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Research

*Methods*

# Transformation of the cucurbit powdery mildew pathogen *Podosphaera xanthii* by *Agrobacterium tumefaciens*

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## Summary

- The obligate biotrophic fungal pathogen *Podosphaera xanthii* is the main causal agent of powdery mildew in cucurbit crops all over the world. A major limitation of molecular studies of powdery mildew fungi (*Erysiphales*) is their genetic intractability. In this work, we describe a robust method based on the promiscuous transformation ability of *Agrobacterium tumefaciens* for reliable transformation of *P. xanthii*.
- The *A. tumefaciens*-mediated transformation (ATMT) system yielded transformants of *P. xanthii* with diverse transferred DNA (T-DNA) constructs. Analysis of the resultant transformants showed the random integration of T-DNA into the *P. xanthii* genome. The integrations were maintained in successive generations in the presence of selection pressure.
- Transformation was found to be transient, because in the absence of selection agent, the introduced genetic markers were lost due to excision of T-DNA from the genome.
- The ATMT system represents a potent tool for genetic manipulation of *P. xanthii* and will likely be useful for studying other biotrophic fungi. We hope that this method will contribute to the development of detailed molecular studies of the intimate interaction established between powdery mildew fungi and their host plants.

## Introduction

Powdery mildew is one of the most common, conspicuous and easily recognizable plant diseases (Fernández-Ortuño *et al.*, 2008). Powdery mildew fungi are obligate biotrophic parasites that are found all over the world and infect a large variety of cultivated plants, such as barley, wheat, grapes, cucurbits, tomatoes, fruits and ornamental plants (Micali *et al.*, 2008; Dean *et al.*, 2012; Zhang *et al.*, 2012). In southern Spain, *Podosphaera xanthii* (synonym *Podosphaera fusca*) has been identified as the sole cause of powdery mildew on cucurbits (Pérez-García *et al.*, 2001; Rivera *et al.*, 2002; Fernández-Ortuño *et al.*, 2006), a disease responsible for significant yield losses worldwide for crops under field and glasshouse conditions (Bardin *et al.*, 1997; Kuzuya *et al.*, 2003; Romero *et al.*, 2004; Pérez-García *et al.*, 2009; Pirondi *et al.*, 2015).

A characteristic feature of the biotrophic interactions between powdery mildew fungi and their host plants is the development of fungal haustoria, parasitism-specific structures that are differentiated from appressorial and mycelial cells, which govern these highly intimate plant–pathogen interactions (Both *et al.*, 2005; Martínez-Cruz *et al.*, 2014). Two major roles of haustoria are uptake of nutrients from the plant cells (Hahn & Mendgen, 1997; Both *et al.*, 2005; Spanu *et al.*, 2010) and delivery of effectors (Meyer *et al.*, 2009; Bindschedler *et al.*, 2011; Micali *et al.*, 2011; Martínez-Cruz *et al.*, 2014). Fungal effectors are proteins with N-terminal signal peptides which are secreted by pathogenic fungi via the endoplasmic reticulum–Golgi apparatus (Lo Presti *et al.*, 2015). Effectors are varied and include proteins that produce cell death (such as necrotrophic fungi), proteins that protect pathogens against host defences by suppressing the host immune response, and proteins that modulate the physiology or structure of host cells to provide an environment for successful infection (such as biotrophic fungi) (Olesen *et al.*, 2003; Lipka & Panstruga, 2005; Opalski *et al.*, 2005; Miklis *et al.*, 2007; Eichmann & Hückelhoven, 2008; Schultheiss *et al.*, 2008; de Jonge *et al.*, 2011).

Studies in a variety of fungal pathogens, such as *Magnaporthe grisea*, *Cladosporium fulvum*, *Blumeria graminis* f. sp. *hordei* and *Golovinomyces orontii*, have identified and analysed several pathogenic effector proteins (Kamoun, 2007; Skamnioti *et al.*, 2008; Gan *et al.*, 2010; Schulze-Lefert & Panstruga, 2011; Lo Presti *et al.*, 2015). By contrast, very little is known about effectors from *P. xanthii*, which hinders our understanding of the pathogenic interaction and our ability to find novel fungal targets for new antifungal phytotherapies. A major issue that limits molecular studies on powdery mildew fungi is their genetic intractability. In model fungal pathogens, analysis of effector

functions can be carried out by genetic manipulation. However, the transformation methods available for filamentous fungi, including protoplast fusion, electroporation and biolistics (Weld *et al.*, 2006; Dubresson *et al.*, 2008; Meyer, 2008; Michielse *et al.*, 2008; Djulic *et al.*, 2011; Jiang *et al.*, 2013), do not work well on powdery mildew fungi and have reduced transformation efficiency (Jiang *et al.*, 2013; Vela-Corcía *et al.*, 2015).

*Agrobacterium tumefaciens*-mediated transformation (ATMT) has been successfully used for the transformation of a wide variety of filamentous fungi, including an obligate biotroph, the flax rust fungus *Melampsora lini* (Lawrence *et al.*, 2010). *Agrobacterium tumefaciens* is able to horizontally transfer DNA from its tumour-inducing plasmid (transferred DNA or T-DNA) and this T-DNA integrates randomly and predominantly as a single copy into the host genome by nonhomologous integration (Michielse *et al.*, 2008; Islam *et al.*, 2012; Jiang *et al.*, 2013). In the present study, we describe a method based on the ability of *A. tumefaciens* to efficiently transform the cucurbit powdery mildew pathogen *P. xanthii*. First, we show the stability of the transformants in the presence of selective pressure. Second, we demonstrate that, in the absence of selection pressure, this transformation is transient due to the excision of the T-DNA from the genome. Finally, we discuss this method as a potent tool for genetic studies of powdery mildew fungi or other biotrophic fungal pathogens, which are necessary to understand their biology and interaction with host plants.

## Materials and Methods

### Growth conditions of plants, fungi and bacteria

We used melon (*Cucumis melo* L.) plants cv Rochet (Semillas Fitó, Barcelona, Spain) susceptible to the *P. xanthii* isolate 2086 in all experiments. Plants were cultivated in a growth chamber with a 16 h : 8 h, light : dark cycle at 25°C. The *P. xanthii* isolate 2086 was cultured and maintained on zucchini (*Cucurbita pepo* L.) cotyledons cv Negro Belleza (Semillas Fitó) in an 8-cm Petri dish under a 16 h : 8 h, light : dark cycle at 22°C for 1 wk (Álvarez & Torés, 1997). The *A. tumefaciens* strains LBA4404 (Ti plasmid pAL4404) (Hiei *et al.*, 1997) and C58C1 (with a disarmed pTiB6 plasmid) (Vergunst *et al.*, 2003) used for *Agrobacterium*-mediated transformation of *P. xanthii*, were grown in lysogeny broth (LB) media containing 50 μg ml$^{-1}$ rifampicin and 50 μg ml$^{-1}$ kanamycin at 28°C. Forty eight hours after inoculation of conidia, a single application of fungicides hygromycin B (100 μg ml$^{-1}$) and carbendazim (600 μg ml$^{-1}$) was applied when required. The transformed colonies were maintained on the cotyledons under selection pressure as described earlier.

### Construction of binary vectors

The T-DNAs of the binary vectors used in our study are displayed in Fig. 1. Plasmids pTAS5-PagsA-gfp-TtrpC (pTAS5) and pPK2-hphgfp were kindly provided by Dr Paul Hooykass (Institute Biology Leiden, Leiden University). The pTAS5 vector contains the hygromycin B phosphotransferase resistance gene (*hph*), under control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (*PgpdA*), and the *gfp* gene, under control of the 1,3-α-D-glucan synthase promoter from *Aspergillus niger* (*PagsA*) (Meyer *et al.*, 2007). The pPK2-hphgfp vector contains the *hph* and *gfp* genes, under control of the *gpdA* promoter (Michielse *et al.*, 2009). For the *P. xanthii* transformation experiments, the plasmids were transferred to *A. tumefaciens* LBA4404 and verified by PCR.

