Molecular Genetics and Genomics (2019) 294:315–328
https://doi.org/10.1007/s00438-018-1510-x

ORIGINAL ARTICLE

# Genetic modification of asexual *Epichloë* endophytes with the *perA* gene for peramine biosynthesis

**Inoka K. Hettiarachchige[1] · Aaron C. Elkins[1] · Priyanka Reddy[1] · Ross C. Mann[1] · Kathryn M. Guthridge[1] · Timothy I. Sawbridge[1,2] · John W. Forster[1,2] · German C. Spangenberg[1,2]**

Received: 5 April 2017 / Accepted: 8 November 2018 / Published online: 15 November 2018


## Abstract

Development of grass–endophyte associations with minimal or no detrimental effects in combination with beneficial characteristics is important for pastoral agriculture. The feasibility of enhancing production of an endophyte-derived beneficial alkaloid through introduction of an additional gene copy was assessed in a proof-of-concept study. Sexual and asexual *Epichloë* species that form symbiotic associations with cool-season grasses of the Poaceae sub-family Pooideae produce bioactive alkaloids that confer resistance to herbivory by a number of organisms. Of these, peramine is thought to be crucial for protection of perennial ryegrass (*Lolium perenne* L.) from the Argentinian stem weevil, an economically important exotic pest in New Zealand, contributing significantly to pasture persistence. A single gene (*perA*) has been identified as solely responsible for peramine biosynthesis and is distributed widely across *Epichloë* taxa. In the present study, a functional copy of the *perA* gene was introduced into three recipient endophyte genomes by *Agrobacterium tumefaciens*-mediated transformation. The target strains included some that do not produce peramine, and others containing different *perA* gene copies. Mitotically stable transformants generated from all three endophyte strains were able to produce peramine in culture and in planta at variable levels. In summary, this study provides an insight into the potential for artificial combinations of alkaloid biosynthesis in a single endophyte strain through transgenesis, as well as the possibility of using novel genome editing techniques to edit the *perA* gene of non-peramine producing strains.

**Keywords** Pasture grass · *Epichloë* · Peramine · Transformation · Metabolic profiling

## Introduction

Cool-season grasses of the Poaceae sub-family Pooideae, including the important temperate forage grasses, tall fescue, and perennial ryegrass, are often infected with clavicipitaceous fungal endophytes that include sexual and asexual *Epichloë* species (Felitti et al. 2006; Posselt et al. 2006; Card et al. 2014). The associations between asexual *Epichloë* species and their host grasses are generally considered to be mutualistic, due to provision of benefits to both partners (Clay 1988). The plant provides certain benefits to the endophyte such as shelter, nutrition, reproduction, and distribution (Scott and Schardl 1993; West 1994; Schardl et al. 2004). The resident endophyte relies entirely on the host plant for dissemination through either seed or growth of vegetative structures (Philipson and Christey 1986; Schardl et al. 2004). Conversely, as part of the mutualistic association, the presence of endophyte provides several advantages to the host plant (Khan et al. 2010). Benefits for the host related to abiotic stress tolerance are obtained through enhanced growth, increased seedling vigour, persistence, particularly under water stress and nutrient deficiency (Ravel et al. 1997; Malinowski and Belesky 1999). The host plant is able to tolerate a broad range of environmental conditions, due to growth advantages provided by the endophyte, which contributes significantly to ecological success (Easton 1999). The endophyte also confers biotic stress tolerance to the host grass, through production of several classes of biologically active alkaloids that are toxic to both arthropod and

Communicated by S. Hohmann.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00438-018-1510-x) contains supplementary material, which is available to authorized users.

✉ German C. Spangenberg
german.spangenberg@ecodev.vic.gov.au

Extended author information available on the last page of the article

mammalian herbivores. Production of secondary metabolites also enhances the resistance of ryegrass to nematodes and some fungal pathogens (Kimmons et al. 1990; Gwinn and Gavin 1992; Schardl and Phillips 1997; Johnson et al. 2003). Of the alkaloids produced by *Epichloë* endophytes, the ergot alkaloids and indole diterpenes are active against vertebrate and invertebrate herbivores (Leuchtmann et al. 2000; Schardl et al. 2013a). Epoxy-janthitrems are tremorgenic mycotoxins, but are much less toxic to grazing vertebrates than lolitrem B, and may also exhibit anti-invertebrate activity (Philippe 2016). In contrast, peramine and lolines are highly active specifically against insect pests but have not been correlated with any toxic symptoms in grazing mammals (Schardl et al. 2013b).

A considerable amount of recent research has been performed on the selection of *Epichloë* endophytes that produce minimal detrimental effects on livestock, while retaining the ability to produce alkaloids that provide adequate control of insects and nematodes (Philippe 2016). For example, novel fungal endophyte strains that lack or produce very low concentrations of lolitrem B and ergovaline such as AR1 and NEA2 (both of which produce peramine as the major alkaloid) have been used to infect perennial ryegrass and have been subjected to commercialisation (Rasmussen et al. 2009). Introduction of peramine biosynthesis into non-peramine-producing strains as well as increased levels in peramine-producing strains would be anticipated to further increase resistance to invertebrate pests and provide corresponding benefits to agriculture.

Peramine was originally identified in extracts of perennial ryegrass which had formed associations with *Epichloë festucae* var. *lolii*, and provides potent protection against herbivorous insect pests such as the Argentinian stem weevil (*Listronotus bonariensis*), an economically important exotic pest of perennial ryegrass in New Zealand (Bush et al. 1997; Schardl et al. 2012, 2013b). Peramine deters feeding of both adults and larvae at concentrations of 0.1 µg/g and 10 µg/g, respectively (Rowan et al. 1990). Furthermore, deterrence of feeding by peramine has the ability to significantly enhance the competitiveness of endophyte-positive ($E^+$) grasses (Schardl et al. 2004). Other possible beneficial properties of peramine production include allelopathy, and inhibition of pathogens or seed predators (Faeth et al. 2002).

A single, multifunctional fungal gene, designated *perA*, is required for peramine biosynthesis (Tanaka et al. 2005). Although *perA* sequences are broadly distributed across both sexual and asexual *Epichloë* species, not all strains share the ability to synthesise peramine in planta (Scott et al. 2007). Presence of an intact copy of *perA* is directly correlated with the capacity to synthesise peramine (Schardl et al. 2013b). DNA sequence analysis has identified a large-scale deletion in the 3′-terminal region of the gene in some endophyte strains which are unable to produce the metabolite (Scott et al. 2007). The presence of miniature inverted repeat transposable elements (MITEs) has been associated with such deletion events (Fleetwood et al. 2011). Of four distinct taxa that colonise perennial ryegrass, peramine-producing strains that belong to *E. festucae* var. *lolii* (*Lp*TG-1; *Lolium perenne* taxonomic group) and *Lp*TG-2 possess intact *perA* genes, while non-peramine-producing strains (belonging to *Lp*TG-3 and *Lp*TG-4) are characterised by partial deletions and premature stop codons (Hettiarachchige et al. 2015). The complete *perA* gene encodes two adenylation (A1 and A2) domains, two thiolation (T1 and T2) domains, a methylation (M) domain, a condensation (C) domain, and a reductase (R) domain, which may be sufficient to produce peramine (Schardl et al. 2013b; Berry et al. 2015). Several variants of *perA* with variability of the number of functional domains have been identified in different *Epichloë* species. Expression of a *perA* variant which lacks the reductase domain may produce a metabolite related to peramine (Schardl et al. 2013b; Berry et al. 2015).

The development of endophyte associations with minimal or no detrimental effects, while retaining beneficial characteristics, is important for successful pastoral agriculture. To achieve this outcome, various different methodologies, from conventional selective breeding to modern biotechnological approaches, have been used in contemporary grass breeding programs (Gundel et al. 2013). Genetic modification of endophytes with useful genes may be used to confer additional valuable attributes on the host, following inoculation with the suitably altered endophyte (Mei and Flinn 2010). The genes required for the biosynthesis of alkaloids provide obvious targets for such modification. For example, ergovaline production has been eliminated from an *Epichloë*–grass association through ablation of a peptide synthetase gene, named *lps*, which is essential for ergopeptine biosynthesis (Panaccione et al. 2001).

