SHORT COMMUNICATION

AGROBACTERIUM-MEDIATED TRANSFORMATION OF THE PLANT PATHOGENIC FUNGUS *VERTICILLIUM ALBO-ATRUM*

C.J. Knight, A.M. Bailey and G.D. Foster

School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK

SUMMARY

We report the successful *Agrobacterium tumefaciens*-mediated transformation of the important plant pathogen *Verticillium albo-atrum*. *V. albo-atrum* was demonstrated to be hygromycin B sensitive and consequently transformations were based on integration of either the hygromycin B resistance gene (*hph*) or *hph* in addition to the fluorescence gene DsRed. The effect of various parameters on transformation efficiency, including spore concentration, acetosyringone concentration, selection media and co-cultivation time were investigated. Transformants were analysed by PCR and Southern analysis, and were found to contain randomly integrated T-DNA that typically inserted as one or two copies. The dual marker system afforded by pCAMDsRed provides an additional and fast method of transformant verification. Development of a successful transformation system for *V. albo-atrum* should facilitate further molecular studies of this important plant pathogen.

Key words: Transformation, *Verticillium albo-atrum*, *V. dahliae,* DsRed, hygromycin resistance

Verticillium albo-atrum along with *V. dahliae* comprises a significant source of disease to crop plants of mainly temperate, but also subtropical and tropical regions as they infect a broad spectrum of host species, from ornamental trees to major crops such as potato, tomato, cotton and tobacco (Pegg and Brady, 2002). Control of the disease is difficult, with fungicides having little effect once infection has begun. Infection often occurs via the roots, or wounds at the base of the plant, followed by entry and spread through the vascular system, ultimately leading to chlorosis, necrosis and wilting of foliage (Fradin and Thomma, 2006). Symptoms can vary between hosts, further confounding successful diagnosis and treatment of infection. Persistence of the fungus in the soil or plant debris as microsclerotia (*V. dahliae*) or dark resting structures (*V. albo-atrum*) facilitates long-term survival in the absence of a suitable host (Pegg and Brady, 2002).

The establishment of a robust transformation system is required for more efficient studies of plant-*V. albo-atrum* interactions, allowing insertion of reporter genes that can be visualised by fluorescence microscopy and also studies of gene function by disruption or silencing. This has been possible for *V. dahliae* following its successful transformation via *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Dobinson *et al.*, 2004) and has facilitated the investigation of several genes in the infection process (Klimes *et al.*, 2008; Rauyaree *et al.*, 2005), but to date transformation of *V. albo-atrum* has not been reported.

ATMT of a fungus was first reported on the yeast *Saccharomyces cerevisiae* in 1995 (Bundock *et al.*, 1995). In the following years, numerous fungal species from phyla including the Ascomycetes, Basidiomycetes and Zygomycetes, have been found to be amenable, to varying degrees, to ATMT in the laboratory, as reviewed in Michielse *et al.* (2005). ATMT often results in a high number of transformants, most of which have single, or low-copy number integrations (de Groot *et al.*, 1998). These features can also make it the method of choice for targeted gene disruptions or insertional mutagenesis through random integration of T-DNA (Betts *et al.*, 2007; Choi *et al.*, 2007; Combier *et al.*, 2003; Leclerque *et al.*, 2004; Tsuji *et al.*, 2003). This publication presents an ATMT method for *V. albo-atrum,* which should provide a useful tool for researchers working on this important pathogen.

In order to identify a suitable selection marker to use for ATMT of *V. albo-atrum*, drug sensitivity profiles were assessed for the antibiotic hygromycin B and the herbicide Basta, both commonly used as selective agents for fungal transformation. Wild type *V. albo-atrum* strain 1974, a UK hop isolate (obtained from D. Barbara, Warwick-HRI), was point-inoculated onto potato dextrose agar (PDA) amended with various concentrations (0-200 μg ml^{-1}) of hygromycin B or Basta. Results demonstrated that whilst *V. albo-atrum* displayed little sensitivity to Basta even at 200 μg ml^{-1}, a concentration of just 25

Corresponding author: G. Foster
Fax: +44.117 3317985
E-mail: Gary.Foster@bristol.ac.uk

µg ml^{-1} hygromycin B was sufficient to decrease colony growth by more than 90%. Hygromycin B therefore appeared to be a suitable selection marker for ATMT of *V. albo-atrum* and a concentration of 50 µg ml^{-1} was consequently used for selection of transformants.

