Short communication

An effector of apple proliferation phytoplasma targets TCP transcription factors—a generalized virulence strategy of phytoplasma?

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SUMMARY

The plant pathogen Candidatus Phytoplasma mali (P. mali) is the causative agent of apple proliferation, a disease of increasing importance in apple-growing areas within Europe. Despite its economic importance, little is known about the molecular mechanisms of disease manifestation within apple trees. In this study, we identified two TCP (TEOSINTE BRANCHED/CYCLOIDEA/Proliferating CELL FACTOR) transcription factors of Malus x domestica as binding partners of the P. mali SAP11-like effector ATP_00189. Phytohormone analyses revealed an effect of P. mali infection on jasmonates, salicylic acid and abscisic acid levels, showing that P. mali affects phytohormonal levels in apple trees, which is in line with the functions of the effector assumed from its binding to TCP transcription factors. To our knowledge, this is the first characterization of the molecular targets of a P. mali effector and thus provides the basis to better understand symptom development and disease progress during apple proliferation. As SAP11 homologues are found in several Phytoplasma species infecting a broad range of different plants, SAP11-like proteins seem to be key players in phytoplasmal infection.

Keywords: apple proliferation, ATP_00189, effector protein, phytohormones, TCP, transcription factor, yeast two hybrid.

Infection with the biotrophic bacterial pathogen Candidatus Phytoplasma mali (P. mali), the causative agent of apple proliferation (AP), can lead to massive yield losses and economic damage in apple production regions (Strauss, 2009). The province of South Tyrol/Alto Adige in northern Italy is the largest interconnected apple-growing region in Europe and has suffered dramatically from apple proliferation outbreaks during the last decade (Berger, 2007; Mattedi et al., 2007). Infected apple trees (Malus x domestica) develop symptoms comprising witches’ brooms, stunting, foliar reddening and undersized, colourless and tasteless fruits (Kartte and Seemüller, 1988; Seemüller and Schneider, 2007). Phytoplasma exhibit a unique life cycle that involves a reproductive phase in a phloem-feeding insect and subsequent transmission into the plant (Christensen et al., 2005). Inside the plant phloem, the bacteria replicate and can be re-transmitted into the phloem-sucking insect to complete their infectious life cycle and enable their dissemination to other host plants (Sugio et al., 2011b). Much progress has been made in unravelling the molecular basis of phytoplasma infection using the Ca. P. asteris strain aster yellow-witches’ broom (AY-WB), mainly by the identification and characterization of the bacterial effectors that play a role in disease manifestation and symptom development in Arabidopsis thaliana (Bai et al., 2009; Kartte and Seemüller, 1988; Lu et al., 2014a,b; MacLean et al., 2011, 2014; Sugawara et al., 2013; Sugio et al., 2011a, b). Although the genome has been fully sequenced (Kube et al., 2008), no functional effector protein of P. mali has been described to date. Therefore, the molecular mechanisms underlying disease manifestation and symptom development in the natural host Malus x domestica remain elusive. A study performed with P. mali identified several genes expressed in Malus x domestica during infection, amongst others a gene that encodes the protein ATP_00189 (GenBank: CAP18376.1), a protein which shares homology to the AY-WB effector SAP11 (Siewert et al., 2014; Sugio et al., 2011a). ATP_00189 contains an N-terminal sequence-variable mosaic (SVM) protein signal sequence (Pfam entry: PF12113) and shares 41% identity to SAP11.

In the present study, we addressed the following questions: (i) whether the SAP11 homologue ATP_00189 of P. mali is differentially expressed in spring and autumn in naturally infected apple trees; (ii) whether sequence variants of this potential effector protein occur within South Tyrol/Alto Adige; (iii) which proteins are
targeted by ATP_00189; and (iv) whether infected apple trees show differential hormonal regulation in spring and autumn.

