Deciphering a Complex Interplay:

Unveiling Molecular Mechanisms in Multitrophic Interactions that Affect the Infection of Plant and Insect Hosts by '*Candidatus* Phytoplasma mali'

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"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales."

-Marie Curie-

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Summary

The small, cell wall less bacterium '*Candidatus* Phytoplasma mali' is associated to apple proliferation disease. Apple proliferation disease causes high economic losses in European apple cultivation areas due to the production of small tasteless fruits and growth abnormalities within the apple trees. '*Ca.* P. mali' itself resides within the phloem of the host plant and is mainly transmitted by two phloem sucking insect species, namely *Cacopsylla melanoneura* and *Cacopsylla picta*. So far, the only possibility to restrict the spread of the pathogen is uprooting of infected trees and insecticide treatments against the insect vectors. Thus, for a better future management of the pathogen spread, it is crucial to decipher the complex multitrophic interplay between the phytoplasma, the insect vectors, the host plant, and the environment.

This work focuses on three research questions within this complex system: (I) Why are some insect vectors more efficient in transmitting '*Candidatus* Phytoplasma mali' than others? (II) How can we improve the detection of phytoplasma within potential insect vectors or plant hosts? (III) How does '*Candidatus* Phytoplasma mali' manipulate its host plant *Malus* x *domestica*?

Testing eggs and nymphs of infected *C. picta* females revealed that within this insect vector '*Ca.* P. mali' is transovarially transmitted. This transovarial transmission renders *C. picta* a very efficient vector of the pathogen. Since all phloem-sucking insect species in an apple orchard can be potential insect vectors, it is important to monitor them for the presence of '*Ca.* P. mali'. This monitoring comprises the PCR testing of several distinct insect species and can be facilitated using a universal endogenous qPCR control, that can be used with all different eukaryotic samples. Such a universal endogenous qPCR control was developed and tested for the use in different PCR assays.

'*Ca.* P. mali' manipulates its host plant by different effector proteins. Among them $SAP11_{CaPm}$, the first identified effector of '*Ca.* P. mali'. $SAP11_{CaPm}$ interacts with different TEOSINTE BRANCHED1/CYCLOIDEA/

PROLIFERATING CELL FACTOR 1 and 2 (TCP) transcription factors.

To understand its role in pathogenesis, interactions with *M.* x *domestica* TCP transcription factors were studied. Additionally, the in-planta effects of early $SAP11_{CaPm}$ expression were unravelled through transcriptomic analyses.

Beside $SAP11_{CaPm}$, a novel potential effector protein of '*Ca.* P. mali', the so-called "Protein in *Malus* Expressed 2" (PME2) was identified and characterized. Interestingly two genetic variants of PME2 were found and one of them provokes detrimental effects on growth and viability when expressed in plant mesophyll protoplasts or in a yeast reporter strain.

In conclusion, this work deciphers parts of the complex interplay between the phytoplasma '*Ca.* P. mali', its plant host *Malus* x *domestica* and its insect vectors. The data set obtained helps to understand the role of *C. picta* as efficient '*Ca.* P. mali' insect vector. It helps to improve the detection of the phytoplasma within different eukaryotic sample material, and to shed light on the function of different effector proteins.

Zusammenfassung

Das kleine, zellwandlose Bakterium ‚*Candidatus* Phytoplasma mali' wird mit der Apfeltriebsuchtkrankheit in Verbindung gebracht. Die Apfeltriebsucht verursacht in den europäischen Apfelanbaugebieten hohe wirtschaftliche Verluste. Typische Symptome sind kleine, geschmacklose Früchte und verschiedene Wachstumsstörungen bei den Apfelbäumen. ‚*Ca*. P. mali' selbst befindet sich im Phloem der Wirtspflanze und wird hauptsächlich durch zwei phloemsaugende Insekten, nämlich *Cacopsylla melanoneura* und *Cacopsylla picta*, übertragen.

Bisher besteht die einzige Möglichkeit, die Ausbreitung des Erregers einzudämmen, im Roden von infizierten Bäumen und in Pflanzenschutzbehandlungen gegen die Vektorinsekten. Um die Ausbreitung des Erregers in Zukunft besser in den Griff zu bekommen, ist es daher von entscheidender Bedeutung, das komplexe multitrophe Zusammenspiel zwischen dem Phytoplasma, den Vektorinsekten, der Wirtspflanze und der Umwelt zu entschlüsseln.

Diese Arbeit konzentriert sich daher auf drei Forschungsfragen: (I) Warum sind einige Vektorinsekten effizienter bei der Übertragung von ‚*Candidatus* Phytoplasma mali' als andere? (II) Wie können wir den Nachweis von Phytoplasmen in potenziellen Vektoren oder Pflanzen verbessern? (III) Wie manipuliert ‚*Candidatus* Phytoplasma mali' seine Wirtspflanze *Malus* x *domestica*?

Eine Studie, in der Eier und Nymphen von infizierten *C. picta*-Weibchen getestet wurden, ergab, dass ‚*Ca*. P. mali' transovariel übertragen wird. Diese transovarielle Übertragung macht *C. picta* zu einem sehr effizienten Überträger des Erregers.

Da alle phloemsaugenden Insektenarten in einer Apfelplantage potenzielle Überträger sein können, ist es wichtig, sie auf das Vorhandensein von ‚*Ca*. P. mali' zu überwachen. Dieses Monitoring umfasst die PCR-Analyse mehrerer verschiedener Insektenarten und kann durch die Verwendung einer universellen endogenen PCR-Kontrolle erleichtert werden. Eine solche universelle endogene qPCR-Kontrolle, die mit verschiedenen eukaryotischen Proben

verwendet werden kann, wurde entwickelt und in verschiedenen PCR-Assays getestet.

‚*Ca*. P. mali' manipuliert seine Wirtspflanze durch verschiedene Effektorproteine. Darunter auch $SAP11_{CaPm}$, der erste identifizierte Effektor von ‚*Ca*. P. mali'. $SAP11_{CaPm}$ interagiert mit verschiedenen TEOSINTE BRANCHED1/ CYCLOI-DEA/PROLIFERATING CELL FACTOR 1 und 2 (TCP) Transkriptionsfaktoren. Um die Rolle von $SAP11_{CaPm}$ in der Pathogenese von ‚*Ca*. P. mali' zu verstehen, wurden Interaktionen mit *M*. x *domestica* TCP-Transkriptionsfaktoren untersucht. Außerdem wurden die Auswirkungen der frühen $SAP11_{CaPm}$-Expression in der Pflanze durch Transkriptomanalysen entschlüsselt.

Neben $SAP11_{CaPm}$ wurde ein neues potenzielles Effektorprotein von ‚*Ca*. P. mali', das sogenannte „Protein in *Malus* Expressed 2" (PME2), identifiziert und charakterisiert. Interessanterweise wurden zwei genetische Varianten von PME2 gefunden, von denen eine bei der Expression in Protoplasten und Hefe-Reporterstämmen zum Absterben der Protoplasten und zu Wachstumsveränderungen bei den Hefekolonien führt.

Zusammenfassend lässt sich sagen, dass diese Arbeit Teile des komplexen Zusammenspiels zwischen dem Phytoplasma ‚*Ca*. P. mali', seiner Wirtspflanze *Malus* x *domestica* und seinen Überträgerinsekten aufklärt. Die erhobenen Daten helfen, die Rolle von *C. picta* als effizienter ‚*Ca*. P. mali'-Vektor zu verstehen, den Nachweis des Phytoplasmas in verschiedenen eukaryotischen Proben zu verbessern und die Funktion verschiedener Effektorproteine aufzuklären.

List of abbreviations

Amp	Antigenic membrane protein
AYWB	Aster Yellow Witches' Broom phytoplasma
BiFC	Bimolecular Fluorescence Complementation
BSL1	Serine/Threonine-Protein Phosphatase BSL1
CaPm	'*Candidatus* Phytoplasma mali'
cDNA	Complementary DNA
CHR8	CHROMATIN REMODELING 8
CIN	CINCINNATA-like
CYC/TB1	Cycloidea/Teosinte Branched1
DET	Differentially Expressed Transcript
ELISA	Enzyme-Linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
F1	First Generation
GO	Gene Ontology
Imp	Immunodominant membrane protein
LAMP	Loop-Mediated Isothermal Amplification
LNA	Locked Nucleic Acids
Md	*Malus* x *domestica* (Apple)
MOS1	Modifier of snc1,1
NYC1	Chlorophyll(ide) *b* reductase NYC1
OEE1/PsbO	Oxygen-Evolving Enhancer Protein 1 (also known as PsbO)
OEE2/PsbP	Oxygen-Evolving Enhancer Protein 2 (also known as PsbP)
PCR	Polymerase Chain Reaction
PME2	Protein in *Malus* Expressed 2
PSII	Photosystem II
qPCR	Quantitative Polymerase Chain Reaction
rDNA	ribosomal DNA
SA	Salicylic Acid
SAP05	secreted AY-WB protein 05
SAP11	secreted AY-WB protein 11
SCY1	Preprotein Translocase Subunit SCY1
TCP	TEOSINTE BRANCHED1/ CYCLOIDEA/PROLIFERATING CELL FACTOR 1 and 2 transcription factor

1. Introduction

Phytoplasma are cell wall-less prokaryotes belonging to the class of *Mollicutes*. Since their first description in 1967, they were believed to be mycoplasma-like organisms. However, research has provided evidence that they constitute a distinct group, leading to the taxon '*Candidatus* Phytoplasma', indicating that those pathogens do not yet fulfil Koch's postulates (Namba, 2019).

The phytoplasma are phloem restricted within plants and are primarily transmitted by phloem-sucking insects. They can survive and multiply in the phloem sap and insect haemolymph. With a genome size ranging from 680 to 1600 kb, phytoplasmas have the smallest genome among bacteria, and their size varies from 200 to 800 nm. Their pleiomorphic shape is due to being surrounded by a single membrane (Bertaccini et al., 2014).

Phytoplasma associated diseases are economically relevant, affecting all families of plants globally, including essential food and feed crops like wheat, rice, and maize, as well as fruit trees and ornamental flowers. Typical symptoms of an infection by '*Candidatus* Phytoplasma' include uncontrolled proliferation of axillary buds, resulting in a so-called witches' broom growth, stunting, bushy growth, leaf yellowing, flower sterility, or phyllody, i.e., the development of green leaf like flowers. Phytoplasma diseases are often named based on the disease symptoms, such as yellows, dwarf, decline or witches' broom (Kirdat et al., 2023; Bertaccini et al., 2014).

Although phytoplasma diseases are generally detrimental and even lethal to host plants, there are instances where an infection is appreciated. For example, more than a thousand years ago in ancient China, paeonies with green flowers were highly valued as the most beautiful flowers, and today, during Christmas time, poinsettias delight our households (Strauss, 2009). While phytoplasma infection in paeonies causes floral virescence (Kirdat et al., 2023), it leads to a bushy growth with free branching of poinsettia plants that would otherwise have long internodes with no lateral shoots

(Nicolaisen and Christensen, 2007).

The phylogenetic characterization of phytoplasma is based on the analysis of the 16S rRNA gene sequence (Hogenhout et al., 2008). So far, 34 ribosomal groups with more than 200 subgroups have been identified, based on a 2.5% dissimilarity of the 16S rRNA sequence (Martini et al., 2019). Currently, 49 '*Candidatus* Phytoplasma' species were officially published and described (Bertaccini et al., 2022).

Among them, '*Candidatus* Phytoplasma mali' ('*Ca.* P. mali'), the pathogen associated to apple proliferation disease, is a devastating pathogen that threatens apple cultivation and is widespread in all important European apple cultivation areas. '*Ca.* P. mali' belongs to the 16SrX group (Seemüller and Schneider, 2004) and can be classified in several different strains, differing in their geographic distribution and virulence (Baric et al., 2011; Schneider and Seemüller, 2009; Paltrinieri et al., 2010; Casati et al., 2010; Casati et al., 2011).

Since there is no direct treatment available against '*Ca.* P. mali', it is considered a quarantine pest in many countries. The European and Mediterranean Plant Protection Organization (EPPO) classifies it in its A2 list as a pest recommended for regulation as a quarantine pest which is present in the EPPO region (European and Mediterranean Plant Protection Organisation, 2020).

1.1 Importance of '*Candidatus* Phytoplasma mali' for South Tyrol

In South Tyrol, the most northern province of Italy, apple (*Malus* x *domestica*) is the economically most important fruit crop. South Tyrolean apple yield accounts for almost 10% of the European and 50% of the Italian apple production. More than 14% of the entire 17,800 ha of apple orchards are organic, making South Tyrol the largest supplier of organic apples in Europe (Autonome Provinz Bozen - Südtirol, 2023).

Since the 1950s, apple proliferation disease has been observed in South Tyrol. Only in 1998, however, the first cases of infected trees on dwarfing M9 rootstocks, the most common rootstock in modern apple orchards, were reported (Barthel et al., 2020b). Typical

symptoms include the production of small, tasteless fruits, early leaf reddening, early bud break, enlarged stipules, and uncontrolled proliferation of axillary shoots, known as witches' brooms. The small and tasteless fruits that grow on infected trees are not marketable, thus causing severe economic damage in apple production (Seemüller et al., 2011a). Apple proliferation was regulated as a quarantine pest in the past; hence, farmers were obligated to uproot and destroy infected trees. Since 2019, its status has changed from a quarantine pest to a regulated non-quarantine pest, meaning that planting material must be free of '*Ca.* P. mali', otherwise movement within the Union territory or introduction from third countries into the European Union is not allowed (Regulation EU 2019/2072, annex IV, part J). However, in South Tyrol it is still strongly recommended to destroy infected material to prevent the spread of the pathogen within apple orchards.

Infections occurred in different waves in South Tyrol. In 2000, a first outbreak with more than 4 % of infected trees was observed, another wave came in 2006 with more than 5 % of infected trees, and seven years later, in 2013, almost 7 % of the trees were infected (Barthel et al., 2020b). In 2022, the infection incidence in South Tyrolean apple orchards ranged between 0.01 % and 0.6 %. Despite this low incidence, a few orchards were found with more than 10 % of symptomatic trees (Österreicher, 2023). It is estimated that the first outbreak in 2000 alone caused economic losses of around 100 million € of economic loss in Italy (Strauss, 2009), and for South Tyrol the damage is approximately at 50 million € for the years 2006 and 2013 (Barthel et al., 2020b).

Interestingly, disease outbreaks were not evenly distributed among the South Tyrolean apple growing area, even though it is the largest interconnected apple growing region in Europe. While the areas Burggrafenamt and Vinschgau in the west were highly affected, with some orchards having more than 60% infected trees, the Eisacktal and Unterland were much less affected (Barthel et al., 2020b). Reasons for these strong outbreaks in some areas could be deduced from the

distribution of two small psyllid species, *Cacopsylla melanoneura* and *Cacopsylla picta*, the insect vectors of '*Ca.* P. mali' (Baric et al., 2011).

1.2 Research Purpose

As outlined above, phytoplasma-associated infections are characterized by complex multitrophic interactions between the bacterium, its plant host, its insect vectors and the environment. For a better understanding of apple proliferation disease, it is essential to shed light on each of these interactions. Figure 1 illustrates the multitrophic interactions and highlights the research topics that have been the focus of this work.

This thesis focuses on the following research questions:

- Why are some insect vectors more efficient in transmitting '*Candidatus* Phytoplasma mali' than others?
  - Hypothesis: The phytoplasma is vertically transmitted by specific female insect vectors, leading to increased infection rates within the insect population and a higher likelihood of transmission to plant hosts.

    Transovarial transmission can be confirmed or excluded by testing eggs and nymphs from infected females for the presence of '*Ca.* P. mali'.
  - Publication: Mittelberger, Cecilia, Obkircher, Lisa, Öttl, Sabine, Oppedisano, Tiziana, Pedrazzoli, Federico, Panassiti, Bernd, Kerschbamer, Christine, Anfora, Gianfranco and Janik, Katrin (2017). The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny. Plant Pathology 66 (6), pp. 1015–1021. https://doi.org/10.1111/ppa.12653.
- How can we improve the detection of phytoplasma within potential insect vectors or plant hosts?
  - Hypothesis: Phloem-sucking insect species in the apple orchard are potential vectors of '*Ca.* P. mali' and should be monitored for the presence of the phytoplasma. To identify these potential insect vectors, it is

necessary to detect the pathogen in several different phloem-sucking insect species. The use of a universal endogenous control for all samples reduces the time and effort required for pathogen detection.

- Publication: Mittelberger, Cecilia, Obkircher, Lisa, Oberkofler, Vicky, Ianeselli, Alan, Kerschbamer, Christine, Gallmetzer, Andreas, Reyes-Dominguez, Yazmid, Letschka, Thomas and Janik, Katrin (2020). Development of a universal endogenous qPCR control for eukaryotic DNA samples. Plant Methods 16 (53). https://doi.org/10.1186/s13007-020-00597-2.

- How does '*Candidatus* Phytoplasma mali' manipulate its host plant *Malus* x *domestica*?
  - Hypothesis: '*Ca*. P. mali', like other phytoplasmas, releases effector

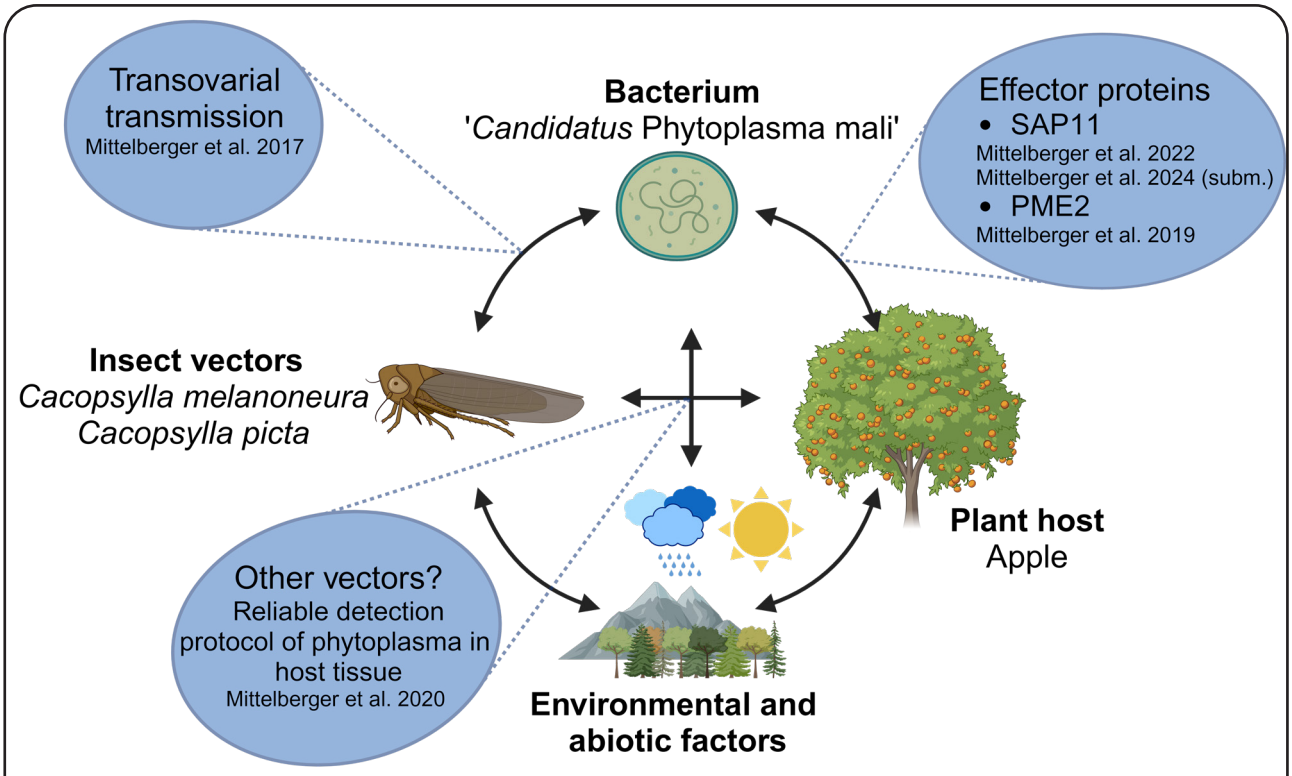

Figure 1: *Multitrophic interactions between the apple proliferation phytoplasma ('*Candidatus *Phytoplasma mali'), its insect vectors, the plant host, and the environment. Research topics of the present thesis are highlighted in blue bubbles.*

proteins through a Sec-dependent secretion system into its host plant.

One of the effector proteins so far identified in '*Ca.* P. mali', $SAP11_{CaPm}$, interacts with different transcription factors, potentially causing specific symptoms and its early in-planta effects are important for the pathogenesis.

To understand the role of $SAP11_{CaPm}$ in pathogenesis, its interactions with *M.* x *domestica* transcription factors and its in-planta effects should be studied using Y2H, BiFC analysis and transcriptome analyses, respectively. Additional potential effector proteins can be identified by *in-silico* analysis through the presence of an *N*-terminal secretion signal. The expression of potential effector proteins in the plant or insect hosts should be verified. Their function will be characterised by yeast two-hybrid (Y2H) screens or bimolecular fluorescence complementation analysis (BiFC) in plant mesophyll protoplasts.

- Publications:
  - Mittelberger, Cecilia, Stellmach, Hagen, Hause, Bettina, Kerschbamer, Christine, Schlink, Katja, Letschka, Thomas and Janik, Katrin (2019). A Novel Effector Protein of Apple Proliferation Phytoplasma Disrupts Cell Integrity of *Nicotiana* spp. Protoplasts. International Journal of Molecular Sciences 20 (18), pp. 1–16. https://doi.org/10.3390/ijms20184613.
  - Mittelberger, Cecilia, Hause, Bettina and Janik, Katrin (2022). The '*Candidatus* Phytoplasma mali' effector protein $SAP11_{CaPm}$ interacts with MdTCP16, a class II CYC/TB1 transcription factor that is highly expressed during phytoplasma infection. PloS one 17 (12), e0272467. https://doi.org/10.1371/journal.pone.0272467.
  - Mittelberger, Cecilia, Moser, Mirko, Hause, Bettina and Janik, Katrin (2024). '*Candidatus*

Phytoplasma mali' SAP11-Like protein modulates expression of genes involved in metabolic pathways, photosynthesis, and defense in *Nicotiana occidentalis* leaves. Submitted to *BMC Plant Biology*, https://doi.org/10.21203/rs.3.rs-3821494/v1.

2. Transmission of '*Candidatus* Phytoplasma mali'

The cell wall less bacterium '*Ca.* P. mali' is primarily transmitted by phloem-sucking insects. In addition to its spread by insect vectors, it can be transmitted via naturally occurring root grafts between neighbouring trees (Ciccotti et al., 2007), by artificially grafting of infected material, or experimentally using dodder (*Cuscuta subinclusa*) bridges (Frisinghelli et al., 2000). However, insect transmission plays the most important role in the field spread of the pathogen. The two psyllid species *C. melanoneura* and *C. picta* are the most important insect vectors of '*Ca.* P. mali' (Frisinghelli et al., 2000; Jarausch et al., 2004; Tedeschi et al., 2002; Mattedi et al., 2007; Mattedi et al., 2008). In north-western Italy the bacterium is also transmitted by the leafhopper species *Fieberiella florii* (Tedeschi and Alma, 2007). The latter species plays no role in transmission in South Tyrol, Germany, or other north-eastern Italian apple cultivation regions (Mattedi et al., 2008; Jarausch and Jarausch, 2010).

The insects acquire the phytoplasma during feeding and ingestion of plant sap. The phytoplasma then moves through the intestine and passes intracellularly through the epithelial cells to circulate within the haemolymph. The phytoplasmas replicate within the insect's body and penetrate specific cells of the salivary glands to be transmitted to the next apple plant on which the insects feed (Weintraub and Beanland, 2006; Jarausch and Jarausch, 2010; Jarausch et al., 2011).

C. melanoneura is more abundant in the South Tyrolean apple orchards, but the population has a low phytoplasma infection rate, while *C. picta* occurs at very low densities in the orchards but with a much higher infection

rate (Baric et al., 2010; Fischnaller et al., 2017). *C. picta* seems to be the predominant vector of '*Ca.* P. mali' not only in South Tyrol but also in German apple cultivation areas (Mayer et al., 2009; Jarausch et al., 2007; Jarausch and Jarausch, 2014).

C. picta was detected in South Tyrol for the first time in 2006, and the strong outbreak of '*Ca.* P. mali' infection in that year is correlated with the introduction of this insect species. At the same time, '*Ca.* P. mali' strain AT-2 was detected for the first time in South Tyrol (Baric et al., 2011), indicating a phytoplasma strain – host insect relation. This is underlined by the fact that most *C. picta* individuals were captured in regions with a high incidence of apple proliferation disease. In these highly affected regions, the phytoplasma strain AT-2 was mainly found. In the Eisacktal, where only *C. melanoneura* occurs as a vector, only the AT-1 strain was detected in infected trees (Baric et al., 2011). Until now, the only measure to restrict the spread of '*Ca.* P. mali' is the use of phytosanitary treatments to reduce the abundance of the transmitting insect vectors. It is thus fundamental to know the insect's life cycle and its occurrence in the orchards to choose the correct timepoint for insecticide treatments.

Both *Cacopsylla* species overwinter on coniferous host plants (Mayer and Gross, 2007; Pizzinat et al., 2011; Barthel et al., 2020a) and are univoltine. Overwintering adults of *C. melanoneura* migrate to the apple orchards already in February, while adults of *C. picta* migrate in March to April, when bud burst takes place (Mittelberger and Janik, 2017; Fischnaller et al., 2017). Both insect species reproduce and feed on apple trees. Individuals of the newly developed spring generations of *C. melanoneura* and *C. picta* leave the orchard by June and by early August, respectively (Fischnaller et al., 2017). Infection rates and densities of the psyllid populations indicate that the last strong outbreak in 2013 was due to high numbers of infected transmitting insects in the orchards. And again, it seemed that *C. picta* was much more efficient in transmitting the phytoplasma than *C. melanoneura*. Indeed, it has been shown a more efficient

phytoplasma multiplication rate in *C. picta* compared to *C. melanoneura* (Pedrazzoli et al., 2007). The phytoplasma levels in the latter insect remained much lower compared to *C. picta* after the acquisition and multiplication period.

However, since especially in the Burggrafenamt and Vinschgau areas where *C. picta* is the predominant vector, some orchards with a high infection rate were found locally (Barthel et al., 2020b), the question arose whether an infected *C. picta* female can transmit the phytoplasma to its offspring. This would explain the efficiency of *C. picta* as a vector for '*Ca.* P. mali' and for the local outbreaks of infection. In 2006 a study investigated whether *C. melanoneura* and *C. pruni*, the insect vector of '*Ca.* P. prunorum', another fruit tree phytoplasma, are capable of transovarial transmission of the pathogens. The results showed a transovarial transmission of '*Ca.* P. prunorum' in *C. pruni*, while none of the tested offspring of *C. melanoneura* was tested positive for '*Ca.* P. mali' (Tedeschi et al., 2006). It has also been shown that transovarial transmission does not occur in *Amplicephalus curtulus*, a cicada that transmits '*Ca.* P. ulmi' (Arismendi et al., 2015). However, there are several reports of transovarial transmission in other phytoplasma insect vectors: the sugarcane white leaf phytoplasma is transmitted to the progenies of *Matsumuratettix hiroglyphicus* (Hanboonsong et al., 2002), the leafhopper *Hishimonoides sellatiformis* vertically transmits the mulberry dwarf phytoplasma (Kawakita et al., 2000) and eggs and nymphs of *Scaphoideus titanus*, the vector of Flavescence dorée phytoplasma ('*Ca.* P. vitis'), were also tested positive for the presence of phytoplasmas (Alma et al., 1997).

Hence, to answer the question whether *C. picta* vertically transmits '*Ca.* P. mali', overwintering adults (remigrants) of *C. picta* were captured in April in apple orchards, morphologically characterised, and reared as single couples of a female and male each in net cages on uninfected apple shoots. After egg deposition, the adults were subjected to PCR analysis for detection of '*Ca.* P. mali'. Eggs as well as larvae from the five developmental stages of the offspring

and adult F1 individuals were analysed by PCR to detect the phytoplasma. Results not only proved that *C. picta* females are able to transmit the pathogen directly to their offspring, but they showed that transovarial transmission is very effective for the spread of the phytoplasma in its insect vector population, since 99.1% of F1 adults deriving from infected remigrant females that transovarially transmitted, were infected. Indeed, phytoplasma concentration in those F1 adults was higher than in their mothers. However, it seems that female remigrants need a sufficiently high phytoplasma level to be competent for transovarial transmission (Mittelberger et al., 2017b).

3. Detection of '*Candidatus* Phytoplasma mali'

The detection of the pathogen within the plant host and the insect vectors is crucial for successful disease management, particularly in the case of phytoplasma diseases. It is essential to utilize pathogen-free planting material (Regulation EU 2019/2072), and identify and control insect vectors since there is no direct treatment available. Additionally, long latency periods and infected but symptomless plants may contribute to the spread of the pathogen, necessitating fast and sensitive detection methods (Baric et al., 2007; Barthel et al., 2021).

After the identification of phytoplasmas as causal disease agents in 1967 (Doi et al., 1967), for a period spanning over two decades, the identification of phytoplasma has relied on diagnostic techniques such as symptom observation, transmission via insects or dodder/grafting onto host plants, and the application of DAPI staining alongside electron microscopy examination of ultrathin sections of phloem tissue. In the 1980s, serological diagnostic techniques such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence emerged (Bertaccini et al., 2019).

With the evolution of DNA-based detection techniques, particularly the polymerase-chain-reaction (PCR), most detection methods for phytoplasmas in plant hosts and insect vectors and respectively for '*Ca.*

P. mali', are now PCR-based (Abou-Jawdah et al., 2019).

The first PCR-based phytoplasma detection techniques were established by amplifying selected regions of the 16S rRNA and/or the 16S-23S intergenic spacer region (Bertaccini et al., 2019; Nejat and Vadamalai, 2013). Advances in PCR detection techniques led to the implementation of real-time quantitative PCR (qPCR)-based protocols, using intercalating dyes or hybridization probes for phytoplasma detection and quantification, allowing for multiplexing (Christensen et al., 2013; Abou-Jawdah et al., 2019; Nair and Manimekalai, 2021). Nowadays, digital PCR technology is also used for detection (Morcia et al., 2020).

In addition to PCR-based detection techniques, some protocols utilize ELISA detection (Brzin et al., 2003) or loop-mediated isothermal amplification (LAMP) (Jonghe et al., 2017; Matić et al., 2022).

Since the molecular detection of phytoplasmas requires DNA extraction from the host tissue (plant or insects), the use of appropriate primers and an accurate PCR assay with quality control steps must be considered for reliable results (Bertaccini et al., 2019).

It is crucial to verify the quality of a sample and the performance of the amplification in PCR analysis (Bustin et al., 2009). Several factors, such as the loss or degradation of template DNA during the extraction process or the presence of inhibiting substances in the sample, can lead to false negative results and should be excluded with appropriate quality control steps. One possible approach is the use of exogenous DNA of a known sequence added to the sample before DNA extraction. This exogenous sequence must then be detected by PCR to exclude any problems in sample preparation or with inhibiting factors during PCR itself. However, an exogenous control does not provide information about the integrity of the target DNA itself; to provide a quality control of the extracted DNA, endogenous PCR controls can be used (Bustin et al., 2009), thereby referring to a known sequence in the sample that is not the target DNA, such as a stretch of plant or insect host DNA (Hodgetts et

al., 2009; Kavanagh et al., 2011). In addition, the endogenous control serves not only to confirm the existence and integrity of extracted DNA and the absence of PCR inhibitory factors but can also be utilized as a reference for the quantification of the target DNA.

Given the wide variety of plants and insects infected by phytoplasmas and the need to search for potential insect vectors of '*Ca.* P. mali' among a diverse range of phloem suckers (Fischnaller et al., 2020), there was a necessity of a universal endogenous qPCR control suitable for detecting DNA from phylogenetically highly distinct eukaryotic hosts.

The endogenous control was designed based on a conserved, short stretch of the nuclear-encoded multicopy *28S* rDNA gene. In addition to the primers for amplification of this stretch, a probe was designed to enable multiplex detection of both host and phytoplasma DNA (Mittelberger et al., 2020). The designed probe incorporates locked nucleic acids (LNA) (Kaur et al., 2006; You et al., 2006)) to enhance binding strength and ensure high temperature melting properties. The developed primer and probe combination (see Table 1) underwent testing on 43 different eukaryotic species. The primers were used in a SYBR-Green assay and, in conjunction with the probe, in TaqMan® assays. To confirm the probe's suitability for multiplexing, an exemplary assay was developed for the simultaneous detection of '*Ca.* P. mali', '*Ca.* P. pyri', and the *28S* rDNA stretch.

The results indicate that the developed primer and probe combination is suitable as a universal endogenous control in both singleplex and multiplex qPCR assays using intercalating dyes, such as SYBR-Green, or hydrolytic probe-based TaqMan® assays.

Table 1: *Primers and probe of the universal endogenous qPCR control for eukaryotic DNA samples Locked nucleic acids (LNA) are indicated by a + prior to the nucleotide.*

Primer name	5'-3' sequence	Reference
UNI28S-fwd	CTACTATCTAGCGAAACC	Mittelberger et al., 2020
UNI28S-rev	AYTAGAGTCAAGCTCAAC	
UNI28S-P	HEX-AAA+G+A+AG+A+C+C+C+T-DAB	

The performance of the UNI28S control is similar to other endogenous controls and it is also applicable to cDNA samples, making it suitable as a reference for gene expression studies.

As the *28S* rDNA gene is a multicopy and not a single-copy gene, it cannot be used for absolute quantification of genome copies in a sample. However, since it is nuclear encoded, it is possible to semi-quantify and compare DNA concentrations in different samples of the same species.

Since their publication, the UNI28S primer pair and probe have been utilized in various studies as endogenous control for the detection of different phytoplasmas (Jović et al., 2021; Krstić et al., 2022).

4. Plant-Phytoplasma Interaction

Currently, the only way to restrict disease outbreaks is by monitoring the spread of phytoplasma in apple orchards and insect vectors and removing infected plants. However, understanding how '*Ca.* P. mali' interacts with its host plant is crucial for improving our knowledge regarding the infection biology and for developing new control techniques.

Phytoplasmas, like other pathogens, use effector proteins to manipulate their hosts (Sugio et al., 2011a; Rashid et al., 2018; Tomkins et al., 2018; Carreón-Anguiano et al., 2023). Unlike other bacterial plant pathogens, phytoplasmas secrete proteins in a Sec-dependent way and lack a type-III secretion system. Potential effector proteins can thus be characterized by a typical *N*-terminal secretion signal (Bai et al., 2009). Some effector proteins, however, lack signal peptides and are secreted through a Sec-independent secretion system (Gao et al., 2023). In addition, some transmembrane proteins of phytoplasmas, such as the immunodominant membrane protein (Imp) and the antigenic membrane protein (Amp), have been shown to interact with actin in insect and plant hosts without inducing negative symptoms in the hosts. Therefore, it has been hypothesized that Imp and Amp

binding to plant and insect actin may have a beneficial effect for phytoplasmas (Boonrod et al., 2012).

Four Sec-dependent secreted effector proteins, namely $SAP11_{CaPm}$, PME2, PM19_00185 and $SAP05_{CaPm}$, as well as six virulence factors belonging to AAA+ ATPases and five FtsH proteases, have been identified in '*Ca.* P. mali' so far (see Table 2). Additionally, it was shown that Imp of '*Ca.* P. mali' binds to actin in plants and could thus support the phloem movement of phytoplasmas (Boonrod et al., 2012; Konnerth et al., 2016; Boonrod et al., 2023).

The focus of a previous study was to identify Sec-dependent secretory proteins within the '*Ca.* P. mali' genome. Janik et al. (2017) identified a homologous gene to the SAP11 effector protein from Aster Yellow Witches' Broom phytoplasma (AYWB, '*Ca.* P. asteris'). The TCP binding partners of $SAP11_{CaPm}$ were identified, and subsequent investigations focused on the early transcriptional responses of the host plant upon $SAP11_{CaPm}$ expression (Mittelberger et al., 2022; Mittelberger et al., 2024, submitted; see Table 2).

Additionally, bioinformatic analysis revealed another gene within the '*Ca.* P. mali' genome with a potential effector function. *In silico* analysis of the protein CAP18323.1, encoded by the gene *atp_00136*, confirmed an *N*-terminal signal peptide that is supposed to confer Sec-dependent secretion (Mittelberger et al., 2019). This interesting candidate effector protein was named "Protein in *Malus* Expressed 2" (PME2, see Table 2).

4.1 The SAP11 Effector Protein

The SAP11 (secreted AYWB protein 11) effector protein from '*Ca.* P. asteris' (Aster Yellow Witches' Broom phytoplasma, AYWB) was one of the first effectors described in phytoplasma (Bai et al., 2009). Studies have shown that $SAP11_{AYWB}$ is binding and destabilizing TEOSINTE BRANCHED1/ CYCLOIDEA/PROLIFERATING CELL FACTOR 1 and 2 (TCP) transcription factors (Sugio et al., 2011b; Sugio et al., 2014; Pecher et al., 2019). Several homologous genes have been identified in other phytoplasma genomes (Wang et al., 2018b; Chen et al., 2022; Zhou

Table 2: *Identified effector proteins and virulence factors in 'Ca. P. mali'.*

Effector protein / Virulence factor	Targets	Function	Reference
$SAP11_{CaPm}$	Class II CIN-like TCPs: MdTCP4a, MdTCP13a, Class II CYC/ TB1 TCP: MdTCP18a	destabilizes class II CIN-like TCPs; downregulates jasmonic acid biosynthesis and enhances lateral shoot outgrowth and early bud break	Janik et al., 2017; Mittelberger et al., 2022; Mittelberger et al., 2024, submitted; Boonrod et al., 2022; Strohmayer et al., 2021; Chang et al., 2018
Protein in *Malus* Expressed 2 (PME2)	unknown	Two variants are known so far ($PME2_{ST}$ and $PME2_{AT}$); Both localize to the plant cell nucleus; $PME2_{ST}$ provokes cell disruption when overexpressed in mesophyll protoplasts, and macroscopic growth aberrations when expressed in yeast reporter strain	Mittelberger et al., 2019
PM19_00185	Md E2 UBC10	Acts as a E3 ubiquitin ligase	Strohmayer et al., 2019
$SAP05_{CaPm}$	SPL transcription factors	Hijacks the 26S proteasome and degrades SQUAMOSA promoter-binding protein-like (SPL) transcription factors through a ubiquitination-independent process; might suppress the plant basal defence	Huang et al., 2021; Liu et al., 2023
AAA+ ATPases and FtsH proteases	unknown	Involved in '*Ca.* P. mali' pathogenicity; '*Ca.* P. mali' strain virulence is genetically distinguishable according to those genes; they may affect some sieve element constituents	Seemüller et al., 2011b; Zimmermann et al., 2015; Seemüller et al., 2017
Immunodominant Membrane Protein (Imp)	G- and F-actin in plants	Does not induce any symptoms; does not interfere with actin polymerization or disrupt the equilibrium between actin monomers and filaments; might be involved in phloem mobility of phytoplasmas and insect transmission	Boonrod et al., 2012; Boonrod et al., 2023

et al., 2021; Orlovskis et al., 2017; Correa Marrero et al., 2023), among them $SAP11_{CaPm}$ in '*Ca*. P. mali' (Janik et al., 2017). Like $SAP11_{AYWB}$, $SAP11_{CaPm}$ binds to different TCPs (Janik et al., 2017; Strohmayer et al., 2021). TCP transcription factors are important for plant morphological development, stress response, and plant immunity (Dhaka et al., 2017). Therefore, they are interesting targets for pathogen effectors (Ceulemans et al., 2021; Stam et al., 2021). Depending on their basic helix-loop-helix (bHLH)-domain sequence, TCPs are divided into class I and class II TCPs. Class II TCPs can be further divided into CINCINNATA (CIN)-like TCPs and CYCLOIDEA/TEOSINTE BRANCHED1 (CYC/TB1)-like TCPs (Li, 2015; Lan and Qin, 2020).

