# Elucidating the molecular mechanisms of epichloae endophyte in plant protection against grass pathogens

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## GENERAL INTRODUCTION

Endophytes are bacterial (including actinomycete) or fungal microorganisms, which spend the whole or part of its life cycle colonizing the inter- and/or intracellular spaces of a healthy host plant without causing apparent symptoms of disease (Sturz et al., 2000; Wilson, 1995). The association between the endophyte and its host plant ranged from latent phytopathogenesis to mutualistic symbiosis (Carroll, 1988).

Epichloae endophytes (holomorphic *Epichloë* spp. and anamorphic *Neotyphodium* spp.) are clavicipitaceous fungi (order Hypocreales, phylum Ascomycota) that form symbioses with many cool-season grasses (Poaceae) in the subfamily Pooideae (Schardl et al., 2008; Schardl, 2010). The Pooideae-epichloae symbioses are systemic (Hinton and Bacon, 1985; White et al., 1997). These fungi are actively colonizing the intercellular spaces of aboveground host tissues such as leaf sheaths, stem, and young inflorescences and seed embryo. Endophyte hypha also colonizes the lateral buds ensuring the continued systemic maintenance of the endophyte. Roots of soil-grown infected grasses however are less favored for colonization. Endophyte hypha in colonized host tissues do not ramify further but they remain metabolically active (Herd et al., 1997)

Epichloae endophytes maintain a constitutive association with their host as they are sustained throughout the life of the host plant. The growth of endophytic hyphae is correlated with the host life cycle. The endophyte synchronizes its growth with that of the host by exhibiting an intercalary division and extension of hyphae, rather than tip growth inside the plant cells (Christensen et al., 2008). In host plant, endophytic hyphae follow a gradient with

high population in the basal regions to low population at the apical portions. For example, the leaf sheaths are highly infected from the basal ends and are greatly reduced at the apical ends.

Most *Epichloë* species and all asexual *Neotyphodium* species are vertically transmissible (Schardl, 2010). Obligately sexual *Epichloë* species transmit only horizontally and they cause a replacement disease called ‘‘choke,’’ whereby seed production of the affected inflorescences is prevented. The fungus ectophytically proliferate in the choked culms to form stromata bearing flask-shaped fruiting structures (perithecia), within which meiosis culminates in ascospore production. The mating between stromata of opposite mating type is meadiated by Dipteran insect symbionts, *Botanophila* spp. Ascospores then mediate infections of new host plants or seeds on neighboring plants (Chung and Schardl, 1997). On the other hand, obligately asexual epichloae (*Neotyphodium* species) fail to produce stromata and are vertically transmitted.

*Epichloë festucae* is associated mainly with *Festucae* spp. and *Lolium* spp. in tribe Poeae and *Koeleria* spp. (Craven et al., 2001; Leuchtmann et al., 1994; Moon et al., 2000). *E. festucae* maintains a balanced or pleiotrophic symbioses with its host grasses ( Schardl et al., 1997). It simultaneously undergoes sexual and asexual life cycles on different tillers of infected host grasses. Thus host-*Epichloë festucae* symbiota exhibit choke disease on only some of the flowering tillers, while the other tillers bear the same fungus in a completely asymptomatic association. Systemic infection extending into developing seeds vertically transmits the endophyte in maternal grass lineages (White el al., 1991).

The symbiosis between grasses and epichloae endophytes is generally considered to be mutualistic because it provides benefits to both partners. The endophyte benefits from host plants by receiving nutrients, shelter and guaranteed transmission to the next host generation.

All of these benefits are a direct consequence of the fungus's strictly symbiotic dependence, with the fungus living within the host's body. On the other hand, endophyte infection on host plant induces increased above-ground biomass, tiller number, seed production, and root growth, and stress tolerance in the grass host. Enhancement of plant tolerance to various biotic stresses constitutes the major ecological benefit that the endophyte imparts to its host. The ability of some epichloae species to protect its host plant against important grass diseases, insect and small animal herbivore, makes them agents of biological plant protection (Schardl and Phillips, 1997).

Epichloae endophytes in symbioses with their host plants are known producers of secondary metabolites that protect the host from insect herbivory. These bioprotective metabolites can be an insect feeding deterrent or toxic to insects. Approximately 45 species of insects belonging to the families Aphididae, Chrysomelidae, Cicadidae, Curculionidae, Gryllidae, Lygaeidae, Miridae, Noctuidae, Pyralidae, Scarabaeidae, and Tenebrionidae are deterred by the *Neotyphodium* and *Epichloë* -infected grasses (as reviewed in Kuldau and Bacon, 2008). Pyrrolopyrazine alkaloid peramine, ergot alkaloid ergovaline and pyrrolizidine loline alkaloids are potent deterrents of insect feeding (Ball et al., 1995; Rowan et al., 1986; Siegel et al., 1990; Wilkinson et al. 2000). Considerable evidences are available showing that a specific alkaloid may singly responsible or may act synergistically with other alkaloids in insect toxicity. Likewise, production of janthitrems by some strains of *Neotyphodium* in perennial ryegrasses also contributed to insect toxicity (Tapper and Lane, 2004). The genetic evidence that the fungal produced secondary metabolite, peramine, protects a perennial ryegrass from insect herbivory by Argentine stem weevil, *Listronotus bonariensis,* has been demonstrated (Tanaka et al., 2005).

Conversely, some epichloid alkaloids have been reported to deter feeding by mammalian herbivore. The antiherbivore effects of the symbiosis are due to the endophyte synthesis of lolitrem B and ergovaline, which are mammalian toxins. Ergovaline is a member of the ergot alkaloid group of mammalian toxins. Although loline alkaloids are most active as toxins and feeding deterrents for a range of insect herbivores, there are reports that some lolines may deter feeding by mammalian herbivore (Bush et al., 1997). In contrast, peramine has not been linked with mammalian toxicity and its primary effect is as an insect feeding deterrent (Rowan et al., 1986).

Protection of the symbiota from infecting pathogens is also one of the reported benefits from this mutualist association, although the effect of epichloae endophytes in disease resistance and susceptibility is poorly understood. Moreover, the reported studies of the biological control effect of endophyte on plant disease suppression showed mixed and inconsistent results. There are studies that present contradictory findings about the contribution of epichloae endophyte in host disease resistance. Endophyte-infected grasses were reported to have an enhanced disease resistance against *Rhizoctonia zeae* (Gwinn and Gavin, 1992), *Sclerotinia homoeocarpa* (Clarke et al., 2006), *Fusarium poae* (Panka et al., 2013), *Puccinia coronuta* (Panka et al., 2004), and *Laetisaria fuciformis* and *Limnomyces roseipellis* (Bonos et al., 2005). On the contrary, Welty et al. (1991) had demonstrated that the endophyte infection did not influence stem rust (*Puccinia graminis*) infection to seedlings of tall fescue grown in a greenhouse. Likewise, plants infected with *N. uncinatum* showed increase winter damage of meadow ryegrass (Wäli et al., 2006). A similar increase in disease incidence from *Drechslera* spp. was observed in *N. uncinatum*- infected meadow fescue (Panka et al., 2004).

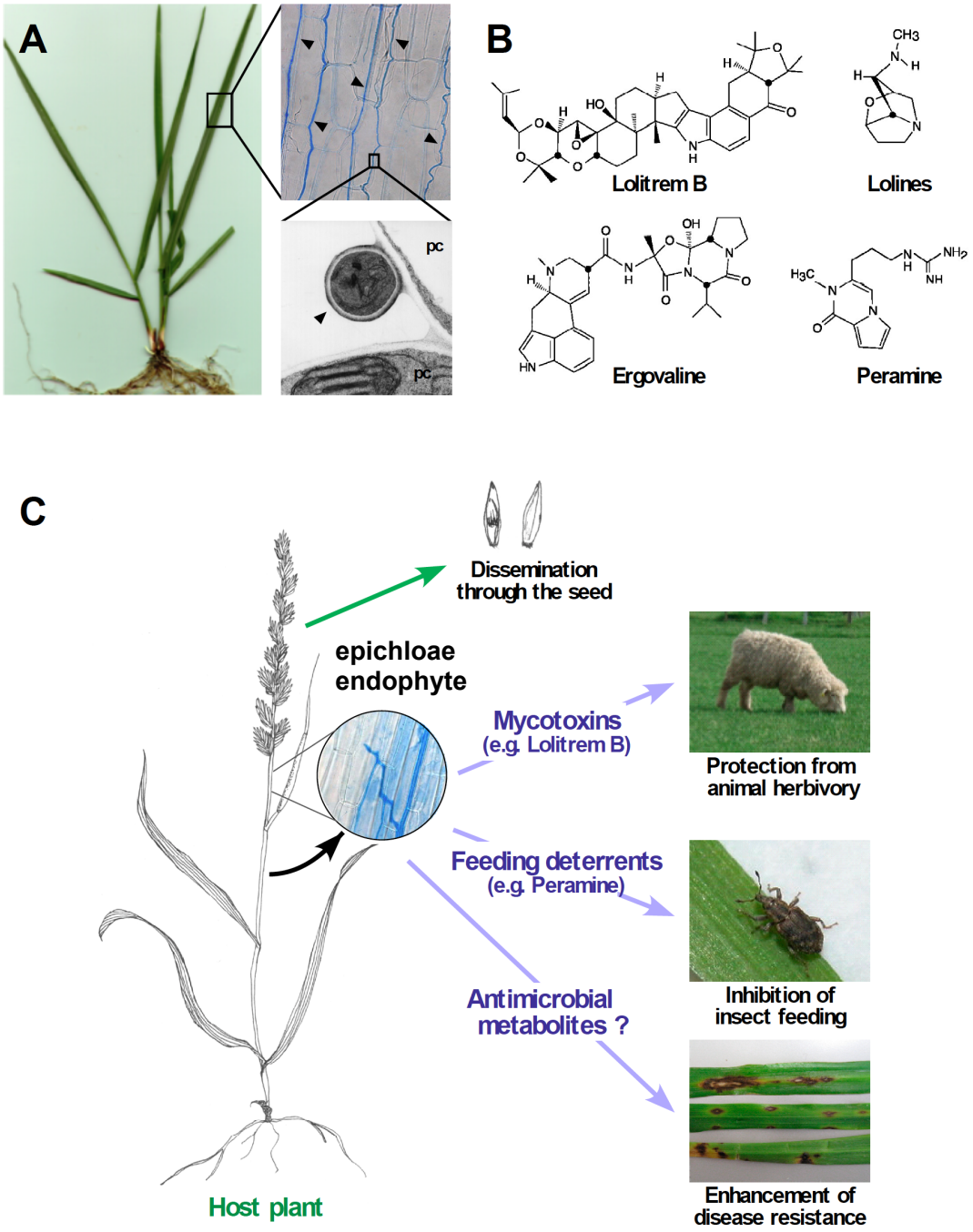



**Fig. 1 (a)** Colonization of *Epichloë festucae* in the intercellular space of host plant perennial ryegrass. **(b)** Secondary metabolites produced by *E. festucae* in host plant. **(c)** Symbiotic interaction between epichloae endophyte and host grass plant. Known benefits for the endophyte (green arrow) and to the host plant (purple arrows) in epichloae-grass symbiosis.

However, in most reported cases of disease suppression or disease enhancement as a result of endophyte-pathogen interaction, specific mechanisms for the observed results were not clarified.

Several mechanisms have been proposed on how epichloae endophytes enhance disease resistance. One is the induction of plant systemic resistance by the endophyte (Nan and Li, 2000; Panka et al., 2013). Tian et al. (2007) reported that endophyte-infected plants have higher defense response enzymes than those without endophytes, demonstrating that the endophyte could apparently induce the plant to produce more enzymes under disease pressure. Alternatively, endophyte-mediated disease suppression effect could involve niche exclusion. Epiphyllous mycelial nets observed in some endophyte-grass associations may play a role in defense against pathogens by niche exclusion (Moy et al., 2000).

Another mechanism of disease control by endophytic symbiont is antibiosis mediated by nonspecific metabolite of endophyte origin. Similar to the production of anti-insect metabolites, epichloae endophytes produce antifungal substances inhibiting growth of some pathogens on culture media (White and Cole, 1985). Siegel and Latch (1991) reported the variation of the inhibitory activity of endophytes in culture, depending on pathogen species and endophytes species combination. Specific chemicals such as indole derivatives, a sesquiterpene, a diacetamide, and a cyclic peptide have been identified from cultures of endophytes as potential fungal inhibitors (Seto et al., 2007; Yue et al., 2000). Alkaloids, on the other hand, have not been associated with the antifungal activity of epichloae endophytes in axenic culture (Siegel and Latch, 1991). Nonetheless, association of disease resistance to specific chemical components has not been established *in planta*.

The three studies presented in this manuscript aim to elucidate the molecular mechanism of plant protection, particularly the production of inhibitory compound, by the *Epichloë festucae* in symbioses with perennial ryegrass (*Lolium perenne*).

In Chapter 1, the inhibitory activity of 14 *E. festucae* isolates, obtained from different temperate grass species and cultivars from different countries, against eight grass pathogens was assessed, *in vitro*. Among the endophyte isolates evaluated, *E. festucae* E437 isolate was identified to be the most active producer of inhibitory metabolite effective to most of the test pathogens on PDA medium. As fungal antagonist, *E. festucae* E437 isolate did not initiate hyphal cell lysis of affected pathogen but interfered with its active apical hyphal growth and differentiation activity. Moreover, perennial ryegrass (*Lolium perenne*) infected with *E. festucae* E437 exhibited reduced grass leaf spot disease symptoms caused by *Drechslera erythrospila.*

In Chapter 2, the gene involved in the production of anti-fungal compound in *E. festucae* isolate E437 was identified. Through plasmid insertion mutagenesis, one mutant that lost antifungal activity against a leaf spot pathogen *D. erythrospila* was identified, isolated and functionally characterized. Analysis of the disrupted gene in the genome of the isolated mutant identified VibA, a homologue of a transcription factor for fungal heterokaryon incompatibility. Deletion mutant of *vibA* lost its antifungal activity against grass pathogens, whereas a non-antifungal endophyte isolate obtained antifungal activity by enhanced expression of *vibA*.

