# Phytopathogenic Mollicutes

An International Journal on Phytoplasma, Spiroplasma and other Phloem-limited Plant Pathogens

*Published by :*
Technology Society of Basic and Applied Sciences

mollicutes.indianjournals.com

Print ISSN : 2249-4669
Online ISSN : 2249-4677

**Volume 13 • Number 1 • June 2023**




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***Indexed in:*** UGC-CARE(Group II), Index Copernicus(2016, ICV - 78.85), NAAS Rating 2021 - 5.41, Scientific Journal Impact Factor (SJIF - 6.02), InfoBase Index(IB Factor - 2.8), EBSCO Discovery, Summon(ProQuest), Google Scholar, CNKI Scholar, J-Gate, Primo & Primo Central, ESJI, I2OR, Indian Science, Cite Factor, DRJI, EZB, IIJIF, Indian Science Abstracts and Agricola.

**Phytopathogenic Mollicutes**
Vol. 13 (1), June 2023, 21-22

doi: 10.5958/2249-4677.2023.00011.7

**Omics**

# Overexpressing a molecular target of SAP11$_{CaPM}$ in apple

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## Abstract

The bacterial effector SAP11$_{CaPM}$ can bind several members of the TCP transcription factor gene family. To investigate the role of the interaction in the infection process, apple plants stably overexpressing the *MdTCP4a* gene were generated and infected with a '*Candidatus* Phytoplasma mali' strain. Preliminary results show a statistically significant lower concentration of the phytoplasma in the aerial parts of the *in vitro* transgenic lines than in the non-transformed "Gala" plants, suggesting that the overexpression of *MdTCP4a* gene could limit phytoplasma multiplication. Interestingly, soil-acclimatized transgenic plants displayed phenotypic characteristics similar to the symptoms of apple proliferation.

**Keywords:** '*Candidatus* Phytoplasma mali', effector proteins, TCP transcription factor, *Malus domestica*

## Introduction

In the last years, research focused on effector proteins allowed the discovery of several new effectors and shed light on their mechanisms of action, providing insight into the complex interplay between phytoplasmas and their plant hosts. Effector proteins secreted by phytoplasmas are diverse and multifunctional, and their effects appear to range from suppressing plant immune responses, altering plant hormone levels, disrupting cell division, and inducing abnormal development. SAP11 is among the first phytoplasma effectors discovered and characterized. Its homolog in '*Candidatus* Phytoplasma mali', often referred to as SAP11$_{CaPM}$, was shown to bind several members of the TCP transcription factor gene family (Janik *et al.*, 2017; Strohmayer *et al.*, 2021; Mittelberger *et al.*, 2022). These genes play a crucial role in regulating plant morphogenesis, flowering, leaf development, and responses to abiotic and biotic stresses (Danisman, 2016). However, there is limited knowledge of their specific functions in apple since most of the data derive from studies on model plant species. In the context of a better understanding of the role of *TCP4a* gene in *Malus domestica* and its deactivation in apple proliferation, transgenic plants stably overexpressing the gene have been generated and characterized. Here it is presented the preliminary results of the infection of the generated transgenic plants with '*Ca.* P. mali', to test the hypothesis that the deactivation of *TCP* gene transcription factors by the bacterial effector SAP11$_{CaPM}$ is crucial for a successful infection.

## Materials and Methods

The full-length coding sequence of the *MdTCP4a* gene of *M. domestica* cultivar Golden Delicious under control of the *Cauliflower Mosaic Virus* 35S promoter was transformed into *M. domestica* cultivar Gala via *Agrobacterium*-mediated transformation. The relative expression of *MdTCP4a* gene in both *in vitro* and soil-acclimatized plants was estimated by a quantitative PCR assay (qPCR). Three transgenic lines and "Gala" for comparison were infected with '*Ca.* P. mali' by *in vitro* micrografting with infected material of *M. domestica* cultivar Golden delicious plants as described by Jarausch *et al.* (1999). A minimum of ten replicates per line was performed. After 40 days of graft contact, scions were separated from the rootstocks and partitioned into two/three subgroups each, and subcultured to generate a final set of 25 single infected plants per line. After 30 additional days of culture DNA was extracted from the aerial parts of the plants and used in a qPCR assay to detect the presence of the phytoplasma as described by Baric and Dalla Via (2004) by amplifying the phytoplasma 16S rRNA gene and the *M. domestica* chloroplast gene coding the tRNA leucine. The Cq values of target and reference, calculated on technical triplicates, were combined to calculate the ΔCq value for each plant and these values were used to estimate the relative phytoplasma titer with the formula $x=2\Delta Cq$ (Silver *et al.*, 2006). The values thus obtained were multiplied by 100,000 to facilitate data visualization.