The proliferation of axillary meristems is enhanced by the binding of SAP11-like effector proteins to TCPs. This, along with another effector protein called TENGU (Hoshi et al., 2009), is responsible for the typical witches' broom symptom characteristic for many phytoplasma diseases (Chang et al., 2018). By targeting class II CIN-like TCPs, SAP11 suppresses the synthesis of jasmonic acid and weakens plant defence against herbivores. This, in turn, makes the plant hosts more attractive to insect vectors (Sugio et al., 2011b). Research has demonstrated that $SAP11_{CaPm}$ can regulate the emission of plant volatile organic compounds (VOCs) (Tan et al., 2016), which may affect the plant's resistance to insects. Additionally, studies have shown that *C. picta* emigrants are attracted to infected *M.* x *domestica* trees (Mayer et al., 2008a; 2008b; 2011).

Transgenic lines of *Arabidopsis thaliana* that overexpress $SAP11_{AYWB}$ exhibit an altered root architecture, with ahairy appearance, which is consistent with the symptoms found in phytoplasma-infected plants. These plants also accumulate cellular phosphate and exhibit a suppressed salicylic acid (SA)-mediated defence response (Lu et al., 2014). Similarly, infected apple trees display a reduced SA accumulation compared to non-infected trees (Janik et al., 2017).

It has been shown that SAP11-like proteins also destabilize class II CYC/TB1 like TCPs of *A. thaliana* (Chang et al., 2018). However, the same study also revealed that $SAP11_{CaPm}$ has

limited ability to destabilize class II CIN like TCPs and is unable to destabilize class II CYC/TB1 AtTCP18.

The SAP11-homologue from '*Ca.* P. mali', $SAP11_{CaPm,}$ binds to the class II CIN-like TCPs MdTCP4a and MdTCP13a (Janik et al., 2017), orthologous sequences of AtTCP4 and AtTCP13 from *A. thaliana*, respectively. These sequences were formerly known as MdTCP25 and MdTCP24 (Tabarelli et al., 2022). In addition to the interaction with the two class II CIN-like TCPs, the Y2H screen with $SAP11_{CaPm}$ as the bait and a cDNA library of *M.* x *domestica* leaf transcriptome as prey revealed an isoform of class II CYC/TB1-like AtTCP18-like, similar to MdTCP18a, and a part of the putative chlorophyll(ide) *b* reductase NYC1 (MdNYC1) as potential targets of $SAP11_{CaPm}$. However, the interaction between these initially identified sequences could not be confirmed (Janik et al., 2017), even though several SAP11 homologs, among them $SAP11_{CaPm}$ from '*Ca.* P. mali' strain PM19, were found to interact with AtTCP18 or its orthologs (Wang et al., 2018b; Chen et al., 2022; Zhou et al., 2021; Chang et al., 2018; Strohmayer et al., 2021). It was a logical step to conduct a detailed analysis of MdTCP18a as a potential target of $SAP11_{CaPm}$, thus reflecting the natural pathosystem using the updated genome of *M.* x *domestica* (Daccord et al., 2017), which provided new sequence and reading-frame information. Hence, the potential interaction between $SAP11_{CaPm}$ and MdTCP18a and MdNYC1 was re-investigated (Mittelberger et al., 2022). The interaction between class II CYC/TB1-like MdTCP18a and $SAP11_{CaPm}$ was confirmed through a Y2H screen and *in planta* by BiFC in *N. benthamiana* mesophyll protoplasts. The interaction was observed in both the cell nucleus and the cytoplasm. The potential interaction with MdNYC1 could not be confirmed, and it remains unclear whether $SAP11_{CaPm}$ destabilizes MdTCP18a or not (Mittelberger et al., 2022).

Additionally, to gain a better understanding of how '*Ca.* P. mali' infection might affect the *TCP* expression, expression levels of *MdTCP18a*, *MdTCP4a*, *MdTCP13a* and $SAP11_{CaPm}$ were determined in leaf samples from *M.* x *domestica* trees. It has been

shown that *MdTCP13a* was stably expressed throughout the season, regardless of infection status, and its expression did not correlate with the expression of $SAP11_{CaPm}$ or the quantity of phytoplasma in infected samples. In contrast, *MdTCP4a* expression showed negative correlation with $SAP11_{CaPm}$ expression and phytoplasma quantity.

The expression of *MdTCP18a,* however, showed a positive correlation with $SAP11_{CaPm}$ expression and the phytoplasma quantity in infected leaf samples. *MdTCP18a* expression was significantly lower in samples collected during spring, both infected and non-infected, when compared to samples collected during autumn. The seasonal dependency of *MdTCP18a* expression may reflect its role in the regulation of bud dormancy (Singh et al., 2021). It is tempting to hypothesise that the effector $SAP11_{CaPm}$, through its binding to MdTCP18a, is responsible for early bud break and lateral shoot outgrowth, which are typical symptoms of '*Ca.* P. mali' infection.

As SAP11 variants occur in various phytoplasma species, the effector appears to be crucial for infecting or colonising host plants. Therefore, it was interesting to investigate the early effects of SAP11 expression in its host plant (Mittelberger et al., 2024, submitted). To achieve this, an infiltration RNA-seq analysis (Bond et al., 2016) was used to identify early transcriptional changes in the plant upon $SAP11_{CaPm}$ expression. *Nicotiana occidentalis* was selected for this study because it has previously been identified as a suitable model plant for '*Ca.* P. mali' infection studies (Boonrod et al., 2022; Luge et al., 2014; Seemüller et al., 2010; Schneider et al., 2014; Boonrod et al., 2012). After transient transformation using *Agrobacterium tumefaciens*, $SAP11_{CaPm}$ expression was detectable in the infiltrated leaves after 24 h and reached its peak after 96 h (Figure 2). However, as a reference genome for *N. occidentalis* is currently unavailable, it was necessary to perform a *de novo* assembly of the transcriptome.

Differential expression analysis identified the initial differentially expressed transcripts (DETs) in $SAP11_{CaPm}$ infiltrated leaves after 24 h, consistent with the expression of $SAP11_{CaPm}$. The highest number of DETs was detectable

120 h after infiltration. The analysis showed that occurrence of $SAP11_{CaPm}$ in plant cells weakens plant defence and photosynthetic processes but enhances expression of genes of the metabolic pathway, which may aid the phytoplasma in propagating within the plant sieve tubes (Figure 2). Regarding the latter, Gene Ontology (GO) enrichment analysis of up- and downregulated DETs revealed that transcripts upregulated due to $SAP11_{CaPm}$ infiltration were assigned to "ATP biosynthetic process" and "proton transmembrane transport". A network analysis confirmed the enhanced ATP biosynthetic process and oxidative phosphorylation, which may help phytoplasma in acquiring metabolites and nutrients from their host (Figure 2). Phytoplasma lack several metabolic genes and are therefore highly dependent on metabolic compounds from their hosts (Oshima et al., 2013; Kube et al., 2012; Namba, 2019).

The downregulated transcripts, 120 h after $SAP11_{CaPm}$ infiltration, were associated with "defence response" and "response to external stimulus", indicating that early $SAP11_{CaPm}$ expression compromises plant defence response, enabling a successful infection of the host plant. In line with that, a functional analysis of the up- and downregulated DETs revealed that more than 25% of the upregulated transcripts in the control-infiltrated samples (compared to the non-infiltration control) are related to defence or stress-related GO-terms, indicating that infiltration alone triggers the plant's defence response. It is a well-known phenomenon that the *Agrobacterium*-mediated infiltration process induces transcriptional changes and alters defence response and phytohormone levels in the infiltrated areas (Pruss et al., 2008; Drapal et al., 2021; Rico et al., 2010; Sheikh et al., 2014). However, in the group of upregulated DETs in $SAP11_{CaPm}$-infiltrated leaves (in comparison to control-infiltration), only few transcripts cluster to defence or phytohormone-related GO terms. On the contrary, in the downregulated transcripts of the same group 28% (72 h) and 19.5% (120 h) of the transcripts are associated with defence or phytohormone-related GO terms, indicating that $SAP11_{CaPm}$ suppresses plant's

defence response.

A few transcripts related to plant immune response showed differential expression at different timepoints following $SAP11_{CaPm}$ infiltration, which may provide insights into the function of $SAP11_{CaPm}$ (Figure 2). One of these transcripts encodes a protein modifier of snc1,1 (MOS1), which was downregulated 24 h after infiltration but upregulated 120 h after infiltration. MOS1 modulates the plant immune response (Li et al., 2010; Zhang et al., 2018), thus, it is possible that the MOS1-directed immune response is suppressed in the early stages of $SAP11_{CaPm}$ expression but becomes active later. Transcripts encoding the serine/threonine-protein phosphatase BSL1, known as a susceptibility factor in *Phytophtora infestans* infection (Wang et al., 2021; Turnbull et al., 2019; Saunders et al., 2012) and the protein CHROMATIN REMODELING 8 (CHR8), known for its role in plant stress responses (Shaked et al., 2006; Ascencio-Ibáñez et al., 2008), were downregulated at 72 h and 120 h after infiltration.

Regarding the 'photosynthetic processes' appearing to be suppressed by SAP 11_{CaPm}, the preprotein translocase subunit SCY1, which is involved in preprotein localization to the thylakoid (Liu et al., 2015; Skalitzky et al., 2011), was downregulated at 24 h and 120 h after infiltration (Figure 2). Two other genes encoding proteins from photosystem II (PSII) were downregulated upon $SAP11_{CaPm}$ expression: the oxygen-evolving enhancer protein 1 (OEE1/PsbO) and OEE2 (PsbP, Figure 2). Both proteins have interesting roles in diverse pathogen infections, as direct targets of effector proteins (Liu et al., 2021; Breen et al., 2023) or by involvement in plant immunity (Fan et al., 2014; Campa et al., 2019). Several studies have demonstrated that phytoplasma infection leads to a decrease in photosynthesis rates and a downregulation of multiple genes involved in photosynthesis (Mittelberger et al., 2017a; Bertamini et al., 2002; Tan et al., 2015; Dermastia et al., 2019). However, it remains unclear whether phytoplasmas directly target the chloroplast as a crucial site for plant defence and stress response, involving steps of phytohormone biosynthesis, or if photosynthesis is simply

compromised due to the altered plant metabolism (Janik et al., 2020; Bittner et al., 2022). However, it has been hypothesised that SAP11 may be involved in the reduction of photosynthesis rates, by regulating the plastid-encoded polymerase PEP. This polymerase is the most important chloroplast transcriptase and regulates *psbD*, which encodes the reaction centre protein D2 of PSII (Janik et al., 2020).

Taken together, the results of the recent study indicate that the early expression of $SAP11_{CaPm}$ in its host plant weakens the plant's defence response, aided by the downregulation of photosynthetic processes, and enhances metabolic pathways probably

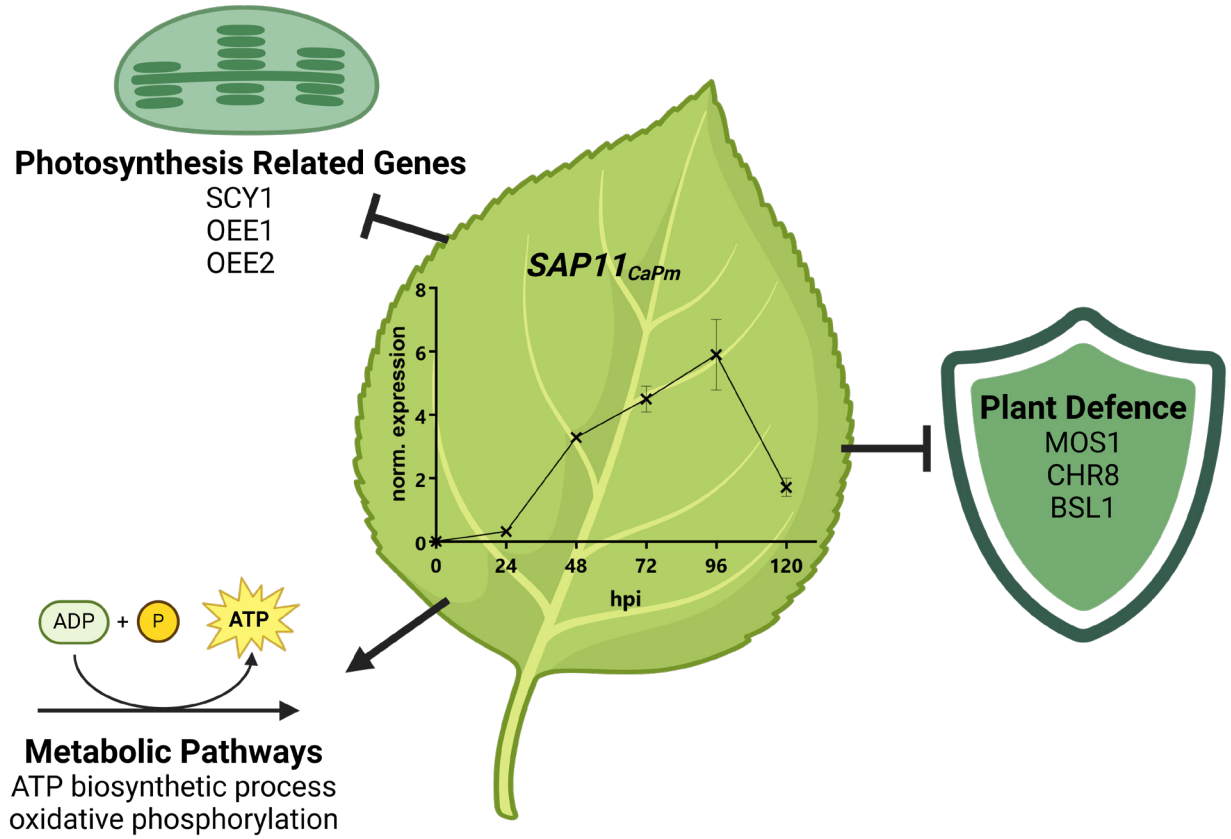

Figure 2: *Early transcriptional changes in leaves upon* $SAP11_{CaPm}$ *expression. The normalized expression of* $SAP11_{CaPm}$ *is detectable after 24 h and reaches its maximum at 96 h. Metabolic pathways such as the ATP biosynthetic process and the oxidative phosphorylation are enhanced upon* $SAP11_{CaPm}$ *expression (indicated by an arrow). Transcripts encoding for genes related to the thylakoid (SCY1) and photosystem (OEE1, OEE2) and related to plant defence (MOS1, CHR8, BSL1) are downregulated (indicated by a bar).*

to provide energy equivalents and metabolic compounds for the benefit of '*Ca.* P. mali'.

4.2 The PME2 Effector Protein

The protein PME2 from '*Ca.* P. mali' has been identified as a potential effector protein, due to its genetic features, such as the *N*-terminal secretion signal, absence of transmembrane regions and an importin α/β-dependent nuclear localization site (NLS) and a nuclear export signal (NES) at the *C*-terminal part (Mittelberger et al., 2019).

The expression of *pme2* was confirmed in infected apple leaf and root tissue, but not in infected *C. picta* individuals. This suggests that PME2 may act as an effector protein in plant hosts. Sequence analysis revealed that the prevalent variant of *pme2* in infected tree samples from South-Tyrol ($pme2_{ST}$) differs from the sequence found in '*Ca.* P. mali' AT strain ($pme2_{AT}$) from Germany. The $PME2_{ST}$ protein sequence contains a duplicated amino acid stretch compared to $PME2_{AT}$. However, both variants translocate to the cell nucleus when expressed in *N. benthamiana* mesophyll protoplasts, despite slight differences in their NLS sequences (Mittelberger et al., 2019).

It was also observed that the expression of $pme2_{ST}$ in *N. benthamiana* and *N. occidentalis* protoplasts, in contrast to the expression of $pme2_{AT}$, reduced protoplast viability by causing cell shrinkage. Furthermore, overexpression of $pme2_{ST}$ in a yeast reporter strain resulted in macroscopic aberrations and a compromised yeast colony growth, rendering the Y2H technique unsuitable for identifying $PME2_{ST}$ interaction partners (Mittelberger et al., 2019).

Even though this study revealed interesting features and functions of PME2, additional research is required to identify the molecular targets of PME2 in the host plant and to understand how PME2 affects symptom development in apple trees.

5. Conclusion

Phytoplasma infections are dependent on a complex multitrophic interaction between

insect vectors, plant hosts, environmental factors, and the bacterium itself (Tomkins et al., 2018; Fondazione Edmund Mach and Laimburg Research Centre for Agriculture and Forestry, 2020). To unravel mechanisms involved in distribution and infection of '*Candidatus* Phytoplasma mali' in apple trees it is important to shed light on the different parts of these interactions (Figure 1).

Since phytoplasma can multiply and reside in the plant and insect host as well, it is important to understand its multiplication and colonization behaviour within both hosts. Former studies proved *C. picta* to be the more efficient vector of '*Ca.* P. mali' in South Tyrol (Baric et al., 2010; Mattedi et al., 2008). However, since the last strong outbreak in 2013, capture numbers of *C. picta* and *C. melanoneura* decreased. Even though *C. melanoneura* is still the predominant psyllid species, '*Ca.* P. mali' infection rates within the population are very low compared to the infection rates of *C. picta* individuals (Fischnaller et al., 2017; Pitzus, 2023). This brought up the first research question, whether a transovarial transmission is possible within *C. picta* and helps the efficient spread of the phytoplasma within this insect species.

Although the results for the vertical transmission of '*Ca.* P. mali' showed that *C. picta* has an enormous potential to spread the pathogen within orchards and is thus much more effective than *C. melanoneura* (Mittelberger et al., 2017b), it remains elusive how the low population density in the years 2014-2017 (Mittelberger et al., 2016; Fischnaller et al., 2017) could be responsible for a distinct number of newly infected trees. Since the insect population within orchards comprises numerous different phloem sucking species it was a logical next step to analyse whether further, so far unknown vector species exist in the local orchards. A first step to identify a potential new vectoring insect is to analyse their phytoplasma acquisition capability. In other words, to test the insects if they carry phytoplasma. Since several thousand individuals of phylogenetically very diverse insect species were captured in the orchards (Fischnaller et al., 2020), it was thus necessary to establish an adequate and

reliable high-throughput protocol for testing those different insect species.

The newly developed universal endogenous qPCR control helps to improve quality assurance steps of PCR assays and hence makes PCR results more reliable. It renders possible to test samples from distinct eukaryotic species with the same PCR setup, i.e. always using the same endogenous control. Additionally, the universal primer and probe set can be easily used in multiplex assays for different PCR-based pathogen detection systems. However, since the *28S* rDNA gene is a nuclear-encoded multicopy gene, only a relative quantification within the same species is possible (Mittelberger et al., 2020).

Beside understanding the transmission of the phytoplasma and a diligent detection of the pathogen in its hosts, it is crucial to understand the pathogen's behaviour within the hosts. As other pathogens, phytoplasmas manipulate their hosts with a set of effector proteins (Carreón-Anguiano et al., 2023; Oshima et al., 2023). Most identified phytoplasmal effector proteins contain a Sec-secretion signal, although recently effector proteins independent of the Sec-secretion have been identified (Gao et al., 2023). So far in '*Ca.* P. mali' four Sec-dependent secreted proteins, namely $SAP11_{CaPm}$ (Janik et al., 2017), PME2 (Mittelberger et al., 2019), PM19_00185 (Strohmayer et al., 2019) and $SAP05_{CaPm}$ (Huang et al., 2021), as well as six AAA+ ATPases and five FtsH proteases (Seemüller et al., 2013), and the transmembrane protein Imp (Boonrod et al., 2012) have been identified as potential effectors.

Several studies have aimed to unravel the function of SAP11, an effector protein that has been identified in several phytoplasma species (Bai et al., 2009; Strohmayer et al., 2021; Sugio et al., 2011b; Sugio et al., 2014; Zhou et al., 2021; Chen et al., 2022; Pecher et al., 2019; Wang et al., 2018a). A SAP11-like effector protein was also found in '*Ca.* P. mali' and an interaction with class II CIN-like TCPs MdTCP4a and MdTCP13a has been proven (Janik et al., 2017). Although, there was a hint that $SAP11_{CaPm}$ may interact with class II CYC/TB1 TCP MdTCP18a the confirmation of the interaction failed. However, an update

of the *M.* x *domestica* genome (Daccord et al., 2017), with new sequence and reading-frame information for MdTCP18a helped to prove the interaction of this transcription factor with $SAP11_{CaPm}$ (Mittelberger et al., 2022). This binding may lead to lateral shoot outgrowth and early bud break, which are typical symptoms of '*Ca.* P. mali' infection in *M.* x *domestica*.

Transcriptomic analysis of the early expression of $SAP11_{CaPm}$ within the host plant, revealed that genes related to plant defence and photosystem are downregulated, while other genes of the metabolic pathway are enhanced (Mittelberger et al., 2024, submitted). Differential gene expression analysis revealed that various transcripts like MOS1, BSL1 or CHR8 that were differentially expressed in $SAP11_{CaPm}$ infiltrated leaf samples are known for their role in plant defence and stress response (Zhang et al., 2018; Saunders et al., 2012; Shaked et al., 2006). Another transcript, SCY1, that is involved in preprotein localization to the thylakoid (Liu et al., 2015; Skalitzky et al., 2011), was downregulated in $SAP11_{CaPm}$ expressing leaf samples. This result provides new insights into the possible effect of $SAP11_{CaPm}$ on the chloroplast and photosynthesis. In line with that, GO enrichment analysis revealed clusters of transcripts associated with defence response to be downregulated upon $SAP11_{CaPm}$ occurrence and associated to the chloroplast as the cellular component.

On the other hand, transcripts clustering to ATP biosynthetic processes and oxidative phosphorylation were upregulated in $SAP11_{CaPm}$ expressing samples, which is consistent with the fact that phytoplasmas lack different metabolic genes, e.g. genes for ATP synthase, glycolysis or oxidative phosphorylation (Namba, 2019). Thus, they are highly dependent on metabolic compounds from their host. Taken together the results of Mittelberger et al., submitted (2024) suggest that the effector protein $SAP11_{CaPm}$ is crucial for a successful colonization of the host plant.

While $SAP11_{CaPm}$ is well studied, little is known about the "Protein in *Malus* Expressed 2" (PME2). PME2 is encoded by the '*Ca.* P. mali' gene *atp_00136*. It contains an *N*-terminal

signal peptide as well as a nuclear localization site and a nuclear export signal within the *C*-terminal part, which are characteristics of Sec-dependent effector proteins. So far two genetic variants, $PME2_{ST}$ and $PME2_{AT}$ could be identified in infected *M.* x *domestica* samples from South Tyrol and in the '*Ca.* P. mali' AT strain from Germany, respectively (Mittelberger et al., 2019).

Expression of $pme2_{ST}$ has detrimental effects on the viability of *Nicotiana* protoplasts and leads to growth aberrations in yeast reporter strains. However, additional work is required to identify targets and PME2's function when expressed in the host plant. It is still unclear whether other variants exist in other '*Ca.* P. mali' strains and how PME2 variants influence '*Ca.* P. mali' strain virulence. In fact, there is still ongoing work in our research group to detangle the function of the two so far identified PME2 effector proteins.

In conclusion, this work sheds light on multiple parts of the complex interplay between '*Ca.* P. mali', its plant host *Malus* x *domestica* and its psyllid insect vectors, by deciphering the role of different effector proteins and the transovarial transmission. In addition, the newly developed universal endogenous primer and probe combination helps to improve phytoplasma detection within the environment.

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7. Eidesstattliche Erklärung

Hiermit erkläre ich, dass die Arbeit mit dem Titel „Deciphering a Complex Interplay: Unveiling Molecular Mechanisms in Multitrophic Interactions that Affect the Infection of Plant and Insect Hosts by ‚*Candidatus* Phytoplasma mali'" bisher weder bei der Naturwissenschaftlichen Fakultät I – Biowissenschaften – der Martin-Luther-Universität Halle-Wittenberg vorgelegt, noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Darüber hinaus erkläre ich, die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet zu haben. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht.

Ritten, am 18.11.2024 Cecilia Mittelberger

8. Appendix

Publication 1

Plant Pathology (2017)

The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny

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Author contributions:

Cecilia Mittelberger: Experimental design and realization of all experiments, capturing, identification and rearing of insects, collection of individuals for further analysis, DNA extraction, PCR analysis, data analysis, evaluation of results, writing of the manuscript

Lisa Obkircher: Assistance in insect collection, DNA extraction and analysis, proofreading of the manuscript

Sabine Öttl: Assistance in molecular identification of the insects, proofreading of the manuscript

Tiziana Oppedisano, Federico Pedrazzoli: Assistance in finding the collection sites for remigrants, rearing and collection of insects that are entitled as FEM individuals in the publication, proofreading of the manuscript

Bernd Panassiti: Assistance in capturing and morphological characterization of remigrants, discussion of rearing method, proofreading of the manuscript

Christine Kerschbamer: Assistance in DNA extraction and PCR analysis, proofreading of the manuscript

Gianfranco Anfora: Discussion of experimental design, proofreading of the manuscript

Katrin Janik: Supervision of the study, discussion of experimental design, methodology and results, assistance in writing the manuscript

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Plant Pathology

The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny

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The phloem-sucking psyllid *Cacopsylla picta* plays an important role in transmitting the bacterium '*Candidatus* Phytoplasma mali', the agent associated with apple proliferation disease. The psyllid can ingest '*Ca.* Phytoplasma mali' from infected apple trees and spread the bacterium by subsequently feeding on uninfected trees. Until now, this has been the most important method of '*Ca.* Phytoplasma mali' transmission. The aim of this study was to investigate whether infected *C. picta* are able to transmit '*Ca.* Phytoplasma mali' directly to their progeny. This method of transmission would allow the bacteria to bypass a time-consuming reproductive cycle in the host plant. Furthermore, this would cause a high number of infected F_1 individuals in the vector population. To address this question, eggs, nymphs and adults derived from infected overwintering adults of *C. picta* were reared on non-infected apple saplings and subsequently tested for the presence of '*Ca.* Phytoplasma mali'. In this study it was shown for the first time that infected *C. picta* individuals transmit '*Ca.* Phytoplasma mali' to their eggs, nymphs and F_1 adults, thus providing the basis for a more detailed understanding of '*Ca.* Phytoplasma mali' transmission by *C. picta*.

Keywords: apple proliferation, epidemiology, insect vectors, phytoplasma & relatives, psyllids, transovarial transmission

Introduction

'*Candidatus* Phytoplasma mali' is the agent associated with apple proliferation (AP), a major threat in several apple-growing regions (Bertaccini *et al.*, 2014). The most important symptom is the production of small, tasteless and colourless fruits, which leads to large financial losses in affected apple-growing areas (Bertamini *et al.*, 2002; Tedeschi *et al.*, 2003; Bertaccini *et al.*, 2014).

Phytoplasmas are transmitted by insect vectors belonging to the taxonomic groups Cicadellidae, Fulgoromorpha and Psyllidae (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2014), and can additionally be transmitted by natural root grafts (Baric *et al.*, 2008). In northern Italy the two phloem-feeding psyllids *Cacopsylla picta* (Hemiptera: Psyllidae) and *Cacopsylla melanoneura* are vectors of '*Ca.* Phytoplasma mali' (Frisinghelli *et al.*, 2000; Tedeschi *et al.*, 2002; Carraro *et al.*, 2008), while in Germany only *C. picta* was found to be able to transmit the pathogen (Mayer *et al.*, 2009; Jarausch *et al.*, 2011).

Studies conducted in Trentino and South Tyrol (northern Italy) show a higher transmission efficiency for *C. picta* compared to *C. melanoneura* (Mattedi *et al.*, 2008). From the end of March to April, remigrant individuals of *C. picta* migrate from their overwintering shelter plants into apple orchards for reproduction and feeding (Mattedi *et al.*, 2008). *Cacopsylla picta* accomplishes one generation per year and the development from eggs to F_1 adults involves five larval instars. *Cacopsylla picta* offspring (emigrants) leave the apple orchards in July and migrate to the overwintering shelter plants (Mattedi *et al.*, 2008).

The life cycle of the bacterium '*Ca.* Phytoplasma mali' is strongly connected to its insect vector and the host plant (Mayer *et al.*, 2008a,b; Bertaccini *et al.*, 2014). The insect vectors acquire the AP phytoplasma by ingesting plant sap from the phloem of infected apple trees (Pedrazzoli *et al.*, 2007; Weintraub, 2007; Mattedi *et al.*, 2008). After ingestion, the phytoplasma move through the food canal of the stylet and invade different cellular tissues of the insects. The bacteria multiply in secretory salivary glands and are translocated into a new plant via the saliva when the infected insect feeds (Hogenhout *et al.*, 2008b).

Currently treatments preventing the spread of transmitting insects and the uprooting of infected trees are the only ways to limit disease spread in affected regions (Baric *et al.*, 2010).

Aside from the acquisition of infected phloem sap by ingestion, another putative way for phytoplasma spread would be a transovarial or 'vertical' pathogen transmission. This type of transmission is well known in plant virus insect vectors (Hogenhout *et al.*, 2008a).

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Transovarial transmission of plant viruses occurs only in the group of viruses that persist and replicate inside their insect vectors (Hogenhout *et al.*, 2008a; Huo *et al.*, 2014). However, until now, transovarial transmission was reported only in 4% of phytoplasma-transmitting insect vectors (Arismendi *et al.*, 2015). Vertical transmission was found in the leafhopper species *Scaphoideus titanus*, the vector of aster yellows phytoplasma (Alma *et al.*, 1997), in *Hishimonoides sellatiformis*, the vector of mulberry dwarf phytoplasma (Kawakita *et al.*, 2000), and in *Matsumuratettix hiroglyphicus*, the vector of sugarcane white leaf phytoplasma (Hanboonsong *et al.*, 2002). Tedeschi *et al.* (2006) showed that females of *Cacopsylla pruni* infected by '*Candidatus* Phytoplasma prunorum' are able to vertically transmit the pathogen and demonstrated infectivity of transovarially infected F_1 individuals. However, those authors could not show transovarial transmission of '*Ca.* Phytoplasma mali' in *C. melanoneura*. The ability of *C. picta* to vertically transmit '*Ca.* Phytoplasma mali' was hypothesized (Tedeschi *et al.*, 2006) but has never been experimentally addressed.

Thus, the aim of this study was to test whether '*Ca.* Phytoplasma mali'-infected *C. picta* are able to vertically transmit the pathogen to their progeny. Specifically, the study aimed to determine whether the number of F_1 individuals deriving from infected or uninfected parental females differs and whether the phytoplasma titre changes between developmental stages of the insect. Additionally, a rearing-feeding-oviposition method was established to study the influence of a potential phytoplasma acquisition on the puncture site of parental insects.

Materials and methods

Insects

Overwintering adults of *C. picta* were collected in April 2016 using the beating tray method (Müther & Vogt, 2003) in two abandoned apple orchards in Valsugana Valley (Trentino, Italy). The collected living individuals were isolated in glass collection tubes, anaesthetized on ice and morphologically characterized using identification keys of Ossiannilsson (1992). The collected winter generation (remigrants) of *C. picta* will henceforth be referred to as 'parental generation', and the reared summer generation (emigrants) as 'F_1 generation'.

Insect rearing

Insects from the natural populations were reared at Laimburg Research Centre (Laimburg) and at Fondazione Edmund Mach (FEM), Italy. A total number of 37 couples (27 at Laimburg and 10 at FEM) of parental females and males of *C. picta* were released into single net-cages under controlled conditions (15–25 °C, natural light and 70–100% relative humidity), on recently (same year) grown apple bud grafts (cv. Golden Delicious on M9 rootstock). Each couple was released on an individual plant. Additionally, 14 parental females (12 at Laimburg, 2 at FEM) were single-caged on grafts. Cages were monitored every day for vitality of parental individuals and the presence of eggs. After egg laying, parental insects were collected, frozen at −80 °C, and subsequently analysed by PCR for detection of '*Ca.* Phytoplasma mali' DNA (described below). Based on the PCR results of *C. picta* parental individuals, plants with eggs were selected and divided into three groups (Fig. 1; Table 1): plants with progeny from an infected parental female; plants with progeny from an uninfected female and an infected parental male (control group 1); and plants with progeny from both uninfected female and male parental individuals (control group 2).

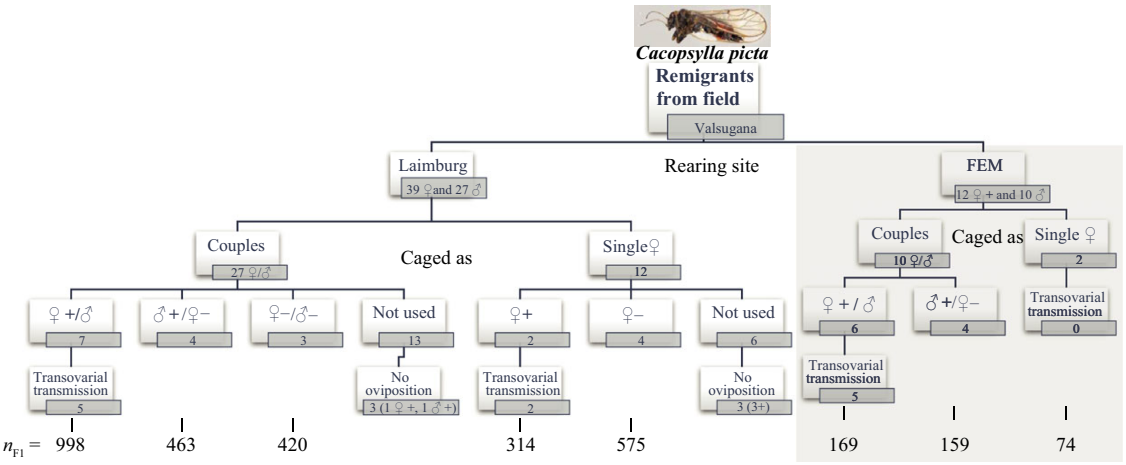

Figure 1 Schematic overview of experimental design. Remigrant *Cacopsylla picta* individuals collected in orchards in Valsugana Valley (Trentino Province, Italy) were caged as couples or single individuals (numbers are given in grey boxes). Rearing experiments were performed at two different sites: at Laimburg Research Centre (Laimburg) and at Fondazione Edmund Mach (FEM). Remigrants were sorted according to their state of infection with '*Candidatus* Phytoplasma mali' as follows: infected females (♀ +), infected males (♂ +), uninfected females (♀ −) and uninfected males (♂ −). Numbers of analysed F_1 individuals (n_{F1}) of the respective parental groups are mentioned underneath the chart. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1 Total number of analysed *Cacopsylla picta* parental and F_1 individuals

Infection state of remigrants	No. of parental females[a]	Developmental stage (F_1)[b]							Total (F_1)
		Egg	L1	L2	L3	L4	L5	Adult	
Infected parental female	17	389	428	131	109	57	268	173	1555
Control group 1, uninfected parental female, infected parental male	8	73	191	33	86	85	91	63	622
Control group 2, uninfected parental individuals	7	244	320	7	93	69	135	127	995
Total	32	706	939	171	288	211	494	363	3172

[a]Remigrant parental individuals were collected in apple orchards in Valsugana Valley and released onto single net-caged apple graftings for oviposition at Laimburg Research Centre or at Fondazione Edmund Mach, Italy.
[b]F_1 individuals were collected from the apple graftings. L1 to L5, nymphal instars.

One or two leaves with eggs were taken from the branches of each group. Using titanium needles and a stereo-zoom microscope (Zeiss Axiozoom V16), all eggs were removed from leaves and pooled in batches of five. Developmental stages of *C. picta* were documented with ZEN PRO microscope software. In the following weeks, assorted samples of each instar-stage (first instars in batches of five, from second instars to F_1 adults as single insects) were collected and frozen at −80 °C. The remaining insects were left on the branches to complete development until the adult stage, when they were collected and frozen at −80 °C.

In total, the study group consisted of 17 infected parental females captured in orchards located in Valsugana Valley (Trentino) and reared in single-net cages (nine at Laimburg, eight at FEM) and a total of 1555 F_1 individuals were analysed. Control group 1 consisted of eight uninfected females (four at Laimburg, four at FEM) caged with an infected parental male. The resulting 622 F_1 individuals were analysed, as well, to study the importance of a potential passive impact of an infected individual on the transmission rate, by locally providing bacterial inoculum to the uninfected host plant. Control group 2 comprised seven uninfected parental females and males, all reared at Laimburg, with 995 F_1 individuals serving as a negative control (Fig. 1; Table 1).

At the end of the experiment, phloem tissue was isolated from root samples of plants where eggs had developed and was stored at −80 °C for subsequent DNA extraction to assess the presence or absence of '*Ca.* Phytoplasma mali'.

DNA extraction and PCR analysis

Isolation of DNA from insects was performed using the DNeasy Blood and Tissue extraction kit (QIAGEN). DNA from remigrants reared at FEM was extracted with the Nucleo Spin tissue kit (Macherey-Nagel). DNA of each insect, egg or first instar batch (five eggs or first instar nymphs were pooled prior to DNA purification) was eluted with 100 µL TE elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0).

Genomic DNA from plants was extracted according to the manufacturer's instructions with a DNeasy Plant Mini kit (QIAGEN) including the following optimization step: plant material (100 mg) was disrupted by addition of 400 µL buffer AP1, for 3 min in a TissueLyser II (QIAGEN). To the disrupted plant material, 4 µL RNase A were added and the mix was incubated for at least 30 min in a water bath at 65 °C. DNA was eluted two times with 50 µL TE buffer as mentioned above.

'*Candidatus* Phytoplasma mali' DNA was detected by SYBR Green real-time PCR with primers rpAP15f-mod and rpAP15r3, as described in Monti *et al.* (2013), targeting the ribosomal protein gene *rpl22*. A 2 µL sample of template DNA was mixed with 5 µL of 2× SYBR FAST qPCR Kit Master Mix (Kapa Biosystems), 2.5 µL nuclease-free water and 0.25 µL each of forward and reverse primer (10 µM). The cycling conditions were as follows: initial denaturation at 95 °C for 20 s; 35 cycles of 95 °C for 3 s and 60 °C for 30 s; and a melting curve ramp from 65 to 95 °C at increments of 0.5 °C every 5 s (CFX384 Touch Real-Time PCR Detection System; Bio-Rad). All insects were tested individually and each sample was tested in triplicate in three independent PCR runs.

Phytoplasma PCR detection limits were carefully determined using a four-point 10-fold dilution series (diluted in TE buffer; 6.5×10^4–6.5×10^1 DNA copies per sample) of the plasmid pJET1.2-*rpl22* containing the subcloned '*Ca.* Phytoplasma mali' *rpl22* PCR amplicon. The dilution series was included in every real-time PCR run. Samples with a mean quantification cycle (C_q) value lower than 30 and a melting curve peak similar to the positive control were considered '*Ca.* Phytoplasma mali'-positive. The phytoplasma titre was quantified based on the four-point plasmid standard curve analysed in parallel with the samples in each PCR run.

