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Optimisation and comparison of transient expression methods to express the green fluorescent protein in the obligate biotrophic oomycete *Plasmopara viticola*

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Summary

Grape downy mildew is caused by *Plasmopara viticola*, an obligate biotrophic oomycete and a major pathogen of grapevine. Studying obligate biotrophic pathogens is difficult as they cannot grow without their host. We therefore attempted to develop a method where the pathogen could be visualized and quantified in planta without killing the host plant. To this end *P. viticola* was transformed with the marker gene gfp coding for the green fluorescent protein. Various transformation methods, namely electroporation, particle bombardment and transformation with *Agrobacterium tumefaciens* were applied. Although some methods yielded positive transformation events, no stable strain of *P. viticola* expressing gfp could be generated. Using the electroporation method, we obtained transient *P. viticola* transformants expressing gfp over 4 generations. In contrast, particle bombardment failed in transforming *P. viticola*. Transformation with *A. tumefaciens* had a low efficiency, only some structures were fluorescent and fluorescence was never observed in the subsequent generations.

K e y w o r d s : *Plasmopara viticola*, oomycete, transformation, obligate biotroph, grapevine.

Introduction

Downy mildews are widespread, severe plant diseases, and are generally favoured by cool, humid weather conditions. The causal organisms, in contrast to true fungi, belong to the phylum *Oomycota* in the kingdom *Stramenopila*. The *Oomycota* are divided into 13 genera, including *Plasmopara* (Thines 2007) which represents at least 23 species (Brandenburger and Hagedorn 2006).

Downy mildew of grapevine (*Vitis vinifera*) is caused by *Plasmopara viticola*. During periods of high humidity, this obligate biotrophic pathogen can infect large areas within a short period of time and cause substantial damage to most parts of the plants including leaves, flowers and young berries. Control is usually achieved by large scale, intensive application of agrochemicals. The economic costs and the negative environmental impact of such disease control methods call for the development of alternative strategies, involving manipulation of host defence mechanisms (Ferreira *et al.* 2004), breeding for resistance (Neuhaus *et al.* 2006) and biocontrol strategies (Musetti *et al.* 2006).

The main means of reproduction and spread of *P. viticola* are asexual sporangiospores generated 5 to 7 d after beginning of the asexual cycle of the pathogen. Sporangiospores released on the plant surface swim towards stomata, encyst there and develop a germ tube which penetrates into the substomatal cavity (Langcake and Lovell 1980, Denzer 1995, Gindro *et al.* 2003). *P. viticola* is a true obligate biotroph completely depending on its host, the grapevine plant, to successfully complete its asexual and sexual cycle. This makes maintenance and manipulation of interactions between plant host and pathogen rather difficult. A host-free system has been established to study the early development of *P. viticola* from sporangiospore release until the formation of a germ tube (Riemann *et al.* 2002). By comparing this host-free system with *P. viticola in planta*, Kiefer *et al.* (2002) have found that the early development of *P. viticola* is specifically and co-ordinately regulated by factors originating from the host plant and by the leaf surface topography (Kortekamp 2003).

In order to have a method to rapidly monitor pathogen development inside the tissue we decided to generate a *P. viticola* strain expressing the reporter gene *gfp. Ustilago maydis* was the first filamentous fungus to be transformed successfully with *gfp* (Spellig *et al.* 1996). Bottin and coworkers (1999) showed for the first time that the *gfp* gene reporter could be used in an oomycete, both as a quantitative reporter of gene induction and as a vital marker allowing the study of development of *Phytophthora parasitica in vitro* and in the host plant. To facilitate the *in planta* tracking of fungi and oomycetes and to measure their biomass, they have been labelled with GFP (Maor *et al*. 1998, Chaure *et al.* 2000, Si-Ammour *et al*. 2003). Labelling oomycetes with GFP requires strong constitutive expression of the transgene which usually results in a cytoplasmic expression in different structures of the organism (hyphae, spores, appressoria) with a limited impact on growth or pathogenicity (Bottin *et al.* 1999, van West *et al.* 1999 a). Expression of *gfp* in oomycetes requires a *gfp* variant that is efficiently translated in oomycetes, an oomycete promoter, and a transformation system that satisfies the requirements of a given experimental objective. *gfp* expression vectors have been developed for all major classes of filamentous fungi and oomycetes (Lorang *et al.* 2001).