The plasmid pPK2-hphgfp was used as a backbone to build the following modified plasmids. The primers used for this purpose are listed in Supporting Information Table S1. The plasmid pPK2Tgfp was created to express a β-tubulin allele (*tub2*) that confers resistance to carbendazim (Vela-Corcía *et al.*, 2014). To construct this plasmid, a pair of primers, Tub-F and Tub-R, were used to amplify the β-tubulin allele from the naturally resistant *P. xanthii* isolate SF60 (ACCN KC333362.1). The pairs of primers PgpdA-F/RPgpdA-tub and Fgfp6-tub/Rgfp6-ApaI were used to amplify the *PgpdA* promoter and the *gfp* gene, respectively, using the backbone plasmid pPK2-hphgfp as a template. The three PCR fragments were joined using overlapping PCR according to following conditions: initial denaturation step at 98°C for 2 min, followed by one cycle of 30 s at 94°C, 30 s at 65°C and 3 min at 72°C, one cycle of 30 s at 94°C, 30 s at 60°C and 3 min at 72°C, one cycle of 30 s at 94°C, 30 s at 55°C and 3 min at 72°C, one cycle of 30 s at 94°C, 30 s at 50°C and 3 min at 72°C, 35 cycles of 30 s at 98°C, 30 s at 48°C and 3 min at 72°C and a final extension step of 10 min at 72°C. The resulting 4.1-kb fragment was digested with *Apa*I and *Kpn*I restriction enzymes (New England Biolabs, Ipswich, MA, USA) and cloned into the plasmid pPK2-hphgfp (which was cut with the same enzymes). This allowed the replacement of the original sequence P*gdpA-hph-gfp* with the new P*gdpA-tub2-gfp* sequence.

Finally, pPK2Tgfp plasmid was transferred to *A. tumefaciens* C58C1 and verified by PCR and digestion pattern before use in transformation experiments of *P. xanthii*.

### *Agrobacterium tumefaciens*-mediated transformation

For transformation experiments, *A. tumefaciens* cells were grown overnight at 28°C in 5 ml of LB media containing 50 μg ml$^{-1}$ rifampicin and 50 μg ml$^{-1}$ kanamycin in an orbital shaker at 200 rpm. Next, 2 ml of this culture was washed twice in induction medium (IM), as described previously (Michielse *et al.*, 2008). The washed cell pellet was resuspended in 5 ml of IM with 200 μM acetosyringone (AS) (Sigma-Aldrich) and grown for 5 h at 28°C in an orbital shaker at 200 rpm. Then, cell suspensions were adjusted to an $OD_{600}$ of 0.6 in IM. Separately, conidia from powdery mildew infected zucchini cotyledons were harvested by immersion of infected leaves in 50 ml of distilled water with 0.01% Tween-20 and adjusted to $1 \times 10^6$ conidia ml$^{-1}$. Next, 100 μl of bacterial suspension were added to 100 μl of conidial suspension, and the mixture co-cultivated for 2 h at 28°C in the dark in an orbital shaker at 90 rpm. After incubation, 15 × 3-μl drops of the suspension containing *A. tumefaciens* cells and *P. xanthii* conidia were deposited per detached zucchini cotyledon and maintained under a 16 h : 8 h, light : dark cycle at 25°C. Twenty four hours after inoculation,

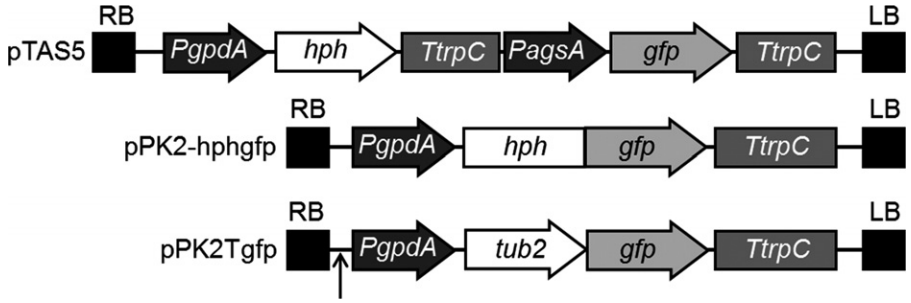


**Fig. 1** A schematic representation of the transferred DNA (T-DNA) in the vectors used in our study. RB, right border; LB, left border; *PgpdA*, *Aspergillus nidulans gpd* promoter; *hph*, hygromycin B resistance gene; TtrpC, *A. nidulans trpC* terminator; *PagsA*, *Aspergillus niger* 1,3-α-D-glucan synthase promoter; *gfp*, green fluorescence protein; *tub2*, β-tubulin gene from the naturally benzimidazole resistant *Podosphaera xanthii* isolate SF60. The arrow denotes the recognition site for EcoRV enzyme used in Southern blot analyses.

the cotyledons were rinsed with 200 μg ml$^{-1}$ cefotaxime to kill *A. tumefaciens* (Dubresson *et al.*, 2008), and 48 h after inoculation, rinsed with 100 μg ml$^{-1}$ hygromycin B or 600 μg ml$^{-1}$ carbendazim (depending on resistance conferred by T-DNA) to select *P. xanthii* transformants. A schematic description of this transformation protocol is shown in Fig. 2.

### Epifluorescence microscopy

Fluorescence microscopy analysis was performed using a Nikon AZ-100 epifluorescence microscope (Nikon, Tokyo, Japan) with a filter suitable for analysis of green fluorescent protein (GFP) and UV fluorescence. For GFP fluorescence, the samples were excited at 460–500 nm wavelengths at a fixed exposure of 2 s. Images were captured with a Nikon digital Slight DS-5Mc camera (Nikon) attached to the microscope, using NIS-ELEMENTS software (Nikon). The images were processed using Adobe PHOTOSHOP CS5 (Adobe Systems Inc., San Jose, CA, USA). Untransformed colonies of *P. xanthii* – those lacking fluorescence – were used as a negative control.

### Molecular analysis of transformants

Genomic DNA from potential *P. xanthii* transformants was isolated using the Puregene DNA purification system (Gentra Systems, Minneapolis, MN, USA). Cotyledon discs with potential transformants were excised, ground in liquid nitrogen into a powder using a mortar with pestle and sand, and homogenized in cell lysis/proteinase K solution. After the protein precipitation step, the genomic DNA present in the supernatant was purified following the manufacturer's recommendations.

The presence of T-DNA in the genomic DNA preparations was first analysed by PCR. To analyse the transformants obtained with the T-DNA of pPK2-hphgfp, fragments of the *gfp* and *hph* marker genes were amplified with the primer pairs gfp6F1/gfp6R1 and hphF1/hphR1, respectively (Table S1). For transformants obtained with the T-DNA of pTAS5, the primer pair PgpdA-F/hphR1 (Table S1) was used to amplify the fragment *PgdpA-hph*. For transformants obtained with the T-DNA of pPK2Tgfp, the primer pairs Tub-F/gfp6R1 and gfp6F1/gfp6R1 (Table S1) were used to amplify the fragment *tub2-gfp*. The amplification conditions were the following: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 4 min, with a final extension at 72°C for 10 min.

For Southern blot analysis, genomic DNA obtained from mixed plant and fungal material was used. For these experiments, 4 μg of genomic DNA from *P. xanthii* transformants or wild-type (WT) isolates was digested with *EcoR*V (New England Biolabs), according to the manufacturer's recommendations, separated on a 1% agarose gels and blotted on positively charged nylon membranes. A fragment of 714 bp of the *gfp* gene was used as a probe. This fragment was obtained by PCR using the primers gfp6F1 and gfp6R1 (Table S1). Cycling conditions consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min. Preparation of DIG-labelled probes, hybridization and chemiluminescent detection was performed according to the manufacturer's instructions (Hoffmann-La Roche, Basel, Switzerland).

In order to analyse the flanking regions of the T-DNA, we used insertion thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The specific left border primers (LB1, LB2 and LB3), specific right border primers (RB1, RB2 and RB3) and degenerate primers (AD1 and AD2) (Sambrook *et al.*, 2001; Combier *et al.*, 2003) used to perform the TAIL-PCR are listed in Table S1. The TAIL-PCR reaction was conducted as described previously (Meirinho *et al.*, 2010). The primary PCR reaction was conducted using 90 ng of genomic DNA as a template in a total volume of 50 μl. To perform the secondary PCR, 1 μl of a 1 : 50 dilution of the primary PCR products was used as a template. The tertiary PCR reactions were performed using 1 μl of a 1 : 10 dilution of the secondary reaction product. The cycler program used in this analysis is detailed in Table S2. The tertiary TAIL-PCR products with an expected difference in size compared with those of secondary PCR (*c.* 100 bp) were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing of the TAIL-PCR products was conducted by Macrogen (Amsterdam, the Netherlands) using the primers LB3 and RB3 (Table S1).