The initial aim of the present study was to study the feasibility of artificially combining biosynthesis of two alkaloids by a single endophyte strain through introduction of a functional *perA* gene copy into an *Lp*TG-3 endophyte strain by *A. tumefaciens*-mediated transformation. Subsequently, the feasibility of enhanced peramine production through introduction of an additional *perA* gene copy/copies into *Lp*TG-1 and *Lp*TG-2 endophyte strains was assessed. Finally, RNA-Seq-based expression analysis of *perA* genes from selected ryegrass endophytes belonging to distinct taxonomic groups was performed, to determine the functional status of the *perA* promoter, and the potential for restoration of truncated *perA* genes by genome editing technology.

## Materials and methods

### Strains and culture conditions

The properties of the selected endophyte strains, in terms of taxon (Hettiarachchige et al. 2015), alkaloid biosynthesis profile, observed growth characteristics, number of *perA* gene copies, and peramine production phenotype are summarised in Table 1. Endophyte cultures were grown either on potato dextrose agar (PDA; Fluka, St. Louis, Missouri, USA), or in PDB at 22 °C in the dark for a period of 7–10 days, depending on growth rate. *A. tumefaciens* strain AGL-1, which was used for transformation, and *Escherichia coli* strain DH5α (Thermo Fisher Scientific, Waltham, MA, USA) which was used during construction and maintenance of plasmid constructs, were grown at 28 °C and 37 °C, respectively, on either LB (Luria–Bertani) agar (5 g yeast extract, 10 g tryptone, 10 g NaCl in 1L of dd$H_2O$) plates or in LB broth. PDA and LB media were supplemented with appropriate antibiotics as necessary.

### Plasmid construction

Gene cassettes were supplied by GeneArt (Regensburg, Germany) as follows: the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd*P) and tryptophan biosynthesis C gene terminator (*trpC*T) of *Aspergillus nidulans*, for cloning of the first reading frame A [RFA-A] cassette; the hygromycin B resistance gene (*hph*) under the control of the *A. nidulans trpC* promoter (*trpC*P) and terminator; the *perA* gene sequence of SE, flanked by *attB*1 and *attB*2 recombination sites.

The Gateway™-enabled destination vector (pEND0002) was constructed through modifications of the T-DNA region of pPZP200 (Hajdukiewicz et al. 1994).

A 2.1 kb *Xba*I/*Kpn*I fragment containing the *hph* expression cassette (from pMA2) was force-cloned across the corresponding sites of pPZP200, so generating pEND0001. The RFA-A cassette (1.7 kb; Invitrogen, Carlsbad, California, USA) was cloned between *gpd*P and *trpC*T at the *Sma*I site of the GeneArt vector, pMA1. Single colonies were screened by restriction endonuclease analysis for the presence of the RFA-A cassette in the desired (sense) orientation. The 2.9 kb *gpd*P-(RFA-A)-*trpC*T cassette was then released by digestion with *Sbf*I and *Hind*III and force-cloned across the corresponding sites of pEND0001 to generate pEND0002.

The 8562 bp *perA* gene fragment containing *attB*1 and *attB*2 sites was excised from the 11ABFUUC peramine pMA vector using the restriction endonucleases *Asc*I and *Pac*I and was cloned into the pDONR 221 (Invitrogen, Carlsbad, California, USA) vector using BP clonase following the manufacturer's recommended method (Invitrogen, Carlsbad, California, USA) to generate the entry clone, pEND0024. This was combined with the destination vector pEND0002 using LR clonase following the manufacturer's recommended method (Invitrogen, Carlsbad, California, USA) to generate a final expression vector, pEND0025 (Supplementary table S1). The structures of all expression clones were verified by Sanger sequencing.

### Sensitivity of non-transgenic endophyte mycelia to hygromycin B

The sensitivity of non-transformed endophyte mycelia to treatment with hygromycin B was determined by plating 400 µL of endophyte culture onto PDA plates containing increasing concentrations of antibiotic (50, 100, 150, 200, 250, and 300 µg/mL), and incubation of plates at 22 °C for 4 weeks.

### *A. tumefaciens*-mediated transformation

*A. tumefaciens* cells (AGL1) were transformed with the binary vectors by electroporation. Transformants were

**Table 1** Properties of *Epichloë* endophytes used for transgenic modification

| Endophyte strain* | SE | NEA11 | NEA12 |
|---|---|---|---|
| Taxon[a] | *E. festucae* var. *lolii* | *Lp*TG-2 | *Lp*TG-3 |
| Alkaloid biosynthesis profile[b] | Lolitrem B<br>Ergovaline<br>Peramine | Ergovaline<br>Peramine | Janthitrems |
| Growth rate[c] | Moderate | Fast | Slow |
| Number of *perA* gene copies[a] | 1 | 2 | 1 |
| Peramine production[b] | + | + | – |

*All isolates are provided by Department of Economic Development, Jobs, Transport, and Resources

[a]Hettiarachchige et al. (2015)

[b]Kaur et al. (2015), peramine production is indicated by "+"

[c]Compared by mycelial growth on PDA in 22 °C in the dark

isolated on LB agar plates supplemented with 20 mg/L rifampicin and appropriate antibiotics for selection of the *A. tumefaciens* strain and binary vector. *A. tumefaciens*-mediated transformation was performed as previously described (Tanaka et al. 2007), with minor modifications.

## Mitotic stability of transformed endophytes

Putative endophyte transformants (appearing 2–4 weeks after transformation) were transferred to fresh selection medium and mitotic stability was determined by sub-culturing five successive times every 2–3 weeks, alternating between media containing and lacking 200 µg/mL hygromycin B. The stability of the putative transformants was also confirmed by PCR detection of the *hph* gene.

## Molecular analysis of transformed endophytes

Genomic DNA was extracted from freeze-dried fungal mycelia of putative transformants using the QIAGEN DNeasy plant mini kit (QIAGEN, Hilden, Germany). PCR analysis was used to confirm the presence of T-DNA using the following primer pairs: *gpd*P-*perA*-F (5′- AGTCCCTGG TAGGCAGCTTT -3′), *gpd*P-*perA*-R (5′- CAGAGGCCA AGATTGTTTGG-3′), *hph*-F (GATGTTGGCGACCTC GTATT) and *hph*-R (GAATTCAGCGAGAGCCTGAC). PCR cycling conditions consisted of an initial denaturation cycle of 10 min at 95 °C, followed by 30 cycles with 30 s denaturation (95 °C), 30 s annealing (59 °C) and 1 min polymerisation (72 °C).

## Metabolic profile analysis of endophyte cultures using liquid chromatography/mass spectroscopy (LC/MS)

### Sample preparation

A randomly selected sub-set of PCR-positive transformants were tested for peramine production in in vitro culture. Five putative transformants, along with the corresponding non-transgenic endophyte strains, were sub-cultured on PDA plates containing 15 mM urea. Four replicates from each endophyte strain were prepared and grown for 8 weeks. Plates were stored at − 80 °C until required. Samples were freeze-dried for 48 h and ground to a fine powder using a genogrinder (SPEX SamplePrep 2010 Geno/GrinderR, Metuchen, USA). The resulting powder was extracted by adding two volumes of 80% (v/v) methanol. Samples were vortexed briefly, sonicated for 10 min, vortexed again, and centrifuged at 10,000 rpm for 10 min. Three extractions were performed for each sample and all supernatants were combined. The solvent was evaporated using a SpeedVac Concentrator (Savant SPD2010, Thermo Fisher Scientific, Waltham, MA, USA). The residue was dissolved in 2 mL of 80% (v/v) methanol and collected in a vial for LC/MS analysis.

### LC/QTOF mass spectrometry

The analytical system used was the Agilent UHPLC 1290 Infinity binary LC system (Agilent Technologies, Santa Clara, California, USA) and Agilent Technologies 6538 UHD Accurate-Mass QTOF Mass Spectrometer with Electrospray Ionisation (ESI) source. Analysis of samples with full-scan acquisition mode in positive ionisation mode was performed. A methanol-only negative control (blank) was run as every sixth sample (five samples followed by a blank) of analysis. The data acquisition software was Agilent MassHunter Qualitative Analysis software (version B.06.00). Chromatographic separation was performed using a Hypersil GOLD™ column (150 × 2.1, 1.9 µm) (Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase was composed of 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). The gradient elution program is detailed in Supplementary Table S2. Peramine standard [Peramine nitrate (BDG Synthesis, Wellington, New Zealand)] was used to construct concentration curves at 1, 20, 40, 80, 200, 400, 800, 1000, 2000, 4000, and 10,000 ng/mL in matrix and peramine concentrations of samples were calculated in parts per million (ppm). Elution time for peramine standard was 3.6 ± 0.05 min.