In Chapter 3, the partial purification of the inhibitory substance produced by the bioprotective E437 isolate of *E. festucae* was done. A methanol-insoluble layer of the culture

filtrate of *E. festucae* showed inhibitory activity against conidial germination of *D. erythrospila*.

# CHAPTER 1

## Characterization of *Epichloë festucae* isolates as potential biocontrol agent against grass pathogens

### INTRODUCTION

In Pooideae-epichloae symbioses, the fungal endophytes benefit from its host plant by accessing nutrients from the host apoplast and by acquiring a means of dissemination through the host seeds. On the other hand, the host plant benefits from the symbiosis through improved nutrient acquisition and tolerance to biotic and abiotic stresses. Grasses infected with epichloae endophyte have shown enhanced resistance to drought (Malinowski et al., 1997; Malinowski and Belesky, 2000), diseases (Kuldau and Bacon, 2008; Latch, 1993) and insect herbivory (Clay et al., 1993; Shiba and Sugawara, 2005; Shiba et al., 2011; Tanaka et al., 2005; Wilkinson et al., 2000). The ability of some epichloae species to protect its host plant against important grass diseases, insect and small animal herbivore, makes them agents of biological plant protection (Schardl and Phillips, 1997).

Protection of the host plant from insect herbivory is the best-characterized benefit from infection by epichloae endophytes. Over 40 invertebrate pests, including insects, mites and nematodes were deterred by grasses infected with epichloae endophytes (Kuldau and Bacon, 2008). Some strains of epichloae endophytes produce anti-insect alkaloids that protect its host plant from insect and small animal herbivory (Bush et al., 1997). Peramine and lolines are well-known epichloid alkaloids reported to have anti-insect activities (Schardl et al., 2006; Shiba and Sugawara, 2005; Wilkinson et al., 2000). Moreover, some strains of epichloae

endophytes produced janthitrems, an indole diterpene, which can be toxic to a wide range of insects (Tapper and Lane, 2004).

While protection of the symbiota from insect and small animal herbivory is the best-studied benefit from this association, the role of epichloae endophytes in plant protection against grass pathogens is poorly understood. There are studies that show contradictory findings about the contribution of epichloae endophyte in host disease resistance. While several reports indicate the enhanced resistance of endophyte-infected grasses against fungal pathogens such as *Sclerotinia homoeocarpa*, *Laetisaria fuciformis*, *Limnomyces roseipellis* and *Rhizoctonia zeae* (Bonos et al., 2005; Clarke et al., 2006; Gwinn and Gavin, 1992), other reports showed no positive effects of endophyte infection on the protection of host plant from fungal diseases (Pańka et al., 2004; Wäli et al., 2006; Welty et al. 1993). Similar to the insect deterrent activity of endophytes which varies between the insect species and the endophyte strains producing different anti-insect metabolites (Kuldau and Bacon 2008), the inconsistent effect of endophyte infection on host disease resistance could be explained by the diverse characteristics of endophyte strains and target pathogens used in previous studies. However, in most reported cases of disease suppression as a result of endophyte-pathogen interaction, specific mechanisms for the observed results were not clarified.

Production and release of antimicrobial compound is one of the reported mechanisms of fungal growth suppression by the endophytes. Siegel and Latch (1991) reported the antifungal activity in culture medium by isolates of grass endophytes. Yue et al. (2000) identified indole derivatives, a sesquiterpene, and a diacetamide from cultures of clavicipitaceous endophytes as potential fungal inhibitors. Nonetheless, in the aforementioned studies, whether those antifungal compound-producing endophyte strains provided plant host

protection against pathogenic fungi was not established. Thus the role of these endophytes that produce bioprotective metabolite in plant disease suppression remains to be elucidated.

Fungi utilized in biocontrol such as the *Trichoderma* species commonly produce cell wall degrading enzymes (Benítez et al., 2004; Kredics et al., 2005). Epichloae endophytes have been reported to secrete hydrolytic enzymes in culture and in endophyte-infected plants (Lam et al., 1995; Reddy et al., 1996). However, the association between the production of cell lytic enzymes and the antifungal activity of epichloae endophyte has not been examined. In general, the behavior of epichloae endophytes as fungal antagonist or how they act on pathogen has not been investigated.

The objectives of this chapter are to assess the *in vitro* inhibitory activity of endophyte isolates against grass pathogens, determine how an *E. festucae* isolate behaves as fungal antagonist and establish the role of an endophyte isolate producing bioprotective metabolite in plant disease suppression.

## MATERIALS AND METHODS

### Biological materials.

Thirteen *Epichloë festucae* strains isolated from meadow fescue (*Lolium pratense*), giant fescue (*L. giganteum*), prairie Junegrass (*Koeleria cristata*), red fescue (*Festuca rubra*), hard fescue (*F. longifolia*), Wallis fescue (*F. valesiaca*), soft fescue (*F. pulchella*) or *Lolium L. spp.* were kindly provided by Prof. Christopher L. Schardl (University of Kentucky, USA) (Table 1). The *E. festucae* isolate Fl1 isolated from *F. trachyphylla* was kindly provided by Prof. Barry Scott (Massey University, New Zealand). All endophyte isolates were grown on potato dextrose agar (PDA) at 23˚C or kept in 4˚C until use. *Drechslera erythrospila* (isolate 638, MAFF No. 305378), *D. siccans* (isolate 962, MAFF No. 305397), *D. dictyoides* (isolate 963, MAFF No. 305398), *Colletotrichum graminicola* (isolate PR-1, MAFF No. 306600), *Bipolaris sorokiniana* (MAFF No. 511217), *Sclerotinia homoeocarpa* (isolate SU16-3, MAFF No. 236941), *Rhizoctonia solani* (isolate 1374, MAFF No. 511374) and *Magnaporthe grisea* (isolate WK3-1) were used as test organisms. Seven of the test fungal grass pathogens were acquired from the collection of the National Institute of Agrobiological Sciences (NIAS, Japan), while the *M. grisea* isolate was kindly provided from the collection of Prof. Yukio Tosa (Kobe University, Japan). All fungal pathogens used in this study were grown on PDA at 23˚C and maintained at 4˚C until use.

Inoculation of endophyte-free seedlings of perennial ryegrass with *E. festucae* strains was performed by the method of Latch and Christensen (1985). Endophyte free seeds of perennial ryegrass cultivar ‘Yatsukaze 2’ were surfaced sterilized and germinated at 22ºC for 1 week in the dark on 3% (w/v) water agar. A small piece of mycelium from the edge of a 1-week old colony was inserted into a slit between the mesocotyl and coleoptile of each seedling. The inoculated seedlings were incubated at 22∞C for 5–7 days in the dark, followed

by 5–7 days in the light. The plants were transferred into root trainers containing commercial potting mix and maintained in the greenhouse for 5–6 weeks before being tested for endophyte infection. The presence of endophyte was confirmed by light microscopic examination of epidermal strips from the outer most leaf sheath stained with aniline blue. Hyphae within epidermal strips were examined using a compound microscope at 100x and 400x magnification. Endophyte- infected or endophyte –free perennial ryegrass (*L. perenne* cv. Yatsukaze 2) was grown in an environmentally controlled growth room at 23˚C with 16/8h light/dark photoperiod.

***In vitro* assay for growth inhibition of fungal pathogens by *E. festucae* isolates.**

A mycelial block of each *Epichloë* isolate (3 mm diameter) was placed on one side of a PDA plate. Fungal endophyte colonies were allowed to grow to a diameter of 10-12 mm for 10 to 21 days before a mycelial plug of grass pathogen (3 mm diameter) was placed at the other side of the PDA plate. The culture was incubated at 23°C for 7-14 days (75-100% of the control plate covered by mycelia of the pathogen) or until either a clear zone of inhibition was observed or the colonies of the two fungi had made contact. Inhibition was determined by measuring the clear zone between the *E. festucae* and the grass pathogen colonies. The dual culture assay for each *E. festucae* isolate and grass pathogen combination was repeated at least three times.

**Inoculation of perennial ryegrass with *D. erythrospila.***

Leaves of perennial ryegrass at approximately 6 cm in length were placed in Petri dishes lined with filter paper moistened with 0.2 μg/ml kinetin. A 10 μl of conidial suspension of *D. erythrospila* (4 x $10^4$ conidia/ml) was inoculated on the abaxial surface of

the leaves. At least five leaf pieces for each treatment were placed in Petri dishes and incubated at 25°C on a 17/7h light/dark cycle. The length of lesions developed on detached leaves was measured 7 days after inoculation. The experiment was repeated at least three times.

**Detection of extracellular protease activity of epichloae isolates.**

To detect extracellular protease activity from the epichloae isolates, a 3 mm mycelial plug from each isolate was separately placed in the center of PDA plate supplemented with 1% (w/v) gelatin (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 23°C (Vermelho et al., 1996). After 10 days of incubation, Petri dishes were stained with 0.1% amido black 10B (Wako Pure Chemicals, Osaka, Japan) for 1 h at 28°C. Extracellular protease activity was detected as a visible halo around the colony of endophyte isolates. The proteinase activity was scored as grade - when no visible halo was present, grade + when a visible proteolysis was limited to <2 mm around the colony, and grade ++ when the zone of proteolysis was 2-4 mm from the margin of the colony.

**Detection of extracellular endoglucanase activity of epichloae isolates.**

A simple endoglucanase detection technique (Sazci et al., 1986) was applied to determine the endoglucanase production activity of the *E. festucae* isolates. A 3 mm mycelial plug of *E. festucae* isolate was placed in a PDA plate containing 1% carboxymethyl cellulose (CMC) (Sigma, St. Louis, MO, USA) and incubated at 23°C. Five days after incubation, Petri dishes were flooded with an aqueous solution of 1% Congo red (Sigma, St. Louis, MO, USA) and agitated at 50 rpm for 15 min. The Congo red solution was then poured off and then the plates were flooded with 1N NaOH. The stained culture plates were then again agitated at 50

rpm for 15 min. This slightly changed the dye color to brownish-red and the enzymatic activity inhibited (Sazci et al., 1986). Extracellular endoglucanase activity was detected as a visible halo around the colony of the endophyte isolates. The hydrolysis activity was scored as grade - when no visible zone of hydrolysis was present, grade + when a visible zone of hydrolysis was limited to <2 mm around the colony, and grade ++ when the zone of hydrolysis was 2-4 mm from the margin of the colony.

**Microscopy.**

Selected *E. festucae* isolate and grass fungal pathogen were subjected to dual culture assay described above. When a clear zone of inhibition was evident, thin mycelial blocks of endophyte and fungal pathogen, directly growing against each other, were selected, excised and stained with calcofluor white staining solution (Fluka, Taufkirchen, Germany), a fluorochrome that binds to polysaccharides. The hyphal morphology of endophyte and pathogens were visualized using a confocal laser scanning microscope FV1000-D (Olympus, Tokyo, Japan). The laser was used as the excitation source at 405 nm, and fluorescence was recorded between 425 nm and 475 nm. The number of hyphal tip with or without stain accumulation was counted from at least 150 hyphal tips.

**Treatment of conidial suspension of *D. erythrospila* with culture filtrate of endophyte isolates.**

A mycelial block (1 x 1 cm) of *Epichloë* isolate grown on PDA was finely chopped and then inoculated in 100 ml flasks containing 50 ml potato dextrose (PD) broth. The flasks were kept in an orbital shaker (100 rpm) at 23°C. Depending on the isolate, culture filtrates were collected from five to seven days after endophyte inoculation. The culture filtrates were

then sterilized using a filter membrane unit (Millex-HA filter unit, 45 µm pore diameter, Millipore, Billerica, MA, USA).

Conidial suspension of *D. erythrospila* was prepared by the method previously described by Paul (1972) and Cromey and Cole (1984). Mycelial plug of *D. erythrospila* grown on PDA was inoculated on sterile ryegrass leaf pieces placed on the surface of tap water agar plates. Plates were incubated at 25˚C with a cycle of twelve hours white light (cool white fluorescent tube) supplemented with near ultraviolet light followed by twelve hours of dark condition. One to two weeks after inoculation, sporulating leaf pieces were transferred in 2 ml tubes and suspended in 1 ml of sterile distilled water. The tubes were agitated with a mechanical vibrator to dislodge the conidia and then leaf pieces were removed. The collected spore suspension was centrifuged at 8,000 rpm for 1 min, and resuspended in distilled water to give a final concentration of 4 x $10^4$ conidia/ml.

Twenty µl of endophyte culture filtrate was added into a sterile biconcave microscope slide and mixed with 5 µl of conidial suspension of *D. erythrospila*. In the control test, conidial suspension was mixed with sterile PD broth. All treatments were replicated three times. The slides were placed in Petri dishes kept at 25°C. After twelve hours, conidial germination was determined and scored from at least 150 conidia per treatment. The replicated experiment was repeated three times.

**Evaluation of the physicochemical properties of antifungal substance produced by *E. festucae* E437 isolate.**

*E. festucae* E437 isolate was cultured in 2.4% potato dextrose broth for 7 days and culture filtrate was collected as described above. Culture filtrate was subjected to different conditions such as incubated at -80˚C overnight, at 95˚C for 10 min and dialyzed overnight

against water using a dialysis tube (Snakeskin dialysis Tubing, Thermo Fisher Scientific, Waltham, MA, USA) with 3500 MWCO (molecular weight cut off). The effect of the treatments of endophyte culture filtrate on conidial germination of *D. erythrospila* was determined as described above.