## Results

### *Agrobacterium* transforms *Podosphaera xanthii*

ATMT of fungi requires first, the co-cultivation of both microorganisms and second, selection of corresponding transformants.

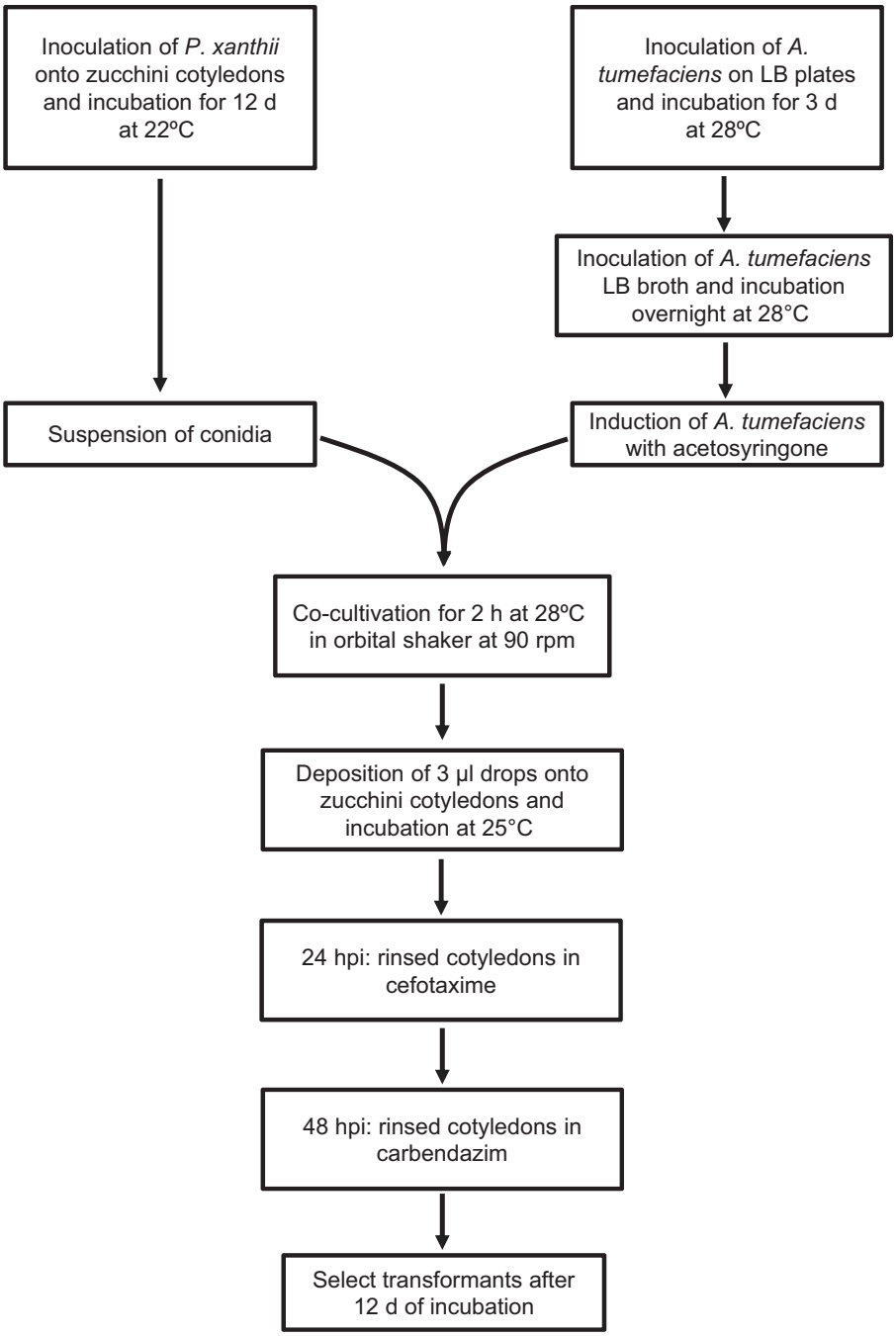


**Fig. 2** Flow diagram of our method for *Agrobacterium tumefaciens*-mediated transformation for *Podosphaera xanthii*. hpi, h post inoculation.

Two main problems need to be taken into account when transforming powdery mildews. First, these fungi are rather sensitive to treatments with liquids, so co-cultivation has to be restricted to 2 h. Second, due to the obligate biotrophic nature of these fungi, selection for transformants has to be performed on its host. For this reason, 3-µl drops of microbial suspensions containing *P. xanthii* conidia and *A. tumefaciens* cells were deposited onto zucchini cotyledons and incubated at 25°C for 24 h, allowing fungal growth while the bacteria are still alive, enabling the T-DNA to be transferred. Following this procedure, we transformed *P. xanthii* conidia with the T-DNA of plasmids pPK2-hphgfp, mediating the expression cassette of HPH (hygromycin B phosphotransferase gene) fused to gene-encoding GFP, and pTAS5, which confers the individual expression cassettes for hygromycin B resistance gene and GFP. Twenty four hours post inoculation (hpi) the cotyledons were rinsed in a solution of 200 µg ml$^{-1}$ cefotaxime to kill *Agrobacterium* and 48 hpi in a solution of 100 µg ml$^{-1}$ hygromycin B, a concentration previously determined to be an efficient selective barrier to *P. xanthii* (Vela-Corcía *et al.*, 2015). Colonies resistant to hygromycin B arose after 10 d post inoculation (dpi) (Fig. 3a,b) with transformation efficiencies of equivalent range (Table 1), whereas no fungal growth was observed on cotyledons inoculated with untransformed conidia (Fig. 3c) or with conidia exposed to

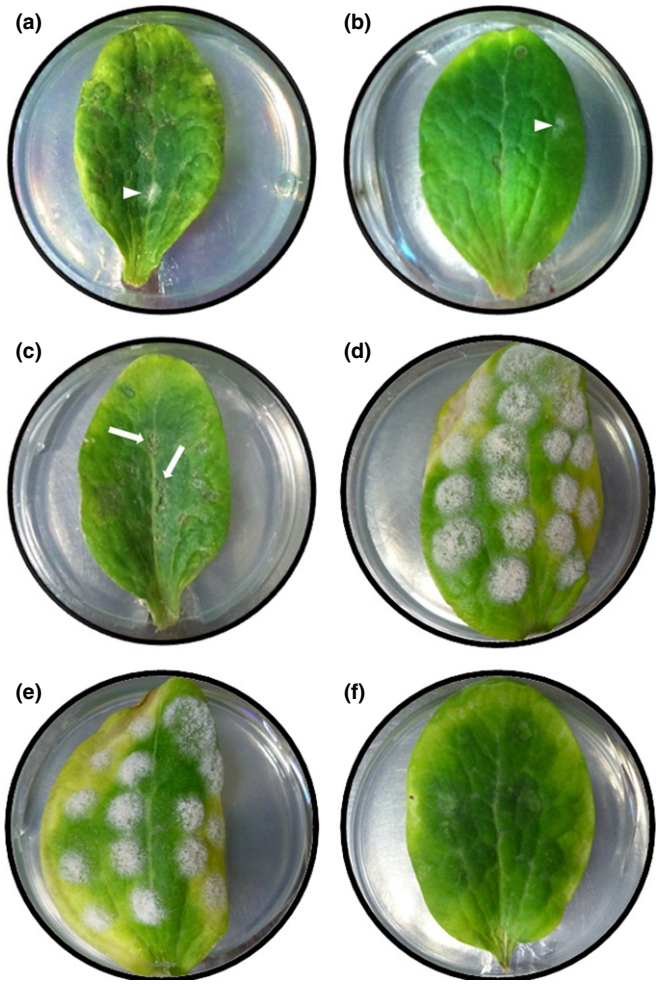


**Fig. 3** *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Podosphaera xanthii* conidia with transferred DNA (T-DNA) of pTAS5 and pPK2-hphgfp: Hygromycin B resistance phenotype. Transformants of *P. xanthii* grown on zucchini cotyledons (arrowheads) and treated with hygromycin B after ATMT transformation of conidia with the T-DNA of (a) pTAS5 or (b) pPK2-hphgfp. (c) No colonies were observed on cotyledons treated with hygromycin B and inoculated with untransformed conidia. In many cases, treatment with hygromycin B induces phytotoxicity to cotyledons in form of necrotic spots (arrows). (d) Untransformed *P. xanthii* colonies growing in absence of hygromycin B. (e) *Podosphaera xanthii* colonies obtained after treatment of conidia with *Agrobacterium* cells without vector, growing in absence of hygromycin B. (f) No colonies were observed on cotyledons treated with hygromycin B and inoculated with conidia treated with *Agrobacterium* cells without vector. Pictures were taken 10 d after ATMT of conidia.