QTOF conditions included a gas temperature 8 L/min at 300 °C; nebulizer pressure 30 psig, capillary voltage 3.5 kV. Signals in the *m/z* (mass-to-charge ratio) 100–1700 range were recorded. MS/MS conditions: collision energy of 20 eV was used to fragment the parent ions in the m/z 100–1700 range. Acquisition rate was1 spectrum/s.

### Data analysis

Data processing was performed with the Agilent MassHunter Qualitative Analysis software (version B.06.00 [6.0.633.10]). Identification of peramine was based on retention time and accurate mass. Identity of peramine was further confirmed from fragmentation data. Confidence of identification was expressed by an overall identification score, the weights of these parameters being: mass score = 100, isotope abundance score = 60, isotope spacing score = 70, MS mass 2.0 mDa + 5.6 ppm, MS isotope abundance 7.5%, and MS/MS mass 5.0 mDa + 7.5 ppm.

Average peramine concentration produced by each transgenic endophyte was compared to that of non-transgenic endophyte and statistical analysis was performed using the Mann–Whitney–Wilcoxon Test (Mann and Whitney 1947).

### Seedling inoculation of transgenic endophytes

Endophyte-devoid ($E^-$) perennial ryegrass cultivar Alto seeds were obtained from New Zealand Agriseeds (NZA), Christchurch, New Zealand. These seeds have been generated from an endophyte-free parental population which has never been inoculated with endophyte. Prior to use for inoculations, DNA was extracted from a representative sample of this seed batch using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and screened using three endophyte-specific SSR markers (NLESTA1QA09, NLESTA1CC05, and NLESTA1NG03) (Kaur et al. 2015; van Zijll de Jong et al. 2008) to confirm the absence of endophyte. A total of 50–100 seedlings were inoculated with each transgenic endophyte strain, together with non-transgenic counterparts. The number of inoculations performed was based on the documented success rates for the respective non-transgenic endophyte strains (K. Guthridge, unpublished).

$E^-$ seeds were surface-sterilised (5% [w/v] NaOCl and Tween 20) for 3 h with shaking at room temperature. Following washing, seeds were plated onto water agar (26 g/L bacteriological agar) and incubated in dark at 20–22 °C for 6–8 days. Fresh endophyte mycelium was inserted into a parallel incision made across the shoot meristem. Inoculated seedlings were incubated in a growth room (23 °C, 80 $\mu Mm^{-2}sec^{-1}$, 8–16 h photoperiod) for 7 days and then transferred to the glasshouse (16 h photoperiod, 60% relative humidity, natural lighting and an average controlled temperature of 25 °C) and planted in 42-cell plant trays filled with potting mix (30 L of seeding raising mix [Van Schaik's Biogro, Dandenong South, Victoria, Australia] mixed with 0.9 L of fine vermiculite, 0.6 L of fine perlite, 25 g of micro nutricote, 20 g of water-holding granules, and 2.5 g of trace elements) and grown for 16 weeks under glasshouse conditions. Non-inoculated $E^-$ Alto seeds were also germinated under the same conditions for use as a control.

### Screening for endophyte presence

Two-to-three tiller samples (c. 0.5 cm from the base) of each endophyte-inoculated and endophyte-free plant were harvested, following 16 weeks growth in soil. DNA extraction and SSR genotyping were performed as previously described using three endophyte-specific SSR markers (NLESTA1QA09, NLESTA1CC05, and NLESTA1NG03) (van Zijll de Jong et al. 2008; Kaur et al. 2015). Four plants from each host–endophyte combination (Alto-SE, Alto-NEA11, and Alto-NEA12) and $E^-$ Alto were then transplanted into 8 cm × 8 cm pots and maintained under glasshouse conditions as described before. When most of the plants had grown to a density of > 30 tillers (one transgenic SE-inoculated plant failed to survive and one non-transgenic SE-inoculated plant exhibited poor growth with a few tillers at this stage and was removed from further analysis), individual plants were split into seven clonal replicates and transplanted into pots of the same size, followed by maintenance under the same glasshouse conditions. Total number of transgenic and non-transgenic samples tested for peramine quantification were 21 for SE (seven clonal replicates of three different host plant genotypes) and 28 for NEA11 and NEA12 (seven clonal replicates of four different host plant genotypes).

## Metabolic profile analysis of Alto–endophyte associations using liquid chromatography/mass spectroscopy (LC/MS)

The pseudostem component of plants was harvested and snap-frozen in liquid nitrogen. Samples were then freeze-dried and extracted twice from 20 mg of each clonal replicate using 80% (v/v) methanol, and the final residue was dissolved in 200 µL of 80% (v/v) methanol and collected in a vial for the LC/MS analysis.

The extracts were analysed using a 100 mm × 2.1 mm Thermo Hypersil Gold 1.9 µm HPLC column fitted to a Thermo Fisher Scientific Vanquish liquid chromatograph (Thermo Fisher Scientific, Waltham, MA, USA). Metabolites were eluted from the column using a gradient mobile phase, (A) 0.1% formic acid in water, (Thermo Fisher Scientific, Waltham, MA, USA) and (B) 0.1% formic acid in acetonitrile (Thermo Fisher Scientific, Waltham, MA, USA) at 0.3 ml/min. The initial conditions (98% A) preceded initiation of a linear gradient to 0% A over 11 min, and this was maintained for 4 min before returning to the initial gradient conditions. The compounds were detected with a Thermo Fisher QExactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operating in the ESI mode with an HESI probe for positive data acquisition. Standards (peramine [Peramine nitrate {BDG Synthesis, Wellington, New Zealand}], ergotamine [Ergotamine D-tartrate {Sigma–Aldrich, St. Louis, MO, USA}], and lolitrem B (Reddy et al. 2015)) were used to construct concentration curves at 1, 10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL in matrix.

For liquid chromatography–mass spectrometry analysis, 3 µL of sample extract was injected onto the LC/MS system. Each extract was analysed using a QExactive Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in FT positive mode over a mass range of 80–1200 amu with resolution set at 35,000. Typical mass accuracy for the alkaloids was 3 ppm or better.

The presence of janthitrem I was confirmed by accurate mass and by MS2 analysis of the ion at *m/z* 646.377 with the same ion at the same retention time in the Alto-AR37 controls (AR37 being used as the positive control for janthitrem I production, as no standards are currently

commercially available for this class of alkaloid). Elution times for peramine, ergovaline, lolitrem B, and janthitrem I were 3.57 ± 0.02 min, 5.37 ± 0.01 min 11.17 ± 0.02 min and 11.19 ± 0.02 min, respectively.

Concentrations of peramine were calculated from the standard curve and average peramine concentration produced by each transgenic endophyte infected plant and the overall average peramine concentration produced by all transgenic endophyte infected plants were compared to the overall average peramine concentration produced by non-transgenic endophyte infected plants.

### Analysis of *perA* gene expression

$E^-$ perennial ryegrass cultivar Alto seeds, and those containing the endophyte strains SE (Standard Endophyte), NEA11, and NEA12 were obtained from New Zealand Agriseeds (NZA), Christchurch, New Zealand. Seeds were surface-sterilised in 5% (w/v) NaOCl with gentle shaking for 3 h. Seeds were then washed thoroughly with sterile water and germinated on wet sterile filter papers in the dark for 2 days and in the light for a further 8 days (23 °C, 80 $\mu Mm^{-2}sec^{-1}$, 8–16 h photoperiod). RNA was extracted from 10-day-old seedlings using a CTAB-based method as previously described (Chang et al. 1993) with minor modifications. Four biological replicates were prepared for each endophyte strain. Libraries were prepared using the Agilent SureSelect strand-specific RNA library preparation kit (Agilent Technologies, Santa Clara, California, USA), and sequencing was performed on an Illumina Hiseq 3000 platform (http://www.illumina.com) (Illumina Inc., San Diego, California, USA).

Raw sequence reads were quality trimmed using the Gydle nuclear program (http://www.gydle.com) (Gydle Inc., Quebec, Canada), such that all reads ≥ 50 bases long were retained using a base quality cut-off value of 20. All high-quality reads for a given endophyte strain were mapped separately to the SE *perA* gene sequence using Bowtie (version 1.0.0) (Langmead et al. 2009) and visualised with Tablet (version 1.15.09.01) (Milne et al. 2013).

## Results

### *A. tumefaciens*-mediated transformation of endophyte mycelia

The sensitivities of non-transgenic SE, NEA11, and NEA12 to the selective antibiotic, hygromycin B, were assessed prior to inception of the transformation experiments, to determine whether the *hph* gene could be used as the selectable marker. Colonies of each strain grew on hygromycin B concentrations up to 150 µg/mL, and at higher concentrations, negligible growth was observed. A higher threshold concentration of hygromycin B (200 µg/mL) was consequently chosen for the selection of putative transformants.