**Statistical analysis.**

Statistical analysis was performed to identify significant differences between samples using statistical analysis package software CropStat ver. 7.2.3 (International Rice Research Institute, Los Baños, Philippines).

## RESULTS

***In vitro* growth inhibition of fungal pathogens by *E. festucae* isolates.**

Growth inhibitory activity of 14 strains of *E. festucae*, isolated from different temperate grass species and cultivars originated from various geographic locations (Table 1), were assayed *in vitro* against several grass pathogens, including *D. erythrospila*, *D. siccans*, *D. dictyoides*, *C. graminicola*, *B. sorokiniana*, *S. homoeocarpa*, *R. solani* and *M. grisea*. Results of dual culture assay showed differences in the spectrum and degree of antifungal activity by the endophyte isolates (Table 2). The antifungal activity of *E. festucae* isolates ranged from non-inhibition of any of the test pathogens to inhibition of at most five fungal pathogens, as in the case of E437 isolate. *E. festucae* E437 isolate inhibited mycelial growth of *D. erythrospila*, *D. siccans*, *D. dictyoides*, *C. graminicola* and *B. sorokiniana* (Fig. 1a-e). *E. festucae* isolates E1017, E186 and E364 had a narrow spectrum of activity inhibiting only

**Table 1.** *Epichloë festucae* isolates used in this study.

| Isolate | Original host | Geographical origin |
|---|---|---|
| E1017 | *Lolium pratense* | Switzerland |
| E1139 | *Lolium giganteum* | Switzerland |
| E1157 | *Koeleria cristata* | France |
| E1168 | *Festuca rubra var* | New Zealand |
| E186 | *Festuca rubra var* | USA |
| E189 | *Festuca rubra var* | USA |
| E2368 | *Lolium L. spp.* | Europe [a] |
| E364 | *Festuca longifolia* | Switzerland |
| E365 | *Festuca longifolia* | Switzerland |
| E435 | *Festuca valesiaca* | Switzerland |
| E437 | *Festuca pulchella* Schrad | Switzerland |
| E498 | *Festuca rubra L.* | Switzerland |
| E903 | *Festuca rubra L.* | Switzerland |
| Fl1 | *Festuca trachyphylla* | Europe [b] |

[a] Wilkinson et al. 2000. [b] Young et al. 2005

**Table 2.** Inhibition of growth of grass pathogens by *E. festucae* isolates in a dual-culture assay.

| *Epichloë festucae* isolate | *Drechslera erythrospila* | *Drechslera siccans* | *Drechslera dictyoides* | *Rhizoctonia solani* | *Bipolaris sorokiniana* | *Magnaporthe grisea* | *Sclerotinia homoeocarpa* | *Colletotrichum graminicola* | Number of pathogens inhibited |
|---|---|---|---|---|---|---|---|---|---|
| E1017 | - | - | - | - | - | - | - | + | 1 |
| E1139 | - | - | - | - | - | - | - | - | 0 |
| E1157 | - | - | - | - | - | - | - | - | 0 |
| E1168 | - | - | - | - | - | - | - | - | 0 |
| E186 | - | - | - | - | - | - | - | + | 1 |
| E189 | - | - | - | - | - | - | - | - | 0 |
| E2368 | - | - | - | - | - | - | - | - | 0 |
| E364 | - | - | - | - | - | - | - | + | 1 |
| E365 | - | - | - | - | - | - | - | - | 0 |
| E435 | - | - | - | - | - | - | - | - | 0 |
| E437 | ++ | ++ | ++ | - | + | - | - | + | 5 |
| E498 | - | - | - | - | - | - | - | - | 0 |
| E903 | - | - | - | - | - | - | - | - | 0 |
| Fl1 | - | - | - | - | - | - | - | - | 0 |

Degree of inhibition was scored as region of no fungal growth between the endophyte and pathogen colonies: - , no inhibition; +, <2 mm; ++, 2-4 mm. Growth of grass pathogen colony without endophyte was >80% of the surface area of PDA plate.

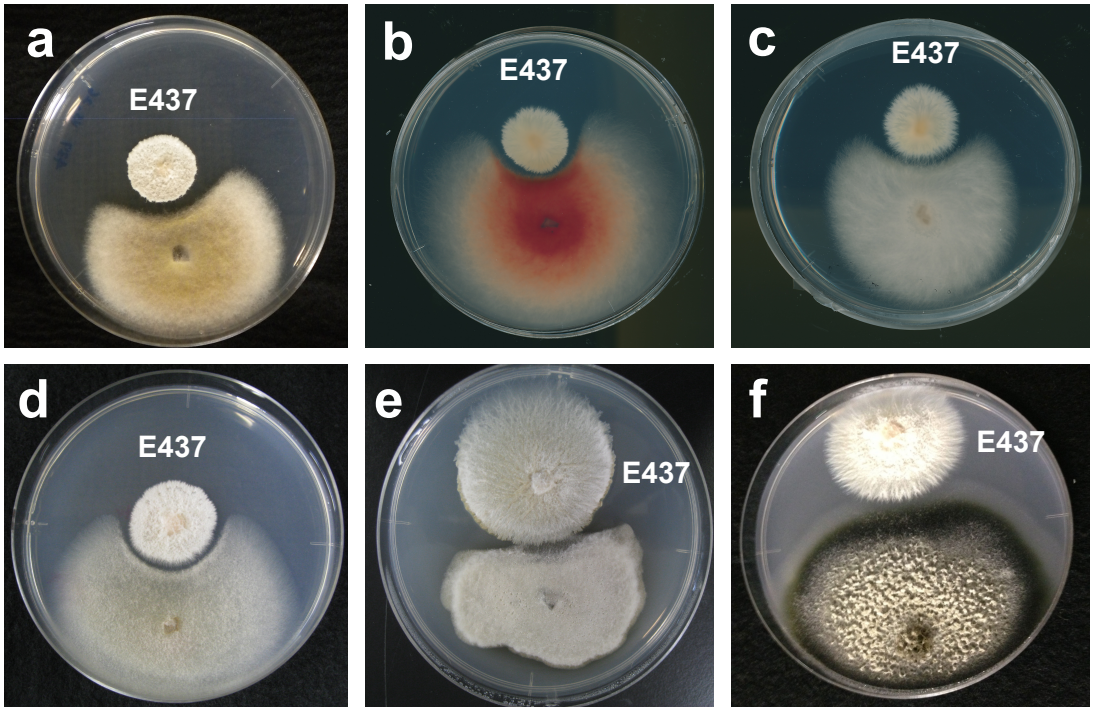



**Fig. 1** Effect of *Epichloë festucae* E437 isolate on mycelial growth of grass pathogens *Drechslera erythrospila* (**a**)*, D. siccans* (**b**), *D. dictyoides* (**c**), *Colletotrichum graminicola* (**d**), *Bipolaris sorokiniana* (**e**) and *Magnaporthe grisea* (**f**) in PD agar plate culture. Colonies of E437 isolate were grown to a diameter of 10-12 mm and a mycelial plug of grass was inoculated. The culture was incubated at 23°C for 7-14 days until either a clear zone of inhibition was observed or the colonies of the two fungi had made contact.

the mycelial growth of *C. graminicola* (Table 2). The degree of antifungal activity of a particular *E. festucae* isolate also differed depending on the fungal pathogen evaluated. The zone of inhibition between E437 and three species of *Drechslera* measured from 2 to 4 mm while it was less than 2 mm for *C. graminicola* and *B. sorokiniana.* A variation of growth inhibitory activity among the endophyte strains isolated from the same grass species and host plant's geographic locations was also observed (Tables 1 and 2). Isolates E364 and E365 were both isolated from *Festuca longifolia* and both originated from Switzerland, however, only E364 exhibited inhibitory activity against *C. graminicola*. Moreover, E186, E364 and E437 were isolated from different grass species of the *Festuca* genus and these endophyte isolates demonstrated varied degrees of inhibitory activity against test grass pathogens.

*C. graminicola* appeared to be the most sensitive to the presence of endophyte as indicated by the number of *E. festucae* isolates that could inhibit the mycelial growth of the pathogen. On the other hand, none of the endophyte isolates tested could inhibit the mycelial growth of *S. homoeocarpa*, *R. solani* and *M. grisea* (Table 2, Fig. 1f). The mycelial colonies of *S. homoeocarpa* and *R. solani* grew fast on PDA and completely covered the endophyte colony 4-5 days after the inoculation of pathogens.

**Effect of *E. festucae* infection on disease symptom of perennial ryegrass caused by *D. erythrospila.***

Detached leaves of perennial ryegrass without endophyte infection or infected with *E. festucae* E437 or Fl1 were inoculated with conidial suspension of *D. erythrospila*. Seven days after the inoculation, the detached leaves of perennial ryegrass, regardless of endophyte status, exhibited leaf spot lesions over the inoculated area (Fig. 2a). However, lesion sizes on E437-inoculated leaves were consistently smaller than those on endophyte-free leaves in three

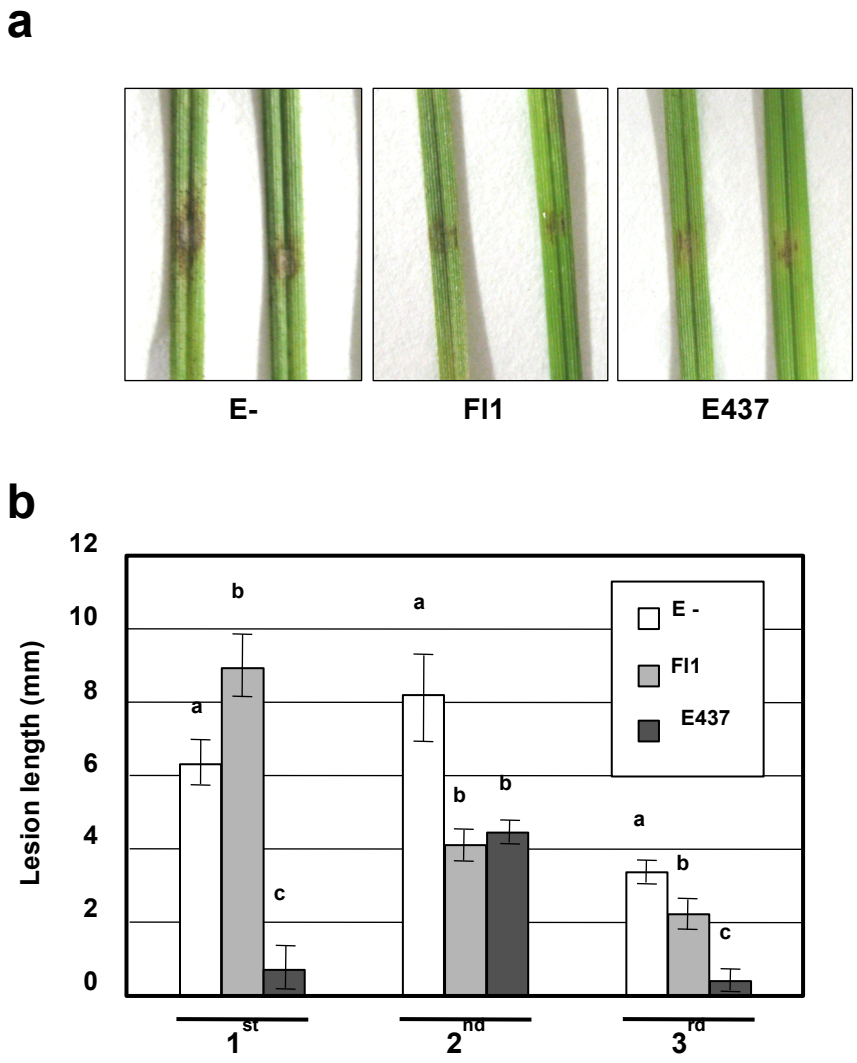



**Fig. 2** Effect of endophyte infection on disease symptom caused by *Drechslera erythrospila*. **(a)** Leaf spot symptoms on endophyte-infected and endophyte-free leaves 7 days after inoculation with *D. erythrospila.* **(b)** Lesion length (mm) of perennial ryegrass leaves infected with *Epichloë festucae* Fl1 or E437 isolate, or endophyte free (E -) perennial ryegrass, 7 days after inoculation with conidial suspension of *D. erythrospila*. The results of three individual experiments are presented (labeled as 1st, 2nd and 3rd). Data are means ± standard error from 5 detached leaves used in each experiment. Different letters indicate significant differences ($p$<0.05). Differences between treatment mean values were compared using least significant difference (LSD) test at $p$<0.05 level, generated using CropStat software.

separate experiments (Fig. 2b). Infection of E437 had a positive effect on reduction of leaf spot disease caused by *D.* erythrospila on host plant. In contrast, though infection of Fl1 reduced disease symptom in 2 out of 3 trials, effect of Fl1-infection on the development of disease symptom was rather inconsistent.

**Protease and endoglucanase production activity of *E. festucae* isolates.**

To verify the relationship between cell lytic enzymes productivity and antifungal activity of *E. festucae*, the ability of the *E. festucae* isolates to produce extracellular proteases was detected on PDA medium with gelatin as substrate. The activity of extracellular proteases was detected as a visible halo around the colony of *E. festucae* isolates. Among the 14 endophyte isolates we tested, only isolates E186, E364, E365 and E437 exhibited proteolytic activity (Table 3, Fig. 3a). The degree of proteolytic activity of these isolates was similar, with 2-4 mm halo observed around the colony. Endophyte isolates producing protease can inhibit mycelial growth of at least one of the test pathogens, except E365. Endoglucanase activity of *E. festucae* isolates was also determined on PDA containing CMC. Staining using Congo red detected lysis of CMC around colony of *E. festucae* isolates (Fig. 3b). All endophyte isolates produced zone of hydrolysis of CMC around the colony (Table 3).