The function of SAP11 is mainly based on three domains: the signal peptide that mediates extra-bacterial translocation of the protein into the surrounding environment; the nuclear localization sequence that targets the protein to the plant nucleus; and the TCP (TEOSINTE BRANCHED/CYCLOIDEA/Proliferating Cell Factor) binding domain that mediates binding to TCP transcription factors (TFs) (Sugio et al., 2014). Although these domains are important for the effector function of this protein, the amino acid sequences of these domains have been described to be poorly conserved between SAP11-like proteins of different phytoplasma species (Sugio et al., 2014). A comparison of SAP11 from AY-WB with ATP_00189 shows that these two proteins share 40% sequence identity. At amino acid positions within the SVM signal peptide and the TCP-binding region that are not identical in both proteins, similar hydrophobic amino acid patterns are evident in ATP_00189 and SAP11 (Fig. S1, see Supporting Information). This might indicate a functional selection based on hydrophobic parts within stretches of the proteins, rather than a selection for exact amino acid motifs. Hydrophobicity-mediated protein functions could, for example, involve membrane binding, protein folding, and polymerization and interaction with host targets. Rümpler et al. (2015) hypothesized that the binding function of MADS-box TFs is determined by a characteristic hydrophobicity pattern, rather than a defined amino acid sequence in the keratin-like domain (K-domain) of these factors. Interestingly, this K-domain is targeted by the phytoplasmal effector SAP54/PHYLL1 which, itself, mimics and binds the K-domain of the TF, and thus primes it for ubiquitin-mediated proteosomal degradation (MacLean et al., 2011, 2014; Maejima et al., 2014; Rümpler et al., 2015). Phytoplasmas are genetically highly dynamic bacteria (Bai et al., 2006; Jarausch et al., 2000; Sugio and Hogenhout, 2012). Sequence analysis of different loci has revealed that P. mali genotypes from different sampling sites within South Tyrol/Alto Adige and within individual trees can be highly variable (Janik et al., 2015). To analyse whether ATP_00189 variants found in South Tyrol/Alto Adige resemble the protein sequence published in 2008 (Kube et al., 2008), DNA from symptomatic apple trees from 20 different orchards was purified using a method described elsewhere (Schlink and Reski, 2002). In all samples, infection with P. mali was confirmed by polymerase chain reaction (PCR) using fO1/rO1 primers (Lee et al., 2000) and by real-time PCR using the probe AP (Mehle et al., 2013). Infection with the other 16SrX group phytoplasmas P. pyri and P. prunorum was ruled out by performing the same real-time PCR with the respective probes (Mehle et al., 2013). The ATP_00189 gene was amplified, subcloned and sequenced (Methods S1, see Supporting Information). The prevalent genetic sequence of ATP_00189 in South Tyrol/Alto Adige (ATP_00189_STAA; Accession: KMS01063), which occurred in all trees and in about 91% of the tested clones, contained three single nucleotide polymorphisms (SNPs) compared with the sequence described by Kube et al. (2008). The ATP_00189 sequence published by Kube et al. (2008) was only detected in one tree, in which the prevalent sequence type ATP_00189_STAA was also present. Two SNPs present at the 5'-end of ATP_00189_STAA lead to amino acid exchanges in the signal peptide (Fig. S1), whereas the third SNP at the 3'-end of the gene does not lead to translational differences, leaving the amino acid sequence of the mature, i.e. signal peptide lacking ATP_00189, protein unaffected. Taking the general genetic dynamics of phytoplasma into consideration (Bai et al., 2006; Jarausch et al., 2000; Sugio and Hogenhout, 2012), the conserved nature of ATP_00189 indicates an importance of this protein for the pathogen.

To determine which proteins are targeted by the SAP11 homologue of P. mali in apple trees under natural conditions, a cDNA library of the leaf transcriptome of Malus x domestica cv. ‘Golden Delicious’ was generated (see Methods S1) and a yeast two-hybrid (Y2H) screen was performed with ATP_00189 as the bait. Sequence analyses of the positive clones in the Y2H screen revealed four different Malus x domestica binding partners of ATP_00189. Three of the four interactors found in this study are homologues of class II TCP TFs from A. thaliana. TCP4-like (GI:657979223), which corresponds to Malus x domestica (Md) MtTCP25 (Xu et al., 2014); TCP13-like (GI:658044279), which is a homologue of MdTCP24 (Xu et al., 2014); and an isoform of TCP18-like (GI:657966084), similar to MdTCP16 (Xu et al., 2014). In addition to the interactions with Malus proteins containing TCP domains, interactions were found between ATP_00189 and a Malus x domestica library clone that shares partial identity with 60 amino acids of the C-terminal part of a putative chlorophyll(ide) b reductase Nyc1, chloroplast-like isoform X2 from Glycine max (GI:571465492). A list of all interacting cDNA fragments identified in the Y2H screen can be found below ‘Accession numbers’.

