The role of cAMP signalling in the symbiosis between *Epichloë festucae* and *Lolium perenne*

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Abstract

In fungal pathogenesis the cAMP signalling cascade is usually essential for virulence. Deletion of the adenylate cyclase gene, the enzyme that synthesises cAMP, often results in an attenuated or avirulent phenotype. Our aim was to identify the signalling mechanisms regulating colonisation of perennial ryegrass (*Lolium perenne*) by the fungal symbiont *Epichloë festucae* Fl1. We have identified genes from several signalling networks, and here report on the outcomes of targeted disruption of the *E. festucae* Fl1 adenylate cyclase gene (*acyA*). A dual genome (endophyte/ryegrass) Affymetrix GeneChip® has been synthesised and we are undertaking large scale transcript profiling of the *L. perenne*/*E. festucae* Δ*acyA* symbiotum to identify target genes regulated by the endophyte cAMP signalling network.

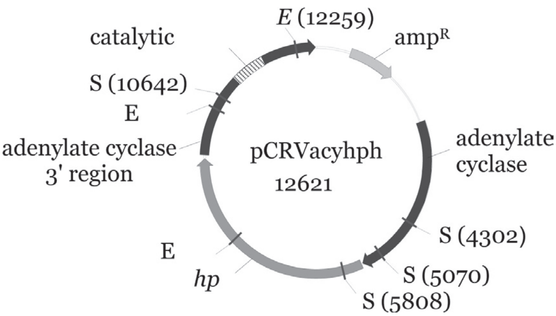
Keywords: cAMP, adenylate cyclase, *acyA*, *Neotyphodium lolii*, *Epichloë festucae*, symbiosis, Affymetrix GeneChip®

Introduction

Signalling pathways facilitate transduction of stimuli from the exterior of cells to the interior, enabling the organism to respond to environmental and biological stimuli by altering growth, differentiation or the production of secondary metabolites. Adenosine 3´5´- cyclic AMP (cAMP) is an integral component of signalling cascades in most organisms. Levels of cAMP are modulated by the activity of two enzymes, adenylate cyclase and phosphodiesterase, responsible for the synthesis and degradation of cAMP respectively. The most well characterised target of cAMP is cAMP-dependent protein kinase, which mediates many of the physiological effects of cAMP in fungi (D'Souza & Heitman 2001) by phosphorylating target proteins such as protein kinases, ion channels and transcription factors. In eukaryotes, cycles of phosphorylation and dephosphorylation are a major mechanism by which cellular pathways are activated or deactivated. In fungi, the cAMP signalling pathway has been shown to play a role in several critical processes in pathogenesis, and deletion of the adenylate cyclase gene results in an attenuated (Klimpel *et al.* 2002) or avirulent phenotype (Brakhage & Liebmann 2005; Choi & Dean 1997; D'Souza & Heitman 2001).

The molecular mechanisms by which fungal endophytes, *Epichloë* and *Neotyphodium* colonise grasses are still largely unknown. In these symbioses, endophytic hyphae adhere to plant cell walls in the intercellular spaces of the host, hyphal growth is coordinated with leaf growth (Tan *et al.* 2001), and the production by endophytes of secondary metabolites within the host is regulated by both environmental and plant factors (Rowan 1993; Spiering *et al.* 2005). We have identified genes from several signalling networks, and here report on the disruption of adenylate cyclase, and the impacts of depleted cAMP on the symbiosis between *E. festucae* Fl1 and *L. perenne.*

Figure 1 Map of *acyA* disruption vector pCRVacyhph. The *acyA* disruption plasmid contains the hygromycin resistance gene (*hph*) flanked by 3kb border sequences from *acyA*. The *hph* gene is controlled by the gpdA promoter and TrpC terminator from *Aspergillus nidulans*.



Material and Methods

Fungi, plants and growing conditions

Fungi were grown in culture on potato dextrose agar (PDA) at 22°C. *L. perenne* cv. Nui plants infected with wild type (w.t.) or hygromycin-resistant *E. festucae* Fl1 transformants were maintained in glasshouse conditions as described (Voisey *et al.* 2007). For production of conidia *E. festucae* isolates were transferred to water agar and incubated at 22°C for 28 days.