As a control of DNA integrity and to normalize amounts of phytoplasma, a region of the single-copy *wingless* (*wg*) gene (Brower & DeSalle, 1998) of *C. picta* was amplified, in parallel to the amplification of the *rpl22* fragment, with primers specific for *C. picta* and other psyllid species, qPSY-WG-F (5′-TC ACGGGCGGCAATG-3′) and qPSY-WG-R (5′-CCCACAGCA CATCAGATCACA-3′). The PCR was performed as described above with 0.25 µM each of the *wg*-specific forward and reverse primer; included in each PCR run was a dilution series (7.3×10^5–7.3×10^2 DNA copies per sample) of the plasmid pJET1.2-*wg* containing the subcloned *wg* gene PCR amplicon. The dilution series and standard curves were prepared as previously described for the pJET1.2-*rpl22* plasmid. Phytoplasma concentration was then calculated in relation to the *wg* gene.

Phytoplasma concentration was quantified within the range of the four-point standard dilution series and samples above the range were diluted in elution buffer and reanalysed if necessary. Samples with a mean C_q value above 30 were considered '*Ca.* Phytoplasma mali'-negative and detection limits could be verified to be comparable as described in Monti *et al.* (2013). Because of batch analysis, quantification of '*Ca.* Phytoplasma mali' in egg or first instar batches was not possible.

Threshold calculation and data analysis was performed using CFX MANAGER software (Bio-Rad), considering only runs with a PCR efficiency between 95% and 105% and a coefficient of determination (R^2) ≥0.99. Three non-template controls (NTC, nuclease-free water) were performed together with each PCR run. Plant material was analysed with the same *rpl22*-based SYBR approach as described above. DNA integrity from plant samples was verified by real-time PCR with a probe for a chloroplast gene as described in Baric & Dalla-Via (2004).

Performance of qPCR for quantification of the *wg* gene was in compliance with the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al.*, 2009; Table S1).

Confirmation of psyllid species identification by PCR-RFLP

Morphological identification of live psyllid specimens is cumbersome and less precise than identification of dead insects. Certain important morphological characteristics, such as wings or terminalia, cannot be properly inspected in detail without harming the insect. To verify the accuracy of morphological psyllid species identification of living parental insects after finalizing the experiments, DNA from all parental individuals was analysed by restriction fragment length polymorphism (RFLP) according to Oettl & Schlink (2015). Only insects morphologically and genetically identified as *C. picta* were considered in this study.

Statistical analysis

For comparison of phytoplasma titre increase in instars, a one-way ANOVA with a Tukey post hoc test was applied, while in all other comparisons, Student's *t*-test was used. All data were statistically analysed using GRAPHPAD PRISM v. 7.0.

Results

Infected *C. picta* females produce infected progeny

Phytoplasma were detected in 30.2% (average per parental female) of the tested egg batches. The percentage detected increased with each developmental stage and, finally, 99.1% of the F_1 adults (average rate per parental female) were found to contain phytoplasma (Fig. 2). Phytoplasma concentration in eggs and first instar batches was very low but unambiguous as characterized by a $C_q < 30$ and a specific amplicon melting peak.

Phytoplasma concentration was determined as the ratio of *rpl22*:*wg* gene copies. The phytoplasma titre increased exponentially from second instar nymphs to the F_1 adult stage. A significant increase was detected between the titre of fourth and fifth instar nymphs ($P = 0.0249$) and between fifth instar nymphs and F_1 adults ($P < 0.0001$). F_1 adults contained 100-fold more phytoplasma than second instars (Fig. 3) and comparable concentrations to those of parental females (Fig. 4).

Five out of 17 infected parental females of the study group did not transmit the phytoplasma to their progeny at all. Parental females that did not transmit phytoplasma to their progeny had a significantly lower phytoplasma titre

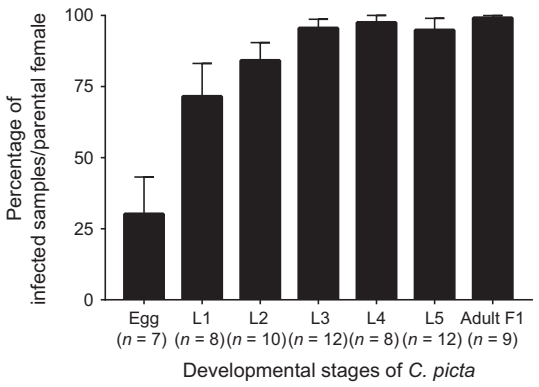

Figure 2 The percentage of *Cacopsylla picta* infected with '*Candidatus* Phytoplasma mali' at different stages of insect development. Columns show the average percentage of egg batches, L1 nymph batches and L2 nymph – F_1 adults produced by infected parental females (n = total number of infected parental females), in which phytoplasma was detectable. Bars show the standard error of the mean.

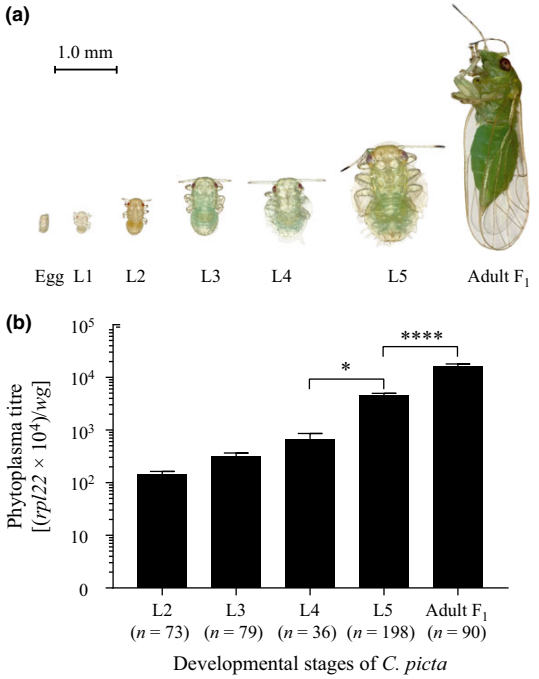

Figure 3 Developmental stages of *Cacopsylla picta* (a) and exponential increase in titre of '*Candidatus* Phytoplasma mali' during insect development (b). (a) Egg, nymphal instars (L1–L5) and F_1 adult of *C. picta*. (b) Phytoplasma titre at different stages of infected *C. picta* is given as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Vertical bars show the standard error of the mean. Statistical differences are indicated by $*P \leq 0.05$, $****P \leq 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com].

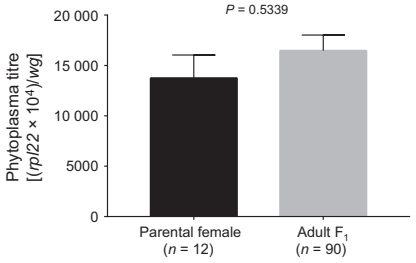

Figure 4 The titre of '*Candidatus* Phytoplasma mali' in *Cacopsylla picta* parental females and F_1 adults, shown as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Error bars indicate the standard error of the mean for each experimental group. There is no significant difference between the two groups ($P = 0.5339 > 0.05$, $t = 0.6242$, d.f. = 100).

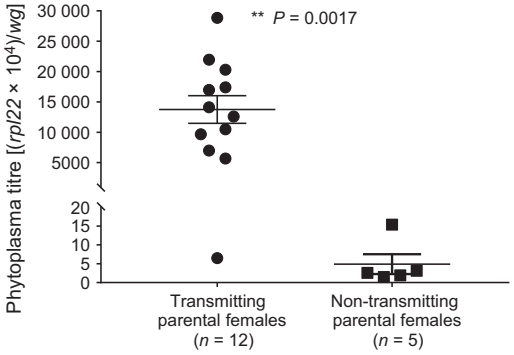

Figure 5 The correlation between transovarial transmission and the bacterial load of parental females. Infected *Cacopsylla picta* parental females were clustered into two groups based on their ability to transovarially transmit '*Candidatus* Phytoplasma mali'. Bacterial load was measured by the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific). Each dot represents the bacterial load of a *C. picta* individual that was able to transmit '*Ca.* Phytoplasma mali' to its progeny. Each square shows the bacterial load of a *C. picta* female that did not transmit '*Ca.* Phytoplasma mali' to its progeny. Horizontal bars show the mean, while vertical bars show the standard error of the mean of each group. Statistical differences are indicated by $^{**}P \leq 0.01$ ($t = 3.805$, d.f. = 15).

($P = 0.0017$, $t = 3.805$, d.f. = 15) than parental females that transmitted '*Ca.* Phytoplasma mali' (Fig. 5). Transmitting parental females contained, on average, 13 759 $(rpl22 \times 10^4)/wg$ gene copies, while non-transmitting insects contained only 5 $(rpl22 \times 10^4)/wg$ gene copies. There was no difference detectable between '*Ca.* Phytoplasma mali' titres of parental females and males (Fig. 6).

Potential phytoplasma acquisition on the bite spot of parental individuals

'*Candidatus* Phytoplasma mali' was detected, at a very low concentration (4.7 $(rpl22 \times 10^4)/wg$ copies), in only one individual (fourth instar nymph) derived from

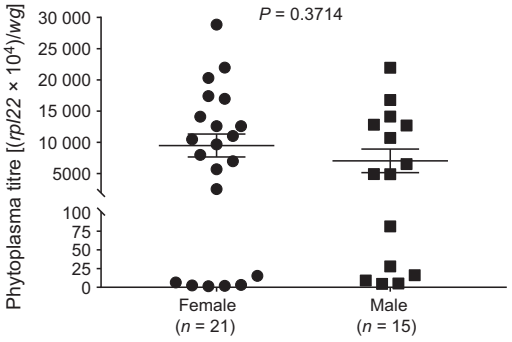

Figure 6 The titre of '*Candidatus* Phytoplasma mali' in infected *Cacopsylla picta* remigrant males and females does not differ. The phytoplasma titre, shown as the ratio of *rpl22* gene copies ('*Ca.* Phytoplasma mali'-specific) to *wingless* (*wg*) gene copies (*C. picta*-specific), of individual remigrant *C. picta* females and males is represented by circles and squares, respectively. Horizontal bars show the mean, while vertical bars show the standard error of the mean for each experimental group. No significant difference between the two groups could be found ($P = 0.371 > 0.05$, $t = 0.9058$, d.f. = 34).

control group 1 (uninfected female and infected male). All other 621 tested individuals tested negative for the phytoplasma.

'*Candidatus* Phytoplasma mali' was not detected in any of the 995 tested F_1 individuals deriving from uninfected *C. picta* females caged with uninfected male remigrants (control group 2).

'*Candidatus* Phytoplasma mali' infection does not affect oviposition rate or the number of eggs of *C. picta* produced

A total of 13 out of the 39 parental females (reared at Laimburg) tested positive, which corresponds to an infection rate of 33.3%. In total, 32 out of 39 (82.1%) parental females laid eggs in the net cages. Oviposition rate of uninfected (84.2%) and infected females (87.5%) caged as couples was nearly the same.

Parental females reared under controlled conditions (Fig. 7), on average, deposited similar numbers of eggs, independent of their infection status ($P = 0.3731$, $t = 0.9201$, d.f. = 14).

Infection status of plant material

All grafts used for the net cages tested negative for '*Ca.* Phytoplasma mali'.

Discussion

The aim of this study was to test whether '*Ca.* Phytoplasma mali'-infected *C. picta* are able to transmit the bacterium to their progeny. To address the principal question of this study, naturally infected *C. picta* individuals were collected and tested for vertical transmission of

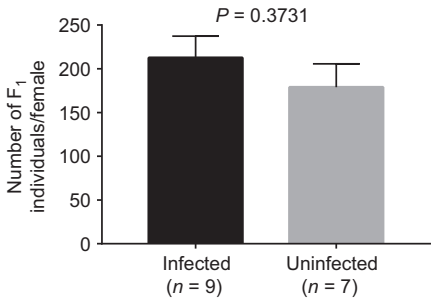

Figure 7 *Cacopsylla picta* females, infected or uninfected with '*Candidatus* Phytoplasma mali', produce similar numbers of offspring. The number of F_1 individuals generated by a single *C. picta* parental female (*n*) was analysed with respect to its infectious state. The black bar represents the number of F_1 individuals derived from infected individuals and the grey bar from uninfected individuals. Each bar shows the mean with the respective standard error of the mean. No significant differences between the two groups could be found ($P = 0.3731 > 0.05$, $t = 0.9201$, d.f. = 14).

'*Ca.* Phytoplasma mali'. As '*Ca.* Phytoplasma mali' was detected in 30.2% (average per parental female) of the egg batches and 99.1% of the F_1 adults, a vertical transmission of the bacteria was demonstrated. Interestingly, both the percentage of infected individuals and the phytoplasma titre gradually increased during the developmental stages, as was hypothesized by Tedeschi *et al.* (2006). Thus, it is reasonable to suggest that all eggs and early instars were actually infected, but contained very low phytoplasma concentrations, below the detection limit of the applied detection system.

The results of this study show that transmission ability is dependent on the phytoplasma load of the respective parental female, indicating that there is a critical threshold of phytoplasma concentration for vertical transmission to occur. However, it remains unclear how phytoplasma distribution in parental females can influence the transmission ability. It is possible that '*Ca.* Phytoplasma mali' must be present in the ovaries of *C. picta* to be vertically transmitted. However, further research is required to determine whether the bacteria actively migrate to the reproductive organs or are distributed all over the insect's body, and are therefore also present in the insect ovaries. Nevertheless, the infectious state of the parental female did not influence the average number of eggs produced.

The possibility that nymphs acquire phytoplasma from the bite spot on the leaf where the parental individual was initially sucking has been discussed by Arismendi *et al.* (2015). This possibility cannot be excluded by the present study as one infected nymph was found that derived from an uninfected parental female caged with an infected male. However, it can be assumed that this method of phytoplasma acquisition is quantitatively negligible in comparison to the demonstrated efficiency of transovarial transmission.

In South Tyrol, the first dramatic outbreak of AP coincided with the appearance of *C. picta*, which was previously absent in the region (Waldner, 2006; Baric *et al.*, 2011). Different studies conducted in Trentino and South Tyrol show that *C. picta* is more efficient than *C. melanoneura* in transmitting the disease and that *C. picta* populations, on average, contain a much higher percentage of infected individuals than *C. melanoneura* (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2007, 2011; Mattedi *et al.*, 2008; Baric *et al.*, 2010; Mittelberger *et al.*, 2016). Interestingly, a correlation between certain '*Ca.* Phytoplasma mali' strains and *C. picta* or *C. melanoneura* has been observed (Baric *et al.*, 2011), but no biological explanation for this correlation has been described so far. Strain differences might be responsible for a differential spread in insects and subsequent transovarial transmission, e.g. caused by bacterial adhesion and distribution properties, as hypothesized by Arismendi *et al.* (2015). Thus, the observed ability of *C. picta* to transmit the pathogen to its progeny could explain why *C. picta* is a more efficient vector of '*Ca.* Phytoplasma mali' than *C. melanoneura*.

In the present study, the phytoplasma titre in newly emerged F_1 adults was similar to that in infected parental individuals. Thus, it can be assumed that transovarially infected F_1 adults are as infective as remigrants (Mattedi *et al.*, 2008; Jarausch *et al.*, 2011). However, transmission trials with transovarially infected *C. picta* would be necessary to determine their transmission efficiency.

This study has shown that infected remigrants of *C. picta* are able to transmit the phytoplasma directly to their progeny. These findings are very important for orchard management, because they emphasize the necessity of reducing remigrant and emigrant individuals to avoid oviposition of infected eggs and the fast, exponential reproduction and spread of highly infectious insect vectors.

To the authors' knowledge, the results of this study are the first that clearly show vertical transmission of '*Ca.* Phytoplasma mali' in its insect vector *C. picta* and thus pave the way for further elucidating the molecular processes of transovarial transmission of '*Ca.* Phytoplasma mali' in *C. picta*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Checklist of MIQE guidelines with details on qPCR performance of *wingless* gene quantification assay.

Publication 2

Plant Methods (2020)

Development of a universal endogenous qPCR control for eukaryotic DNA samples

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Cecilia Mittelberger: Design and performance of the qPCR validation experiments, comparison of the different internal controls and different qPCR chemistries, PCR analysis of different eucaryotic tissues for proof of principle, evaluation and analysis of all results, writing the manuscript

Lisa Obkircher: Assistance with the PCR analysis, DNA extraction from different eukaryotic tissues, revision of the manuscript

Vicky Oberkofler, Alan Ianeselli: Design of the probe, proofreading of the manuscript

Christine Kerschbamer: Assistance with DNA extraction and PCR analysis, proofreading of the manuscript

Andreas Gallmetzer, Yazmid Reyes-Dominguez: Evaluation of the probe performance on an independent qPCR system, discussion of the results, revision of the manuscript

Katrin Janik: Supervision of the whole work, discussion of the methodology and results, assistance in writing the manuscript

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Plant Methods

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Development of a universal endogenous qPCR control for eukaryotic DNA samples

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Abstract

Background: Phytoplasma are obligate intracellular plant-pathogenic bacteria that infect a broad range of plant species and are transmitted by different insect species. Quantitative real-time PCR (qPCR) is one of the most commonly used techniques for pathogen detection, especially for pathogens that cannot be cultivated outside their host like phytoplasma. PCR analysis requires the purification of total DNA from the sample and subsequent amplification of pathogen DNA with specific primers. The purified DNA contains mainly host DNA and only a marginal proportion is of phytoplasmal origin. Therefore, detection of phytoplasma DNA in a host DNA background must be sensitive, specific and reliable and is highly dependent on the quality and concentration of the purified DNA. DNA quality and concentration and the presence of PCR-inhibitors therefore have a direct impact on pathogen detection. Thus, it is indispensable for PCR-based diagnostic tests to validate the DNA preparation and DNA integrity before interpreting diagnostic results, especially in case that no pathogen DNA is detected. The use of an internal control allows to evaluate DNA integrity and the detection of PCR-inhibiting substances. Internal controls are generally host-specific or limited to a defined group of related species. A control suitable for the broad range of phytoplasma hosts comprising different insect and plant species is still missing.

Results: We developed a primer and probe combination that allows amplification of a conserved stretch of the eukaryotic 28S rDNA gene. The developed endogenous qPCR control serves as a DNA quality control and allows the analysis of different eukaryotic host species, including plants, insects, fish, fungi, mammals and human with a single primer/probe set in single- or multiplex assays.

Conclusions: Quality and performance control is indispensable for pathogen detection by qPCR. Several plant pathogens are transmitted by insects and have a broad range of host species. The newly developed endogenous control can be used with all so far tested eukaryotic species and since multiplexing is possible, the described primer and probe set can be easily combined with other PCR-based pathogen detection systems.

Keywords: Quantitative real-time PCR, Pathogen detection, Endogenous control, Reference gene, Phytoplasma

Background

Polymerase chain reaction (PCR), first described by [1] and implemented as technique by [2], has become a broadly used technology in molecular biology in the last decades. The amplification of specific DNA in a given sample is a central technique important for many scientific and diagnostic methods and approaches [3]. The basic principle of PCR is the detection of DNA sequences specific for a certain organism, virus or an artificial DNA fragment. The DNA is amplified by using two oligonucleotides, so called primers, that specifically anneal to the flanking regions of the DNA of interest. In the presence of free nucleotides, a DNA polymerase then amplifies the

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stretch between the primers and the thereby exponentially amplified DNA (the amplicon) is stained with DNA intercalating dyes and can be detected or quantified by various methods [4].

The integrity of the target DNA, i.e. the template is crucial for a successful PCR reaction [5]. However, in case the template DNA was (i) lost or (ii) degraded during the sample preparation procedure or (iii) amplification inhibiting substances are present in the sample, the PCR might fail although the DNA of interest was present in the sample. Such failures lead to false-negative results and must be avoided to provide highly reliable PCR results. To analyze the unwanted loss of DNA during a purification procedure exogenous DNA of known sequence can be added to a sample prior to DNA extraction [6]. This specific exogenous DNA must then be detected by PCR, as an internal amplification control in sufficient amounts after extraction to ensure that DNA was successfully purified from the sample. The use of exogenous controls also allows to analyze the presence of potential PCR inhibitors in the sample. Nonetheless, this method is based on the addition of an external DNA fragment and does not deliver information about the integrity of the target DNA that is of real interest [7].

Beside exogenous PCR controls, endogenous controls can be used. An endogenous PCR control provides information about the successful purification of DNA from the actual sample and the absence of inhibiting substances in the extracted DNA [8]. Endogenous thus means that the control DNA is present in the sample but is not the target i.e. host DNA during pathogen detection or reference genes (or RNA/cDNA, respectively) in expression studies [9]. Successful amplification of the endogenous control allows to deduce that the DNA (i) was successfully purified from the sample, (ii) was not degraded during the procedure and that (iii) the PCR sample does not contain certain substances in a quantity that hampers successful DNA amplification. Furthermore, the amplification of a target of known quantity (e.g. the number of copies per genome or a housekeeping gene that is invariantly expressed) can be utilized as a reference for quantification of the target DNA [10]. So far, no universal endogenous control has been described that can be used for phylogenetically highly diverse samples.

Phytoplasma infect several hundred plant species and cause severe damages in a wide range of crop plant species [11, 12]. They are transmitted by different insect vectors [11] and thus phytoplasma surveys often involve the detection of these bacteria in different plant and insect species. Nowadays, most tools for phytoplasma detection are based on quantitative PCR (qPCR) techniques, using intercalating DNA dyes (SYBR-Green) or hybridization probes (TaqMan®) [13]. Several qPCR protocols are available for the detection of phytoplasmas using different host specific endogenous internal controls [13–19]. Some of the available host specific internal controls can be used simultaneously with the phytoplasma specific primers in a multiplex qPCR assay [14, 15, 18, 19], for others it is necessary to perform a separate qPCR run [20]. However, a universal endogenous control for the simultaneous detection of phytoplasma specific targets and phylogenetically different host species DNA is still missing.

The aim of this work was the development of an endogenous control that can be used as an indicator of DNA purification and integrity for several different eukaryotic host species. This control was sought to be combined with the PCR-based detection of phytoplasma to establish a reliable detection method in different (potential) hosts using TaqMan® chemistry.

In this study a method is described that comprises the amplification of a conserved, short stretch of the 28S rDNA gene as a universal endogenous control. Beside the primers a hydrolysis probe was designed containing locked nucleic acids (LNA) to design a short probe with high temperature melting properties and improved binding strength even in single mismatch nucleotides [21, 22]. The combination of probe and primers allows fluorometric single- or multiplex amplicon detection using TaqMan® qPCR assays.

Results

The objective of this study was the development of a qPCR assay that incorporates the amplification of an endogenous control gene to the pathogen detection in different host DNA samples. Thus, a highly conserved region that might be suitable for the development of universal primers and probe among phylogenetic distinct species was identified within the 28Sr DNA stretch. The newly developed primer and probe combination was first optimized for qPCR application using SYBR-Green and TaqMan® chemistry and then tested in different assays to validate its performance.

Validation of qPCR performance

The assay validation was performed following the MIQE [7] guidelines (see Additional file 1: Table S1). The primers UNI28S-fwd and UNI28S-rev (Fig. 1) were used in a SYBR Green qPCR assay to determine the optimal reaction and cycling conditions.

Using the primers at annealing temperatures from 50 to 60 °C uniform amplicons could be generated (see Additional file 1: Table S2). Specific melting curve peaks were observed in all samples (see Additional file 1: Fig. S1) and the broad annealing temperature range indicates that the amplification is robust [23]. To determine the optimal

Fig. 1 Consensus sequence of conserved 28S rDNA stretch. Consensus of sequence alignment of the conserved region of 28S V. The sequence is depicted from 5′ (left) to 3′ (right). Primer and probe binding sites are indicated by blue (primers) and red (probe) bars

primer concentrations for the assay (i.e. concentrations that allow reliable amplification in the absence of primer dimer formation) a primer concentration matrix was prepared. This analysis revealed that a primer concentration of 250 nM forward and 250 nM of the reverse primer in the final reaction allowed amplification of a specific amplicon as characterized by a low C_q value and specific melting curves and did not lead to primer dimer formation (see Additional file 1: Table S3 and Fig. S2). Higher primer concentrations increased the fluorescent signal during amplification (characterized by a lower C_q) but led to primer dimer formation. The addition of $MgCl_2$ to the PCR mix did not improve, but impaired amplification as characterized by an increasing C_q value (see Additional file 1: Fig. S3). Serial dilutions of pJET1.2-28S revealed that the linear dynamic range (LDR) in which a PCR efficiency between 95 and 105% and an R^2 of 0.99 could be achieved, was between 6.38×10^3 and 6.38×10^6 copies per reaction in the SYBR-Green based qPCR assay (Fig. 2).

For optimization of probe-based qPCR, primer performance was analyzed in combination with the described hydrolysis probe. Since primer dimers are not interfering with the amplicon detection, the primer concentration was increased to 400 nM of each primer in order to enhance the amplification reaction (see Additional file 1: Fig. S2).

The LDR as well as the limit of detection (LOD) were determined by using a tenfold standard dilution series of pJET1.2-28S between 5×10^6 and 5×10^{-3} copies per reaction. Dilutions containing the theoretical amount of less than one copy per reaction served to reach the C_q plateau in which no amplification occurs or in which the plasmid quantity cannot be linearly correlated to the obtained C_q. The LDR could be reliably detected and ranged between 54.24 ($C_q = 29.91$) and 287,818 ($C_q = 18.04$) plasmid copies per reaction (Fig. 3a).

To determine if the UNI28S primers and probe can be used for multiplexing, an exemplary multiplex TaqMan® qPCR assay was performed to detect two economically relevant phytoplasma species: apple proliferation (AP) phytoplasma ('*Candidatus* Phytoplasma mali') and pear decline (PD) phytoplasma ('*Candidatus* Phytoplasma pyri') together with the eukaryotic 28S rDNA in different concentrations. The LDR, with a range of 64.51 ($C_q = 29.67$) to 447,235 ($C_q = 17.43$) plasmid copies per reaction, for the detection of the 28S rDNA in a multiplex assay, was found to be comparable to the singleplex assay (Fig. 3a, b). The performance of the 28S amplification was 100% ± 5% (max) and an $R^2 > 0.99$ in the singleplex and the multiplex assay (Fig. 3c, d).

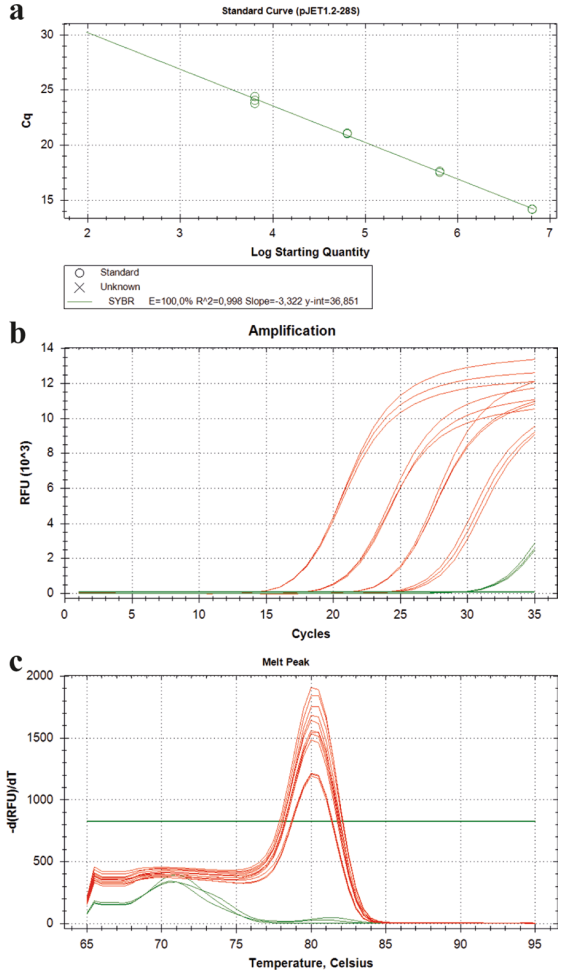

Fig. 2 Amplification with UNI28S primers in a SYBR-Green assay. Amplification of 28S using the UNI28 primers in a SYBR-Green assay. A serial dilution of pJET1.2-28S was prepared covering tenfold dilutions from 6.38×10^6 to 6.38×10^3 copies per reaction. Each dilution step was run as a triplicate. Red lines depict the samples containing the plasmid template in different dilutions and the green line represents the no template control (NTC; nuclease free water instead of template)

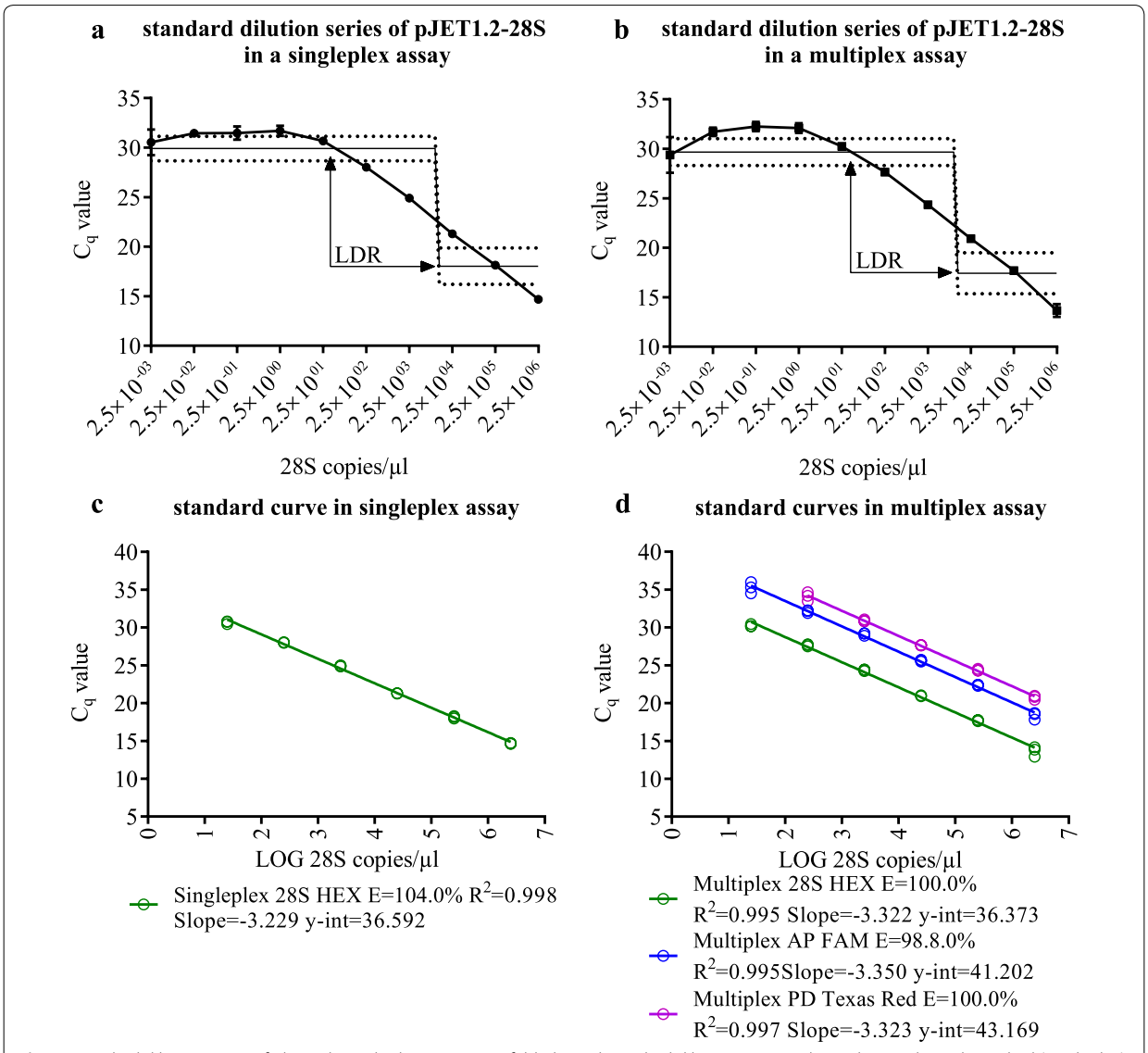

Fig. 3 Standard dilution series of plasmid standards. 10 point tenfold plasmid standard dilution series with **a** only 28S plasmid standard (singleplex) or **b** a combination of three different plasmid standards (multiplex) run in triplicates using a TaqMan® assay and standard curves of the 28S plasmid and plasmid standards with phytoplasmal target including performance parameters of **c** singleplex and **d** multiplex assay The linear dynamic range is depicted as interpolation line with 95% confidence interval (dotted line) and calculated by a 4-point logarithmic nonlinear regression analysis. Error bars represent the SEM of the three technical triplicates

The LOD was found to be five 28S copies per reaction (2.5 copies/µl), since this was the lowest number of 28S copies that could reliably detected in repeated assays using the pJET1.2-28S calibration curve.

For determination of the repeatability each sample was run in triplicates and the standard deviation of the measurement was calculated. Intraassay variation was determined for five independent runs using four different template concentrations (Fig. 4). The average C_q-value variation of the technical triplicates was maximum 5.1% (based on the average C_q-value) within the same run (Fig. 4). Based on the comparison of five independent runs, using the same four-point standard curve measured in triplicates, that had a similar intercept (38.74±0.35), the average C_q-value of each individual standard dilution did not vary more than 3.7% (based on the average C_q-value of all five independent runs) between the assays (Fig. 4). PCR

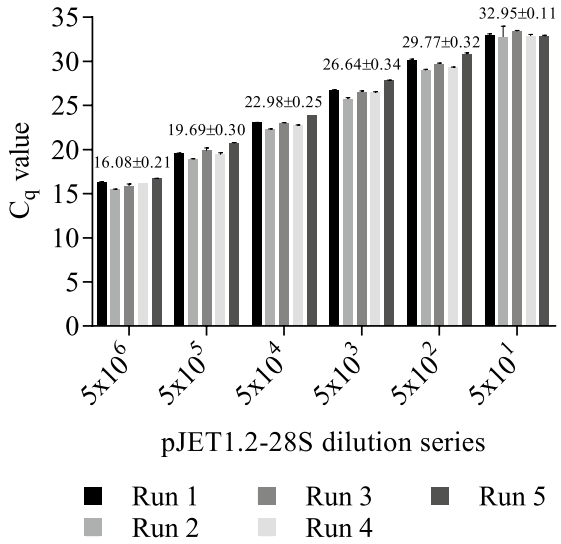

Fig. 4 Intraassay and interassay variation. Intraassay and interassay variation of five independent TaqMan® qPCR assays. A six-point serial dilution series of pJET1.2-28S was used as the template for amplification. The bars represent the mean C_q-value of three technical replicates ± SEM (error bars, intraassay variation) and the values over the grouped columns show the mean C_q value ± SEM of the five independent runs (interassay variation). Differences were analyzed using Two-way ANOVA with Tukey's multiple comparison posttest

amplification efficiency ranged between 95.8 and 101.7%, with an $R^2 \geq 0.99$.

The primers were designed to amplify a 28S rDNA fragment of eukaryotic organisms. Specificity was therefore determined with DNA from prokaryotic *E. coli* as a negative control. Primer and probe combination in assays with *E. coli* DNA as the template did not generate an amplicon under the described PCR conditions (Table 1). The primer and probe combination amplified the 28S rDNA fragment in all 43 tested eukaryotic species (Table 1) and in cDNA samples from *Malus* × *domestica*, *Vitis vinifera* and *Cacopsylla picta*.

The quantification of the 28S amplicon in 23 different species, using the same mass of template DNA showed huge differences between the tested species (Table 1). While in 1 ng of *Boletus edulis* DNA more than 900,000 copies of the 28S fragment were detected, in the same quantity of a human blood DNA sample only 410 copies were amplified.

Comparison with different other internal controls

To validate whether the performance of the qPCR using the UNI28S primers and probe is comparable to the performance of internal controls that are currently used in phytoplasma detection assays, exemplarily the amplification performance of two different apple specific gene fragments was compared to the performance of the qPCR with 28S rDNA as the target. An apple specific chloroplast DNA gene fragment of the tRNA leucine (*trnL*) gene [24] and a fragment of the single-copy gene 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) [25] were used for this analysis. While a reliable detection of *ACO* in highly diluted apple DNA templates (1:10,000) was difficult and only reliable in up to 1:1000 diluted samples, 28S and *cpLeu* reliably amplified even 1:10,000 diluted samples (see Additional file 1: Table S4) and showed thus a lower LOD than *ACO* (Fig. 5).

TaqMan® assay vs SYBR-Green assay

Four-point tenfold dilution series from different samples (*A. alni, L. stri, E. vitis, Ginkgo biloba, Malus* × *domestica, Vitis vinifera*, *Malus* × *domestica* cDNA, *Vitis vinifera* cDNA), tested in triplicates, were used as templates for the detection of the 28S fragment in a TaqMan® and SYBR-Green assay (Fig. 6). The quantification results from the two assays are comparable. Nevertheless, when dealing with undiluted samples with a high DNA amount, it was not always possible to get reliable results when using SYBR-Green (see Fig. 6, *A. alni*, *Malus* × *domestica* cDNA, *Vitis vinifera* cDNA) and quantification results from five diluted samples differed significantly between the two assays.

Limitations of the SYBR-Green assay

Even though primer concentrations were optimized for SYBR-Green based qPCR assays, reactions with a high template DNA amount showed problems during amplification (Fig. 7). It was necessary to dilute those samples up to 100-fold to gain reliable results when using SYBR-Green for 28S amplification. Those problems appeared especially with big insect species, e.g. *Aphrophora alni*, where DNA was extracted from the whole insect body and thus the extracted DNA amount was relatively high (182.5 ± 1.4 ng μl^{-1}) compared to smaller insect species (e.g. *Empoasca vitis*, 4.4 ± 0.6 ng μl^{-1}) or DNA from 100 mg of plant tissue (e.g. *Vitis vinifera*, 23.9 ± 0.1 ng μl^{-1}).

In contrast to the SYBR-Green assay, TaqMan® assays worked reliably even in samples with a high DNA background.