The full-length genes of MdTCP24 and MdTCP25 (plant transcription factor database http://planttfdb.cbi.pku.edu.cn/ id MDP0000692406 and id MDP0000442611) were de novo amplified from Malus x domestica DNA. The Nyc1 and MdTCP16 genes contain introns and might have different splice variants. Thus, the identified fragments of these genes were amplified from cDNA. Using the de novo subcloned prey vectors, interaction could be shown for ATP_00189 and the full-length MdTCP24 and MdTCP25 in co-transformed yeast (Fig. 1). The expression of ATP_00189 was confirmed by immunoblot analysis using an antibody against the LexA-tag, which was coupled to ATP_00189 (Fig. S2, see Supporting Information). In planta interactions of ATP_00189 with MdTCP24 and MdTCP25 were verified by bimolecular fluorescence complementation (BiFC) in Nicotiana benthamiana mesophyll protoplasts (Fig. 2). Mesophyll protoplasts...
were prepared as described by Sheen (2002). ATP_00189 and the MdTCP encoding cDNAs were subcloned into the BiFC vectors pE-SPYNE and pE-SPYCE, respectively (Walter et al., 2004). The BiFC vectors contain the information for the N-terminal (pE-SPYNE) or C-terminal (pE-SPYCE) halves of yellow fluorescent protein (YFP). An interaction of the proteins leads to the reconstitution of YFP by confocal laser scanning microscopy, 19%–24% of the randomly visualized protoplasts showed a strong YFP signal predominantly in the nucleus (Fig. 2 and Table S1, see Supporting Information). The putative interaction of NYC1 and the MdTCP16 isoform fragments, indicated by the initial Y2H library screen, could not be confirmed, in either ATP_00189 co-transformed yeast carrying the cDNA of these genes or BiFC analyses. As a negative control, a member of the Malus x domestica class I TCP TF family (MdTCP34) was used as a proxy for this TF subclass (see below), for which no interactor was found in the Y2H library screen. Accordingly, no interaction between MdTCP34 and ATP_00189 was observed in the co-transformed yeast (Fig. 1). In BiFC experiments, only 2% of the counted protoplasts showed very weak signals of ATP_00189 interaction with MdTCP34 (Table S1), whereas the majority of protoplasts did not exhibit any YFP fluorescence (Fig. 2).

In a genome-wide screen of Malus x domestica, 52 TCP genes were identified (Xu et al., 2014). These TFs share the so-called TCP domain which mediates nuclear localization, DNA binding and protein–protein interaction (Cubas et al., 1999; Kosugi and Ohashi, 2002). TCP proteins can be divided into two classes based on sequence similarities (Cubas et al., 1999; Kosugi and Ohashi, 2002), and are involved in the regulation of diverse plant cellular processes, such as branching or floral and leaf development (Martin-Trillo and Cubas, 2010). In analogy with the identified A. thaliana interaction partners of SAP11 (Sugio et al., 2011a), ATP_00189 binds Malus x domestica homologues of two TCP protein members of the CINCINNATA (CIN)-related TCP group II, namely MdTCP25 (a homologue of A. thaliana TCP4) and MdTCP24 (a homologue of A. thaliana TCP13/PFT1). A TCP4 homologue of Malus x domestica was shown to bind the FLOWERING LOCUS T (MdFT1) involved in apple flowering (Kotoda et al., 2010; Mimida et al., 2011), indicating a function of TCP4 apple homologues in developmental processes, such as fruit ripening, where the expression of MdTCP25 has been shown (Xu et al., 2014). TCP4 of A. thaliana has been shown to be involved in leaf development, thereby negatively regulating cellular mitotic processes, leaf growth and abscisic acid (ABA) responses in A. thaliana (Danisman et al., 2012; Sarvepalli and Nath, 2011a,b). Furthermore, it was shown that TCP4 of Arabidopsis regulates jasmonic acid (JA) biosynthesis by activating the expression of LOX2 (lipoxygenase 2) (Danisman et al., 2012; Schommer et al., 2008), an enzyme that catalyses one of the first steps of JA biosynthesis in A. thaliana (Vick and Zimmerman, 1983). SAP11 binds and destabilizes TCP4 and other CIN-TCP proteins, and thus interferes with LOX2-mediated JA accumulation in A. thaliana. This reduction in JA levels, in turn, enhances oviposition of the leafhopper Macrosteles quadrilineatus on the infected plant, and thus supports bacterial dissemination to other host plants (Sugio et al., 2011a). In our study, we showed that ATP_00189 binds MdTCP25 of Malus x domestica and might thus be involved in the down-regulation of JA biosynthesis, leading to diminished JA responses (Sugio et al., 2011b, 2014) and increased ABA levels and responses (Sarvepalli and Nath, 2011b). Our results further demonstrated the binding of ATP_00189 to MdTCP24, a Malus x domestica homologue of TCP13 from A. thaliana, another CIN-TCP group II protein, also known as PTF1. This TF is nuclear-encoded, but located in plastids (Baba et al., 2001), thus most likely playing a role in plastid gene expression and regulation. PTF1 is involved in phosphate tolerance, carbon metabolism and

