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Phleomycin resistance gene as a reliable selection marker for *Agrobacterium tumefaciens*-mediated transformation of the citrus postharvest pathogen *Penicillium digitatum*

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Abstract:

***Penicillium digitatum* exists in nature as a causative agent of green mould disease in citrus fruits at the postharvest stages. Inspections of the molecular mechanism of the host invasion by *P. digitatum* usually require suitable selection markers for genetic manipulation. In this study, we recruited the phleomycin resistance gene as a selection marker for the genetic transformation of *P. digitatum* using *Agrobacterium tumefaciens*. The results showed that the growth of the wild strain *P. digitatum* PdVN1 from fungal spores is quite sensitive to phleomycin and is inhibited at a concentration of 50 µg/ml, whereas growth from fungal mycelium is more tolerant and completely suppressed at a concentration of 200 µg/ml. Under optimised conditions, the *A. tumefaciens*-mediated transformation (ATMT) efficiency of *P. digitatum* with the phleomycin resistance marker could reach over 1000 transformants per 10^6 spores. All the tested transformants presented the integration of T-DNA in their genomes and were mitotically stable for the phleomycin resistance. Furthermore, the results also revealed the success for heterologous expression of the *DsRed* fluorescent gene in the fungus, in which the strong red fluorescent signal could be observed over the whole fungal mycelium and spores. Our work demonstrates for the first time that the phleomycin resistance gene can serve as a reliable selection marker for *A. tumefaciens*-mediated transformation of the postharvest pathogen *P. digitatum*. This selection marker can be exploited for T-DNA insertional mutagenesis and for functional investigations of target genes involved in citrus decay by *P. digitatum*.**

Keywords: ***Agrobacterium*-mediated transformation, citrus postharvest pathogen, *DsRed* fluorescent reporter gene, *Penicillium digitatum*, phleomycin resistance marker.**

Classification number: **3.5**

Introduction

Penicillium digitatum causes green mould disease on citrus fruits and has been reported to be the most destructive pathogen during the postharvest stages. This pathogen can infect fruits through wounded points on the citrus peel prior to the colonization of the whole fruits for severe decay. *P. digitatum* grows on an infected citrus peel and can be seen as white mycelium, which later turns olive colour due to fungal sporulation [1-3]. The molecular mechanism of citrus infection by *P. digitatum* is still unclarified. Up to date, only a few genes required for pathogenicity of *P. digitatum* have been characterized [4-6]. The development of new genetic tools with reliable selection markers is essential and can help to dissect more about the infection process of this pathogenic fungus.

Agrobacterium tumefaciens is a soil-borne bacterium, which has the capability of transferring the T-DNA from the tumour-inducing (Ti) plasmid into plants and fungi [7]. The *Agrobacterium tumefaciens*-mediated transformation (ATMT) was reported for the first time in filamentous fungi by de Groot, et al. in 1998 [8]. At the time, this transformation method became popular and useful for fungal research community. Currently, the ATMT methods have been demonstrated to be highly efficient for genetic transformation of a large number of filamentous fungi [8-10]. ATMT represents a simple method for genetic

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transformation with high efficiency and fungal spores can be used directly as the transformation material [10, 11]. The common antifungal agents, including hygromycin, nourseothricin, and phleomycin, have been broadly exploited for genetic transformation of filamentous fungi [3, 8, 10-12]. Although phleomycin was employed for the transformation of different fungi and plants [13-16], so far it has not been tested on the citrus postharvest pathogen *P. digitatum*. Recently, ATMT has been demonstrated to be an effective transformation method in *P. digitatum* using two dominant selection markers conferring the resistance to hygromycin and nourseothricin. The ATMT efficiencies with these two selection markers reached the yields of 60-1240 transformants for 10^6 spores depending on the examined strains of *P. digitatum* [3, 17]. In addition, the *DsRed* fluorescent gene originated from the reef coral *Discosoma* sp. has been commonly used as a model reporter gene for genetic transformation of numerous filamentous fungi including *P. digitatum* [3, 11, 18-21]. In this study, we report for the first time that the phleomycin resistance gene can be employed as a selectable marker for genetic transformation of *P. digitatum* using *A. tumefaciens*. Our work shows that the transformation efficiency with the phleomycin resistance marker could achieve over 1000 transformants for 10^6 spores.