*Agrobacterium* cells without vector (Fig. 3f). In many cases, we observed that treated cotyledons suffered severe damage due to hygromycin B exposure (Fig. 3c).

Next, we evaluated if the hygromycin-resistant colonies expressed the other phenotypic features conferred by the T-DNA, the emission of fluorescence (Fig. 4). To analyse this, we observed randomly selected hygromycin B resistant colonies using a fluorescence microscope. Observing colonies transformed with T-DNA of pPK2-hphgfp, we detected fluorescent signal corresponding to GFP expression, whereas untreated wild type colonies did not show any fluorescence (Fig. 4). Fluorescent

**Table 1** *Agrobacterium*-mediated transformation efficiency of *Podosphaera xanthii*

| Plasmids | Inoculated points[a] | Assays | | | | | | Mean[b] |
|---|---|---|---|---|---|---|---|---|
| | | 1st | 2nd | 3rd | 4th | 5th | 6th | |
| pPK2-hphgfp | 60 | 4[c] | 2 | 5 | 5 | 1 | 3 | 3.3 ± 0.66 |
| pTAS5 | 60 | 2 | 1 | 3 | 1 | 1 | 4 | 2.0 ± 0.51 |
| pPK2Tgfp | 60 | 2 | 3 | 2 | 1 | 1 | 4 | 2.2 ± 0.47 |

[a]In each assay 60 drops containing *P. xanthii* conidia and *Agrobacterium tumefaciens* cells were deposited onto zucchini cotyledons (see the Materials and Methods section for details).
[b]Mean of transformed colonies obtained per assay ± SE.
[c]Number of colonies resistant to the corresponding fungicide, hygromycin B (pPK2-hphgfp, pTAS5) or carbendazim (pPK2Tgfp) obtained in each assay.

signals were observed to be homogeneous throughout the entire hyphae (Fig. 4b). Moreover, we saw some conidiophore with fluorescent signals (Fig. 4c). However, fluorescent signals of transformants obtained with T-DNA of pTAS5 were completely different from those observed with T-DNA of pPK2-hphgfp (Fig. 4). Observing in detail those colonies, we detected an irregular distribution of fluorescent signal (Fig. 4e), which is in contrast to that seen in colonies obtained with T-DNA of pPK2-hphgfp (Fig. 4a–c). We observed that the fluorescent signal was near fungal penetration points and that the fluorescence intensity decreased as it moved away from these penetration points (Fig. 4f).

In our observations, we noticed that hygromycin B was phytotoxic to cotyledons. For that reason, we built the plasmid pPKTgfp (Fig. 1), a derivative of pPK2-hphgfp but with the *hgh* allele replaced with the allele *tub2* of *P. xanthii* isolate SF60, that confers resistance to the nonphytotoxic fungicide carbendazim (Vela-Corcía *et al.*, 2015). To test the functionality of our new construct, conidia of the carbendazim-sensitive *P. xanthii* isolate 2086 were transformed using the procedure described earlier. After 14 d of inoculation, colonies were visible on cotyledons inoculated with the transformation mixture (Fig. 5a) or the carbendazim-resistant isolate SF60 (Fig. 5c), but not on cotyledons inoculated with the sensitive isolate 2086 (Fig. 5b) or with conidia exposed to *Agrobacterium* cells without vector (Fig. 5f). In addition, the replacement of the selection marker did not alter the fluorescence pattern provided by the backbone plasmid, that is, we observed a homogenous distribution of fluorescence within the hyphae of *P. xanthii* (Fig. 6). As expected no phytotoxic damage was observed when carbendazim was used as the selective agent.

## Molecular analysis of transformants

It is expected that the plasmids used in our study would integrate the T-DNA into the genome of fungal cells after nonhomologous recombination. For molecular characterization of transformants and to avoid contamination with plasmid DNA, resistant colonies were transferred to new cotyledons for several generations in the presence of the selection agent according to the corresponding T-DNA resistance and maintained on the cotyledons as

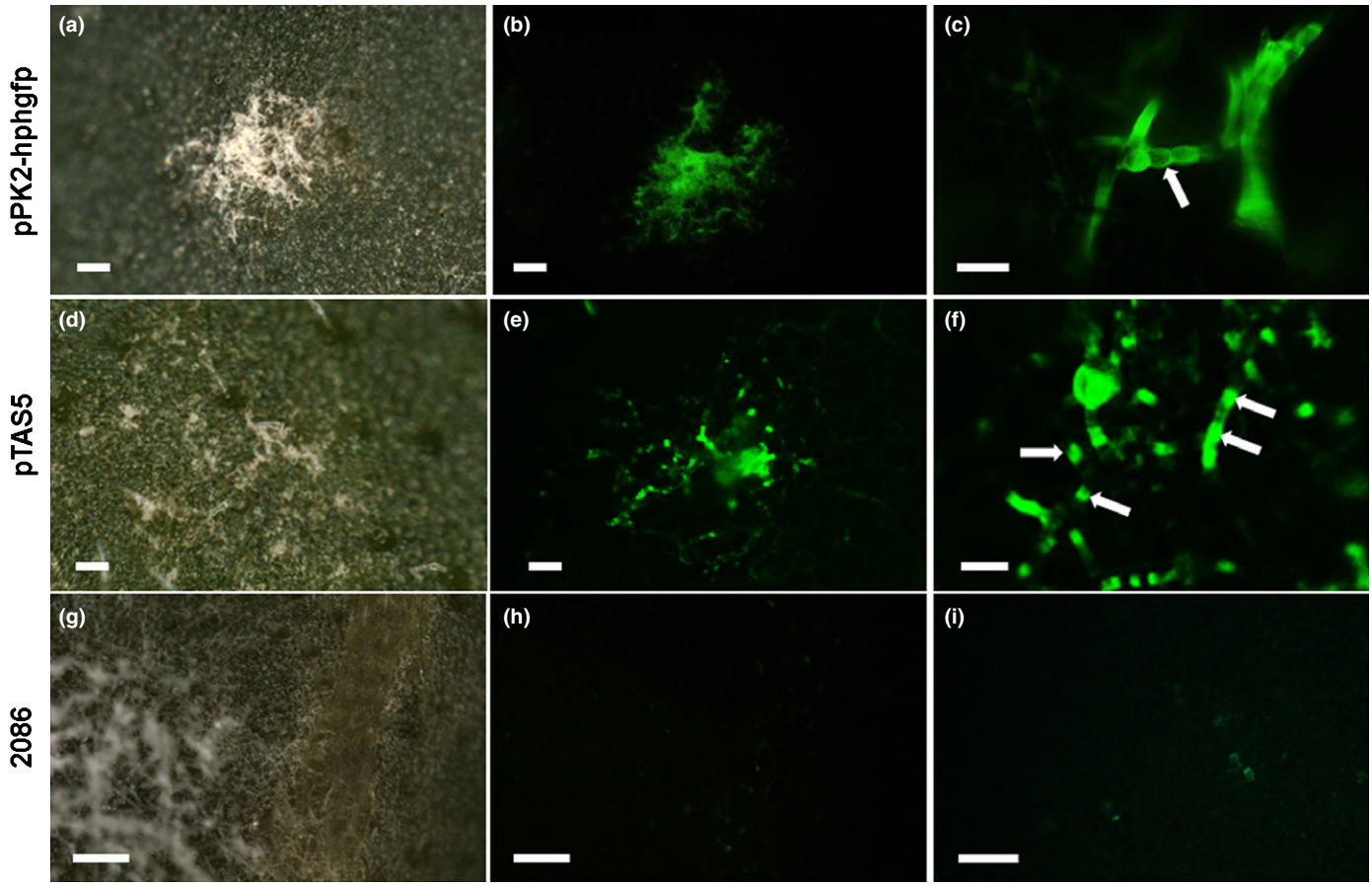


**Fig. 4** *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Podosphaera xanthii* conidia with T-DNA of pTAS5 and pPK2-hphgfp: GFP fluorescence phenotype. Transformants of *P. xanthii* grown on zucchini cotyledons treated with hygromycin B after ATMT of conidia with the T-DNA of (a–c) pPK2-hphgfp or (d–f) pTAS5. (a) A *P. xanthii* transformant growing in the presence of hygromycin B. (b, c) Homogenous distribution of the fluorescence signal in hyphae and conidiophores (arrows). (d) A transformant colony growing in the presence of hygromycin B. (e, f) Irregular distribution of the GFP signal, mostly concentrated near the penetration points (arrow). Wild-type isolate 2086 (WT) growing in the absence of hygromycin B (g) does not emit fluorescence (h, i). Pictures were taken 10 d after ATMT of conidia. Bars: (a, b) 80 μm; (c, f) 50 μm; (d, e, g, h) 500 μm; (i) 250 μm.