Two binary vectors were used for fungal transformations. pBIN7.1 contains the hygromycin B resistance gene (*hph*) under the control of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator (Amey *et al.*, 2002) whilst pCAMDsRed contains the DsRed (Express) gene under the control of the *gpdA* promoter and the *trpC* terminator, and *hph* driven by the *trpC* promoter (Eckert *et al.*, 2005).

Agrobacterium tumefaciens containing the appropriate vector was grown at 28ºC for 48 h in LB medium containing 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} rifampicin. Fifty ml of induction media (IM), with or without 200 µM acetosyringone was inoculated with the *A. tumefaciens* culture to an OD_{600nm} of 0.15. The culture was then incubated at 28ºC until the OD_{600nm} reached 0.25. Conidia were harvested from PDA plates, adjusted to 1×10^8 spores ml^{-1} and then mixed with an equal volume of bacteria (200 µl total) before plating onto induction media agar plates (with or without 200 µM acetosyringone) overlaid with cellophane discs and the plates incubated for 36-48 h at room temperature. The cellophane discs were then transferred to PDA selection plates containing 50 µg ml^{-1} hygromycin B and 75 µg ml^{-1} Timentin. *V. albo-atrum* transformants typically appeared after 5-7 days.

Putative transformants were subcultured to fresh selection plates (PDA supplemented with hygromycin B and Timentin) and single colonies obtained by repeated streaking on selection plates. Transformants containing the DsRed gene were also screened for expression using a DM-LB Leica microscope fitted with a red-shifted TRITC filter (emission 545/30 nm, excitation 620/60 nm). As can be seen from Fig. 1, ATMT of *V. albo-atrum* using pCAMDsRed results in obvious DsRed expression in spores and hyphae. The use of a dual marker