**Effect of the co-culturing of antifungal *E. festucae* isolate on the hyphal growth of *D. erythrospila.***

To observe the effect of antifungal endophyte isolate on the hyphal morphology of affected pathogen species, *D. erythrospila* cultured with or without *E. festucae* isolate E437 was stained with calcofluor white, a fluorochrome that binds to polysaccharides (e.g. chitin) in the cell walls of fungi. When singly cultured E437 and *D. erythrospila* were stained with

**Table 3.** Production of extracellular protease and endoglucanase by *E. festucae* isolates.

| *E. festucae* isolate | *Protease activity* [a] | *Endoglucanase activity* [b] |
|---|---|---|
| E1017 | - | + |
| E1139 | - | + |
| E1157 | - | + |
| E1168 | - | + |
| E186 | ++ | + |
| E189 | - | + |
| E2368 | - | + |
| E364 | ++ | + |
| E365 | ++ | + |
| E435 | - | + |
| E437 | ++ | + |
| E498 | - | + |
| E903 | - | + |
| Fl1 | - | + |

[a] Degree of proteolytic activity on agar plates containing gelatin as substrate (region of visible halo around the colony): 1, no visible halo was present; +, <2 mm around the colony; ++, 2-4 mm from the margin of the colony. [b] Zone of CMC hydrolysis: +, visible halo was present around the colony.

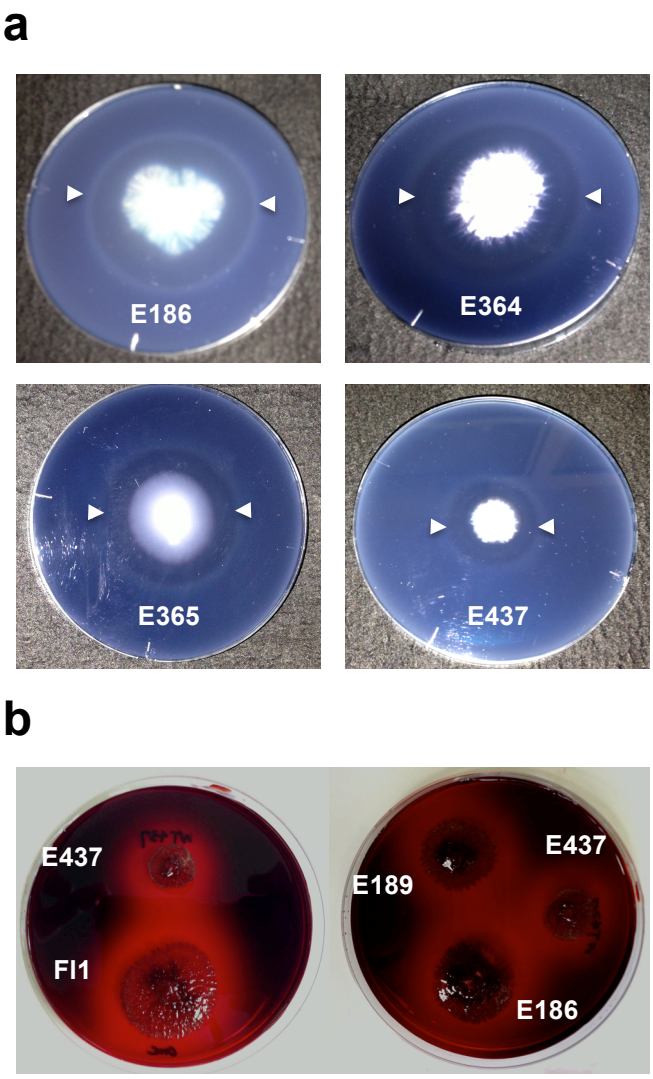



**Fig. 3** Representative data showing the detection of extracellular activity of protease **(a)** and endoglucanase **(b)** around the colony of *Epichloë festucae* isolates. **(a)** *E. festucae* isolates were grown for 10 days on PDA containing with 1 % (w/v) gelatin as substrate. Activity of extracellular proteases was detected as visible halo (indicated by arrowheads) around the colony of *E. festucae* isolates. **(b)** *E. festucae* isolates were grown on PDA plates containing 1% carboxymethyl cellulose (CMC). At five days after incubation, Petri dishes were stained with 1% Congo red. Activity of the extracellular endoglucanase was detected as visible halo around the colony of *E. festucae* isolates.

calcofluor white, accumulation of stain at the hyphal tips of the two fungi was evident, indicating the normal hyphal tip growth of fungal cells (Fig. 4). However, when the *D. erythrospila* was co-cultured with E437, the number of *D. erythrospila* mycelial tips with accumulated calcofluor white stain significantly decreased (Fig. 4b and c), whereas those of E437 remained unchanged (Fig. 4a and c). Similar effects of E437 on *C. graminicola* hyphal morphology and accumulation pattern of calcofluor white were observed, while there was no obvious effect of E437 on growth pattern of *M. grisea*.

**Effect of culture filtrate of *E. festucae* isolates on the conidial germination of fungal pathogen.**

All of the *D. erythrospila* conidia germinated within 12 hours in PD broth. In contrast, conidial germination of *D. erythrospila* was significantly inhibited in the culture filtrate of E437. When culture filtrates of other isolates of *E. festucae* were tested for their inhibitory activity, none of them could inhibit the conidial germination of *D. erythrospila.* (Fig. 5b)

**Evaluation of the physicochemical properties of antifungal substance produced by *E. festucae* isolate E437.**

Several tests were performed to characterize the antifungal compound produced by *E. festucae* E437 isolate. Culture filtrate of E437 was either incubated at -80˚C overnight, at 95˚C for 10 min or dialyzed overnight using a dialysis tube with 3500 MWCO (molecular weight cut off), and the inhibitory activity on conidial germination of *D. erythrospila* was determined. Freeze or heat treatment of culture filtrate did not significantly reduce its inhibitory activity against the test pathogen (Fig. 5c). In contrast, more than 95% of *D. erythrospila* had germinated in dialyzed E437 culture filtrate.

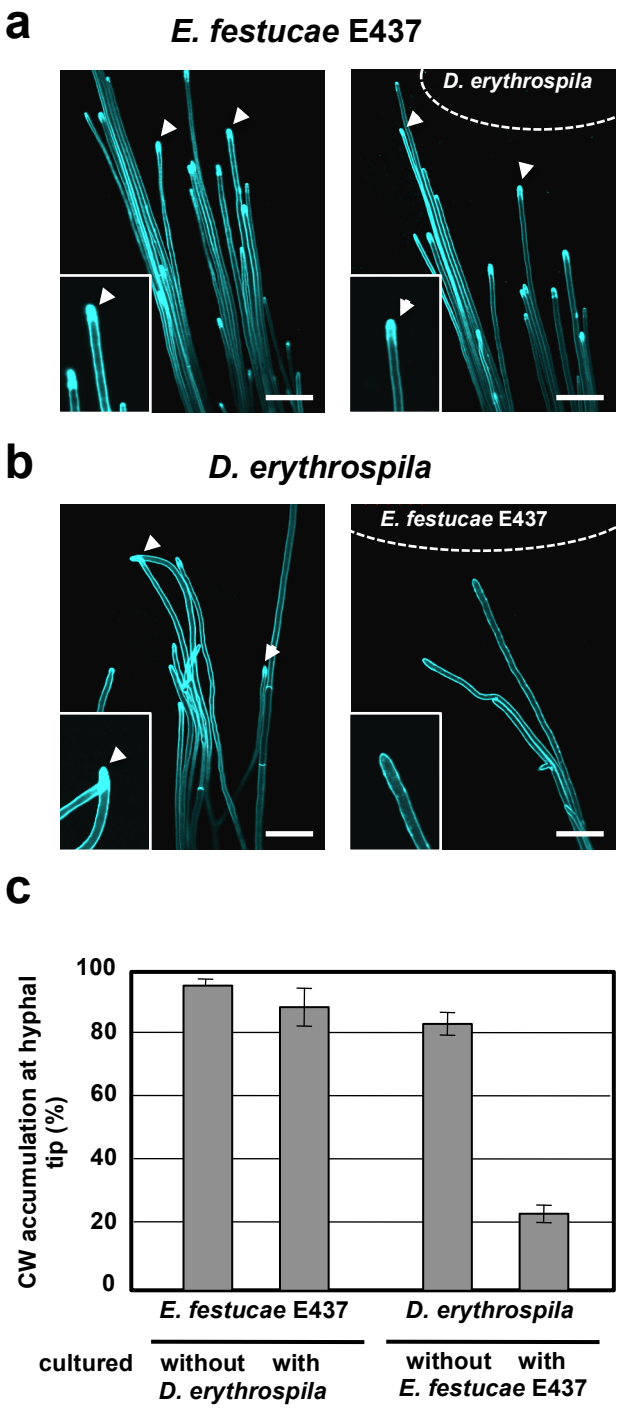



**Fig. 4** Hyphal morphology of *Epichloë festucae* E437 isolate and *Drechslera erythrospila* grown on PD agar. Hyphae were stained with calcofluor white (CW) and monitored with confocal laser microscopy. **(a)** Hyphal morphology of E437 cultured with (right) or without (left) *D. erythrospila* on PD agar. **(b)** Hyphal morphology of *D. erythrospila* cultured with (right) or without (left) E437 on PD agar. Arrowheads indicate hyphal tips with the accumulation of CW stain. Bars = 30 µm. **(c)** Effect of co-culture with E437 on the accumulation of CW stain at hyphal tips of *D. erythrospila*. The number of hyphal tips with or without accumulation of CW stain was counted using a confocal laser scanning microscope. Data are means ± standard error from at least 150 hyphal tips from 3 sites (> 50 tips/site).

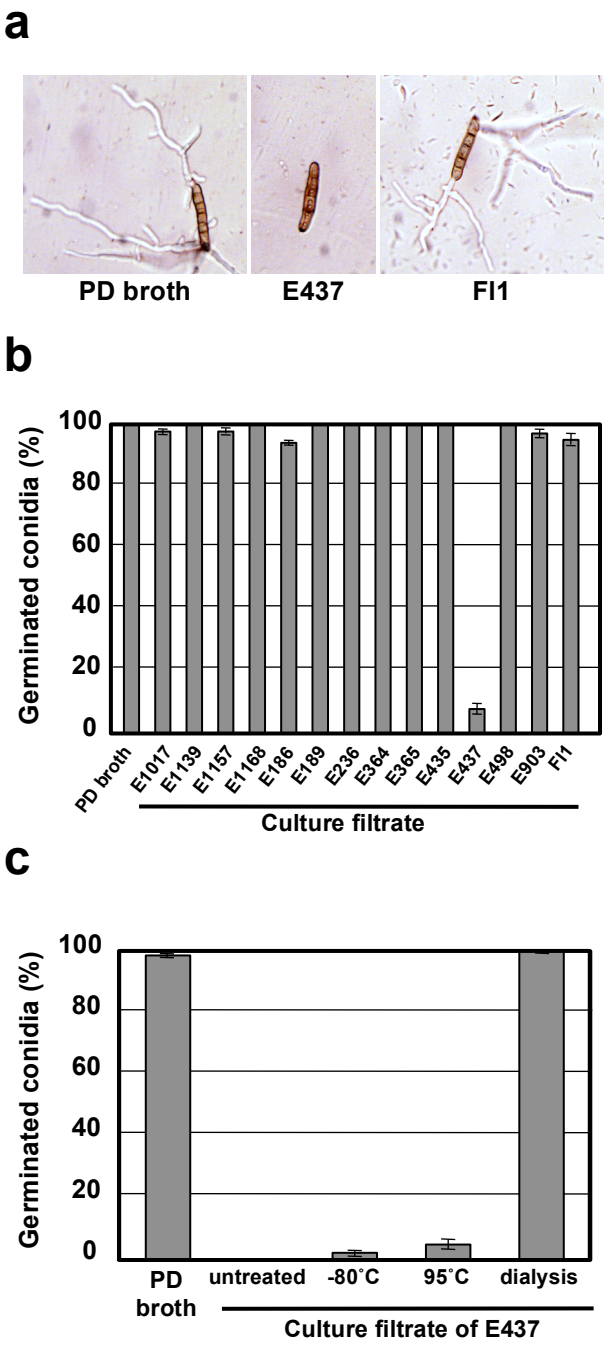



**Fig. 5** Effect of culture filtrate of endophyte isolates on conidial germination of *Drechslera erythrospila*. **(a)** Conidial suspension of *D. erythrospila* was incubated in PD broth or culture filtrate prepared from *Epichloë festucae* E437 or Fl1 isolate for 12 h. **(b)** Percentage of germinated conidia after 12 h incubation in PD broth or in culture filtrate of *E. festucae* isolates. **(c)** Effect of the various treatments on the antifungal activity of culture filtrate from E437 isolate against *D. erythrospila*. Culture filtrate of E437 was incubated in -80˚C overnight (-80˚C), in 95˚C for 10 min (95˚C) or dialyzed overnight against water using a dialysis tube with 3500 MWCO (dialysis), and mixed with conidial suspension of *D. erythrospila*. The frequency of germinated conidia was calculated from at least 150 spores.

