it simplifies enormously the downstream processing, which might represent approximately 80% of the overall production costs (Wilken and Nikolov, 2012). Crude extracts are active probably because they are highly enriched in AFPs. They are obtained using an acidic buffer that selectively extracts basic proteins, yielding solutions in which half of the content is AFPs. Thus, AFP physicochemical properties assist their recovery of active products through an easy and efficient process that reduces significantly production costs.

Moreover, we show that topical application of plant extracts enriched either in AfpA or AfpB, as well as the purified proteins, confer protection on plants of agronomical interest against relevant fungal pathogens. The amount of required antifungal proteins to completely arrest fungal infection is significantly small, in the low micromolar range, which suggests economically feasible spray applications of easily produced crude plant extracts. Previously, we already reported that AfpA and AfpB are efficient in controlling B. cinerea infections in tomato plants, being AfpA more effective than AfpB (Shi et al., 2019; Garrigues et al., 2018). Our results now confirm this data, and they provide a simplified system for obtaining large amounts of active proteins for foliar applications to control gray mold in tomato crops. Although it still needs to be confirmed, AfpA and AfpB might be also active controlling B. cinerea infection on other important crop plants. Given that this fungus is a broad spectrum phytopathogen infecting almost all vegetable and fruits crops, the use of these antifungal proteins as an alternative or complement to botrycides would have a significant economic and ecologic impact, considering that they represent 10% of the fungicide market (Dean et al., 2012).

We also show that AfpA and AfpB are able to protect rice leaves against the infection by blast fungus, a hemiobiotrophic fungus considered the most important fungal plant pathogen because of its economic importance (Dean et al., 2012). Finding molecules that can mitigate the devastating effects of the blast disease on rice production is of great relevance since half of the world population relies on rice as staple food. There is a previous report on the efficacy of AFP from A. giganteous on rice blast (Vila et al., 2001). Our data, together with this previous result, suggest that AFPs in general might be an interesting alternative to control blast disease in rice. Additionally, the fact that AfpA and AfpB are efficient in two different pathosystems, B. cinerea-tomato plants and *Magnaporthe oryzae* -rice plants, and against fungi with different pathogenic strategies, suggests that they can be broadly used as ecofriendly fungicides in crop protection through foliar applications. Further studies are needed to characterize their effectiveness in greenhouse or field assays, their persistence over time and their effects on plant performance, including plant development and productivity.

Notably, plant extracts enriched in AfpA and AfpB also protect postharvest tomato fruits against Botrytis gray mold disease, in agreement with previous evidences on the effectiveness of AfpA on the control of postharvest decays of fresh fruits (Gandía et al., 2020; Garrigues et al., 2018). Given the fact that both proteins have potent fungicidal activity against a broad spectrum of fungal pathogens, our results suggest that AFPs can be widely applied as surface-coat on the management of postharvest decay of fresh fruits. However, the control efficacy of each protein needs to be characterized for each pathosystem since differences have been already reported. For instance, AfpA controls the green mold disease caused by *P. digitatum* in orange fruits at concentrations as low as 0.15 μM, whereas AfpB showed no significant protection at any tested concentration (Garrigues et al., 2018). More specificity was observed for AfpA that controls the blue mold rot caused by *P. expansum* infection in Golden Delicious but not in Royal Gala apple fruits (Gandía et al., 2020).

Finally, we also prove that plant AfpA-enriched extracts, and to a lower extent AfpB-enriched extracts, are effective in controlling the infection of rice seeds from *F. pro-*

liferatum . Although it needs to be experimentally determined, this protection could potentially be extended to other seeds and against other Fusarium sps. Our observations suggest that AfpA, and also AfpB, represent an alternative for protecting seeds from fungal infections, that cause important losses in grain crops through inhibition of germination and contamination with mycotoxins. Fusarium sps are mycotoxigenic fungi that produce dangerous mycotoxins commonly found in cereal grains, not only important components of human diets but also animal feed (Summerell, 2019; Bennett and Klich, 2003). Plant protein extracts enriched in the antifungal proteins, or directly purified proteins, could be used for surface coating seeds for controlling fungal infections. Controlling Fusarium sps, and other mycotoxigenic fungi such as Aspergillus sps to whom AfpA and AfpB are also active (Garrigues et al., 2018, 2017), would avoid mycotoxins entering in the food chain for the benefit of human health. Altogether, our results providing a sustainable and efficient production system in plant biofactories, and demonstrating the efficacy of plant products, represent an important advance in the use of AfpA and AfpB as environmental friendly and effective "green fungicides" in crop and postharvest protection.

EXPERIMENTAL PROCEDURES

Plasmid constructs

To produce AFPs in *N. benthamiana* leaves, we used the previously described TMV-derived expression system (Shi et al., 2019). We prepared two different constructs for the expression of AfpA, one of them targeting the protein to the apoplastic space and the other to the vacuole (Figure 1a). The codon optimized cDNA encoding the mature AfpA protein (XP˙016603682.1) extended in N-terminal with the secretory signal of tobacco AP24 protein (XP˙009782398.1) was synthesized by IDT (Leuven, Belgium). For the apoplastic construct, the AfpA ORF was PCR amplified using the primers CP:AP24˙f and AfpA:CP˙r (Table S1), and assembled through Gibson reaction into the intermediate pMTMVi-N plasmid as previously described (Shi et al., 2019). For the vacuolar construct, the AfpA ORF was extended in C-terminal with the vacuolar sorting signal (VS) through two consecutive rounds of PCR amplification, the first one using AP24˙f and AfpAVS˙r primers, and the second one using CP:AP24˙f and VS:CP˙r primers. The obtained DNA fragment was then assembled into the intermediate pMTMVi-N plasmid. Equally, the AfpBVS fragment was obtained through double PCR using AP24˙f and AfpBVS˙r primers, and then CP:AP24˙f and VS:CP˙r primers. Next, the TMV-cDNA fragments in the intermediate plasmids were assembled into the pGTMV plasmid through a second Gibson reaction, as previously described (Shi et al., 2019). All the constructs were verified by nucleotide sequencing.

Agroinoculation of *N. benthamiana* leaves

TMV recombinant clones in pGTMV plasmids were delivered into plants via agroinfiltration using Agrobacterium tumefaciens strain GV3101 carrying the pSoup helper plasmid (Hellens et al., 2000). Overnight cultures diluted at 0.1 OD600 in induction medium (10 mM MES, 10 mM MgCl2, 200 µM acetosyringone) and incubated for 3h at room temperature were used for vacuum infiltration of whole plants. *N. benthamiana* plants used for agroinfiltration experiments were grown at 4-5 leaf stage in the greenhouse at 24 °C with a 14h light- 10h dark photoperiod. Leaves of inoculated plants were harvested at 7 dpi for protein purification.

Protein analysis and purification

Proteins were extracted from apoplastic fluids from fresh leaves by vacuum infiltration using phosphate saline buffer (PBS) supplemented with 0.02% (v/v) Silvet L-77. Intracellular protein extracts were obtained from frozen leaves using acid buffer (84 mM citric acid, 30 mM Na2HPO4, 6 mM ascorbic acid, 0.1% (v/v) 2-mercaptoethanol, pH 2.8). Extracts were clarified by centrifugation at 16000 g for 15 min at 4 °C. Protein concentration was determined using the Bio-Rad protein assay and bovine serum albumin (BSA) as standard.

Protein preparations were separated in tricine-SDS-PAGE (18%), and stained using Coomassie blue or transferred to nitrocellulose membrane (Protran 0.2 µM) for immunodetection. AfpB was detected using antiserum against P. chrysogenum PAFB kindly provided by Dr. F Marx (Huber et al., 2018b), and AfpA with the antisera previously reported (Garrigues et al., 2018). AfpBVS protein was purified by filtering plant extracts using Amicon Ultra-15 10K Centrifugal filters (Millipore), and monitoring purity by tricine-SDS-Gel. Protein concentrations were determined by spectrophotometry at 280 nm, or by comparing band intensities in complex protein extracts with known amount of purified proteins using the Quantity Tools Image Lab™ Software (version 5.2.1) included in the Chemi-Doc™ Touch Imaging System (Bio-Rad).

Antifungal assays

Growth inhibition assays against the *P. digitatum* CECT209796 (PHI26) were performed in 96-well microplates, as described previously (Garrigues et al., 2017). Total protein extracts and purified proteins were dialyzed against water and used at the indicated concentrations in antifungal assays.

