Attempts of a Transient Transformation of Grapevine

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***Abstract* - *Agrobacterium*-mediated transient transformation is routinely performed for the analysis of gene function in several plant species. There have been some reports of successful application of *Agrobacterium*-mediated transient transformation also to grapevines. In order to analyze the function of six potential '*Candidatus* Phytoplasma solani' ("bois noir" phytoplasma, BN) effector genes, a grapevine *Agrobacterium*- mediated transient transformation protocol was employed. Although *Agrobacterium*-mediated transient transformations of tobacco and potato are routinely performed in the laboratory and although the exaction following the published protocols or varying several parameters and the ability to infiltrate indicator stain Bromophenol blue in grapevine leaves, *Agrobacterium*-mediated transient transformation of grapevine did not work in the experimental conditions tested.**

***Key words* - *Agrobacterium*, grapevine, transient transformation**

I. Introduction

Plant microbial pathogens produce specific molecules, called effectors that alter their hosts in order to successfully invade and multiply in plants. Phytoplasmas are limited to the phloem sieve cells of their plant hosts. Thus, effectors are released into the cytoplasm of sieve cells upon secretion by phytoplasmas. The effectors may interact with plant components in the sieve cell or may unload from the phloem to interact with target molecules in companion, mesophyll, and other plant cells. It is generally supposed that effectors improve the pathogen fitness. Thus, it can be speculated that phytoplasma effectors induce various physiological and morphological changes during infection of their hosts, and phytoplasmas gain fitness advantages from inducing such changes (Sugio et al. 2011).

Using bioinformatics tools, six potential '*Candidatus* Phytoplasma solani' (Quaglino et al. 2013) ("bois noir" phytoplasma, BN) effectors were identified. In order to study their influence on physiological and morphological changes of grapevine (*Vitis vinifera* L.), it was tried to introduce a grapevine *Agrobacterium*-mediated transient transformation protocol.

Sufficient acceptable levels of expression of the markers β-glucuronidase (GUS) and green fluorescent protein (GFP) were achieved using vacuum agro-infiltration of grapevine leaves from plants grown *in vitro* in the presence of additional virulence factors (carried on plasmid pCH32) in the *Agrobacterium* strain (Santos-Rosa et al. 2008).

Grapevine leaf tissues of *in vitro* grown plants were successfully transiently transformed with fluorescent markers targeted to cytoplasm (free GFP and mRFP1), endoplasmatic reticulum (GFP::HDEL), chloroplast (GAPA1::YFP) and mitochondria (b::GFP) that were visualized by confocal microscope (Zottini et al. 2008).

Further on, transient overexpression of stilbene synthase in *in vitro* grapevine leaves before infection with downy mildew (*Plasmopara viticola*) was achieved (Santos-Rosa et al. 2008); and transient silencing of the grapevine polygalacturonase-inhibiting protein (PGIP) gene was shown by agroinfiltration with a double strand RNA (dsRNA) construct for RNA interference in leaves of grapevine plants grown *in vitro* (Bertazzon et al. 2011).

Moreover, *Agrobacterium*-mediated transient gene transfer of m*gfp*$_5$-ER gene construct as visual reporter gene was demonstrated on non-detached leaves of greenhouse grown grapevines obtained from green cuttings by vacuum infiltration. Following the optimized protocol, up to half of the infiltrated leaf surface displayed green fluorescent foci found in the intercoastal areas (Ben-Amar et al. 2013). Monitoring of transient Green Fluorescent Protein expression daily achieved for 2 weeks post-infiltration with the highest expression level on day 6 (Ben-Amar et al. 2013). Evidence of GFP silencing in transgenic GFP expressing grapevine via agro-infiltration was found (Ben-Amar et al. 2013).

As the *in planta* infiltration system described by Ben-Amar et al. (2013) was extensively optimized and as the authors claim to provide a powerful tool to explore easily gene function in grapevine avoiding tissue culture steps and the labor intensive generation of transgenic plants, it was decided to follow their protocol.

II. Material and Methods

Plant material

Two-node cuttings of wooden or green shoots of grapevine of cultivar Chardonnay were prepared by cutting just below the lower node and a few centimeters above the upper node. The

lower bud was cut off. In the case of green cuttings, leaf lamina was trimmed (Fig. 1).

Figure 1. Two-node cuttings of a green (left) and wooden (right) shoots.

Wooden cuttings were soaked in water for half an hour. Afterwards, bottom end was dipped in auxin powder and the cuttings were planted in the mixture of fine vermiculite (2-3 mm) and middle vermiculite (2-6 mm) in the ratio 1:2. Cuttings were kept in a sprouter for 4 weeks, where they were heated from below to develop roots (Fig. 2).