## DISCUSSION

Dual culture assay of different isolates of *E. festucae* against several grass fungal pathogens showed differences on the range and degree of antifungal activity by the epichloae isolates. The observed range of antifungal activity depends on the endophyte isolate - grass pathogen combination (Table 2). The endophyte E437 isolate was found to be the most active producer of inhibitory metabolite effective to some of the tested pathogens on PDA medium. The three species of *Drechslera* were the most sensitive against E437, followed by *C. graminicola* and *B. sorokiniana.* Moreover, among the endophyte strains used in this study, only culture filtrate of E437 could significantly inhibit the conidial germination of *D. erythrospila* (Fig. 5b). Differences in *in vitro* antifungal activity, between and within species of clavicipitaceous endophytes have also been reported by other studies (Christensen et al. 1991; Siegel and Latch 1991). Among strains of *E. festucae*, Christensen (1996) had noted differences on the inhibitory activity of the isolates against *D. erythrospila* and *R. zeae* where the degree of inhibition varied among the two test pathogens. Similar results have been obtained by Li et al. (2007) who conducted dual-culture tests of 14 isolates of *N. gansuense* against pathogens of *Achnatherum inebrians* (drunken horse grass). They reported that not all of their *Neotyphodium gansuense* isolates could inhibit the mycelial growth and spore germination of the plant pathogenic fungi tested. The observed differences range and magnitude on inhibitory activity of test *E. festucae* strains may be explained by the inherent genetic differences among the endophyte strains, supposed to produce a range of antifungal compounds. Ren et al. (2009) have showed a difference in magnitude of inhibitory activity not only between epichloae species, but also among morphotypes of the same species. In their study, the morphological types of epichloae isolates expressing a range of antifungal activity reflect the physiological and genetic differences among the isolates. Another possible

explanation is that the quantity and type of antifungal compound produced by an endophyte isolate could be affected by different cultural or growth condition (Christensen and Latch, 1991).

*E. festucae* E437 isolate exhibited strong protease and endoglucanase activity in culture medium. The role of extracellular proteases and endoglucanases in plant protection by biological control agents, particularly of the *Trichoderma* species has been reported (de la Cruz et al., 1995; Elad and Kapat, 1999; Kredics et al., 2005). Overproduction of an inducible extracellular protease by *Trichoderma* strains resulted in an up to five-fold increase in the biocontrol efficiency against *R. solani* (Pozo et al., 2004). Similarly, overexpressing $\beta$-1,4-endoglucanase gene in *T. longibrachiatum* resulted to enhanced biocontrol activity of the transformants against *Pythium ultimum* on cucumber (Migheli et al., 1998). Unexpectedly in this study, even the epichloae isolates with no inhibitory activity to any of the tested pathogens, exhibited active endoglucanase and protease activity in culture medium (Tables 2 and 3). Although E437 isolate exhibited strong hydrolytic enzyme production activity in culture medium, hyphal cell lysis was not observed on the affected pathogen, *D. erythrospila*, when co-cultured with E437 (Fig. 4). Epichloae endophytes have been reported to secrete proteins such as fungal subtilisin-like proteinase and invertase in both culture and in endophyte-infected plants (Lam et al., 1995; Lindstrom and Belanger, 1994; Reddy et al., 1996). Moy et al. (2002) detected fungal $\beta$-1, 6-glucanase in the apoplast of an endophyte-infected grass implying its role in providing nutrition to endophyte, *in planta*. They also suggested the possible function of $\beta$-1, 6-glucanase in degrading cell walls of other fungi encountered by the endophyte within the plant. Although the techniques that were used in this study were mainly qualitative, results showed that the ability of the epichloae isolates to

produce extracellular proteases and endoglucanase is not necessarily associated with its antimicrobial activity in culture medium.

Conidial germination of *D. erythrospila* was significantly inhibited in the culture filtrate of E437 isolate (Fig. 5). Characterization of E437 culture filtrate showed that dialysis of the filtrate using a 3500 MWCO dialysis membrane but not freeze or heat treatment of the culture filtrate significantly reduced its inhibitory activity against conidial germination of *D. erythrospila* (Fig. 5c). These results implied that the antifungal compound produced by *E. festucae* isolate E437 in culture is not a proteinaceous cell lytic enzyme, but a low molecular weight metabolite. This is consistent with the observed effect of E437 on the hyphal morphology of *D. erythrospila* (Fig. 4). When co-cultured with endophyte E437 isolate, cell lysis was not observed on the hyphal tips of *D. erythrospila.* However, there was significant reduction on accumulated calcoflour white, a flourochrome stain known to bind to polysaccharides such as chitin in the cell walls of fungi, at the pathogen's hyphal tips. This observation implies that the potential inhibitory compound in E437 does not initiate hyphal cell lysis of affected pathogen but interferes with its active apical hyphal growth and differentiation activity. Several antifungal compounds produced by cultures of *Epichloë* and *Neotyphodium* species have been isolated. Yue et al. (2000) identified indole-3-acetic acid and indole-3-ethanol, a sesquiterpene and a diacetamide from cultures of epichloae isolates as antifungal metabolites against grass pathogens. Seto et al. (2007) reported cyclic peptide, named epichlicin, from *E. typhina* inhibiting the spore germination of *Clasdosporium phlei*.

Detached ryegrass leaves regardless of endophyte infection status exhibited lesions when inoculated with grass leaf spot pathogen, *D. erythrospila*. However, significantly smaller lesions were observed on leaves infected with E437 isolate (Fig. 2). These results were consistent with other research findings in which clavicipitaceous endophytes improved

disease resistance in host plants (Gwinn and Gavin, 1992; Li et al., 2007; Tian et al., 2008). Between two endophyte strains with opposing ability to inhibit the growth of *D. erythrospila* in culture medium, E437-infected plants showed more consistent reduction in lesion sizes than Fl1 endophyte isolate. This result showed that the inhibitory metabolite produced by the epichloae E437 isolate is possibly involved in the disease resistance of the host plant. However, infection by Fl1 also showed positive effect for the reduction of disease symptom in some experimental replicate (Fig. 2). A possible explanation for this might be, apart from antimicrobial substance production, endophyte Fl1 isolate uses a different strategy in protecting its host from pathogens. Different mechanisms adapted by endophytes in protecting their host plants have been suggested. Competition for plant space and resources (Moy et al., 2000), induction of plant defense responses (Schulz and Boyle, 2005; Tian et al., 2008) and stimulation of a higher production of phenolics and emission of volatile organic compounds (Pańka et al., 2013) are some of the reported mechanisms by which endophytes may counteract pathogen development. It is also possible that epichloae endophytes might produce inhibitory substances that differ *in vitro* and *in planta* (Christensen, 1996).

Results of this study support the conceptual premise that selective strains of epichloae endophytes produce antibiotic substances that inhibit pathogen growth and conidial germination, thus suppress disease development. In addition, differences in the magnitude of inhibitory activity between strains of a single species of epichloae endophyte, *E. festucae* were observed, suggesting that some isolates have the potential to produce greater quantities of one or more antifungal compounds. In this regard, one of the important issues that warrant further research is to clarify the genetic basis of the variation in antibiotic substance production among epichloae strains. Genetic basis of the difference in production of antibiotic substances among epichloae strains is important to fully understand the role of endophytes in

plant protection and their promise for further practical use in biological control. The gene involved in the biosynthesis and regulation of inhibitory substances produced by an epichloae endophyte against infecting grass pathogens is presented and discussed in the next chapter.

# CHAPTER 2

## Isolation and functional analysis of a gene involved in the biosynthesis and regulation of inhibitory substances produced by *E. festucae*

### INTRODUCTION

The production and release of anti-insect metabolites is one of the known mechanisms of how epichloae endophytes protect its host from insect herbivory. Epichloae endophytes in association with their grass hosts are noted to synthesize bioprotective alkaloids such as peramine and lolines, and another class of compound, the janthitrems that increase resistance of its hosts to insect feeding (Patterson et al., 1991; Riedell et al., 1991; Rowan and Gaynor, 1986; Tapper and Lane, 2004). Moreover, the genetic basis of the biosynthesis of endophyte-derived anti-insect metabolites had been recently elucidated (Tanaka et al., 2005).

Several studies have demonstrated that some strains of *Epichloë/Neotyphodium* species inhibit the growth of several grass pathogens in culture medium (Siegel and Latch, 1991; White and Cole, 1985). Yue et al. (2000) identified indole-3-acetic acid and indole-3-ethanol, a sesquiterpene and a diacetamide from cultures of epichloae isolates as antifungal metabolites against grass pathogens. Seto et al. (2007) reported that cyclic peptide, epichlicin, produced by *E. typhina* inhibits the spore germination of *Clasdosporium phlei*. Thus far, however, the association of disease resistance to these chemical components has not been established *in planta*.

Similar to the insect deterrent activity of endophytes that varies between the insect species and the endophyte strains producing different anti-insect metabolites (Tapper and

Lane, 2004; Tintjer and Rudgers, 2006), differences in the magnitude and range of microbial inhibitory activity were also reported among individual strains of a single species of epichloae endophyte and the infecting pathogen species (Christensen et al., 1991; Li et al., 2007; Ren et al., 2009; Siegel and Latch, 1991). This observation suggests that some epichloae strains have the potential to produce greater quantities of one or more antifungal compounds. Moreover, the inhibitory role of endophyte-derived metabolites depends on the endophyte isolate- grass pathogen species combination.

In contrast to the recent advances in the genetic basis of the biosynthesis of endophyte-derived anti-insect metabolites, so far, however, there is no report on the genes involved in the biosynthesis and regulation of epichloae-derived antimicrobial substances. Unlocking the genetic basis of the difference in the production of antibiotic substances among epichloae strains will provide deeper understanding of the role of endophytes in plant protection and their promise for further practical use in biological control.

The objective of this study is to identify and functionally characterize the gene involved in the production of the anti-fungal compound in *E. festucae* isolate E437.

## MATERIALS AND METHODS

### Fungal strains and growth conditions.

*Epichloë festucae* strains E437 and Fl1 were kindly provided by Prof. Christopher L. Schardl (University of Kentucky, USA) and Prof. Barry Scott (Massey University, New Zealand). The cultures of *E. festucae* (Table 1) were grown on 2.4% potato dextrose agar and maintained at 23˚C or kept in 4˚C until use. The test fungal grass pathogens, *Drechslera erythrospila* (isolate 638, MAFF No. 305378), *D. siccans* (isolate 962, MAFF No. 305397), *D. dictyoides* (isolate 963, MAFF No. 305398), *Colletotrichum graminicola* (isolate PR-1, MAFF No. 306600), *Sclerotinia homoeocarpa* (isolate SU16-3, MAFF No. 236941), and *Rhizoctonia solani* (isolate 1374, MAFF No. 511374) were acquired from the collection of the National Institute of Agrobiological Sciences (NIAS, Japan). *Magnaporthe grisea* isolate (WK3-1) was kindly provided from the collection of Prof. Yukio Tosa (Kobe University, Japan). All test fungal grass pathogens were grown on PDA at 23˚C and maintained at 4˚C until use.

### Nucleic acid isolation, Southern blot hybridization, PCR, and quantitative RT-PCR (qPCR) analysis.

Fungal genomic DNA was isolated from freeze-dried mycelium using the method described previously (Byrd et al., 1990) or using an Extract-N-Amp plant PCR kit (Sigma, USA) according to the manufacturer's instructions. Genomic digests were transferred to positively charged nylon membranes (Hybond-$N^+$, GE Healthcare, UK) by capillary transfer

**Table 1**. *Epichloë festucae* strains used in this study

| *E. festucae* | Relevant characteristics | References |
|---|---|---|
| E437 | Wild type | Niones and Takemoto (2014) |
| Fl1 | Wild type | Young *et al.* (2005) |
| 830 | E437/pNPP1; Hyg $^{R}$ | This study |
| *ΔvibA*-1 | E437/*Δvib-1*:: P*trpC-hph*; Hyg $^{R}$ | This study |
| *ΔvibA*-2 | E437/*Δvib-1*:: P*trpC-hph*; Hyg $^{R}$ | This study |
| *ΔvibA*-3 | E437/*Δvib-1*:: P*trpC-hph*; Hyg $^{R}$ | This study |
| P*tef*:*vibA*-1 | E437/pNPP154, Hyg $^{R}$ | This study |
| *ΔvibA/VibA*-12 | *ΔvibA*/pSF17, pNPP152, Hyg $^{R}$;Gen$^{R}$ | This study |
| P*tef*:*VibA-GFP*-7 | E437/pNPP155, Hyg $^{R}$ | This study |
| P*vibA*:*GFP*-1 | E437/ pNPP153, Hyg R | This study |

and fixed by UV cross-linking in a UV cross-linker (CL-1000, Ultra-Violet Products Ltd., UK). The filter was probed with [a-$^{32}$ P]dCTP-labeled probes (3000 Ci/mmol, MP Biomedicals, USA). Probe labeling, hybridization and detection conditions were performed as described previously (Tanaka et al., 2006). Standard PCR amplifications of genomic or plasmid DNA templates were performed with PrimeStar HS DNA polymerase (Takara, Japan). Sequences of PCR primers used in this study are provided in Table 2.

Total RNA was isolated from frozen mycelium using TRIzol reagent (Invitrogen, USA) and reverse transcribed with ReverTra Ace (Toyobo, Japan). Quantitative RT-PCR was performed in the LightCycler Quick System 350S (Roche Applied Science, Germany) using Thunderbird SYBR qPCR Mix (Toyobo, Japan) with gene-specific primers (Table 2). The thermo cycle conditions were performed as described previously (Tanaka et al., 2013). The thermo cycle conditions used were: one cycle at 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 5 s and 72°C for 30 s, using single fluorescence acquisition. The melting curve was generated by holding the sample at 65°C for 30 s, followed by ramping up to 95°C at a rate of 0.2°C s−1 using continuous fluorescence acquisition. The relative expression level of each target was normalized to that of actin (*actG*).