Discussion

This study describes a new primer and probe combination that allows amplification of a conserved stretch of the eukaryotic 28S rDNA gene. The amplification of this gene fragment can be used for sensitive detection of eukaryotic DNA, coming from phylogenetic highly distinct species, for different purposes. The use of internal

Table 1 Amplification results of eukaryotic species

Taxon	Species	Tested tissue	Mean C_q	Stdv C_q	28 S copies reaction^{-1}
Arthropod	*Anaceratagallia ribauti*	Whole insect	16.40	0.17	2,605,892
Arthropod	*Aphrophora alni*	Whole insect	8.65	0.72	561,670,136
Arthropod	*Asymmetrasca decedens*	Whole insect	16.85	0.27	1,916,323
Arthropod	*Cacopsylla melanoneura*	whole insect	16.52	0.02	14,145,566
Arthropod	*Cacopsylla picta*	Whole insect	16.24	0.07	17,121,902
Arthropod	*Cicadula quadrinotata*	Whole insect	14.51	0.08	9,706,053
Arthropod	*Cixius nervosus*	Whole insect	10.22	0.72	189,127,348
Arthropod	*Dicranotropis hamata*	Whole insect	13.84	0.19	15,409,055
Arthropod	*Edwardsiana rosae*	Whole insect	15.18	0.04	6,085,580
Arthropod	*Emelyanoviana mollicula*	Whole insect	16.18	0.14	3,042,300
Arthropod	*Empoasca vitis*	Whole insect	19.31	0.16	348,147
Arthropod	*Eriosoma lanigerum*	Whole insect	15.91	0.18	119,623,912
Arthropod	*Ixodida*[a]	Whole insect	21.54	0.65	133,850
Arthropod	*Laodelphax striatella*	Whole insect	12.81	0.23	31,543,702
Arthropod	*Macrosteles quadripunctulatus*	Whole insect	20.38	0.12	165,800
Arthropod	*Macrosteles cristatus*	Whole insect	16.53	0.16	2,381,296
Arthropod	*Macrosteles laevis*	Whole insect	16.02	0.11	3,407,075
Arthropod	*Macrosteles ossiannilssoni*	Whole insect	15.15	0.06	6,213,481
Arthropod	*Macrosteles sexnotatus*	Whole insect	17.33	0.07	1,373,854
Arthropod	*Psammotettix alienus*	Whole insect	16.20	0.09	3,007,348
Arthropod	*Psammotettix confinis*	Whole insect	15.06	0.07	6,598,274
Arthropod	*Stictocephala bisonia*	Whole insect	9.42	0.90	330,858,020
Arthropod	*Zygina flammigera*	Whole insect	16.78	0.17	2,011,619
Arthropod	*Zyginidia pullula*	Whole insect	15.88	0.38	3,745,691
Fish	*Salmo salar*[a]	Meat	20.69	0.11	240,091
Fungi	*Boletus edulis*[a]	Stem part	15.42	0.28	9,270,902
Fungi	*Saccharomyces cerevisiae*[a]	Colony	17.97	0.25	1,584,262
Mammal	*Bos primigenius taurus*[a]	Meat	25.08	0.31	11,466
Mammal	*Capreolus capreolus*[a]	Meat	22.57	0.57	65,416
Mammal	*Equus ferus caballus*[a]	Hair	22.95	0.39	50,274
Mammal	*Homo sapiens*[a]	Blood	26.57	0.93	4,103
Mammal	*Mus musculus*[a]	Blood	24.09	0.23	22,820
Mammal	*Ovis gmelini aries*[a]	Meat	22.19	0.36	84,923
Mammal	*Sus scrofa*[a]	Meat	23.66	1.05	30,811
Plant	*Ginkgo biloba*[a]	Leaf	19.89	0.23	418,889
Plant	*Lycopersicon esculentum*[a]	Leaf	15.40	0.05	8,605,181
Plant	*Malus × domestica*[a]	Root	20.73	1.69	233,529
Plant	*Nicotiana occidentalis*[a]	Leaf	16.95	0.15	2,989,473
Plant	*Olea europaea*[a]	Leaf	20.28	1.18	319,704
Plant	*Pinus cembra*[a]	Leaf	17.68	0.31	1,932,346
Plant	*Prunus armeniaca*[a]	Leaf	16.25	0.25	5,216,449
Plant	*Pyrus communis*[a]	Leaf	20.72	0.53	235,152
Plant	*Vitis vinifera*[a]	Leaf	18.04	0.21	1,509,260

Amplification results of the 28S rDNA amplicon in 43 different eukaryotic species with threshold cycle values (C_q) and quantification results based on the tenfold standard dilution series of pJet1.2-28S

[a] Template DNA was diluted to 5 ng/µl

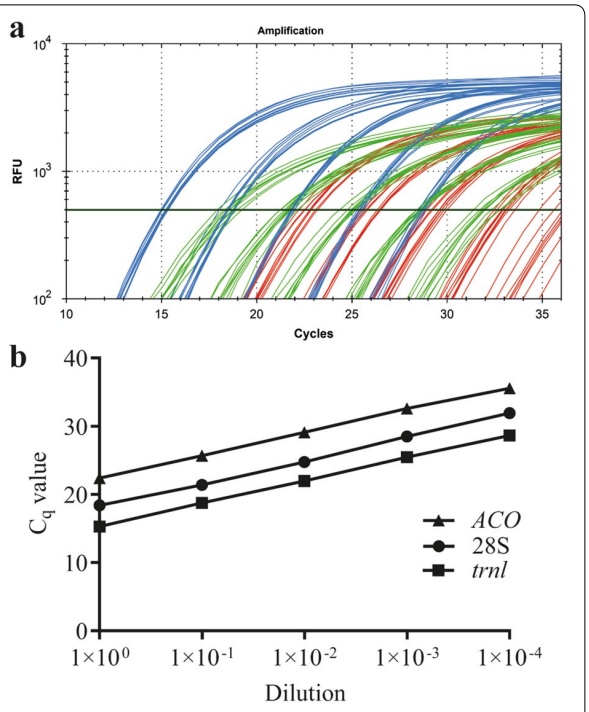

Fig. 5 Comparison between different endogenous controls. Amplification curves of 28S (green), *cpLeu* (blue) and *ACO* (red) reference genes of a tenfold dilution series (undiluted—1:10,000) from three different apple root samples, measured as triplicates (**a**) and the five point tenfold dilution series of sample 1 (**b**) measured in triplicates with the three reference genes

controls in qPCR applications is particularly important to verify DNA integrity and the absence of PCR inhibitors from the sample. PCR-based detection of pathogen specific DNA in a background of eukaryotic DNA is a common technique for diagnostic purposes. Pathogen detection can fail in a given sample due to the absence of the pathogen in the sample or due to impairments during DNA preparation or PCR performance. These impairments can be of different origin, such as due to low quality DNA extraction, DNA degradation, presence of inhibitors in the DNA sample or a high DNA background to only mention a few. Factors leading to PCR impairment can hamper pathogen detection or lead to false-negative results. It is therefore indispensable to ensure that DNA has been successfully extracted from the respective tissue and that PCR inhibitors are absent in the DNA. Such a quality control can be achieved by amplifying a known DNA target that has been added to the sample prior to DNA extraction (exogenous control; exCtrl) or by amplifying a DNA target present in the extracted sample DNA (endogenous control; enCtrl). Successful amplification of the enCtrl indicates that intact DNA has been successfully extracted and that PCR inhibition does not occur. Furthermore, it allows relative, non-spectrometric DNA quantification. EnCtrls are thus preferable over exCtrls because they allow verification of DNA integrity and prove the absence of PCR inhibitors [9]. Moreover, enCtrls can be used for the relative quantification of pathogens in a given sample [13].

When working with phytoplasma, it is important to have reference genes for a broad range of plant or insect host species available. Although several protocols are available for the detection of different phytoplasma species in single- or multiplex assays [14, 16–18, 24], all of them use internal endogenous controls with a rather narrow host range. Only the 18S rDNA gene has been reported as a universal endogenous qPCR control, working with a broad range of plant species [26]. However, a universal enCtrl that amplifies a conserved DNA target present in diverse eukaryotic species including plants and insects has not been reported so far.

For reverse transcription (RT)-qPCR applications several reference genes are available as enCtrl. However, available sequences for primer and probes of different reference genes are not suitable for a universal detection among phylogenetic distinct species. In most cases sequences and assays are species specific, restricted to certain taxa or optimized for particular gene expression studies [27, 28]. Among the (RT)-qPCR enCtrl the 28S rDNA gene was found to be stably expressed under different experimental conditions [29], however the use as internal control in gene expression studies is controversially discussed [30]. The 28S rDNA gene is nuclear-encoded but not a single copy gene. Therefore, absolute quantification of genome copies in a sample is not possible if the number of 28S rDNA gene copies in the respective genome is unknown. But, since the 28S rDNA gene is nuclear-encoded it is possible to relatively quantify host DNA between different samples deriving from the same species. This would not be possible amplifying a target gene that is organelle-encoded (e.g. in the mitochondrial or chloroplast genome) since the number of organelles can be tissue-dependent. The comparison between the amplification curves of *trnL*, *ACO* and 28S illustrates the difference regarding the difference between a nuclear-encoded single-copy (*ACO*), a nuclear-encoded multi-copy (28S) and an organelle-encoded (*trnL*) gene. *TrnL* is encoded in the chloroplast, an organelle that occurs in high (but varying) numbers in each cell. One copy of *ACO* represents a single cell and the *28S* is nuclear encoded, but multi-copy, which means that the 28S copy-number does not vary between the cells of a given species but the absolute number of this gene per cell is unknown or needs to be determined. The amplification of the 28S target in a sample therefore generates lower C_qs than the amplification of *ACO*

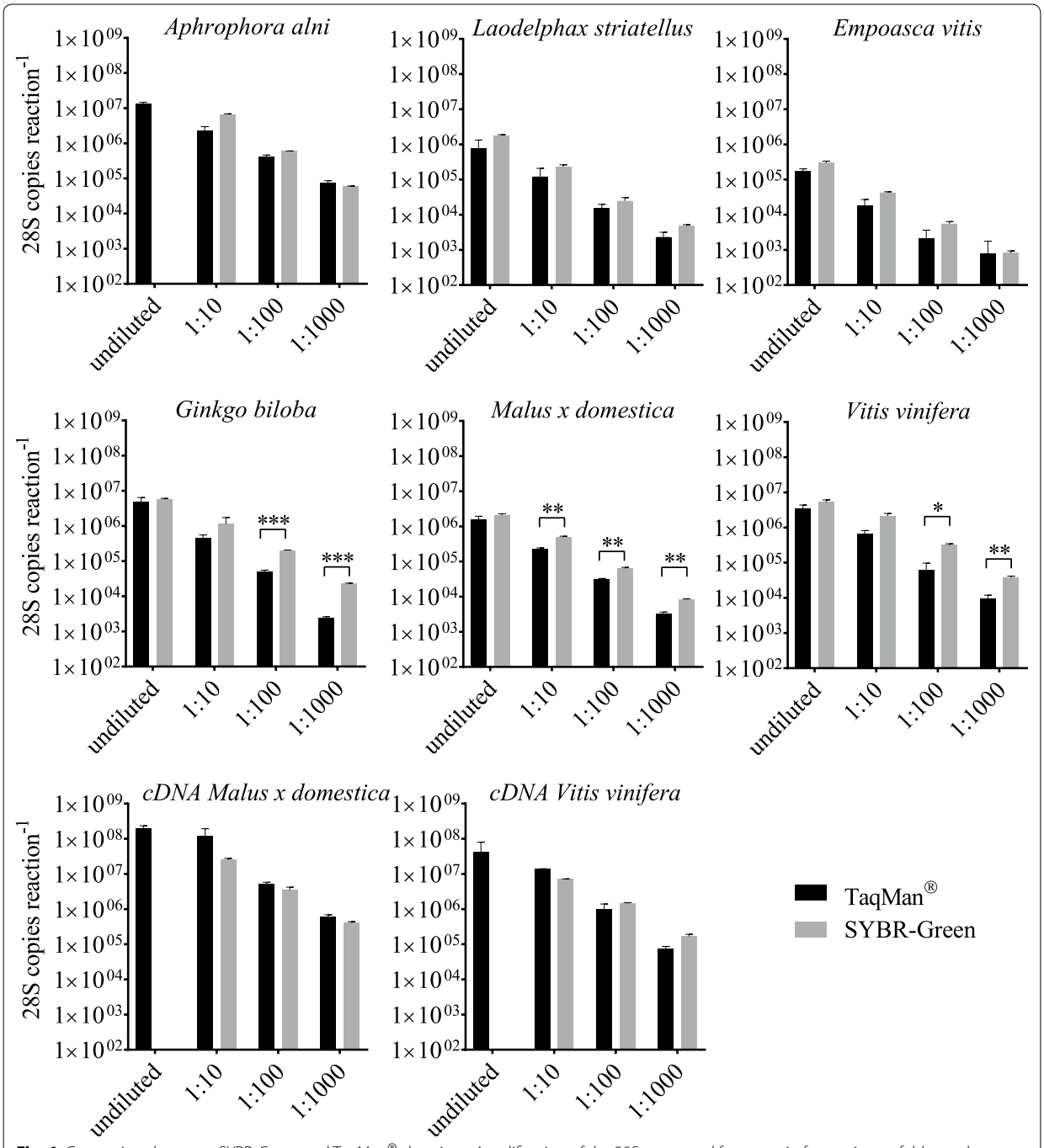

Fig. 6 Comparison between SYBR-Green and TaqMan® chemistry. Amplification of the 28S conserved fragment in four-point tenfold sample dilutions of *Aphrophora alni*, *Laodelphax striatellus*, *Empoasca vitis*, *Ginkgo biloba*, *Malus* × *domestica*, *Vitis vinifera* and cDNA samples of *Malus* × *domestica* and *Vitis vinifera* run as triplicates. Two different amplification chemistries were used, i.e. SYBR-Green and TaqMan®. Error bars represent SEM and significant differences were determined by multiple t-test with Holm-Sidak correction indicated by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

but higher C_qs compared to *trnL* as seen in Fig. 5. Due to its universality, the newly developed 28S-based enCtrl can be used in high throughput qPCR applications where several different species shall be analyzed regarding the presence of phytoplasma e.g. when searching for potential insect vectors. The host DNA quantity, as a proxy

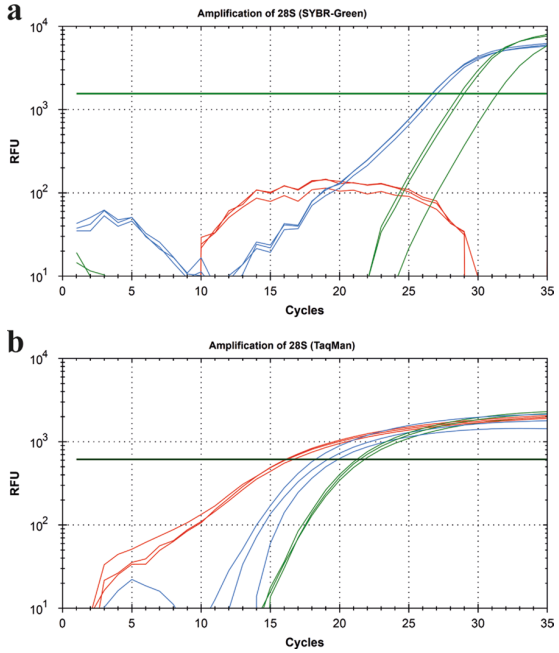

Fig. 7 Limitations of SYBR-Green assay. Amplification curves of 28S fragment in an undiluted (red), 1:10 (blue) and 1:100 (green) diluted *Aphrophora alni* sample using a SYBR-Green (**a**) and TaqMan® (**b**) assay. Each dilution step was run as a triplicate. The undiluted sample did not show reliable amplification curves when using SYBR-Green

for DNA purification efficacy can be compared between samples from the same species but not between DNA samples derived from different species. In case that it is necessary to determine the 28S rDNA gene copy number present in the genome of a certain eukaryotic species, Southern Blot analysis can be performed. However, the determination of 28S rDNA gene copy number per genome or cell was not part of this study. Quantification of DNA using the described method is limited to eukaryotic DNA, that means that DNA of prokaryotic origin present in the sample is not detected. For the detection of phytoplasma this is of minor relevance since the quantity of total prokaryotic DNA in the sample is very low and thus neglectable. However, quantification of the 28S rDNA gene in a mixture of eukaryotic (host) and prokaryotic (phytoplasma) DNA is therefore rather an estimation than an exact quantification of total DNA present in the sample. Accurate quantification is achieved only for the eukaryotic DNA proportion in the sample. Beside the application of the newly developed universal enCtrl during phytoplasma detection, it can be used in combination with other qPCR assays whenever an enCtrl for a eukaryotic host species is necessary. Since it is also applicable for amplification of cDNA targets it might be also applicable for certain gene expression studies, however the suitability of the 28S rDNA gene as an accurate housekeeping gene should be carefully assessed [30–33].

A careful evaluation of qPCR results is crucial, because many factors can influence PCR performance and thus the accuracy of the results [34, 35]. It is recommended to analyze standards, i.e. serial dilutions of a sample containing the target DNA, in parallel in every assay [7, 36, 37]. As shown in Fig. 6 absolute quantification is depending on the chemistry applied in the qPCR. Even though the primers are the same in both assays and the PCR performance for both assay types was in the range of $E = 100\% \pm 5\%$ and $R^2 \geq 0.99$ the SYBR results showed significantly higher absolute quantities than the TaqMan® assay at some dilutions. This might be explained by the fact that SYBR intercalates with all double stranded DNA present in the sample and does not stain the specific amplicon only. This might cause non-specific fluorescence especially in templates that contain a high amount of background DNA or in templates in which primer dimer formation occurs. Latter can lead to "false-high" results when using diluted DNA samples. These technical restrictions can lead to a narrow LDR when using SYBR. The LOD is not necessarily affected since the melting curve allows discrimination between the specific amplicon and primer dimers. However, a differential quantification between the fluorescence derived from the specific and the non-specific signal is impossible. In samples with high DNA background, even if the sample is free from inhibitors, SYBR can create false-negative results due to its unspecific DNA-binding characteristics. Detection in samples with high DNA concentrations is thus very likely to fail in any qPCR analysis that is based on intercalating DNA dyes. In TaqMan® assays only the specific amplicon creates fluorescence. Background DNA and primer dimer thus do not lead to a non-specific fluorescence in this assay.

We used primer and probes described for the detection of two different phytoplasma species [42] in combination with the UNI28S primer and probe to evaluate the performance of the latter in a three-fluorophore multiplex assay. Performance validation of the UNI28S primers and probe combination was done according to the MIQE guidelines [7] with a plasmid standard dilution curve. The use of standard dilution curves renders it possible to measure and evaluate qPCR performance parameters, such as PCR efficiency. The semilogarithmic standard dilution curve should display a linear range with a low variation in the single points, cover the whole range of quantified DNA amount and display a high correlation coefficient and a slope near − 3.33, which is the slope of the LDR from the semilogarithmic transformation of the copies per reaction (y) and the measured C_q (x) in which an increment of one C_q corresponds to the doubling of

DNA present in the sample (Fig. 3). A slope of this value is thus a numeric indicator that doubling of DNA in each cycle occurred and thus stands for 100% amplification or PCR efficiency (E) [38].

Outside the LDR, detection might be still possible, but the absolute quantity cannot be determined with the linear regression correlation between C_q and template concentration in the LDR. Minimum and maximum, i.e. the start and end point of the LDR, can be determined by 4-point logarithmic nonlinear regression analysis as depicted in Fig. 3a, b. The LOD, which is the lowest concentration that can be detected with reasonable certainty [7] is an indicator of PCR sensitivity. In our analytical assays an LOD of five template copies per reaction could be reliably detected. This number is almost equal to the most sensitive theoretically possible LOD of three copies per reaction [7]. However, it needs to be mentioned that the LOD is a value that needs to be determined in every novel PCR setting, especially when using different master mixes, cyclers, etc.

The LOD as numeric value for PCR sensitivity is an important and characteristic parameter. However, it was only of secondary relevance in our practical settings, since the quantity of 28S was not limiting. For the detection of low quantities of a target, i.e. for pathogen diagnostics the pathogen specific LOD is crucial and should be determined in a negative control DNA background of host DNA to mimic the actual assay situation. While the LOD is important for diagnostic approaches to detect plant pathogens, it is less important for reference gene detection. Nevertheless, it must be considered that if the host DNA amount is already very low, the probability to detect pathogen DNA in that respective sample is even lower. Comparison of the total DNA amount between samples is thus important to guarantee a certain diagnostic standard. In our experimental setting an accurate quantification of 28S rDNA copies and the determination of the LDR (but not the LOD) was therefore rather important to allow the comparison of DNA quantities from samples analyzed in independent assays. While the detection limit did not play a major role, high concentrations of DNA hampered SYBR-Green based detection of the target DNA. This is most probably because of the limited availability of DNA intercalating SYBR-Green dye in the reaction. The presence of high amounts of double stranded template DNA before the amplification can lead to a saturating incorporation of SYBR-Green dye. This would lead to a depletion of SYBR-Green available for the intercalation into newly formed amplicons during PCR amplification and thus prevent the formation of an increasing fluorescent signal during the reaction. The LOD of the 28S amplification using SYBR-Green was about 1000-fold higher compared to the LOD in the TaqMan® assay.

The LDR of the SYBR-Green assay was determined between 6.38×10^3 and 6.38×10^6 and is thus also suitable for quantification in a decent range. However, with the limitation that very high DNA concentrations significantly hampered the amplification. Our results clearly indicate that the assay type strongly impacts the confidence interval in which target quantification can be achieved. Even if the same primers are used and these primers amplify in a broad melting temperature range, the master mix impacts the PCR performance as indicated by our SYBR-Green results with varying $MgCl_2$ concentrations. These findings underline the importance to carefully evaluate and improve every PCR assay with appropriate standards and clearly define its characteristics in every new setting and choose the appropriate assay for the respective scientific question. It might be even more important to know the limitations for every respective assay to be able to interpret results. Although different studies report a comparable sensitivity of TaqMan® and SYBR-Green based qPCR [39, 40] this is not a general rule, since it strongly depends on how neat the respective PCR assay has been established and under what conditions the assays are performed.

The described UNI28S primers and probe were successfully used in a multiplexing approach and are routinely used in our lab as an enCtrl during phytoplasma detection in different plant and insect species. Since the novel primers and hydrolysis-probe amplify a conserved fragment of the 28S rDNA gene in many diverse eukaryotic species, they can thus be used for many different research purposes.

Conclusion

A universal endogenous control for phylogenetic distinct eukaryotic template DNA is now available for qPCR assays. Performance and quality control of qPCR runs is crucial for reliable results. EnCtrls in qPCR assays proof the integrity of template DNA and the absence of PCR inhibitors. Together with plasmid standards enCtrls can be useful for quantification purpose. The newly described and validated primer and probe combination is suitable as endogenous control in a broad range of eukaryotic species, and the single- as well as multiplex protocol can be used for pathogen detection based on qPCR assays.

Methods

Primer and probe design

To identify a highly conserved, short stretch in the eukaryotic genome, fragment V of the nuclear-encoded 28S rDNA was amplified with the respective forward

and reverse primers described in [41]. The amplicons of approximately 760 bp were sequenced and aligned using Geneious version 11.1.4. (https://www.geneious.com). A conserved sequence stretch was identified, and suitable qPCR primers and a probe were designed that allow amplification of an 84 bp region (Fig. 1) generating an amplicon within the size range recommended for SYBR and probe-based assays [23]. To create a primer pair that universally amplifies in eukaryotic species, a wobble nucleotide was incorporated in the reverse primer UNI28S-rev, while for the probe development LNAs were integrated to increase the binding specificity and strength. The designed sequences for primer pair UNI28S-fwd/UNI28S-rev and the probe UNI28S-P are depicted in Table 2.

qPCR setup

Different primer and probe concentrations were tested to determine the optimal working concentration in singleplex and multiplex TaqMan® assays and in SYBR-Green assays. All qPCR reactions were run on a CFX384 Touch™ or CFX96 Touch™ real-time PCR detection system (Bio-Rad) in HardShell® Bio-Rad Plates sealed with Microseal 'B' Film (Bio-Rad). PCR evaluation was performed using the CFX Manager software (Bio-Rad).

Sequences for primer and probes that were used as internal standards in this study can be found in Table 2.

Criteria for a high-quality qPCR run with the possibility of the quantification of a target were defined according to the MIQE Guidelines [7].

SYBR-Green assay

The primer performance was analyzed based on the recommendations of [23]. The optimal annealing temperature was determined by running a qPCR at annealing temperature gradient from 50 to 60 °C. A primer concentration matrix was performed with varying, symmetric and asymmetric primer concentrations, i.e. concentrations ranging from 125 nM to 500 nM. To analyze the effect of $MgCl_2$ on the PCR performance, 1.5 mM, 3 mM, 4.5 mM or 6 mM $MgCl_2$ were added to the reaction (note: the SYBR FAST qPCR Kit Master Mix already contains 2.5 mM $MgCl_2$ in the 1× working concentration and the $MgCl_2$ was additionally added to the mix). If not indicated differently, PCR reactions with SYBR-Green were performed as follows: 250 nM UNI28S-fwd, 250 nM UNI28S-rev, 1X SYBR FAST qPCR Kit Master Mix (Kapa Biosystems) with 2 µl template DNA, adjusted to a total volume of 10 µl with nuclease-free water. Cycling conditions were as follows: initial denaturation at 95 °C C for 20 s followed by 35 cycles of 95 °C for 3 s and annealing at 60 °C for 30 s. The generated amplicon was melted from 65 to 95 °C with an increment of 0.5 °C per 5 s.

Probe-based singleplex assay

The following TaqMan® qPCR mastermix reagent concentrations were adjusted based on the recommendations of [23]. For the amplification of the 28S fragment in a total reaction volume of 10 µl the following components were combined: 2 µl of template DNA, 5 µl 2X iQ™ Multiplex Powermix (Bio-Rad), 400 nM of each primer (UNI28S-fwd, UNI28S-rev) and 200 nM of the probe (UNI28S-P). The primers were synthesized and supplied by Microsynth AG (Switzerland) and the probe, with incorporated LNAs, was supplied by EuroClone S.P.A. (Italy). The following cycling conditions were applied: initial denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 1 min.

As an example for other internal controls the apple specific chloroplast DNA gene tRNA leucine (*trnL*) [24] and the *Malus* × *domestica* single-copy gene 1-amino-cyclopropane-1-carboxylate oxidase (*ACO*) [25] were amplified in a 10 µl reaction volume using 5 µl 2X iQ™ Multiplex Powermix (Bio-Rad), 200 nM qMd-cpLeu-F and qMd-cpLeu-R primer [24] or qMd-ACO-F/

Table 2 Primer and probes of endogenous reference genes used in this study

Target gene	Target species	Primer name	5′–3′ sequence	References
28S	Eukaryotes	UNI28S-fwd	CTACTATCTAGCGAAACC	This work
		UNI28S-rev	AYTAGAGTCAAGCTCAAC	
		UNI28S-P	HEX-AAA + G+A + AG + A+C + C+C + T-DAB	
cpLeu	*Malus* × *domestica*	qMD_cpLeu-fwd	CCTTCATCCTTTCTGAAGTTTCG	[24]
		qMD_cpLeu-rev	AACAAATGGAGTTGGCTGCAT	
		qMD_cpLeu	HEX-TGGAAGGATTCCTTTACTAAC-TAMRA	
aco	*Malus* × *domestica*	Md-ACO-F	CCAGAATGTCGATAGCCTCGTT	[25]
		Md-ACO-R	GGTGCTGGGCTGATGAATG	
		Md-ACO	HEX-TACAACCCAGGCAACG	

A + prior to the nucleotide code indicates that the following nucleotide is an LNA

qMd-ACO-R primer pair [25] together with 200 nM of the respective probe qMd-cpLeu or qMd-ACO. Both probes are conjugated at the 5′- end to HEX reporter dye. Cycling conditions were the same as for 28S probe-based assay.

Probe-based multiplex assay

The multiplex qPCR was run in a total reaction volume of 10 µl with 2 µl of sample DNA and 5 µl 2X iQ™ Multiplex Powermix, 900 nM of each SAD-fwd (5′-TGGTTAGAG CACACGCCTGAT-3′) and SAD-rev (5′-TCCACTGTG CGCCCTTAATT-3′) primer, 200 nM of qAP-IGS probe (5′FAM- CAAAGTATTTATCTTAAGAAAACAAGCT-3′) and 200 nM of qPD-IGS probe (5′TexRed- AATATT TATTTTAAAAAAAAGCTCTTTG-3′) [42, 43] together with 400 nM of each UNI28S-fwd and UNI28S-rev and 200 nM of UNI28S-P. PCR conditions were the same as described for the 28S probe-based singleplex assay.

Plasmid standards for quantification

The amplified 28S rDNA fragment was subcloned into the plasmid vector pJET1.2 via the CloneJET PCR Cloning Kit (ThermoFisher) and transformed into electrocompetent MegaX DH10B $T1^R$ *E. coli* (Invitrogen). The plasmid was extracted from *E. coli* with the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Additionally, to remove genomic DNA impurities, that might affect accurate photometric plasmid quantification, the eluates were run on an agarose gel and the plasmid band was gel extracted (QIAquick Gel Extraction Kit, Qiagen). The plasmid DNA concentration was measured with PicoGreen® (ThermoFisher) on a NanoDrop™ 3300 fluorospectrometer (ThermoFisher). Plasmids were diluted to a concentration of 2.5×10^8 plasmid copies μl^{-1}. Plasmid copy content was calculated based on the molecular weight of the plasmid, applying the following formula, considering that one nucleotide has a molecular weight of 327 g/mol:

$$\text{plasmid copy content}\left[\frac{\text{copies}}{\mu\text{l}}\right] = \frac{\text{plasmid concentration}\left[\frac{\text{ng}}{\mu\text{l}}\right]}{\text{molecular weight of the plasmid}\left[\frac{\text{ng}}{\text{copy}}\right]}$$

With the adjusted plasmid solution, a tenfold standard dilution series in AE buffer (Qiagen) for quantification of the 28S rDNA fragment was prepared. The dilution series of 2.5×10^6–2.5×10^0 28S copies μl^{-1} was analyzed together with each qPCR run to determine qPCR performance parameters such as the linear dynamic range (LDR), amplification efficiency, coefficient of determination (correlation coefficient, R^2) and the limit of detection (LOD). As described for the 28S fragment, phytoplasmal target sequences of '*Candidatus* Phytoplasma mali' (apple proliferation phytoplasma; AP) and '*Candidatus* Phytoplasma pyri' (pear decline phytoplasma; PD) were subcloned into pJET1.2 plasmid vector and prepared from *E. coli* strain DH10B $T1^R$. The subcloned amplicons were amplified with SAD-fwd and SAD-rev primer pair, according to [42] using DNA from infected apple or pear as template. A tenfold dilution series of the three template plasmids (pJET1.2-28S, pJET1.2-AP and pJET1.2-PD) in the range of 2.5×10^7–2.5×10^0 plasmid copies μl^{-1} (of each plasmid) was prepared in AE-buffer (Qiagen). The standard dilution series was used to analyze the performance of every multiplex qPCR run.

DNA, RNA extraction and cDNA synthesis

To test the specificity of the primer pair and probe, samples from 43 different eukaryotic species were tested. DNA from horse hairs, beef, pork, mutton, roe venison, salmon filet, tick, leafhopper and psyllid species, yeast and bacteria (as a negative control) was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and DNA from plant and fungi species was extracted with the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. DNA was eluted in 100 µL AE buffer provided with the extraction kits. DNA quantity was measured with a NanoDropTM 1000 spectrophotometer. RNA from apple and grapevine was extracted as described in 44 [44]. The extracted RNA was pretreated with TURBO DNA-free™ Kit (Invitrogen). cDNA was synthesized using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen). For comparability of 28S copy numbers DNA was diluted to a final concentration of 5.0 ng μl^{-1} prior use as template in the qPCR assay.

Statistical analysis

All statistical analyses were performed using GraphPad Prism® 7.01.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13007-020-00597-2.

Additional file 1. Additional figures and tables.

Abbreviations

AP: Apple proliferation; enCtrl: Endogenous control; exCtrl: Exogenous control; LDR: Linear dynamic range; LNA: Locked nucleic acids; LOD: Limit of detection; PD: Pear decline.

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Authors' contributions
TL and KJ contributed conception and design of the study; CM, LO, VO, AI, AG, YRD and KJ developed the methodology; CM, LO, VO, AI, CK, AG, YRD and KJ performed the formal analysis; CM and KJ performed the statistical analysis and wrote the first draft of the manuscript; TL and KJ were responsible for funding acquisition. All authors contributed to manuscript revision. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All vertebrate tissues used for DNA extraction were commercially available fish or meat products. Human and mouse DNA were provided from Hannover Medical School.

Consent for publication
Not applicable.

Competing interests
The Research Centre Laimburg has a granted patent (Grant no: 102018000003299) directed to the sequences of the primers UNI28-fwd and Uni28-rev as well as the hydrolysis probe UNI28-P described in this study. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential competing interests.

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A Novel Effector Protein of Apple Proliferation Phytoplasma Disrupts Cell Integrity of *Nicotiana spp.* Protoplasts

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Cecilia Mittelberger: Design and performance of the study, yeast transformation, Y2H screen with $PME2_{Kube}$ and targeted screen with both variants, verification of the expression in *C. picta*, expression in yeasts and plants, evaluation of all results, writing the manuscript

Hagen Stellmach: Assistance with the protoplast extraction, assistance with the confocal microscopy, proofreading of the manuscript

Bettina Hause: Discussion of methods and results, proofreading of the manuscript

Christine Kerschbamer: Assistance with Y2H screens and transformations, proofreading of the manuscript

Katja Schlink: Supervision of $PME2_{AT}$ identification, discussion of methods and results

Thomas Letschka: Discussion of methods and results

Katrin Janik: Supervision and design of the study, first Y2H Screen with $PME2_{AT}$, discussion of methods and results, assistance in writing the manuscript

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Article

A Novel Effector Protein of Apple Proliferation Phytoplasma Disrupts Cell Integrity of *Nicotiana* spp. Protoplasts

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Abstract: Effector proteins play an important role in the virulence of plant pathogens such as phytoplasma, which are the causative agents of hundreds of different plant diseases. The plant hosts comprise economically relevant crops such as apples (*Malus* × *domestica*), which can be infected by '*Candidatus* Phytoplasma mali' (P. mali), a highly genetically dynamic plant pathogen. As the result of the genetic and functional analyses in this study, a new putative P. mali effector protein was revealed. The so-called "Protein in *Malus* Expressed 2" (PME2), which is expressed in apples during P. mali infection but not in the insect vector, shows regional genetic differences. In a heterologous expression assay using *Nicotiana benthamiana* and *Nicotiana occidentalis* mesophyll protoplasts, translocation of both PME2 variants in the cell nucleus was observed. Overexpression of the effector protein affected cell integrity in *Nicotiana* spp. protoplasts, indicating a potential role of this protein in pathogenic virulence. Interestingly, the two genetic variants of PME2 differ regarding their potential to manipulate cell integrity. However, the exact function of PME2 during disease manifestation and symptom development remains to be further elucidated. Aside from the first description of the function of a novel effector of P. mali, the results of this study underline the necessity for a more comprehensive description and understanding of the genetic diversity of P. mali as an indispensable basis for a functional understanding of apple proliferation disease.

Keywords: phytoplasma; effector protein; apple; pathogenicity; virulence; apple proliferation

1. Introduction

Phytoplasma are small, biotrophic bacteria that cause hundreds of different plant diseases and are involved in their infection cycle not only in plant hosts, but also in insect vectors. '*Candidatus* Phytoplasma mali' (P. mali), the causal agent of apple proliferation (AP) disease, has caused significant economic losses in apple production in Northern Italy (one of Europe's main production areas) in the last decades [1]. Phytoplasma are obligate plant and insect symbionts that exhibit a biphasic life cycle comprising reproduction in certain phloem-feeding insects as well as in plants [2,3]. Within their plant host, phytoplasma colonize the phloem. By ingestion of phloem sap, insect vectors acquire the phytoplasma, with the colonization of those insects enabling the transmission of the pathogen between host plants [3,4]. Although several concepts of phytoplasma effector biology were able to be unraveled for the '*Candidatus* Phytoplasma asteris' strain Aster Yellow Witches' Broom (AY-WB) in the model plant *Arabidopsis thaliana* [5–12], the understanding of effector-driven changes induced by P. mali remain limited. Genetic and functional homologues of AY-WB phytoplasma protein SAP11 could be identified in P. mali [10,13]. Recently a novel effector was described that exhibits E3 Ubiquitin ligase function and

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affects the plant's basal defense [14]. Furthermore, the immunodominant membrane protein Imp of P. mali was shown not to be involved in symptom development but is considered to play a role during plant colonization [15]. A role of phytoplasmal HflB proteases and an AAA+ ATPase in AP virulence has been hypothesized but not yet clarified [16–18]. P. mali encodes genes for a Sec-dependent protein secretion system, whereas genes encoding components of other secretion systems, such as the type three secretion system, are mainly lacking [19,20]. Secreted phytoplasma proteins may directly interact with cellular host components and thus manipulate the cell's metabolism [3]. Potential effector proteins may thus be identified by the presence of a characteristic *N*-terminal secretion signal.

The aim of this study was to characterize the function of the phytoplasmal "Protein in *Malus* Expressed 2" (PME2) from P. mali that exhibits genetic features indicating that it acts as an effector protein in plants. To unravel PME2s potential role as an effector, this study analyzed (1) whether it is genetically conserved; (2) whether it is expressed during infection; (3) where it is translocated within the plant cell; and (4) if it induces morphological changes within the expressing plant cells.

To address these questions, we analyzed the expression of PME2 in P. mali-infected *Malus* × *domestica* leaf and root tissue, and in infected *Cacopsylla picta* (i.e., insect species transmitting P. mali). In infected *Malus* × *domestica* we found two distinct genetic variants of *pme2*. In addition, heterologous overexpression of PME2 in mesophyll protoplasts of *Nicotiana* spp. was used to gain insights into the subcellular localization of PME2 as well as its effects on plant cell integrity. These data were complemented by the expression of PME2 in yeast. With the data presented here, the first steps into unraveling the molecular mechanism of PME2 function were taken, but further experiments in the future will be indispensable.

2. Results

2.1. In Silico Analysis of PME2 Indicates Effector Potential

Bioinformatic analysis of conserved hypothetical proteins encoded in the P. mali genome [19] revealed that CAP18323.1, encoded by the gene *atp_00136*, contains interesting features that might confer effector function. Neural networks and hidden Markov prediction models (Transmembrane Helices Hidden Markov Model; TMHMM) were applied to analyze CAP18323.1 for the presence of a signal peptide and the presence of transmembrane regions (SignalP v. 3.0 [21], TMHMM [22]). Since phytoplasma phylogenetically belong to Gram-positive bacteria [3], a prediction algorithm trained on this bacterial group was applied. The N-terminal amino acid-stretch 1–31 contains a signal peptide that is supposed to confer Sec-dependent secretion of the protein (Figure 1). Further transmembrane regions were not predicted, indicating that CAP18323.1 is not inserted in a membrane. Upon translation, *N*-terminal signal peptides are cleaved [23]. At the C-terminal part of CAP18323.1 an importin α/β-dependent nuclear localization site (NLS) and a nuclear export signal (NES) were predicted [24,25]. The absence of transmembrane regions in the mature protein, the predicted localization in the plant cytoplasma or the nucleus (WoLF PSORT, [26]), and the small size of about 16 kDa (Analysis Tool on the ExPASy Server, [27]) indicate that CAP18323.1 may exhibit an effector function (Figure 1).

2.2. Atp_00136 (Pme2) is Expressed in P. Mali-Infected Malus × Domestica but not in the Insect Vector C. Picta

Subsequently, it was analyzed whether *atp_00136* was expressed in apple trees infected with P. mali. Leaf and root samples of P. mali-infected and non-infected *Malus* × *domestica* cv. "Golden Delicious" trees were taken in May and October. Expression of *atp_00136* was analyzed with *atp_00136*-specific primers and *Malus* × *domestica* cDNA derived from mRNA. Expression of *atp_00136* was confirmed in P. mali-infected leaf and root tissue by the detection of distinct amplicons at the expected size in the respective samples (Figure 2).