Figure 2. Wooden cuttings in the sprouter.

After 4 weeks, plants were transferred into soil (Fig. 3) and kept in greenhouse for another 4 to 6 weeks under standard greenhouse conditions with 16 h light/24°C and 8 h dark/22°C.

Figure 3. Plants in soil in greenhouse.

Eight to 12 week old plants were used for transient transformation experiments. Non-detached leaves were used as the target organ for vacuum infiltration of either *Agrobacterium tumefaciens* harboring potential phytoplasma effector genes or indicator stain.

Transient transformation procedure

Non-detached leaves of greenhouse-grown grapevine plants were either wounded using razorblade or sonicated (Fig. 4) or left intact prior to exposure to vacuum.

Figure 4. Sonicator (Iskra Pio d.o.o., Slovenia).

The plants were placed upside down in a desiccator chamber (Fig. 5) with the leaves completely immersed either in the solution of the indicator stain or in the suspension of *Agrobacterium tumefaciens* harboring potential phytoplasma effector genes, optionally containing Break-thru S240. Two different indicator stains were tested: bromophenol blue and methylene blue. Bromophenol blue was prepared as 0.01% or 0.1% water solution. Methylene blue was prepared as 0.1% of Methylene blue in 0.1% Sodium tetraborate solution.

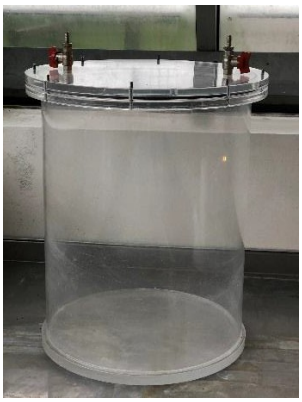

Figure 5. Desiccator chamber.

Vacuum was applied using a 71 l vacuum pump (Pro Virtus d.o.o., Slovenia) (Fig 6.), and followed by a quick release.

Figure 6. The 71 l vacuum pump (Pro Virtus d.o.o., Slovenia).

III. RESULTS

During the investigation, all the combinations of several experimental conditions were tested (Table 1).

Table 1: Experimental conditions for grapevine infiltration

Optimization parameters	
Parameters	***Tested options***
Age of the leaf	fully expanded, not fully expanded
Wounding	none, veins, lamina
Sonication	0s, 1s, 3s, 10s, 30s
Concentration of Break-thru S240 (v/v)	0%, 0.1%, 0.5%, 1.5%
Vacuum pressure	0.5 bar, 1 bar
Time of exposure to vacuum pressure	30 s, 1 min, 2 min, 5 min, 10 min
Type of indicator stain	Bromophenol blue, Methylene blue
Concentration of indicator stain	0.01%, 0.1%
Bacterial suspension OD	0.5, 1, 1.5

Wounding of veins or lamina by razor blade resulted in severely damaged leaves that dried out post infiltration.

Also the sonication longer than 3 seconds resulted in severe damage on sonicated leaves (Fig. 7), while no damage was observed when leaves were sonicated for 3 seconds or less.

Figure 7. Damage on leaves, sonicated longer than 3 seconds.

Three seconds of sonication of non-wounded leaves immersed in the 0.1% solution of bromophenol blue containing 1.5 % (v/v) of Break-thru S240 for 2 minutes under 1 bar of vacuum pressure (Fig. 8) resulted in blue patches on leaves (Fig. 9 and Fig. 10) that fainted out in the next days.

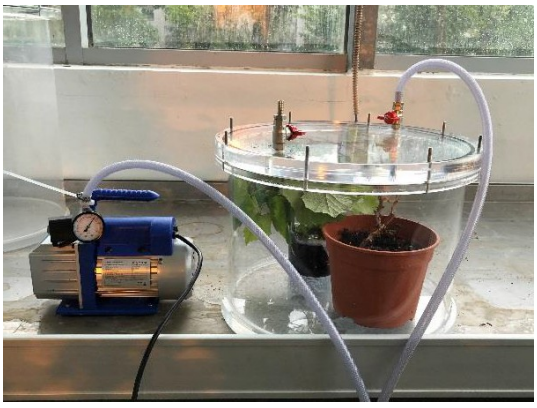

Figure 8. Grapevine plant exposed to vacuum pressure.

The occurrence of blue patches did not correlate with leaf size and/or age (Fig. 9 and Fig. 10).