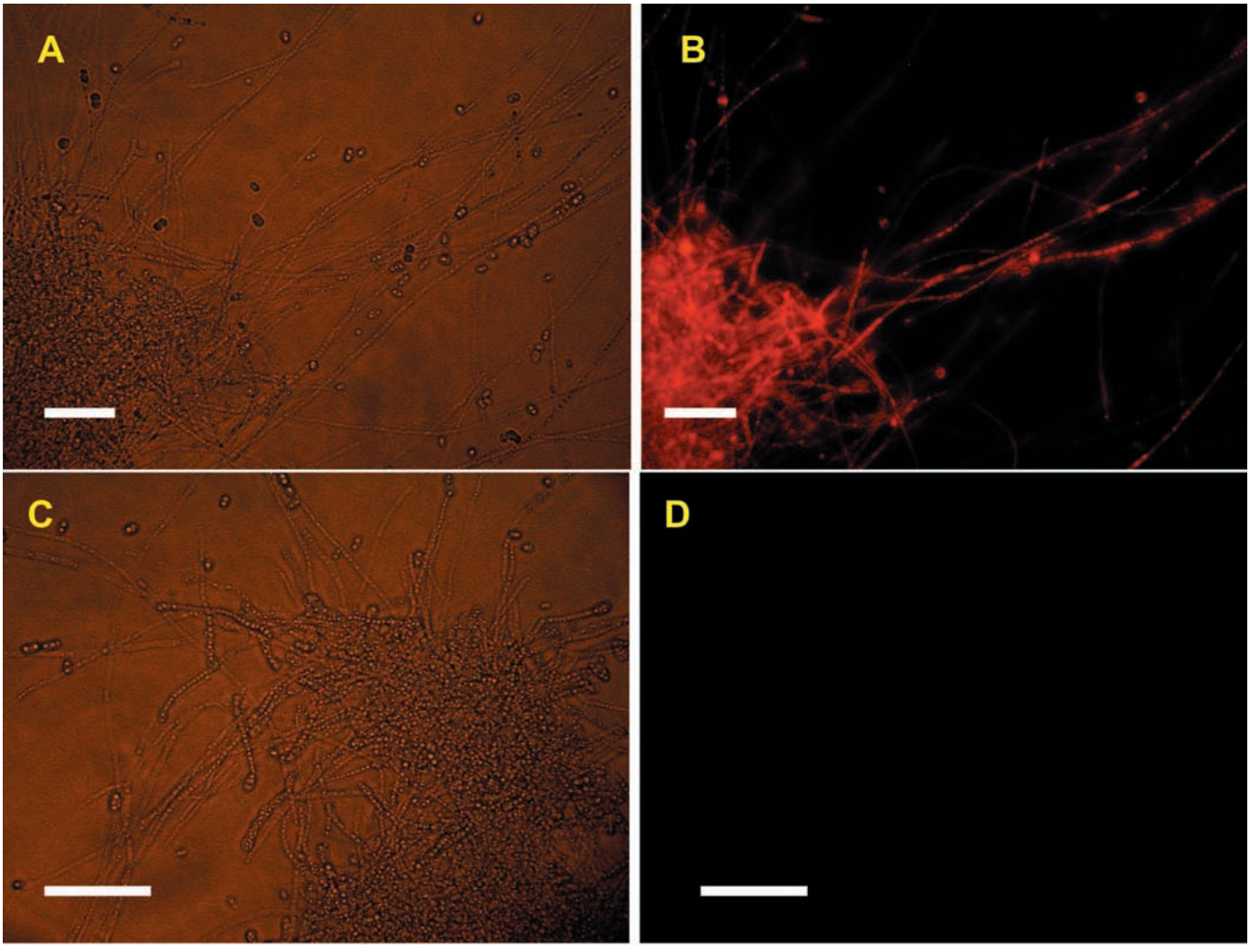

Fig. 1. The bright red fluorescence observed in pCAMDsRed transformed *V. albo-atrum* (A and B) clearly indicates successful DsRed expression in hyphae and spores. (A) white light (B) epifluorescence. Wild type *V. albo-atrum* shown under (C) white light and (D) epifluorescence with no discernible red fluorescence. Scale bar represents 200 µm.