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All the common genetic transformation methods have been successfully used to transform oomycetes. Chemical transformation was first described on protoplasted *Phytophtora* species (Bailey *et al*. 1991, Judelson and Michelmore 1991) and has been much improved in the meantime (McLeod *et al*. 2006). Microprojectile bombardment and Agrobacterium-mediated transformation were also successfully used to transform *Phytophtora infestans* with GUS (Cvitanich and Judelson 2003, Vijn and Govers 2003). Electroporation is the most applied and successful technique to transform a large number of fungi and oomycete species. Using electroporation and specific vectors, several oomycetes such as *Phytophtora* (Si-Ammour *et al*. 2003), *Pythium aphanidermatum* (Weiland 2003) and more recently *Plasmopara halstedii* (Hammer *et al.* 2007) have been transformed.

The aim of this study was to develop a transformation system for the obligate biotrophic oomycete grape downy mildew. Here we present the results with electroporation, particle bombardment and Agrobacterium-mediated transformation of *P. viticola*.

Materials and Methods

Plant material: Leaves of the grapevine cultivar Chasselas which is highly susceptible to downy mildew isolate NCCR1 (Hamiduzzaman *et al.* 2005) were used for the experiments. Plants were grown from seeds (obtained from Syngenta, Stein, Switzerland) in 1.60 l pots containing TKS1 growing substrate (Klasmann Deilmann, Germany). The plants were cultivated in a growth chamber with a 16 h light/8 h dark period, 65 % relative humidity and 650 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination.

Spore production for infection and transformation: *P. viticola* isolate NCCR1 was grown and maintained on 'Chasselas' leaves. Sporangia were harvested by suction from sporulating lesions with a disposable pipette tip (with filter) attached to a vacuum device. Sporangia that remained on the surface of the filter were transferred to a 1.5 ml Eppendorf tube and suspended in distilled water 30 min before transformation. For all the transformation procedures, sporangia were suspended in distilled water. Therefore, the final suspension consisted of a mixture of sporangia and sporangiospores. The highest obtainable sporangiospore concentration was reached about 30 min. after suspension of the sporangia. This time point was chosen for the electroporation since the absence of cell wall in sporangiospores favours transformation efficiency. Transformation efficiency was assessed by determining the ratio of fluorescent/non-fluorescent sporangia using a haemocytometer.

Inoculation of grapevine leaves: Ten µl drops of sporangia suspension (2.4 x 10^6 sporangia$\cdot ml^{-1}$) were applied to the lower surface of detached leaves on humid filter paper and kept at high relative humidity in Petri dishes sealed with Parafilm in a growth chamber with a 16 h light/8 h dark period and 650 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination.

Harvesting of transformed sporangia: Green fluorescing sporangia were harvested under low magnification using a binocular dissecting microscope (Nikon SMZ 1000) equipped with filters GFP-L (ex. 480; em. 510) and GFP-B (ex.480; em. 535/50) by suction into a thin Pasteur pipette filled with a cotton filter.

Vectors for transformation of *P. viticola*: Vector P34GF, which contains a enhanced *gfp* (Pang *et al*. 1996), was prepared as follows. The transformation vector p34GFN used to express *gfp* in *Phytophtora* species (Si-Ammour *et al.* 2003) was modified to remove the geneticin (*nptII*) resistance gene. Geneticin cannot be used as selective marker due to its toxic effect on grapevine plants. For vector construction, the Ham34Pro-*gfp*-Ham34Ter cassette was excised with *HindIII* and *BamHI* from the p34GFN vector and cloned into the *BamHI* and *HindIII* sites of pUC18, resulting in P34GF. Non-linearised vector was used for transformations.

