Towards the elimination of ergot alkaloid biosynthesis genes in *Neotyphodium Coenophialum*

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Abstract

Neotyphodium coenophialum strain e19 from tall fescue cv. Kentucky 31 carries *dmaW*1 and *dmaW*2, two gene homologues that encode dimethylallyltryptophan synthase, the enzyme for the first step in ergot-alkaloid biosynthesis. In our effort to disrupt both homologues and ultimately obtain marker-free mutants, we are using a marker-exchange strategy employing the Cre/loxP site-specific recombination system. Of 1522 transformants obtained and screened, three were likely *dmaW2* disruptants because they gave no PCR product from the wild-type locus, but yielded the larger PCR fragment from the disruption construct. The putative *dmaW*2-knockouts were also transformed with pKAES186, a plasmid with a cassette containing the *cre* and *ble* genes in between loxP sequences. The transformants obtained were screened for the presence of *hph*, *cre* and *ble* genes. The preliminary results indicate a loop-out of the *hph* gene. The transformants inoculated into endophyte-free tall fescue preserved their compatibility with the plant. The fungus grown from these plants will be further analysed for the presence of *hph*, *cre* and *ble* genes.

Keywords: Cre/LoxP, dimethylallyltryptophan synthase, *dmaW*, *Epichloë*, ergot alkaloids, *Festuca arundinacea*, gene knockouts, *Lolium arundinaceum, Neotyphodium coenophialum*, tall fescue

Introduction

In its mutualistic association with the important forage grass, tall fescue (*Lolium arundinaceum*), the endophyte *Neotyphodium coenophialum* can have opposing agricultural consequences, improving plant productivity and tolerance to biotic and abiotic stresses, but producing ergot alkaloids associated with livestock toxicosis. One possible way to eliminate this problem is to modify the fungus making it unable to produce ergot alkaloids (Wang *et al.* 2004). This modification requires introduction of several antibiotic resistance genes, used as selective markers of transformation. The presence of these resistance markers will impede release of plants due to regulatory issues and public concerns. In order to eliminate the resistance genes we chose to use the Cre/loxP system as a method to develop marker free transformants.

Neotyphodium coenophialum has two homologues — *dmaW1* and *dmaW2* — encoding dimethylallyltryptophan synthase (DMAT synthase), the first pathway specific step in ergot alkaloid biosynthesis (Tsai *et al.* 1995). Several transformation methods were used to replace the native *dmaW2* allele with a mutant *dmaW2* allele disrupted by the hygromycin resistance gene (*hph*) flanked by loxP sites to allow excision by the Cre recombinase. The elimination of the *hph* marker by Cre was tested.

Material and Methods

Strains and culture conditions

Neotyphodium coenophialum e19 was isolated and maintained as described in Wang *et al.* (2004). For fungal DNA extraction, mycelial culture was grown on cellophane sheets on potato dextrose agar (PDA) at 21°C for 5–6 days. The fungal DNA was from fresh mycelium isolated following the method described by Al-Samarrai and Schmid (2000), and measured using DNA-binding fluorochrome Hoechst 33258 in a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia Biotech, San Francisco, California). For fungal transformation, cultures were grown in potato dextrose broth (PDB), shaking at 200 rpm at 21°C for 10 days.

Plasmids used for transformation

Plasmid pKAES174, containing the 5’ and 3’ regions of the *dmaW2* locus flanking the *loxP-hph-loxP* cassette used in transformations, are described in detail elsewhere (Machado 2004). The plasmid pKAES186, was generated by similar techniques, and contains a cassette with the *cre* (cyclization recombination) and *ble* (bleomycin and phleomycin resistance) genes between loxP sites.