![Fig. 1 ATP_00189 binds class II TCP (TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTOR) transcription factors (TFs) MdTCP24 and MdTCP25. A yeast two-hybrid (Y2H) screen was performed using the binding domain (BD)-coupled ATP00189 expression plasmid (pLexA-N-ATP_00189) and an expression plasmid carrying the activation domain (AD) coupled to the full-length MdTCP of Malus x domestica (pGAD-HA-ccdB constructs), identified in the Y2H screen. MdTCP34 was not identified as an interaction partner in the Y2H screen and serves as a negative control. Interaction between ATP_00189 and the respective TCP TF complements the auxotrophy for histidine and adenine. In the absence of interaction, co-transformed yeast does not grow on adenine- and histidine-depleted selection plates.](image-url)
root growth in maize (Li et al., 2011) and in ABA-regulated transcriptional responses in the chloroplast in Arabidopsis (Yambursenko et al., 2015). SAP11 has been shown to degrade TCP13/PTF1 (Sugio et al., 2011a) and affects phosphate metabolism, anthocyanin accumulation and root architecture in A. thaliana (Lu et al., 2014b). Commonly observed symptoms of apple proliferation, such as reddening and altered root growth (Kunze, 1979), might thus be induced by SAP11-mediated TCP13/PTF1 degradation in P. mali-infected plants.

As AY-WB in A. thaliana affects JA biosynthesis via SAP11, we were interested in whether hormonal changes occur in apple proliferation. To reveal which hormonal pathways are affected by P. mali, we analysed the phytohormonal levels at two different time points. Pools of leaves from symptomatic and healthy apple trees of the variety Malus x domestica cv. ‘Golden Delicious’ were harvested in May, when phytoplasma could not be detected in the canopy, and in October, when the bacteria have colonized the leaves and express atp_00189 (Fig. 3, top panel I). Phytohormone quantification was performed using liquid chromatography-mass spectrometry analysis (Vadassery et al., 2012). In infected and control trees, levels of cis-12-oxo-phytodienoic acid (OPDA), (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), salicylic acid (SA) and ABA were determined (Fig. 3, bottom panel II). In May, OPDA, JA-Ile and SA were significantly higher in AP-infected trees than in controls (Fig. 3, bottom panel II, a–c). JA and SA levels in infected trees were not increased any further in October; indeed, they tended to be below the levels of the non-infected control (Fig. 3, bottom panel II, b, c). From May to October, levels of SA, OPDA and JA-Ile in the leaves of healthy controls increased, whereas the levels in P. mali-infected apple trees did not change significantly (Fig. 3, bottom panel II, a–c). Similarly, Musetti et al. (2013) detected a reduced expression of JA marker genes in infected trees in the autumn. From May until October, ABA levels decreased in P. mali-infected and control trees, but the amount of ABA in infected trees remained significantly higher in October compared with the control group (Fig. 3, bottom panel II, d). These results support several findings of Zimmermann et al. (2015), who showed an increased ABA accumulation for one time point after infection, which we also observed in samples harvested in October, and
increased SA levels that we detected in material collected in May. In the study of Zimmermann et al., however, no effect on JA-Ile and OPDA was observed. This could be because the authors chose another sampling time point. Our results suggest that the regular, seasonal increase of JA-Ile, OPDA and SA is impeded by phytoplasmal infection, either by direct interference with their synthesis and/or indirectly through phytohormonal crosstalk, as antagonistic actions between ABA, SA and JA have been described (Anderson...
et al., 2004; Mohr and Cahill, 2007; Yasuda et al., 2008). Phytoplasm mali disappears from the apple tree crown during the winter and recolonizes the aerial parts of the tree beginning in spring (Baric et al., 2011; Loi et al., 2002; Pedrazzoli et al., 2008; Seemüller et al., 1984). Hence, the bacterial titre in the crown is low until June and increases throughout the growing season. On the one hand, this might explain the induction of SA and JA in spring when the first phytoplasmas recolonize the aerial parts and are recognized by the plant. On the other, the increasing bacterial concentration may explain their growing impact on leaf phytohormone levels in autumn. The observed hormonal changes in infected apple trees indicate a phytoplasm-mediated effect on JA, ABA and SA signalling. It remains to be clarified whether altered JA levels and action have a similar effect on the P. mali leafhopper vectors Cacopsylla picta or C. melanoneura, as has been shown for oviposition or feeding of the leafhoppers Macrosteles quadrilineatus and Empoasca in the Arabidopsis and tobacco systems, respectively (Kallenbach et al., 2012; Sugio et al., 2011a).