The expression vector (pEND-*perA*) generated by replacing the Gateway RFA-A cassette in vector pEND0002 by the *perA* gene of SE was transformed into *A. tumefaciens* strain AGL1. PCR analysis was performed to confirm the presence of the transformed plasmid (data not shown). The *A. tumefaciens* strain was co-cultivated with finely cut endophyte mycelium from the experimental strains SE, NEA11, and NEA12. Hygromycin B-resistant colonies started to develop 2–4 weeks after transformation, suggesting that all three endophyte strains (and hence taxa) were receptive to *A. tumefaciens*-mediated T-DNA transfer. The number of resistant transformants generated for SE, NEA11, and NEA12 was 39, 43, and 78 per 1 g of mycelia, respectively. The negative controls that were used, being the corresponding non-transgenic endophyte or *A. tumefaciens* strain, did not give rise to resistant colonies, providing confidence that genuine transformation events were selected.

Stable transformation events were confirmed through the ability of these putative transformants to grow after sub-culturing onto fresh selection plates containing higher hygromycin concentrations (300 µg/mL). Successful expression of *hph*, a commonly used selectable marker for fungal transformation, suggests that the *A. nidulans trpC* promoter is effective for the expression of transgenes in the target endophyte strains.

### PCR analysis and mitotic stability assessment

A total of 36 hygromycin B-resistant transformants (12 from each endophyte strain) that were selected on antibiotic selection media were subjected to PCR to identify the presence of introduced *perA* and *hph* genes. Primers for the *perA* gene were designed that flank both the *gpd* promoter and the introduced *perA* gene, to permit discrimination from the endogenous genes that are present in all three strains. The expected sizes of PCR products for *perA* (201 bp) and *hph* (414 bp) were observed for all transformants, confirming the presence of stable transformation events. No amplification products were produced by non-transgenic endophyte strains.

PCR-positive transformants maintained the ability to grow on hygromycin B after alternate sub-culturing on to PDA, with and without hygromycin B, for a minimum of five sub-cultures, so confirming mitotic stability. The stability of the putative transformants was also confirmed by PCR detection of the *hph* gene.

## Metabolic profiling

The ability of transformants to produce peramine in in vitro culture was assessed following molecular characterisation of transformants. For this purpose, a total of five randomly selected PCR-positive transformants from each endophyte strain were tested. The presence of peramine was identified based on retention time and accurate mass, while further confirmation was achieved using multiple mass spectrometry (MS/MS). All five transformants containing the *perA* gene for each endophyte strain that was analysed demonstrated the presence of a specific peak with a retention time of $3.8 \pm 0.15$ min, and were well within 6.5 ppm of the *m/z* 248.15063 $[M+H]^+$, so confirming the ability of transformants to produce peramine in culture. This result demonstrates that the presence of a functional copy of the *perA* gene is capable of restoring production to an otherwise non-producing strain, NEA12, and further confirms the sole responsibility of the *perA* gene for peramine biosynthesis. Non-transgenic SE and NEA11 also produced the same specific peak with the expected retention time, while no specific peak was observed for non-transgenic NEA12, as expected. The successful expression of the *perA* gene suggests that the *A. nidulans gpd* promoter is effective for the expression of transgenes in the target endophyte strains, which represent three distinct taxa.

The identity of peramine as a metabolite was further confirmed based on mass spectrometric fragmentation pattern. The fragment ion spectra of *m/z* (mass-to-charge ratio) 248.15063 $[M+H]+$ for 13 out of 15 of the transformants included all of the distinct fragment ions *m/z* 149, *m/z* 175, *m/z* 206, and *m/z* 231 (Fig. 1). The remaining two transformants generated only *m/z* 175 and *m/z* 248 of those ions, probably due to low abundance. Non-transgenic SE and NEA11 generated ions only with *m/z* 175 (data not shown).

## Level of peramine production

Variable levels of peramine production were observed for transgenic SE, NEA11, and NEA12 endophyte strains containing *perA* genes in in vitro culture. Differences between expression levels of transformants of the same strain could be due to variability of the number of *perA* gene copies that have been integrated, as well as the influence of the genomic site of integration in endophyte genomes. Very low levels of peramine were detected for non-transgenic SE and NEA11 (Fig. 2).

A significant difference ($p < 0.05$) in peramine production was observed for all transgenic SE and NEA11 when compared to the corresponding non-transformants. This result is consistent with the observation that small amounts of peramine are produced by endophytes in culture, compared to higher levels detected in $E^+$ grass tissue. The activity of the constitutive *gpd* promoter, rather than the native *perA* promoter, was presumably responsible for the elevated level of peramine biosynthesis observed in culture for transgenic SE and NEA11 when compared to their non-transgenic counterparts. A higher level of peramine production was observed for most NEA12 transformants when compared to SE and NEA11 transformants.

## Seedling inoculation of transgenic endophytes

Inoculation of ryegrass seedlings with PCR-verified transgenic endophyte strains that produce peramine in culture, together with the corresponding non-transgenic strains, was performed to determine the capacity to form effective associations and produce peramine in planta. Putative associations were screened 16 weeks after transfer to soil with *Epichloë*-specific SSR marker assays, as explained above. All three combinations of non-transgenic and transgenic endophyte strains established associations with their hosts, suggesting that transgene presence did not exert inhibitory effects on inoculation ability. The infection frequencies obtained are summarised in Table 2.

$E^+$ plants (both non-transgenic and transgenic) were transferred to pots from cell trays and maintained under glasshouse conditions, followed by re-analysis 12 weeks later using the same SSR marker assay, to assess vegetative stability of the association. PCR-based screening for the presence of transgenic sequences was also confirmed. For each strain, four transgenic and four corresponding non-transgenic endophyte infected plants were used for the detection of peramine, ergovaline, lolitrem B, and janthitrem I. Analysis of LC/MS data revealed that all strains produced the expected alkaloid profiles.

Interestingly, transgenic NEA12-infected plants were able to produce both peramine and janthitrem I, while non-transgenic NEA12-infected plants produced only janthitrem I. Variable levels of peramine production were observed for non-transgenic and transgenic SE and NEA11 as well as transgenic NEA12-infected plants: such variation may be due to genotypic differences between host plants. The levels of peramine production detected for transgenic NEA12 infected plants were low compared to those of SE and NEA11 (Fig. 3). This observation differs from that made for peramine production in culture (Fig. 2) where most NEA12 transformants produced high level of peramine and suggests that in planta peramine production in SE and NEA11 is primarily due to the activity of the endogenous gene copies under the up-regulated native *perA* promoter. Conversely, the low level of peramine produced in planta by transgenic NEA12-infected plants is due to control by the constitutive fungal promoter.

Plants with expected alkaloid profile were split into seven clonal replicates, grown for another 12 weeks and