described earlier. The colonies from the third-generation were used for molecular analysis. To test for integration of T-DNA, genomic DNA was obtained from mixed plant and fungal material from colonies transformed with the T-DNA of pPK2-hphgfp, pPK2Tgfp and pTAS5. The genomic DNA was isolated, purified and used for PCR analysis with primer pairs designed to amplify different fragments of the T-DNA of each plasmid (Fig. 7). As expected from the phenotypic analysis, amplification signals of the expected size were obtained for all transformants but not for untransformed colonies: 0.8 kb for *gfp* and 0.7 kb for *hph* amplicons in T-DNA of pTAS5 (Fig. 7a), 2.2 kb for the *PgpdA::hph* amplicon in T-DNA of pPK2-hphgfp (Fig. 7b), and 2.1 kb for the *tub2::gfp* fragment contained in T-DNA of pPK2Tgfp (Fig. 7c). The sequencing and alignment of the *tub2::gfp* fragment from the second-generation colonies indicated the presence of the T-DNA in *P. xanthii* transformants. In addition, sequencing chromatograms of the amplicons obtained from β-tubulin-specific primers confirmed the presence of two alleles, the resistant allele with the amino acid substitution E198A responsible for the resistance to carbendazim and the sensitive allele (Fig. S1).

Confirmation of T-DNA integration was obtained by Southern blot hybridization analysis (Fig. 8). Genomic DNA from four-third-generation carbendazim-resistant colonies was isolated and digested with *EcoRV* and resolved on a 1% agarose gel before transfer to a membrane. Hybridization with a probe that targets the *gfp* allele gave an unique band that varied in size depending on the transformant, indicating a single T-DNA insertion event at different integration sites into the fungal genome.

In order to analyse the integration site and the flanking regions of T-DNA, we conducted a TAIL-PCR analysis using genomic DNA isolated from the transformants. After three rounds of PCR with specific and degenerate primers, we obtained several bands from each transformant, and selected those of the predicted sizes that changed between the second and third PCR reactions for sequencing (Fig. S2). The results of the TAIL-PCR sequencing confirm that T-DNA integration occurred at different integration sites as observed by comparison of the sequences. In analysing the sequence results, we observed the loss of some nucleotides at both borders (Table S3). Additionally, BLAST searches revealed different integration sites for the transformants, including chitin deacetylase, carbohydrate esterase, and pectin

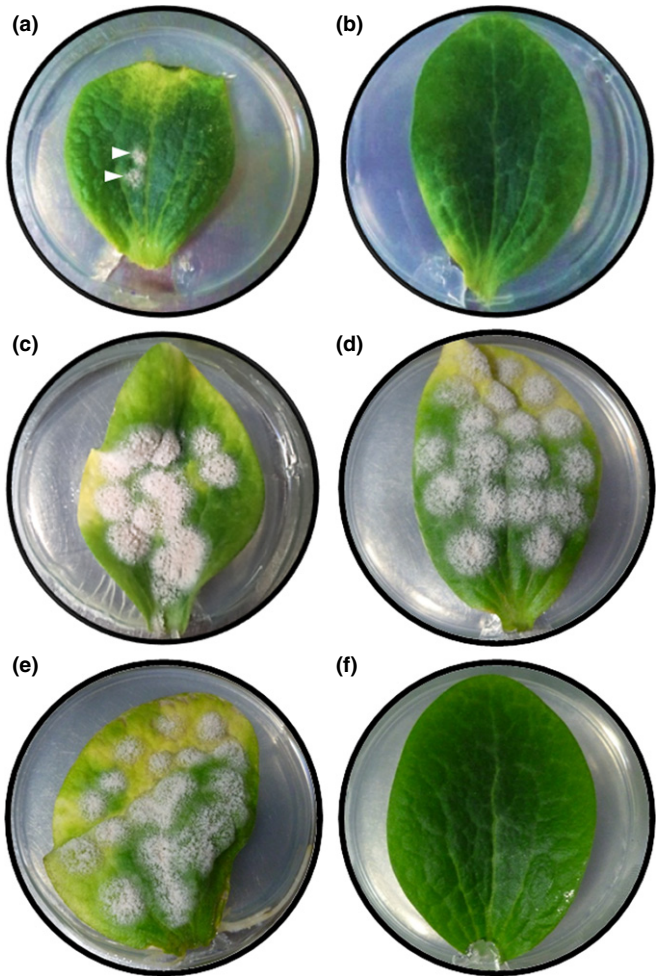


**Fig. 5** *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Podosphaera xanthii* conidia with transferred DNA (T-DNA) of pPK2Tgfp: Carbendazim resistance phenotype. Transformants of *P. xanthii* grown on zucchini cotyledons treated with carbendazim after ATMT of conidia with the T-DNA of pK2Tgfp. (a) *Podosphaera xanthii* transformants that are resistant to carbendazim (arrowheads). (b) Isolate 2086 is unable to grow in the presence of carbendazim. (c) Carbendazim-resistant isolate SF60 growing in the presence of carbendazim. (d) Isolate 2086 growing in absence of carbendazim. (e) Isolate 2086 treated with *Agrobacterium* cells without vector growing in absence of carbendazim. (f) No colonies were observed on cotyledons treated with carbendazim and inoculated with conidia of isolate 2086 treated with *Agrobacterium* cells without vector. Pictures were taken 14 d after ATMT of conidia.

lyase-like genes and one hypothetical protein gene (Table 2). Taken together, these results suggest successful integration of the T-DNA at different sites in the *P. xanthii* genome.

## Mitotic stability of transformants

Two important aspects that characterize a successful transformation protocol are the preservation of the fitness of the transformants and the stable inheritance to subsequent generations of the genotype gained from the transformation. To check the integration stability of the T-DNA, spores of the first generation of fluorescent colonies were transferred to fresh cotyledons without selective pressure. Only one colony with fluorescence was

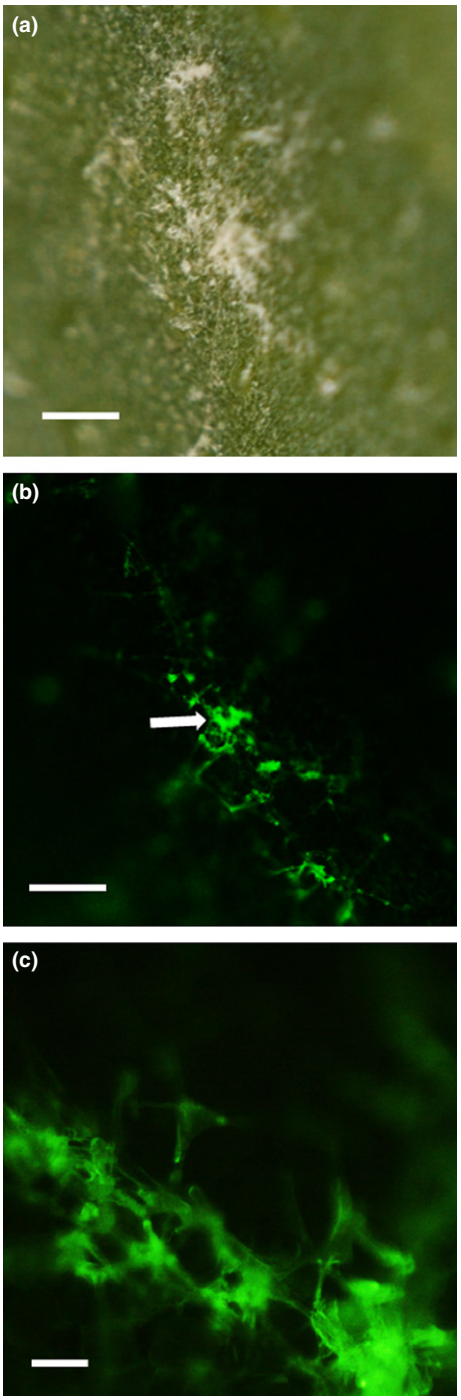


**Fig. 6** *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Podosphaera xanthii* conidia with transferred DNA (T-DNA) of pPK2Tgfp: GFP fluorescence phenotype. A transformant of *P. xanthii* grown on zucchini cotyledons treated with carbendazim after ATMT transformation of conidia with the T-DNA of pPK2Tgfp. (a) Transformant growing in the presence of carbendazim. (b) The GFP signal is homogenously distributed in the hyphae of the colony (arrow). (c) A closer view of the area marked by the arrow in (b). Bars: (a, b) 250 μm; (c) 100 μm. GFP, green fluorescent protein.