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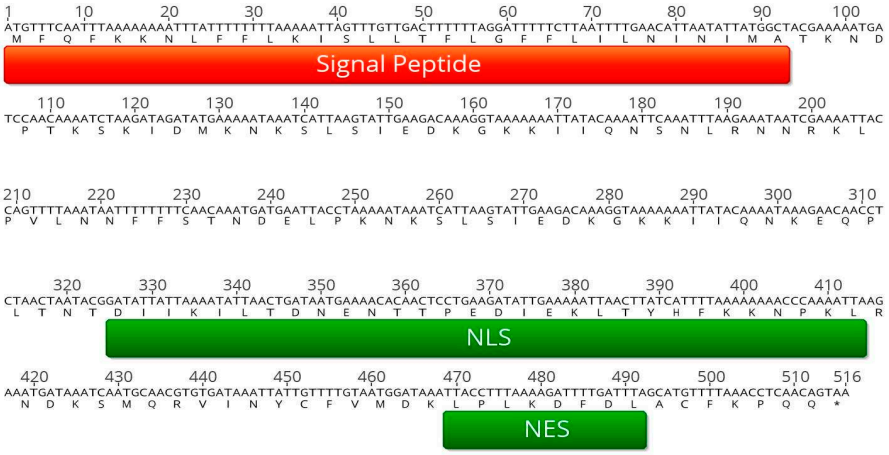

Figure 1. Results of the in silico analysis. Sequence analysis of *atp_00136* revealed the presence of an *N*-terminal signal peptide (indicated in red), as well as a nuclear localization signal (NLS), and a C-terminal nuclear export signal (NES), both indicated in green. Graphs were generated with Geneious Prime 2018 version 11.1.4.

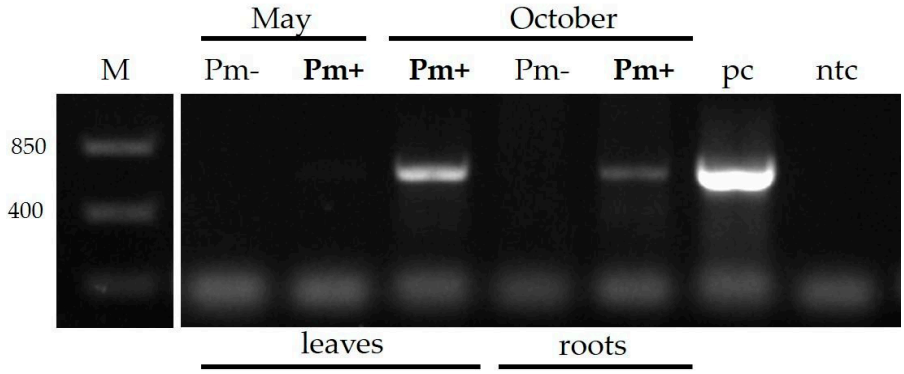

Figure 2. Expression of *pme*2 (CAP18323.1) in '*Candidatus* Phytoplasma mali' (P. mali)-infected *Malus* × *domestica*. Transcripts of *pme*2 were detected by PCR using cDNA from *Malus* × *domestica* infected with P. mali. A discrete band of the size indicative for the *pme*2 transcript was detected in P. mali-infected (**Pm+**) but not in non-infected (Pm–) leaves and roots harvested in October. DNA derived from an infected *Malus* × *domestica* served as a positive control (pc) and water as the non-template control (ntc).

Using quantitative PCR (qPCR) the expression levels of *atp_00136* and P. mali in the samples were quantified. The results show that *atp_00136* is only expressed in tissue colonized by P. mali (Table 1). Since identified expressed genes were named in a chronological manner, *atp_00136* was named "Protein in *Malus* Expressed 2" (*pme2*) based on the general recommendations for bacterial gene nomenclature [28].

To analyze if *pme2* was expressed in the transmitting insect vectors during infection, three P. mali-infected *C. picta* individuals were analyzed for the expression of the potential effector. In the RNA/cDNA of all infected individuals, P. mali-specific transcripts of the ribosomal protein *rpl22* were detected, but expression of *pme2* was not detectable.

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Table 1. Detection of *atp_00136* in cDNA samples from infected and non-infected leaf tissue from May and October 2011. In May phytoplasma were only detectable in the roots but not in the leaves. *atp_00136* was only detectable in P. mali-infected and colonized tissue. Cq values are given as the mean value of three repeated qPCR runs.

Month	Status	Pool	cDNA Integrity (*tip41*)	Phytoplasma (*16S*)	*atp_00136*
May	non-infected	1	26.38	N/A	N/A
		2	26.38	N/A	N/A
Oct	non-infected	3	26.58	N/A	N/A
		2	26.59	N/A	N/A
		3	26.58	N/A	N/A
May	infected	1	26.56	N/A	N/A
		2	26.53	N/A	N/A
		3	26.61	N/A	N/A
Oct	infected	1	26.71	23.67	28.00
		2	26.44	23.18	27.66
		3	26.48	23.34	28.34

2.3. *Genetic Variability of* Pme2

Cloned amplicon sequencing revealed that the prevalent variant of *pme2* from infected trees in South Tyrol (North-East Italy) differs compared to the *pme2* sequence of the P. mali AT strain from Germany [19]. In a total of 20 samples from naturally infected apple trees in the regions Burggraviato and Val Venosta, a prevalent, conserved sequence of *pme2* was identified ($pme2_{ST}$; accession number MN224214). This conserved variant exhibits a single nucleotide polymorphism (SNP) in the sequence stretch before the NLS, and two SNPs within and one SNP after the NLS compared to the AT strain (Figure 3). All four SNPs in the $pme2_{ST}$ variant lead to nonsynonymous missense substitutions at the protein level as compared to the *pme2* sequence published previously [19] ($pme2_{AT}$). The NLS of $pme2_{ST}$ has a slightly higher prediction score than the NLS of $pme2_{AT}$. The most striking difference between $pme2_{AT}$ and $pme2_{ST}$ is a stretch of 120 bp in $pme2_{ST}$ which is absent in $pme2_{AT}$. This stretch is a partial duplication of a fragment also present in $pme2_{AT}$ (Figure 3). In three *Malus* × *domestica* samples, a very sporadic sequence of *pme2* could be detected that did not contain the $pme2_{ST}$ characteristic sequence duplication but showed strong sequence similarity to $pme2_{AT}$. The sporadic sequence contains six SNPs at positions 218 (A > T), 220 (A > G), 322 (A > G), 331 (A > C), 344 (C < T), and 427 (T > G) that lead to nonsynonymous missense mutations (accession number MN224215) compared to $pme2_{AT}$. However, in the trees in which these very sporadic *pme2* sequences were found, $pme2_{ST}$ could also be detected, indicating the presence of a mixed population of different P. mali strains.

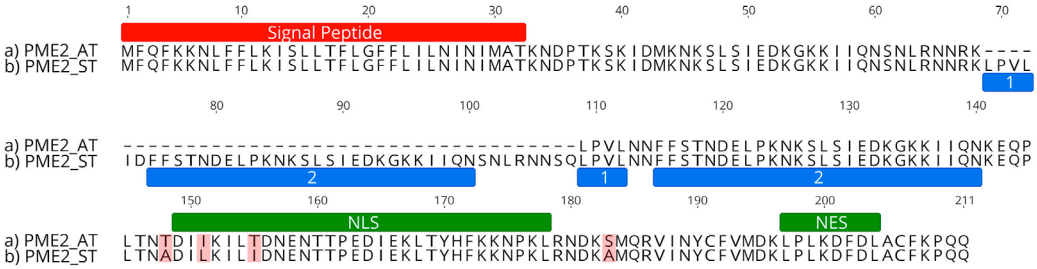

Figure 3. Sequence comparison of $PME2_{ST}$ and $PME2_{AT}$. The protein variants (**a**) $PME2_{AT}$ and (**b**) $PME2_{ST}$ contain the same *N*-terminal signal peptide sequence (red). $PME2_{ST}$ (**b**) contains a duplicated amino acid stretch (the replicative sequences 1 and 2; marked in blue) of a partial sequence also present in $PME2_{AT}$ (**a**). Both variants show slight differences in and directly before the nuclear localization signal sequences (NLS, green). The nuclear export signal sequence (NES, green) is identical in both protein variants. Amino acid differences of $PME2_{ST}$ to the $PME2_{AT}$ variant are shown in black, whereas similarities are shown in grey. Graphs were generated with Geneious Prime 2018 version 11.1.4.

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2.4. $PME2_{ST}$ and $PME2_{AT}$ Translocate to the Nucleus of Nicotiana spp. Protoplasts

To identify the subcellular localization of the PME2 protein in the plant cell, mesophyll protoplasts of *Nicotiana occidentalis* and *N. benthamiana* were transformed, with expression vectors coding for $PME2_{AT}$ and $PME2_{ST}$ tagged with GFP or mCherry-fluorescent protein to allow subcellular tracking. The *N*-terminal signal part was not considered for these studies, since it is removed from the processed, mature CAP18323.1 protein. *N. occidentalis* and *N. benthamiana* can be infected with P. mali. Upon infection, both *Nicotiana* species show disease-specific symptoms and are thus appropriate model plants for P. mali effector studies [15,29]. Confocal microscopy analysis revealed that overexpressed $PME2_{AT}$ and $PME2_{ST}$ are translocated to the nucleus of *Nicotiana* spp. protoplasts. This translocation was independent of the used tag and *Nicotiana* species (Figure 4 and Figures S1–S3). The in vivo results therefore confirm the in silico prediction that $PME2_{ST}$ and $PME2_{AT}$ are translocated to the nucleus of potential host cells.

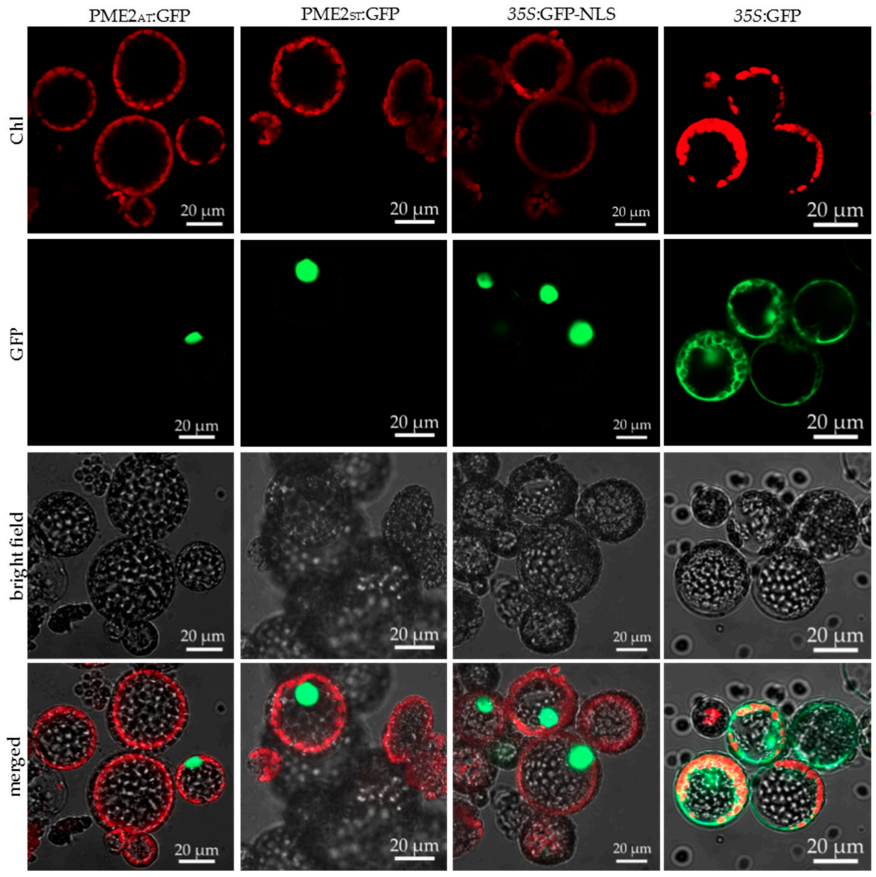

Figure 4. $PME2_{ST}$ and $PME2_{AT}$ are translocated to the nucleus of mesophyll protoplasts. Mesophyll protoplasts of *Nicotiana benthamiana* were transformed with the plasmid pGGZ001 encoding C-terminal GFP-tagged $PME2_{ST}$ (first column), $PME2_{AT}$ (second column), GFP *N*-terminally fused to a NLS sequence (third column), or GFP only as a control for nuclear localization (fourth column). Expression of the transgenes was under the control of a *35S* promoter. The upper panel shows autofluorescence of chloroplasts (Chl), the second panel the signal derived from the GFP, and the third panel the bright field image and the last panel an overlay of all images (merged). Microscopic analysis was performed with a Zeiss LSM 800. Corresponding images after expression of mCherry-tagged PME2 and of use of *Nicotiana occidentalis* mesophyll protoplasts are presented in Figures S1–S3. Bars represent 20 µm.

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A leaf infiltration assay using *Agrobacterium* strain EHA105 transformed with PME2 encoding expression vectors did not result in detectable expression or phenotypic alterations of either $PME2_{AT}$ or $PME2_{ST}$ in both *Nicotiana* species. Nonetheless, positive controls expressing the fluorophore tag only and leaves infiltrated with the P. mali SAP11-like effector protein ATP_00189 [13] as control showed strong signals (Figure S5), indicating that PME2 expression might be somehow blocked or is immediately degraded by the plant.

2.5. $PME2_{ST}$ but not $PME2_{AT}$ Affect Cell Integrity of Nicotiana spp. Protoplasts

Protoplasts transformed with the $PME2_{ST}$ expression vector often showed shrinkage, and only about 50% of the *N. benthamiana* protoplasts were viable 20 h post-transformation compared to the transformation control expressing the fluorophore only or a GFP with NLS (Figure 5a). The shrunk cells lysed and only the remaining cell debris was microscopically detectable (Figure 4). The effect on protoplast integrity was observed in protoplasts expressing $PME2_{ST}$:GFP and $PME2_{ST}$:mCherry, and thus was independent of the fluorophore used as a tag for microscopic analyses. Similar results were obtained using *N. occidentalis* as the heterologous $PME2_{ST}$ expression system. The mCherry-tagged $PME2_{ST}$ induced a weak but significant reduction of viability in *N. occidentalis* protoplasts (Figure 5b). The GFP-tagged $PME2_{ST}$ showed the same tendency but to a stronger extent, i.e., it reduced cell viability by about 50%, which is similar to the effect seen in *N. benthamiana* protoplasts. Cell viability stain with fluorescein diacetate (FDA) showed similar results, i.e., that *N. benthamiana* protoplasts transformed with the $PME2_{ST}$-expressing vector showed a significantly reduced viability (Figure 6). Shrunk cells were positive for propidium iodide (PI) staining (Figure S4), indicating that these cells were dead.

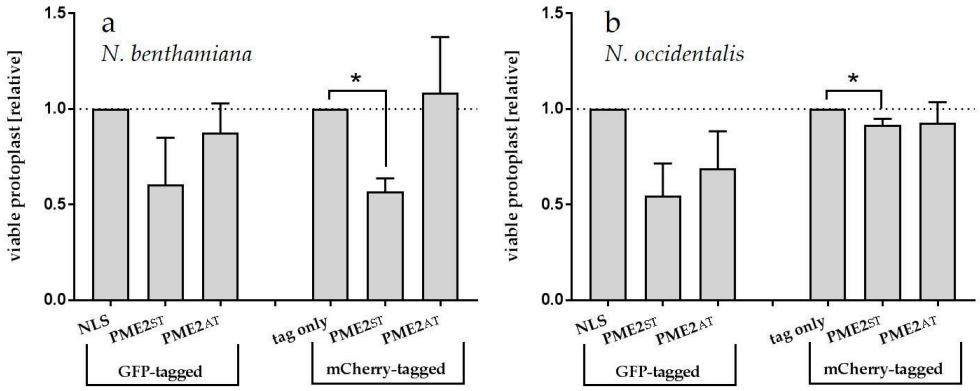

Figure 5. $PME2_{ST}$ overexpression reduces viability of (**a**) *N. benthamiana* and (**b**) *N. occidentalis* mesophyll protoplasts. For each assay, 20,000 mesophyll protoplasts were transformed with the plasmid pGGZ001 encoding $PME2_{ST}$, $PME2_{AT}$ (tagged with GFP or mCherry), the GFP-tagged control for nuclear localization (NLS), or the mCherry tag (tag only) and viable protoplasts were counted. Overexpression of the transgenes was under the control of a *35S* promoter. Data represent the mean viability +/– SE of 3–4 independent experiments. The respective control (NLS or tag only) was set at 1 to allow comparison between different experiments. Differences between the groups were determined applying a one way-ANOVA analysis. Significant differences ($p < 0.05$) between groups are indicated with an asterisk (*).

Interestingly, $PME2_{AT}$ did not have an effect on protoplast integrity in *N. benthamiana* nor in *N. occidentalis* protoplasts (Figure 5).

2.6. A Yeast Two-Hybrid Screen Was Unsuitable for the Elucidation of $PME2_{ST}$ Function

Upon expression of $PME2_{ST}$, the yeast reporter strain *Saccharomyces cerevisiae* NMY51 showed several macroscopic aberrations in colony growth (Figure 7a). However, at the microscopic level

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when visualizing the yeast cell wall with calcofluor white, no phenotypic differences between yeast cells expressing $PME2_{AT}$, $PME2_{ST}$, and empty bait vector pLexA-N could be detected (Figure 7b). Considering the effect of mere $PME2_{ST}$ expression on growth of the yeast reporter strain, the relevance of any identified interaction in a yeast two-hybrid screen remains highly questionable and the assay was therefore not performed.

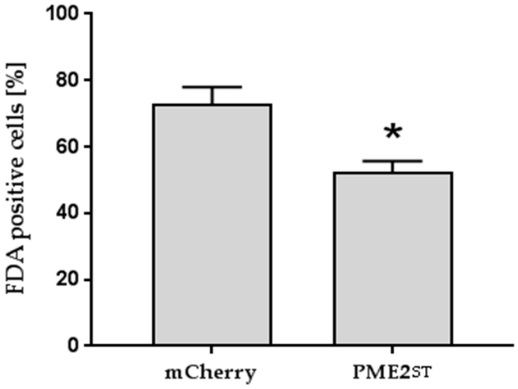

Figure 6. $PME2_{ST}$ overexpression reduces viability of *N. benthamiana* mesophyll protoplasts. Mesophyll protoplasts were transformed with the plasmid pGGZ001 encoding mCherry-tagged $PME2_{ST}$ (PME_{ST}) or the mCherry tag only (mCherry) and stained with fluorescein diacetate (FDA) to detect viable cells. Data represent the mean percentage of FDA-positive stained cells +/− SE ($n = 3$). The statistical difference between the two groups was determined by using a Student's *t*-test and is indicated with an asterisk (*$p < 0.05$).

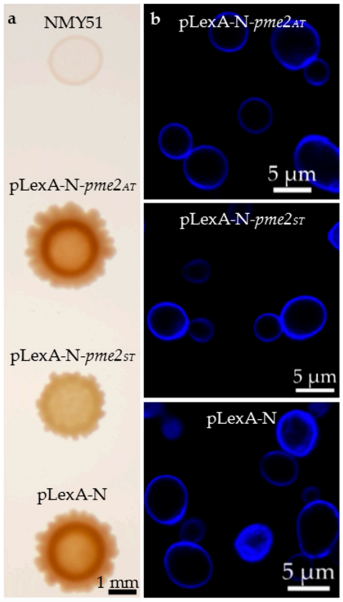

Figure 7. $PME2_{ST}$ overexpression in the yeast reporter strain NMY51 leads to macroscopic aberrations. *Saccharomyces cerevisiae* strain NMY51 was transformed with the yeast two-hybrid (Y2H) bait vector pLexA-N, which encodes tryptophan auxotrophy, expressing $PME2_{AT}$, $PME2_{ST}$, or the empty vector only, and drop-plated onto SD-trp plates. In comparison to the empty pLexA-N vector and the vector expressing PME_{AT}, colonies expressing PME_{ST} showed reduced growth and remained white (**a**). Yeast cells stained with calcofluor white did not show any phenotypic differences on single-cell level (**b**). Calcofluor white fluorescence was visualized on a confocal microscope (LSM 800, Carl Zeiss AG, Oberkochen, Germany).

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3. Discussion

The results of this study show that $PME2_{ST}$ (a variant of CAP18323.1 previously annotated as "conserved hypothetical protein") affects plant cell integrity. Based on our findings and the definition that effectors are secreted pathogen proteins altering host-cell structure and function [30], we propose defining PME2 as a phytoplasmal effector. Interestingly, two different variants of PME2 were identified and both variants translocate to the nucleus of plant cells, but only the newly described regional variant $PME2_{ST}$ subsequently affects protoplast integrity. The small size of about (at a maximum) 21 kDa ($PME2_{ST}$: 21 kDa and $PME2_{AT}$: 16 kDa; both considering the mature protein without the signal peptide) indicates that PME2 can be translocated from the phloem and target adjacent tissues or be distributed systemically in the plant [3]. Subcellular localization using microscopy requires the use of fluorescent tags that are attached to the protein of interest. Tagging can affect subcellular localization of the protein; however, we used two different tags (GFP and mCherry) to analyze whether tagging influences the target localization. In cells expressing the tag only, a localization of the fluorescent signal in the cytoplasm could be observed. PME2 was localized only in the nucleus and since no signal was visible in other cell compartments, it can be assumed that the observed localization is effector-mediated (see also [31]). Only protoplasts transformed with $PME2_{ST}$ showed significant cell disruption as indirectly quantified by counting the remaining viable cells and FDA staining of the protoplasts. Shrunk cells were positive for the PI stain but did not show a GFP signal. The lack of the GFP-signal might be caused by a disruption of the nucleus, protein degradation, and/or leakage of the signal into the surrounding medium. Cells expressing $PME2_{ST}$ were intact, indicating that the effector either exhibits a dose-dependent or delayed effect on cell integrity.

Both variants of PME2 contain an *N*-terminal signal peptide, a nuclear localization signal (NLS), and a nuclear export signal (NES). It is a common feature of nuclear proteins to contain both NLS and NES and these signals coordinate the translocation of the protein between nucleus and cytoplasm [32]. Nuclear targeting of proteins containing a classical NLS is mediated by the importin α/β heterodimer through NLS-dependent binding to the importin α subunit and importin β–mediated attachment to the nuclear pore complex [33,34]. The SNPs in the NLS region of $PME2_{ST}$ lead to a (slightly) higher sequence-based NLS prediction; thus, the differences might show a stronger translocation to the nucleus. The NES signal (which indicates that shuttling of PME2 between nucleus and cytoplasm might occur) is the same in both variants. Even though nucleocytoplasmic distribution is predicted, PME2 was only detected in the nucleus. Many proteins containing NLS and NES appear to be localized in the nucleus because the rate of import to the nucleus is higher than the rate of export to the cytoplasm [35]. It remains thus unclear if PME2 is strictly limited to the nucleus or if a constant shuttling between nucleus and cytosol occurs.

Bacterial effectors that translocate to the nucleus, the so-called nuclear effectors, can affect master switches of the host immune machinery or alter host transcription to the benefit of the pathogen [31]. Effectors from different phytoplasma species target plant–host transcription factors or affect gene expression on the transcriptional level to alter the host metabolism to their own benefit [4,12,13,36,37]. However, none of these effectors have yet been reported to exhibit such detrimental effects during in planta expression. The effector protein BR1 of the phloem colonizing squash leaf curl geminivirus shuttles between the cytoplasm and the nucleus of protoplasts [38]. Upon binding to the second movement protein BL1, BR1 shuttles to the cytoplasma [39] and the concerted action between BR1 and BL1 mediates cell-to-cell movement of the virus within the phloem and to adjacent cells [35,38,40,41]. To unravel BR1 function it was necessary to identify its interaction partner, a general approach to investigate effector function. Yeast two-hybrid (Y2H) screens have been successfully applied to determine phytoplasmal effector targets on the molecular level [10,13]. These screens allow the screening of a protein of interest (effector) against a library containing hundreds of thousands of different potential interaction partners of a certain host species [42,43]. Successful interaction is monitored by a genetic reporter system that complements certain auxotrophies in the recombinant yeast reporter strain. However, a Y2H with $PME2_{ST}$ is not suitable since $PME2_{ST}$ expression strongly

affected the Y2H yeast reporter strain. This effect on yeast cells further supports the finding that $PME2_{ST}$ exhibits a strong effect not only on plant, but also on yeast cells, even though the latter do not have relevance as phytoplasma host cells. Since $PME2_{ST}$ exhibits such a strong effect on the expressing host and non-host cells, alternative approaches must be applied to unravel its molecular function. *Nicotiana* spp. leaf infiltration assays with recombinant *Agrobacterium* strains expressing PME2 failed. It remains furthermore elusive as to whether PME2 exhibits effects on the host plant phenotype. Considering the $PME2_{ST}$ effects on protoplasts it can be assumed that a systemic overexpression would lead to overwhelming deleterious effects in transgenic plants that express this effector. The results show that PME2 is expressed in roots and leaves of infected *Malus* × *domestica*, but not in infected individuals of its insect vector *C. picta*, underlining the hypothesis that PME2 plays a role as an effector protein in plant cells. However, it needs further clarification if expression is fine-tuned in a tempo-spatial manner in the plant host.

A neatly coordinated and local expression during infection might have very local effects and might not lead to cell disruption as seen in heterologous overexpression experiments. It is hypothesized that phytoplasma are able to degrade plant cell walls or generate holes in plant cell membranes to expedite cell-to-cell effector translocation [4]. Infection with P. mali induces cytochemical modifications and injuries of the affected phloem cells [44,45]. It is speculated that plasma membrane integrity is affected by until-now unknown P. mali effector(s) and that plasma membrane disruption is involved in the observed phloem damage induced by virulent P. mali strains [45]. However, since molecular indications are lacking, interpretation of the mode of PME2 action remains speculative. Subsequent approaches to analyze PME2 function should comprise assays that do not depend on functional living cells.

Since PME2 is translocated to the nucleus it is possible that it directly targets the host DNA by mimicking DNA regulatory elements, such as transcription factors or repressors. Some plant pathogen effectors bind host DNA and thus modulate gene expression [46,47]. An example of these effectors are TAL effectors of the plant pathogen *Xanthomonas*. TAL effectors bind promoter elements and regulate plant host expression to the benefit of the pathogen [48–51]. The effector AvrBs3 of *Xanthomonas* translocates to the nucleus where it acts as a transcription factor and affects the size of mesophyll cells [52]. Bioinformatic prediction and sequence comparison did not indicate that PME2 has similarity with currently known transcription factors or other gene expression regulating factors in plants.

Both P. mali strains from which the two different PME2 variants were derived cause infection and typical disease symptoms in *Malus* × *domestica*. Thus, the effect of $PME2_{ST}$ on cell integrity seems to be dispensable for infection and symptom development but might affect strain virulence. However, a direct comparison between the two strains regarding their virulence is missing. It might also be possible that another effector of P. mali strain AT (unknown at the time of this research), mimics and thus complements the function that $PME2_{AT}$ is lacking.

Some P. mali strains strongly differ regarding their virulence potential in *Malus* × *domestica* and several studies addressed the genetic identification of virulence factors or certain genetic determinants that account for these differences [17,18,53–55]. Since phytoplasma cannot be genetically manipulated, determining the importance of an effector during infection often involves tortuous experimental paths. In this study we provide the first characterization of the P. mali effector PME2 and its effect on cells of potential plant hosts. We report an interesting difference between two variants of PME2 that occur in Italy and Germany, claiming that further full genomic sequence analysis is required to better understand how P. mali manipulates its host on the molecular level.

4. Materials and Methods

4.1. *Verification of* Pme2 *Expression in* Malus × Domestica *and* C. Picta

For the verification of *pme2* expression by PCR in infected apple root and leaf samples RNA was extracted from the plant tissue as described in [13]. Extracted RNA was subjected to DNase treatment

using DNAfree Turbo reagent (Ambion, Austin, TX, USA) and cDNA synthesis was performed using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA). The generated cDNA was diluted 1:200 in nuclease free water and cDNA integrity was checked in all samples by performing a control PCR targeting the house-keeping gene transcript putative tip41-like family (transcript identifier: Mdo.1349) using the primers 5'-ACATGCCGGAGATGGTGTTTGG-3' (forward) and 5'-ACTTCCAGAGTACGGCGTTGTG-3' (reverse). Contamination with genomic DNA was checked by performing a PCR with primers amplifying a fragment within the non-coding region trnL of chloroplast DNA using the primers B49317 and A49855 [56]. No DNA contamination was detected in any of the cDNA samples, and the amplification of the putative tip41-like transcript fragment was positive, thus confirming the integrity of the generated cDNA. PCR reactions to verify *pme2* expression were set up in a total reaction volume of 10 µL, using 2 µL of diluted cDNA (1:200) as template, 0.05 µL GoTaq® DNA Polymerase (Promega, Madison, WI, USA), 2 µL of 5X Green GoTaq® Reaction Buffer (Promega, Madison, WI, USA), 0.2 µL dNTP-mix (40 mM), 1 µL of forward primer ATP00136_forw_EcoRI (10 µM, 5'-CCCCCCGAATTCATGTTTCAATTTAAAAAAAATTTA-3'), and 1 µL of reverse primer ATP00136_rev_SalI (10 µM, 5'-CCCCCCGTCGACATTATTACTGTTGAGGTTTAA-3'). Cycling conditions were applied as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 44.9 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. PCR products were visualized on 1% agarose gel. Additionally, *pme2* expression level was detected by qPCR based on SYBR-Green chemistry using the primer pair ATP00136_GW_fwd (5'-CACCATGACGAAAAATGATCCAACAAA-3')/ATP00136_nostopp_rev (5'-CTGTTGAGGTTTAAAACAT-3') in a total reaction volume of 20 µL using 4.0 µL of diluted cDNA (1:200) as a template together with 10.0 µL 2× SYBR FAST qPCR Kit Master Mix (Kapa Biosystems/αmann-La Roche, Basel, Switzerland), 1.0 µL of each primer (10 µM), and 4.0 µL of nuclease free water. qPCR conditions were as follows: an initial denaturation step at 95 °C for 20 s followed by 34 cycles of 95 °C for 3 s and 60 °C for 30 s and a melting curve ramp from 65 to 95 °C, at increments of 0.5 °C every 5 s (CFX384 Touch Real-Time PCR Detection System; BioRad, Hercules, CA, USA). Data analysis was performed using the CFX ManagerTM software (BioRad, Hercules, CA, USA).

To control whether *pme2* is expressed in infected individuals of the insect vector *C. picta*, RNA of six potentially infected and two uninfected F1 individuals was extracted with the ZR Tissue & Insect RNA MicroPrep™ kit (ZymoResearch, Irvine, CA, USA) according to the manufacturer's instructions. Extracted RNA was subjected to DNase treatment using DNAfree Turbo reagent (Ambion, Austin, TX, USA) and RNA integrity was controlled with an RNA ScreenTape on a TapeStation 2200 (both Agilent, Santa Clara, CA, USA). cDNA was synthesized with the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA). Together with the cDNA synthesis a control was performed lacking the reverse transcriptase (-RT). Here, 2 µL of diluted cDNA (1:200) were used as template in a total qPCR reaction volume of 10 µL, together with 5 µL 2× SYBR FAST qPCR Kit Master Mix (Kapa Biosystems/Hoffmann-La Roche, Basel, Switzerland), 2 µL of nuclease free water, and 0.5 µL of forward and reverse primer (10 µM). The primer combination qPSY-WG-F and qPSY-WG-R, targeting the species-specific *wingless* gene [57], was used to determine cDNA integrity. P. mali infection was detected in three of the six individuals with primer pair rpAP15f-mod and rpAP15r3, targeting the ribosomal protein gene *rpl22* [58]. *Pme2* expression was checked with primer pair ATP00136_GW_fwd and ATP00136_nostopp_rev using the same qPCR conditions as described for the qPCR detection in *Malus* × *domestica* leaf samples.

4.2. Amplification, Subcloning, and Sequencing of atp_00136

DNA was purified from leaves from P. mali infected *Malus x domestica* cv Golden Delicious trees (10 trees from Burggraviato and 10 trees from Val Venosta) using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was diluted 1:10 in water and 2 µL template were used in a total PCR reaction volume of 50 µL as follows: *atp_00136* was amplified using 0.02 U/µL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific,

Waltham, MA, USA) using HF-buffer supplied by the manufacturer, 400 µM dNTPs, and 0.5 µM of each primer (forward: 5'-CCCCCCGAATTCATGTTTCAATTTAAAAAAAATTTA-3'; reverse: 5'-CCCCCCGTCGACATTATTACTGTTGAGGTTTAA-3'). DNA was denatured at 98 °C for 30 s followed by 30 cycles of denaturation for 10 s at 98 °C, amplification for 30 s at 49.3 °C, and elongation at 72 °C for 30 s. The PCR was finalized by a terminal elongation step at 72 °C for 5 min. The PCR product was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and 1 µg of purified PCR product was digested with 4 U EcoRI and SalI following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA), ligated into equally digested pUC19 using T4-Ligase (Thermo Fisher Scientific, Waltham, MA, USA) and transformed into MegaX DH10B™ T1R cells (Life Technologies, Carlsbad, CA, USA). At least five clones from each tree were sequenced with pUC19 specific primers (GATC Biotech, Constance, Germany) and analyzed to see different variants of the gene indicating a mixed infection.

4.3. Subcloning of Pme2 into GreenGate Expression Vectors

The genes $pme2_{ST}$ and $pme2_{AT}$ were subcloned into the GreenGate-entry module pGGC000 [59] using the primer pair ATP00136pP_CBsaI_fw (5'-AACAGGTCTCAGGCTCCATGACGAAAAATGATCCAACAAA-3') and ATP00136pP_DBsaI_rv (5'-AACAGGTCTCACTGACTGTTGAGGTTTAAAACAT-3'). Using different components from the GreenGate-kit plant, transformation constructs coding for $pme2_{AT}$-*linker-GFP* or $pme2_{AT}$-*linker-mCherry* and $pme2_{ST}$-*linker-GFP* or $pme2_{ST}$-*linker-mCherry*, driven by the *35S* promoter and flanked at the 3'-end by the *RBCS* terminator, including kanamycin as the plant resistance marker, were designed. The following modules were assembled by GreenGate reaction in a total volume of 15 µL: 150 ng pGGA004 (*35S*), 150 ng pGGB003 (B-dummy), 150 ng pGGC000-$pme2_{AT}$ or pGGC00-$pme2_{ST}$, 150 ng pGGD001 (*linker-GFP*) or pGGD003 (*linker-mCherry*), 150 ng pGGE001 (*RBCS*), 150 ng pGGF007 (*pNOS*:Kan^R:*tNOS*), and 100 ng pGGZ001 (empty destination vector). Subsequently, 1.5 µL 10× CutSmart Buffer (New England Biolab, Ipswich, MA, USA), 1.5 µL ATP (10 mM), 1.0 µL T4 DNA Ligase (5 u/µL) (Thermo Fisher Scientific, Waltham, MA, USA), and 1.0 µL BsaI-HF®v2 (20,000 u/mL) (New England Biolab, Ipswich, MA, USA) were added to the module mixture, and 30 cycles of 2 min at 37 °C and 2 min at 16 °C each, followed by 50 °C for 5 min and 80 °C for 5 min were performed. Subsequently, 5 µL of the reaction mixture were used for heat-shock transformation of *ccdB*-sensitive One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). For the assembly of positive controls, the modules pGGC012 (*GFP-NLS*) or pGGC014 (*GFP*) or pGGC015 (*mCherry*) were used instead of the above mentioned pGGC000 modules. The correct assembly of the plant transformation constructs was confirmed by sequencing. Plasmid-DNA for protoplast transformation was obtained as described elsewhere [60], using the NucleoSnap® Plasmid Midi preparation kit (Macherey-Nagel, Düren, Germany) and PEG precipitation.

4.4. Protoplast Isolation and Transformation

Protoplasts of *N. benthamiana* and *N. occidentalis* were isolated from four- to five-week-old plants, cultivated under long photoperiod conditions (16 h/8 h, 24 °C/22 °C, 70% rH) and transformed as described in [60] using 10 µg plasmid-DNA per 20,000 protoplasts. After 18 h, at least 100 protoplasts of each transformation were checked for the occurrence of GFP or mCherry-fluorescence using a confocal laser scanning microscope (LSM800, Zeiss, Oberkochen, Germany) with an excitation wavelength of 488 nm for GFP and 561 nm for mCherry. The detection wavelength of GFP was set between 410 nm and 575 nm and of mCherry between 575 nm and 650 nm. Autofluorescence of chlorophyll was detected between 650 nm and 700 nm. After 20 h the number of intact protoplasts/mL was determined by counting in a Fuchs-Rosenthal chamber. Protoplast transformation and viability determination was repeated independently four times.

Only experiments in which at least 20% of the protoplasts in the control setup were viable after transformation were considered for further evaluation. Significant outliers were removed from the

data set using the GraphPad QuickCalcs Outlier calculator online tool (https://www.graphpad.com/quickcalcs/Grubbs1.cfm; status of information 16th September 2019). Greisser Greenhouse correction on raw data and one-way-ANOVA with a Tukey Posttest were performed to analyze statistical differences between groups (GraphPad Prism 7.01., GraphPad Software, San Diego, CA, USA). To allow a better visual comparison, data were normalized to each respective control, which was set to 1.

Additionally, protoplast viability was visualized by propidium iodide (PI) and counted by fluorescein diacetate (FDA) staining in three independent repetitions. For the first, 20 µL of protoplasts transformed with GFP tagged expression vectors were mixed with 20 µL of PI solution (10 µg/mL PI in 0.65 M mannitol). FDA staining was done according to [61] using 20 µL of protoplasts transformed with either mCherry tagged $PME2_{AT}$ expression vectors or a vector expressing only mCherry and 20 µL of FDA solution (0.1 mg/mL FDA in 0.65 M mannitol). Fluorescence of PI, mCherry, and GFP was recorded using a LSM800 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) with excitation and detection wavelengths for GFP and mCherry as described above and for PI excitation at 561 nm and detection between 560 nm and 640 nm.

4.5. Nicotiana spp. Leaf Infiltration

For subcellular localization of PME2, the two GreenGate expression vectors, as well as GFP and GFP-NLS expression vectors as positive controls, were subcloned by electroporation into *Agrobacterium tumefaciens* strain EHA105. As an additional control, we subcloned a GreenGate expression vector expressing the SAP11-like P. mali effector protein ATP_00189 [13] with an *N*-terminal fused GFP tag into *A. tumefaciens* strain EHA105. The transgenic *A. tumefaciens* clones were cultured for 2 days at 28 °C in liquid selective LB medium. Subsequently, 0.5 OD/mL were resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 200 µM acetosyringone, pH 5.7) and infiltrated with a blunt syringe into leaves from four- to five-week-old *N. occidentalis* and *N. benthamiana*. Fluorescence was recorded after 48 h and 72 h using the confocal laser scanning microscope (LSM800, Zeiss, Oberkochen, Germany) with excitation for GFP at 488 nm and detection between 410 nm and 546 nm and excitation for mCherry at 561 nm and detection between 562 and 624 nm.

4.6. Expression in Yeast

For a potential Y2H, $pme2_{AT}$ and $pme2_{ST}$ were subcloned into bait-vector pLexA-N as described in [13,62] with primer pair ATP00136_forw_EcoRI/ATP00136_rev_SalI. The bait-plasmids pLexA-N-$pme2_{ST}$ and pLexA-N-$pme2_{AT}$ were transformed into *S. cerevisiae* strain NMY51. Growth aberrations of yeast colonies on selective SD-trp plates were observed and recorded by photographing.

For calcofluor white staining, yeast cells were grown overnight in SD-trp liquid media. Subsequently, 2 mL of the overnight culture were centrifuged, supernatant removed, and the cells resuspended in clear phosphate-buffered saline (PBS) buffer. Then, 10 µL of a 5 mM calcofluor white solution (Biotium, Fremont, California) were added to the cell suspension and incubated for 20 min at room temperature. The yeast cell wall was visualized by a confocal laser scanning microscope (LSM800, Zeiss, Oberkochen, Germany) with excitation at 405 nm and detection wavelength between 400 nm and 560 nm.