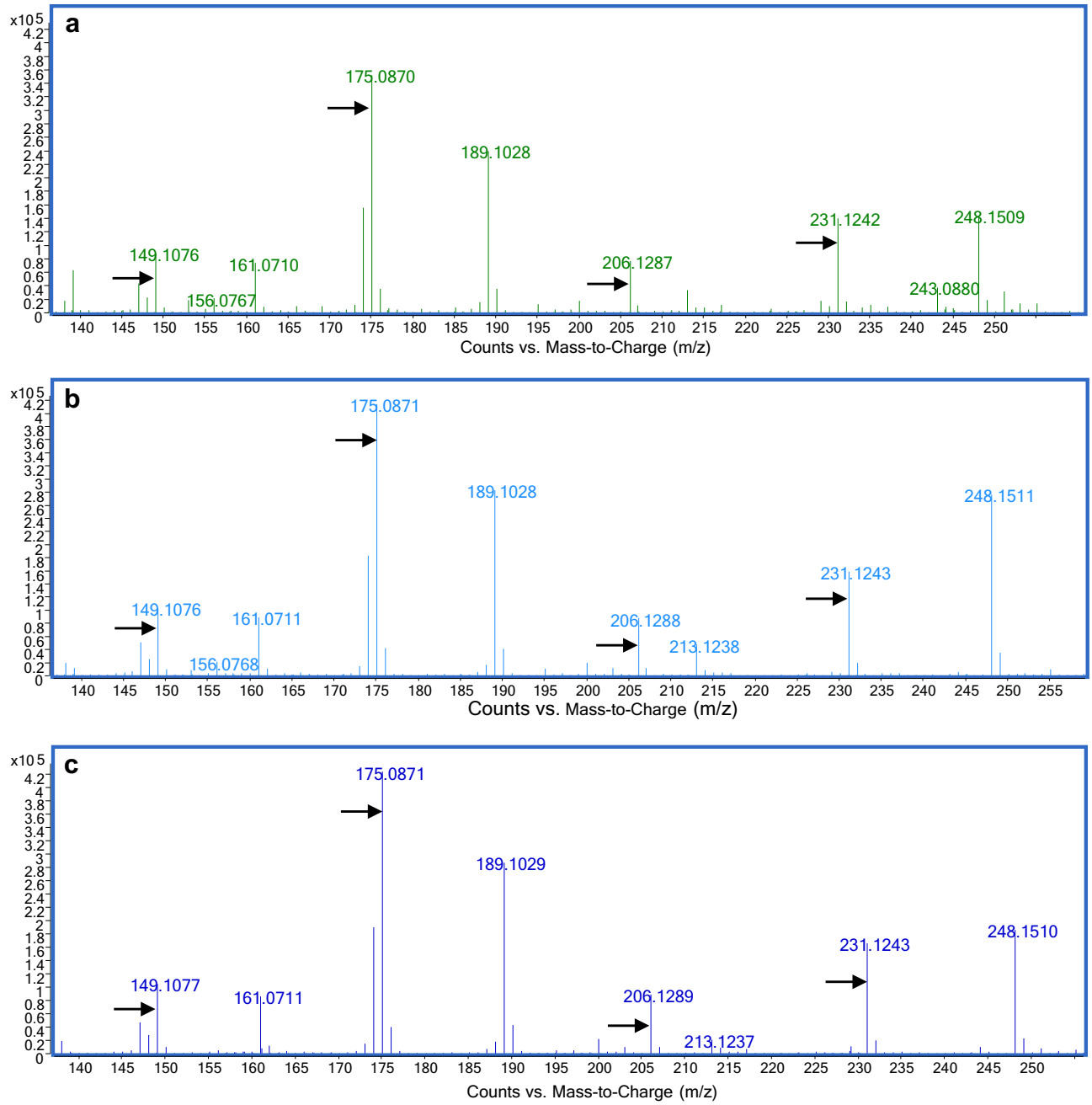


**Fig. 1** Mass fragment spectrum derived from the parent ion of peramine. **a** Transgenic SE_1, **b** Transgenic NEA11_1, and **c** Transgenic NEA12_1. The distinct fragment ions *m/z* 149, *m/z* 175, *m/z* 206, and *m/z* 231 are indicated by arrows

re-analysed, producing alkaloid profiles consistent with the previous results. Only one transgenic NEA11-infected plant produced a significantly high ($P < 0.05$) peramine, while no significant difference ($P < 0.05$) in peramine production was detected for the other three transgenic NEA11-infected plants when compared to the corresponding non-transgenic NEA11. Furthermore, no significant difference was observed between overall average levels of peramine produced by non-transgenic and transgenic NEA11. Of three transgenic SE-infected plants that were analysed, two produced a significantly higher level of peramine, and one produced significantly less peramine when compared to the corresponding non-transgenic counterparts. As a consequence, the overall average peramine produced by transgenic SE *in planta* was significantly higher than that of non-transgenic SE (Fig. 3). However, this effect may be mostly due to the genotypic effect of the host plant on peramine production.

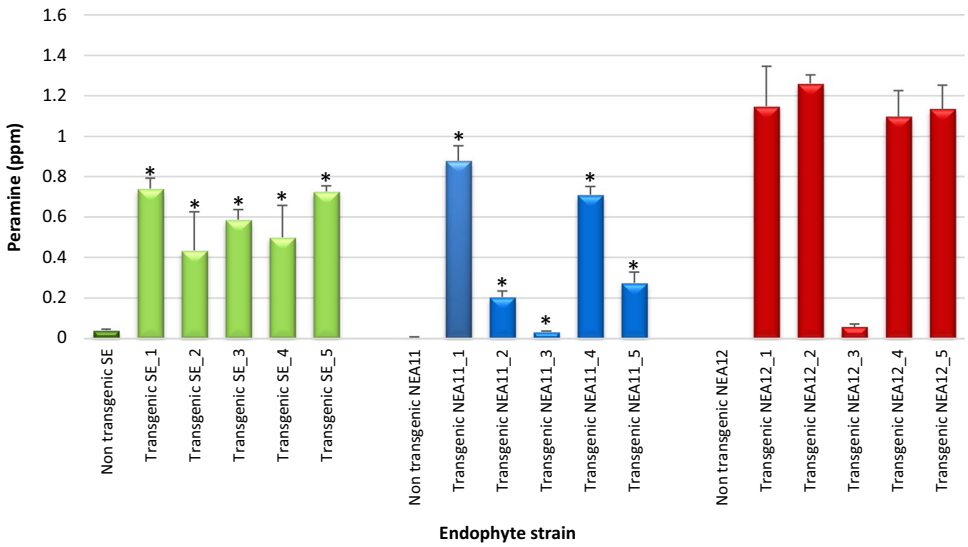


**Fig. 2** Variation in peramine production by non-transgenic and transgenic SE, NEA11, and NEA12 endophyte strains in in vitro culture. Each bar shows the average peramine concentration (ppm) produced by four replicates of each endophyte strain and error bars denote standard errors of the mean. *Indicates a significant statistical difference ($p < 0.05$) between transgenic and corresponding non-transgenic endophyte samples. Non-transgenic NEA12 is not shown as it did not produce any peramine in culture

**Table 2** Infection frequencies obtained for different endophyte strains

| Endophyte strain | Transgenic | | | Non-transgenic | | |
|---|---|---|---|---|---|---|
| | SE | NEA11 | NEA12 | SE | NEA11 | NEA12 |
| Infection frequency (%) | 16.5 | 27.5 | 8.5 | 23 | 39 | 8 |

## Expression of the *perA* gene in different Alto–endophyte associations

The level of peramine produced in planta under the control of the *A. nidulans gpd* promoter by transgenic NEA12-infected plants was very low compared to the other peramine-producing endophytes used in this study, despite the widespread use of a fungal constitutive promoter. Consequently, an expression analysis of the *per*A gene was performed to test the possibility of introducing peramine production into a non-peramine-producing strain using the native promoter and novel genome editing techniques.

High-quality reads from each Alto–endophyte association were mapped to the SE-derived *perA* gene sequence, numbering 1294, 3791, and 523 for Alto-SE, Alto-NEA11, and Alto-NEA12 associations, respectively. Read distribution along the gene length demonstrated full coverage for SE and NEA11 and partial coverage for NEA12 (Fig. 4). Transcription of the *per*A gene up to coordinate 5475 bp was observed for NEA12, which was beyond the premature stop codon identified at coordinate 2923 bp from gene sequence analysis. Expression of the truncated *per*A gene of NEA12 confirms functionality of the native promoter, supporting the potential for using genome editing techniques to restore full gene function.

# Discussion

## *A. tumefaciens*-mediated transformation of the *perA* gene

The SE-derived *perA* gene sequence was used to construct an expression vector under the control of the *A. nidulans gpd* promoter, which is known to function across a broad range of Ascomycete species (Mikkelsen et al. 2003). The expression vector was constructed using Gateway™ cloning, which provides a simple and highly efficient system for gene cloning and rapid target gene transfer between vectors (Zhu et al. 2009). Modifications of the conventional vectors by introduction of the Gateway cassette have been used extensively for functional studies in filamentous fungi (Oliveira et al. 2008; Shafran et al. 2008; Zhu et al. 2009).