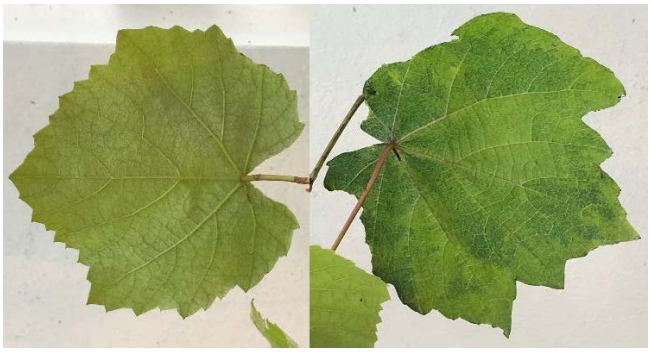

Figure 9. A bromophenol blue infiltrated (right) and a non-infiltrated control (left) fully expanded leaf.

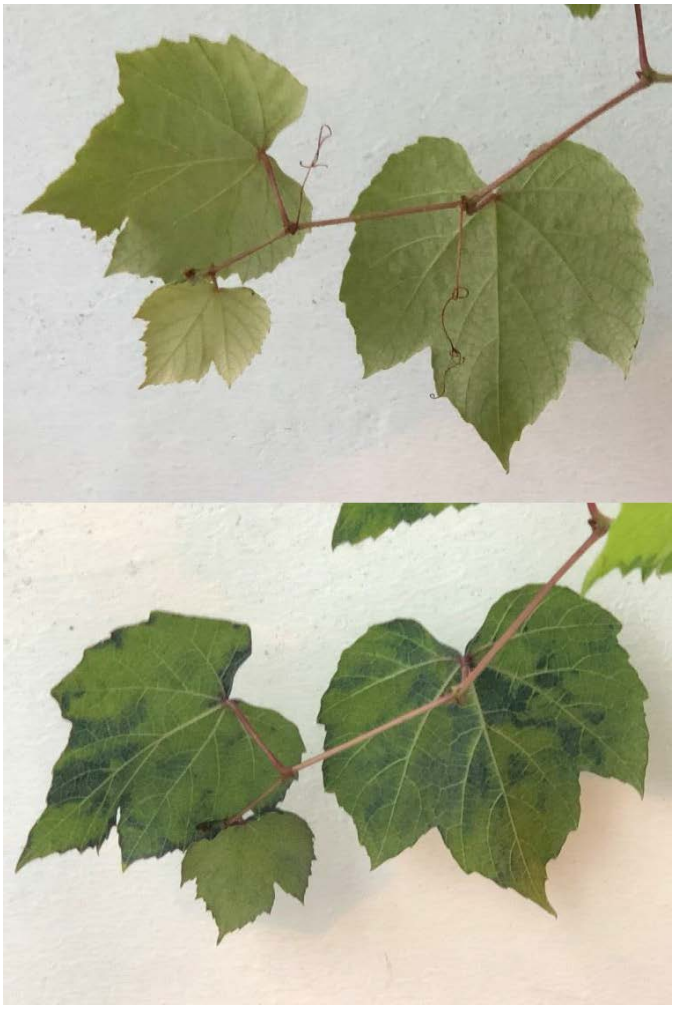

Figure 10. Young not fully expanded leaves before (upper panel) and after (bottom panel) bromophenol blue infiltration.

No difference in the occurrence of blue patches was observed when using 2 min, 5 min, 10 min of exposure to vacuum pressure.

Usage of methylene blue (Fig. 11) resulted in much fainter intensity of blue patches as compared to usage of bromophenol blue at the same experimental conditions.

Figure 11. A fully expanded leaf before (left) and after (right) methylene blue infiltration.

Although the infiltration of indicator stain bromophenol blue resulted in the occurrence of blue patches on leaves, Agrobacterium-mediated transient transformation of grapevine did not work in any of our experimental conditions.

IV. Discussion

Transient expression of foreign genes by *Agrobacterium* infiltration in grapevine plants would be a versatile technique for functional analysis. Although *Agrobacterium*-mediated transient transformations of tobacco and potato are performed routinely in the laboratory and although it was either exactly followed the published protocols or varied several parameters and although it was possible to infiltrate indicator stain bromophenol blue in grapevine leaves, *Agrobacterium*-mediated transient transformation of grapevine did not work in the reported experimental conditions.

Functional analyses of six potential '*Candidatus* Phytoplasma solani' effector genes would be the most desired in grapevine plants, as BN is associated with severe symptoms on grapevine. As A*grobacterium*-mediated transient transformation of grapevine did not work in the described experimental conditions, the functional analyses of potential BN effector genes will be performed on other host plants.

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