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## Results

The relative expression of *MdTCP4a* gene of the transgenic lines varies between *in vitro* and *ex vitro*: the first show a moderate increase or slight decrease compared to non-transformed shoots, while the in soil-acclimatized ones display a 6- to 10-fold increase of the *MdTCP4a* gene transcript. These results reflect on the plants' phenotype as well: the 35S::*MdTCP4a* plants did not display phenotype changes after more than a year of micropropagation while kept *in vitro*, shortly after the soil acclimatisation the transgenic lines displayed loss of apical dominance, smaller leaves and shorter stems compared to the non-transformed (Figure 1). Interestingly, these phenotypic characteristics disappeared approximately six months after the appearance.


**Figure 1.** Phenotype characteristics of transgenic apple plants (cv Gala) overexpressing the *MdTCP4a* gene two months after the soil acclimatisation, compared to a non-transformed "Gala" plant. Line IA.2 shows smaller and crinkled leaves compared to non-transformed, while lines IB.3 and IIIA show a loss of apical dominance and are shorter than the non-transformed.

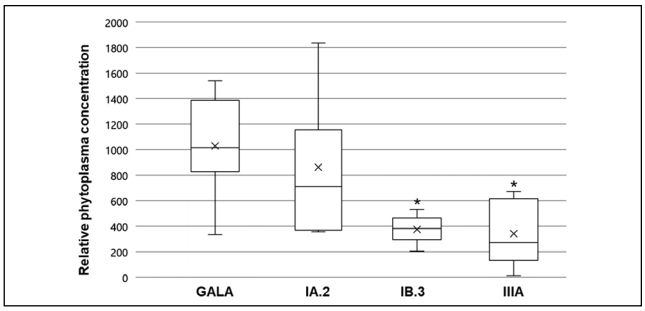


**Figure 2.** Box plot illustrating the '*Ca*. P. mali' titer measured for three 35S::*MdTCP4a* transgenic lines (IA.2; IB.3; IIIA) and the control (non-transformed "Gala") via qPCR. Boxes represent the distribution of first and third quartiles, while the error bars refer to the total distribution of non-outliers' samples. "X" and the horizontal lines inside the boxes indicate mean and median values, respectively. Asterisk indicates a statistically significant difference ($p < .05$).

Results of the micrografting infection tests, displayed as a box plot in Figure 2, indicate that the transgenic line IA.2 does not show a significant difference in the phytoplasma concentration compared to non-transformed, while lines IB.3 and IIIA display a significantly lower quantity ($p < .05$ per one-way ANOVA analysis with Tukey's Honest Significant Difference post hoc test).

## Discussion

Soil-acclimatized transgenic plants showed dramatic phenotypic changes during the first three months including smaller stems, loss of apical dominance, and small, crinkled leaves. Interestingly, these phenotype characteristics resemble some of the typical symptoms of apple proliferation, which include small leaves, development of shoots from axillary buds, which give rise to secondary shoots that originate witches' brooms and, often, stunting (Schmid, 1975; Seemüller, 1990). The phenotype characteristics disappeared three months after the acclimatization, indicating the establishment of a physiological condition after the disequilibrium induced by the acclimatisation process. This phenomenon is consistent with the hypothesis of post-transcriptional tight regulation of *TCPs* operated by miR319 (Palatnik *et al*., 2003).

Interestingly, two out of three *in vitro* transgenic lines infected with '*Ca*. P. mali' showed a statistically significant lower concentration of the phytoplasma in the plant's aerial parts than non-transformed "Gala". The concentration of phytoplasma in the aerial parts of the plant is strongly correlated with the severity of the symptoms displayed by infected plants (Carraro *et al*., 2004). Nonetheless, these promising results need to be confirmed by increasing the sample size and performing *ex vitro* infection screening, thus allowing the phenotype characterization of infected plants.

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