obtained in the first pass (second generation) but failed to produce a new generation of fluorescent derivatives. This transformant was obtained by transformation with T-DNA of pPK2-

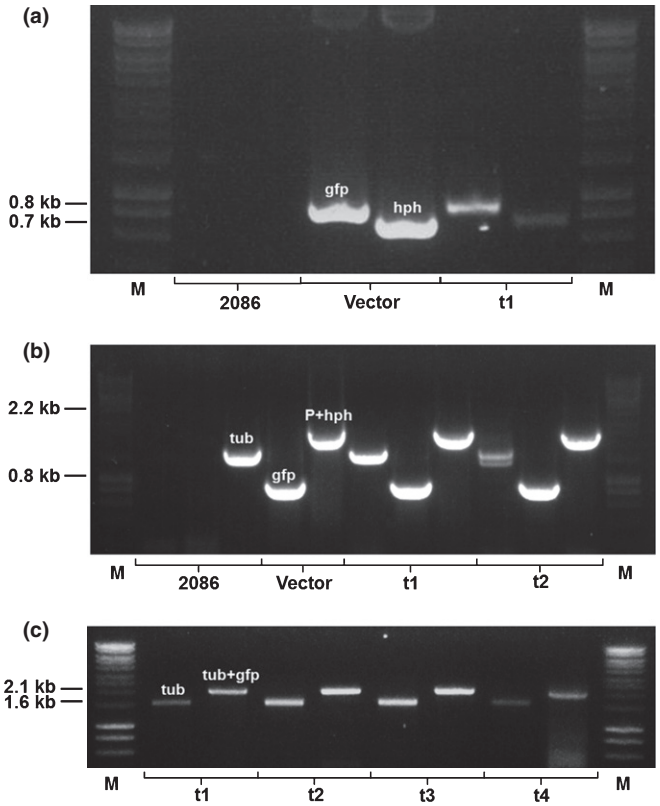


**Fig. 7** Molecular analysis of *Podosphaera xanthii* transformants obtained after *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the transferred DNA (T-DNA) of pTAS5, pPK2-hphgfp and pK2Tgfp. Genomic DNA of the potential transformants was subjected to PCR amplification of different marker genes that are contained in the T-DNA regions. The size of the expected PCR product is indicated on the left. (a) Amplification of *gfp* and *hph* fragments in a transformant obtained with pTAS5. (b) Amplification of *tub2* (tub), *gfp* or a fragment containing parts of the promoter *PgpdA* and the *hph* gene (P + hph) in two pPK2-hphgfp transformants. (c) Amplification of *tub2* (tub) or a fragment containing parts of the *tub2* and *gfp* genes (tub + gfp) in four pPK2Tgfp transformants. Lanes: M, molecular weight marker HyperLadder 1 kb (Bioline, London, UK); 2086, isolate 2086 wild-type carbendazim-sensitive genomic DNA (40 ng); vector, plasmid DNA (20 ng); t1–t4, genomic DNA of different transformants.

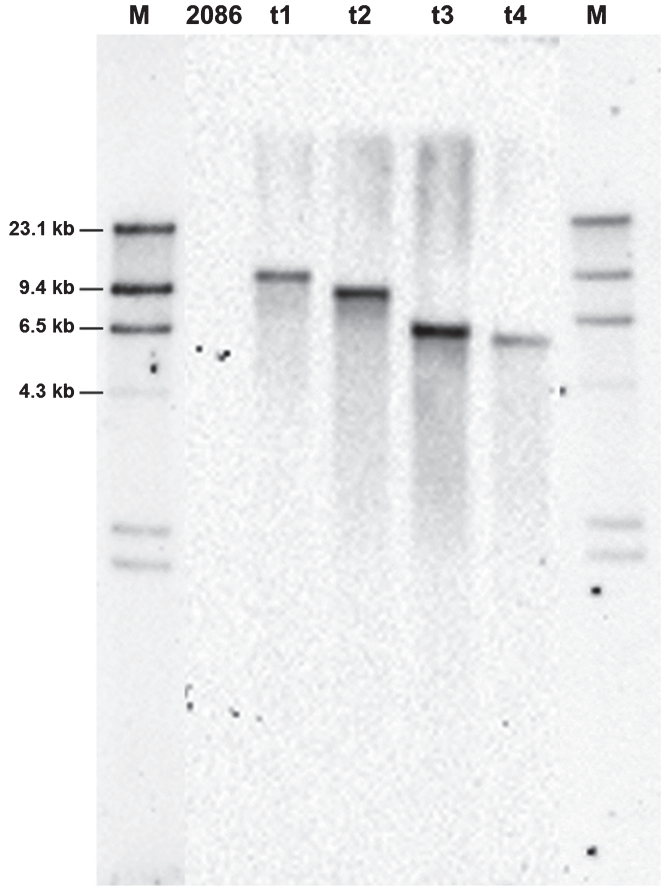


**Fig. 8** Southern blot analysis of transferred DNA (T-DNA) sequences in the genomes of *Podosphaera xanthii* transformants obtained after *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the T-DNA of pK2Tgfp. Genomic DNA isolated from carbendazim-resistant *P. xanthii* transformants (4 μg) was digested with *Eco*RV and probed with a fragment of the *gfp* gene. Lanes: M, DNA Molecular Weight Marker II DIG-labelled (Hoffmann-La Roche); 2086, isolate 2086 wild-type carbendazim-sensitive; t1–t4, carbendazim-resistant transformants. Numbers on the left indicate the positions of molecular weight markers (kb).

hphgfp (Fig. S3). In parallel, we transferred multiple conidia from the first generation of resistant colonies to new cotyledons that were treated or not with the respective selection agent. These transfers were carried out successively to obtain different generations of the transformants. In the case of colonies treated with the corresponding selection agent, we obtained an increase in the number of resistant colonies (Fig. 9) that also presented GFP fluorescence. By contrast, in the absence of fungicide, the resistant phenotype fades after one generation. These findings indicate that the stability of the constructs was associated with the presence of selective pressure.

In order to check the biological fitness of the transformants, the growth of different generations of transformants was compared with that of WT (Fig. S4). On the one hand, the first generation of transformants has a growth pattern similar to that of the 2086 WT isolate; however, transformants treated with hygromycin B grew slower than the untreated WT (Fig. S4). On the other hand, colonies transformed with TDNA of pPK2Tgfp (arrowhead) have retarded growth compared with SF60 WT after the addition of carbendazim (Fig. S4). At 10 dpi, the second-generation transformants recovered fitness, and the growth pattern was similar to that of the WT (Fig. S4). Similarly, the fifth-generation transformants showed an aspect very similar to WT isolates in the presence of the selection agent (Fig. S4), Once again, these findings suggest successful integration of T-DNA in the *P. xanthii* genome under fungicide pressure.