Pb34GF was constructed by the insertion of the *BamHI*/EcoRI fragment from p34GFN containing the *Ham34*Pro-*gfp-Ham34*Ter cassette into the binary plasmid p3300. Pb34 GF was electroporated into *Agrobacterium tumefaciens* strains GV3101. All DNA manipulations were performed using standard procedures (Sambrook *et al.* 1989) and *E. coli* strain DH5α was used for general cloning and was grown at 37°C in Luria-Bertania (LB) medium (Sambrook *et al.* 1989). DNA for electroporation experiments was prepared in *E. coli* strain DH5α and purified using the JETstar 20 Plasmid Midiprep Kit (Genomed).

Culture conditions of *A. tumefaciens*: *A. tumefaciens* GV3101 cells were grown overnight at 28 °C in low-salt LB medium (amended with 5 $g \cdot l^{-1}$ NaCl) containing 100 $\mu g \cdot ml^{-1}$ rifampicin and 50 $\mu g \cdot ml^{-1}$ kanamycin. Subsequently, 1 ml of the culture was washed twice with 1 ml induction medium (IM) (Murashige and Skoog salts and 40 mM 2-(N-morpholino) ethanesulphonic acid (MES), pH 5.4, 10 mM glucose, 0.5 % (w/v) glycerol) supplemented with 75 µM acetosyringone (AS), 10 × diluted in fresh IM + AS and grown for another 5 h at 28 °C. The final OD_{600} of the cultures was adjusted to approximately 0.2. Before co-cultivation, the cells were washed twice with an equal volume of sterile distilled water.

Electroporation: Sporangia of *P. viticola* were suspended to a concentration of 2.4 x 10^6 sporangia/ml in distilled water. After 30 min, 15 µg of p34GF vector DNA were added to 500 µl of the suspension and electroporated using the Gene Pulser Xcell (Bio-Rad). Rapidly, 500 µl of distilled water were added to the cuvette and the electroporated suspension was used to inoculate leaves of Chasselas at a concentration of 1.2 x 10^6 sporangia$\cdot ml^{-1}$. During 10 d, the inoculated leaves were monitored for GFP fluorescence using a binocular dissecting microscope (Nikon SMZ 1000) with filters GFP-L (ex. 480; em. 510) and GFP-B (ex.480; em. 535/50). Alternatively, the samples were monitored using a confocal microscope (Leica TCS 4D).

Transformation of *P. viticola* with *A. tumefaciens*: For transfer of the T-DNA from *A. tumefaciens* to *P. viticola*, 1 ml of bacterial suspension was added to 5 ml of water containing sporangia at a concentration of 1 x 10^6 sporangia$\cdot ml^{-1}$. After co-cultivation for 5 h at 22 °C in the dark on a rotary shaker (30 rpm), the suspension containing both *P. viticola* and *A. tumefaciens*

was inoculated onto grapevine leaves. Twenty four hours later the leaves were rinsed with a solution containing 200 µg·ml^{-1} cefotaxim to kill *A. tumefaciens*. During 10 d, the inoculate leaves were monitored for GFP fluorescence as mentioned above.

Particle bombardment: The preparation of 0.4 µm gold particles (BioRad) coated with plasmid P34GF was performed following the manufacturer's protocol. Bombardment was performed with a Helios Gene Gun (Bio-Rad). The bombardment pressure was around 9 kPa according to Cvitanich and Judelson (2003) and the distance to target was 3 cm.