Materials and methods

Microbial strains and cultivation media

Escherichia coli DH5α and *Agrobacterium tumefaciens* AGL1 were used for plasmid propagation and fungal transformation, respectively. These bacteria were grown in Luria-Bertani medium. The fungal strain *P. digitatum* PdVN1, isolated in Vietnam [3], was grown on the potato dextrose agar (PDA) medium.

Preparation of fungal spore suspensions

The wild strain *P. digitatum* PdVN1 and transgenic strains were grown on the PDA plates at 25°C for 4-5 days. Fungal spores were collected as mentioned earlier [3]. The spore suspensions were adjusted to the concentration of 10^6 spores/ml for later use. The spore concentration was monitored with a hemocytometer under microscopy.

Extraction of fungal genomic DNA

Fungal strains were grown in the potato dextrose broth (PDB) medium at the temperature of 25°C at 200 rpm for 3 days and fungal mycelia from the cultures were collected by filtration through Miracloth (Calbiochem, Darmstadt, Germany). Genomic DNA was extracted from the fungal biomass as previously described [22].

Examination of the susceptibility of P. digitatum to phleomycin

A PDA agar plug with the diameter of 4 mm containing fungal mycelium or 10 µl of spore suspension (10^6 spores/ml) of the wild strain PdVN1 was placed on the PDA medium supplemented with phleomycin (50-200 µg/ml). The plates were incubated at a temperature of 25°C for 3-4 days to examine growth of the fungus.

PCR amplification

PCR amplifications of the phleomycin resistance marker and the *DsRed* reporter gene were performed with specific primer pairs (Table 1). GoTaq® Green Master Mix (Promega, Madison, USA) was used for PCR screening. PCR procedure includes 94°C (3 min); 30 cycles of 94°C (30 s), 58-60°C (30 s), 72°C (1-2 min); 72°C (7 min). PCR products were analysed on 0.7% agarose gels.

Table 1. The primers used in this study.

Primer name	Sequence (5'-3')	Product size (bp)	Source
DsRed-F	AACTCGAGCACGTGCTTAAGGATATCATGGCCTCCTCCGAGG	729	[19]
DsRed-R	AAGGATCCCCGCGGGAGCTCGATATCCTACAGGAACAGGTGGTGGC		
Phleo-F	GGGCTCGAGAGGCCTCCGGTGACTCTTTCTGGC	1250	[11]
Phleo-R	TCGGTCAGTCCTGCTCCT		

Agrobacterium tumefaciens-mediated transformation of P. digitatum

The binary vector pPK2-Red2 was transformed into the bacterium *A. tumefaciens* AGL1 by electroporation using the Gene Pulse Xcell™ Electroporation System (Bio-Rad, California, USA). *A. tumefaciens* AGL1 carrying the binary vector was used for genetic transformation of *P. digitatum* as previously described [3]. PDA plates supplemented with phleomycin (200 µg/ml) and cefotaxime (300 µg/ml) were used for selection of fungal transformants and for suppression of the *A. tumefaciens* cells, respectively. The plates were kept at the temperature of 25°C for 4-5 days to collect transformants.

Examination of transgenic strains

Fungal transformants as transgenic strains were cultivated on PDA containing phleomycin at the concentration of 200 µg/ml to confirm the ability of phleomycin resistance. Afterwards, these transformants were examined for mitotic stability with several mitotic generations on PDA. The transformants were then grown in PDB for 3 days at 25°C and their mycelia were collected for genomic DNA extraction. T-DNA integrations into the genome of the pathogenic fungus were verified by PCR with two independent primer pairs listed in Table 1. The selected transformants were grown directly on microscopic slides containing droplets of PDA as reported by Vu, et al. (2018) [3] and the expression of the *DsRed* fluorescent gene in these transformants was analysed under Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany).