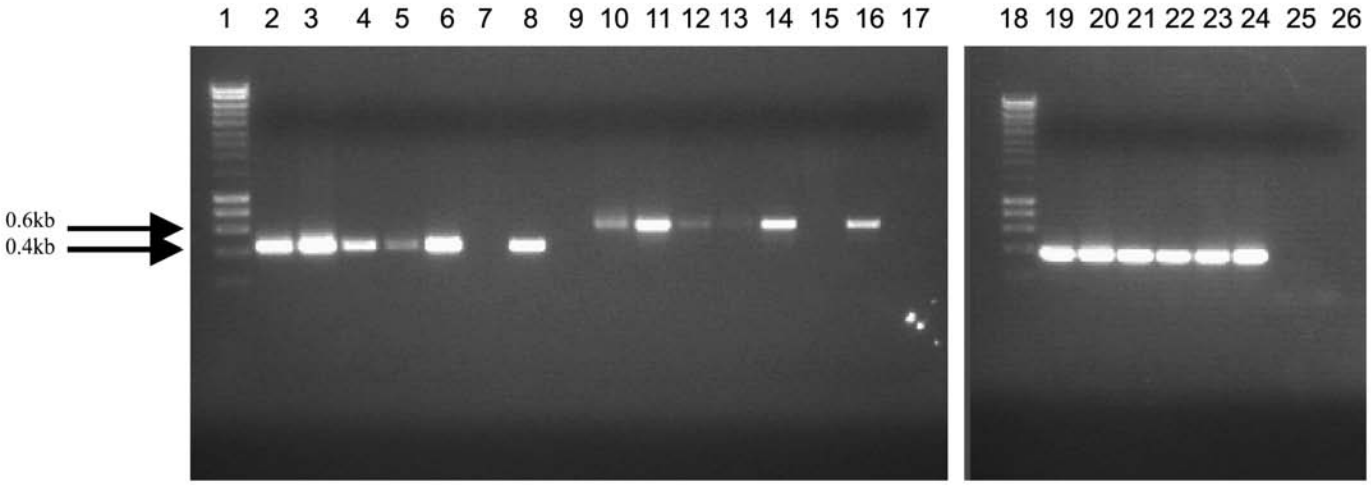

Fig. 2. PCR of typical pCAMDsRed transformants showing presence of DsRed and *hph* genes. Lane 1, DNA hyperladder I (Bioline), Lanes 2-6, transformants 1-5 amplified using DsRed primers. Lane 7, wild type *V. albo-atrum* amplified with DsRed primers. Lane 8, pCAMDsRed plasmid (positive control) amplified with DsRed primers. Lane 9, water with DsRed primers. Lanes 10-14, transformants 1-5 amplified with hygromycin B primers. Lane 15, wild type *V. albo-atrum* with hygromycin B primers. Lane 16, pCAMDsRed plasmid (positive control) with hygromcyin B primers. Lane 17, water with hygromycin B primers. Lane 18, DNA hyperladder I. Lanes 19-23, transformants 1-5 with ITS primers. Lane 24, wild type *V. albo-atrum* with ITS primers. Lane 25, pCAMDsRed plasmid with ITS primers. Lane 26, water with ITS primers.

system such as hygromycin B resistance and DsRed allows rapid verification that hygromycin B resistant colonies are a result of a transformation event rather than spontaneous resistance as colonies can also be screened for DsRed fluorescence *in situ*.

For molecular confirmation of transformation, fungal genomic DNA was extracted as described by Keon and Hargreaves (1998) and used in a standard PCR reaction with primers HygF (5'-GCGTGGATATGTCCTGCGGG-3') and HygR (5'-CCATACAAGCCAACCACGG-3') (Amey *et al.*, 2002) to amplify a 598 bp region of the *hph* gene. Primers DsRedF (5'-AGGACGTCATCAAGGAGTTC-3') and DsRedR (5'-CAGCCCATAGTCTTCTTCTG-3') amplified a 414 bp fragment of the DsRed gene. Species specific ITS Primers that amplify a ~300 bp ITS region were used to confirm that fungal DNA was of *V. albo-atrum* origin - V. aaF (5'-CCGCCGGTACATCAGTCTCTTTATTTATAC-3') and V. aaR (5'-GTGCTGCGGGACTCCGATGCGAGCTGTAAT-3') (Nazar *et al.*, 1991). Fig. 2 shows that bands of the appropriate size (598 bp for *hph* and 414 bp for DsRed) were consistently produced in transformants, and absent in wild type *V. albo-atrum* DNA.

Southern analysis was used to determine T-DNA copy number in transformants. Ten µg of genomic DNA were digested with *Hin*dIII, separated by electrophoresis and transferred onto Hybond N^+ membrane. Hybridisation was conducted according to standard protocols (Sambrook *et al.*, 1989) using either a [α-^{32}P]dCTP labelled *hph* PCR product (for pBIN7.1 transformants) or DsRed PCR product (for pCAMDsRed transformants). T-DNA was found to integrate randomly into the fungal genome, and in low copy number (Fig. 3). This is typical of ATMT of other fungal species (de Groot *et al.*, 1998).

A range of different parameters were then investigated to optimise the transformation process. To select the best *Agrobacterium*, strains LBA1126, GV3101 and LBA4404 containing the plasmid pBIN7.1 were evaluated for transformation competency in *V. albo-atrum*. All strains were able to generate hygromycin B resistant

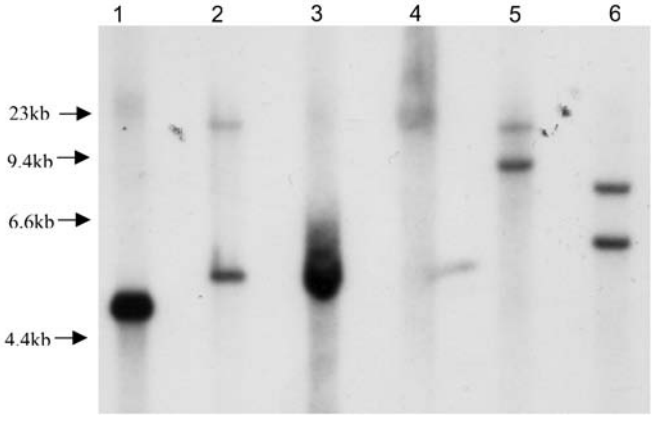

Fig. 3. Southern analysis of *V. albo-atrum* colonies resulting from ATMT with pBIN7.1. Genomic DNA was digested with *Hin*dIII and probed with the *hph* gene. The presence of a single hybridising signal in most lanes is indicative of a single T-DNA insertion event in these transformants. The different size of each band in each transformant is indicative of independent random insertion events. No hybridisation signal was seen with wild type *V. albo-atrum* DNA.