Plant protection assays against fungal infection

Tomato leaf protection assays against B. cinerea infection were performed as previously described (Shi et al., 2019). Fungal biomass in inoculated leaves in the presence of AFPs was determined at 3 dpi by quantitative PCR using specific primers for B. cinerea CutA gene (Z69264) and normalized to tomato actin gene (U60480.1) (Table S1). DNA (15 ng per qPCR) was obtained using the Maxwell RSC plant DNA kit (Promega). Q-PCR analyses were carried out in 96-well optical plates in a LightCycler 480 System (Roche) using SYBR green.

For tomato fruit protection assays, cherry tomatoes were purchased in a local grocery. They were then treated with a 1.5 % (v/v) bleach solution for 5 min and profusely washed with abundant sterile water. Next, tomatoes were inoculated by placing 15 µl drops of B. cinerea conidia suspensions (5x105 conidia/ml) onto needle-prick wounds. To assess the protective effect of AFPs, conidial suspensions were applied in the presence of purified AFPs or plant protein extracts containing AFPs at 10 µM concentrations. Inoculated tomatoes were incubated at room temperature and high humidity in a hermetic box for 9 days to determine the percentage of incidence. We analyzed 6 tomatoes per treatment in 4 independent experiments.

Rice seed protection assays against Fusarium spp. were done on Oryza sativa var. Nipponbare seeds infected with *F. proliferatum* as previously described (Gómez-Ariza et al., 2007; Bundó et al., 2014). Protection conferred by AFPs was assayed on seeds inoculated with 20 µl of spore suspensions at 5x104 /ml in the presence of purified AFPs or plant extracts containing AFPs at 10 µM concentrations. At 7 dpi, seedlings were categorized in 3 groups and counted: non-germinated seeds, seedlings showing growth retardation, and seedlings showing normal growth. We analyzed 12 seeds per treatment in 4 independent experiments.

Blast disease protection was done using a detached leaf assay previously described (Coca et al., 2004). Leaves were collected from 4 leaves-Nipponbare rice plants and drop-inoculated with spore suspensions of *Magnaporthe oryzae* Guy-11 GFP. Protection conferred by AFPs was tested by infecting with drops containing the fungal spores along with the antifungal proteins at 10 µM concentrations. The progression of symptoms was followed visually. Lesion area was measured by image analysis using the Fiji ImageJ2 package. We analyzed three infection points on three leaves from three independent plants in at least three independent experiments.

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General discussion

Plant diseases caused by pathogenic fungi are one of the major factors limiting worldwide crop production, and destroying plant products after postharvest, endangering seriously food security. Mycotoxigenic fungi also contaminate plant products used for food or animal feeding with harmful toxins for human health. The control of pathogenic fungi is usually based on the application of synthetic fungicides, that are not always effective and safe for human consumption and environmentally friendly. Moreover, the indiscriminate use of fungicides has led to the proliferation of fungicide-resistant strains, not only in plant pathogens but also in fungi causing human diseases. Currently, there are only a few classes of licensed fungicides for agricultural applications. AMPs have attracted much attention as potential biofungicides that can substitute or complement the chemical fungicides. Several challenges need to be overcome before AMPs can be used in agriculture, including production at low price and high amounts in a safe manner, as well as the well understanding of their mode of action, their effectiveness, their persistence and their effects on plants and fruits to be treated with them, without forgetting of the unintended effects on beneficial fungi.

In this study, we have developed cost-effective and safe systems for producing AMPs at commercial-level yields in short times. We developed an efficient system for producing AFPs using a TMV vector for transient expression in *N. benthamiana* plants (Chapter I). This system was further improved by targeting AFP accumulation in vacuoles allowing us to reach yields up to 1.2 mg AFP per gram of leaf (Chapter IV). Moreover, we developed another system based on targeting AMPs to LDs that was used for the production of the linear synthetic PAF102 peptide. This peptide was a recalcitrant peptide to be produced biotechnologically, and not efficiently produced using the *N. benthamiana* production system. The strategy of targeting PAF102 in LDs was developed first in rice seeds (Chapter II), and then, transferred to *P. pastoris* system where we produced commercial amounts of PAF102 in in shorter times (Chapter III). Finally, we also demonstrated that plant produced AFPs can be used for protecting plants from fungal infections, particularly plant AFPs showed efficiency on controlling Botrytis gray mold in tomato plants and fruits, blast disease in rice plants, and *Fusarium* infections in rice seeds (Chapter IV). Collectively, this work contributed to the advance of AMPs towards its development as novel fungicides with applications in crop and postharvest protections.

1. Efficient production of AFPs in plants

Plants are excellent biofactories for producing proteins and other metabolites of interest for industry, cosmetic and pharma applications. They offer many advantages as production systems: they are economic to grow with low investment cost, production can be easily scaled up, and are free of animal and human pathogens. These advantages make plant-based production systems very suitable for the production of AMPs to be used in agriculture, that require huge amount of AMPs at very low-cost and in a safe manner. Although AMPs have been produced previously in plants, yields reported were not high enough for commercial applications (Bundó *et al*., 2014; Holásková *et al*., 2018; Lee *et al*., 2011; Montesinos *et al*., 2016, 2017). Several factors can be impeding the efficient accumulation of AMPs in plants, including their cytotoxic effects and their low stability in plants. Our work demonstrates that transient expression using a TMV vector in *N. benthamiana* plants is a very efficient strategy to produce AFPs. We were able to produce several AFPs at yields of commercial relevance, up to 1.2 mg of protein per gram of fresh leaves, breaking the yield barrier of AMPs in plants. Other reports already tested plant transient expression systems using viral vectors for the production of AMPs without reaching the high values that we obtained for AFPs (Patiño-Rodríguez *et al*., 2013; Zeitler *et al*., 2013). Several reasons might be contributing to the high yields

obtained for AFPs, including the high stability of these AMPs that favors high accumulation levels, as well as the subcellular compartment where they were accumulated to avoid cytotoxic effects. Our studies demonstrate that vacuolar targeting is the most appropriate strategy to reach high yields of bioactive, stable proteins.

Using the developed system, we biotechnologically produced for the first time AfpA, the most potent antifungal protein so far reported. AfpA yield was about 0.179 mg/g of fresh leaves. Yield was higher for the AfpB reaching eight times more amount of protein (1.2 mg/g leaves). We found a tight correlation between protein activity and yield. In this regards, AfpA is a highly active protein that reached the lowest yield; whereas proteins as GFP or the *P. digitatum* Sca protein, which have not bioactivity, were the ones reaching the highest yield (up to 4 mg/g leaves).

One of the main advantages of the production system is the simplicity of extraction and purification of the AFPs from plant material. AFPs were targeted to a particular subcellular compartment, either the apoplastic space or vacuoles. Extracting from apoplasts is an extremely simple procedure, since extracellular fluids can be easily obtained by vacuum infiltration of harvested leaves and sieving, yielding highly AFP-rich solutions, in which more than half of the protein content is AFPs. On the other hand, vacuolar AFPs can be also easily obtained using an acidic extraction buffer that selectively extract these proteins on the basis of their physico-chemical properties, yielding also AFP-rich solutions with more than half of content is AFPs. Starting from these protein extracts, either ECFs or acidic extracts, a simple chromatographic step is required to purified basic AFPs to homogeneity, or alternatively by simple size filtration with centrifugal filters. Even better, plant AFP-enriched extracts can be directly used without further purification since they were effective against plant pathogens. This greatly reduces the cost of downstream processing that accounts in most of the cases for about 80% of the production costs (Gottschalk, 2007). In addition, the production process can be easily scaled up by simple spraying agrobacteria cultures to deliver the viral vector in producing plants, allowing the fast inoculation of hundreds plants to produce large amount of AFPs at cost-effective prices for agricultural applications.

2. Production of linear AMPs in rice seeds and *P. pastoris* LDs.

Linear AMPs are difficult to be biotechnologically produced, mainly due to the lack of stability of these peptides and/or their toxicity to host cells. Initially, we explored the production of CecA and PAF102 in *N. benthamiana* leaves using the TMV-derived vector system. However, we failed to get good amounts of these peptides due to cytotoxic effects on leaves that showed dead appearance few days after agroinfiltration. Therefore, we developed an alternative system targeting them to LDs as plant oleosin fusion proteins, given that CecA was previously produced on LDs from rice seeds (Montesinos *et al*., 2016).