**Preparation of deletion, complementation, and overexpression constructs.**

The base vector for deletion construct pNPP150, was prepared as follows: The 1.1-kb *Spe*I/*Not*I fragment of herpes simplex virus thymidine kinase (HSVtk) gene was amplified from pGKO2 vector (Khang et al., 2005) with primers Spe-HSVtk-F and HSVtk-Nt-R and cloned into *Spe*I/*Not*I of pPN94 (Takemoto et al., 2006) to generate pPN94-HSVtk. The 2.5-

kb TEF promoter-HSVtk-*trpC* terminator cassette was amplified with primers 94-pTEF-F and TrpC-94-R and cloned into the *Hpa*I site of pSF15.15 (Takemoto et al., 2006) to generate pNPP150. The *E. festucae vibA* deletion construct pNPP151 was prepared as follows. A 1.0-kb fragment 5' of *vibA* was amplified from E437 genomic DNA with primers IF830KO5-F2 and IF830KO5-R2. A 1.2-kb fragment 3' of *vibA* was amplified from the genomic DNA of *E. festucae* strain E437 with primers IF830KO3-F and IF830KO3-R. A 1.5-kb *trpC* promoter-*hph* cassette was amplified from pNPP150 with primers hph-F and hph-R. Then these three PCR products were cloned into a linearized pNPP150 vector (amplified with the primers 94-pTEF-F and pNPP150-R) using In-Fusion HD Cloning kit (Clontech, USA).

To reintroduce the *E. festucae vibA* gene into the Δ*vibA* deletion strain, complementation construct pNPP152 was prepared as follows: The 5.0-kb *E. festucae vibA* gene including the 2-kb upstream and 1.1-kb downstream regions of its *vibA* coding sequence (Fig. 2A) was amplified from E437 genomic DNA with primers EI-830comp-F1 and 830comp-EV-R1, and digested with *Xba*I and *Eco*RV and subsequently cloned into *Xba*I/*Eco*RV site of pBlueScriptII KS (+) (Stratagene, USA).

The *vibA* overexpression construct pNPP154 was prepared by ligating a *Xba*I/*Eco*RI digest of a 1.8-kb *vibA* fragment amplified from cDNA of *E. festucae* strain E437 with primers OEx-vib1-*Xba*I-F1 and OEx-vib1-*EcoR*I-R1 into the *Xba*I/*Eco*RI site of pPN94.

**Table 2.** Primers used in this study.

| Primer | Sequence |
|---|---|
| EfVib1-F | AACAACTCGATCTGTCTGCG |
| EfVib1-R | GCAACTTCCAGGTTGCTGAC |
| EfactG.1 | AATACGACGAGAGCGGTCCC |
| EfactG.2 | CAGCCCATTGCTTCCCCTTG |
| Spe-HSVtk-F | CCCGGGACTAGTATGGCTTCGTACCCCTGC |
| HSVtk-Nt-R | AATTGCGGCCGCTCAGTTAGCCTCCCCCAT |
| 94-pTEF-F | TCGATAAGCCAGGTTGGTAGCAAACGGTGG |
| TrpC-94-R | TCATTAATGCAGGTTCCATCTTAGTAGGAA |
| IF830KO3-F | TGAGTCGTATTAATTGAGCGATTGGACCTA |
| IF830KO3-R | AACCTGGCTTATCGATTGTCTCTTCGACTG |
| IF830KO5-F2 | TTAGGTGACACTATAGACAAGAACAGACAG |
| IF830KO5-R2 | CAGCTGCTCGAGTTCCGAGTCGGCTAGGCA |
| hph-F | AATTAATACGACTCACTATA |
| hph-R | GAACTCGAGCAGCTGAAGCT |
| pNPP150-R | TATAGTGTCACCTAAATCGTATGTGTATGA |
| EI-830comp-F1 | GGGCCCGAATTCCCTGTCAAATGTTCGTGG |
| 830comp-EV-R1 | GGCCCCGATATCTAATTCGAGGCTGTGGCT |
| OEx-vib1-XbaI-F1 | GGGGCCTCTAGAATGACGGAGCTTCGAGGA |
| OEx-vib1-EcoRI-R1 | GGCCCCGAATTCTTATGCTGCGGTCCATGA |
| B-EGFP-F | GGATCCATGGTGAGCAAGGGCGAG |
| EGFP-Nt-R | GCGGCCGCTTTACTTGTAC |
| Spe1-EGFP-F1 | ACTAGTATGGTGAGCAAGGGCGAG |
| EGFP-Nt-R | GCGGCCGCTTTACTTGTAC |
| 830-promoter-EcoRI-F1 | GGGCCCGAATTCGTGTATCGTCGCAAGCCGCG |
| 830-promoter-BamHI-R1 | GGCCCGGGATCCGGTGGCCGCTGGGTACGCTG |
| BI-3GA | CCCGGGGGATCCGGTGCTGGTGCTGGTGCT |
| IF94GFP-Vib1-F | AACCTCTAGAGGATCATGACGGAGCTTCGAGGA |
| IF94GFP-Vib1-R | CACCAGCACCGGATCCTGCTGCGGTCCATGAATA |
| pII99-2 | TTGAGTGAGCTGATACCG |
| pII99-3 | GGCTGGCTTAACTATGCG |
| 830-F1 | CAACGAGGCCAACGAGACAC |
| 830-F2 | TGCATTTCCCGTACTGTCGG |
| 830-R1 | CGGCCATTGTTGGCAGTTGC |
| hph-2 | ACTCGTCCGAGGGCAAAG |
| PtrpC-2 | CAAATTTTGTGCTCACCG |

Restriction enzyme sites and linker sequence for In-fusion reaction are in red and blue letters, respectively.

To express GFP under the control of *vibA* promoter, a *Bam*HI/*Not*I 0.7-kb EGFP fragment was prepared by digesting a PCR product that was amplified with primers B-EGFP-F and EGFP-NI-R from pNPP1 (Kayano et al., 2013). An *Eco*RI/*Bam*HI 2.0-kb *vibA* promoter region was prepared by digesting a PCR product amplified with primers 830-promoter-*Eco*RI-F1 and 830-promoter-*Bam*HI-R1. Both DNA fragments were cloned into the *Eco*RI-*Not*I site of pNPP1 to generate pNPP153. The base vector for the expression of GFP fusion protein pNPP140 was prepared as follows: A *Bam*HI/*Not*I 0.7-kb 3GA-EGFP fragment amplified with primers BI-3GA and EGFP-NI-R from pNPP9 (Takemoto et al., 2011) was cloned into the *Bam*HI/*Not*I site of pPN94. Three copies of glycine-alanine (GA) at the N-terminus of the EGFP act as spacer between the GFP and the test protein.

To express *vibA* tagged with GFP under the control of the TEF promoter, the 1.8-kb *vibA* fragment was amplified using the primers IF94GFP-Vib1-F and IF94GFP-Vib1-R. The purified PCR product was ligated into the *Bam*HI site of pNPP140 using In-Fusion HD Cloning kit to generate pNPP155. Constructs used in this study are listed in Table 3.

**_E. festucae_ transformation and molecular analysis of transformants.**

Protoplasts of *E. festucae* were prepared according to the method previously described (Young et al., 2005). Protoplasts were transformed with 5 µg of either circular (pSF17, pNPP152, pNPP153, pNPP154 and pNPP155) or linear PCR-amplified product (pNPP151 with primers pII99-2 and pII99-3) as described previously (Itoh et al., 1994). For restriction enzyme-mediated integration mutagenesis, 5 µg of *Bam*HI-linearized pNPP1, 12 units of *Bam*HI and 20 µl of K buffer for restriction enzyme were added to 100 µl of protoplast

mixture. Transformants were selected on YPS/PDA medium containing hygromycin (150 μg/ml) or geneticin (400 μg/ml). For the selection of *vibA* knockout transformant, 1 μM 5-fluoro-2-deoxyuridine (F2dU) was added to PDA media and *HSVtk* gene was used as a negative selection marker against ectopic transformants (Khang et al., 2005). The putative transformants for *E. festucae vibA* gene replacement were confirmed by amplifying the *vibA* gene using internal *vibA* gene primers 830-F1 and 830-R1 (Table 2).

**DNA sequencing and bioinformatics.**

DNA fragments were sequenced by the dideoxynucleotide chain termination method using Big-Dye ver. 3 chemistry (Applied BioSystems, USA). Products were separated on an ABI 3130 analyzer (Applied Biosystems). Sequence data were analyzed and annotated in MacVector program (ver. 11, MacVector Inc., USA). The sequence of *vibA* locus was obtained from *E. festucae* E2368 genome sequences kindly provided by Prof. Christopher L. Schardl (University of Kentucky, USA, http://csbio-l.csr.uky.edu/endophyte/). Deduced protein sequences of fungal VibA (VIB-1) were collected from the fungal genome resources of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and Broad institute (http://www.broad.mit.edu/annotation/fungi/fgi/). Protein sequences were aligned using ClustalW ver. 2.1 (Larkin et al., 2007) with default settings.

**Table 3.** Plasmids used in this study.

| Vector name | Base vector | Restriction site used | Insert | Primers used to amplify insert | References | Note |
|---|---|---|---|---|---|---|
| pBlueScriptII KS(+) | | | | | Stratagene | Base vector for cloning; $Amp^R$ |
| pNPP1 | | | | | Kayano et al. 2013 | $Amp^R/Hyg^R$ |
| pPN94 | | | | | Takemoto et al. 2006 | $Amp^R/Hyg^R$ |
| pSF15 | | | | | Takemoto et al. 2006 | $Amp^R/Hyg^R$ |
| pSF17 | | | | | Tanaka et al. 2008 | $Amp^R/Gen^R$ |
| pNPP9 (pPN94-NoxR-GFP) | | | | | Takemoto et al. 2011 | $Amp^R/Hyg^R$ |
| pNPP140 (pPN94-3GA-GFP) | pPN94 | *Bam*HI/*Not*I | 3GA-GFP | BI-3GA and EGFP-NI-R | This study | GFP expression, $Amp^R/Hyg^R$ |
| pNPP150 | pSF15 | *Hpa*I | P*tef*-HSVtk-T*trpC* | Spe-HSVtk-F and HSVtk-Nt-R, 94-pTEF-F and TrpC-94-R | This study | P*tef*:*HSVtk*, $Amp^R/Hyg^R$ |
| pNPP151 (pNPP150 *vibA* KO) | pNPP150 | - | 5' *VibA*-P*trpC-hph*-3'*VibA* | IF830KO5-F2 and IF830KO5-R2, IF830KO3-F and IF830KO3-R | This study | *VibA* KO, $Amp^R/Hyg^R$ |
| pNPP152 (pBS-*VibA*) | pBlueScript II KS(+) | *Xba*I/*Eco*RV | P*vibA*:*VibA* | EI-830comp-F1 and 830comp-EV-R1 | This study | *VibA* complementation |
| pNPP153 (pNPP1 P*vibA*:GFP) | pNPP1 | *Bam*HI /*Not*I | P*vibA*, EGFP | 830-promoter-EcoRI-F1 and 830-promoter-BamHI-R1, B-EGFP-F and EGFP-NI-R | This study | Expression of GFP under the control of *VibA* promoter, $Amp^R/Hyg^R$ |
| pNPP154_(pPN94 *VibA*) | pPN94 | *Xba*I/*Eco*RI | *EfVibA* fragment | OEx-vib1-XbaI-F1 and OEx-vib1-EcoRI -R1 | This study | Overexpression of *vib-1,* $Amp^R/Hyg^R$ |
| pNPP155_(pPN94-*VibA*-GFP) | pNPP140 | *Bam*HI | Ef*VibA* | IF94GFP-Vib1-F and IF94GFP-Vib1-R | This study | Expression of VibA-GFP under the control of TEF promoter, $Amp^R/Hyg^R$ |

**Mycelial and conidial germination inhibitory assay.**

Dual culture assay using mycelial block was performed to determine the antifungal activity of *E. festucae* transformants against the grass pathogens. Mycelial blocks of *E. festucae* strains were placed on quarter positions of a PDA plate and allowed to grow for 7 days before a mycelial plug of grass pathogen (3 mm diameter) was placed in the center of the PDA plate. The culture was incubated at 23°C until either a clear zone of inhibition was observed or the colonies of the two fungi have made contact. Inhibition was determined by measuring the clear zone between the *E. festucae* and the grass pathogen colonies. Conidial germination inhibitory assay was performed as follows. Mycelia of *E. festucae* transformants were inoculated in PD broth and cultured in an orbital shaker (100 rpm) at 23°C. After 7 days of incubation, the culture filtrate was harvested and then sterilized using a filter membrane unit (Millex-HA filter unit, 45 μm pore diameter, Millipore, USA). Twenty μl of endophyte culture filtrate was added into a sterile biconcave microscope slide and mixed with 5 μl of conidial suspension of *D. erythrospila* (1 $x10^4$ spores/ml). In the control test, conidial suspension was mixed with sterile PD broth. All treatments were replicated at least three times. The slides were placed in Petri dishes and kept at 25°C. After 12 hours, germination rate of conidia was scored from at least 150 conidia. The replicated experiment was repeated at least three times.

**Detection of extracellular protease activity of epichloae isolates.**

Extracellular protease activity of the epichloae isolates was detected as follows (Vermelho et al., 1996). A 3 mm mycelial plug from each isolate was separately placed in the

center of a PDA plate supplemented with 1% (w/v) gelatin (Becton Dickinson, USA) and incubated at 23°C. Extracellular protease activity was detected as a visible halo around the colony of endophyte isolate.