Interestingly, increased LOX activity in P. mali-infected apple tree leaves correlates with the recovery phenomenon, characterized by a type of resilience against P. mali in the tree canopy (Carraro et al., 2004; Musetti et al., 2013; Patui et al., 2013; Seemüller et al., 1984; Schmid, 1975); this emphasizes the importance of decreased JA levels for the success of phytoplasm infection. However, biotrophic pathogens are generally sensitive to SA-mediated responses (Glazebrook, 2005; Thomma et al., 2001). SA induction can lead to systemic acquired resistance, which results in a broad-spectrum resistance against pathogens and thus plays an important role in plant immunity ( Ryals et al., 1996). The latest results of a study with SAP11-transgenic A. thaliana plants indicate a role of SAP11 in the down-regulation of SA responses (Lu et al., 2014b). As SA accumulation in P. mali-infected apple trees is reduced, as shown in this study, it is plausible to consider a similar function for ATP_00189 as well.

In our study, we focused on the characterization of the Malus x domestica binding partners of P. mali ATP_00189, a homologue of the well-characterized AY-WB effector SAP11, to elucidate the molecular processes underlying the disease progress of AP. Interestingly, we found that ATP_00189 shares targets with SAP11, supporting the idea that the principles of SAP11-mediated TCP factor degradation found in A. thaliana might also be valid during P. mali infection in apple trees. Infected apple trees show altered hormonal levels compared with controls, indicating an effect of P. mali on the hormone system of Malus x domestica. Integrating our data with results achieved in AY-WB SAP11 studies in A. thaliana, we hypothesize that, during infection of its natural host, the apple tree, ATP_00189 of P. mali has similar functions to SAP11 revealed in its host, the model plant A. thaliana. Given the fact that SAP11 homologues are found in many different phytoplasm species and that the motifs of this protein seem to be conserved throughout different phytoplasm species, it is likely that SAP11 and its relatives play a pivotal role in phytoplasmal infection. Targeting this protein and its derivatives might be a suitable approach for future infection prevention or therapy of economically important phytoplasmal diseases.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Fig. S1 Comparison of amino acid sequences of SAP11 from aster yellow-witches’ broom (AY-WB) phytoplasma (WP_011412651.1) and ATP_00189 (CAP18376.1). Sequences of the SAP11 protein from AY-WB, the SAP11-like protein (AP) from apple proliferation phytoplasma as described by Kube et al. (2008) and the SAP11-like protein AP_STAA (the main variant found in northern Italy, South Tyrol/Alto Adige) share stretches of identical amino acid motifs or amino acids of similar hydrophobicity. This indicates similar functions of the proteins. The colour and height of the bars in the respective panels indicate the relative levels of hydrophobicity of adjacent amino acids within the sequence (Hydrophobicity). Sequence identities of AP and AP_STAA with the AY-WB reference protein are highlighted by green boxes above the sequences. Differences in the signal peptide between AP and AP_STAA are indicated by red letters in a box. Analysis was performed using Geneious software (http://www.geneious.com; Kearse et al., 2012).

Table S1 In planta interaction of ATP_00189 with MdTCP24, MdTCP25 or MdTCP34. Mesophyll protoplasts of *Nicotiana benthamiana* were co-transfected with the binary bimolecular fluorescence complementation (BIFC) vectors pE-SPYNE and pE-SPYCE harbouring ATP_00189 (fused to the N-terminal subunit of yellow fluorescent protein (YFP)) and MdTCP24, MdTCP25 or MdTCP34 (each fused to the C-terminal subunit of YFP), respectively. Interaction leads to the generation of YFP fluorescence. Transformation with pE-SPYNE-ATP_00189 alone served as a negative control. Protoplasts were randomly counted and the number and percentage of YFP-positive protoplasts for each co-transfection were determined.

Methods S1 Supplementary Material and Methods Section. Several methods mentioned a legend is thus not intended.