Results and discussion

Penicillium digitatum PdVN1 is highly susceptible to phleomycin

We examined the susceptibility of the wild strain *P. digitatum* PdVN1 to phleomycin by growing the fungus on the medium containing this antifungal agent at different concentrations. The results showed that fungal spores were more susceptible and totally suppressed by phleomycin at a concentration of 200 µg/ml. In contrast, fungal mycelium was more resistant to low concentrations of this agent. At a concentration of 200 µg/ml, phleomycin could completely inhibit growth of the fungus with both the inoculation materials as spores and mycelium (Fig. 1A). Therefore, this concentration of phleomycin was used for transformation of *P. digitatum* PdVN1 in order to inhibit the growth of untransformed fungal cells. For transformation of *P. digitatum*, the binary vector pPK2-Red2 was employed. This vector contains a transfer DNA (T-DNA) region, which harbours two different cassettes for expression of the phleomycin resistance gene and the *DsRed* gene under the regulation of the constitutive *gpdA* promoter from the model filamentous fungus *Aspergillus nidulans* (Fig. 1B). Recently, pPK2-Red2 has also been successfully exploited for genetic transformation of the penicillin-producing fungus *Penicillium chrysogenum* using phleomycin as the selection agent [11].

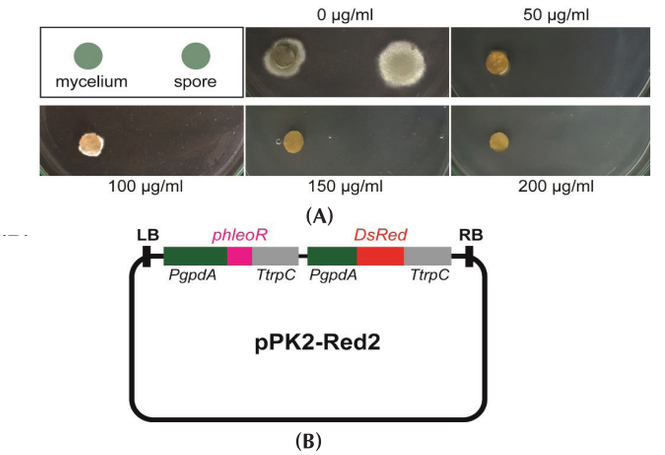

Fig. 1. The susceptibility of *P. digitatum* PdVN1 to phleomycin and the map of the binary vector pPK2-Red2. (A) The wild strain PdVN1 (mycelium, spores) was grown on the PDA medium supplemented with different concentrations of phleomycin (50-200 µg/ml). **(B)** The map of the binary vector pPK2-Red2 showing the T-DNA structure containing the expression cassettes for the phleomycin resistance gene *(phleoR)* and the *DsRed* fluorescent reporter gene. This T-DNA region is restricted by the left border (LB) and right border (RB).

Phleomycin resistance gene represents a reliable selection marker for transformation of the citrus postharvest pathogen P. digitatum