transformants, however LBA4404 had a tendency to form clumps when grown in liquid culture, making an accurate determination of the optical density difficult, resulting in a large variation in transformation efficiency. Some researchers have successfully used LBA4404 with Ascomycete fungi, for example Aimi *et al.* (2005), although they did not compare its efficiency to that of other strains, so it is unclear if higher transformation rates could have been achieved with alternative strains. In our studies it was observed that strain LBA1126 provided the most reliable and consistent results when used for ATMT of *V. albo-atrum*.

The effect of varying fungal spore concentrations on transformation efficiency was investigated. A spore concentration of 1×10^5 spores ml^{-1} did not produce any transformants, whereas concentrations of 1×10^6 spores ml^{-1} and 1×10^7 spores ml^{-1} resulted in transformation frequencies of 0-22 and 13-61 transformants per ml respectively. The highest transformation efficiency was obtained when 1×10^8 spores ml^{-1} was used (205-503 transformants per ml). Spore concentration was not extended beyond 1×10^8 spores ml^{-1} as a concentration higher than this would have resulted in a breakdown of selection conditions and this concentration (1×10^8 spores ml^{-1}) yielded a high number of transformants sufficient for most purposes.

Decreasing the co-cultivation from 48 to 24 h resulted in an approximately ten-fold decrease from an average of 280 transformants per 1×10^8 spores ml^{-1} to an average of 20 transformants per 1×10^8 spores ml^{-1}. As has been observed with ATMT of other fungal species (Covert *et al.*, 2001; Maruthachalam *et al.*, 2008), co-cultivation periods longer than 48 h resulted in excessive mycelial growth, confounding identification of transformants.

In order to induce *A. tumefaciens vir* genes necessary for ATMT, acetosyringone is typically added to fungal transformations at a concentration of 200 µM (de Groot *et al.*, 1998; Covert *et al.*, 2001; dos Reis *et al.*, 2004). To investigate if this concentration is optimal for ATMT of *V. albo-atrum*, transformations were conducted using varying concentrations of acetosyringone (0-400 µM) in IM and co-cultivation plates. *V. albo-atrum* transformants were produced at concentrations of acetosyringone as low as 10 µM. However, despite repeated attempts no transformants were ever generated in the absence of acetosyringone. It was found that a concentration of 100 µM acetosyringone sometimes resulted in the highest numbers of transformants. However, this was rather variable and more consistent results were seen when 200 µM acetosyringone was used. Concentrations of acetosyringone above 200 µM resulted in decreased *A. tumefaciens* growth and overgrowth of fungi on co-cultivation plates.

A suitable medium for selection of fungal transformants should allow unambiguous differentiation between wild type colonies and transformants. A range of growth media were selected for comparison, i.e. potato dextrose agar (PDA), yeast malt glucose agar (YMG), malt mycological peptone agar (MMP), cornmeal agar (CMA) and malt extract agar (MEA). Growth of wild type and transformed colonies were compared on each medium at a range of hygromycin B concentrations (0, 25, 50, 100 and 200 µg ml^{-1}). As expected, the growth of the hygromycin resistant transformant was noticeably greater than that of wild type *V. albo-atrum* in the presence of hygromycin B. On MEA, growth of the transformant was lower at all concentrations than on the alternative media. At high concentrations of hygromycin B, the growth of transformants on YMG, CMA and MMP was slowed in comparison to lower concentrations. This effect does not appear to be as severe on PDA, and therefore PDA appears to be the best at giving consistent good growth of *V. albo-atrum*, as well as clear selection of transformants.