As mentioned earlier, in the absence of selection pressure, the putative *P. xanthii* transformants rapidly lost the phenotypes associated with the genetic markers introduced by ATMT. To determine the reasons why these phenotypes were lost, new transformants were generated using the T-DNA of pPK2Tgfp that were transferred to new cotyledons that were treated or not with carbendazim for several generations and then subjected to Southern blot analysis. As previously indicated, to avoid contamination

**Table 2** BLAST analysis of thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) products obtained from flanking sequences of transferred DNA (T-DNA) regions of four *Podosphaera xanthii* transformants obtained after *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the T-DNA of pK2Tgfp

| Transformant | Border | Description | Total score (%) | Sequence identity (%) | Accession no. |
|---|---|---|---|---|---|
| t1 | LB | Chitin deacetylase *Blumeria graminis* f. sp. *hordei* DH14 | 59 | 42 | CCU76842.1 |
| | RB | No homology | – | – | – |
| t2 | LB | Hypotethical protein *Candida glabrata* strain CBS138 | 2 | 89 | XP_446971.1 |
| | RB | No amplification | – | – | – |
| t3 | LB | Carbohydrate esterase family protein *Glonium stellatum* | 38.1 | 68 | XM_008602592.1 |
| | RB | Hypothetical protein *Ascochyta rabiei* ArDII | 35.4 | 39 | KZM23709.1 |
| t4 | LB | Pectin lyase-like protein *Aureobasidium numibiae* CBS 147.97 | 37 | 62 | KEQ69858.1 |
| | RB | No homology | – | – | – |

LB, left border; RB, right border.

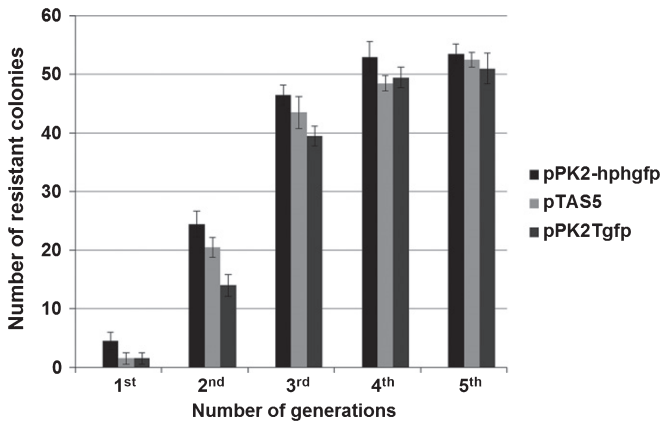


**Fig. 9** Mitotic stability of *Podosphaera xanthii* transformants obtained after *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the transferred DNA (T-DNA) of pTAS5, pPK2-hphgfp and pK2Tgfp. Transformants were selected on zucchini cotyledons treated with hygromycin B (pTAS5, pPK2-hphgfp) or carbendazim (pK2Tgfp) and subcultured (60 single-spore colonies) up to five generations in the presence of the corresponding antifungal agent. The resistance phenotypes of *P. xanthii* transformants are stable in the presence of a selective pressure because the number of resistant colonies progressively increases in successive generations. Values represent the mean of resistant colonies obtained in four independent experiments, and bars are the ± SE.

with plasmid DNA, only transformants from the third generation were analysed. As shown in Fig. 10, the hybridizing bands corresponding to the T-DNA were observed only in those transformants exposed to carbendazim. When this pressure was eliminated, the bands were no longer observed, suggesting that the excision of the T-DNA from the genome of *P. xanhii*. Furthermore, the hybridizing bands were different in size and different from the bands obtained for the transformants analysed in Fig. 8, indicating the random integration in the genome of the T-DNA in independent transformants.

## Discussion

A major bottleneck for genetic studies on powdery mildew fungi is imposed by the genetic intractability of these fungi. Attempts to manipulate a range of fungal species have generated a repertoire of diverse methods with variable efficiency: protoplast fusion, electroporation, biolistic and *Agrobacterium*-mediated transformation. Most of these methods, however, have been futile when applied towards the transformation of powdery mildews, likely because of the technical limitations derived from their biotrophic lifestyle. Particle bombardment is the first technique that has successfully delivered transgenes into powdery mildew fungi (Christiansen *et al.*, 1995; Chaure *et al.*, 2000). Unfortunately, this methodology has not been developed further due to a lack of reproducibility. Versatile electrotransformation also has been recently used to transform the cucurbit powdery mildew *Podosphaera xanthii* (Vela-Corcía *et al.*, 2015). Nevertheless, there are two major limitations to this technique including the poor reliability and stability of the transformants, and the damage it causes to conidia (Jiang *et al.*, 2013). In this study, we tested the use of *A. tumefaciens* as a vector to introduce transferred DNA (T-DNA) constructs into *P. xanthii* conidia. Our results provide strong evidence that the *A. tumefaciens*-mediated transformation (ATMT) system is a harmless and reliable method that can overcome the technical limitations of other techniques that impede the transformation of *P. xanthii*.

The ATMT method has been successfully used in non-biotrophic pathogens that are capable of growing in artificial media (de Groot *et al.*, 1998; Gouka *et al.*, 1999; Sugui *et al.*, 2005; Michielse *et al.*, 2008; Islam *et al.*, 2012) and, a few years ago, it was also used for the first time in an obligate biotroph, the flax rust fungus *Melampsora lini* (Lawrence *et al.*, 2010). However, to the best of our knowledge, there are no reports describing use of the ATMT method in powdery mildew fungi. Thus, in order for our study to be successful, we had to overcome the technical limitation of co-culturing the microbes. Two important aspects of this step in the protocol are the time required for co-cultivation, 48 h in most cases (Figueiredo *et al.*, 2010; Rodrigues *et al.*, 2013), and that *P. xanthii* conidia shrink after 3 h in water. As a result, the total co-cultivation time includes both the 2 h liquid co-cultivation and the 24 h co-cultivation on the cotyledons; other ATMT systems apply the co-cultivation mix after 4–5 h of co-cultivation (Dubresson *et al.*, 2008). After this incubation period, although the number of transformants was relatively low compared with other filamentous fungal species (Duarte *et al.*, 2007; Michielse *et al.*, 2008; Wang & Li, 2008), we obtained an average of two to three transformants for each T-DNA construct

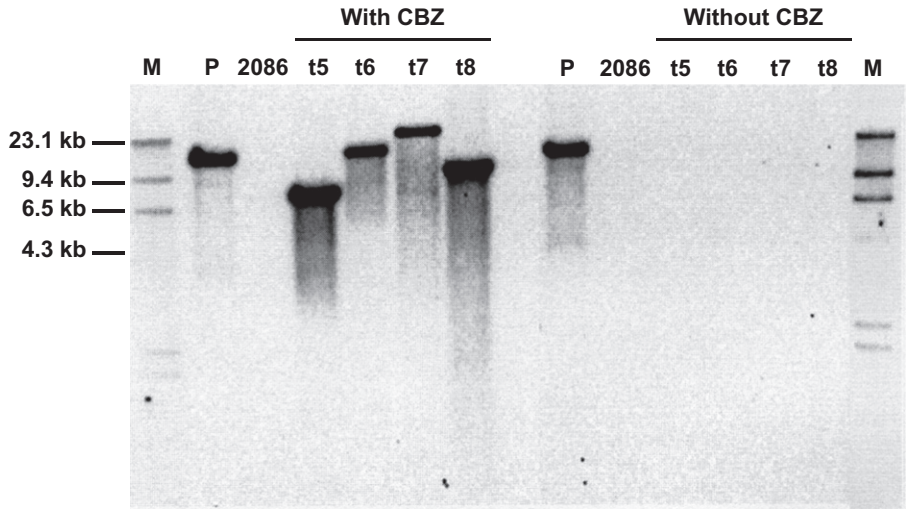


**Fig. 10** Southern blot analysis of transferred DNA (T-DNA) sequences in the genomes of third-generation *Podosphaera xanthii* transformants grown in presence or absence of selection pressure. New transformants were obtained after *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the T-DNA of pK2Tgfp and transferred to new cotyledons to obtain third-generation transformants. Genomic DNA was isolated from third-generation of carbendazim-resistant *P. xanthii* transformants grown in the presence of carbendazim (with CBZ) and from third-generation transformants grown in the absence of fungicide (without CBZ). Genomic DNA (4 μg) was digested with *Eco*RV and probed with a fragment of the *gfp* gene. Lanes: M, DNA Molecular Weight Marker II, DIG-labelled (Hoffmann-La Roche); P, plasmid pPK2Tgfp digested with *Eco*RV; 2086, isolate 2086 wild-type carbendazim-sensitive; t5–t8, carbendazim-resistant transformants. Numbers on the left indicate the positions of molecular weight markers (kb).

and transformation experiment, demonstrating the reproducibility and efficiency of our method. We conclude that the limiting factor for the ATMT method is the fragility of *P. xanthii* conidia in aqueous solutions and not the transformation itself.