Fungal transformation

Protoplasts were prepared as described by Murray *et al.* (1992), except that mycelium was treated with 35 mg Gluconex, 25 mg bovine serum albumin, 50 mg Driselase, 8 mg Zymicase, and 50 mg beta-D-glucanase in 10 mL of osmotic medium. Protoplast yield per mL averaged 6 x 10^7. The protoplasts were prepared and transformed by electroporation with *Sal*I-linearized pKAES174 (0.5–5.0 x 10^7 protoplasts and 4–6 µg DNA in 70 µL) by the method of Tsai *et al.* (1992). Alternatively, they were transformed with the linearized plasmid by Ca^{2+}/polyethylene glycol treatment (0.5–5.0 x 10^7 protoplasts and 1–2 µg DNA in 100 µL) (Panaccione *et al.* 2001). The treated protoplasts were then mixed with 7 mL of complete regeneration medium (CRM) (Panaccione *et al.* 2001) and poured on top of 20 mL CRM plates containing hygromycin B at 48 µg/mL. The transformation plates were incubated at 21°C for 4–5 weeks. The fungal colonies were then transferred to PDA with hygromycin B (48 µg/mL) for faster growth, sporulation and single spore isolation.

PCR screening for transformants

DNA from single spore isolated transformants was extracted by using DNeasy 96 Plant Kit (Qiagen, Valencia, California). PCR amplification was carried out using several sets of primers specific for *dmaW-2*:

dmaWe19copy2.1d (5’-AGAAACAGACAGGGCTATTC-3’),
dmaWe19copy2.5u (5’-CTCGCCGGCATGCGTCAAAA-3’),
dmaW144.15d (5’-CGAATGTAGATTACAATGGG-3’), and
dmaWcopy2.3u (5’-GCAGTTTGGAGTATCTTTAG-3’),

as well as primers specific for the hygromycin gene:

HYG_HY1 (5’-CGAAGAATCTCGTGCTTCAGCTTCGATGTA-3’), and
HYG_YG1 (5’- CCATTGTCCGTCAGGACATTGTTG-3’).

Oligonucleotide primers were from Integrated DNA Technologies Inc. (Coralville, Iowa). Each 25 µL PCR reaction mixture included 1.25 mM each dNTP, 5 µM each of the two primers, 1X PCR buffer, 2 µL DNA template extract and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, New Jersey). The reaction mixtures were held at 95°C for 9 min, and then subjected to 35 cycles of the following program: 94°C for 30 sec, 61°C for 35 sec, 72°C for 3 min 10 sec.

The plasmid pKAES186, containing a cassette with *cre* and *ble* genes between loxP sites was introduced into the *dmaW*2 knockout mutant. Isolation of the protoplasts from the *dmaW*2 knockout mutant was described above. The protoplasts were transformed by the PEG method (Panaccione *et al.* 2001). Transformants were selected on CRM plates containing 25 µg/mL phleomycin and screened by PCR with primer set dmaWe19copy 2.1d and dmaWe19copy2.5u specific for *dmaW2*, a set of primers specific for the *hph* gene, HYG_HY1 and HYG_YG1 as well as the following primers to detect *ble* and *cre*:

ble seq1 pKAES183: (5'- GAAGCTTCACCTGTCTCTCG-3'),
seq15out ble(u) (5'-CTTCGCATCTGGGCAGATGATG-3'),
cre (d)Cre(-) (5'-TCCAATTTACTGACCGTA-3'), and
(u)Cre-XbaI (5'-CATGTCTAGACTAATCGCCATCTTCCAG CAGGCG-3').

Seedling inoculations

Tall fescue seedlings were inoculated as described by Latch and Christensen (1985). 'Kentucky 31' endophyte free seeds were surface sterilised and germinated on water agar for 4–5 days. The fungal mycelium was grown on PDA cellophane plates. The seedlings were wounded at or near the shoot apical meristem, and fungal mycelium was placed inside the wound. After inoculation, the seedlings were incubated at 21°C for 5 days in the dark and then in the light for 4–6 days. Then the seedlings were planted in soil and placed in the greenhouse. At the three-tiller stage, the seedlings were analysed for the presence of endophyte by tissue-print-immunoblot (TPIB) as described by Gwinn *et al.* (1991). The preparation of *N. coenophialum* antigen and production of polyclonal rabbit antibodies against it are described by An *et al.* (1993).

