Agrobacterium tumefaciens T-DNA mediated transformation of *Epichloë festucae*

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Abstract

Although much is known about the signals and mechanisms that lead to pathogenic interactions between plants and fungi comparatively little is known about fungal-plant mutualistic symbiotic interactions. The association between *Epichloë festucae* and perennial ryegrass is a powerful experimental system to understand the molecular basis for fungal-plant mutualistic interactions. We have adopted a forward genetics approach to dissect this symbiosis. Previously we described the use of plasmid mutagenesis to isolate a symbiotic mutant. We describe here the development of an *Agrobacterium tumefaciens* mediated T-DNA transformation system for *E. festucae*.

Keywords: *Epichloë festucae*, *Agrobacterium tumefaciens*, T-DNA, transformation

Introduction

In the association between *E. festucae* and perennial ryegrass the endophyte grows systemically in the intercellular spaces of vegetative and reproductive tissues as infrequently branched hyphae parallel to the axis of the leaf (Tan *et al*. 2001; Christensen *et al*. 2002). Growth of the hyphae is strictly intercellular and synchronised with that of the host grass throughout the life cycle of the plant. To identify *E. festucae* genes that are required for the establishment and maintenance of a mutualistic interaction with the grass host we have initiated a forward genetics approach (mutant phenotype to gene) to isolate mutants that disrupt this highly regulated symbiosis. In a plasmid mutagenesis screen using the technique of restriction enzyme-mediated integration (REMI) (Schiestl & Petes 1991) we isolated an *E. festucae* mutant (FR2) that induces a stunting phenotype and premature senescence of the host grass (Tanaka *et al*. 2006). While this mutant was the result of single site plasmid insertion that was linked to the mutant phenotype, REMI frequently results in multiple-copy and multi-site integrations and the generation of untagged deletions in the fungal genome (Michielse *et al*. 2005; Elliott & Howlett 2006). The development of *Agrobacterium tumefaciens* mediated T-DNA mutagenesis in filamentous fungi (Bundock *et al*. 2002; de Groot *et al*. 1998) overcomes many of these limitations as the insertions are frequently single copy and when deletions are generated they tend to be small (Elliott & Howlett 2006). The aim of this work was to develop *A. tumefaciens* mediated T-DNA mutagenesis in *E. festucae* as an alternative to REMI to generate symbiotic mutants.

Methods

Biological material and growth conditions

Cultures of *Epichloë festucae* wild-type strain Fl1 were grown on 2.4% potato dextrose (PD) agar at 22°C. *A. tumefaciens* strain EHA105 (PN1828; Rif^R) containing a disarmed agropine plasmid, pTiBo542, (Hood *et al*. 1993) plus either pBSYT6 (PN1976) or pBSYT7 (PN4015), were grown in either LB or IM medium at 28°C and where appropriate supplemented with rifampacin (50 µg/ml) or kanamycin (50 µg/ml). *Escherichia coli* strain DH5α was used for all plasmid transformations using ampicillin (50 µg/ml) for selection.

Induction medium (IM) contained per litre: 0.01 M $K_2HPO_4.3H_2O$, 0.01 M KH_2PO_4, 2.57 mM NaCl, 9 µM $FeSO_4.7H_2O$, 3.78 mM $(NH_4)_2SO_4$, 10 mM glucose, 0.7 mM $CaCl_2$, 2 mM $MgSO_4$, glycerol (0.5%), buffered to pH 5.3 with 40 mM 2-[*N*-Morpholino] ethanesulfonic acid (MES) (Bundock *et al*. 2005).

Molecular biology

Genomic DNA from *E. festucae* was isolated from freeze-dried mycelium using previously described methods (Yoder 1988). Genomic digests separated by electrophoresis were transferred to positively charged nylon (Roche) membranes by capillary transfer and DNA fixed by UV-crosslinking. The filters were probed with [α-^{32}P]-dCTP (3000 Ci/mmole, Amersham) labelled probes and signals detected as previously described (Young *et al*. 1998).