Results

Electroporation: A construct containing the *ham34* promoter of the oomycete *Bremia lactucae* fused to the coding sequence of the *gfp* gene, P34GF, was introduced into the *P. viticola* strain NCCR1 by electroporation. Electroporation was performed 30 min after adding the sporangia suspension to the water because the number of released sporangiospores was highest at this time point (data not shown). Multiple variables associated with introducing DNA into *P. viticola* by electroporation were tested (Table). The best results were obtained by electroporating germinated sporangia 30 min after start of germination (500 µl of 2.4 x 10^6 sporangia·ml^{-1}) with 15 µg of p34GF vector DNA at 550 V, 100 µF and 350 Ω pulse duration. The synthetic *gfp* gene used in this study was constructed to improve *gfp* expression in plants (Pang *et al.* 1996). The replacement of the serine at position 65 with a threonine yielded 100- to 120-fold brighter fluorescence than wild-type *gfp* upon excitation with 490-nm light. Introducing the vector p34GF into sporangiospores of *P. viticola* resulted in a general fluorescence of the oomycete (Figs 1 and 2 a-d). Successful transformations using p34GF were already obtained from the first experiment and the protocol described in material and methods was optimized until a maximal number of transformants was achieved. Because of constitutive activity of the *B. lactucae* promoter, *P. viticola* could be visualized by fluorescence microscopy (Figs 1 and 2).

Table

Parameters tested and efficiency of *Plasmopara viticola* transformation by electroporation. To asses the transformation efficiency, voltage, resistance and capacitance of electroporation were varied. Efficiency was measured either by transformants per assay or per µg of vector DNA

Number of sporangia	2,.4x10^5 , 2.4x10^6 and 2.4.x10^7
Voltage (V)	from 350 to 800
Resistance (Ω)	from 300 to 750
Capacitance (µF)	from 25 to 700
Transformants per assay[a]	15 - 25
Transformants per µg of vector DNA[a]	1 – 1.66

[a] Minimum-maximum range taken from 45 experiments in optimal conditions (550V, 100µF, 350 Ω)

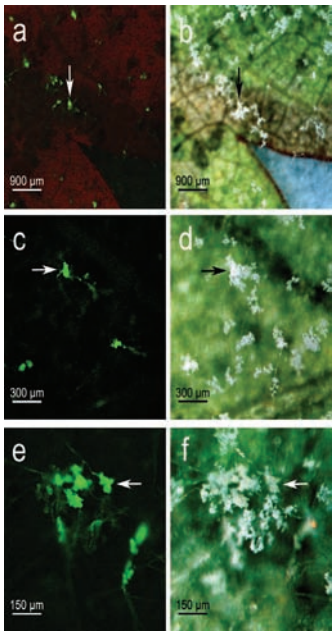

Fig. 1: GFP expression in *P. viticola* during sporulation on grapevine leaves cv. 'Chasselas'. **a**, **c**, **e**: Micrographs of sporangiophores taken under blue light excitation U.V.+ filter GFP-L (EX 480/40; BA 510). **b**, **d**, **f**: Same as a, c, e, but under bright field. **a-b**: First generation of transformed *P. viticola*. **c-d**: Second generation of transformed *P. viticola*. **e-f**: Third generation of transformed *P. viticola*. Arrows show transformed sporangia expressing *gfp*.