We first produced PAF102 in the LDs of rice seeds at moderate yields, reaching amounts about 20 μg of peptide per gram of seed that corresponds to 5.1 nmoles/g of seed. PAF102 yields were on the average of reported yields for small peptides in rice seeds (0.03–10 nmoles/g of seed) (Montesinos *et al*., 2016; Suzuki *et al*., 2011; Takagi *et al*., 2005, 2008, 2010; Wang *et al*., 2013; Yasuda *et al*., 2005). However, this was the first time that the rationally designed PAF102 was produced biotechnologically, demonstrating that targeting LDs is an excellent strategy to produce biologically active peptides. Production of peptides in rice seeds have several advantages, including that can be stored at room temperature for long times and that production can be uncoupled from processing. Interestingly, the LDs are accumulated on the rice bran that

is the by-product of the rice grain milling process. The accumulation of PAF102 in the rice bran adds an extra value to this by-product assisting their exploitation to bring PAF102 to the market.

The production of AMPs in rice seeds has however the inconvenience of the long development times, given that to obtain seeds is required to complete the plant cycle, that in the case of rice takes around four months. To speed the production process, we implemented the plant oleosin fusion technology in the *P. pastoris* system. This yeast system is an interesting production system because it is easy to genetically manipulate and grows fast in short times to high cell densities, allowing the production of grams of recombinant protein per liter of culture (Ahmad *et al*., 2014). The main difficulty was that yeast cells have not a high content in LDs, thus to enhance *P. pastoris* LD content, the Arabidopsis *dgat1* gene was coexpressed with the oleosin-CecA or oleosin-PAF102 genes. DGAT1 is the limiting-enzyme in the biosynthesis of triacylglycerols and a target to increase oil content in cells (Liu *et al*., 2012). Using this system, CecA was not accumulated in *P. pastoris* cells due to susceptibility to host proteases, but PAF102 was accumulated in LDs at high levels, reaching commercially relevant yields of up to 180 mg/l of yeast culture in only 4 days.

Production of PAF102 in LDs, either in rice seeds or in *P. pastoris*, has the additional advantage of facilitating the downstream purification of the peptide since LDs are easily recovered by flotation in dense solutions. And the recovered PAF102 was biologically active against pathogenic fungi. Our results provide an efficient production system of the rationally designed antifungal peptide PAF102 that exhibit a strong activity against important phytopathogens for agricultural and clinic applications.

3. Efficiency of *in planta*-produced AFPs in plant protection against fungal infections

The final objective of this study was to characterize the efficacy of *in planta*-produced AFPs in the control of plant diseases caused by fungal pathogens. AFPs are known to confer protection against fungal infections when produced *in situ* through transgenic strategies (Coca *et al*., 2004; Girgi *et al*., 2006; Li *et al*., 2008; Moreno *et al*., 2005; Narvaez *et al*., 2018; Oldach *et al*., 2001). Also, there are few examples in the literature reporting the efficiency of AFPs in the control of fungal diseases in plants when applied topically, most of them related with *A. giganteous* AFP that proves efficiency on the control of *M. oryzae* infection in rice leaves (Vila *et al*., 2001), *B. cinerea* in geranium plants (Moreno *et al*., 2003), *F. oxysporum* in tomato plants (Theis *et al*., 2005), *Alternaria alternate* in banana fruits (Barakat, 2014). More recently and simultaneous to this thesis work, AfpA was reported by our collaborators to be efficient in controlling *B. cinerea* infection in tomato plants (Garrigues *et al*., 2018), *P. digitatum* in orange fruits (Garrigues *et al*., 2018) or *P. expansum* in apple fruits (Gandía *et al*., 2020).

In our study, we investigated whether the *in planta*-produced AfpA and AfpB can confer protection against relevant fungal pathogens, including *M. oryzae* and *B. cinerea* considered the most important plant pathogens (Dean *et al*., 2012). We demonstrated that both proteins, AfpA and AfpB, are able to control *B. cinerea* infection on tomato plants at low concentrations, being AfpA more effective than AfpB. Moreover, AfpA and AfpB are also efficient in the protection of rice leaves against blast fungus infections, in this case being AfpB more efficient than AfpA. Interestingly, the protection can be afforded not only by purified proteins, but also by AfpA- or AfpB-enriched plant extracts, which can be easily obtained from our production system. Our studies suggest that plant extracts enriched with AfpA or AfpB can be used through spray application to protect crop plants. The strategy could be economically feasible thanks to the production platform for AFPs that we developed in this thesis. Further studies on the time lasting AFP

protective effects on plants and on the effects that AFPs might have on plant performance are needed before developing these AFPs as fungicides for agricultural applications.

We also showed that AfpA and AfpB can be applied for the control of *B. cinerea* in tomato fruits, either as purified proteins or as total plant protein extracts containing them. These results, together with reported results on the efficiency of AfpA in controlling *P. digitatum* infection on orange fruits (Garrigues *et al*., 2018) and *P. expansum* on apple fruits (Gandía *et al*., 2020), suggest that application of these proteins can be also a good strategy for postharvest protection of commodities. The amount of protein required for treating fruits is significantly lower than for plant treatments in fields or greenhouses, making even more feasible the application of these molecules in postharvest protection.

Finally, we also proved that AfpA, and to a lower extent AfpB, are effective controlling *F. proliferatum* infection of rice seeds. This result suggest that these antifungal proteins can be also applied to the protection of seeds against seed-born fungal pathogens, most of them mycotoxigenic fungi. Seeds can be surface coated with a AFP-containing solution to prevent fungal infections, avoiding seed viability losses, as well as mycotoxin contaminations.

Overall, this Ph.D thesis developed a sustainable and efficient production system for AFPs in *N. benthamiana* leaves, and another system for the production of linear AMPs using the oleosin fusion technology in rice seeds and yeast. Also, we proved the antifungal activity of these biofactory products. Furthermore, we showed that AFPs produced in plants can be used for protecting plants from fungal infections. These results represent a significant advance on the usage of AFPs as environmentally friendly and effective fungicides in agriculture applications.

Conclusions

Conclusions

-We have developed a system based on *N. benthamiana* leaves and a new TMV-derived vector for the transient production of AFPs. The system yields large amounts of proteins in short time periods (7 days).

-We have proven that the system can be simplified for large-scale production through spray application of agrobacteria for viral vector delivery, and by uncoupling protein processing from production storing leaves at room temperature for long periods of time.

-We have demonstrated that subcellular compartments where AFPs are accumulated have an important impact on protein yield, probably due to toxicity towards plant cells. We found that vacuolar targeting of AFPs is the most appropriate compartment for reaching high yields.

- Using the developed *N. benthamiana* production system, we can produce up to 0.170 mg per gram of fresh leaf of the highly active AfpA, and eight times more of AfpB (1.2mg/g).

-We have also demonstrated that our production system is even more efficient for non-bioactive proteins, such as the GFP or *P. digitatum* Sca protein, reaching yields of about 4mg of protein per gram of fresh leaf.

-*In planta*-produced AFPs exhibit equivalence in terms of antifungal activity to their counterparts from fungal origin.

-Plant protein extracts containing AfpA and AfpB are active against fungal pathogens without requiring further protein purification.

-Purified AfpA and AfpB, as well as plant protein extracts enriched with these two proteins, are efficient in controlling Botrytis gray mold disease in tomato leaves and fruits, blast disease in rice plants and *Fusarium proliferatum* infection in rice seeds.

-For the rationally designed antifungal peptide PAF102 that was recalcitrant to be produced biotechnologically, we developed an alternative production system based on targeting its accumulation in lipid droplets (LDs) using the oleosin fusion technology.

-PAF102 was produced at moderate yields in LDs of rice seeds reaching 20 μg of peptide per gram of grain.

-We efficiently implemented the plant oleosin technology in the *P. pastoris* system for the production of PAF102 in LDs to commercially relevant yields up to 180 mg/l of yeast culture in only 4 days.

-The accumulation of PAF102 in LDs of rice seeds or yeast facilitated its downstream extraction and recovery by flotation on density gradients, with the recovered PAF102 being biologically active against pathogenic fungi.

-Cecropin A was not produced in LDs of *P. pastoris* because it is susceptible to the yeast proteases.