Cloning and disruption of the adenylate cyclase gene

A PCR fragment of the *N. lolii* Lp19 adenylate cyclase gene (*acyA*) was cloned from DNA amplified using degenerate primers with homology to the adenylation domain of non-ribosomal peptide synthetases (Johnson *et al.* 2007b). The 300bp sequence was labelled with digoxigenin (DIG, Roche) and used as a probe to identify homologous clones from a lambda library (Fleetwood *et al.* 2007) of *N. lolii* Lp19 genomic DNA. A single homologous clone was recovered and the DNA insert sequenced. BLASTX analysis indicated that the entire 6057bp insert comprised most of the adenylate cyclase gene barring the first 750bp of the open reading frame (ORF). Alignment of the gene with the adenylate cyclase genes of other fungi revealed a high degree of sequence conservation with adenylate cyclases from *Metarhizium anisopliae* and *Gibberella zeae*. Analysis of the conceptual adenylate cyclase protein confirmed that key motifs consistent with fungal Class III adenylate cyclase genes were present, including the adenylate cyclase catalytic core, the protein phosphatase 2C box1 and box2 residues, and the Ras-associated domain.

To disrupt *acyA*, the hygromycin resistance gene (*hph*) was inserted upstream of the putative catalytic domain of the adenylate cyclase gene. A gene replacement vector, pCRVacyhph (Fig. 1), was constructed using the Gateway multi-site system (Invitrogen). The *hph* cassette from pAN7 (Punt *et al.* 1987) was flanked either side with 3kb of DNA with homology to *acyA* using the procedure described in Fleetwood *et al.* (2007) . Gene disruption experiments were performed in *E. festucae* strain Fl1 (originally an isolate from *Festuca longifolia*). Homologous recombinants of *E. festucae* Fl1 were obtained by PEG-mediated transformation of protoplasts. Protoplasts were prepared using the method of Young *et al.* (1998), except that 10 mg/ml of Glucanex (Interspex) was used to digest the cell walls for 3 hours at 30°C with shaking (100 rpm). *E. festucae* strain Fl1 was transformed using 5 µg of each plasmid by the method of Vollmer & Yanofsky (1986) with modifications (Itoh *et al.* 1994). Transgenic colonies were selected on PDA containing 150µgmL^{-1} of hygromycin B.

DNA isolation and analysis of *E. festucae* transformants

DNA was isolated from *E. festucae* transformants using the methods of Yoder (1988) and Al-Samarrai and Schmid (2000). PCR reaction and cycling conditions were conducted according to manufacturer's instructions (Invitrogen).

Characterisation of *E. festucae* isolates growing in *L. perenne*

Epichloë endophytes were artificially inoculated into *L. perenne* seedlings according to the method of Latch & Christensen (1985). The phenotype of *E. festucae* Fl1Δ*acyA* mutants in *L. perenne* cv. Nui was determined by straining epidermal peels from infected leaf sheath material with 0.15% (w/v) aniline blue as described by Christensen *et al.* (2002).

Transcript Profiling using an *N. lolii*/ *L. perenne* Affymetrix GeneChip®

A NimbleExpress Affymetrix GeneChip® was developed to compare the transcript profiles of *E. festucae* Fl1Δ*acyA*42 with the wild type strain. The majority of the sequences tiled on the microarray originated from EST libraries from *N. lolii* Lp19 and and *L. perenne*. Development of the GeneChip® is described in detail in Voisey *et al.* (2007). *L. perenne* plants infected with wild type or *E. festucae* Fl1Δ*acyA*42 were grown in triplicate in climate controlled conditions, and the pseudostem harvested for extraction of RNA as described (Johnson *et al.* 2007a). Analysis of RNA quality, labelling, chip hybridisations, washes and GeneChip® scanning followed recommended procedures and were conducted at the Centre for Genomics and Proteomics, School of Biological Sciences, University of Auckland, New Zealand. Microarray data were processed as described in Voisey *et al.* (2007).

Results and Discussion

Disruption of the *acyA* gene in *E. festucae* Fl1

The *acyA* gene was deleted in *E. festucae* Fl1 as this species is closely related and more amenable to transformation than *N. lolii*. The vector pCRVacyhph was used to insert the hygromycin resistance cassette upstream of the catalytic domain of the *acyA* gene. Approximately 50 colonies were assessed by PCR for the *hph* insertion event. Three colonies with the expected gene replacement, Δ*acyA*34, Δ*acyA*42 and Δ*acyA*47, were identified and confirmed by Southern analysis (data not presented). Two further colonies, *acyA*19 and *acyA*49, with an intact *acyA* gene plus in ectopic insertion of the gene replacement vector were identified for use as transformation controls.