In this study, we employed the binary vector pPK2-Red2 [11] for evaluating the genetic transformation of *P. digitatum* PdVN1. This vector harbours the phleomycin resistance marker, in which the *Sh ble* gene conferring phleomycin resistance is regulated by the constitutive *gpdA* promoter from the filamentous fungus *A. nidulans* (Fig. 1B). The *Sh ble* gene isolated from *Streptoalloteichus hindustanus* encodes a protein binding to phleomycin and subsequently inhibits its DNA cleavage activity [14, 16]. Our results showed that the ATMT method using the phleomycin resistance marker is efficient for genetic transformation of *P. digitatum* PdVN1. With the transformation conditions optimized for the ATMT including a temperature of 25°C, time of 72 h for the co-cultivation step, acetosyringone (AS) concentration of 200 µM for induction, and spore concentration of 10^6 spores/ml, the efficiency for transformation of *P. digitatum* PdVN1 could reach over 1000 transformants per 10^6 spores (Fig. 2). The transformation efficiency with the phleomycin resistance marker in this study is similar to the ones with two other selection markers conferring the resistance to hygromycin and nourseothricin (approximately 1240 transformants per 10^6 spores) that we previously reported for the ATMT method in *P. digitatum* [3].

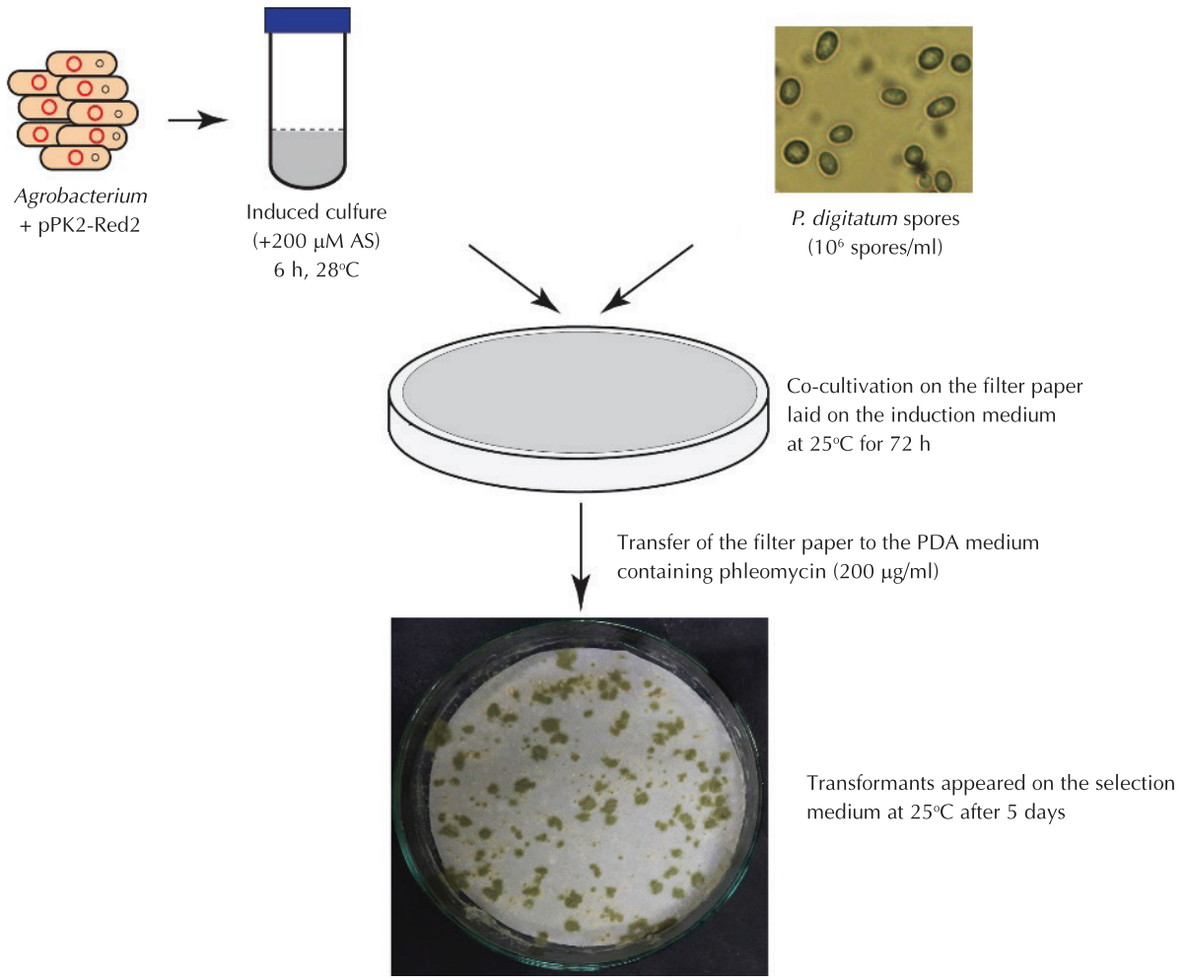

Fig. 2. The optimized procedure for *A. tumefaciens*-mediated transformation of *P. digitatum* PdVN1 using the phleomycin resistance marker. The induced *Agrobacterium* cells containing the binary vector pPK2-Red2 were mixed with fungal spores for co-cultivation on the cellulose membrane covered on the induction medium (IM). This step promoted the transfer of T-DNA fragment from *Agrobacterium* into the fungal genome. The membrane was then shifted to the PDA medium supplemented with a suitable concentration of phleomycin for selection of transformants.

The transgenic strains are mitotically stable for maintaining the T-DNA structure