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Annex I

Supplemental Material for Chapter I

SUPPORTING INFORMATION

Efficient production of antifungal proteins in plants using a new transient expression vector derived from tobacco mosaic virus

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Running title: A new viral system for efficient AFP production

Keywords: antifungal proteins, viral vector, tobacco mosaic virus, plant biofactory, *Nicotiana benthamiana*, Gibson assembly.

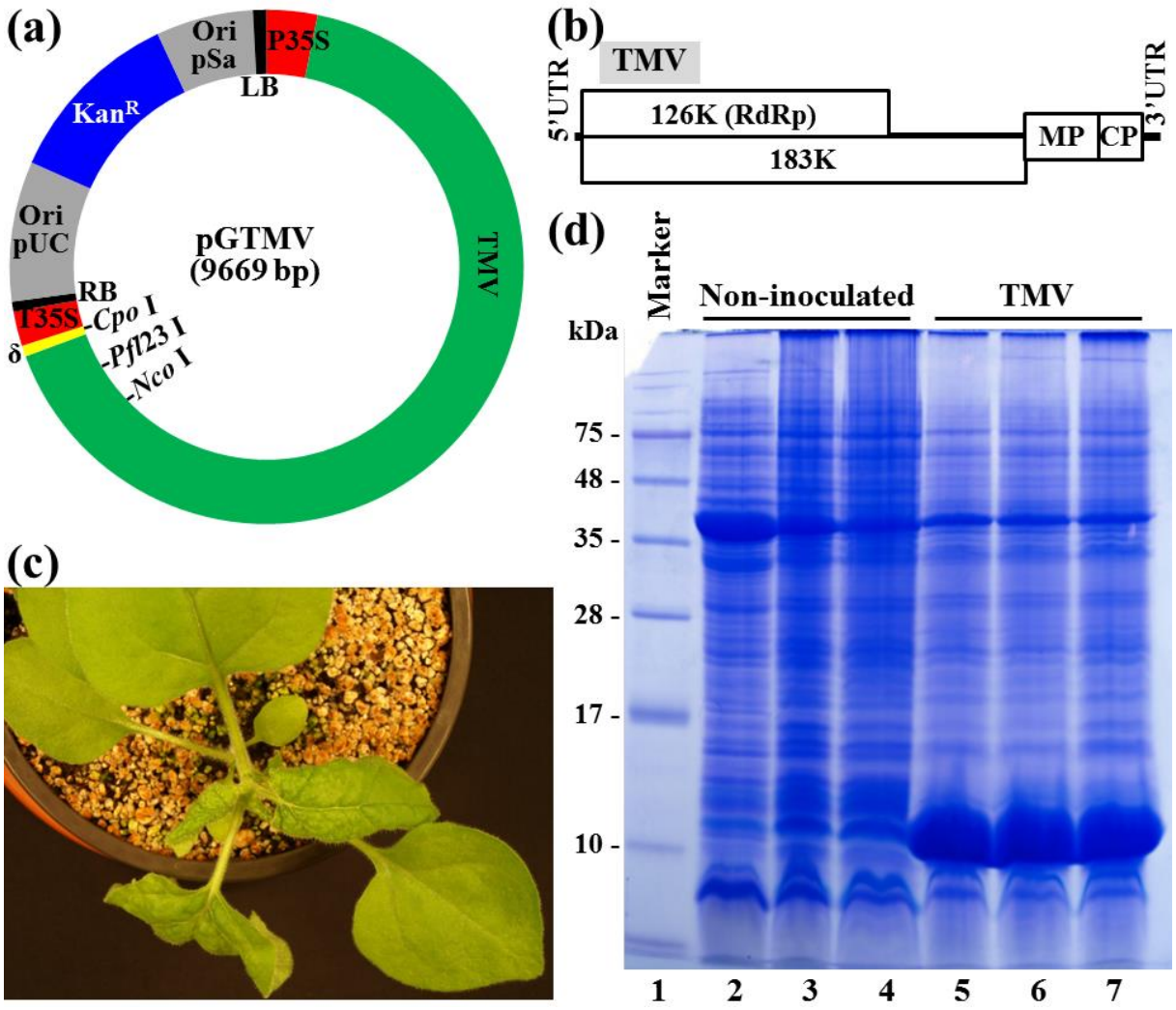

Figure S1. *Tobacco mosaic virus* (TMV) infectious clone. (a) Schematic representation of plasmid pGTMV that contains the whole TMV cDNA. See more details in the legend to Figure 1. (b) Schematic representation of wild-type TMV genome. Boxes represent the 126K and 183K replication proteins, movement protein (MP) and coat protein (CP). Lines represent the 5' and 3' untranslated regions (UTRs). (c) *Nicotiana benthamiana* plant infected with wild-type TMV. Picture was taken 5 days post-inoculation (dpi). (d) Coomassie blue-stained polyacrylamide gel showing the protein content of *N. benthamiana* leaves from plants mock-inoculated (lanes 2 to 4) or agroinoculated with TMV (lanes 5 to 7). Lane 1, marker proteins with sizes in kDa indicated on the left.

Figure S2. Full sequences of plasmid pGTMV, which contains a full-length TMV infectious clone (GenBank accession number MK087763), and intermediate plasmids pMTMVi-N and pMTMVi-M, which can be used to construct recombinant TMV clones unable and able to move systemically throughout the plant, respectively.

>pGTMV (9669 bp)

GCGGCCGCGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAA
ATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCC
ACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTC
CACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCA
TTTGGAGAGGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTA
CAATGGCATACACACAGACAGCTACCACATCAGCTTTGCTGGACACTGTCCGAGGAAACAACTCCTTGGTCAATG
ATCTAGCAAAGCGTCGTCTTTACGACACAGCGGTTGAAGAGTTTAACGCTCGTGACCGCAGGCCCAAGGTGAACT
TTTCAAAAGTAATAAGCGAGGAGCAGACGCTTATTGCTACCCGGGCGTATCCAGAATTCCAAATTACATTTTATA
ACACGCAAAATGCCGTGCATTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATATCTGATGATGCAAATTC
CCTACGGATCATTGACTTATGACATAGGCGGGAATTTTGCATCGCATCTGTTCAAGGGACGAGCATATGTACACT
GCTGCATGCCCAACCTGGACGTTCGAGACATCATGCGGCACGAAGGCCAGAAAGACAGTATTGAACTATACCTTT
CTAGGCTAGAGAGAGGGGGGGAAAACAGTCCCCAACTTCCAAAAGGAAGCATTTGACAGATACGCAGAAATTCCTG
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CCTTGTCCATGACGTTTTACCTGCATACTAAGCTTGCCGTTCTAAAGGATGACTTACTGATTAGCAAGTTTAGTC
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CCGGCGGCCGGCCGCAACAGATCGTTTTATAAATTTTTTTTTTTGAAAAAGAAAAAGCCCGAAAGGCGGCAACCT
CTCGGGCTTCTGGATTTCCGATCCCCGGAATTAGATCCGTTTAAACTACGTAAGATCGATCT**TGGCAGGATATAT**
TGTGGTGTAAACGTTCCTGCGGCGGTCGAGATGGATCT**TGGCAGGATATATTGTGGTGTAAAC**GTTCCT

TMV-MK087763 is in bold. ***Cauliflower mosaic virus* (CaMV) 35S promoter** is in red with the transcription +1 nucleotide on yellow background. **Hepatitis delta virus (HDV)-derived ribozyme** is in red. **CaMV 35S terminator** is in fuchsia with the processing and polyadenylation site underlined. *E. coli* pUC replication origin is in blue on gray background. *Kanamycin* selection marker is in blue on dark gray background (complementary strand). *A. tumefaciens* pSa replication origin is in blue on gray background. *A. tumefaciens* T-DNA **RB** with **overdrive** (underlined) is in blue on yellow background and **double LB** is in blue on red background.

