Characterisation of fungal endophytes present in *Elymus canadensis* (Canada wildrye)

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Abstract

Elymus canadensis (Canada wildrye - CWR) is a native perennial cool season bunch grass tolerant to a range of soils, winter hardy and able to grow across the United Sates and as far North as Southern Alaska. Canada wildrye is often used for prairie restoration, conservation and erosion stabilisation. Young CWR plant tissue is palatable and nutritious to grazing animals. CWR has been reported to harbour a sexual endophytic fungus, *Epichloë elymi*, but some accessions have been identified that have not produced stroma. We isolated and characterised the epichloë endophytes from three endophyte-infected CWR accessions collected from Mexico and Texas. We established that the endophytes present in these CWR accessions are of hybrid origin, with *E. elymi* and *E. amarillans* ancestral genomes, and are therefore considered to be asexual isolates. The endophytes were examined for their alkaloid potential, particularly the detrimental ergot alkaloids, with inconclusive results.

Keywords: *Elymus canadensis*, Canada wildrye, hybrid, *Epichloë elymi*, *Epichloë amarillans*

Introduction

Clavicipitaceous fungal endophytes of the genera *Epichloë* and *Neotyphodium* form symbiotic associations with cool season grasses from the subfamily Pooideae. Members of the epichloë family consist of sexual (*Epichloë*) and asexual (*Neotyphodium*) species. Many *Neotyphodium* spp. are of hybrid origin, which is recognisable by the presence of multiple gene copies (Craven *et al.* 2001; Moon *et al.* 1999, 2000, 2004; Schardl & Craven 2003; Schardl *et al.* 1994; Tsai *et al.* 1994).

Elymus canadensis, (Canada wildrye, CWR), is a member of the Triticeae host tribe that is known to harbour a clavicipitaceous fungal endophyte (Bultman & White 1988; White & Bultman 1987; Schardl & Leuchtmann 1999; Vinton *et al.* 2001). Previous studies have indicated that epichloë endophytes identified in *Elymus* species are *Epichloë elymi*, which are of sexual origin and have the ability to form stroma and choke the developing inflorescence (Bultman & White 1988; White & Bultman 1987; Schardl & Leuchtmann 1999). In a study of endophyte-infected CWR prairie grasses, stroma formation did not occur. However, the endophytes were not subjected to phylogenetic analysis (Vinton *et al.* 2001).

Many epichloë endophytes provide bioprotection to their hosts by producing alkaloids that have anti-insect and anti-mammalian properties. More recently, the genes required for the biosynthesis of the alkaloids known as peramine, lolines, indole-diterpenes and ergot alkaloids have been sequenced (Damrongkool *et al.* 2005; Spiering *et al.* 2005; Tanaka *et al.* 2005; Wang *et al.* 2004; Young *et al.* 2006). However, *E. elymi* is only known to produce the insect feeding deterrent peramine (Clay & Schardl 2002; Schardl & Leuchtmann 1999; Siegel *et al.* 1990).

In this study, CWR accessions were collected from Texas (NFCWR1 and NFCWR3), and Mexico (NFCWR2). These *Elymus* species were endophyte-infected but stroma did not form during cultivation. The objective of this research was to isolate the endophytes present in the NFCWR accessions and characterise them based on their phylogenetic origin and toxin potential.

Methods

Culture growth

Fungal isolates (Table 1) were isolated directly from endophyte-infected pseudostems (Moon *et al*. 2002). Cultures were maintained on Potato dextrose (PD) agar at 22°C.

Molecular biology

Genomic DNA was isolated from ~0.5 mm of tissue from the base of individual plant pseudostems of unknown endophyte status. The samples were placed in a 1.2 mL collection tube in 96 well format containing a 5 mm steel BB then freeze-dried overnight. The samples were ground in liquid nitrogen using a TissueLyser (Qiagen). The genomic DNA was extracted using the Magattract 96 DNA plant core kit (Qiagen) as per the manufacturer's instructions. Genomic DNA from pure endophyte cultures was isolated using the DNeasy Plant mini kit (Qiagen) according to the manufacturer's instructions.

PCR was used to detect endophyte genomic DNA sequences within total DNA extracted from grass samples. The PCR was based on primers that anneal to the endophyte translation elongation factor 1-α (*tef1*) (tef1-exon1d-1, GGGTAAGGACGAAAAGACTCA and tef1-exon4u-1, CGGCAGCGATAATCAGGATAG) (Craven *et al.* 2001). Phylogenetic analysis was performed using primers designed to the ITS region (ITS4, TCCTCCGCTTATTGATATGC and ITS5, GGAAGTAAAAGTCGTAACAAGG), *tef1* and *tub2* (T1.1, GAGAAAATGCGTGAGATTGT and T1.2, CTGGTCAACCAGCTCAGCAC) genes. PCR reactions were performed in 25 or 50 µL reaction volumes containing 5-100 ng of DNA, 1x green reaction buffer (Promega), 200 µM each dNTPs, 200 nM of each primer, 1 U Go Taq (Promega). The PCR programme consisted of 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 1 min. The PCR products were separated on 2% agarose in 1x TBE (Invitrogen), stained with ethidium bromide and visualised under UV light.