Vector construction

Plasmid pYT6 (Kan^R) was prepared by Yasuo Itoh by cloning a *Hin*dIII/*Sal*I hygromycin resistant gene cassette (P*glaA-hph*), from pCWHyg1 (Young *et al*. 1998) into pCAMBIA1380 digested with *Hin*dIII/*Xho*I. . Plasmid pBSYT6 (Kan^R, Amp^R) was prepared by cloning a *Bam*HI/*Spe*I digest of pBlueScript KS+ into *Bgl*II/*Spe*I digested pYT6. Plasmid pBSYT7 (Kan^R, Amp^R) was prepared by removing the hygromycin resistance cassette from pBSYT6 by digestion with *Sac*I/*Xba*I and replacing with a *Spe*I/*Hin*dIII geneticin resistance cassette (P*trpC-nptII*) from pSF17.1. pSF17.1 was prepared by Simon Foster by ligating a 1.75-kb PCR product containing the P*trpC-nptII*-T*trpC* cassette from pII99 into *Sma*I cut pSP72 (Promega). The PCR product was generated by PCR overlap extension to eliminate the *Nco*I site in *nptII* and to truncate the P*trpC*.

Transformation conditions

A. tumefaciens strain EHA105 was grown overnight in LB medium at 28°C, harvested by centrifugation and resuspended in IM medium to A600 of 0.15 and then grown for a further 6 h

Table 1 Different hyphal growth and morphology classes of T-DNA mutants of *E. festucae*.

Phenotype	Number	%
Wild type	386	85.4
Small colony	13	2.9
Reduced aerial hyphae	37	8.2
Increased aerial hyphae	2	0.4
Premature senescence	14	3.1
Total	452	100

Figure 1 Physical maps of pBSYT6 and pBSYT7 vectors used for *A. tumefaciens* mediated T-DNA transfer of *E. festucae*.

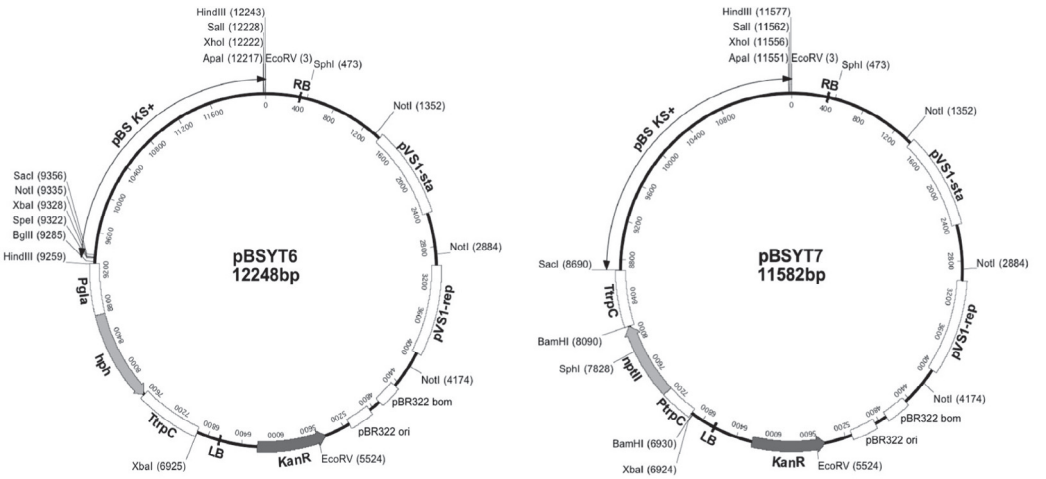


Figure 2 T-DNA transformed hyphae of *E. festucae* growing on PD agar containing hygromycin.