*A. tumefaciens*-mediated transformation is effective for the transformation of filamentous fungi due to versatility of choice in starting material (such as protoplasts, hyphae, spores, or blocks of mycelial tissue), high transformation efficiency, and a less labour-intensive nature than protoplast-requiring transformation protocols (de Groot et al. 1998; Mullins et al. 2001; Amey et al. 2002; Fitzgerald et al. 2003; Meyer et al. 2003; dos Reis et al. 2004). Growth of endophyte mycelia was completely inhibited by hygromycin B at 200 µg/mL, similar concentrations (200–300 µg/mL) having

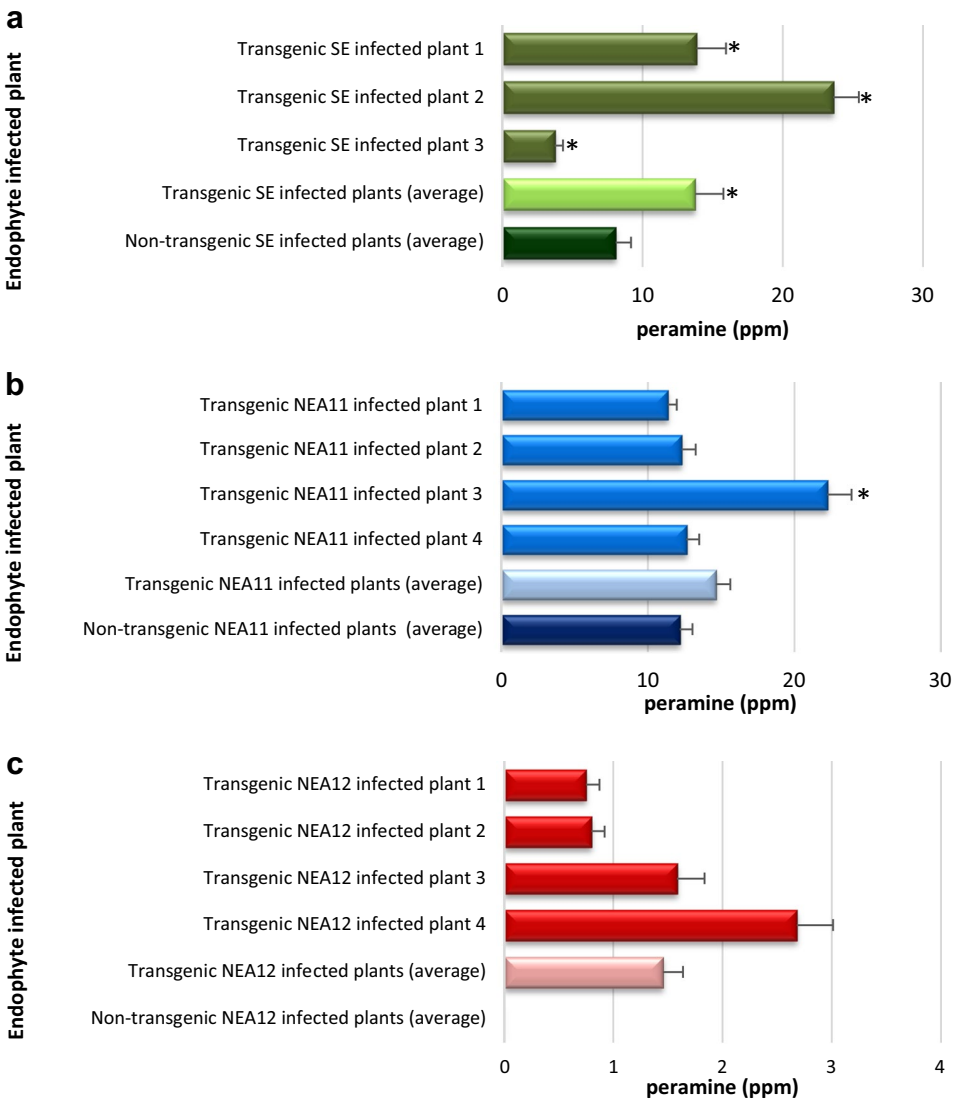


**Fig. 3** Variation in peramine production by non-transgenic and transgenic SE, NEA11, and NEA12 in planta. Error bars denote standard errors of the mean. *Indicates a significant statistical difference ($p < 0.05$) in peramine production between transgenic and corresponding non-transgenic endophyte infected plants. **a** First three bars from the top show average peramine concentration produced by each transgenic SE-infected plant ($n = 7$). The fourth and fifth bars show the overall average peramine concentration produced by transgenic and non-transgenic SE-infected plants ($n = 21$), respectively. **b, c** First four bars from the top show average peramine concentration produced by each transgenic NEA11- and NEA12-infected plant ($n = 7$), respectively. The fifth and sixth bars show the overall average peramine concentration produced by transgenic and non-transgenic NEA11- and NEA12-infected plants, respectively ($n = 28$)

been successfully used for *A. tumefaciens*-mediated transformation of both *E. festucae* var. *lolii* and other ascomycete species (Mikkelsen et al. 2001; Panaccione et al. 2001; Knight et al. 2009).

## Metabolic profile analysis of transformants

PCR-positive transformants were analysed for the production of peramine in in vitro culture. Restoration of peramine production by NEA12 transformants confirms that the peramine-devoid phenotype of NEA12 is due to the effects of the deletion and premature stop codon within the *perA* gene sequence. It also confirms the sole responsibility of the *perA* gene for peramine biosynthesis, consistent with the outcomes of other studies (Tanaka et al. 2005).

Endophytes utilise nitrogen both for growth and for the biosynthesis of alkaloids, which are nitrogen-rich compounds (Faeth and Fagan 2002). Alkaloid levels have been reported to be potentially enhanced by increased nitrogen availability in $E^+$ grasses, especially under moderate water stress (Faeth et al. 2002). In contrast, reductions of both alkaloid concentrations and endophyte prevalence have been found in some studies when plants were grown at high nitrogen levels (Rasmussen et al. 2009). However, the highest level of loline biosynthesis in culture for *E. uncinata* has been obtained when urea is provided as the sole nitrogen source (Blankenship et al. 2001). Therefore, transformants containing *perA* gene in this experiment were grown on PDA containing urea for the analysis of peramine production. All sample extractions were performed under cold conditions to minimise the possibility of false negatives, and additionally, the absence of false positives was verified by analysis of negative controls between each of the five samples.

Methods that have been commonly used so far for peramine identification include mass spectroscopy, ultraviolet spectroscopy, and thin-layer chromatography (Rowan 1993).

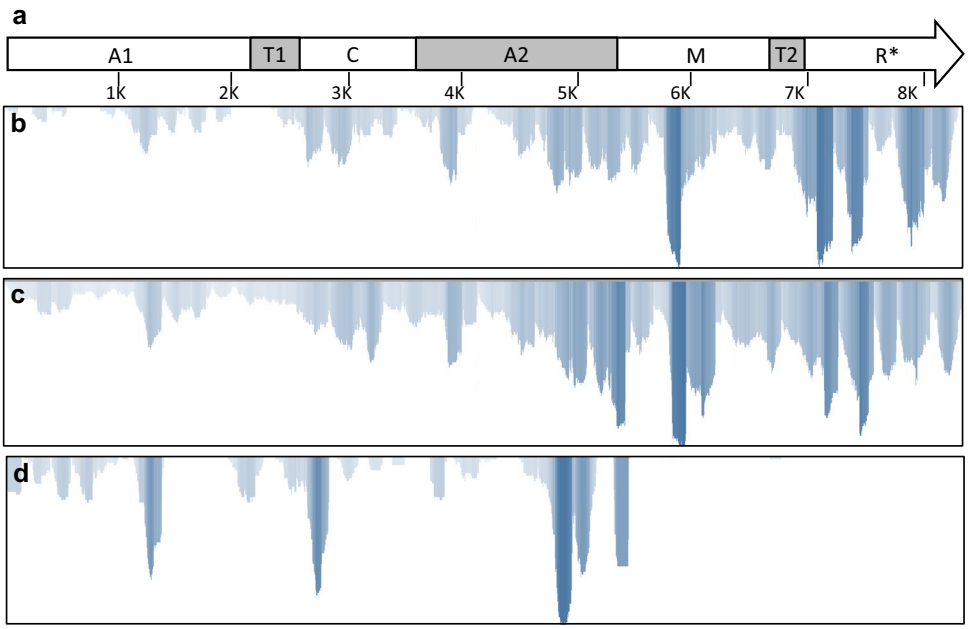


**Fig. 4** Tablet output showing Illumina HiSeq-derived reads mapped to the *perA* gene of SE: **a** full-length *perA* gene (Berry, Takach, et al. 2015), **b** Alto-SE, **c** Alto-NEA11, and **d** Alto-NEA12 associations

Of these, detection of peramine by MS/MS has been found to be c. 50-fold more sensitive than UV-based methods (Koulman et al. 2007). Peramine production by transformants generated in this study was analysed using LC/MS, and the metabolite was identified based on retention time and accurate mass measurements. In the mass spectrum, the fragments exhibited *m/z* values of 206 ($C_{11}H_{16}O_1N_3$) corresponding to loss of diazomethane, *m/z* 231 corresponding to loss of ammonia ($NH_3$) and two more distinct ions with *m/z* of 175 and 149. These results were consistent with the published mass spectral data (Koulman et al. 2007).