>pMTMVi-N (2376 bp)
AAGATTACAAACGTGAGAGACGGAGGGCCCATGGAACTTACAGAAGAAGTCGTTGATGAGTTCATGGAAGATGTC
CCTATGTCGATCAGGCTTGCAAAGTTTCGATCTCGAACCGGAAAAAAGAGTGATGTCCGCAAAGGGAAAAATAGT
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*CGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCG*CTCGA**G**GTCCTGCAACTTGAG
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CCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA
GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACG
CGCAGAAAAAAAGGATCTCAAGAAGATCCTTTTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGT
CTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGC
CCCAGTGCTGCAATGATACCGCGAGAGCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGA
AGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA
GTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCG
TTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAA
GCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCA
GCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCA
TTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC
AGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGA
TCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGA
GCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTC
CTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGTCTCG

TMV-MK087763 genome fragment is in bold. The ATG to AGA mutation of the TMV CP initiation codon is indicated. Unique restriction sites ***Nco*I** and ***Pfl*23II** are on green background. A polylinker is inserted between TMV positions 5756 and 6177. The polylinker consists of two unique restriction sites ***Age*I** and ***Xho*I** (yellow background) separated by a blue-white *LacZ* marker (in blue and italics). Two ***Bsa*I** recognition sites to release the TMV fragment, once manipulated, are indicated on blue background. Hybridization sites for PI and PII oligonucleotides to alternatively produce the manipulated TMV fragment by PCR are doubly underlined. Plasmid also contains pUC replication origin (in gray) and ampicillin resistance marker (on gray background).

>pMTMVi-M (3373 bp)

AAGATTACAAACGTGAGAGACGGAGGGCCCATGGAACTTACAGAAGAAGTCGTTGATGAGTTCATGGAAGATGTC
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TTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGG
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CGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCTCGAGGTCCTGCAACTTGAG
GTAGTCAAGATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTTTT
TCCCTCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATATACATCCGCAGG
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GCGTTCAGGCGGAAGGCCTAAACCAAAAAGTTTTGATGAAGTTGAAAAAGAGTTTGATAATTTGATTGAAGATGA
AGCCGAGACGTCGGTCGCGGATTCTGATTCGTATTAAATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTG
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CGCAGGGTAGACGACGCTACGGTTGCAATTCGGTCTGCTATAAATAATTTAGTTAATGAACTAGTAAGAGGTACT
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AGGTGCTGAAATATAAAGTTTGTGTTTCTAAAACACACGTGGTACGTACGATAACGTATAGTGTTTTTCCCTCCA
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TACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAA
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GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGAC
TGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTC
AAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC
TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAA
ATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGGTCTCG

TMV-MK087763 genome fragment is in bold. The ATG to AGA mutation of the TMV CP initiation codon is indicated. Unique restriction sites ***Nco*I, *Pfl*23II** and ***Cpo*I** are on green

background. A polylinker is inserted between TMV positions 5756 and 6177. The polylinker consists of two unique restriction sites ***Age*I** and ***Xho*I** (yellow background) separated by a blue-white *LacZ* marker (in blue and italics). 3' fragment of tomato mosaic virus (ToMV) genome (CP promoter, CP ORF and 3' UTR) is on gray background (**transcription initiation** and **start** and **stop** codons are indicated). **HDV-derived ribozyme** is in red. Two ***Bsa*I** recognition sites to release the manipulated TMV fragment are indicated on blue background. Hybridization sites for PI and PIII oligonucleotides to alternatively produce the manipulated TMV fragment by PCR are doubly underlined. Plasmid also contains pUC replication origin (in gray) and ampicillin resistance marker (on gray background).

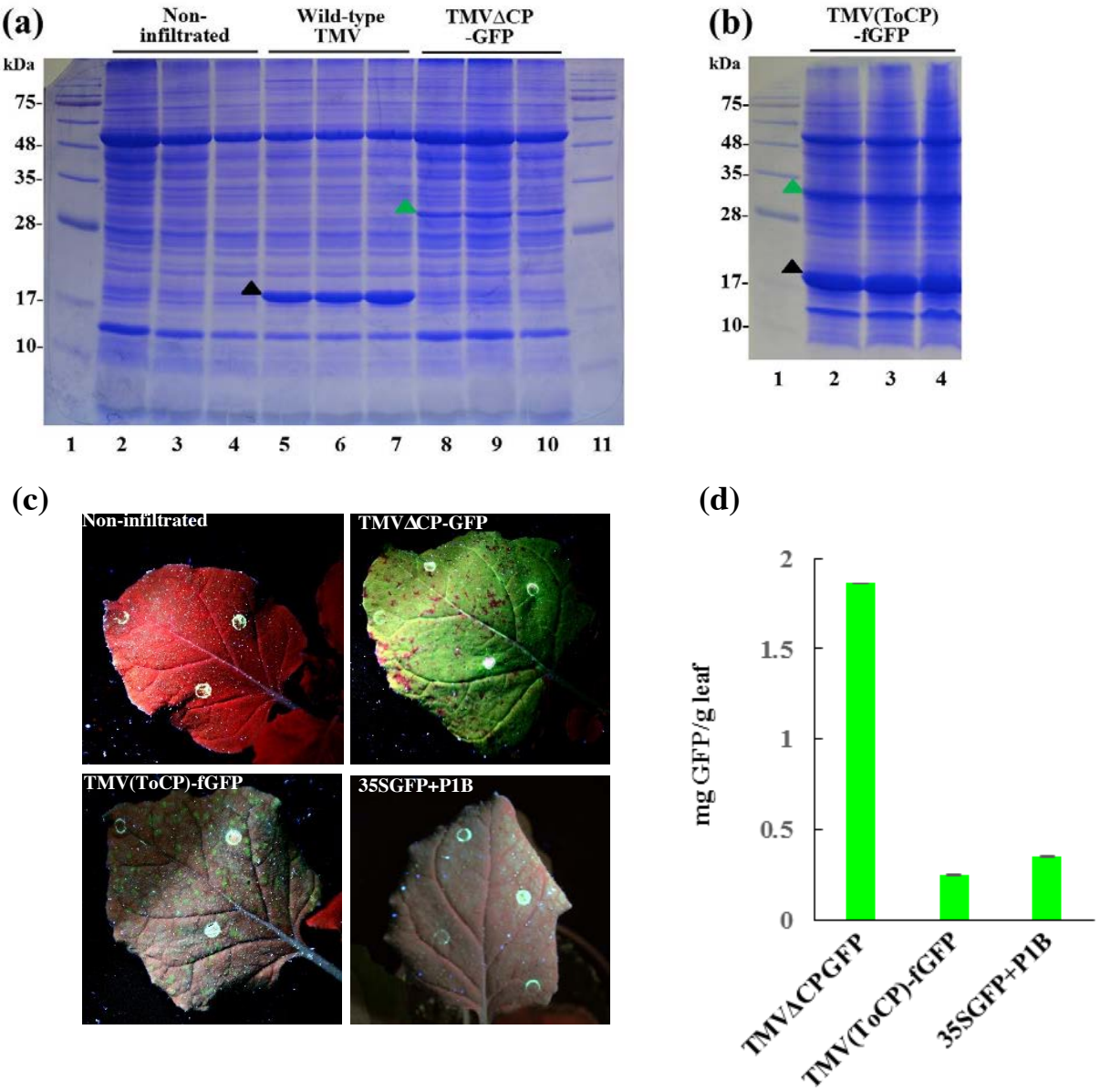

Figure S3. Analysis of GFP produced in *N. benthamiana* plants using the TMV vector system. Total proteins from plant leaves were separated by SDS-PAGE in 12.5% polyacrylamide gels and stained with Coomassie blue. (a) Lanes 1 and 11, and (b) lane 1, marker proteins with the sizes in kDa on the left. (a) Proteins from tissues of three independent plants non-infiltrated (lanes 2 to 4), or infiltrated with wild-type TMV (lanes 5 to 7) or TMVΔCP-GFP (lanes 8 to 10). Tissues were collected 7 dpi. (b) Proteins from upper non-inoculated tissues from three independent plants agroinoculated with TMV(ToCP)-fGFP (lanes 2 to 4). Tissues were collected 13 dpi. Green and black arrowheads points to GFP and TMV CP. (c) Appearance of *N. benthamiana* leaves agroinfiltrated with the indicated TMV vectors, or the binary vector pGWB5 along with the silencing suppressor P1b from *Cucumber vein yellowing virus* (CMYV) at 7 dpi under UV light. (d) Comparative analysis of GFP production in the agroinfiltrated leaves with the two viral vectors and a simple 35S promoter using the P1b silencing suppressor at 7 dpi.

Figure S4. Sequence of TMV-derived recombinant viruses TMVΔCP-GFP, TMVΔCP-AfpB, TMVΔCP-AfpBKDEL, TMVΔCP-UP, TMVΔCP-UPKDEL, TMVΔCP-UPVS, TMVΔCP-AgAFP and TMV(ToCP)-fGFP.