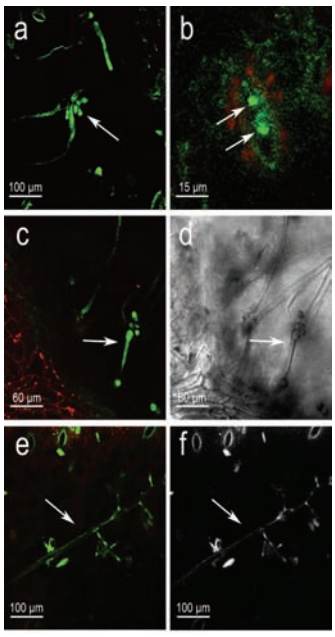

Fig. 2: Confocal images of grapevine cv. 'Chasselas' leaves infected with *P. viticola* expressing *gfp*. **a**, **b**, **c**, **e**: Confocal migrographs. **d**, **f**: Transmitted light of c and e. **a**: Arrow show sporangiophores initiation of *P.viticola* expressing *gfp* (*P. viticola* transformed by electroporation). **b**: Arrows show fluorescent structures of transformed *P. viticola* (*P. viticola* transformed by electroporation) in a stomatal opening. **c**, **d**: Arrows show a sporangiophore of *P. viticola* (transformed by electroporation) expressing *gfp*. **e**, **f**: Arrows show hyphae of *P. viticola* expressing *gfp* on the surface of the leaf (*P. viticola* transformed with *A. tumefaciens*).

Since *P. viticola* is an obligate biotroph, it cannot be grown *in vitro*. Therefore, the selection of transformants expressing *gfp* has to be performed visually. Fluorescent sporangia were sucked from leaves under a binocular dissecting microscope and U.V. light (Fig. 1 a, c, e) using a thin Pasteur pipette. Thus, the transformants expressing *gfp* were isolated and taken to the fourth generation by transferring the fluorescent sporangia to new uninfected leaves of Chasselas. Over 4 generations the number of transformants expressing *gfp* was reduced by approximately 33 % at each generation. It was not possible to obtain a fifth fluorescing generation. These 4 generations were composed of transformed and non transformed *P. viticola* (Fig. 1 a, c, e). As observed with the vast majority of transformed pathogens, the fitness of the *gfp* expressing organisms is often lower than the fitness of untransformed ones (Si-Ammour *et al.* 2003) (data not shown). This might explain the observed delay in sporulation with our GFP-transformants (data not shown).

Transformation with *A. tumefaciens*: To establish an efficient transformation method for *P. viticola with A. tumefaciens*, we constructed the binary vectors Pb34 GF. It carries a T-DNA that contains the Ham34Pro-*gfp*-Ham34Ter cassette. *A. tumefaciens* strain GV 3101 containing Pb34 GF was co-cultivated with *P. viticola* sporangia suspension during 2-4 h. This protocol was adapted from the method used to transform *Phytophtora infestans* by Vijn and Govers (2003). Due to the short life time of sporangiospores in suspension, it was difficult to increase the time of co-cultivation. Subsequently, the sporangia suspension was used to inoculate grapevine leaves. Examination of the infected leaves by confocal microscopy revealed fluorescent structures of *P.viticola* growing inside the leaves (Fig. 2 b). Rarely, some fluorescent structures such as hyphae or sporangiophores were observed (Fig. 2 e). Although fluorescence of GFP was observed in the transformants, an efficient transfer of the fluorescent phenotype to the next generation of *P.viticola* failed. Induction of *A. tumefaciens* strains with acetosyringone did not enhance the transformation efficiency.

Particle bombardment: The grapevine leaves were inoculated 3-6 days before the bombardment to transform *P. viticola* at different developmental stages around the time of sporangiophore formation. Initials tests, aimed to establish the viability of *P. viticola* following bombardment at different development stages of development showed that maximal recovery was achieved when leaves were bombarded 3 to 4 days after inoculation. This time point corresponds to the aggregation of hyphae in the substomatal cavity that will give rise to sporangiophores on the leaf surface. When the oomycete structures emerge from the stomata, nuclei are expected to be localised at this point and therefore should be hit more easily during the bombardment. The set of parameters tested for the transformation of *P. viticola* through microprojectile bombardment was adjusted according to previous investigations of fungi and oomycetes transformation (Christiansen *et al.* 1995, Cvitanich and Judelson 2003). The experiment was carried out with the plasmid P34GF but none of the emerging sporangiophores and sporangia following the bombardment were fluorescent. The new sporangia were used to inoculate new leaves but no GFP fluorescence was observed.