5. Conclusions

In this study we identified and characterized the novel P. mali effector protein PME2. This effector contains an NLS and an NES sequence and translocates to the nucleus of *N. benthamiana* mesophyll protoplasts. Two naturally occurring genetic variants of PME2, namely $PME2_{ST}$ and $PME2_{AT}$, differ regarding their ability to induce cellular modifications in yeast and plant cells. When overexpressed, the variant $PME2_{ST}$ affects yeast growth and reduces the viability of *Nicotiana* spp. mesophyll protoplasts. These findings indicate that PME2 might play a role for P. mali virulence in plants. Despite the similarities between both PME2 variants, this effect was not observed in yeast or

protoplasts expressing $PME2_{AT}$. The results of our study show for the first time that a phytoplasmal effector causes detrimental effects when overexpressed in protoplasts.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/18/4613/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AP	Apple proliferation
AY-WB	Aster Yellow Witches' Broom
C. picta	*Cacopsylla picta*
FDA	Fluorescein diacetate
GFP	Green fluorescent protein
N.	*Nicotiana*
NES	Nuclear export signal
NLS	Nuclear localization signal
PI	Propidium iodide
P. mali	*Candidatus* Phytoplasma mali
PME2	Protein in Malus Expressed 2
qPCR	quantitative PCR
SNP	Single nucleotide polymorphism
Y2H	Yeast two-hybrid

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Publication 4

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The *'Candidatus* Phytoplasma mali' effector protein $SAP11_{CaPm}$ interacts with MdTCP16, a class II CYC/TB1 transcription factor that is highly expressed during phytoplasma infection.

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Author contributions:

Cecilia Mittelberger: Design and performance of the study, targeted Y2H screen, BiFC experiments, evaluation of all results, writing the manuscript

Bettina Hause: Discussion of methods and results, Proofreading of the manuscript

Katrin Janik: Supervision and design of the study, first Y2H screen, discussion of methods and results, assistance in writing the manuscript

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RESEARCH ARTICLE

The '*Candidatus* Phytoplasma mali' effector protein SAP11$_{CaPm}$ interacts with MdTCP16, a class II CYC/TB1 transcription factor that is highly expressed during phytoplasma infection

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Abstract

'*Candidatus* Phytoplasma mali', is a bacterial pathogen associated with the so-called apple proliferation disease in *Malus* × *domestica*. The pathogen manipulates its host with a set of effector proteins, among them SAP11$_{CaPm,}$ which shares similarity to SAP11$_{AYWB}$ from '*Candidatus* Phytoplasma asteris'. SAP11$_{AYWB}$ interacts and destabilizes the class II CIN transcription factors of *Arabidopsis thaliana*, namely AtTCP4 and AtTCP13 as well as the class II CYC/TB1 transcription factor AtTCP18, also known as BRANCHED1 being an important factor for shoot branching. It has been shown that SAP11$_{CaPm}$ interacts with the *Malus* × *domestica* orthologues of AtTCP4 (MdTCP25) and AtTCP13 (MdTCP24), but an interaction with MdTCP16, the orthologue of AtTCP18, has never been proven. The aim of this study was to investigate this potential interaction and close a knowledge gap regarding the function of SAP11$_{CaPm}$. A Yeast two-hybrid test and Bimolecular Fluorescence Complementation *in planta* revealed that SAP11$_{CaPm}$ interacts with MdTCP16. MdTCP16 is known to play a role in the control of the seasonal growth of perennial plants and an increase of *MdTCP16* gene expression has been detected in apple leaves in autumn. In addition to this, *MdTCP16* is highly expressed during phytoplasma infection. Binding of MdTCP16 by SAP11$_{CaPm}$ might lead to the induction of shoot proliferation and early bud break, both of which are characteristic symptoms of apple proliferation disease.

Introduction

'*Candidatus* Phytoplasma mali' ('*Ca*. P. mali') is the bacterial pathogen associated with the so-called apple proliferation disease [1]. Apple trees affected by this disease show different growth aberrations like uncontrolled shoot proliferation or enlarged stipules, early bud break and flowering and so-called witches' brooms. Symptomatic trees produce small, tasteless, and

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unmarketable fruits, which causes economic losses and threatens apple production [2]. Phytoplasmas are wall-less, phloem-limited bacteria, that need insect vectors for their transmission and use effector proteins to manipulate their host plants [3–6]. '*Ca*. P. mali' is transmitted by certain psyllids [7] and encodes several potential effector proteins that appear to be released into *Malus* plants by a Sec-dependent secretion system [8,9]. So far, the best characterized effector protein is $SAP11_{CaPm}$, which shows similarity to $SAP11_{AYWB}$ from '*Candidatus* Phytoplasma asteris' that is associated with Aster Yellow Witches' Broom disease in aster [10]. Despite of being the best described effector, $SAP11_{CaPm}$'s function is not fully unraveled yet, and there are still knowledge gaps regarding its interaction partners in *Malus* × *domestica*, the natural host of '*Ca*. P. mali'.

The development of witches' brooms, i.e., uncontrolled branching, is a common symptom not only for apple proliferation, but also for several other phytoplasma diseases. Overexpression of $SAP11_{AYWB}$ and SAP11-like proteins from other phytoplasma species resulted in an increased formation of lateral shoots [11–15]. Interestingly, $SAP11_{AYWB}$, $SAP11_{CaPm}$ and other SAP11-like proteins bind and destabilize different TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 1 and 2 (TCP) transcription factors [12,14,16].

TCP transcription factors are highly conserved in all land plant lineages and contain a basic helix-loop-helix (bHLH) domain, responsible for DNA binding [17]. TCPs are classified according to sequence differences in the bHLH-domain in class I and class II subfamilies [18]. The class II TCPs are further subdivided in CINCINNATA (CIN)-like TCPs and in CYCLOIDEA/TEOSINTE BRANCHED1 (CYC/TB1)-like TCPs [19]. TCPs of both classes play a key role in morphological development of plants, stress adaptions and plant immunity [20] and are thus interesting targets of diverse pathogen effector proteins [21,22].

A total of 52 TCP-domain containing genes were identified in apple [23]. For CIN-like class II TCPs, MdTCP25 (orthologue to AtTCP4) and MdTCP24 (orthologue to AtTCP13), an interaction with $SAP11_{CaPm}$ has been shown [8]. Additionally, a yeast-two-hybrid (Y2H) screen using $SAP11_{CaPm}$ revealed cDNA fragments of MdTCP16, a AtTCP18-like CYC/TB1 class II TCP and of the putative chlorophyllide b reductase NYC1 as putative interaction partners [8]. An interaction between $SAP11_{CaPm}$ and the corresponding full-length gene products of the MdTCP16- and MdNYC1-fragment could not be confirmed at that time. Shortly after, the genome of *Malus* × *domestica* was *de novo* assembled and updated [24]. This sheds new light on the full-length ORFs including *MdTCP* encoding genes and led to some new sequence and reading-frame information regarding the assigned full-length genes of *MdTCP16* and *MdNYC1*.

AtTCP18 is a key regulator of shoot branching [25–27] and is also known as BRANCHED1 (BRC1). BRC1 is used as a common term for all AtTCP18 orthologues in other plant species. Its expression is repressed by cytokinin, gibberellin, phytochrome B and sugar, and is promoted by auxin, an important regulator of apical dominance, strigolactones and a low red to far-red light ratio [28]. BRC1 influences not only the plant architecture, but also the seasonal growth of perennial plants, such as temperate fruit trees by responding to photoperiodic changes [29]. In addition, BRC1 interacts with the FLOWERING LOCUS T protein in Arabidopsis and represses floral transition in axillary meristems [30].

Since several other SAP11-like proteins interact with AtTCP18-like TCPs [11,13,15,31,32] and $SAP11_{CaPm}$ from '*Ca*. P. mali' strain PM19 showed an interaction with AtTCP18 in an Y2H screen [16], the aim of this study was a detailed analysis of the two potential interactions between $SAP11_{CaPm}$ with either MdTCP16 (i.e. the orthologue of AtTCP18) or MdNYC1. Moreover, to gain a better understanding of the role of MdTCP16, MdTCP24 and MdTCP25 in the host plant during phytoplasma-infection, the expression of these *TCPs* was determined by qPCR analysis of non-infected and infected *Malus* × *domestica* leaf samples in spring and autumn.

Materials and methods

Yeast-two-hybrid screen

In a previous study cDNA sequences that were partially similar to genes encoding *MdTCP16* and *MdNYC1* were identified as interactors of $SAP11_{CaPm}$ from '*Ca*. P. mali' strain STAA (Accession: KM501063) by a Y2H screen [8]. These cDNA nucleotide sequences were obtained by sequencing the prey vectors in the identified yeast colonies and blasting against the NCBI nt-database [33,34]. The sequence, identified as *MdTCP16*, shared 100% sequence identity with the reference sequence XM_008376500.2 and covers 69.5% of the coding sequence for protein XP_008374722.1 (S1 Fig). Primers were designed to amplify the coding sequence of XP_008374722.1 (S1 Table). These primers contained overhangs that attached *Sfi*I-restriction sites to the amplicon for subsequent cloning into the pGAD-HA Y2H prey vector.

The sequence identified as *MdNYC1* shared 100% sequence identity to the reference sequence XM_029109831.1 and covers 13.7% of the C-terminal end of XP_028965664.1. Primers were designed to amplify the coding sequence of XP_028965664.1 and of the C-terminal part identified as an interactor of $SAP11_{CaPm}$ in the Y2H screen. Both amplicons were subcloned into pGAD-HA prey vector via their primer-attached *Sfi*I-overhangs.

The Y2H prey vector was co-transformed with pLexA-N-$SAP11_{CaPm}$ bait vector into the *Saccharomyces cerevisiae* strain NMY51 cells [8,35]. Growth was monitored four days after transformation on selective SD plates lacking the amino acids adenine, leucine, histidine and tryptophane.

Bimolecular fluorescence complementation analysis

Primers, specific for *MdTCP16* and $SAP11_{CaPm}$ from '*Ca*. P. mali' strain STAA and with *attB*-site overhangs were designed (S1 Table) and fragments were amplified in a total of 50 μL reaction volume using 10 ng template and a final concentration of 1 x iProof HF Buffer, 200 μM dNTPs (50 μM each nucleotide), 0.5 μM of each primer and 0.02 U/μl iProof DNA Polymerase (Bio-Rad, Hercules, CA). The cycling conditions were 30 sec of initial denaturation at 98˚C followed by 35 cycles of 98˚C for 10 sec, 60˚C for 30 sec and 72˚C for 90 sec. The final extension was carried out at 72˚C for 5 min. The amplified fragments were analyzed on a 1.2% agarose-gel and extracted with Kit Montage Gel Extraction columns (Millipore, Bedford, MA). The purified amplicons were used in a BP-reaction for the creation of Gateway-Entry vectors using 1 μL purified amplicon, 100 ng of donor vector (pDONR221-P1P4 or pDONR221-P3P2), 1 μL BP Clonase™ II enzyme (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Depending on the N- or C-terminal location of the split-YFP in pBiFC vectors [36], *MdTCP16* and $SAP11_{CaPm}$ entry-vectors with or without stop-codon were combined in a LR-reaction using Gateway™ using LR Clonase™ II enzyme mix (Invitrogen) and following the manufacturer's instructions.

Nicotiana benthamiana leaf mesophyll protoplasts were isolated for bimolecular fluorescence complementation analysis (BiFC) from leaves of four-week-old plants and protoplasts were transformed with the pBiFC vectors as described in Janik et al. (2017) [37]. In detail, leaf pieces of two *N. benthamiana* leaves were vacuum infiltrated for 30 min with 10 mL of enzyme solution containing 0.4 M mannitol, 20 mM KCl, 20 mM 4-morpholineethanesulfonic acid (MES), 1.5% (w/v) cellulase R-10, 0.4% (w/v) macerozyme R-10, 10 mM $CaCl_2$, and 1 mg/mL bovine serum albumin. The leaf pieces were then incubated in the dark at room temperature for 4 h and afterwards gently shaken for 30 min in the dark to release the protoplasts. The protoplast-leaf pieces solution was filtrated through a cell strainer into two 15 mL round bottom centrifuge tubes and centrifuged at 200 x g and 4˚C for 1 min. The supernatant was carefully removed, and the first pellet was resuspended in 3 mL buffer W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES at pH 5.7). The second pellet was carefully resuspended with

the first suspension. The protoplast suspension was incubated in the dark on ice for 40 min for sedimentation of the protoplasts. The protoplast pellet was again carefully resuspended in 3 mL buffer W5 and incubated for another 40 min in the dark on ice. The protoplast pellet was resuspended in 2 mL buffer MMG (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES at pH 5.7) and protoplast density was counted in a Neubauer improved cell counting chamber. The protoplast solution was diluted with buffer MMG to a final concentration of 100,000 protoplasts per mL. 10 μg of pBiFC vector plasmid DNA, obtained by plasmid preparation with NucleoSnap Plasmid Midi kit (Macherey-Nagel, Düren, Germany) followed by a PEG precipitation, were mixed with 200 μL of protoplast solution. 220 μL of PEG-transformation-solution (0.2 M mannitol, 0.1 M $CaCl_2$, 40% (w/v) PEG 4000) were added to the protoplast-DNA mix and mixed by carefully shaking the tube. After 5 min of incubation at room temperature, 880 μL of buffer W5 were carefully added to the suspension. The protoplast transformation solution was centrifuged for 1 min at 4˚C and the pellet was resuspended in 200 μL buffer WI (0.5M mannitol, 20mM KCl, 4mM MES). The tubes were then placed horizontally in the dark for incubation at room temperature overnight. Transformed protoplasts were analyzed 16h after transformation using a confocal laser scanning microscope (Zeiss LSM800, Carl Zeiss Microscopy, Oberkochen, Germany). Transformation rate and BiFC rate were determined as a mean value of three independent repetitions of protoplast transformation.

Plant material

Trees in the greenhouse: Apple rootstocks M9 were graft inoculated with '*Ca*. P. mali' strain PM6 (AT2-subtype) [38] infected or non-infected control scions of *Malus x domestica* cultivar (cv.) Golden Delicious and kept in greenhouse without temperature or light control. These small, one-year-old plants had a maximum height of about 40 cm.

Trees in the foil tunnel: Fully grown, ca. 2 m high, naturally infected *Malus x domestica* cv. Golden Delicious trees were grown and kept in an insect safe foil tunnel. Trees were infected with the locally predominant AT2 strain as determined by Sanger sequencing. Since it was a natural infection it cannot be ruled out, however, that a mixed infection with other strains was present in these trees. Nevertheless, this situation resembles the one in the field. Leaf samples for the analyses were taken in May and October 2011 as described in Janik et al. 2017 [8]: For each time-point pools of leaves from non-infected and infected trees (eleven trees/pool in May and six trees/pool in October), respectively, were assembled. Each of the pools of infected trees comprised material from trees representing the same symptom intensities. Equal amounts of material from each tree were pooled.

The phytoplasma levels from pooled leaf and root samples are reported in S2 Fig.

RNA extraction and cDNA synthesis

Greenhouse samples were collected in May and October 2021 from single apple trees. Leaf discs from five non-infected and five infected, one year old apple trees, grown in greenhouse were excised, and immediately frozen in liquid nitrogen. Leaf discs were grinded with mortar and pestle under liquid nitrogen. 100 mg of leaf powder was used for RNA extraction following protocol A of the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). RNA was eluted in 50 μL elution buffer. RNA concentration was measured with a Spectrophotometer (NanoPhotometer® N60, Implen, München, Germany). 1 μg or 2 μg of RNA were reverse transcribed into cDNA by using SuperScript™ IV VILO™ Master Mix with ezDNase enzyme (Invitrogen), following the protocol that includes gDNA digestion with DNase enzyme. Samples that contained all reagents and RNA except the reverse transcriptase ("No-RT controls") were performed in parallel. Synthesized cDNA was stored at -80˚C.

Sampling of naturally infected trees of *Malus* × *domestica* cv. Golden Delicious was done in May and October 2011. Three pools of leaves from control or '*Ca*. P. mali'-infected trees (6–11 trees/pool) were tested. RNA extraction and cDNA preparation is described in Supplementary Material and Methods of Janik et al. (2017) [8]. Synthesized cDNA was stored at -80˚C.

DNA extraction

DNA of *Malus* × *domestica* leaf samples was extracted from approx. 50 mg grinded leaf tissue, using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands), following the manufacturer's instruction. DNA was eluted in 50 μL elution buffer.

qPCR analysis

The transcription factors *MdTCP16*, *MdTCP25*, *MdTCP24* and the effector protein $SAP11_{CaPm}$ were amplified with specific qPCR primer pairs (S1 Table) using a SYBR-Green qPCR assay. Reactions were run in a total of 10 μl, using 2 x Universal KAPA SYBR® FAST master mix (KAPABIOSYSTEMS, Wilmington, MA), 20 pmol of forward and reverse primer and 2 μl of 1:50 diluted cDNA samples. Additionally, qPCR master mixes for the reference genes *GAPDH*, *tip41* and *EF1α* were prepared for all samples, using the same reagent and template concentrations, and cycling conditions as mentioned above. All targets were amplified on the same 384well plate in one qPCR run on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), using three technical replicates per sample. Cycling conditions were as follows: 95˚C for 20 sec, followed by 35 cycles with 95˚C for 3 sec and 60˚C for 30 sec, melt curve from 65˚C to 95˚C with an increment of 0.5˚C/5 sec.

For determining the qPCR efficiency of every primer-combination, a four-point dilution series (1:10, 1:50, 1:100, 1:200) of a cDNA sample mixture was analyzed in each qPCR run.

Phytoplasma quantity was determined by the detection of the phytoplasma specific *16S* gene together with the *Malus* specific single copy gene *ACO* as described by Baric et al. (2011) [39]. In brief, 2 μl of template DNA were analyzed in a total multiplex reaction volume of 20 μl, using 2x iQ^{TM} supermix master mix (Bio-Rad), 18 pmol of each qAP-16S forward and reverse primer, 4 pmol qAP-16S probe, 4 pmol of each qMD-ACO forward and reverse primer and 4 pmol of qMD-ACO probe. The '*Ca*. P. mali' specific qAP-16S probe was 5'-labeled with the reporter dye FAM, while the *Malus* specific qMD-ACO probe was 5'-labeled with VIC. Cycling conditions were as follows 95˚C for 3 min followed by 35 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Reactions were run in triplicates on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For determining the qPCR efficiency, a five-point dilution series (undiluted, 1:10, 1:50, 1:100, 1:200) of a sample mixture of DNA from infected *Malus* × *domestica* roots was analyzed in each qPCR run.

Data analysis

Normalized expression was calculated according to Taylor et al. (2019) [40] considering qPCR efficiency (E) of each run, following the formula $1+E^{\Delta Cq}$ for relative quantity. Statistical analysis was performed with GraphPad Prism 7.05 (GraphPad Software Inc., La Jolla, CA).

Results

Yeast-two-hybrid and bimolecular fluorescence complementation analyses

The Y2H test using $SAP11_{CaPm}$ from '*Ca*. P. mali' strain STAA fused to the GAL4-binding domain as bait revealed an interaction between MdTCP16 and $SAP11_{CaPm}$. An interaction

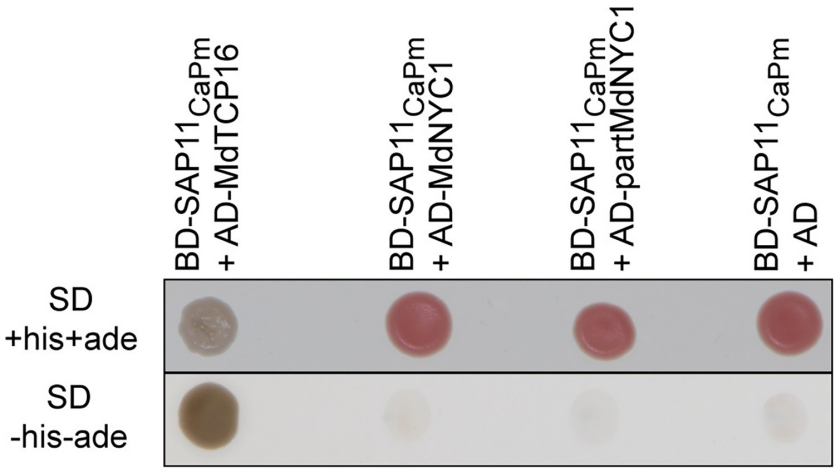

Fig 1. SAP11$_{CaPm}$ interacts with MdTCP16 but not with MdNYC1 in yeast. Interaction between the bait SAP11$_{CaPm}$ fused to a DNA binding domain (BD) and the prey protein from *Malus* × *domestica* fused to an activation domain (AD) is indicated by growth of the *Saccharomyces cerevisiae* reporter strain NMY51 on SD minimal medium lacking the amino acids histidine (his) and adenine (ade). The white color of BD-SAP11$_{CaPm}$ + AD-MdTCP16 grown on full medium is an additional indication for a strong interaction, since the ADE2 reporter gene is activated upon interaction, while in absence of a protein-protein interaction and thus no ADE2 activation, a red colored intermediate accumulates in the adenine metabolic pathway.

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between SAP11$_{CaPm}$ and MdNYC1 (neither the fragment nor the full-length protein) was not detectable (Fig 1).

For further confirmation of the interaction between SAP11$_{CaPm}$ and MdTCP16, the sequences of SAP11$_{CaPm}$ and MdTCP16 were subcloned into different pBiFC-2in1 vectors [36] by Gateway-cloning [41]. The pBiFC-2in1 vectors were transformed into *N. benthamiana* mesophyll protoplasts [37] and interaction was analyzed by confocal microscopy 16 h after transformation. Up to 92.5% of the transformed protoplasts showed a YFP signal, resulting from the interaction between SAP11$_{CaPm}$ and MdTCP16 (Fig 2). The YFP-signal was localized

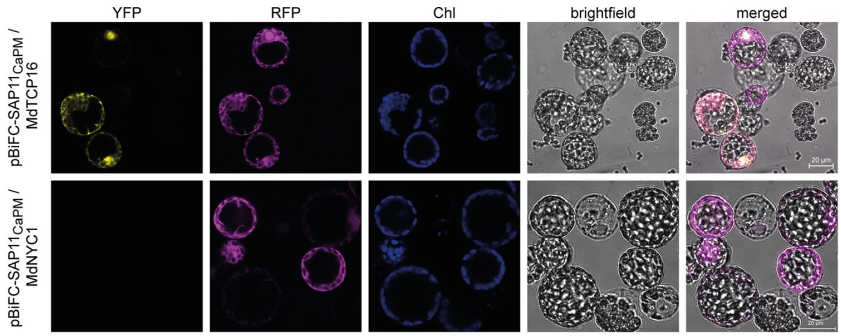

Fig 2. SAP11$_{CaPm}$ interacts with MdTCP16 *in planta*. *Nicotiana benthamiana* mesophyll protoplasts were co-transformed with a BiFC expression vector encoding SAP11$_{CaPm}$ and MdTCP16. SAP11$_{CaPm}$ and MdTCP16 interact in the nucleus and in the cytoplasm as indicated by the occurrence of a YFP signal in these two cellular compartments. The co-expression of SAP11$_{CaPm}$ and MdNYC1 did not show any YFP signal. The RFP signal (depicted in magenta) indicates a successful transformation of protoplasts with the BiFC vector. Chl (depicted in blue) shows the autofluorescence of chlorophyll within the chloroplasts. Microscopic analysis was performed with a Zeiss LSM 800 confocal microscope. Bars represent 20 μm for all micrographs.

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to the cell nucleus and occurred additionally in the cytoplasm. The co-expression of $SAP11_{CaPm}$ and MdNYC1 fused to both YFP-halves did not reveal YFP fluorescence after transformation of protoplasts, confirming the Y2H results (Fig 2).

Taken together, these results show that $SAP11_{CaPM}$ interacts with MdTCP16 in addition to the previously shown interaction with MdTCP24 and MdTCP25 [8].

Expression of TCPs in *Malus* × *domestica* during infection

It was unclear how an infection with '*Ca*. P. mali' affects the expression of the three TCP-encoding genes being the targets of its effector $SAP11_{CaPm}$. Thus, to analyze the expression of *MdTCP16*, *MdTCP24* and *MdTCP25* during infection in *Malus* × *domestica* host plant, qPCR assays were used to determine expression levels of the respective TCPs and the effector protein $SAP11_{CaPm}$ (Fig 3).

MdTCP24 was stably expressed throughout the season and its transcript levels did not change upon infection, regardless of whether the trees were grown in the field or in the greenhouse (Fig 3A and 3B). Also, *MdTCP25* in leaves from greenhouse plants was stably expressed throughout the season, but in naturally infected samples the expression was significantly higher in samples taken in spring than in those taken later in the season, for both non-infected and infected trees. Furthermore, regarding field-grown trees in spring, *MdTCP25* expression is significantly lower in naturally infected samples compared to non-infected samples (Fig 3A and 3B). The expression of *MdTCP16* was lower than those of *MdTCP24* and *MdTCP25*. *MdTCP16* transcript levels were low in both infected and non-infected samples from spring and higher in samples from autumn. Furthermore, it was tendentially, but not significantly higher in infected autumn samples than in non-infected autumn samples (Fig 3A and 3B). $SAP11_{CaPm}$ expression could not be detected in naturally infected spring samples, but it was detectable in the leaves from greenhouse samples throughout the season (Fig 3C–3E). $SAP11_{CaPm}$ expression in leaves tends to be increased when phytoplasma level in the same tissue is high (Fig 3F).

The expression of *MdTCP16* significantly correlated positively with the expression of $SAP11_{CaPm}$ in leaves from trees of the greenhouse ($R^2 = 0.40$) and there was a trend of this finding also in leaves from trees cultivated in the foil tunnel ($R^2 = 0.52$). *MdTCP16* expression also strongly correlated positively ($R^2 = 0.97$) with the phytoplasma quantity in leaf samples (Fig 3C and 3F, Table 1). *MdTCP16* expression furthermore negatively correlates ($R^2 = 0.74$) with the phytoplasma concentration (3F, Table 1). This is opposite to the findings for *MdTCP25*, whose expression correlated negatively with $SAP11_{CaPm}$ quantity in leaf samples of trees from the greenhouse ($R^2 = 0.15$) and from the foil tunnel ($R^2 = 0.78$) (Fig 3D). The negative correlation between *MdTCP25* and $SAP11_{CaPm}$ expression was significant in leaves from the foil tunnel, whereas in the samples from greenhouse tree a trend of a negative correlation was observed (Fig 3D). *MdTCP24*, in contrast, did neither correlate with the expression of $SAP11_{CaPm}$ nor with the phytoplasma quantity (Fig 3E and 3F).

Discussion

It is known that $SAP11_{AYWB}$ from AYWB-phytoplasma interacts and destabilizes AtTCP18 in *Arabidopsis thaliana* [12], but it was so far unknown if $SAP11_{CaPm}$ from '*Ca*. P. mali' can interact with MdTCP16, the orthologous of AtTCP18 in *Malus* × *domestica*, the actual host plant of this phytoplasma species. It has been shown previously that $SAP11_{CaPm}$ from strain PM19, that shares 99.2% sequence identity to $SAP11_{CaPm}$ from strain STAA used in this study, interacts with AtTCP18 in a Y2H test [16]. However, to understand the potential effects of the effector protein $SAP11_{CaPm}$, it is important to unravel the targets not only in model plants but in the

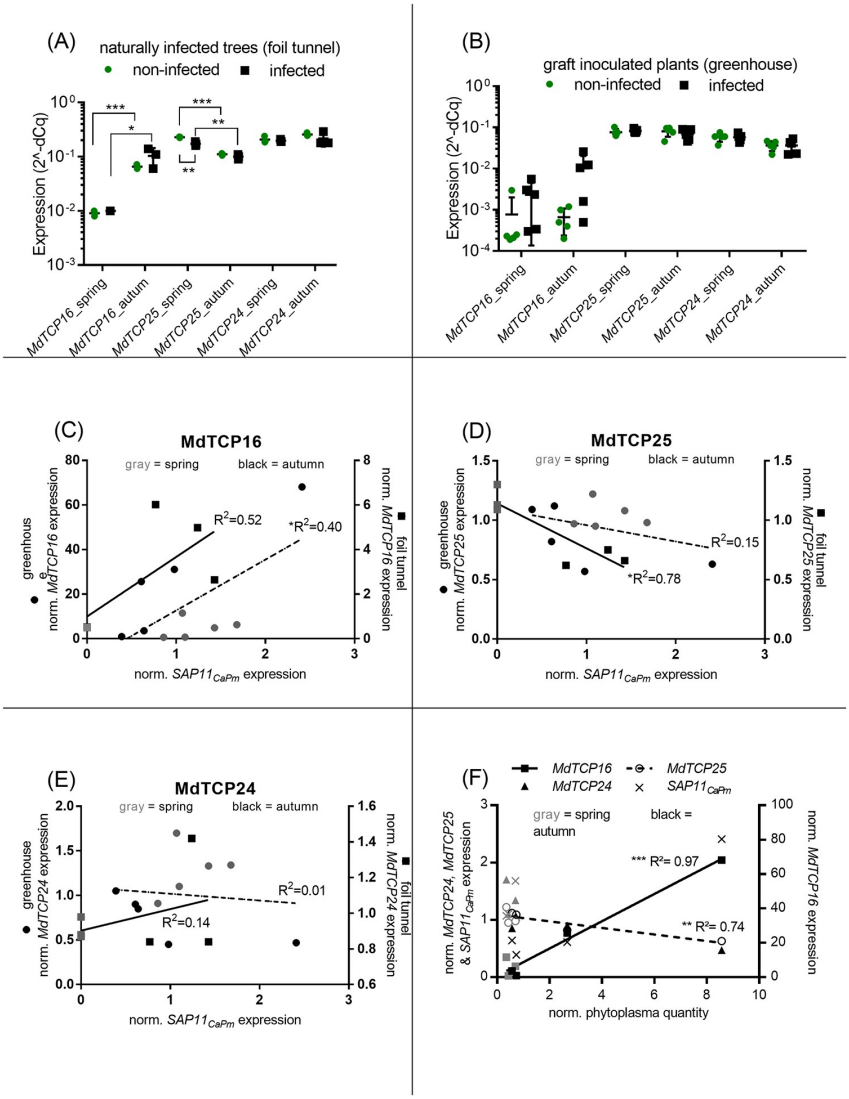

Fig 3. *MdTCP16* expression increases from spring to autumn, is slightly higher in infected samples and strongly correlates to phytoplasma levels in leaves from infected *Malus* × *domestica*. *MdTCP* expression in spring and autumn of non-infected (green) and naturally infected (black) apple leaves (A) or graft inoculated and grown in greenhouse plants (B). Naturally infected samples (A) comprise one leaf pool from non-infected trees, and three leaf pools from infected trees. Graft inoculated greenhouse plants (B) comprise leaf samples from 5 non-infected and 5 infected trees, each represented by a data point. Correlation of *MdTCP* and $SAP11_{CaPm}$ expression (C, D, E) in infected *Malus* × *domestica* leaf-samples from spring (grey, n = 8) and autumn (black, n = 8). Lines in graphs C, D, E and F show linear regression of the respective samples either from the greenhouse (dashed line) or from the foil tunnel (solid line). Samples in C, D and E were grouped regarding their growing conditions and plotted to different y-axis due to the differences in the concentration ranges of the different sample subsets. Correlation of *MdTCP*, $SAP11_{CaPm}$ expression and phytoplasma levels (F) in graft-inoculated *Malus* × *domestica* leaf-samples from spring (grey, n = 3) and autumn (black, n = 4). Statistical analysis was performed with multiple t-test. Statistical differences were determined using the Holm-Sidak method, with alpha = 0.05 and linear regression analysis, using GraphPad Prism 7.05 (GraphPad Software Inc.). For pools comprising only one biological replicate, technical replicates accounted to the statistical analysis. Significant differences between groups are indicated with asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Data points with very similar values might overlap in the graph and can thus misleadingly appear as a single data point. In only seven out of the ten samples from the greenhouse phytoplasma concentration could be determined (F). This was due to a lack of sample material for the DNA preparation which is necessary for phytoplasma detection.

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Table 1. Linear regression analysis of TCP expression depending on phytoplasma quantity or $SAP11_{CaPm}$ expression. 95% confidence interval (CI), goodness of fit and significance level (alpha = 0.05) of linear regression, calculated with GraphPad Prism 7.05 (GraphPad Software Inc.), between normalized phytoplasma quantity and $SAP11_{CaPm}$, *MdTCP16*, *MdTCP25* and *MdTCP24* expression (left panel) and between normalized $SAP11_{CaPm}$ expression and *MdTCP*s expression in leaves from greenhouse (middle panel) and from foil-tunnel, respectively (right panel).

	normalized phytoplasma quantity (greenhouse)				normalized $SAP11_{CaPm}$ expression (greenhouse)			normalized $SAP11_{CaPm}$ expression (foil tunnel)		
	$SAP11_{CaPm}$	*MdTCP16*	*MdTCP25*	*MdTCP24*	*MdTCP16*	*MdTCP25*	*MdTCP24*	*MdTCP16*	*MdTCP25*	*MdTCP24*
95% CI										
Slope	-0.020 to 0.358	6.195 to 9.67	-0.096 to -0.019	-0.196 to 0.006	0.194 to 45.46	-0.405 to 0.13	-0.603 to 0.452	-0.912 to 6.262	-0.658 to -0.0958	-0.320 to 0.562
Y-intercept	0.143 to 1.439	-5.168 to 6.743	0.955 to 1.22	0.903 to 1.595	-38.49 to 18.16	0.762 to 1.43	0.435 to 1.75	-2.0 to 3.99	0.907 to 1.38	0.535 to 1.27
X-intercept	-infinity to -0.498	-0.995 to 0.585	12.01 to 54.6	7.031 to +infinity	-78.23 to 1.014	3.4 to +infinity	2.708 to +infinity	-infinity to 0.456	1.923 to 10.28	-infinity to -1.071
Goodness of Fit										
R^2	0.513	0.965	0.743	0.540	0.403	0.150	0.0135	0.517	0.776	0.127
P value	0.07	<0.001	0.01	0.06	0.049	0.269	0.750	0.107	0.02	0.489
Deviation from zero?	Not Significant	Significant	Significant	Not Significant	Significant	Not Significant	Not Significant	Not Significant	Significant	Not Significant
Equation	Y = 0.169*X + 0.791	Y = 7.932*X + 0.788	Y = -0.057*X + 1.087	Y = -0.095*X + 1.249	Y = 22.83*X-10.16	Y = -0.1377*X + 1.097	Y = -0.07555*X + 1.094	Y = 2.675*X + 0.9928	Y = -0.3771*X + 1.141	Y = 0.1209*X + 0.9024
Number of X values	7	7	7	7	10	10	10	16	16	16

95% confidence interval (CI), goodness of fit and significance level (alpha = 0.05) of linear regression, calculated with GraphPad Prism 7.05 (GraphPad Software Inc.), between normalized phytoplasma quantity and $SAP11_{CaPm}$, *MdTCP16*, *MdTCP25* and *MdTCP24* expression (left panel) and between normalized $SAP11_{CaPm}$ expression and *MdTCP*s expression in leaves from greenhouse (middle panel) and from foil-tunnel, respectively (right panel).

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natural host plant and to understand the induced changes in TCP expression in the native pathosystem.

Our data show unequivocally that $SAP11_{CaPm}$ interacts with MdTCP16, both yeast and in planta, whereas another candidate (MdNYC1) does not interact (Figs 1 and 2). $SAP11_{CaPm}$ also binds to MdTCP25 and MdTCP24 from *Malus* × *domestica* and degrades different AtTCPs at the protein level, among them the orthologue of MdTCP25, i.e. AtTCP4 [16], but it is not known, whether binding of $SAP11_{CaPm}$ affects the stability of MdTCP16, since it was not able to destabilize the *Arabidopsis thaliana* orthologue AtTCP18 [11,31]. Interestingly, our study indicates that *MdTCP16* expression is induced during phytoplasma infection and might then be counter-regulated by $SAP11_{CaPm}$ at the protein level. A degradation of MdTCP16 induced by $SAP11_{CaPm}$ at the protein level could induce *MdTCP16* expression on the transcriptional level via a gene-product mediated feedback loop regulation [42]. The MdTCP16 protein might act like a repressor and negatively affects expression of the *MdTCP16* gene, meaning that skimming the proteinaceous gene product leads to an increase of its gene expression. That might explain why *MdTCP16* transcriptional expression levels are by trend increased in infected plants and that phytoplasma level in leaves is positively correlated with the expression of this transcription factor (Fig 3A–3E) which has been shown also for '*Ca*. P. ziziphi' infected *Ziziphus jujube*, another phytoplasma pathosystem where the *ZjTCP7* that

encodes a MdTCP16 orthologue of *Z. jujube*, is upregulated in leaves [43]. It is worth noting that–in contrast to *MdTCP16* –*MdTCP25* expression is adversely i.e., negatively and *MdTCP24* expression not at all correlated with $SAP11_{CaPm}$ expression during infection. This implies that other regulatory mechanisms might be involved in the regulation of *MdTCP25* and *MdTCP24* expression.

Whatever pathway is affected during *MdTCP16* expression, it seems reasonable to speculate that it is important for the phytoplasma to attack MdTCP16 in its plant host.

The increased *MdTCP16* expression in autumn samples might be an indication that this TCP is involved in the seasonal control of branching. *MdTCP16* is mainly expressed in axillary and flower buds and only weakly in leaves, stems and shoot tips of apple trees [44]. Short photoperiods lead to the expression of *BRC1* –an orthologue of *MdTCP16* –controlling a complex network that regulates bud dormancy [45]. Interestingly, early bud break is a symptom of Phytoplasma-infected apple trees. It is tempting to speculate that '*Ca*. P. mali' infection affects *MdTCP16* expression via $SAP11_{CaPm}$ in axillary buds and is thus involved in the induction of early bud break. Our study was, however, focused on gene expression analyses in leaves, thus based on the current data availability it would be very speculative to hypothesize about $SAP11_{CaPm}$'s function in other plant tissues.

No $SAP11_{CaPm}$ expression was detected in leaf samples from naturally infected trees in spring, but the effector was detectable in leaves from greenhouse trees already in spring. This discrepancy might be explained by differences in environmental and physiological circumstances between the fully grown naturally infected trees and the small and young trees from the greenhouse. It is possible that the phytoplasma colonization of the canopy occurs earlier or that the bacterial colonization is more uniform in the foliage part of the smaller greenhouse plants.

'*Ca*. P. mali' infection leads to increased soluble sugar content in phloem sap [46] and in leaves [47]. Sugar promotes the bud outgrowth by acting as a repression-signal for *BRC1* expression [25,48]. This is neither in line with the increased *MdTCP16* expression that we find in phytoplasma infected apple leaves, nor with what has been described for *ZjTCP7* expression in phytoplasma infected Chinese jujube [43]. Thus, other factors might outcompete the repressing effect of increased sugar levels on *MdTCP16* expression, such as an increased auxin level in leaves of infected apple trees [49]. Auxin induces the *MdTCP16* expression but blocks the axillary bud outgrowth in non-infected plants [50,51].