>TMV (with the ATG to AGA mutation of CP start codon)

GTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACAATGGCAT
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AGCGTCGTCTTTACGACACAGCGGTTGAAGAGTTTAACGCTCGTGACCGCAGGCCCAAGGTGAACTTTTCAAAAG
TAATAAGCGAGGAGCAGACGCTTATTGCTACCCGGGCGTATCCAGAATTCCAAATTACATTTTATAACACGCAAA
ATGCCGTGCATTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATATCTGATGATGCAAATTCCCTACGGAT
CATTGACTTATGACATAGGCGGGAATTTTGCATCGCATCTGTTCAAGGGACGAGCATATGTACACTGCTGCATGC
CCAACCTGGACGTTCGAGACATCATGCGGCACGAAGGCCAGAAAGACAGTATTGAACTATACCTTTCTAGGCTAG
AGAGAGGGGGGAAAACAGTCCCCAACTTCCAAAAGGAAGCATTTGACAGATACGCAGAAATTCCTGAAGACGCTG
TCTGTCACAATACTTTCCAGACATGCGAACATCAGCCGATGCAGCAATCAGGCAGAGTGTATGCCATTGCGCTAC
ACAGCATATATGACATACCAGCCGATGAGTTCGGGGCGGCACTCTTGAGGAAAAATGTCCATACGTGCTATGCCG
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TGACGTTTTACCTGCATACTAAGCTTGCCGTTCTAAAGGATGACTTACTGATTAGCAAGTTTAGTCTCGGTTCGA
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ACGACAGATTAGTGACTGAGTACAAGGCCTCTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAAGAAA
CGGAAGTGATGTACAATGCACTTTCAGAATTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTTTT
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TCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTTTTTCCCTCCACTTAAATCGAAGGGTTGTGTCTTGG
ATCGCGCGGGTCAAATGTATATGGTTCATATACATCCGCAGGCACGTAATAAAGCGAGGGGTTCGAATCCCCCCG
TTACCCCCGGTAGGGGCCCA

>TMVΔCP-GFP (sequence that replaces nucleotides 5757 to 6176 in TMV)
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CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC
ACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTAC
CCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC
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GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGC
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TACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTG
ACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTGA

>TMVΔCP-AfpB (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue)
ATGTCCAACAACATGGGCAACCTCAGGTCCTCCTTCGTCTTCTTCCTCCTGGCCCTGGTGACCTACACTTATGCA
AGTAAATACGGAGGACAATGCAGTCTGAAACACAACACGTGCACGTACCTGAAGGGTGGAAGAAACGTTATTGTC
AACTGCGGTTCGGCTGCCAATAAGAGGTGCAAGTCTGATCGCCACCACTGTGAATACGATGAGCACCACAGGAGG
GTTGACTGCCAGACTCCAGTTTGA

>TMVΔCP-AfpBKDEL (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue; KDEL is in green)
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AGTAAATACGGAGGACAATGCAGTCTGAAACACAACACGTGCACGTACCTGAAGGGTGGAAGAAACGTTATTGTC
AACTGCGGTTCGGCTGCCAATAAGAGGTGCAAGTCTGATCGCCACCACTGTGAATACGATGAGCACCACAGGAGG
GTTGACTGCCAGACTCCAGTT**AAGGACGAACTC**TGA

>TMVΔCP-UP (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue)

ATGTCCAACAACATGGGCAACCTCAGGTCCTCCTTCGTCTTCTTCCTCCTGGCCCTGGTGACCTACACTTATGCA
GTTGACAGCCCTCAGTGGACCATCCAGAACGCCCAGCGTGTCTGCAACCCCCAAGACACCTCCTGCACCTGGACC
TTCAGCATCTACCCTGGCGCCGGTGCCGCCACTCCCTGCACCTACGTGGTTGAAGGCAGCCCTGCCTCCCGCGCC
AACGGTGGCCCCGTCACCTGCGGCGCATACACCGTCACTTCTGGCTGGAGCGGCCAGTTCGGTGCGGACAATGGC
TTCACCACTCTCTCTGTTGTCGACAACAACTCTCGCCAGATCATCTGGCCTGCTTATACCGATAAGCAGCTCGCT
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>TMVΔCP-UPKDEL (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue; KDEL is in green)
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GTTGACAGCCCTCAGTGGACCATCCAGAACGCCCAGCGTGTCTGCAACCCCCAAGACACCTCCTGCACCTGGACC
TTCAGCATCTACCCTGGCGCCGGTGCCGCCACTCCCTGCACCTACGTGGTTGAAGGCAGCCCTGCCTCCCGCGCC
AACGGTGGCCCCGTCACCTGCGGCGCATACACCGTCACTTCTGGCTGGAGCGGCCAGTTCGGTGCGGACAATGGC
TTCACCACTCTCTCTGTTGTCGACAACAACTCTCGCCAGATCATCTGGCCTGCTTATACCGATAAGCAGCTCGCT
GGCGGTGCGGTTGTCAAGCCTGACCAGAGCTACGCCCCTGCTGCTCTCCCT**AAGGACGAACTC**TGA

>TMVΔCP-UPVS (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue; VS is in red)
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TTCAGCATCTACCCTGGCGCCGGTGCCGCCACTCCCTGCACCTACGTGGTTGAAGGCAGCCCTGCCTCCCGCGCC
AACGGTGGCCCCGTCACCTGCGGCGCATACACCGTCACTTCTGGCTGGAGCGGCCAGTTCGGTGCGGACAATGGC
TTCACCACTCTCTCTGTTGTCGACAACAACTCTCGCCAGATCATCTGGCCTGCTTATACCGATAAGCAGCTCGCT
GGCGGTGCGGTTGTCAAGCCTGACCAGAGCTACGCCCCTGCTGCTCTCCCT**GGCAACGGCCTCCTGGTCGACACC**
ATGTGA

>TMVΔCP-AgAFP (sequence that replaces nucleotides 5757 to 6176 in TMV; AP24 is in blue)
ATGTCCAACAACATGGGCAACCTCAGGTCCTCCTTCGTCTTCTTCCTCCTGGCCCTGGTGACCTACACTTATGCA
GCCACATACAATGGCAAATGCTACAAGAAGGATAATATCTGCAAGTACAAGGCACAGAGCGGCAAGACTGCCATT
TGCAAGTGCTATGTCAAAAAGTGCCCCCGCGACGGCGCGAAATGCGAGTTTGACAGCTACAAGGGGAAGTGCTAC
TGCTGA

>TMV(ToCP)-fGFP
(sequence that replaces nucleotides 5757 to 6176 in TMV; FLAG epitope in purple; linker in gray)
ATG**GACTACAAGGACGACGATGACAAA**GCAGCCGCTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTG
CCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCC
ACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACC
ACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCC
ATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTG
AAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTG
GGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAAC
GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTAC
AAGTGA
(ToMV sequence that replaces nucleotides 6366 to 6395 in TMV)
GAACCAAATCCTCAAAAAGAGGTCCGAAAAATAATAATAATTTAGGTAAGGGGCGTTCAGGCGGAAGGCCTAAAC
CAAAAAGTTTTGATGAAGTTGAAAAAGAGTTTGATAATTTGATTGAAGATGAAGCCGAGACGTCGGTCGCGGATT
CTGATTCGTATTAAATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTGTTTTTGTCATCTGTATGGGCTGA
CCCTATAGAATTGTTAAACGTTTGTACAAATTCGTTAGGTAACCAGTTTCAAACACAGCAAGCAAGAACTACTGT
TCAACAGCAGTTCAGCGAGGTGTGGAAACCTTTCCCTCAGAGCACCGTCAGATTTCCTGGCGATGTTTATAAGGT
GTACAGGTACAATGCAGTTTTAGATCCTCTAATTACTGCGTTGCTGGGGTCTTTCGATACTAGGAATAGAATAAT
CGAAGTAGAAAACCAGCAGAATCCGACAACAGCTGAAACGTTAGATGCTACCCGCAGGGTAGACGACGCTACGGT
TGCAATTCGGTCTGCTATAAATAATTTAGTTAATGAACTAGTAAGAGGTACTGGACTGTACAATCAAAATACTTT
TGAAAGTATGTCTGGGTTGGTCTGGACCTCTGCACCTGCATCTTAAATGCATAGGTGCTGAAATATAAAGTTTGT
GTTTCTAAAACACACGTGGTACGTACGATAACGTATAGTGTTTTTCCCTCCACTTAAATCGAAAGGGTAGTGTCT
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CGGAACCCCCGGGTTGGGGCCCA