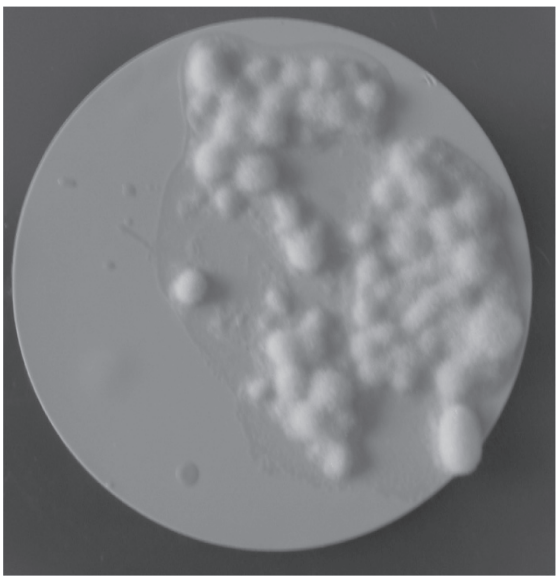
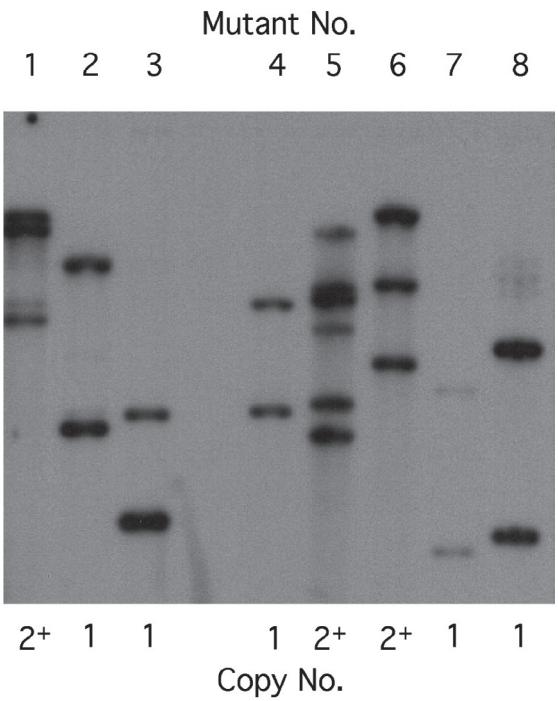


Figure 3 Molecular analysis of T-DNA mutants of *E. festucae*. Southern hybridization of *Sst*I (*Sac*I) genomic digests of *E. fesucae* T-DNA mutants numbers Ag352 (lane 1), Ag353 (lane 2), Ag357 (lane 3), Ag358 (lane 4), Ag378 (lane 5), Ag380 (lane 6), Ag396 (lane 7) and Ag397 (lane 8) probed with [^{32}P]-labelled pBSYT6.

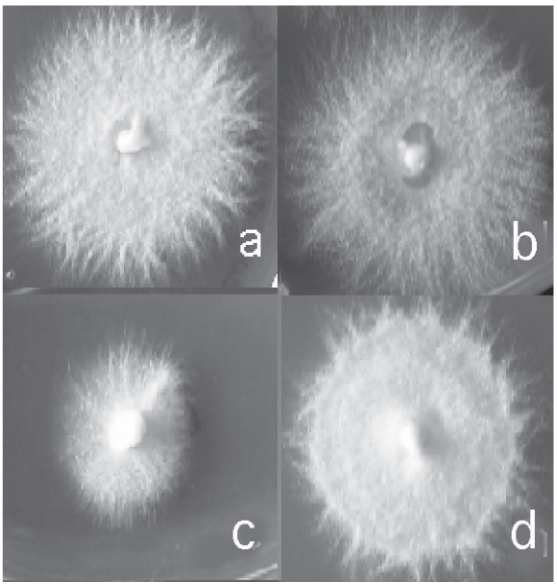


at 28°C in the presence of acetosyringone (200 μM). Mycelial cultures (4 ml) of *E. festucae* were grown in PD medium in Universal bottles for 6 days at 22°C, washed twice in 5 ml of IM medium by centrifugation and washing of the pellet, and finally resuspended in 100 μl of the *A. tumefaciens* culture. This mixture was then spread on a nitrocellulose membrane (Millipore 0.4 μm HA, 47 mm) on IM agar medium containing acetosyringone (200 μM) and incubated at 22°C for 2 days. The membrane was then transferred to a PD agar plate containing hygromycin (150 μg/ml) and cefataxime (200 μg/ml) and incubated for a further 14-20 days. The resulting transformants were nuclear purified by sub-culturing mycelium from the edge of a colony to PD medium containing either hygromycin (150 μg/ml), or geneticin (200 μg/ml).