Taken together, BRC1 and its homologues seem to be important molecular targets of SAP11-like effector proteins from different phytoplasma species. The results of this study prove the interaction of $SAP11_{CaPm}$ and the MdTCP16 transcription factor. SAP11-like proteins might be crucial for the successful phytoplasma colonization of the canopy in spring by downshifting BRC1. In the context of the current knowledge, it can be assumed that this downregulation is involved in the formation of lateral shoot outgrowth and early bud break, which are typical symptoms of '*Ca*. P. mali' infection, and eponymous to the diseases name "apple proliferation" [31]. However, the factors involved in *BRC1*/*MdTCP16*/*AtTCP18* gene regulation are not easy to detangle, since they seem to be species- and tissue- dependent and regulated in a complex manner. By expressing SAP11-like proteins, phytoplasma target different members of the plant TCP family which serve as molecular hubs, to manipulate their plant hosts in a very sophisticated -but not yet fully understood- manner.

Supporting information

S1 Fig. Reference Sequence of XM_008376500.2. The sequence includes the CDS for XP_008374722.1 (*MdTCP16*) with TCP domain and the identified part in the Y2H screen. (PDF)

S2 Fig. Phytoplasma concentration of leaf and root samples. The normalized phytoplasma concentration is given as the ratio of the '*Ca*. P. mali' specific *16S* gene copies and the *Malus x domestica* single-copy gene *ACO*. Phytoplasma concentration was quantified in seven infected leaf samples from greenhouse plants (three from spring and four from autumn), in three naturally infected pooled leaf samples (one from spring, two from autumn) and in one naturally infected pooled root sample (autumn).
(PDF)

S1 Table. Primers used in this study. Lowercase letters indicate bases for Gateway-*attB* site overhangs or *Sfi*I restriction site overhangs.
(PDF)

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Publication 5

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'*Candidatus* Phytoplasma mali' SAP11-Like protein modulates expression of genes involved in energy production, photosynthesis, and defense in *Nicotiana occidentalis* leaves.

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Bettina Hause: Contribution to the study design, interpretation of the data, revision of the manuscript

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'Candidatus Phytoplasma mali' SAP11-Like protein modulates expression of genes involved in energy production, photosynthesis, and defense in *Nicotiana occidentalis* leaves

Cecilia Mittelberger[1], Mirko Moser[2], Bettina Hause[3] and Katrin Janik[1*]

Abstract

Background *'Candidatus* Phytoplasma mali', the causal agent of apple proliferation disease, exerts influence on its host plant through various effector proteins, including $SAP11_{CaPm}$ which interacts with different TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL FACTOR 1 and 2 (TCP) transcription factors. This study examines the transcriptional response of the plant upon early expression of $SAP11_{CaPm}$. For that purpose, leaves of *Nicotiana occidentalis* H.-M. Wheeler were Agrobacterium-infiltrated to induce transient expression of $SAP11_{CaPm}$ and changes in the transcriptome were recorded until 5 days post infiltration.

Results The RNA-seq analysis revealed that presence of $SAP11_{CaPm}$ in leaves leads to downregulation of genes involved in defense response and related to photosynthetic processes, while expression of genes involved in energy production was enhanced.

Conclusions The results indicate that early $SAP11_{CaPm}$ expression might be important for the colonization of the host plant since phytoplasmas lack many metabolic genes and are thus dependent on metabolites from their host plant.

Keywords Apple proliferation, Plant defense, RNA-seq, SAP11

Background

'Candidatus Phytoplasma mali' ('*Ca*. P. mali') is a plant pathogen, associated to Apple proliferation disease in apple (*Malus* x *domestica* Borkh.). This cell wall-less bacterium belongs to the class of Mollicutes and has one of the smallest genomes among all so far fully sequenced phytoplasma species [1]. Phytoplasmas reside in the plant phloem and are transmitted by phloem sucking psyllids. '*Ca*. P. mali' manipulates its host plant by secreting effector proteins via a sec-dependent secretion system [2]. Several effector proteins are known from different phytoplasma [3]. So far, in '*Ca*. P. mali' four effector

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proteins, namely $SAP11_{CaPm}$ [4], PME2 [5], PM19_00185 [6] and $SAP05_{CaPm}$ [7], as well as the virulence factor AAA+ATPase AP460 [8] have been identified as host manipulating factors. While little or nothing is known about PME2's, $SAP05_{CaPm}$´s and PM19_00185´s function in apple trees, the potential function of the '*Ca*. P. mali' SAP11, homolog of $SAP11_{AYWB}$ (from '*Candidatus* Phytoplasma asteris'), has been also described in apple [4, 9–11]. $SAP11_{AYWB}$ binds and destabilizes three different TCP (TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL FACTOR 1 and 2) transcription factors and is involved in the development of different symptoms [12, 13]. In contrast to $SAP11_{AYWB}$, $SAP11_{CaPm}$ localizes not only to the cell nucleus, but also to the cytoplasm [14]. However, it has been shown that it binds (similar as $SAP11_{AYWB}$) two class II CIN-like TCPs, namely MdTCP4a (orthologue to AtTCP4) and MdTCP13a (orthologue to AtTCP13) [4], formerly known as MdTCP25 and MdTCP24 respectively [15] as well as to the class II CYC/TB1 TCP MdTCP18a (orthologue to AtTCP18) (formerly known as MdTCP16 as described in [16]). $SAP11_{CaPm}$-binding to its TCP-interaction partners causes severe growth aberrations, early bud break and hormonal disbalance within the plant [14]. Effector binding of MdTCP4a and MdTCP13a is supposed to be at the basis of the changes in jasmonate (JA) and abscisic acid (ABA) levels observed in infected plants and might be the reason for the development of late flowers, leaf reddening and altered root architecture [4]. The binding of MdTCP18a is supposed to counteract the *MdTCP18a* upregulation in infected plants, leading to an early bud break and uncontrolled shoot outgrowth [16].

The stable overexpression of $SAP11_{AYWB}$ in *Arabidopsis* plants resulted in a total of 59 upregulated and 104 downregulated genes as revealed by RNA-seq [17]. From the 59 upregulated genes, 18 genes were functionally annotated as inorganic phosphorus (P_i) starvation-induced genes. In the group of downregulated genes, *LIPOXYGENASE2* (*LOX2*), a gene encoding an enzyme involved in JA biosynthesis, and *PATHOGENESIS-RELATED GENE1 (PR1)* and *ELICITOR-ACTIVATED GENE3-1 (ELI3-1)*, two salicylic acid (SA) responsive genes, were found. This indicates that $SAP11_{AYWB}$ suppresses the defense response while enhancing bacterial growth in *Arabidopsis* plants. In addition, it has been shown that defense response to insect vectors is also reduced in $SAP11_{AYWB}$ overexpressing *Arabidopsis* plants [18]. *Nicotiana occidentalis* H.-M. Wheeler plants directly infected with '*Ca*. P. mali' showed 157 proteins with an increased and 173 with a decreased expression compared to healthy plants. This highlights the fact that a single effector, such as SAP11, is only partially involved in the pathogen induced transcriptional changes [19]. The proteins encoded by genes with an increased expression comprised mainly the alpha-linolenic acid synthesis, while those with downregulation were involved in porphyrin and chlorophyll metabolism [19]. This was in line with increased JA levels and leaf yellowing of infected plants.

Even though such studies help to understand possible functions of $SAP11_{CaPm}$, only little is known so far about the very early role of $SAP11_{CaPm}$ during early infection of plants with '*Ca*. P. mali'. Thus, the aim of this study was to gain a better understanding of the transcriptional changes that occur in the plant host during early occurrence of the effector protein. Infiltration RNA-seq [20] was used to unravel expression networks and effector function in healthy plants upon expression of $SAP11_{CaPm}$. Moreover, *N. occidentalis* H.-M. Wheeler was chosen since it has been described as the appropriate model plant to study '*Ca*. P. mali' effector functions [10, 19, 21–23]. Therefore, the gene encoding the effector protein $SAP11_{CaPm}$ was transiently expressed by agroinfiltration in *N. occidentalis* H.-M. Wheeler leaves and differential gene expression was analyzed until 5 days post infiltration in the respective leaf tissue. Transcriptional changes in the infiltrated leaves revealed that $SAP11_{CaPm}$ affects mainly genes involved in defense responses, photosynthesis, and pathways involved in the production of energy equivalents at early time points of its occurrence in the cells.

Methods

Plant material and agroinfiltration

Nicotiana occidentalis H.-M. Wheeler seeds were kindly provided by Kajohn Boonrod from RLP AgroScience GmbH, Neustadt, Germany [10, 21]. Seedlings were grown in a plant growth chamber (Percival AR22L, Percival Scientific, Perry, IA, USA) under long photoperiod conditions (16 h/8 h, 24 °C/22°C, 70% rH). Four to five-week-old plants were used for agroinfiltration.

For agroinfiltration the coding sequence of the mature $SAP11_{CaPm}$ effector protein from '*Ca*. P. mali' strain STAA (Accession: KM501063) was subcloned into the GreenGate-entry module pGGC00 [24] using the primer pair ATP00189pP_Cfw (AACAGGTCTCAGGCTCCATGTCTCCTCCTAAAAAAGATTCTA) / ATP00189pP_Drv (AACAGGTCTCACTGATTTTTTTCCTTTGTCTTTATTGTTA).

Transformation constructs coding for $SAP11_{CaPm}$:GFP under the control of CaMV *35 S* promoter and flanked by the RBCS terminator and a plant kanamycin resistance marker were assembled using modules from the GreenGate-kit [24]. In detail, a GreenGate reaction containing 150 ng pGGA004 (*p35S*), 150 ng pGGB003 (B-dummy), 150 ng pGGC000- $SAP11_{CaPm}$, 150 ng pGGD001 (linker-GFP), 150 ng pGGE001 (*tRBCS*), 150 ng pGGF007 (*pNOS::KanR: tNOS*), and 100 ng pGGZ001 (empty destination vector) was combined in a total volume of 15 µL.

For the GreenGate reaction 1.5 µL 10× CutSmart Buffer (New England Biolab, Ipswich, MA, USA), 1.5 µL ATP (10 mM), 1.0 µL T4 DNA Ligase (5 u/µL) (Thermo Fisher Scientific, Waltham, MA, USA), and 1.0 µL BsaI-HF®v2 (20,000 u/mL) (New England Biolab, Ipswich, MA, USA) were added to the module-mixture, and 30 cycles at 37 °C and at 16 °C for 2 in each, followed by 50 °C for 5 min and 80 °C for 5 min were performed. Subsequently, 5 µL of the reaction mixture were used for heat-shock transformation of ccdB-sensitive One Shot® TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA, USA). A second vector containing only the GFP gene fused to a nuclear localization signal was assembled, using the pGGC012 module (GFP-NLS). The correctness of the assembled plant expression vectors was confirmed by sequencing.

The validated GreenGate expression vectors, *35 S::SAP11*$_{CaPm}$*:GFP* and *35 S::GFP-NLS* were transferred together with *pSOUP* helper plasmid into electrocompetent *A. tumefaciens* strain EHA105. The *A. tumefaciens* clones were cultured for 2 days at 28 °C in selective LB medium. For infiltration 0.5 OD/mL were resuspended in infiltration medium (10 mM MgCl2, 10 mM MES, 200 µM acetosyringone, pH 5.7), regenerated for 4 h at 28 °C and infiltrated with a blunt syringe into three leaves from four- to five-week-old *N. occidentalis* H.-M. Wheeler plants. Six plants were infiltrated with the effector expressing *35 S::SAP11*$_{CaPm}$*:GFP* construct and six plants were infiltrated with *35 S::GFP-NLS* serving as controls. Additional six plants were not infiltrated and used as non-infiltrated controls. The infiltrated area was marked with a pen on the adaxial leaf side and six leaf discs with a size of 1 cm² (two/infiltrated area) were excised immediately after infiltration from one plant of each variant. Leaf disc excision was repeated on different plants in a 24-h-rhythm. Leaf discs were immediately flash frozen in liquid nitrogen. Infiltration and leaf disc sampling were repeated with three independent plant sets, grown at three different time points.

cDNA library construction and sequencing

A total of 36 leaf discs samples of plants from all three treatments for the timepoints 0 h, 24 h, 72 h and 120 h were sent on dry ice for RNA extraction, library preparation and sequencing to StarSEQ (Mainz, Germany). Additionally, 15 samples were prepared from different growth stages of untreated *N. occidentalis* H.-M. Wheeler plants and send on dry ice to StarSEQ. RNA of those samples together with RNA of the 36 leaf disc samples was pooled and used for the de novo transcriptome assembly.

The stranded RNA sequencing library was prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolab, Ipswich, MA, USA). The library for the de novo transcriptome assembly was sequenced on a Illumnia NextSeq500 platform in 2×150 nt paired-end mode. The libraries of the 36 leaf samples were sequenced on the same platform in 1×75 nt single-end mode.

Quality assessment of the reads was performed using the FASTQC tool [25].

Adapter trimming of paired end reads was done with the FASTQ Toolkit (BaseSpace Labs) retaining reads with a minimum read length of 32 nt.

De novo transcriptome assembly of *Nicotiana occidentalis*

The workflow for the de novo assembly of the transcriptome and the RNA-seq analysis was carried out as follows: First, all adapter trimmed paired end reads were quality trimmed with a sliding window of 4 nt, a minimum phred quality score of 20 and a minimal read length of 75 nt using the tool Trimmomatic v.0.36 [26]. The de novo assembly was then performed in strand-specific mode using Trinity v. 2.9 [27]. The whole de novo assembled transcriptome was first annotated using Trinotate v.3.2.0 [28] with the help of Transdecoder v.5.5.0 [29] to estimate all possible coding regions. Since the annotation contained several transcripts not belonging to *Nicotiana*, the whole transcriptome was decontaminated using the MCSC Decontamination method [30] filtering for transcripts belonging to the order of *Solanales.* The remaining decontaminated transcriptome was reannotated with Trinotate v.3.2.1 using homology search to SwissProt sequence database with Blast 2.12.0+ [31], to PFAM database for protein domain identification with HMMER (hmmer v.3.3.2) [32] and for the prediction of a signal peptide with SignalP v.5.0.b [33] and of a transmembrane domain with tmhmm v.2.0c [34]. The annotated transcripts were visualized using the build in TrinotateWeb tool.

Differential expression analysis, go enrichment analysis

Transcripts were quantified using Trinity v.2.11.0 build in Salmon (v.1.4.0) [35] pipeline. Selection of differentially expressed transcripts (DETs) was afterwards performed with the Trinity v.2.11.0 build in DESeq2 pipeline [36], where parameters for filtering are set to >4-fold change and a false discovery rate (FDR) < 0.001.

Lists of DETs were annotated by homology search with Blast 2.12.0+ [31] against standard nucleotide collection database (nt) with an e-value cut off set to 0.001.

The lists of DETs were further analyzed and subset by Venn diagrams using jvenn [37].

Gene ontology (GO) assignments were first extracted from Trinotate output and then all lists of up and downregulated DETs and the Venn subsets were functional enriched with the Trinity v2.11.0 build in GOseq [38] pipeline, using the *de-novo* assembled and

decontaminated *N. occidentalis* H.-M. Wheeler transcriptome as background.

The functional enrichment was visualized using RStudio 2022.07.1 (RStudio, PBC) with R v4.2.0 [39] and the Bioconductor packages goseq v.1.48.0 [40] and rrvgo v.1.8.0 [41].

For a detailed analysis of enriched transcripts, the GO assignments were filtered with a script for defence or stress related terms and phytohormone related terms. In detail, files with GO assignments were searched using strings for defence/stress ("stress", "defence", "immune") and for phytohormone related terms ("salicylic", "auxin", "gibberel*", "jasmonic", "ethylene", "cytokinine", "abscisic", "brassinosteroi*"; asterisks in the term indicate placeholder for any letter).

For further functional characterization of up- and downregulated transcripts the different subsets were analyzed with STRING database v.12.0 [42] using the whole *N. tabacum* L. genome as background for network analysis. In detail the protein sequences of DETs were uploaded to the multiple sequences search interface, annotated with STRING by homology search within the *N. tabacum* L. genome and network was visualized with protein interactions based on functional and physical protein associations. The network was then clustered with MCL (Markov Cluster Algorithm) [43] clustering using an inflation parameter of 4. Enriched gene ontologies and KEGG pathways of the biggest cluster were downloaded.

RNA extraction, cDNA synthesis and qPCR

For validation of DETs, the agroinfiltration approach was repeated with a new set of plants, using three biological replicates. The excised leaf discs were immediately flash frozen in liquid nitrogen, grinded using a mortar and pistil, and 100 mg of frozen leaf powder was used for RNA extraction with Spectrum™ Plant Total RNA Kit (Merck, Darmstadt, Germany) following protocol A of the manual. RNA concentration was measured with a spectrophotometer (Implen N60). Using 2 µg of RNA, genomic DNA removal and cDNA synthesis was performed with SuperScript™ IV VILO™ Master Mix with ezDNase™ enzyme.

To find suitable and stable expressed reference genes, primer pairs for NbPP2a, NbNQO, NbGAPDH and NbEF1a, identified as reference genes in *N. benthamiana* Domin [44] as well as the endogenous universal qPCR control UNI28S [45] were selected. They *in-silico* matched (tested with Geneious R11.1.5) to transcripts within the *de-novo* assembled *N. occidentalis* H.-M. Wheeler transcriptome and were thus tested in a qPCR assay. The qPCR data of all candidates were analyzed by RefFinder [46] and the two most stable genes, *PP2a* and *NQO* were used as reference genes.

Diluted cDNA was used for qPCR assays with three technical replicates, using SYBR chemistry. In detail, 2 µL of template were used in a 10 µL reaction mixture containing 5 µL 2x SYBR FAST qPCR Kit Master Mix (Kapa Biosystems), 2.6 µL nuclease-free water and 0.20 µL each of forward and reverse primer (10 µM). All qPCR reactions were run on a CFX384 Touch Real-Time PCR Detection system, using the following conditions: initial denaturation at 95 °C for 20 s; 35 cycles of 95 °C for 3 s and 60 °C for 30 s; and a melting curve ramp from 65 to 95 °C at increments of 0.5 °C every 5 s. Melting curve analysis was performed to confirm the generation of the correct amplicon. Specific melting curves for every target amplified by SYBR green qPCR are provided in Additional file 1.

To determine qPCR efficiency of the respective target together with each qPCR run a five-point serial dilution of *N. occidentalis* H.-M. Wheeler cDNA (1:10, 1:20, 1:50, 1:100, 1:200) was analyzed. As an additional quality control of qPCR, a three-point serial dilution (1:10, 1:50, 1:100) was analyzed, amplifying the reference genes *NbNQO* and *NbPP2a*. Data analysis was performed using CFX Manager Software (Bio-Rad) and RStudio 2022.07.1 (RStudio, PBC) with R v4.2.0 [39] using the MCMC qPCR package (v.1.2.4) [47] applying an informed model.

Results

Expression of SAP11$_{CaPm}$ in *Nicotiana occidentalis*

To analyze early effects of *SAP11*$_{CaPm}$ expression on the transcriptome of *N. occidentalis* H.-M. Wheeler, leaves were infiltrated with *A. tumefaciens* harboring a construct encoding SAP11$_{CaPm}$ fused to GFP. As controls, infiltration with nuclear localized GFP (GFP-NLS) and non-infiltrated plants were used. Samples were taken every 24 h up to 120 h and subjected to RNA-seq. To verify the expression of *SAP11*$_{CaPm}$, leaf samples later used for RNA-seq as well as leaf samples from a second independent experiment, were analyzed by RT-qPCR (Fig. 1). Within 24 h after infiltration, the first transcript accumulation was detectable. In the leaf samples set used for RNA-seq, the expression reached its maximum 96 h after infiltration and decreased 120 h post infiltration. Coherently, the analysis on the number of reads obtained by RNA-seq and indicative for expression of *SAP11*$_{CaPm}$ showed this kinetics. In the second independent leaf set *SAP11*$_{CaPm}$ expression was stable between 48 h and 96 h after infiltration and dropped only slightly after 120 h.

To verify the presence of SAP11$_{CaPm}$ fused to GFP in *N. occidentalis* H.-M. Wheeler cells, leaves were examined using confocal laser scanning microscopy. The occurrence of SAP11$_{CaPm}$:GFP as well as of GFP-NLS from the control-infiltrations became visible at 48 h after infiltration, thereby lagging behind the rise of transcripts (Fig. 2). SAP11$_{CaPm}$:GFP was observed to localize to the

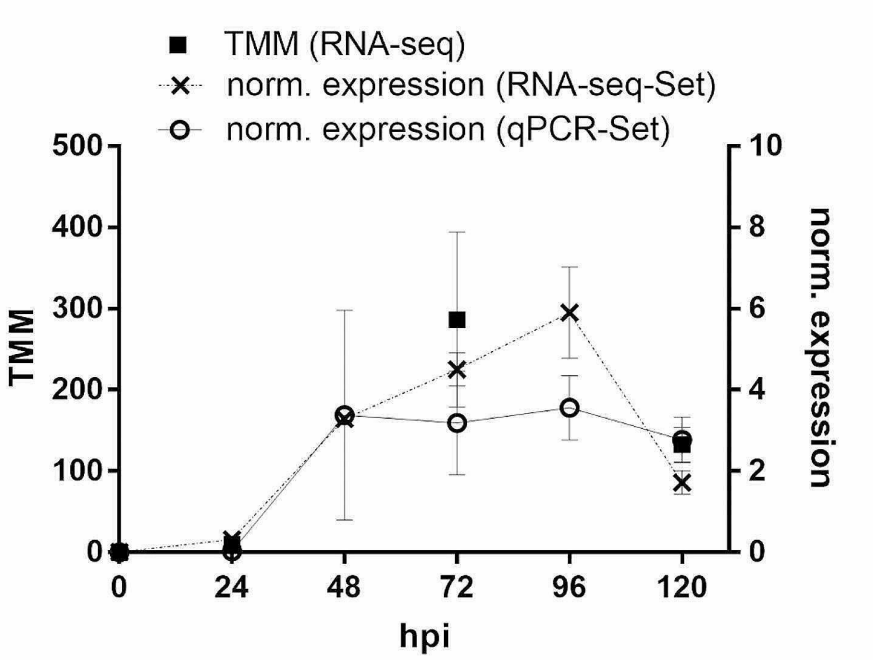


Fig. 1 Expression of $SAP11_{CaPm}$ in leaf samples analyzed by RNA-seq and qPCR. Beside the RNA-seq sample set a second independent sample set was analyzed only by qPCR. The RNA-seq set was analyzed at 0 h, 24 h, 72 h and 120 h, while the qPCR set was analyzed at every timepoint. Data represent the mean +/- SEM of 3 biological replicates. TMM = trimmed mean of M values [48]

cell nucleus and the cytoplasm of infiltrated *N. occidentalis* H.-M. Wheeler cells, while the GFP-NLS in control-infiltration localized only to the cell nucleus.

De novo transcriptome assembly

A total of 54,704,261 (GC content: 43%) adapter and quality trimmed paired end reads from a pool of 51 RNA samples from *N. occidentalis* H.-M. Wheeler plants infiltrated or not were used for the de novo transcriptome assembly with Trinity v2.9. The clean reads were assembled, resulting in 166,787 transcripts, with an average length of 1,034 bp and an N_{50} of 1,504 bp.

The transcriptome was further decontaminated from sequences originating from species other than the order *Solanales* using the Model-based Categorical Sequence Clustering MCSC decontamination pipeline, that is based on the Model-based Categorical Sequence Clustering (MCSC) algorithm [30]. The decontaminated transcriptome contained 153,640 transcripts with an average length of 1,076 bp, N_{50} of 1,559 bp and a GC content of 39.57%.

This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GKBG00000000. The version described in this paper is the first version, GKBG01000000.

The sequencing dataset used in this study is available in the NCBI repository with BioProject ID PRJNA871046.

Differential expression analysis

The RNA-seq libraries obtained from leaf samples infiltrated with *A. tumefaciens* to express either $SAP11_{CaPm}$:*GFP* or *GFP-NLS* or non-infiltrated were subjected to transcriptome analysis. To get insights into $SAP11_{CaPm}$-mediated changes, differential expression analysis was done using DEseq2 [36] within the Trinity Package (v2.11.0) [27], making pairwise comparisons of non-infiltrated (ni), control-infiltrated (ctrl) and $SAP11_{CaPm}$ infiltrated ($SAP11_{CaPm}$) samples at different time points (Fig. 3).

Differentially expressed transcripts (DETs) were identified, which occurred over time within a treatment group or between treatment groups at the same time point (Fig. 4).

The first DETs in infiltrated leaves were detectable at 24 h after infiltration (Fig. 4A, D). In comparison to non-infiltrated leaves, control-infiltrated *N. occidentalis* H.-M. Wheeler leaves showed 318 upregulated and 115 downregulated transcripts. Only a few DETs were detectable between control-infiltrated and the $SAP11_{CaPm}$-infiltrated samples: 34 transcripts were downregulated in $SAP11_{CaPm}$ expressing samples compared to the control-infiltration. Seven of these 34 transcripts were upregulated in control-infiltrated samples compared to the non-infiltrated samples.

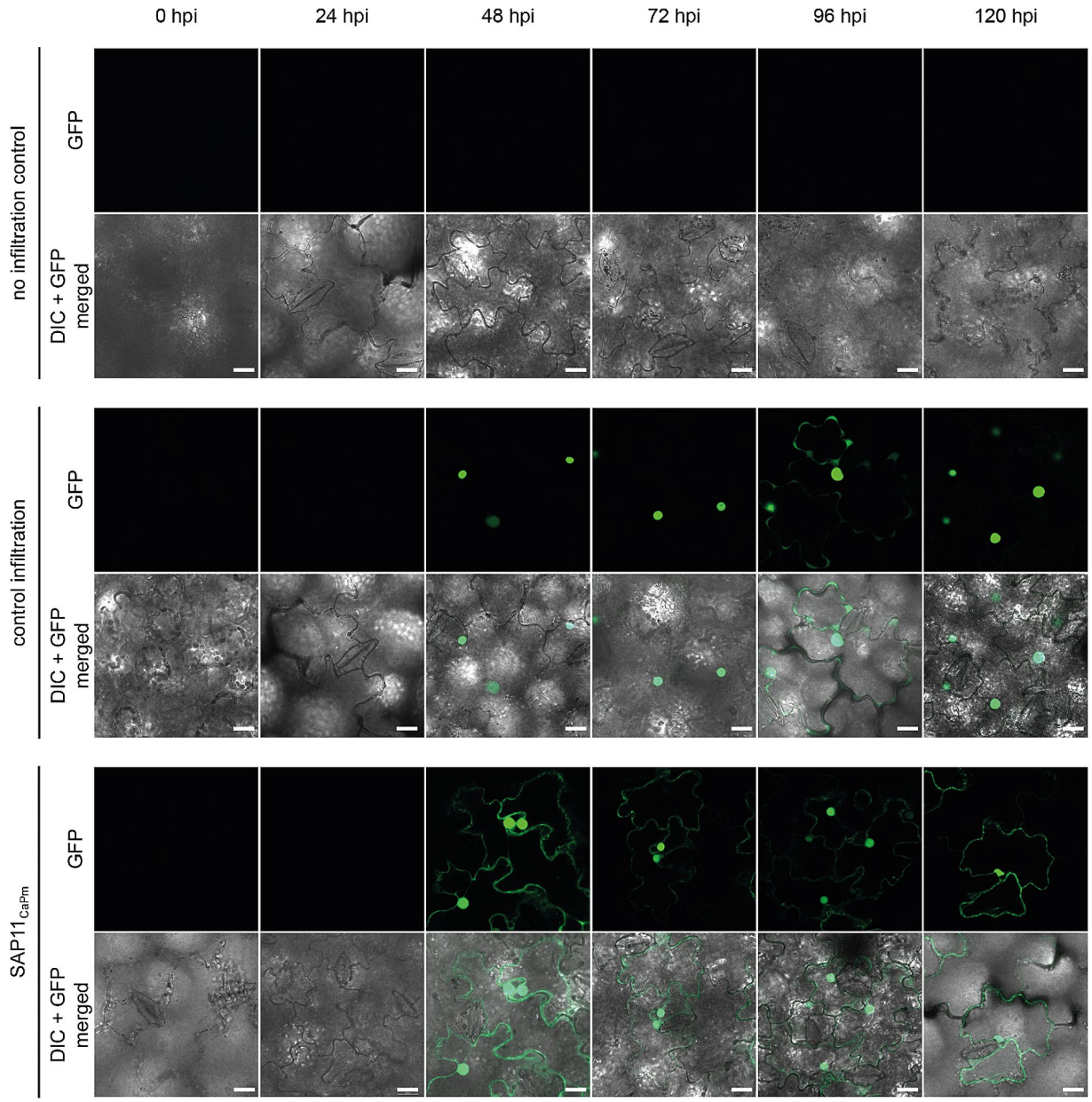


Fig. 2 Detection of GFP-NLS (green fluorescent protein fused to a nuclear localization sequence, control infiltration) and $SAP11_{CaPm}$:GFP ($SAP11_{CaPm}$) in infiltrated N. occidentalis leaves. No infiltration control shows images of leaves that were not infiltrated. GFP fluorescence was visualized by confocal laser scanning microscopy. Bars represent 20 µm. GFP = only GFP channel, DIC + GFP = Differential interference contrast microscopy merged to the GFP channel

At 72 h after infiltration (Fig. 4B, D). a more substantial number of DETs were evident. Control-infiltrated leaves exhibited 1026 upregulated and 181 downregulated transcripts compared to non-infiltrated leaves. In contrast, $SAP11_{CaPm}$-expressing leaves showed only two upregulated and 25 downregulated transcripts compared to control-infiltrated leaves. Six out of these 25 downregulated transcripts were upregulated in control-infiltrated leaves.

The highest number of DETs was observed at 120 h after infiltration (Fig. 4C, D).

Control-infiltrated samples had 1983 upregulated and 1184 downregulated transcripts compared to non-infiltrated leaves. The comparison between $SAP11_{CaPm}$ infiltration and control-infiltration revealed that 193 transcripts were upregulated, and 440 transcripts were downregulated due to $SAP11_{CaPm}$ infiltration. Among

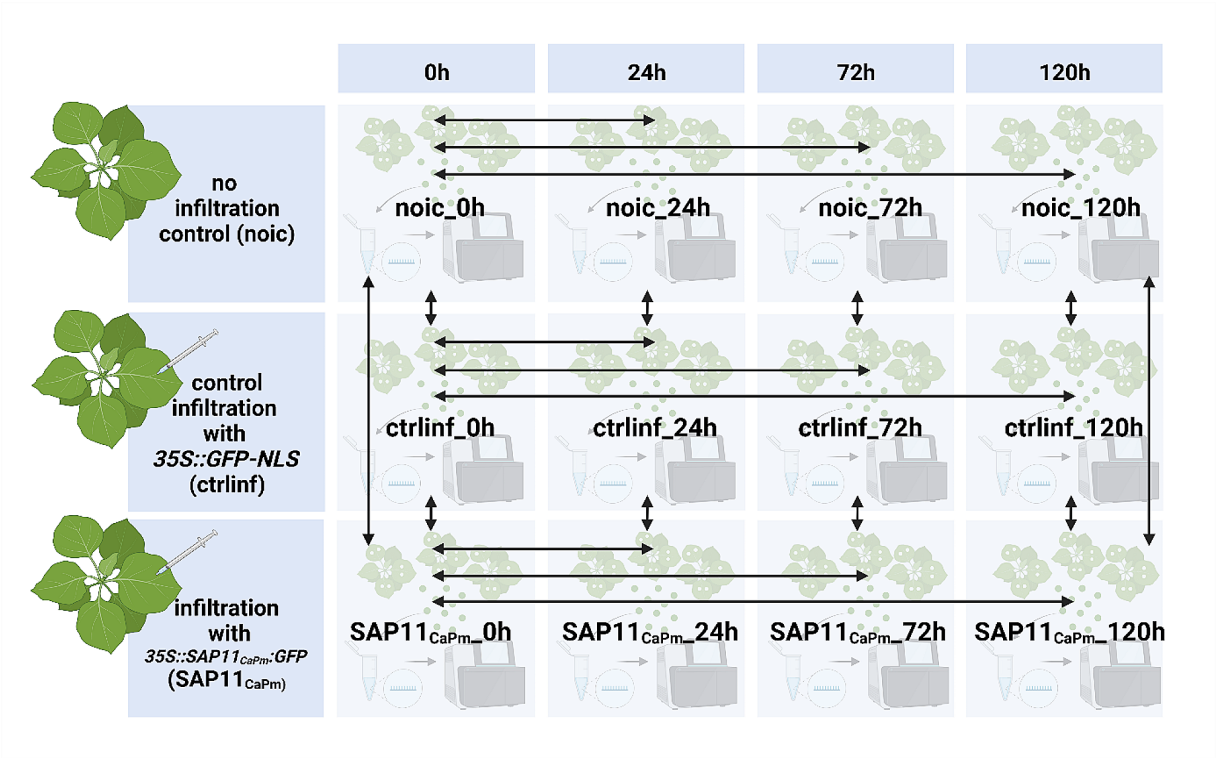


Fig. 3 Identification of DETs in leaf samples of three different treatment groups. The first control group was not infiltrated (ni), the second control group was infiltrated with GFP-NLS under control of 35S promoter (ctrl) and the third group was infiltrated with 35S::SAP11$_{CaPm}$:GFP (SAP11$_{CaPm}$). Arrows show the comparisons that were made with DEseq2 [36]

these 44 of the upregulated transcripts were downregulated between non-infiltrated samples and control-infiltration and 73 downregulated transcripts were upregulated in the control-infiltrated leaves (Fig. 4C).

Table 1 shows transcripts that resulted differentially expressed at two different timepoints upon SAP11$_{CaPm}$ expression (compared to ctrl). Interestingly, a transcript encoding a protein modifier of *snc1,1* (MOS1) was downregulated 24 h after infiltration (L2FC -11.54) but upregulated 120 h after infiltration (L2FC 12.40). Three transcripts were downregulated at 24 h and 120 h, but only for one of them, i.e. the preprotein translocase subunit SCY1, information regarding its function is available. The acyl-CoA thioesterase 2 was downregulated at 24 h and 72 h after infiltration with SAP11$_{CaPm}$ (L2FC −11.38).

Six genes were downregulated at 72 h and 120 h after infiltration with SAP11$_{CaPm}$ (Table 1) in contrast to control-infiltration. Among those genes, genes encoding the serine/threonine-protein phosphatase BSL1, the protein CHROMATIN REMODELING 8 (CHR8) and a NTRC-like thioredoxin reductase were detected. Despite the downregulation in SAP11$_{CaPm}$ infiltrated samples after 72 h and 120 h, the NTRC-like thioredoxin reductase and the pre-mRNA-splicing factor prp12 and THO complex subunit 4D-like were upregulated upon infiltration (in the absence of SAP11$_{CaPm}$): the NTRC-like thioredoxin reductase was upregulated after 72 h and 120 h, the pre-mRNA-splicing factor prp12 after 72 h and THO complex subunit 4D-like after 24 h and 72 h.

The putative DUF21 domain-containing protein At3g13070, as well as BSL1 and the pre-mRNA-splicing factor prp12 were not only downregulated in SAP11$_{CaPm}$ infiltrated samples in comparison to control-infiltration but also in comparison to the not-infiltrated samples.

qPCR validation of selected DETs

A total of 15 transcripts, that were differentially expressed between control-infiltration and SAP11$_{CaPm}$ infiltration, were selected as candidates for qPCR validation (Table 2).

RT-qPCR analyses from the 15 selected genes revealed high variance between biological replicates and no significant differences could be detected in the selected comparisons. The expression pattern, however, followed the same trend as those in RNA-seq data (Fig. 5). Taken together, these validation results of selected genes, the SAP11$_{CaPm}$ expression verified by qPCR (Fig. 1) and the RNA-seq data obtained, provide novel insight into the

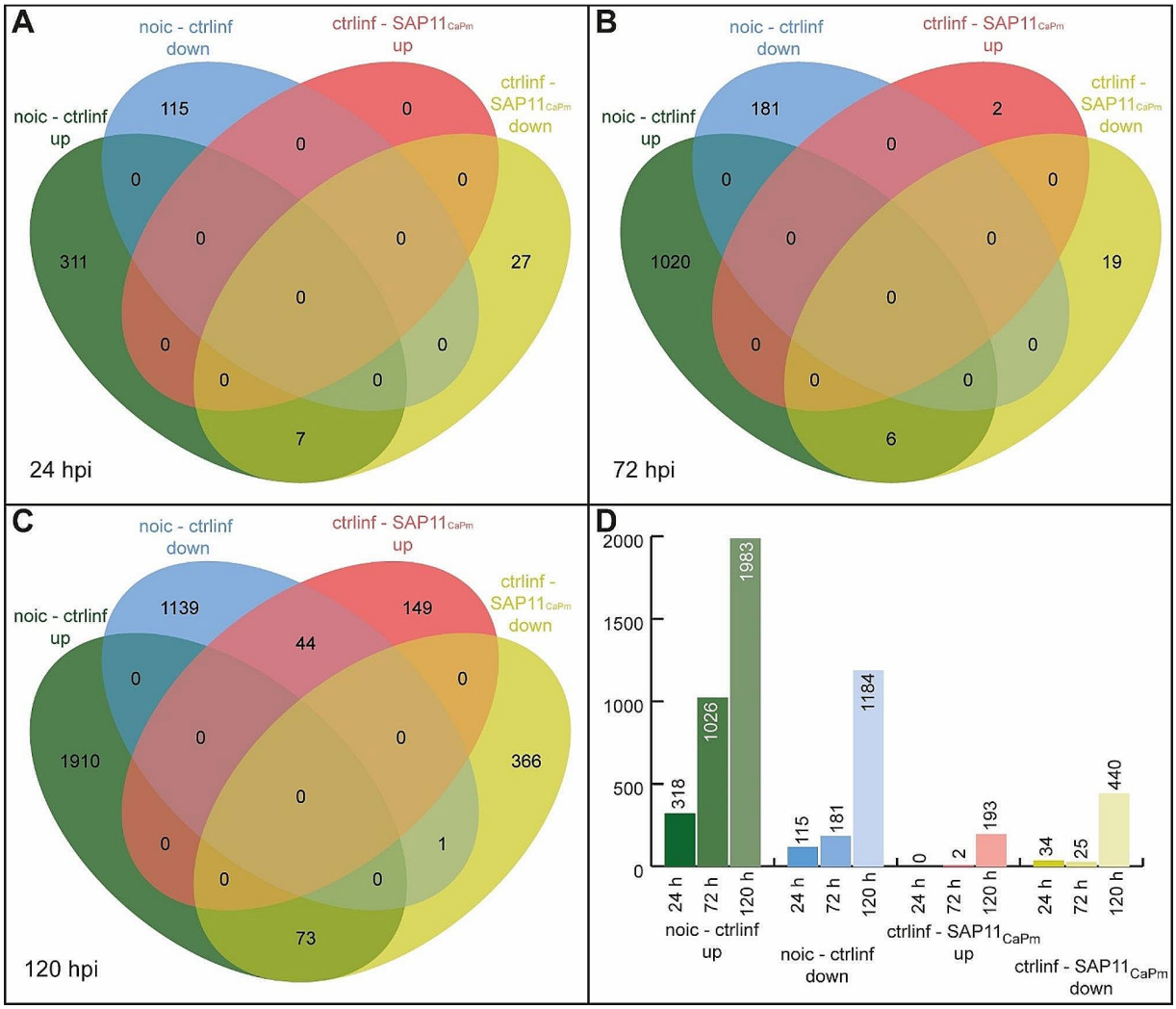


Fig. 4 Numbers of DETs at log2fold change (L2FC) of ≥ ± 2; 4-fold differential expression, p-value cutoff for FDR < 0.001. The VENN diagram shows DETs detected at (**A**) 24, (**B**) 72 and (**C**) 120 h after infiltration. Up- and downregulated transcripts in control-infiltrated samples and $SAP11_{CaPm}$ infiltrated samples were analyzed using jvenn [37]. (**D**) The barplot summarizes for each time point the total number of up- (green) and downregulated (blue) transcripts upon control- infiltration in comparison to non -infiltration and up- (red) or downregulated (yellow) transcripts due to infiltration with $SAP11_{CaPm}$ in comparison to control-infiltration

early effects of $SAP11_{CaPm}$ on the transcriptome of *N. occidentalis* H.-M. Wheeler.