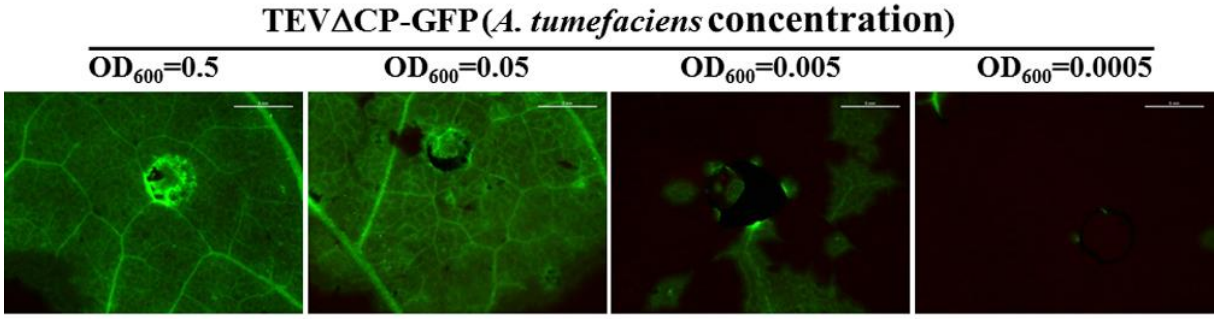


Figure S5. Dilution analysis of TMVΔCP-GFP infectivity in *N. benthamiana*. *A. tumefaciens* cultures to express TMVΔCP-GFP at the indicated OD_{600} were used to infiltrate *N. benthamiana* leaves. GFP fluorescence in the agroinfiltrated areas was detected at 7 dpi using a stereomicroscope. Representative pictures of three biological replicates are shown. Bars represent 5 mm.

Table S1. Primers used in this work.

Primer name	Sequence
PI	5’-TTACAAACGTGAGAGACGGAGGGCC-3’
PII	5’-GGAGGGAAAAACACTATGCGTTATC-3’
PIII	5’-GTAGAGAGAGACTGGTGATTTCAGC-3’
CP:AP24_fwd	5’-CTCAGTTCGTGTTCTTGTCAATGTCCAACAACATGGGC-3’
AP24AfpB_rev	5’ -TCCTCCGTATTTACTTGCATAAGTGTAGGT-3’
AP24AfpB_fwd	5’ -CACTTATGCAAGTAAATACGGAGGACAATGC-3’
AfpB:CP_rev	5’-CTACCTCAAGTTGCAGGACCTCAAACTGGAGTCTGGCAG-3’
AfpBKDEL_rev	5’-CCCGGGTTACTAGAGTTCGTCCTTAACTGGAGTCTGGCAGTC-3’
KDEL:CP_rev	5’-CTACCTCAAGTTGCAGGACCTCAGAGTTCGTCCTTAACTG-3’
AfpD:CP_rev	5’-CTACCTCAAGTTGCAGGACCTCAAGGGAGAGCAGCAGG-3’
AfpDKDEL_rev	5’-CCCGGGTTACTAGAGTTCGTCCTTAGGGAGAGCAGCAGGGGC-3’
AfpDVS_rev	5’-TCACATGGTGTCGACCAGGAGGCCGTTGCCAGGGAGAGCAGCAGGGGC- 3’
VS:CP_rev	5’-CTACCTCAAGTTGCAGGACCTCACATGGTGTCGACCAG-3’
AgAFP:CP_rv	5’-CTACCTCAAGTTGCAGGACCTCAGCAGTAGCACTTCCC-3’

Annex II

Supplemental Material for Chapter II

Supplementary Table 1. Primers used in this study

Primers used for cloning and PCR analysis. Restriction sites are underlined.	
NarIPAF103_fwd	5´ATTA**GGCGCC**CACCACCACCACCACCACCACCGCAAGAAGT3’
PAF103NarI_rev	5’GTGCAC**GGCGCC**ATTACTAGAGTTCGT3´
promOle18_fwd	5’GG**GAATTC**GATGGTCAGCCAATACATTGATCCGTT3’
Ole18_rev	5’TCT**GTCGAC**GATGTCTTGGTGCCGGC3’
HindIII_tNos_rev	5’TG**AAGCTT**GTTTGACAGGTTATCATCGGATCTAGTAACATAG3’
PstI_tNos_fwd	5’TT**CTGCAG**CCCGGGGATCGTTCAAACATTTG3’
promGluB1_fwd	5'GG**GGTACC**TCTAGACAGATTCTTGCTACCAA3'
promGluB4_fwd	5'GG**GGTACC**TACAGGGTTCCTTGCGTGAAGAA3'
promGlb1_fwd	5’**GGTACC**TGGAGGGAGGGAGAGGGGAGAGATG3’
SacI_tNos_rev	5'CC**GAGCTC**GTTTGACAGCTTATCATCGGATCTA3‘

Primers used for RT-PCR analysis	
OsEF1a_fwd	5’GTGCTCGACAAGCTCAAGGCCG3’
OsEF1a_rev	5’GTCTGATGGCCTCTTGGGCTCG3’
SPGluB1PAF_fwd	5’TGGCGAGTTCCGTTTTCTCT3’
SPGluB1PAF_rev	5’GTTCGTCCTTCCAGAACCACT3’
SPGluB4PAF_fwd	5’TGGCGACCATAGCTTTCTCTC3’
SPGlb1PAF_fwd	5’AGCAAGGTCGTCTTCTTCGC3’
SPGlb1PAF_rev	5’CCACTTCTTGCGGCGG3’

Supplementary Table 2. Estimation of transgene copy number by qPCR analysis. Values correspond to the Ct mean and standard deviation of qPCR analysis. The amplicons were the rice single copy *SPS* gene and the t-Nos region of the transgene.

		SPS		*Tnos*		
		Mean	SD	Mean	SD	Copy number
	Wt	22.06	0.11	31.75	0.24	0.69
	EV	22.34	0.20	31.33	0.28	0.71
pOle18:Ole18-PAF102: Tnos	**1**	25.49	0.08	25.95	0.20	0.98
	3	22.14	0.07	23.18	0.03	0.95
	6	22.20	0.09	24.11	0.07	0.92
	5	22.40	0.08	22.56	0.05	0.99
	7	21.91	0.02	19.90	0.05	1.10
pGluB4:PAF103: Tnos	**1.2**	22.09	0.06	20.82	0.02	1.06
	3.1	22.43	0.10	19.47	0.05	1.15
	3.4	21.98	0.10	19.72	0.02	1.11
	1.4	22.11	0.08	20.04	0.06	1.10
pGluB1:PAF103:Tnos	**5**	22.28	0.09	19.70	0.06	1.13
	8	25.79	0.06	22.51	0.27	1.15
	1	22.67	0.10	20.76	0.12	1.09
	3	22.52	0.05	20.51	0.16	1.10
	13	22.78	0.08	21.38	0.19	1.07
pGlb1:PAF103:Tnos	**1**	31.67	0.01	21.78	0.22	1.45
	6	26.81	1.25	22.80	1.19	1.18
	7.1	25.46	0.01	22.45	0.24	1.13
	7.2	25.60	0.66	22.01	0.00	1.16

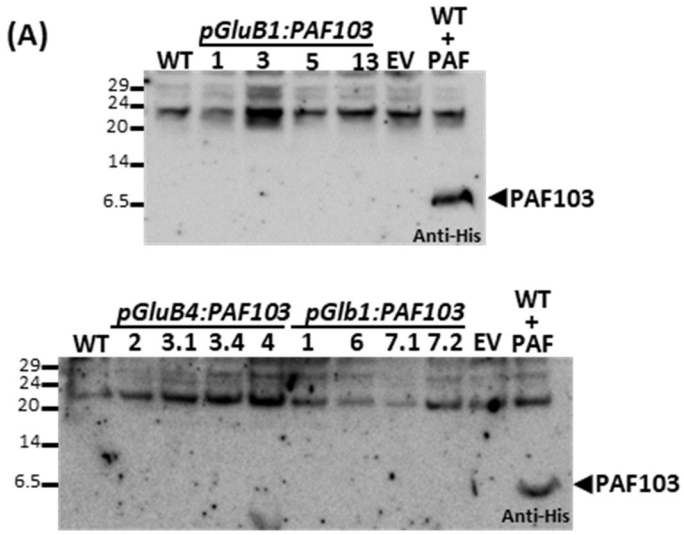
>>*PAF103*

ggatccatggccCACCACCACCACCACCACCACCGCAAGAAGTGGTTCTGGGCCG
GCCCGGCCCGCCGCAAGAAGTGGTTCTGGGCCGGCCCGGCCTGGCGCAAGAAGTG
GTTCTGGAAGGACGAACTCTAGTAAtggatcc