Discussion

GFP has been shown to be a useful tool serving as a reporter protein in many molecular biology studies and particularly as a vital marker for visualizing plant-pathogen interactions (Spellig *et al.* 1996, Maor *et al.* 1998; Bottin *et al.* 1999, van West *et al.* 1999 a, Lorang *et al.* 2001, Si-Ammour *et al.* 2003, Hammer *et al.* 2007). The expression of β-glucuronidase (GUS) reporter gene in plant pathogenic fungi also allows to observe the interaction with plants (Snoeijers *et al.* 1999) but the major advantage of GFP compared to GUS is that it allows the direct observation in living tissues without the addition of an exogenous substrate.

Another advantage of pathogen-expressed GFP is the possibility to quantify the infection by measuring the emitted fluorescence (Si-Ammour *et al.* 2003). Microscopic observation often call for long staining procedures (Hamiduzzaman *et al.* 2005) although recently improved shorter methods have been described (Diez-Navajas *et al.* 2007). Methods based on Real Time PCR technology are an other possibility but they are quite expensive (Valsesia *et al.* 2005).

Here, we show that *P. viticola* is amenable to genetic transformation using different methods. However, no stable transformants could be generated. The reasons for the loss of fluorescence from the transformed *P. viticola* are still unclear and our data are not sufficient to explain this observation. It seems that neither deletion nor methylation are the causes of inactivation of integrated genes as it was shown for *Phytophthora* (Judelson and Whittaker 1995). According to van West *et al.* (1999b), this observation could be explained by an internuclear silencing process but it remains matter of speculation concerning our results. More surprising is the results obtained recently by Gaulin *et al.* (2007). They showed that a silencing construct introduced into *Phytophtora parasitica* could be lost but the silencing process still worked. As it was shown by Fotheringham and Holloman (1990) in true fungi, transgenes may be present in large extrachromosomal structures. The non-duplication of these structures could explain why the transgene has been lost during the next generations.

In summary, our studies suggest that the promoter *Ham34* is constitutive in *P. viticola*. The unstable integration of a transgene in *P.viticola* is similar to what has been observed recurrently during oomycete transformations (Gaulin *et al.* 2007, Hammer *et al.* 2007, van West *et al.* 1999 b, Judelson and Whittaker 1995). Our results also demonstrate the possibility to transform *P. viticola* by using an electoporation method and an appropriate vector to study the gene functions during the life's cycle and/or the infection process. A major problem during the transformation of an obligate biotroph is the selection of trans-

formants. Growing *P. infestans in vitro* transformed with the selectable marker gene neomycine phosphotransferase (*nptII*) allows the use of an antibiotic such as geneticin for the selection of transformants (Si-Ammour *et al.* 2003). *In planta*, the common antibiotics are usually toxic at useful concentrations. One possibility would consist in using another selection system, for example, genetically modified plants resistant to an antibiotic such as kanamycin (Bornhoff *et al.* 2005.) and a genetic construct carrying a selective marker resistance to kanamycin for oomycete transformation. Although generating transgenic grapevine is difficult and time consuming, this might represent a solution to improve the transformation efficiency of *P. viticola* because a selection pressure could be applied.

In conclusion, we feel that although generating transgenic grapevine is difficult and time consuming, this might represent a solution to improve the transformation efficiency of *P. viticola* because a selection pressure could be applied. The higher efficiency observed with electroporation is likely due to the absence of a cell wall in sporangiospores facilitating this procedure because permeability is achieved more easily. Additionally, electroporation is a rapid process and can be used on a sporangia suspension containing a high concentration of sporangiospores. The limiting parameter for the Agrobacterium-mediated transformation is probably the prolonged period of incubation required to achieve gene transfer and in comparison the relatively short period of survival of sporangiospores in solution. With the biolistic method, the projectiles have to either cross at the least the epidermal cell layer and the oomycete cell wall to reach their target, or at least to go through the cell wall of sporangia initials emerging from the stomata. Since not every hit also leads to a successful transformation, they are likely a very rare event in such a situation and might easily be overseen.

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