Five transformants as transgenic strains named Pd-R1, Pd-R2, Pd-R3, Pd-R4, and Pd-R5 were randomly selected and cultivated on the PDA medium containing phleomycin. The young mycelia of these strains were cut and transferred to new plates containing the PDA medium without phleomycin for three successive mitotic generations prior to re-growing on the PDA medium supplemented with the selection agent as phleomycin. Our results indicated that all five tested transformants still grew well on the selection medium supplemented with phleomycin in comparison to the wild strain PdVN1 (WT), which was not able to survive on this medium (Fig. 3A). These transgenic strains were then analysed by PCR with two different primer pairs, which amplify specifically the phleomycin resistance cassette and the *DsRed* fluorescent gene (Table 1). The results showed that the T-DNA structure carrying the cassettes for expression of the *Sh ble* gene conferring phleomycin resistance and the *DsRed* reporter gene was integrated in the genomes of all five strains (Fig. 3B). Interestingly, these results were also similar to the transformation data obtained from *P. chrysogenum* when the binary vector pPK2-Red2 and phleomycin as the selection agent were used for genetic transformation of this penicillin-producing fungus [11]. In fact, the mitotic stability of T-DNA structures in fungal genomes had been reported in several filamentous fungi [7-9, 19].

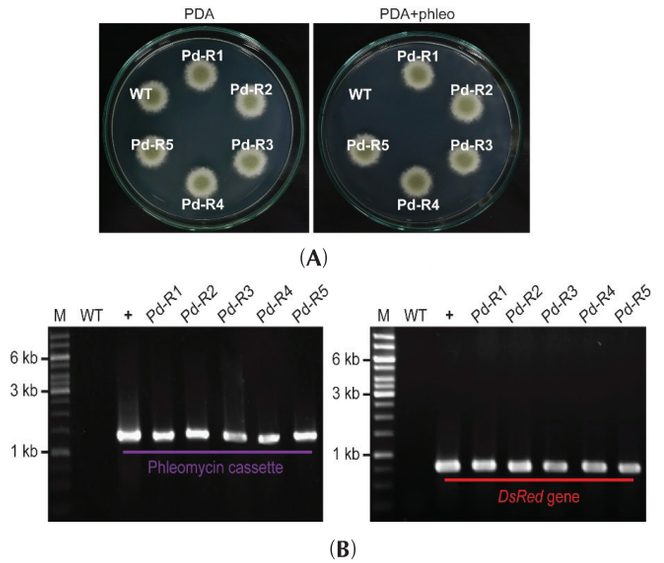

Fig. 3. Confirmation of the phleomycin resistant transformants. (**A**) Five randomly selected transformants (Pd-R1, Pd-R2, Pd-R3, Pd-R4, Pd-R5) were grown simultaneously on PDA and PDA containing 200 µg/ml of phleomycin (PDA+phleo). The *P. digitatum* PdVN1 strain (WT) was used as reference control. (**B**) The examined transformants were verified by PCR using the primer pair amplifying the phleomycin resistance marker or the *DsRed* fluorescent reporter gene. Total DNA isolated from *P. digitatum* PdVN1 (WT) and the purified plasmid pPK2-Red2 were used as DNA templates for controls, respectively.