PCR products were cloned directly into pGEM-Teasy (Promega) and used to transform *E. coli* XL1 blue cells. Plasmid DNA was isolated from twelve independent colonies per amplified gene using QIAprep spin miniprep kit (Qiagen) and sequenced. PCR fragments were directly sequenced after purification with the QIAquick PCR purification kit (Qiagen). The sequence data were edited using Sequencher 4.6 (Gene Codes). Phylogenetic analysis was performed as per Craven *et al*. 2001 using maximum parsimony that utilised the branch and bound search in PAUP (Swofford 1998).

Ergot alkaloid analysis

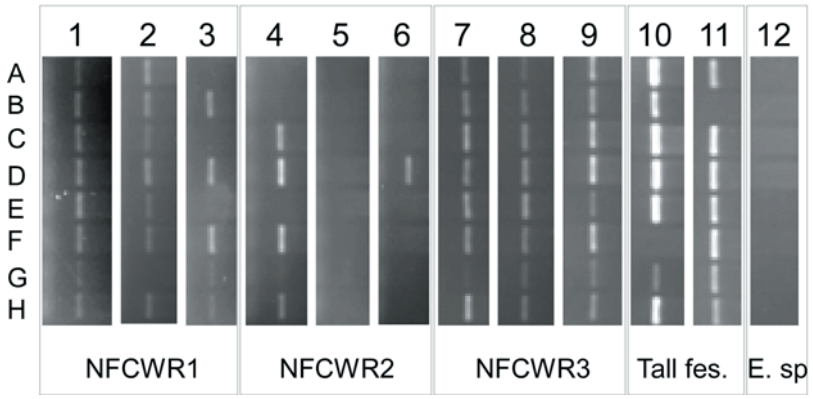
The presence of ergot alkaloids in endophyte-infected plant material was analysed using the Phytoscreen PT ergot alkaloid kit, a competitive based ELISA (Agrinostics Ltd, Watkinsville, Ga). Freeze dried plant material (100 mg) was extracted and mixed gently for 3 h. The extract was allowed to settle overnight

Table 1 Biological cultures

Strain name	Species	Host plant	Location
NFE1000	EcaTG-1	*Elymus canadensis*[1]	Texas
NFE1001	EcaTG-1	*E. canadensis*[1]	Mexico
NFE1002	EcaTG-1	*E. canadensis*[1]	Texas
EC1	*Epichloë elymi*	*E. canadensis*	Oklahoma
EC4	*E. elymi*	*E. canadensis*	Oklahoma

[1] NFCWR plants

Figure 1 High throughput analysis of grasses for endophyte infection.
A PCR screen for endophyte detection in CWR plants. PCR was performed with the Tef primers. The samples have been arranged to represent the format (96 well plate) of DNA isolation. Columns 1-3 contain samples infected with NFE1000, 4-6 with NFE1001, 7-9 with NFE1002, 10-11 endophyte-infected tall fescue, 12 Elymus species; A12, *E. pendulinus*; B12, *E. nevskii*; C12, *E. breviaristatus*; D12, *E. sibiricus*; E12, *E. mutabilis*; F12, *E. lanceolatus*; G12, *E. trachycaulus*; H12, *E. wawawaiensis*.



at 4°C. A 1 mL sample was removed and centrifuged at 8000 rpm for 3 min. A 50 µL aliquot was assayed for ergot alkaloids according to the manufacturer's instructions.

Results and Discussion

Identification of endophyte-infected Canada wildrye

Seventy two plants from three NFCWR accessions were screened for endophyte infection. Since the narrow stems of the CWR plants were not suitable for analysis with an endophyte specific immunoblot, the plants were screened for endophyte infection using aniline blue staining of leaf peels and PCR with primers specific to epichloë endophytes. The fragile nature of the CWR stems made leaf peels very labour intensive and difficult to obtain consistently. However, PCR was successful in detecting the presence of epichloë specific sequences from total DNA extracted from the base of CWR pseudostems (Fig. 1). PCR of the three CWR accessions indicated endophyte infection rates of 83%, 21% and 96% (Fig. 1). Other *Elymus* species and tall fescue were screened for endophyte infection which indicated 0% and 88% infection respectively (Fig. 1). Seed storage prior to germination may have resulted in the loss of endophyte infection of the other *Elymus* species.