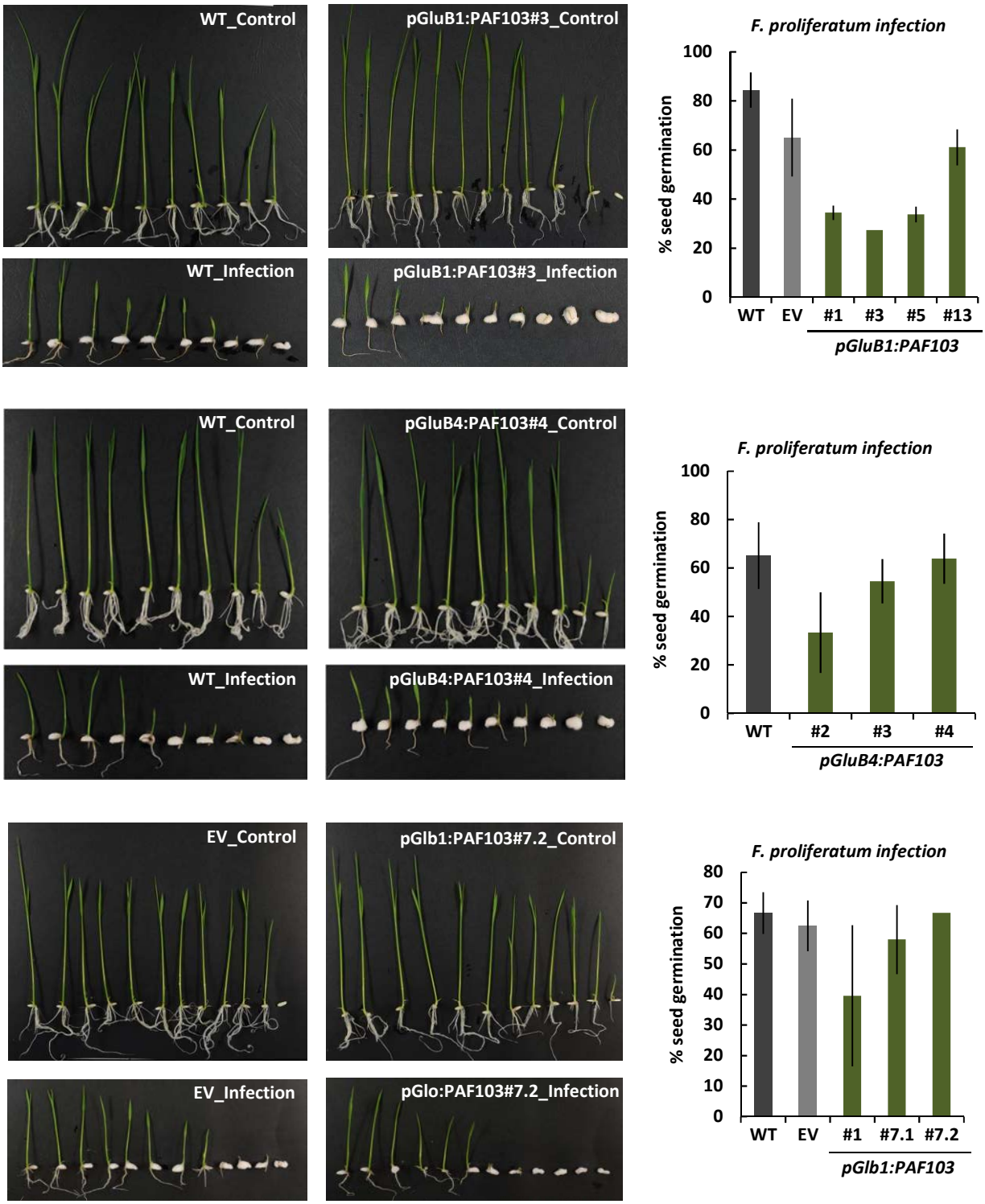
>>*PAF102*

gtcgacaCCGACCACCGAGAACCTCTACTTCCAGGGCCACCGCAAGAAGTGGTTC
TGGGCCGGCCCGGCCCGCCGCAAGAAGTGGTTCTGGGCCGGCCCGGCCTGGCGCA
AGAAGTGGTTCTGGTAGTAAtctgcag

Supplementary Figure 1. DNA sequence of the synthetic *PAF103* and *PAF102* genes. Underlined sequences are the His-tag and KDEL-extension encoding sequences. Blue color sequences are the protease recognition site (PRS). Green color sequences are the restriction enzyme recognition sites used for cloning purposes.



Supplementary Figure 2. PAF103 does not accumulate in rice seeds. Immunoblot analysis of PAF103 using anti-His monoclonal antibodies, in PB enriched fractions purified from wild-type (WT), or from transgenic homozygous mature seeds carrying the empty vector (EV), or the indicated transgenes. As a positive control, synthetic PAF103 peptide was added to WT extracts and run in parallel (WT+PAF).



Supplementary Figure 3. Fungal infection assays of *PAF103* transgenic seeds with the phytopathogen *F. proliferatum*. Phenotypical appearance of wild-type (WT) and transgenic seedlings carrying the empty vector (EV) or the indicated transgenes, at 7 days after germination under control conditions or inoculated with *F. proliferatum* spore suspension (10^3 spores/ml). Pictures are representative of at least 3 independent lines per construct, and at least 3 independent assays. The graphs show the mean and standard deviation values of germination rate of 3 independent assays.

Annex III

Supplemental Material for Chapter III

Supplementary Table 1. Primers used in this study

EcoRI_Ole18fwd	5'CG**GAATTC**ATGGCGGATCGCGACCGCGC3'
Ole18rev_wo stop	5'CTTTACTCATTGGCGAGGATGTCTTGGTGCC3'
GFPfwd	5'GACATCCTCGCCAATGAGTAAAGGAGAA3'
XbaI_GFPrev	5'CC**TCTAGA**TTATTTGTATAGTTCATCCATGCCATGT3'
XhoI_Ole18rev_with stop	5'CG**CTCGAG**TTACGAGGATGTCTTGGTGC3'
XhoI_CecArev	5'CG**CTCGAG**TTATCACTTGGCGATTTGGG3'
XhoI_PAF102rev	5'CG**CTCGAG**TTACTACCAGAACCACTTCT3'
Dgat1_fwd	5'GGCCGTCTCGGATCGGTACCATGGCGATTTTGGATTCTG3'
Dgat1_rev	5'TTCTGAGATGAGTTTTTGTTTCATGACATCGATCCTTTTC3'

Annex IV

Supplemental Material for Chapter IV

Table S1. Primers used in this work

Primer name	Sequence
CP:AP24_f	5’-CTCAGTTCGTGTTCTTGTCAATGTCCAACAACATGGGC-3’
AfpA:CP_r	5’-CTACCTCAAGTTGCAGGACCTTA CTTACGAAAATCACAAGTAACC-3’
AP24_f	5’-CACCATGTCCAACAACATGGGCAAC-3’
AfpAVS_r	5’-TCACATGGTGTCGACCAGGAGGCCGTTGCCCTTACGAAAATCACAAGTAACC-3’
VS:CP_r	5’-CTACCTCAAGTTGCAGGACCTCACATGGTGTCGACCAG-3’
AfpBVS_r	5’-TCACATGGTGTCGACCAGGAGGCCGTTGCCAACTGGAGTCTGGCAGTC-3’
BcCutA_f	5’-AGCCTTATGTCCCTTCCCTTG-3’
BcCutA_r	5’-GAAGAGAAATGGAAAATGGTGAG-3’
SlActin_f	5’-GGTGTGATGGTGGGTATGG-3’
SlActin_r	5’-GCTGACAATTCCGTGCTC-3’