Therefore, in carrying out these series of experiments to determine a suitable selectable marker for transformation of *V. albo-atrum*, the fungus was found to be highly sensitive to the antibiotic hygromycin B, which was consequently used at a concentration of 50 µg ml^{-1} for transformant selection. We note that hygromycin B has also been used at this concentration for transformant selection of the closely related *V. dahliae* (Dobinson *et al.*, 2004) and this concentration is comparable to that used for ATMT of other Ascomycete species (Grimaldi *et al.*, 2005; Gorfer *et al.*, 2007; Aimi *et al.*, 2005). ATMT with binary vectors containing the *hph* gene successfully resulted in the generation of transformants, at frequencies comparable to the 50-500 transformants per 1×10^6 spores reported for *V. dahliae* (Dobinson *et al.*, 2004) and that of other Ascomycete species such as *Leptosphaeria biglobosa* and *L. maculans* where 20-40 per 1×10^7 spores were reported (Eckert *et al.*, 2005).

Experiments designed to further optimise the ATMT system for *V. albo-atrum* confirmed that PDA allowed the best isolation of transformants during selection. PDA has been used by other researchers working with *V. dahliae* and *V. albo-atrum* (Cherrab *et al.*, 2002; Qin *et al.*, 2006; Robb *et al.*, 1991) and other Ascomycete fungi (Aimi *et al.*, 2005; Weld *et al.*, 2006). As reported previously for other fungi (Michielse *et al.*, 2005), a positive correlation was seen in this study between spore concentration and ATMT efficiency. Similarly, it was found that increasing the co-cultivation time resulted in increased ATMT efficiency. Co-cultivations of 24-48 h are typical with fungal ATMT (Sugui *et al.*, 2005; Amey *et al.*, 2002; Mullins *et al.*, 2001). Whilst 48 h co-cultivation was found to result in more *V. albo-atrum* transformants than 24 h, it was also noted that if a high concentration of spores is used (1×10^8 spores ml^{-1}) a co-cultivation period of 48 h could result in overgrowth of the fungus by the time it is transferred to selection. This problem has been documented by other researchers in

the literature (Covert *et al.*, 2001), and makes identification of transformants difficult. Therefore, it is best to modify parameters such as co-cultivation time and spore concentration accordingly.

Experiments investigating the effect of varying acetosyringone concentrations demonstrate that concentrations below 200 µM can successfully generate transformants. Indeed, transformants were produced when as little as 10 µM acetosyringone was used. No transformants were ever produced in the absence of acetosyringone. The effect of acetosyringone concentration on transformation efficiency does not appear to be strictly dose dependent. Despite lower concentrations of acetosyringone sometimes generating more transformants than higher concentrations, a concentration of 200 µM acetosyringone was found to provide the most consistent results in terms of transformation efficiency. This concentration is also almost universally used in fungal ATMT (Mullins *et al.*, 2001; Chen *et al.*, 2000; Covert *et al.*, 2001; dos Reis *et al.*, 2004). Concentrations above this appeared to have a bacteriostatic effect on *A. tumefaciens*, as reported in other studies (Amoah *et al.*, 2001; Sheng and Citovsky, 1996).

Use of the dual marker plasmid pCAMDsRed greatly facilitated transformant isolation as the bright red fluorescence was simple to observe even on primary transformation plates and so could help to rapidly confirm true transformants. DsRed was found to represent an excellent reporter gene for the fungus, and has the potential to greatly facilitate studies of *V. albo-atrum in planta.* Use of such a fluorescent marker could provide an additional, less destructive method of visually quantifying and localising aspects of the disease process, as well as additional benefits of the reduced autofluorescence associated with DsRed systems compared with GFP (Eynck *et al.*, 2007; Vallad and Subbarao, 2008).

In conclusion, the important plant pathogen *V. albo-atrum* (strain 1974) was successfully transformed using *A. tumefaciens* mediated transformation with both pBIN7.1 and pCAMDsRed. The development of an efficient transformation system for this pathogen is a useful tool in the study of *V. albo-atrum*. ATMT based approaches such as T-DNA mutagenesis and targeted knockouts can be used to further our understanding of the molecular mechanisms of this fungus and its interactions with its numerous hosts. In addition, the use of the dual marker system afforded by pCAMDsRed provides an effective method of visualising and studying the *V. albo-atrum* disease process *in planta*.

ACKNOWLEDGEMENTS

We thank Dez Barbara for the *V. albo-atrum* isolate and Martin Urban for pCAMDsRed. C Knight was funded by a NERC studentship.

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Received June 15, 2009
Accepted July 15, 2009