Results

Homologous recombination in *N. coenophialum* e19

The plasmid pKAES174 contained a 341 bp deletion from the *dmaW*2 coding region (starting from the ATG initiation codon), which was replaced by the *hph* gene flanked by loxP sites. This plasmid was introduced into *N. coenophialum* e19 by electroporation and polyethylene glycol (PEG) transformation. The 1522 transformants obtained were screened by PCR for homologous recombination with *dmaW*2-specific primers. These were designed to amplify two different fragments: a 1.5 kb fragment specific for wild- type and a 2.5 kb fragment from the *dmaW*2 mutant. The presence of only the 2.5 kb band indicated that a recombination event took place and the 2.5 kb fragment replaced the 1.5 wild-type fragment (Fig. 1). In each PCR screen, wild-type *N. coenophialum* was used as a positive control, and the negative control was *N. uncinatum* (an endophyte of meadow fescue), which lacks *dmaW*. Of 1522 transformants obtained and screened, three were likely *dmaW*2 disruptants because they gave no PCR product from the wild-type locus, but yielded the fragment from the disruption construct (Fig. 2). Two of these knockout mutants (*dmaW*2-ko) were designated e7133 and e7134, and further investigated.

Introduction of transformants into tall fescue cultivar Kentucky 31

The *dmaW*2-ko mutants e7133 and e7134, an ectopic transformant, and wild-type *N. coenophialum* e19 were introduced into 'Kentucky 31' seedlings. Seedling infection frequencies for the e7133 and e7134 were higher (67%) than those for ectopic transformants (27%) and for the wild-type (54%), but it is unknown if there was a significant difference between these values.

Figure 1 Map of the *dmaW*2 locus before (A) and after (B) marker-exchange gene disruption, and after subsequent Cre-mediated loss of *hph* (C). A primer set, indicated as two arrows in each panel, was used for PCR-amplification of a 1.5 kb fragment from wt *dmaW*2 (A), a 2.5 kb fragment from *dmaW*2-ko with *loxP-hph-loxP* (B), and a 1.2 kb fragment from *dmaW*2-ko after elimination of *hph* (C).

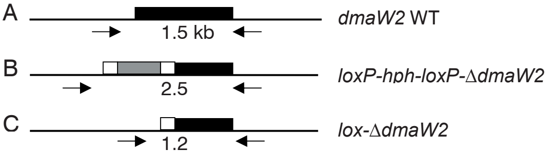


Figure 2 PCR screening for homologous recombinants in which mutated *dmaW*2 substituted for the wild type gene. PCR with primer sets indicated in Fig. 1 amplified a 1.5 kb fragment from wt *dmaW*2 or a 2.5 kb fragment from *dmaW*2-ko with loxP-*hph*-loxP (ko). Nc (*N. coenophialum*) as positive control, Nu *(N. uncinatum*) as negative control, Ec- ectopic transformant M- 1 kb ladder size marker.

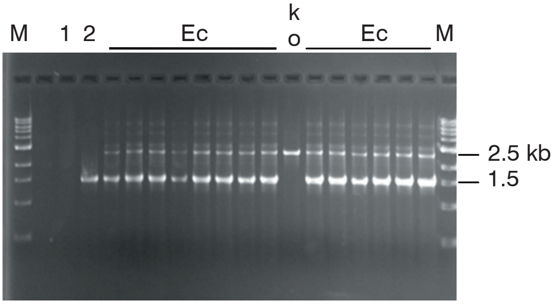
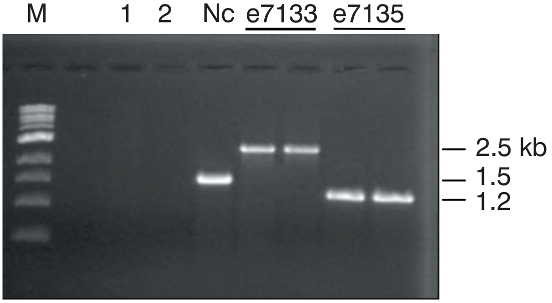