A major issue during transformation of an obligate biotrophic pathogen is the use of resistance markers for the selection of transformants. For transformation of most fungi, a selective agent is applied to the culture medium (de Groot *et al.*, 1998; Gouka *et al.*, 1999; Michielse *et al.*, 2008; Islam *et al.*, 2012). However, in the case of obligate biotrophs, such as powdery mildews, the selection agent has to be applied onto the plant host as well, which may also prevent efficient transformation. In plants, common antifungals are usually phytotoxic at useful concentrations (Dubresson *et al.*, 2008). Thus, we decided to replace the hygromycin B resistance cassette because this antibiotic damages cotyledons and causes difficulties in the selection and maintenance of resistant colonies.

For this, we decided to replace the hygromycin B resistance cassette with the β-tubulin allele from the *P. xanthii* SF60 isolate resistant to carbendazim. This β-tubulin variant differs from β-tubulin gene from the 2086 isolate by a point mutation (E198A) that confers resistance to benzimidazole fungicides, a group of broad-spectrum systemic fungicides that interact with tubulin and interfere with cell division (Davidse, 1986; Qiu *et al.*, 2011; Fan *et al.*, 2013; Vela-Corcía *et al.*, 2014). As an agricultural fungicide, the cotyledons treated with carbendazim are not damaged, as occurs with hygromycin B treatment. This change allows us to analyse and maintain the resistant colonies and, more importantly, provides a good resistance marker for future studies.

The fluorescence patterns of green fluorescent protein (GFP) vary between transformants depending on the T-DNA construct used; pPK2-hphgfp and pPK2Tgfp express GFP homogenously in the cytoplasm of all fungal hyphae and in some conidia, a phenomenon previously observed in *Fusarium oxysporum* (Islam *et al.*, 2012). However, the pTAS5 vector has an irregular fluorescent signal and is concentrated mostly near penetration points. The distribution of this signal could be explained by the activity of the promoters that express the *gfp* gene of each vector: a constitutive promoter for pPK2-hphgfp and pPK2Tgfp, and the *agsA* promoter for pTAS5. The gene *agsA* encodes a putative α-glucan synthase, which is strongly induced in response to fungal cell wall stress (Meyer *et al.*, 2007; Damveld *et al.*, 2008; Yoshimi *et al.*, 2013). As occurs with other powdery mildews, *P. xanthii* develops haustoria in plant cells, getting exposed to pathogenesis-related proteins (PR) of the plant with antifungal properties (Nookaraju & Agrawal, 2012), such as the fungal cell wall degrading enzymes chitinases and β-glucanases (Rivera *et al.*, 2002; Nookaraju & Agrawal, 2012; Zhang *et al.*, 2012). So, it is conceivable that this stress on the fungus could trigger the expression of the *agsA* promoter, leading to the concentration of the fluorescent signal underneath the hyphae, near the penetration sites: the tightest fungus–plant interfaces (Weßling *et al.*, 2012; Weis *et al.*, 2013).

In addition, molecular analyses show that randomly selected transformants contain the artificial constructs. Further detailed analysis of carbendazim-resistant colonies revealed interesting results. From second-generation carbendazime-resistant colonies, we obtained a fragment comprising resistant β-tubulin and *gfp* genes. From sequencing chromatograms, we observe that all colonies presented the E198A mutation (GCG), indicating that our colonies were transformed but that the transformants also have a second nucleotide in the same position that corresponds to sensitive β-tubulin (GAG). This phenomenon is a consequence of the existence of two β-tubulin alleles in each colony, sensitive

and resistant. This result is expected because we did not replace wild-type β-tubulin and randomly integrated the resistant β-tubulin allele. As has been observed in other fungal species, such as *Aspergillus flavus* and *Trichoderma viride*, the presence of both β-tubulins alleles confers resistance to benzimidazole fungicides because the resistant β-tubulin is a dominant marker, even when β-tubulin alleles have the same expression levels (Seip *et al.*, 1990; Goldman *et al.*, 1993).

Continuing with our molecular analysis, we next analysed the integration pattern of T-DNA in four carbendazim-resistant colonies. Using Southern blot, we confirmed the presence of a single copy of T-DNA in the *P. xanthii* genome in the resistant colonies. As has been observed with other transformation studies using *A. tumefaciens*, more than one T-DNA copy is an infrequent occurrence (Kemppainen *et al.*, 2008; Michielse *et al.*, 2008). Additionally, the different sizes of the hybridizing fragments indicate integration pattern of the T-DNA into different sites in the genome. This observation was confirmed after performing TAIL-PCR analyses to obtain the T-DNA flanking regions. As observed in our Southern blot assay, we obtained a different pattern of amplicons for the four selected transformants and the sequence analyses finally confirmed different genomic T-DNA integration sites. Previous studies have shown that transformation with *A. tumefaciens* results in random integration of T-DNA into fungal genomes (Mullins *et al.*, 2001). An interesting aspect of our sequence analysis was the examination of the border sequences of the T-DNA regions, where most of the border sequences were truncated. Truncation of T-DNA border sequences is common in *Agrobacterium*-mediated transformation (de Groot *et al.*, 1998; Mullins *et al.*, 2001; Chambers *et al.*, 2014) and explains why amplification by TAIL-PCR sometimes fails. These results demonstrate that the phenotypes observed in the transformants, fungicide resistance and GFP expression, were due to integration of T-DNA into the *P. xanthii* genome.

Our results show that *P. xanthii* is easily transformed with *A. tumefaciens*, but the stability of the transformants needs further studies. Analysis of the mitotic stability of the transformants led us to observe that T-DNA remains in the fungal genome only in the presence of fungicide pressure, with the only exception of one colony in the second generation. This is a phenomenon that occurs with variable frequency in filamentous fungi. Similar to our system, *Mucur miehei* transformants maintained T-DNA only in presence of fungicide (Monfort *et al.*, 2003); in *Aspergillus sojae*, after PCR analysis, 40% of colonies were mitotically stable under selection pressure (Mora-Lugo *et al.*, 2014); and in *Backusella lamprospora*, the T-DNA is lost in successive generations (Nyilasi *et al.*, 2008). In our system, maintaining the selection pressure in successive generations leads to an increased number of resistant colonies within each generation, reaching a maximum of 85–90% of resistant colonies in the fifth generation; however, if this selection pressure is removed, T-DNA is rapidly excised. A possible explanation for this relative stability is that powdery mildews have a high number of transposons in their genomes (Spanu *et al.*, 2010), which may reduce the stability of T-DNA.

In conclusion, we have designed a protocol for *A. tumefaciens*-mediated transformation (ATMT) of *P. xanthii* that permits the feasible and robust transformation of the powdery mildew cucurbit pathogen. Unlike previous methods developed for powdery mildews, we demonstrate the reproducibility of our methodology by obtaining different transformants with different constructs. Further experiments are needed to determine if it is possible to modulate selected *P. xanthii* genes by overexpressing, misexpressing, downregulating or deleting them. We believe that ATMT is a strong tool that can revolutionize the emerging field of powdery mildew effector research and it is a necessary step to fully understand fungal pathogenesis of plant–powdery mildew interactions. Finally, due to the simplicity of our method, we anticipate that similar protocols may be easily adapted for other obligate biotrophic pathogens or mutualistic symbionts.

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## Author contributions

J.M-C., D.R. and A.P-G planned and designed the research; J.M-C and D.R. performed the experiments; and J.M-C., D.R., A.d.V. and A.P-G. analysed the data and wrote the paper.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Sequence chromatograms of *tub2::gfp* fragment and *tub2* genes amplified from four *Podosphaera xanthii* transformants obtained with the T-DNA of pPK2Tgfp by ATMT.

**Fig. S2** TAIL-PCR analysis of flanking sequences of T-DNA regions of four transformants obtained with the T-DNA of pPK2Tgfp by ATMT.

**Fig. S3** GFP fluorescent phenotype of a second generation *Podosphaera xanthii* transformant containing the T-DNA of pPK2-hphgfp and grown without the selective agent.

**Fig. S4** Appearance of *Podosphaera xanthii* transformants after different generations.

**Table S1** Oligonucleotides used in this study

**Table S2** Conditions used for thermal asymmetric interlaced polymerase chain reaction protocol

**Table S3** T-DNA border flanking sequences of four selected *Podosphaera xanthii* transformants obtained after ATMT transformation with the plasmid pK2Tgfp

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