Gene ontology (GO) enrichment and analysis

All groups of up- and downregulated DETs (Fig. 4) were analyzed using the Trinity v2.11.0 build in pipeline for GOseq [38]. The results were separated by the three sub-ontologies of GO: Molecular Function (MF), Cellular Component (CC) and Biological Process (BP) (Fig. 6). The datasets were further analyzed with the rrvgo package [41] which groups GO terms based on their semantic similarity and results were represented by a scatter plot (see Additional file 2).

After 24 h and 72 h no significant (FDR < 0.5) enriched GO terms were found in the group of $SAP11_{CaPm}$ downregulated transcripts in comparison to control-infiltration. The two upregulated transcripts in $SAP11_{CaPm}$ expressing samples at 72 h were assigned to the term "regulation of protein metabolic process" (BP). Enriched terms in the group of downregulated transcripts in $SAP11_{CaPm}$ expressing leaves compared to control-infiltration at 120 h comprised transcripts that were categorized into "cellular process", "biosynthetic process" or "response to external stimulus" (Fig. 6). Enriched GO terms in MF were mainly related to binding processes, such as "mRNA binding", "organic cyclic compound binding" or "magnesium chelatase activity", whereas CC

Table 1 Annotations of DETs, that are differentially regulated at two different time points (A and B) upon $SAP11_{CaPm}$ expression (compared to ctrl)

Time point A	L2FC	Time point B	L2FC	Annotation
24 h	-12.35	120 h	-9.51	XM_019400596.1 PREDICTED: *Nicotiana attenuata* preprotein translocase subunit SCY1
24 h	-12.03	120 h	-9.31	XM_019375308.1 PREDICTED: *Nicotiana attenuata* putative DUF21 domain-containing protein At3g13070
24 h	-11.31	120 h	-10.25	XM_009759060.1 PREDICTED: *Nicotiana sylvestris* angio-associated migratory cell protein (LOC104210218)
24 h	-11.38	72 h	11.26	XM_016613005.1 PREDICTED: *Nicotiana tabacum* acyl-CoA thioesterase 2-like (LOC107791023)
24 h	-11.54	120 h	12.40	XM_009765541.1 PREDICTED: *Nicotiana sylvestris* protein MODIFIER OF SNC1 1 (LOC104215684)
72 h	-12.14	120 h	-10.69	XM_019370017.1 PREDICTED: *Nicotiana attenuata* serine/threonine-protein phosphatase BSL1 (LOC109207134)
72 h	-11.45	120 h	-10.19	XM_009610029.3 PREDICTED: *Nicotiana tomentosiformis* protein CHROMATIN REMODELING 8 (LOC104102344)
72 h	-3.49	120 h	-9.25	XM_009774985.1 PREDICTED: *Nicotiana sylvestris* dedicator of cytokinesis protein 7 (LOC104223519)
72 h	-12.11	120 h	-8.89	XM_016623444.1 PREDICTED: *Nicotiana tabacum* thioredoxin reductase NTRC-like (LOC107800295)
72 h	-2.38	120 h	-9.11	XM_019377213.1 PREDICTED: *Nicotiana attenuata* pre-mRNA-splicing factor prp12 (LOC109213418)
72 h	-12.23	120 h	-10.20	XM_016605885.1 PREDICTED: *Nicotiana tabacum* THO complex subunit 4D-like (LOC107784716)

enriched terms were "plastid", "chloroplast" or "membrane-bounded organelle" (Fig. 6). Employing the rrvgo analysis within the set of 366 transcripts (Fig. 4; ctrl-$SAP11_{CaPm}$ down) unaffected by infiltration but downregulated during $SAP11_{CaPm}$ expression unveiled a cluster associated with both "defence response" and "response to external stimulus". The group of 149 upregulated transcripts (Fig. 3) in $SAP11_{CaPm}$ expressing samples after 120 h compared to the control-infiltration mainly contained transcripts that were assigned to BP GO terms like "proton transmembrane transport", "reverse transcription involved in RNA−mediated transposition" and "ATP biosynthetic process". The enriched MF GO terms were related to "endodeoxyribonuclease activity" and the enriched CC GO terms were mainly allocated to "mitochondrial protein−containing complex" and "proton−transporting ATP synthase complex, coupling factor F(o)". The rrvgo analysis of all 193 upregulated transcripts at 120 h showed a cluster assigned to "ATP biosynthetic process" and "proton transmembrane transport" (Additional file 2).

In the group of upregulated transcripts found only in the control-infiltrated samples at 24 h, 72 h and 120 h after infiltration, GO terms for the category BP are enriched such as "defence response", "response to biotic stimulus" or "response to stress. Downregulated transcripts after 24 h were enriched in BP GO terms related to "homeostasis", while 72 h after infiltration the enriched BP terms were assigned to "photosynthesis, light harvesting", "protein−chromophore linkage" or "electron transport chain". At 120 h after infiltration the most enriched BP terms were "starch metabolic process", "cation transport" and "photosynthetic electron transport chain" (Fig. 6). The rrvgo analysis revealed a cluster of transcripts related to "ATP biosynthetic process" that is, in contrast to infiltration with $SAP11_{CaPm}$ (ctrl - $SAP11_{CaPm}$), upregulated upon control-infiltration (Additional file 2). For both timepoints, 72 h and 120 h most of the enriched CC terms are chloroplast related ("thylakoid membrane", "photosystem", "chloroplast").

Functional analysis of groups

The GO annotations were selectively refined to include transcripts associated with defense or stress terms and phytohormone-related terms. As depicted in Fig. 7, the distribution of defense/stress and phytohormone-related terms in single DET groups is illustrated. Over 25% of the upregulated transcripts in the control-infiltrated samples were identified as defense or stress-related. Notably, in the downregulated DETs of control-infiltrated samples, the proportion of defense/stress-related transcripts decreases over time. In the group of upregulated DETs in $SAP11_{CaPm}$-infiltrated leaves compared to control-infiltrated samples, almost no defense/stress or phytohormone-related transcripts were observed after 72 h (0%) or 120 h (0.5%). Conversely, in the downregulated transcripts of the same group, 28% of the transcripts (72 h) and 19.5% (120 h) are associated with defense/stress or phytohormone-related terms. The Additional file 3 lists all transcripts that were assigned to defense/stress or phytohormone-related terms.

Table 2 Selected transcripts for qPCR validation with annotation and L2FC changes

Sample A	Sample B	Accession Nr.	Description	L2FC
ctrl_120h	$SAP11_{CaPm}$_120h	XM_019373029.1	PREDICTED: *Nicotiana attenuata* calmodulin-binding receptor-like cytoplasmic kinase 3 (LOC109209712)	-2.3
ctrl_120h	SAP11_120h	XM_009804809.1	PREDICTED: *Nicotiana sylvestris* probable leucine-rich repeat receptor-like protein kinase At5g49770 (LOC104248540)	-9.5
ctrl_120h	SAP11_120h	XM_019390873.1	PREDICTED: *Nicotiana attenuata* proline dehydrogenase 2	13.3
ctrl_120h	SAP11_120h	XM_019378812.1	PREDICTED: *Nicotiana attenuata* protein PHYLLO	12.4
ctrl_120h	SAP11_120h	XM_019370442.1	PREDICTED: *Nicotiana attenuata* calmodulin-7 (LOC109207504)	-10.9
ctrl_120h	SAP11_120h	JF897607.1	*Nicotiana benthamiana* chloroplast PsbP1 precursor (psbP1) mRNA	-11.2
ni_120h	SAP11_120h	XM_016603442.1	PREDICTED: *Nicotiana tabacum* probable WRKY transcription factor 31 (LOC107782559)	12.6
ctrl_120h	SAP11_120h	XM_016603442.1	PREDICTED: *Nicotiana tabacum* probable WRKY transcription factor 31 (LOC107782559)	13.1
ctrl_120h	SAP11_120h	XM_019379586.1	PREDICTED: *Nicotiana attenuata* oxygen-evolving enhancer protein 1	-11.2
ctrl_120h	SAP11_120h	XM_019372421.1	PREDICTED: *Nicotiana attenuata* SKP1-like protein 21 (LOC109209196)	-8.9
ctrl_24h	SAP11_24h	XM_019403282.1	PREDICTED: Nicotiana XXXattenuate polyadenylate-binding protein-interacting protein 7 (LOC109237039)	-11.4
ctrl_120h	SAP11_120h	XM_016640507.1	PREDICTED: *Nicotiana tabacum* protein kinase APK1A	12.7
ctrl_72h	SAP11_72h	XM_016623416.1	PREDICTED: *Nicotiana tabacum* probable xyloglucan endotransglucosylase/hydrolase protein 6 (LOC107800268)	-2.8
ctrl_120h	SAP11_120h	XM_009778335.1	PREDICTED: *Nicotiana sylvestris* TMV resistance protein N-like (LOC104226353)	-10.7
ctrl_120h	SAP11_120h	XM_016646486.1	PREDICTED: *Nicotiana tabacum* protein LUTEIN DEFICIENT 5	-9.7
ctrl_120h	SAP11_120h	XM_016646486.1	PREDICTED: *Nicotiana tabacum* protein LUTEIN DEFICIENT 5	12.0
ctrl_120h	SAP11_120h	XM_019407249.1	PREDICTED: *Nicotiana attenuata* patatin-like protein 2 (LOC109240587)	-10.0

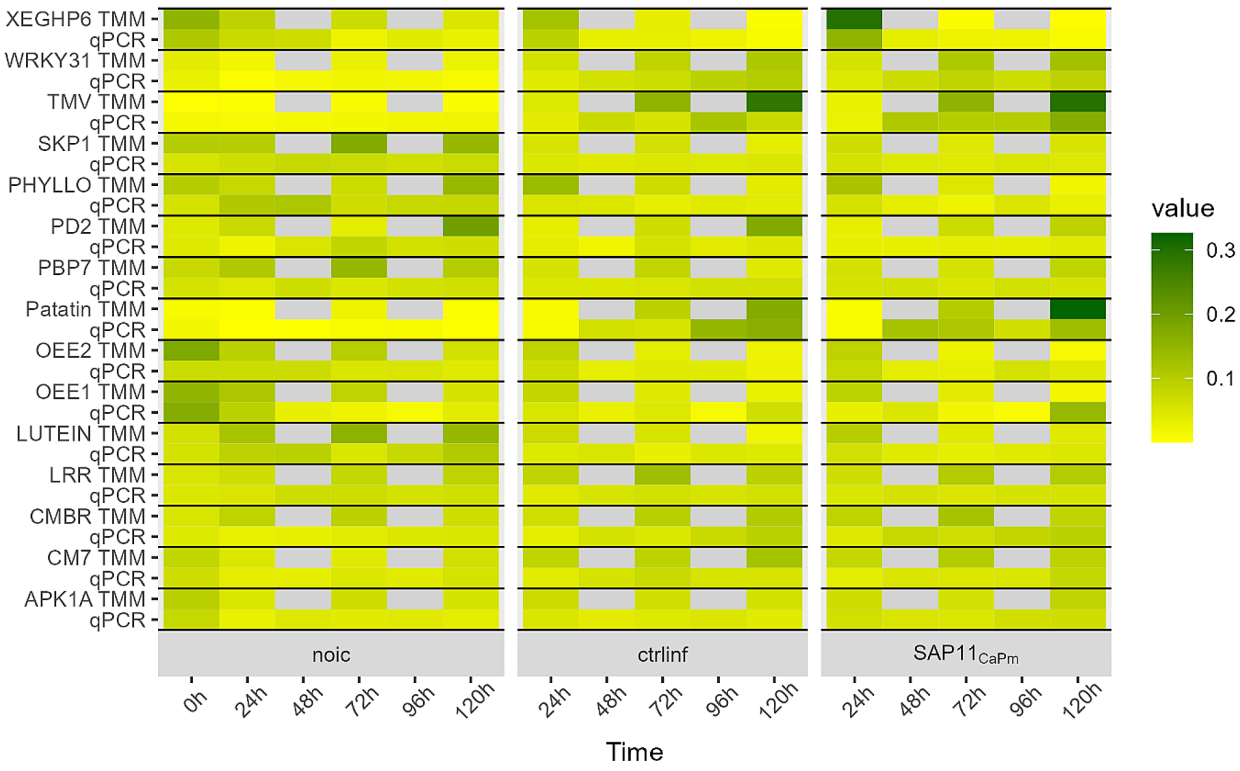


Fig. 5 Heatmap of transcript accumulation as determined by RT-qPCR analysis and as TMM from RNA-seq data. Values are given as sum normalized values

Fig. 6 GO enrichment in samples taken 120 h after infiltration. The GO enrichment is separated by biological process (green), molecular function (blue) and cellular component (yellow). The y-axis represents the top 10 GO terms, while the x-axis displays the percentage of enriched GO terms within each category. The size of the filled circles corresponds to the number of transcripts associated with the GO term, and the color of the circles reflects the adjusted p-value

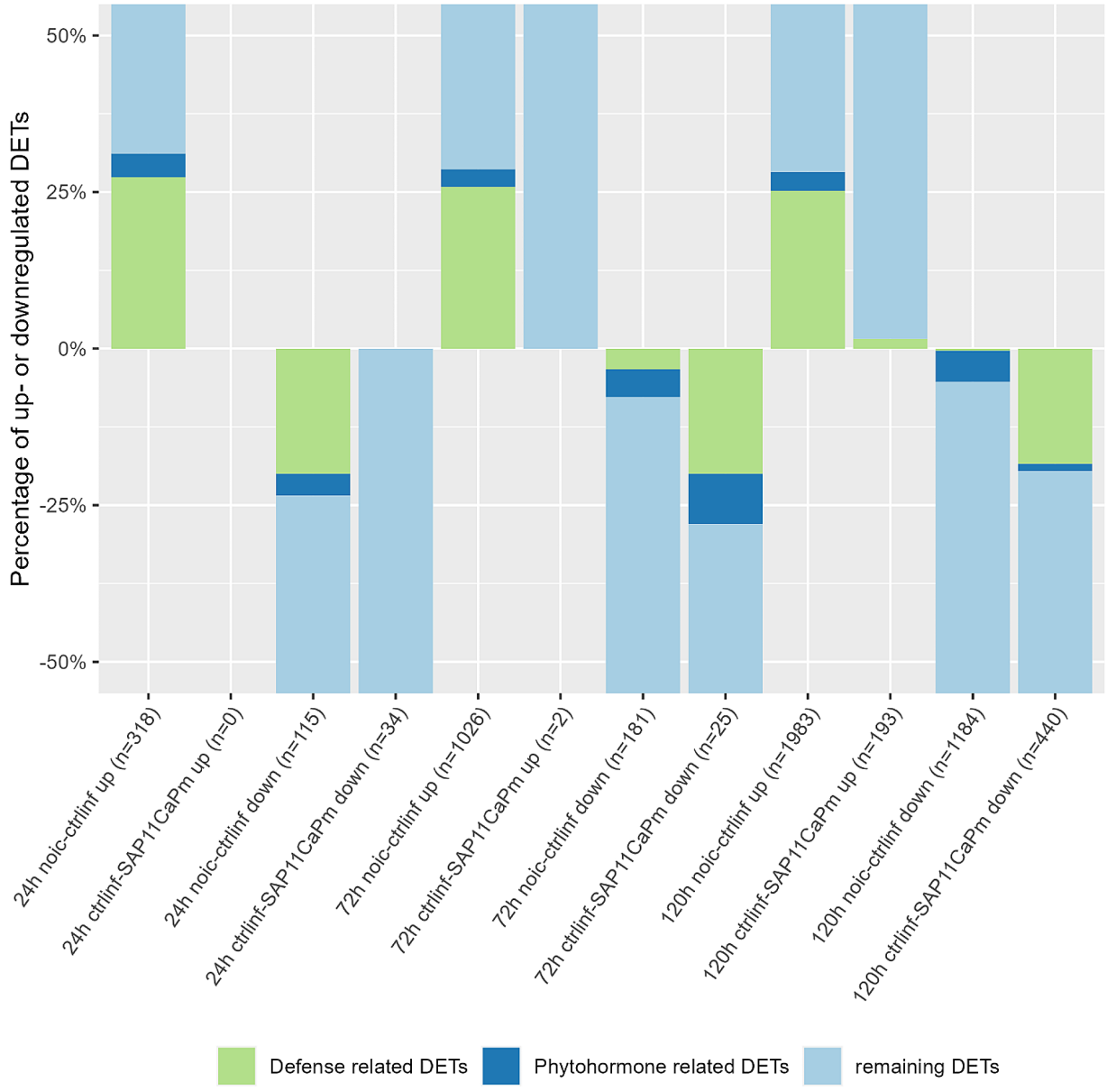


Fig. 7 Analysis of GO annotations. Percentage of defense and phytohormone related differentially expressed transcripts, according to GO annotation

Network analysis

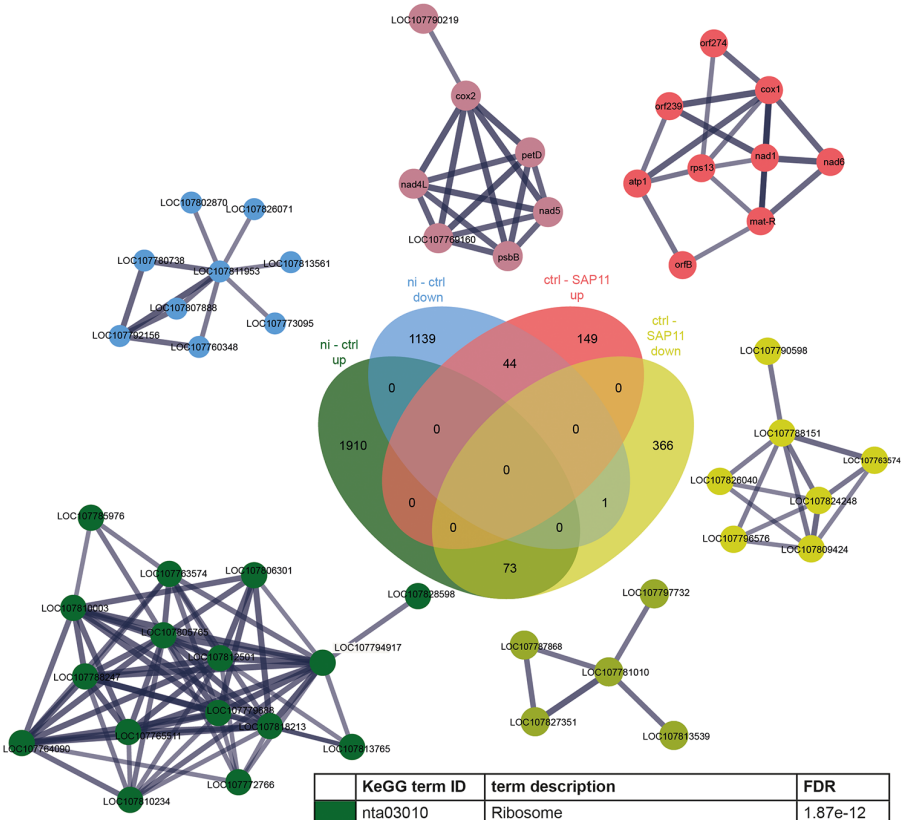
A network analysis of the different groups of DETs was performed using STRING [42].

It showed that KEGG pathways related to ribosome was upregulated upon infiltration (Fig. 8, ni – ctrl up), while the starch and sucrose metabolism was downregulated due to the infiltration process itself (Fig. 8, ni – ctrl down).

The main network cluster in the group of downregulated transcripts upon $SAP11_{CaPm}$ infiltration (ctrl vs. $SAP11_{CaPm}$) showed enriched KEGG pathways related to ribosome, while the main cluster of up regulated transcripts is enriched in oxidative phosphorylation and metabolic pathways. Detailed information about KEGG enriched transcripts and annotation of the main network cluster transcripts can be found in Additional file 4 and Additional file 5.

Discussion

Pathogen-derived effectors are important players in the process by which a pathogen modulates its host's metabolism. To get insights into the role of $SAP11_{CaPm}$, an effector produced by '*Ca*. P. mali', the causal agent of apple proliferation disease, early changes in gene expression



	KeGG term ID	term description	FDR
	nta03010	Ribosome	1.87e-12
	nta00500	Starch and sucrose metabolism	1.92e-19
	nta01110	Biosynthesis of secondary metabolites	1.99e-11
	nta01100	Metabolic pathways	3.94e-09
	nta01100	Metabolic pathways	2.02e-06
	nta00195	Photosynthesis	3.09e-05
	nta00190	Oxidative phosphorylation	0.00016
	nta00190	Oxidative phosphorylation	3.82e-08
	nta01100	Metabolic pathways	0.0168
	nta03010	Ribosome	0.0049
	nta00260	Glycine, serine and threonine metabolism	1.53e-05
	nta00450	Selenocompound metabolism	0.00035
	nta01200	Carbon metabolism	0.00035
	nta01110	Biosynthesis of secondary metabolites	0.00044
	nta00630	Glyoxylate and dicarboxylate metabolism	0.0017
	nta00270	Cysteine and methionine metabolism	0.0036
	nta01100	Metabolic pathways	0.0036
	nta01230	Biosynthesis of amino acids	0.0092

Fig. 8 Network analysis of up- and downregulated transcripts. STRING analysis was followed by MCL (Markov Cluster Algorithm) clustering. The main network clusters within each group with their KEGG annotations (see coloring) are shown

upon expression of $SAP11_{CaPm}$ were analyzed by infiltration RNA-seq [20]. For this, *SAP11*$_{CaPm}$ was transiently expressed by agroinfiltration in *N. occidentalis* H.-M. Wheeler leaves, since it has been shown that this model plant can be infected with '*Ca*. P. mali' [10, 19, 21–23]. Most importantly, transiently expressed $SAP11_{CaPm}$ fused to GFP was detected in the nucleus of leaf cells, thereby occurring in the same subcellular compartment in which transcription factors occur to manipulate gene expression. This location is thus important evidence that *N. occidentalis* gene expression might be actively altered by $SAP11_{CaPm}$. A reference genome of *N. occidentalis* H.-M. Wheeler is, however, not available, thus we opted for an approach where the *N. occidentalis* H.-M. Wheeler transcriptome was assembled de novo and used as a reference for the differential expression analysis.

A major problem with de novo assembling are biological contaminations of samples [30, 49]. These contaminations might derive from bacteria residing on the plant's surface or be of human origin [50]. To filter and exclude these contaminations from our de novo assembled *N. occidentalis* H.-M. Wheeler transcriptome we used a pipeline based on the MCSC algorithm [30].

The transcript levels of $SAP11_{CaPm}$ increased continuously until 96 h after infiltration, while it decreased at 120 h after infiltration. This result was slightly different compared to a second independent experiment, in which the expression remained stable and unchanged after 48 h and until 120 h after infiltration. Those differences in $SAP11_{CaPm}$ expression strength might affect the putative changes in host's gene expression profiles, although the protein was detectable to almost similar levels up to 120 h after infiltration. A group of differentially expressed transcripts in RNAseq was selected for the validation of their expression by RT-qPCR. Some of the qPCR results were very variable between biological replicates. Even though obtained from the same experimental setup, independent biological samples can inherently vary in their gene expression due to differences in cellular heterogeneity, or other factors. This inherent variability between biological replicates is a common source of variation in transcriptome analyses [51]. Nevertheless, the comparison of RNA-seq data with qPCR data of selected genes from the independent leaf samples, showed a common trend in both data sets. This corroborates findings derived from RNA-seq data by an independent method. In infected apple trees, the natural host plant, $SAP11_{CaPm}$ expression is not stable throughout the year [16] and the degree of colonization by phytoplasma, plant growth and climatic conditions might influence the spatio-temporal expression of the effector. Nonetheless, using another natural host of '*Ca*. P. mali', allowing transient expression assays, helps to unravel the transcriptional changes in the plant due to the action of one single effector protein. The highest number of DETs was detected at 120 h after infiltration. Interestingly, six genes were commonly downregulated at 72 h and 120 h after infiltration; four of these transcripts were allocated to defense-related terms in the GO annotation. Among them, the genes encoding the protein phosphatase BSL1 and chromatin remodeling8 (CHR8) were identified. The protein phosphatase BSL1 belongs to the BSU1 family and contributes to the brassinosteroid signalling. BSL1 interacts with the *Phytophtora infestans* effector protein PiAVR2 and acts as a susceptibility factor by affecting the balance between growth and immunity in plants [52–54].

CHR8 belongs to the switch2/sucrose non-fermenting2 (SWI2/SNF2) chromatin remodeling gene family that is involved in DNA damage response (Shaked et al. 2006). It has been shown that CHR8 is upregulated during an artificial infection of *A. thaliana* with cabbage leaf curl virus [55] as well as during genotoxic stress [56], indicating its potential role in plant stress response.

The gene encoding the modifier of snc1,1 (MOS1) was downregulated at 24 h and upregulated at 120 h after $SAP11_{CaPm}$ infiltration. MOS1 regulates the nucleotide binding site-Leu-rich repeat (NB-LRR) type R protein SNC1 [57]. MOS1 interacts with AtTCP15, while AtTCP15 directly binds to SNC1 and thereby modulates the plant immune response [58]. Due to that binding, MOS1 enhances the activity of SNC1 and helps to reinforce the defense response. Based on the results of this study it can be assumed that the MOS1 triggered immune response is suppressed in the early stage of $SAP11_{CaPm}$ expression, while it recovers later.

Our results show that the occurrence of $SAP11_{CaPm}$ led to a downregulation of plant defense (Fig. 7). Nonetheless, it should be noted that the infiltration process itself induces several transcriptional changes within the infiltrated leaf area [59, 60]. Agroinfiltration induces host defense and alters phytohormone levels [59, 61, 62]. This is in line with the findings in Fig. 7. Transcripts related to plant defense and phytohormones are upregulated in control-infiltrated leaves. However, when comparing control-infiltration to $SAP11_{CaPm}$ infiltration, it is evident that defense and phytohormone related transcripts are downregulated. The regulation of phytohormones is key to several phytoplasma diseases and plays a role in the regulation of plant defence responses against phytoplasmas [63]. The observed downregulation observed in this study of phytohormone transcripts thus indicates that $SAP11_{CaPm}$ is able to suppress plant defense response.

At the same time, however, the ATP biosynthetic processes are upregulated. Phytoplasmas are highly dependent on metabolic compounds from their hosts since they lack different metabolic genes, among them genes for ATP synthase, glycolysis and for oxidative phosphorylation [64–67]. In the same line, the ATP biosynthetic process and the oxidative phosphorylation are upregulated upon $SAP11_{CaPm}$ expression in *N. occidentalis* H.-M. Wheeler. This might reflect the enhanced levels of glycerolipid and glycerophospholipid metabolites and the numerous differentially expressed carbohydrate metabolism genes shown for sweet cherry virescence (SCV) phytoplasma infected sweet cherry trees [68, 69].

It can be assumed that an increase of the expression of genes involved in energy production in the host plant helps the phytoplasma to obtain sufficient metabolites and nutrients necessary for proliferation and colonization.

In contrast to metabolic genes, transcripts assigned to the chloroplast were downregulated 120 h upon $SAP11_{CaPm}$ infiltration. Photosynthesis rates have been shown to be reduced in many different phytoplasma infections

[70–72]. In line with that, several transcriptomic and proteomic studies of different phytoplasma infected plants have shown that numerous genes involved in photosynthesis are downregulated during infection [73]. The finding that SCY1 is strongly downregulated after 24 h and consistently at 120 h opens new insights on the possible SAP11 impact on chloroplast. SCY1 is involved in preprotein localization to the thylakoid [74] and mutant plants for SCY1 show impairment in thylakoid biogenesis [75] with a chloroplast-to-nucleus retrograde signal whit impaired production of nuclear-encoded chloroplast proteins [76] and chlorotic phenotypes [77]. During periods of darkness, SCY1 levels increase in the chloroplast which augments the import of nuclear-encoded proteins [78]. .

It is still unknown whether phytoplasma effector proteins directly target the chloroplast or if photosynthesis is compromised due to the changed plant metabolism [79]. A potential role of SAP11 in reducing the activity of photosystem II (PSII) by binding the AtTCP13 transcription factor has been suggested [79]. AtTCP13 is also known as PTF1, a transcription factor that regulates gene expression in chloroplasts via the plastid-encoded polymerase PEP [80]. PEP is the major chloroplast transcriptase and regulates the expression of *psbD* encoding the reaction center protein D2 of PSII. Since in *A. thaliana ptf1* mutants the expression of *psbD* is reduced [81], it can be assumed that SAP11 binding to TCP13 has a similar effect.Even though *psbD* was not downregulated in this study, two other genes encoding proteins from PSII were downregulated upon $SAP11_{CaPm}$ expression, namely the oxygen-evolving enhancer proteins 1 (OEE1/PsbO) and OEE2 (PsbP). Interestingly, OEE2/PsbP is directly targeted by a *Plasmopara viticola* RXLR effector protein [82, 83], while OEE1/PsbO binds to HIPM (HrpN-interacting protein from *Malus* spp.), a susceptibility gene for *Erwinia amylovora* infection in apple [84]. *Nicotiana benthamiana* Domin *psbP* knockout mutants showed a reduced growth and bleached leaves and were less susceptible to *Phytophtora capsici* infection [82]. In the same line, transgenic grapevine lines overexpressing *psbP* were more susceptible to *P. viticola* infection [82], indicating that PsbP downregulation enhances immunity. In contrast OEE1/PsbO and OEE2/PsbP proteins were more abundant in leaves of powdery mildew resistant cucumbers than in susceptible ones [85].

Conclusions

Our findings revealed a downregulation of defense-related genes, suggesting a suppression of the plant's immune response by $SAP11_{CaPm}$. Moreover, the modulation of genes involved in oxidative phosphorylation, as well as upregulation of ATP biosynthetic processes, hinted at a potential strategy employed by the phytoplasma via its effectors to exploit host metabolic pathways for its proliferation and colonization.

Additionally, the downregulation of transcripts related to chloroplast, such as SCY1, OEE1/PsbO or OEE2/PsbP suggest a potential link between $SAP11_{CaPm}$ and the compromise of photosynthetic processes, possibly through interactions with chloroplast-related factors.

Our study contributes to the understanding of $SAP11_{CaPm}$'s impact on phytoplasma host plants. However, questions regarding the direct targeting of chloroplasts and the intricate mechanisms leading to photosynthesis reduction and the effect on ATP biosynthetic processes and oxidative phosphorylation remain open. Future research elucidating these aspects will advance our understanding of phytoplasma-plant interactions and will result in new strategies for mitigating the impact of phytoplasma infections in agricultural settings.

Abbreviations

ABA	Abscisic Acid
ATP	Adenosine Triphosphate
BP	Biological Process
BSL1	Serine/Threonine-protein phosphatase BSL1, BSU1-like 1
BSU1	BRI1 SUPPRESSOR 1
'*Ca.* P. mali'	'*Candidatus* Phytoplasma mali'
CaMV 35S	Cauliflower Mosaic Virus 35 S promoter
CC	Cellular Component
cDNA	Complementary DNA
CHR8	Chromatin Remodeling 8
DET	Differentially Expressed Transcript
DUF21	Domain of Unknown Function 21
ELI3-1	ELICITOR-ACTIVATED GENE3-1
FDR	False Discovery Rate
GC	Guanine-Cytosine (content in DNA)
GFP	Green Fluorescent Protein
GO	Gene Ontology
JA	Jasmonate
KEGG	Kyoto Encyclopedia of Genes and Genomes
L2FC	Log2 Fold Change
LOX2	LIPOXYGENASE2
MF	Molecular Function
MOS1	Modifier of snc1,1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NB-LRR	Nucleotide-Binding Leucine-Rich Repeat
NTRC	NADPH-dependent thioredoxin reductase C
nt	Nucleotide collection database
OEE1/PsbO	Oxygen-Evolving Enhancer Protein 1
OEE2/PsbP	Oxygen-Evolving Enhancer Protein 2
PEP	Plastid-Encoded Polymerase
PiAVR2	*Phytophthora infestans* effector protein AVR2
PR1	PATHOGENESIS-RELATED GENE1
qPCR	Quantitative Polymerase Chain Reaction
RNA-seq	RNA sequencing
RBCS	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SA	Salicylic acid
SCV	Sweet Cherry Virescence phytoplasma
SEM	Standard Error of the Mean
SWI2/SNF2	SWITCH2/SUCROSE NON-FERMENTING2
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, and PROLIFERATING CELL FACTORS
TMM	Trimmed Mean of M values
tRBCS	Ribulose-1,5-bisphosphate carboxylase/oxygenase terminator

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions

CM contributed to the study design, performed all experiments, analyzed, and interpreted the data and wrote and revised the manuscript. MM analyzed and interpreted the data and revised the manuscript. BH contributed to the study design, interpreted the data, and revised the manuscript. KJ contributed to the study design, helped in analyzing and interpreting the data, contributed to the writing and revision of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available in the NCBI repository with BioProject ID PRJNA871046 and contains 13 BioSample datasets (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA871046). The Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GKBG00000000 (https://www.ncbi.nlm.nih.gov/nuccore/2428580569).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Publisher's Note

Curriculum Vitae

Mittelberger Cecilia

Education:

2018	Application for acceptance as a doctoral candidate at the Faculty of Natural Sciences I at the Martin Luther University Halle-Wittenberg
2017	State examination for the professional qualification as an agronomist (section A) in Italy (Dottore Agronomo e Forestale Sezione A)
2015	Master's degree in International Horticultural Sciences, with honours at the University of Bologna (Laurea Specialistica in Ortofrutticoltura Internazionale, con lode) Classe delle lauree specialistiche n.77/S-Scienze e Tecnologie Agrarie
2011-2014	Master of Horticultural Science at the Technical University of Munich
February 2012 - March 2013	Erasmus stay at the University of Bologna
May 2013 – December 2013	Practical work at Laimburg Research Centre for the Master's thesis entitled „Qualitative and Quantitative Analysis of Chlorophyll Catabolites in Plants Infected by Phytoplasma"
Degree:	**Master of Science, grade: 1.0 with honours**
2008-2011	Studied Horticultural Sciences at the Technical University of Munich, Germany
Degree:	**Bachelor of Science, grade: 1.3**
2005-2008	Technical secondary school for agriculture (Fachoberschule für Landwirtschaft), Auer, Italy
Degree:	**High school diploma (Matura), grade: 100/100 points**
2003-2005	Two years at Laimburg Technical College (Fachschule Laimburg, Pfatten/Vadena, Italy), specialising in horticulture then entrance examination for the 1st & 2nd class of the technical secondary school for agriculture (Fachoberschule für Landwirtschaft, Auer/Ora, Italy)

Professional experience:

02/2023 till today	Owner and farm manager of the direct marketing farm „Föranerhof" in Ritten/Renon (Italy)
12/2017 till today	Employee at the Laimburg Research Centre (Pfatten/Vadena, Italy)

	in the workink group Functional Genomics with research focus on apple proliferation disease
Summer semester 2020 + 2021	Teaching assistant for the lecture Plant Genetics at the Free University of Bozen-Bolzano (Italy)
10/2018	One-month research stay at the Leibniz Institute of Plant Biochemistry in Halle (Germany) in the research group Jasmonate Function & Mycorrhiza
02/2015 - 12/2017	Project collaborator in the APPL2.0 project at the Laimburg Research Centre (Pfatten/Vadena, Italy) with research focus on apple proliferation disease
01/2014 - 02/2015	Technical assistant in the chemical laboratory of the Agricultural High School in Auer/Ora (Italy)
05/2013 -12/2013	Collaboration in the project „BIOPHYTIROL - Degradation Products of Chlorophyll as Biomarkers for the Early Eetection of Infestation of Native Crops by Microbial Pests" at the Laimburg Research Centre (Pfatten/Vadena, Italy)
11/2012 - 08/2013	Collaboration in the project „Scientific Research on New Breeding Methods for Ornamental Plants" at the young plant nursery A. Psenner (Bozen/Bolzano, Italy). Writing the project report
11/2011 - 12/2012	Research assistant at the Chair of Economics of Horticulture and Landscaping at the Technical University of Munich, Germany
11/2010 - 03/2011	Research assistant at the Chair of Agricultural Economics at the Technical University of Munich, Germany
2009-2010	Internships as part of university education:
August 2010 - September 2010	South Tyrolean Advisory Council for Fruit and Viticulture (Südtiroler Beratungsring für Obst- und Weinbau) (Lana, Italy): Insights into the problems of apple cultivation. Carrying out assessments and surveys on various diseases (apple proliferation, scab, codling moth, fruit skin moth)
August 2009 - October 2009	Young plant nursery A. Psenner (Bozen/Bolzano, Italy):
February 2009 - March 2009	Performed ELISA tests on mother plants for young plant production and produced in vitro cultures of *Pelargonium* and *Lantana* for virus clearance and breeding.

Soft Skills:

Language Skills	**German:** Mother tongue **Italian:** Fluent in written and spoken Italian, Bilingualism certificate A (C1) of the Province of Bolzano (2013) **English:** Fluent in speaking and writing, Cambridge English Level 2 Certificate in ESOL International (First), Grade A, Council of Europe Level C1 (2017)

IT Skills	Very good knowledge of all common Microsoft Office applications
	Image processing programmes: Adobe Lightroom, Photoshop, InDesign, Illustrator, BioRender Statistical programmes: R, SPSS, Graph Pad Sequence analysis: Geneious Programming languages: Basic knowledge of Phyton and awk
Awards	Prize of the Mayor of the City of Freising for the best Master's thesis in the Faculty of Agricultural and Horticultural Sciences (TUM, Germany, July 2015)
	Special prize from the Federal Association of University Graduates/ Engineers in Horticulture and Landscape Architecture - BHGL (Germany, July 2015)
	Award for academic excellence 2014 of the initiative „Proexcellentia - The best academics in South Tyrol" of the Stiftung Südtiroler Sparkasse (Italy, December 2014)
Voluntary Activity	Since 2020: Councillor in the municipality of Ritten/Renon (Italy)

Ritten/Renon, 18.11.2024 Mittelberger Cecilia

Publication List

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Janik, K.; **Mittelberger, C.** and Moser, M. (2020). Lights out. The chloroplast under attack during phytoplasma infection? *Annual Plant Reviews* (3), pp. 1–28. https://doi.org/10.1002/9781119312994.apr0747.

Mittelberger, C.; Stellmach, H.; Hause, B.; Kerschbamer, C.; Schlink, K.; Letschka, T. and Janik, K. (2019). A Novel Effector Protein of Apple Proliferation Phytoplasma Disrupts Cell Integrity of Nicotiana spp. Protoplasts. *International Journal of Molecular Sciences* 20 (18), pp. 1–16. https://doi.org/10.3390/ijms20184613.

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Mittelberger, C.; Obkircher, L.; Oettl, S.; Oppedisano, T.; Pedrazzoli, F.; Panassiti, B.; Kerschbamer, C.; Anfora, G. and Janik, K. (2017). The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny. *Plant Pathology* 66 (6), pp. 1015–1021. https://doi.org/10.1111/ppa.12653.

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