Results

Two binary vectors, pBSYT6 and pBSYT7, containing fungal selectable markers conferring resistance to hygromycin and gentamycin respectively, were developed for *A. tumefaciens* mediated T-DNA transformation of *E. festucae* (Fig. 1). Plasmid

Figure 4 T-DNA induced colony morphology mutants of *E. festucae*. Wild-type (A), and T-DNA mutants Ag78 (B), Ag149 (C) and Ag243 (D).



pBSYT6 was constructed by cloning pBlueScript KS+ into pYT6, to provide a ColE1 origin of replication and bacterial selectable marker (Amp^R), to facilitate plasmid rescue in *E. coli* of T-DNA junction sequences containing *E. festucae* DNA. Plasmid pYT6 contains a hygromycin resistance cassette between the left and right T-DNA borders, a Kan^R bacterial selectable marker, pBR322 (ColE1) origin of replication (*ori*), the *cis* acting site required for conjugational transfer (*bom*; basis of mobility), and the pVS1-*rep* and pVS1-*sta* sequences, which confer stability to the plasmid in an *A. tumefaciens* background, even under non-selectable conditions (Deblaere *et al*. 1987). Plasmid pBSYT7 was constructed by replacing the hygromycin resistance cassette in pBSYT6 with a geneticin resistance cassette from pII99.

Incubation of *E. festucae* hyphae with *A. tumefaciens* strain EHA105 containing a disarmed derivative of the agropine type Ti plasmid, pTiBo542, and pBSYT6, gave rise to numerous Hyg^R colonies after incubating the mating mixture at 28°C for about 2 weeks (Fig. 2). Following nuclear purification of these transformants by sub-culturing the edge of the growing colony onto fresh selectable medium, DNA was isolated from a selection of eight arbitrarily chosen transformants, and a Southern blot of an *Sst*I digest probed with pBSYT6 (Fig. 3). Five of the eight (63%) transformants examined had a single copy T-DNA insertion, and each insertion was at a unique site in the genome.

A collection of 452 T-DNA mutants was generated and the hyphal morphology and growth of each classified into one of four different phenotypes (Table 1). 66/452 (14.6%) of the mutants were altered in either colony growth or morphology compared to wild-type (Fig. 4A), and included reduced aerial hyphae (Fig. 4B), smaller colony size (Fig. 4C), increased aerial hyphae (Fig. 4D) and premature senescence (not shown).

Discussion

We describe here the development of a method to generate *A. tumefaciens* T-DNA induced mutants of *E. festucae*. Many filamentous fungi are now amenable to this transformation method (Michielse *et al*. 2005). For the method to be effective as a mutagenesis system it is essential that insertion is relatively random. From the small number of transformants examined to date it would appear that each *E. festucae* mutant had the T-DNA integrated at a unique site, with around 63% having a single copy insertion. Single copy insertions are the preferred outcome for genetic analysis and molecular rescue of the flanking *E. festucae* sequences. However, it is also important that the mutant phenotype is linked to the T-DNA insertion. Genetic analysis of a large set of T-DNA induced mutants from *Cryptococcus neoformans* demonstrated that just 50% of the mutants showed linkage (Walton *et al*. 2005). In the same study they showed that there was a bias toward insertions into promoters of the genes, a result consistent with what has been observed in *Arabidopsis thaliana* (Alonso *et al*. 2003; Pan *et al*. 2005) Despite this limitation, insertions in the promoter region frequently give rise to mutant phenotypes. Recently, Elliott & Howlett (2006) reported the isolation of a T-DNA induced mutant of *Leptosphaeria maculans* that had reduced pathogenicity. Molecular analysis showed that the single copy insertion in this mutant was located in the promoter region of two bi-directionally transcribed genes; encoding an alcohol dehydrogenase-like gene (*Adh4L*) and a 3-ketoacyl-CoA thiolase (*Thiol*). Ectopic expression of the *Thiol* gene recapitulated the mutant phenotype, indicating that T-DNA induced alteration of expression of this gene was responsible for the pathogenicity defect.

The isolation here of *E. festucae* morphological mutants demonstrates that T-DNA mutagenesis will be a useful method for tagging and isolating genes important for the symbiotic interaction between *E. festucae* and perennial ryegrass.

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