Figure 3 PCR analysis indicating elimination of *hph* by Cre recombinase: A PCR test for *N. coenophialum dmaW*2-ko transformed with pKAES186. The *dmaW*2-ko transformed with pKAES186 (e7135) has lost *hph*, as indicated by the 1.2 kb PCR product. PCR from *N. coenophialum* wt (Nc) DNA amplified a 1.5 kb fragment. PCR from *dmaW*2-ko (e7133) amplified a 2.5 kb fragment. M is 1 kb marker, 1 (*N. uncinatum* DNA) and 2 (water) are the negative controls.



Elimination of *hph* in *dmaW*2-ko

The *dmaW*2-ko construct has *hph* flanked by loxP sites, which are the substrate for the activity of Cre recombinase. The *dmaW*2-ko mutant e7133 was transformed with pKAES186 carrying a cassette containing *ble* and *cre* genes flanked by the loxP sites. The plasmid was designed with the *cre* gene following an inducible promoter (*lolC* from *N. uncinatum*), which should cause *cre* expression *in planta*. Expression of *cre* would determine the excision of sequences located between loxP sites, namely, *hph*, *cre* and *ble*. If the *hph* gene is eliminated by Cre, a PCR test would result in a 1.2 kb PCR fragment, compared to the 2.5 kb fragment from *dmaW*2-ko and 1.5 kb fragment in the wild-type *dmaW*2 (Fig. 1). The transformants were screened by PCR for the construct integration and presence of *cre* and *ble* genes. Additional PCR screening was done to verify the elimination of *hph* with the same set of primers used for *dmaW*2-ko screening as well as the *hph* gene. The preliminary results indicated that *cre* was expressed in culture and excised the *hph* gene with the retention of a loxP site (Fig. 3).

Seedling inoculations

The new transformant designated e7135 was inoculated into tall fescue seedlings as described above. At the three tiller stage, the seedlings were analysed by TPIB to detect the endophyte. Endophyte-infected plants were obtained, but with a lower frequency (20%) than obtained earlier with the *dmaW*2-ko mutants e7133 and e7134.

Discussion

The ergot alkaloids produced by *N. coenophialum* are a major problem linked with fescue toxicosis in livestock. Wild-type strain e19 from tall fescue cv. Kentucky 31 harbours *dmaW*1 and *dmaW*2, two homologous genes for the enzyme catalysing the first ergot-alkaloid biosynthesis step. Our long term goal is to have a modified strain of *N. coenophialum* that does not produce ergot alkaloids and is useful for field purposes. The presence of a foreign gene in the modified *N. coenophialum* could be a public concern. For the elimination of these genes the Cre/loxP site-specific recombination system was used. The function of Cre in *N. coenophialum* ATCC62374 was tested by Machado (2004). The results showed that the system is efficient, but the modified strain lost its compatibility with the plant. One possible explanation for the loss of compatibility might be repeated subculturing in the laboratory. This was not the case with *N. coenophialum* strain e19 used in this study. The strain is less amenable for laboratory work, but the transformation did not affect its compatibility with the plant. The seedling infection frequency for the *dmaW*2-ko mutants was 67%, ectopic transformants gave 27% infection, and for the wild-type we obtained 54% infection. These high values are surprising, compared to the usual frequency (15-25%) that is obtained with routine inoculations of tall fescue with other *N. coenophialum* isolates.

Our preliminary results indicated that the *hph* gene was removed in culture by expression of the Promoter$_{lolC}$-*cre* construct. Additional testing of these mutants, after reisolation from the inoculated plants, will indicate whether or not the other foreign genes (*ble* and *cre*) could also be eliminated.

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