As expected based on *perA* gene sequence structure, no production of peramine was observed for non-transgenic NEA12, while very low levels of peramine production were observed for non-transgenic SE and NEA11. Consistently, production of low amounts of peramine in culture has been previously reported for *E. festucae* var. *lolii* and *E. typhina* endophytes, in contrast to higher levels detected in $E^+$ grass tissue (Rowan 1993; Tanaka et al. 2005). In contrast to the respective non-transgenic counterparts, substantial production of peramine in culture was apparent for transformants of all three endophyte strains, albeit at variable levels. Activity of the constitutive *gpd* promoter was presumably responsible for the elevated level of peramine biosynthesis observed in culture.

## Expression of peramine in transgenic endophyte-containing plants

Subsequently, in planta peramine production was determined. Non-transgenic and transgenic endophyte strains of all three taxa established associations with their hosts suggesting that transgene presence did not exert inhibitory effects on inoculation ability. Infection frequency is based on endophyte identity as well as on host plant genotype and frequencies obtained in this study are consistent with the previous studies (Kaur et al. 2015).

As observed in vitro, failure to produce peramine is rescued in transgenic Alto-NEA12 associations when compared to non-transgenic counterparts, albeit at low levels compared to SE- and NEA11-containing plants. Expression under control of the native *perA* promoter may be up-regulated *in planta* (Khan et al. 2010), as compared to a constitutive fungal promoter. A significantly higher average level of peramine production was detected for transgenic SE-infected plants as compared to non-transgenic SE-containing plants. However, this effect may be due to the severely restricted growth of one Alto non-transgenic SE association. No significant increases in average level of peramine production were observed when comparing transgenic NEA11-containing plants to their counterparts that contain non-transgenic NEA11, consistent with a low level of additional peramine biosynthesis from the transgene.

## Transcriptional analysis of the *perA* gene

Isolation of *perA* gene sequences from perennial ryegrass-derived endophytes revealed the presence of full-length copies in *Lp*TG-1 and *Lp*TG-2 strains, while representatives of *Lp*TG-3 and *Lp*TG-4 lack the 3′-terminal region of *perA*, reducing the length of the gene from 8505 bp to 7282 bp (Hettiarachchige et al. 2015). *Lp*TG-3 strains also contain a premature stop codon at coordinate 2923 bp within the *perA* gene sequence (Hettiarachchige et al. 2015).

Mapping of RNA-Seq reads to the full-length *perA* genes of SE and NEA11 in the present study demonstrated the expression of all functional domains, as evident from metabolic profiling and gene sequence analysis. The full-length *perA* gene encodes a multifunctional protein with two adenylation and thiolation domains and single condensation, methylation, and reductase domains (Tanaka et al. 2005). Interestingly, mapping of reads to the 5′-portion of the truncated *perA* gene in NEA12 revealed expression, suggesting that the cognate promoter is active in *Lp*TG-3 strains. This observation supports the potential for using genome editing approaches to restore full gene function in this genetic background in future. Chemotypic analysis of *E. festucae* E2368 has revealed that isolates lacking the reductase domain are unlikely to produce peramine, although all the other domains are present (Young et al. 2012; Schardl et al. 2013b). It has been suggested that *E. festucae* E2368 has the ability to produce a peramine-related metabolite (Schardl et al. 2013b). This transcriptional analysis strongly supports the hypothesis that the truncated *perA* gene is expressed, and also suggests the NEA12 *perA* gene encodes at least one functional domain.

## Conclusions

The beneficial properties of endophytes in agricultural applications may be enhanced through the use of naturally occurring strains with desirable qualities, or through targeted genetic modification of endophytes. The results of this proof-of-concept study clearly demonstrate that production of two alkaloids may be artificially combined in a single endophyte strain through transgenesis, so producing a novel strain with enhanced beneficial properties for grassland agriculture. Future experimental studies are needed to test the feasibility of improving levels of in planta peramine production through the use of a stronger exogenous promoter. This study further demonstrates the potential for use of genome editing techniques to produce large amounts of peramine under the control of the native promoter, based on effective expression of a truncated gene copy.

**Acknowledgements** The authors thank Dr. Simone Rochfort for support with the metabolic profiling experiment, and Dr. Emma Ludlow for careful critical reading of the manuscript. Funding for this work was provided by the Victorian Department of Economic Development, Jobs, Transport and Resources, the Dairy Futures Cooperative Research Centre, and the DairyBio Initiative.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.



## References

Amey RC, Athey-Pollard A, Burns C, Mills PR, Bailey A, Foster GD (2002) PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. Mycol Res 106:4–11

Berry D, Takach JE, Schardl CL, Charlton ND, Scott B, Young CA (2015) Disparate independent genetic events disrupt the secondary metabolism gene *perA* in certain symbiotic *Epichloë* species. Appl Environ Microbiology 81:2797–2807

Blankenship JD, Spiering MJ, Wilkinson HH, Fannin FF, Bush LP, Schardl CL (2001) Production of loline alkaloids by the grass endophyte. *Neotyphodium uncinatum* in defined media. Phytochemistry 58:395–401

Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol 114:1–7

Card SD, Rolston MP, Lloyd-West C, Hume DE (2014) Novel perennial ryegrass-*Neotyphodium* endophyte associations: relationships between seed weight, seedling vigour and endophyte presence. Symbiosis 62:51–62

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Clay K (1988) Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology 69:10–16

de Groot MJA, Bundock P, Hooykaas PJJ, Beijersbergen AGM (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842

dos Reis MC, Fungaro MHP, Duarte RTD, Furlaneto L, Furlaneto MC (2004) *Agrobacterium tumefaciens*-mediated genetic transformation of the entomopathogenic fungus *Beauveria bassiana*. J Microbiol Methods 58:197–202

Easton HS (1999) Endophyte in New Zealand ryegrass pastures, an overview. In: Woodfield DR, Matthew C (eds) Ryegrass endophyte: an essential New Zealand symbiosis. In: Proceedings of a New Zealand Grassland Association Symposium, Napier, New Zealand Grassland Research and Practice Series. pp 1–9

Faeth SH, Fagan WF (2002) Fungal endophytes: Common host plant symbionts but uncommon mutualists. Integr Comp Biol 42:360–368

Faeth SH, Bush LP, Sullivan TJ (2002) Peramine alkaloid variation in *Neotyphodium*-infected Arizona fescue: Effects of endophyte and host genotype and environment. J Chem Ecol 28:1511–1526

Felitti S, Shields K, Ramsperger M, Tian P, Sawbridge T, Webster T, Logan E, Erwin T, Forster J, Edwards D, Spangenberg G (2006) Transcriptome analysis of *Neotyphodium* and *Epichloë* grass endophytes. Fungal Genet Biol 43:465–475

Fitzgerald AM, Mudge AM, Gleave AP, Plummer KM (2003) *Agrobacterium* and PEG-mediated transformation of the phytopathogen *Venturia inaequalis*. Mycol Res 107:803–810

Fleetwood DJ, Khan AK, Johnson RD, Young CA, Mittal S, Wrenn RE, Hesse U, Foster SJ, Schardl CL, Scott B (2011) Abundant degenerate miniature inverted-repeat transposable elements in genomes of epichloid fungal endophytes of grasses. Genome Biol Evol 3:1253–1264

Gundel PE, Pérez LI, Helander M, Saikkonen K (2013) Symbiotically modified organisms: nontoxic fungal endophytes in grasses. Trends Plant Sci 18:420–427

Gwinn KD, Gavin AM (1992) Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. Plant Dis 76:911–914

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25:989–994

Hettiarachchige IK, Ekanayake PN, Mann RC, Guthridge KM, Sawbridge TI, Spangenberg GC, Forster JW (2015) Phylogenomics of asexual *Epichloë* fungal endophytes forming associations with perennial ryegrass. BMC Evol Biol 15:72

Johnson LJ, Johnson RD, Schardl CL, Pinaccione DG (2003) Identification of differentially expressed genes in the mutualistic association of tall fescue with *Netyphodim coenophialum*. Physiol Mol Plant Pathol 63:305–317

Kaur J, Ekanayake P, Tian P, van Zijll de JE, Dobrowolski M, Rochfort S, Mann R, Smith K, Forster J, Guthridge K, Spangenberg G (2015) Discovery and characterisation of novel asexual *Epichloë* endophytes from perennial ryegrass (*Lolium perenne* L.). Crop Pasture Sci 66:1058–1070