## RESULTS

### Isolation of an *E. festucae* mutant that lost its anti-fungal activity against temperate grass pathogens.

In Chapter 1, *E. festucae* isolate E437 exhibited antifungal activity against grass pathogens including *Drechslera erythrospila*, *D. siccans*, *D. dictyoides*, *Colletotrichum graminicola* and *Bipolaris sorokiniana*. To isolate E437 mutants with reduced antifungal activity, plasmid insertion mutagenesis (restriction enzyme mediated integration, REMI) was done (Kuspa and Loomis, 1992). The pNPP1 plasmid linearized with *Bam*HI was transformed with the protoplast of *E. festucae* E437. From the *in vitro* dual culture assay of 1200 independent plasmid insertion mutants against *D. erythrospila*, one mutant was isolated, designated 830, which lost its ability to inhibit the mycelial growth of the pathogen (Fig. 1a). Mutant 830 also could not inhibit the colony growth of *D. siccans*, *D. dictyoides*, *C. graminicola*, and *B. sorokiniana* (Fig. 2). The culture filtrate of mutant 830 had no inhibitory effect on the germination of pathogen conidia (Fig. 1b).

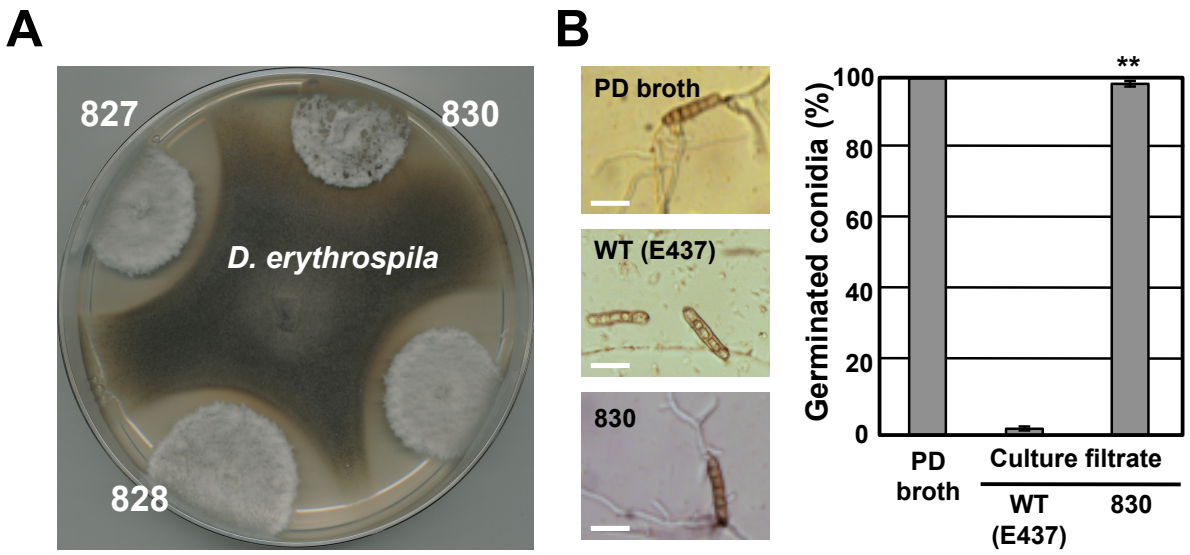



**Fig. 1** Isolation of mutant 830 which lost the inhibitory activity of mycelial growth of grass pathogens. **(a)** Loss of inhibitory activity of mutant 830 on mycelial growth of *D. erythrospila*. Colonies of restriction-enzyme-mediated DNA integration (REMI) transformants were grown to a diameter of 10-12 mm and a mycelial plug of *D. erythrospila* was inoculated. The culture was incubated at 23°C until either a clear zone of inhibition was observed or the colonies of the two fungi had made contact. **(b)** Percentage and morphology of germinated conidia of *D. erythrospila* 12 h after incubation in culture filtrate of *E. festucae* wild type (WT) E437 or mutant 830. Potato dextrose (PD) broth served as control. The frequency of germinated conidia was calculated from at least 150 spores. Data are means ± standard error from three biological replicates (>50 spores/experiment). Data marked with asterisks are significantly different from the control (WT E437) as assessed by the two-tailed Student's *t* test: $^{**}p < 0.01$. Bars = 30 µm.

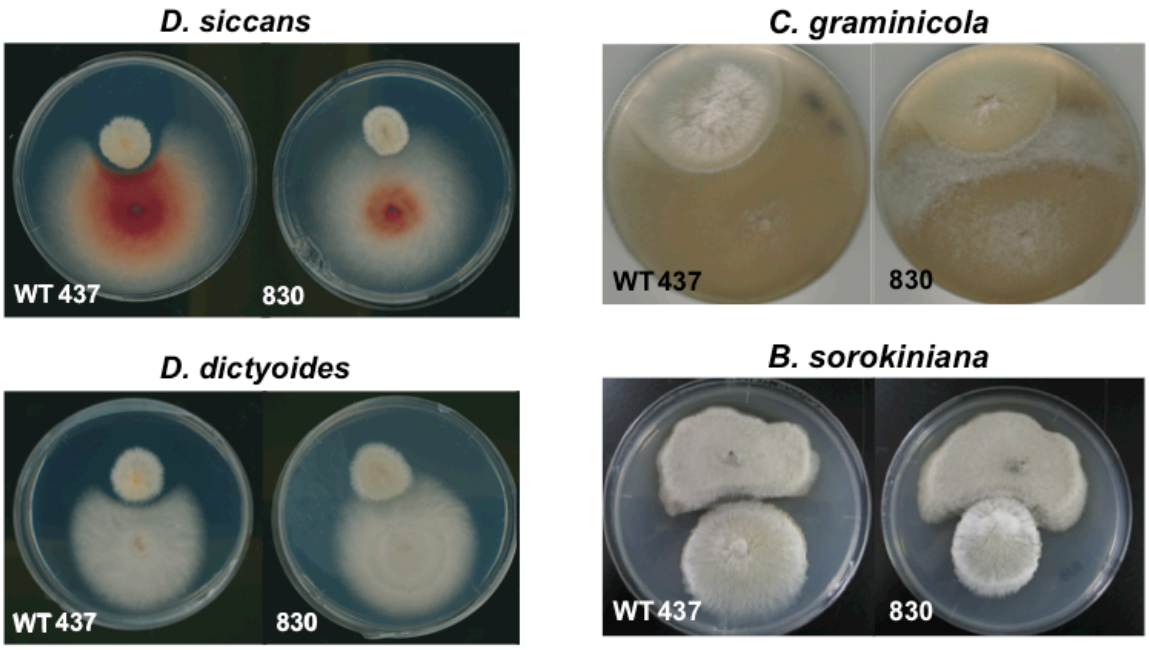



**Fig. 2** Mutant 830 lost inhibitory activity on the colony growth of *D. siccans*, *D. dictyoides*, *C. graminicola* and *B. sorokiniana.* Colonies of *E. festucae* wild type E437 (WT E437) and mutant 830 were grown to a diameter of 10-12 mm and a mycelial plug of grass pathogen was inoculated. The culture was incubated at 23°C until either a clear zone of inhibition was observed or the colonies of the two fungi have made contact.

**Mutant 830 contains a plasmid insertion in the promoter region of *vibA* gene.**

Genomic DNA of mutant 830 was digested with the restriction enzyme *Eco*RV that was absent from the transformation vector pNPP1, and Southern blot analysis was performed with pNPP1 as probe. Only one hybridizing band was observed, indicating the presence of a single vector integration site in the genome of this mutant (Fig. 3a and b). To identify the site of pNPP1 insertion, the genomic DNA of mutant 830 was digested with *Eco*RI or *Cla*I. Two hybridized bands were detected in either *Eco*RI- (over 12 kb and approx. 4 kb) or *Cla*I- (approx. 11 kb and 8 kb) digested genomic DNA of mutant 830 (Fig. 3b). The weak hybridizing 4-kb *Eco*RI fragment, which was expected to have hygromycin resistance (*hph*) gene cassette (Fig. 3a), was isolated from the gel and self-ligated. The chromosomal DNA flanking the *hph* cassette was PCR- amplified using the primers hph2 and Ptrpc-2 and was sequenced. Analysis of this sequence, with genome sequence of *E. festucae* isolate E2638, showed that pNPP1 was integrated at the *Bam*HI site of an expected promoter region of a gene encoding a transcription factor homologous to VIB-1 of *Neurospora crassa* (Xiang and Glass, 2002). Single insertion of pNPP1 vector in this *Bam*HI site was further confirmed by a series of PCR analyses with various primer combinations (Fig. 4). The deduced protein encoded by this gene shared 47% sequence identity with the VIB-1 (Accession No. Q9C2N1) of *N. crassa,* a transcriptional regulator that is essential for the expression of the genes involved in non-self recognition and vegetative incompatibility (or heterokaryon incompatibility, HI) mediated by allelic differences (Dementhon et al., 2006; Xiang and Glass, 2002). The *vib-1* homologue of *E. festucae* is hereon referred to as *vibA*.

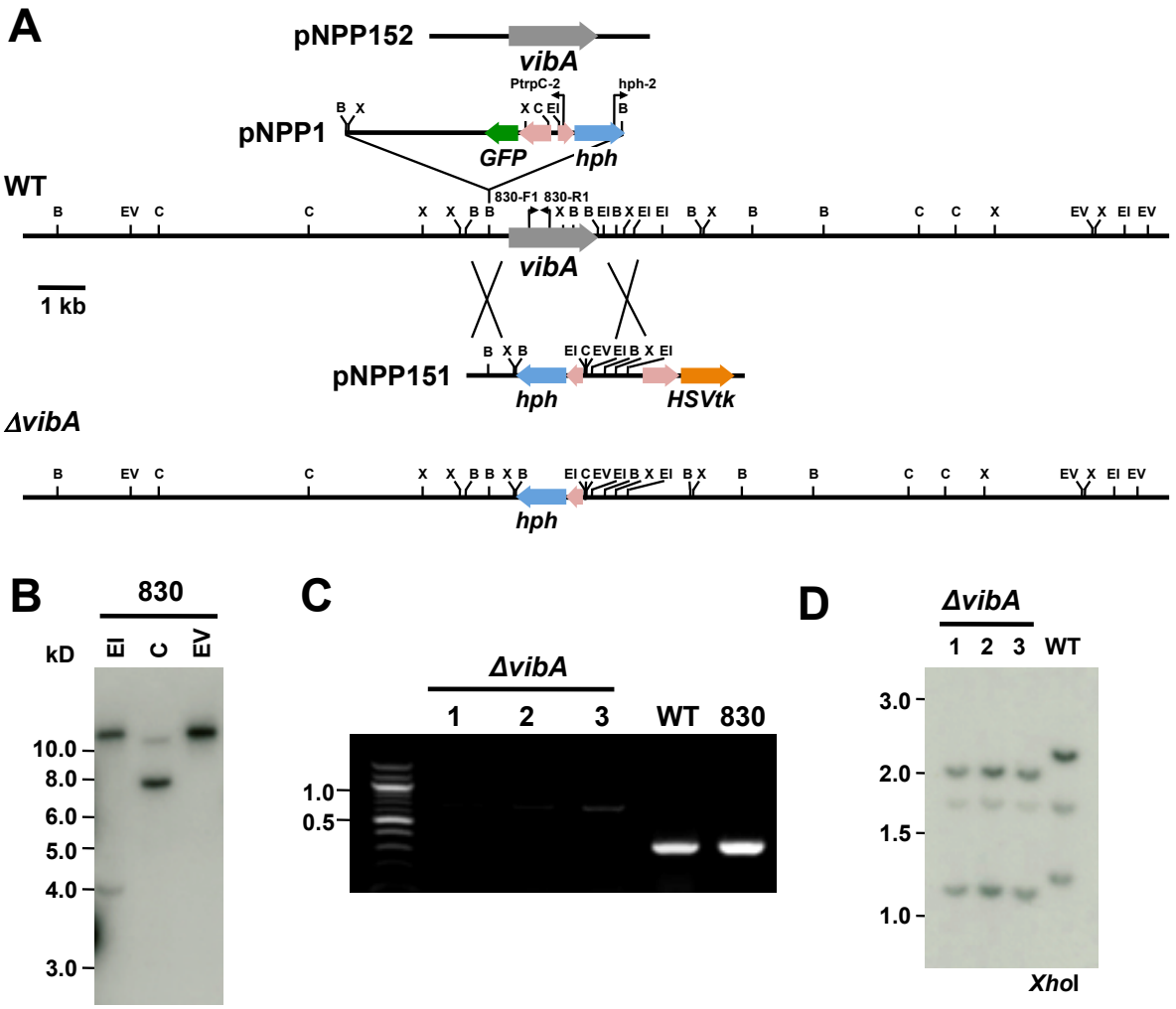



**Fig. 3** Deletion of *E. festucae vibA*. **(a)** Physical maps of the *E. festucae vibA* wild type genomic region, integration site of pNPP1 in mutant 830, linear insert of replacement construct pNPP151, *E. festucae vibA* mutant genomic region, and insert of complementation construct pNPP152 showing restriction enzyme sites for *Bam*HI (B), *Eco*RI (EI), *Eco*RV (EV), *Cla*I (C) and *Xho*I (X). **(b)** Autoradiograph of DNA gel blots of *Eco*RI (EI) *Cla*I (C) or *Eco*RV (EV) genomic digests of a mutant 830 of *E. festucae* E437 strain probed with [$^{32}$P]-labeled pNPP1. Sizes in kilobases of markers are indicated at left. **(c)** PCR-based verification of *vibA* mutants. Internal *vibA* gene primer pair, 830-F1 and 830-R1, was used to amplify the band. The locations of primers are indicated in A. **(d)** Autoradiograph of a Southern blot of *Xho*I genomic digests of *E. festucae* wild type E437 (WT) and *vibA* deletion strains (Δ*vibA*), probed with [$^{32}$P]-labelled pNPP151. Sizes in kilobases of markers are indicated at left.