The endophytes, NFE1000, NFE1001 and NFE1002 (Table 1), were isolated from the NFCWR stems and maintained in culture. The NFE culture morphology is white and cottony and the isolates did not produce conidia on PD agar. However, water agar is more conducive to producing conidia.

Two sexual epichloë endophytes were isolated from stroma present on choked CWR plants from Oklahoma, USA. The ITS sequence (DQ899096) revealed the cultures were *E. elymi*, a sexual isolate known to be present in *Elymus canadensis*. The *E. elymi* culture morphology is also white and cottony but the fungus grows faster than the NFE isolates.

Phylogenetic analysis

PCR and sequence analysis of the ITS region revealed that the NFE isolates were more similar to *E. amarillans* than *E. elymi*. While the ITS sequence analysis is informative, only one progenitor copy is maintained after interspecific hybridisation (Ganley & Scott 2002) and therefore ITS phylogenetic analysis is used in combination with other sequence analysis. PCR and sequence analysis of *tub2* and *tef1* genes revealed that the NFE isolates contained multiple alleles. This indicates the endophyte is of hybrid origin. The CWR choke isolates contained one copy of *tub2* and *tef1* and BLASTN analysis supported the identification of these isolates as *E. elymi*.

The PCR bands from the NFE isolates were cloned into pGEM-Teasy to sequence individual fragments. The *tub2* and *tef1* sequences from the NFE isolates formed two distinct copies where each gene copy was identical between each isolate, apart from one nucleotide difference in NFE1001 *tub2* copy 1. BLASTN analysis of each *tef1* and *tub2* allele indicated that the likely ancestral genomes of the NFE isolates were *E. elymi* and *E. amarillans*. Maximum parsimony trees for the

tub2 and *tef1* genes (data not shown) grouped the NFE isolates with *E. amarillans* (copy 1) and *E. elymi* (copy 2). These data indicate that the NFE endophytes are the result of interspecific hybridisations between *E. amarillans* and *E. elymi* and would be considered asexual and unable to form stroma on host plants. *Hordeum bogdanii* was recently shown to contain an endophyte with a very similar hybridisation event (Moon *et al.* 2004).

Ergot alkaloid analysis

Endophyte-infected CWR plants were analysed for ergot alkaloids using a competitive ELISA that detects the ergoline ring moiety common to ergot alkaloids. Two endophyte-infected plants from each NFE group were tested. The analysis of the CWR plants showed that the ergot alkaloid levels of five plants were very low, ranging from 1.4 – 66 ppb, but one plant resulted in 477 ppb. This initial screen for ergot alkaloids was inconclusive as one plant had similar levels to that of Kentucky 31 endophyte-infected grass (365 – <2000 ppb ergot alkaloids) while the remaining plants would be considered close to the lowest level of detection. Additional sampling and HPLC analysis will confirm the ergot alkaloid status of these plants.

Many epichloë endophytes have been screened for the production of bioprotective alkaloids (Christensen *et al.* 1993; Clay & Schardl 2002; Siegel *et al.* 1990; Spiering *et al.* 2002). Such data indicate that only *E. festucae* is capable of producing all four classes of alkaloids, but no one isolate has been identified that produces all four alkaloids at once (Clay & Schardl 2002; Siegel *et al.* 1990). *E. elymi* has been reported to produce the anti-insect alkaloid, peramine, but has not been shown to produce ergot alkaloids, lolines and indole-diterpenes. Some *E. amarillans* have the ability to produce ergovaline and peramine but are not known to produce lolines and indole-diterpenes. Based on this information, it is unlikely that a hybrid endophyte consisting of *E. elymi*/*E. amarillans* ancestral parents would have the capability to produce indole-diterpenes or lolines but they would be highly likely to produce peramine. The NFE isolates could have the potential to produce ergot alkaloids if this capability was present in the ancestral *E. amarillans* parent. The recent sequencing of the biosynthetic genes required for the production of peramine (Tanaka *et al.* 2005), lolines (Spiering *et al.* 2005), indole-diterpenes (Young *et al.* 2006) and ergot alkaloids (Damrongkool *et al.* 2005; Wang *et al.* 2004,) will make PCR screening a quicker and simpler option for assessing the alkaloid potential of all epichloë endophytes. However, it is still important that the PCR data are supported by alkaloid analysis.

The identification and alkaloid characterisation of asexual endophytes present in CWR will enable us to utilise these endophytes within the CWR breeding programme in a similar vein to the work that has been achieved with tall fescue and novel endophytes (Bouton *et al*. 2002).

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