Successful expression of the DsRed fluorescent reporter gene in P. digitatum using the phleomycin resistance marker

We cultivated all five transgenic strains together with the wild strain PdVN1 directly on the sterile microscopic slides containing droplets of the PDA medium. After 3 days of incubation at 25°C, the slides were checked under the fluorescence microscope using the filter set with the excitation/emission of 558/583 nm for detection of the DsRed signal. The results showed that the expression of the *DsRed* reporter gene in all five strains was very strong and the red fluorescent signal was observed in both fungal hyphae and spores (Fig. 4).

The random integration of T-DNA from a binary vector into fungal genome could create insertion mutants, which resulted in a large collection of the mutants for further identifications of potential genes involved in metabolism, differentiation and pathogenicity in fungi [9, 10, 14]. Therefore, the transfer of the T-DNA structure containing the phleomycin resistance marker from the binary vector pPK2-Red2 into the citrus postharvest pathogen *P. digitatum* PdVN1 may be a good approach for the construction of insertion mutant libraries. Furthermore, the *DsRed* reporter gene in the binary vector can also be replaced with a gene of interest for overexpression in this pathogenic fungus.

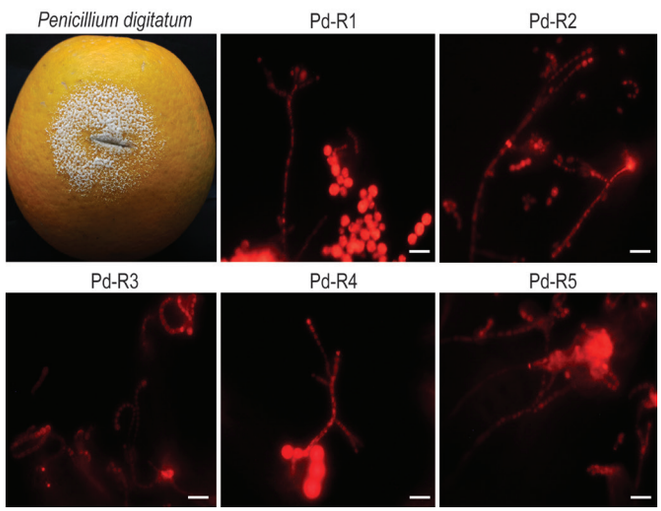

Fig. 4. Examination of the DsRed expression in the transgenic strains. All five transgenic strains (Pd-R1 to Pd-R5) generated from the wild strain *Penicillium digitatum* PdVN1 were grown directly on microscopic slides containing the PDA medium. Fungal mycelia were observed under the Axioplan fluorescence microscope. The scale bars indicate the same sizes of the images.

Conclusions

In this study, we have successfully employed the phleomycin resistance gene as a reliable selection marker for the genetic transformation of the citrus postharvest pathogen *P. digitatum* using the bacterium *A. tumefaciens*. The transformation efficiency of *P. digitatum* PdVN1 under the optimized conditions could reach over 1000 transformants per 10^6 spores. The transformants could maintain the ability for phleomycin resistance after several mitotic generations. We further succeeded in heterologous expression of the *DsRed* reporter gene in *P. digitatum* using a binary vector carrying the phleomycin resistance marker. This selection marker in combination with the optimized ATMT method for *P. digitatum* can be exploited for gene targeting or for the generation of insertion mutants by T-DNA integration events for screening the potential genes required for fungal pathogenicity on citrus.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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