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Characterisation of novel secondary metabolism
genes in plant-endophytic *Epichloë* fungi

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ABSTRACT

Plant-endophytic fungi of the genus *Epichloë* are symbionts of cool season grasses. *Epichloë* infections are generally asymptomatic, with endophyte and host growth synchronised to form an interconnected hyphal network in the intercellular spaces separating host cells. In return for the sustenance received from the host apoplast, *Epichloë* spp. produce a range of bioprotective secondary metabolites (SM) that deter both vertebrate and invertebrate herbivores. Peramine is an *Epichloë*-derived insect-deterring SM produced by the two-module non-ribosomal peptide synthetase (NRPS) PerA, encoded by the gene *perA*. The *perA* gene is widespread across *Epichloë*, but peramine production is much more discontinuous. Through an *Epichloë*-wide survey it is shown that the peramine chemotype of *Epichloë* isolates can be accurately predicted through a combination of PCR and sequencing methods. Furthermore, the protein encoded by the widespread peramine-negative *perA-ΔR* allele is analysed in detail. The *perA-ΔR* allele has a transposable element inserted into the 3' end of the gene, and was previously assumed to be non-functional. Evidence for ongoing *perA-ΔR* functionality is presented, with the PerA-ΔR protein shown to exhibit altered substrate specificity relative to PerA. A PerA/PerA-ΔR domain swap is shown to restore peramine production to PerA-ΔR, providing insight into the functional changes that separate this protein from PerA.

Another major project investigated the role SM genes may play in *Epichloë* sexual development. Many *Epichloë* spp. are transmitted vertically through infection of the host seed. However, sexual *Epichloë* spp. may also undergo a reproductive cycle that involves formation of pre-sexual stromata structures over developing host inflorescences. This sexual cycle culminates in horizontal transfer of the endophyte to endophyte-free host plants. Deletion of the symbiotically regulated endophyte gene *irlA* induced a symbiosis-defective phenotype *in planta*, and this observation led to the identification of a novel *Epichloë* SM cluster. Synteny analysis and comparison to previously characterised fungal SM gene clusters defines a five-gene cluster centred on *irlA* that is shown to be upregulated in stromata. A model is

proposed whereby this SM cluster controls initiation of the proliferative hyphal growth associated with stromata formation.

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COMMON ABBREVIATIONS

| | |
|------------------|--------------------------------------------------------------------------------------|
| 10AA | The 10 amino acid A-domain substrate specificity-conferring code |
| 4'PP | 4'-phosphopantetheine |
| 4'PPTase | 4'-Phosphopantetheinyl transferase |
| A-domain | NRPS AMPylation domain |
| aa | amino acid(s) |
| ABI | Applied Biosystems Inc. |
| AFR | Adenylate-forming reductase |
| Amp | Ampicillin |
| AMP | Adenosine monophosphate |
| Amp ^R | Ampicillin resistance |
| ATP | Adenosine triphosphate |
| BBE | Berberine bridge enzyme |
| BLAST | Basic local alignment search tool |
| BLASTn | BLAST search against a nucleotide sequence database with a nucleotide sequence query |
| BLASTp | BLAST search against a protein sequence database with a protein sequence query |
| bp | Base pairs |
| C-domain | NRPS condensation domain |
| cDNA | Complementary DNA |
| CDS | Coding sequence |
| CDYE | Czapek Dox yeast extract |
| CLSM | Confocal laser-scanning (fluorescence) microscopy |
| CRISPR | Clustered regularly-interspaced short palindromic repeats |
| DEPC | Diethylpyrocarbonate |
| DIG | Digoxigenin |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | deoxyribose nucleoside triphosphate |

| | |
|-----------------------|--------------------------------------------------------------|
| E ⁻ | Not infected by endophyte |
| EAS | Ergot alkaloid synthesis |
| EDTA | Ethylene diamine tetra-acetic acid |
| eGFP | Enhanced green fluorescent protein |
| FAD | Flavin adenine dinucleotide |
| g | Acceleration due to gravity on Earth (9.8 m s ²) |
| gDNA | Genomic DNA |
| Gen | Geneticin |
| Gen ^R | Geneticin resistance |
| GFP | Green fluorescent protein |
| h | Hour(s) |
| <i>hph</i> | Hygromycin resistance-conferring gene |
| Hyg | Hygromycin |
| Hyg ^R | Hygromycin resistance |
| IDT | Indole-diterpene |
| IFR | Isoflavone reductase |
| IRL | Isoflavone reductase-like |
| kb | kilo base pairs |
| LB | Lysogeny broth |
| LCMS | Liquid chromatography-mass spectrometry |
| LOL | Loline |
| M | Moles per liter |
| M-domain | NRPS methylation or methyltransferase domain |
| min | Minute(s) |
| mRNA | Messenger RNA |
| MS or MS ¹ | Mass spectrometry |
| MS ² | Tandem mass spectrometry |
| NAD | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCM | Nitrocellulose membrane |
| <i>nptII</i> | Geneticin resistance-conferring gene |

| | |
|-----------------|------------------------------------------------------------------------------------------------------------------------------|
| NRP | Non-ribosomal peptide |
| NRPS | Non-ribosomal peptide synthetase |
| P5C | 1-pyrroline-5-carboxylate |
| PAMP | Pathogen-associated molecular pattern |
| PAX | Paxilline |
| PCP | Peptide carrier protein |
| PCR | Polymerase chain reaction |
| PD | Potato dextrose |
| PEG | Polyethylene glycol |
| PER | Peramine |
| PIP | Pinoresinol-lariciresinol reductase/Isoflavone reductase/ Phenylcoumaran benzylic ether reductase family proteins |
| PKS | Polyketide synthetase |
| PLP | Pyridoxal phosphate |
| PP _i | Phyrophosphate |
| qPCR | Quantitative polymerase chain reaction |
| R-domain | Reductase domain |
| RNA | Ribonucleic acid |
| RNA-seq | RNA sequencing |
| RNase | Ribonuclease |
| RPM | Revolution per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |
| s | Second(s) |
| SAM | S-adenosylmethionine |
| SDS | Sodium dodecyl sulfate |
| SM | Secondary metabolism OR secondary metabolite |
| SNP | Single nucleotide polymorphism |
| T-domain | NRPS thiolation domain (aka PCP domain) |
| TBE | Tris-boric acid-EDTA |

| | |
|-----------|----------------------------------------------------------------------------------------------|
| tBLASTn | BLAST search against a translated nucleotide sequence database with a protein sequence query |
| TE | Trace elements (or thioesterase; context dependent) |
| TE-domain | NRPS type I thioesterase domain |
| TEII | Type II thioesterase |
| TF | Transcription factor |
| TM | Trans-membrane |
| TSP | Trans-species polymorphism |
| v/v | Volume:volume ratio |
| w/v | Weight:volume ratio |
| WGA | Wheat germ agglutinin |
| wt | Wild type |