Phenotypic characterisation of *E. festucae* Fl1Δ*acyA* in culture

Saprophytic growth of *E. festucae* Fl1Δ*acyA* strains in culture was substantially reduced when compared with the wild type or strains with an ectopic insertion of the *hph* cassette (data not shown). The growth rate of *E. festucae* Fl1Δ*acyA*42 could be fully complemented by addition of 7.5mM cAMP to the media, indicating that cAMP depletion is the cause of the slower growth rate (data not shown). The wild type and ectopic strains (*acyA*19, *acyA*49) did not change in radial colony diameter in response to cAMP supplementation.

In addition, disruption of *acyA* substantially increased the production of conidia in culture by *E. festucae* FL1 when compared with the wild-type or isolates with ectopic integrations of the gene disruption vector. This data suggested that the cAMP signalling pathway negatively regulates production of conidia in *E. festucae*, which contrasts with other fungi such as *Aspergillus fumigatus* (Liebmann *et al.* 2003), *A. nidulans* (Fillinger *et al.* 2002) and *Magnaporthe grisea* (Choi & Dean 1997), where disruption of adenylate cyclase reduced the number of conidia.

Phenotype of mutant endophyte strains growing *in planta*

To evaluate the role of cAMP signalling during infection of plants by epichloë endophytes, mycelia of isolates Δ*acyA*34, Δ*acyA*42 and Δ*acyA*47 were artificially inoculated into cut seedlings of *L. perenne*. Both Δ*acyA*34 and Δ*acyA*42 were capable of colonising *L. perenne*, although the success rate (in terms of the number of plants colonised) was much lower than the wild type (data not shown). This suggests that although not essential for infectivity, cAMP may regulate processes that increase the efficacy of the infection process. Conversely, strain Δ*acyA*47 was not infectious. The reason for this has not yet been determined. The visual phenotype of *L. perenne* infected with strains Δ*acyA*34 and Δ*acyA*42 was identical to those infected with wild type *E. festucae*, however the plants were heavily colonised in comparison with plants infected by the wild type strain. Hyphae of Δ*acyA*42 and 34 had a hyper-branching phenotype consistent with continued development of lateral branches after host growth had ceased. Together these data suggest that cAMP signalling is not essential for infectious growth of endophytes in ryegrass, rather is involved in the regulation of hyphal growth in plants. Further work is required to confirm that cAMP levels have been reduced in endophytes as a result of *acyA* disruption, and that the phenotype of the Δ*acyA* strains can be restored to the wild type by ectopic integration of the wild type *acyA* gene, thus confirming that the phenotype observed is due to *acyA* disruption.

Effects of *acyA* disruption on expression of plant and endophyte genes during symbiosis

Large-scale transcript profiling experiments using the *N. lolii*/ *L. perenne* GeneChip® have been conducted to identify genes that are regulated by cAMP during symbiosis (Voisey *et al*., 2007). We have compared the expression profiles of endophyte and plant genes in pseudostem material infected with wild type *E. festucae* with those of plants infected with the mutant endophyte strain, Δ*acyA*42 (Voisey *et al*. 2007). The data for each species (endophyte and plant) have been normalised independently using Robust Multi-array Average (RMA , Irizarry *et al.* 2003) to account for differences in fungal biomass between plants. Data analysis to identify differentially-expressed genes is currently in progress. Preliminary results indicate that many genes were either up or down-regulated in response to disruption of *acyA* in endophyte-infected plants. In particular a number of endophytic proteases are down-regulated in Δ*acyA*42 in comparison with the wild type association suggesting that proteases are positively regulated by the cAMP signalling network in wild type associations between *E. festucae* Fl1 and *L. perenne*. The majority of differentially–expressed genes in Δ*acyA*42 are of unknown function, and functional annotation of these sequences and their role in symbiosis will be a priority for this team in the future.

ACKNOWLEDGEMENTS

Thanks are due to Catherine Tootill for technical assistance (Grasslands Research Centre, Palmerston North, AgResearch). We would like to acknowledge Liam Williams at the University of Auckland Microarray Facility at the Centre for Genomics and Proteomics (New Zealand) for analysis of RNA quality, labelling of cRNA, and GeneChip® hybridisation and scanning. This research was funded by the New Zealand Foundation for Research Science and Technology (C10X0203) and AgResearch AR&C project 5598/1.

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