Khan A, Bassett S, Voisey C, Gaborit C, Johnson L, Christensen M, McCulloch A, Bryan G, Johnson R (2010) Gene expression profiling of the endophytic fungus *Neotyphodium lolii* in association with its host plant perennial ryegrass. Australas Plant Pathol 39:467–476

Kimmons CA, Gwinn KD, Bernard EC (1990) Nematode reproduction on endophyte-infected and endophyte-free tall fescue. Plant Dis 74:757–761

Knight CJ, Bailey AM, Foster GD (2009) *Agrobacterium*-mediated transformation of the plant pathogenic fungus *verticillium alboatrum*. J Plant Pathol 91:745–750

Koulman A, Lane GA, Christensen MJ, Fraser K, Tapper BA (2007) Peramine and other fungal alkaloids are exuded in the guttation fluid of endophyte infected grasses. Phytochemistry 68:355–360

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25

Leuchtmann A, Schmidt D, Bush LP (2000) Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë*/*Neotyphodium* endophytes. J Chem Ecol 26:1025–1036

Malinowski DP, Belesky DP (1999) Tall fescue aluminum tolerance is affected by *Neotyphodium coenophialum* endophyte. J Plant Nutr 22:1335–1349

Mann HB, Whitney DR (1947) On a test whether one or two random variables is stochastically larger than the other. Ann Math Stat 18:50–60

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent Pat Biotechnol 4:81–95

Meyer V, Mueller D, Strowig T, Stahl U (2003) Comparison of different transformation methods for *Aspergillus giganteus*. Curr Genet 43:371–377

Mikkelsen L, Roulund N, Lübeck M, Jensen DF (2001) The perennial ryegrass endophyte *Neotyphodium lolii* genetically transformed with the green fluorescent protein gene (*gfp*) and visualization in the host plant. Mycol Res 105:644–650

Mikkelsen L, Sarrocco S, Lübeck M, Jensen DF (2003) Expression of the red fluorescent protein DsRed-Express in filamentous Ascomycete fungi. FEMS Microbiol Lett 223:135–139

Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, Shaw PD, Marshall D (2013) Using tablet for visual exploration of second-generation sequencing data. Brief Bioinform 14:193–202

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91:173–180

Oliveira JM, van der Veen D, de Graaff LH, Qin L (2008) Efficient cloning system for construction of gene silencing vectors in *Aspergillus niger*. Appl Microbiol Biotechnol 80:917–924

Panaccione DG, Johnson RD, Wang J, Young CA, Damrongkool P, Scott B, Schardl CL (2001) Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. Plant Biol 98:12820–12825

Philippe G (2016) Lolitrem B and indole diterpene alkaloids produced by endophytic fungi of the genus *Epichloë* and their toxic effects in livestock. Toxins 8:1–16

Philipson MN, Christey MC (1986) The relationship of host and endophyte during flowering, seed formation and germination of *Lolium perenne*. NZ J Bot 24:125–134

Posselt UK, Barre P, Brazauska G, Turner LB (2006) Comparative analysis of genetic similarity between perennial ryegrass genotypes investigated with AFLPs, ISSRs, RAPDs and SSRs. Czech J Genet Plant Breed 42:87–94

Rasmussen S, Parsons AJ, Newman JA (2009) Metabolomics analysis of the *Lolium perenne-Neotyphodium lolii* symbiosis: more than just alkaloids? Phytochem Rev 8:535–550

Ravel C, Michalakis Y, Charmet G (1997) The effect of imperfect transmission on the frequency of mutualistic seed-borne endophytes in natural populations of grasses. Oikos 80:18–24

Reddy P, Deseo MA, Forster JW, Rochfort S, Spangenberg GC (2015) Isolation of secondary metabolites from perennial ryegrass-endophyte associations. In: Proceedings of the 9th International Symposium on Fungal Endophytes of Grasses and 1st International Symposium on Plant Microbiomes, Melbourne, Australia, 28th September–1st October 2015

Rowan DD (1993) Lolitrems, peramine and paxilline: Mycotoxins of the ryegrass/endophyte interactions. Agric Ecosyst Environ 44:103–122

Rowan D, Dymock J, Brimble M (1990) Effect of fungal metabolite peramine and analogs on feeding and development of argentine stem weevil (*Listronotus bonariensis*). J Chem Ecol 16:1683–1695

Schardl CL, Phillips TD (1997) Protective grass endophytes - where are they from and where are they going. Plant Dis 81:430–438

Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol 55:315–340

Schardl CL, Young CA, Faulkner JR, Florea S, Pan J (2012) Chemotypic diversity of *Epichloë*, fungal symbionts of grasses. Fungal Ecol 5:331–344

Schardl CL, Florea S, Pan J, Nagabhyru P, Bec S, Calie PJ (2013a) The E*pichloae*: alkaloid diversity and roles in symbiosis with grasses. Curr Opin Plant Biol 16:480–488. https://doi.org/10.1016/j.pbi.2013.06.012

Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ, Haws DC, Moore N, Oeser B, Panaccione DG, Schweri KK, Voisey CR, Farman ML, Jaromczyk JW, Roe BA, O'Sullivan DM, Scott B, Tudzynski P, An Z, Arnaoudova EG, Bullock CT, Charlton ND, Chen L, Cox M, Dinkins RD, Florea S, Glenn AE, Gordon A, Güldener U, Harris DR, Hollin W, Jaromczyk J, Johnson RD, Khan AK, Leistner E, Leuchtmann A, Li C, Liu J, Liu J, Liu M, Mace W, Machado C, Nagabhyru P, Pan J, Schmid J, Sugawara K, Steiner U, Takach JE, Tanaka E, Webb JS, Wilson EV, Wiseman JL, Yoshida R, Zeng Z (2013b) Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genet 9:e1003323

Scott B, Schardl CL (1993) Fungal symbionts of grasses: evolutionary insights and agricultural potential. Trends Microbiol 1:196–200

Scott B, Takemoto D, Tanaka A, Young CA, Bryant MK, May KJ (2007) Functional analysis of the *Epichloe festucae*-perennial ryegrass symbiosis. In: Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses. Grassland Research and Practice Series 13:433–441

Shafran H, Miyara I, Eshed. R, Prusky D, Sherman A (2008) Development of new tools for studying gene function in fungi based on the Gateway system. Fungal Genet Biol 45:1147–1154

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57:1036–1050

Tanaka A, Wrenn RE, Takemoto D, Scott B (2007) *Agrobacterium tumefaciens* T-DNA transformation of *Epichloë festucae*. New Zealand Grassland Association: Endophyte Symposium:469–472

van Zijll de Jong E, Dobrowolski MP, Sandford A, Smith KF, Willocks MJ, Spangenberg GC, Forster JW (2008) Detection and characterisation of novel fungal endophyte genotypic variation in cultivars of perennial ryegrass (*Lolium perenne* L.). Aust J Agric Res 59:214–221

West CP (1994) Physiology and drought tolerance of endophyte-infected grasses. In: Bacon CW, White JF (eds). CRC Press, Boca Raton

Young CA, Takach JE, Mittal S, Andreeva K, Florea S, Schardl CL (2012) Epichloae, endophytes of cool season grasses: implications, utilization and biology. In: Proceedings of the 7th International Symposium on Fungal Endophytes of Grasses, Lexington, Kentucky, USA, pp 77–83

Zhu T, Wang W, Yang X, Wang K, Cui Z (2009) Construction of two Gateway vectors for gene expression in fungi. Plasmid 62:128–133

## Affiliations

**Inoka K. Hettiarachchige[1] · Aaron C. Elkins[1] · Priyanka Reddy[1] · Ross C. Mann[1] · Kathryn M. Guthridge[1] · Timothy I. Sawbridge[1,2] · John W. Forster[1,2] · German C. Spangenberg[1,2]**

Inoka K. Hettiarachchige
inoka.hettiarachchige@ecodev.vic.gov.au

Aaron C. Elkins
aaron.elkins@ecodev.vic.gov.au

Priyanka Reddy
priyanka.reddy@ecodev.vic.gov.au

Ross C. Mann
ross.mann@ecodev.vic.gov.au

Kathryn M. Guthridge
kathryn.guthridge@ecodev.vic.gov.au

Timothy I. Sawbridge
tim.sawbridge@ecodev.vic.gov.au

[1] Agriculture Victoria, AgriBio, Centre for AgriBioscience, 3083 Bundoora, VIC, Australia

[2] School of Applied Systems Biology, La Trobe University, 3086 Bundoora, VIC, Australia