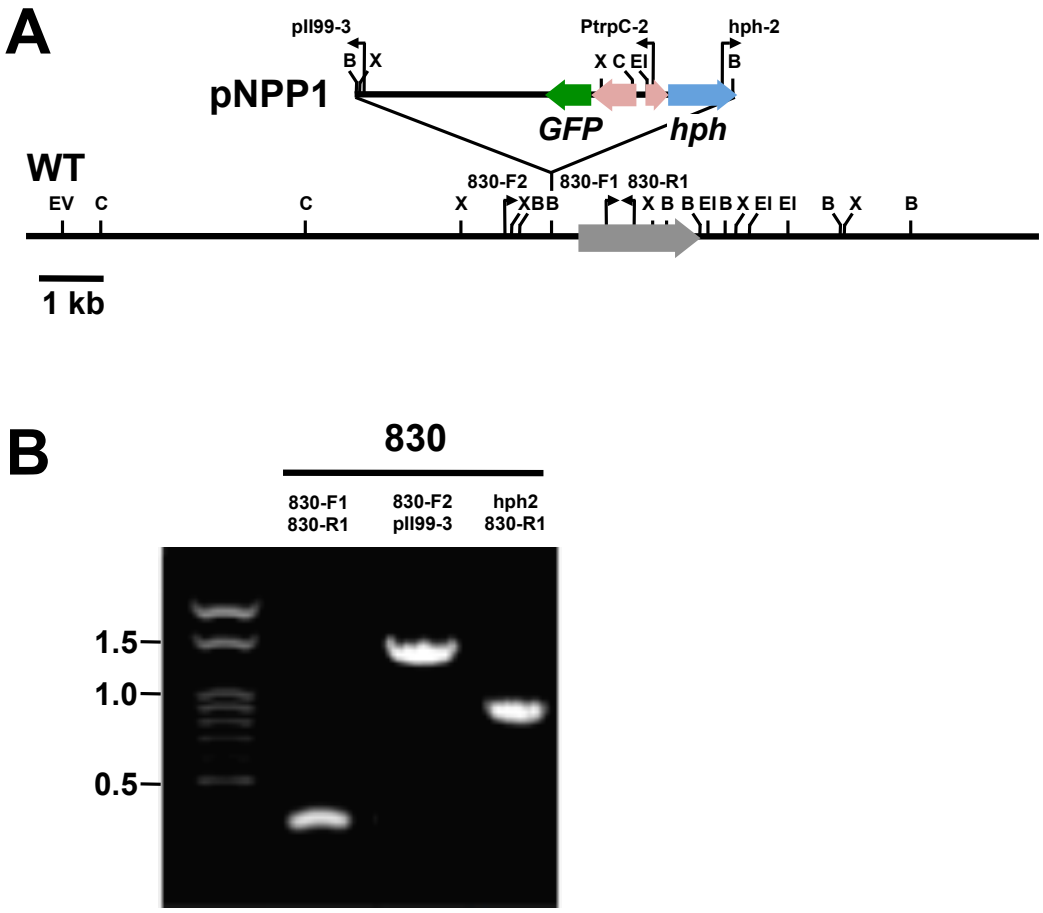



**Fig. 4 (a)** Physical map of the integration site of pNPP1 in the genome of E. festucae mutant 830, showing restriction enzyme sites for *Bam*HI (B), *Eco*RI (EI), *Eco*RV (EV), *Cla*I (C) and *Xho*I (X). **(b)** Confirmation tests through a series of PCR using various primer combinations to establish the integrity of insertion of pNPP1 vector in the *Bam*HI site of *E. festucae* genome. Locations of primers used are indicated in the physical map **(a)**.

*E. festucae* VibA is a putative transcription factor containing NDT80/PhoG DNA binding domain (Fig. 5). The VibA-GFP expressed under the control of TEF promoter that showed obvious nuclear localization (Fig. 7) further supported that VibA would act as a regulator of gene expression. VibA homologue can be found as a single copy gene in genomes of Ascomycota fungi except some species of hemiascomycetous yeasts, but obvious homologue was absent from Basidiomycota and Zygomycota (Figs. 5 and Fig. 8).

**Deletion of *E. festucae vibA* loses antifungal activity of the endophyte.**

To confirm that the plasmid insertion in the promoter region of *vibA* was responsible for the reduced antifungal activity of mutant 830, a *vibA* deletion mutant was generated by replacing the *vibA* gene with the *hph* gene via homologous recombination (Fig. 3a). Transformants obtained were subjected to *in vitro* dual culture assay against *D. erythrospila,* from which candidate *vibA* mutants that failed to inhibit the mycelial colony growth of the pathogen were further selected. Ten out of 15 transformants tested lost their ability to inhibit the colony growth of the pathogen, a phenotype similar to mutant 830. Three of the putative *vibA* deletion mutants were then confirmed by PCR and Southern hybridization to verify the integrity of the target gene replacement events (Fig. 1c and d). The 340-bp band amplified with the internal *vibA* gene primers, 830-F1 and 830-R1, was detected in the wild type E437 and mutant 830, but not in *vibA* deletion mutants (Fig. 1a and c). Moreover, DNA gel blot analysis of *Xho*I genomic digests of the transformants and wild type E437 probed with deletion construct pNPP151 confirmed that these transformants contained a replacement at

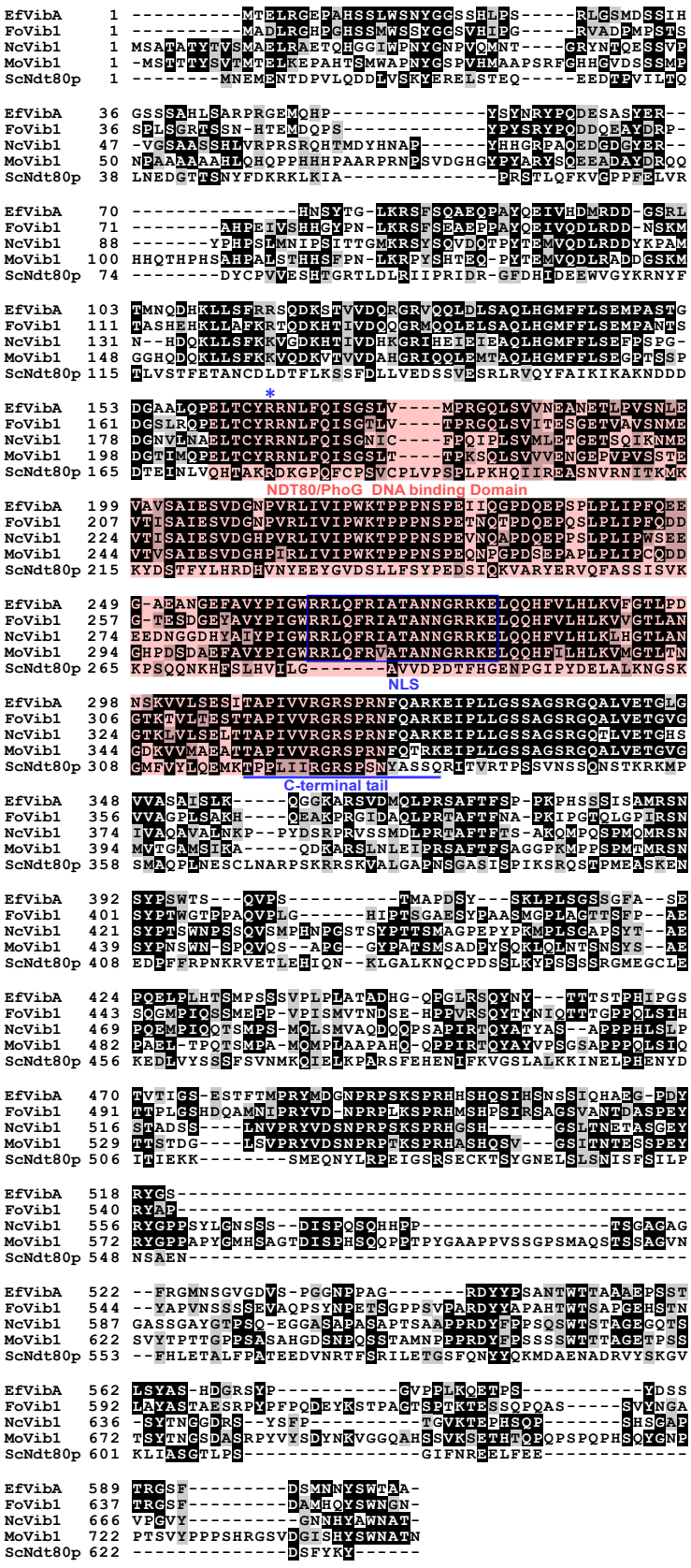



**Fig. 5** The deduced protein sequence of EfVibA was aligned with Vib1 of *Fusarium oxysporum* (FoVib1, FOXB_17342), *Magnaporthe oryzae*, (MoVib1, MGG_00729), *Neurospora crassa* (NcVib1, NCU03725.5) and *Saccharomyces cerevisiae* Ndt80p by ClustalW ver. 2.1 (Larkin et al., 2007) with default settings. NDT80/PhoG DNA binding domain is indicated in red box. Predicted bipartite nuclear localization signal (NLS) was in indicated blue box. C-terminal tail of Ndt80p predicted to make contact with the DNA major groove (Lamoureux et al., 2002) is underlined, and Arginine residue required for the function of Ndt80p in *Candida albicans* (Yang et al., 2012) is indicated by asterisk.

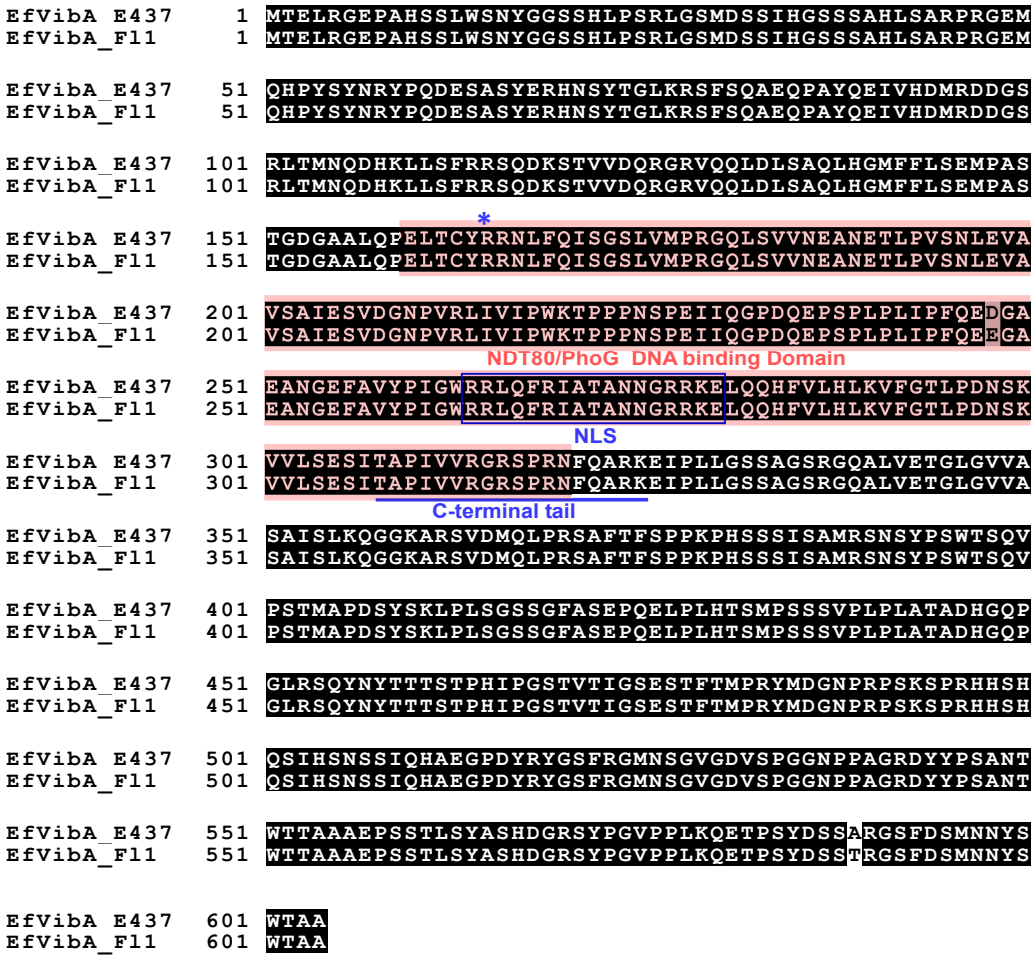



**Fig. 6** The deduced protein sequences of EfVibA of *E. festucae* isolate E437 was aligned with that of *E. festucae* isolate Fl1 by ClustalW ver. 2.1 (Larkin et al., 2007). NDT80/PhoG DNA binding domain is indicated in red box. Predicted bipartite nuclear localization signal (NLS) is indicated in blue box. C-terminal tail of Ndt80p predicted to make contact with the DNA major groove (Lamoureux et al., 2002) is underlined, and Arginine residue required for the function of Ndt80p in *Candida albicans* (Yang et al., 2012) is indicated by the asterisk.

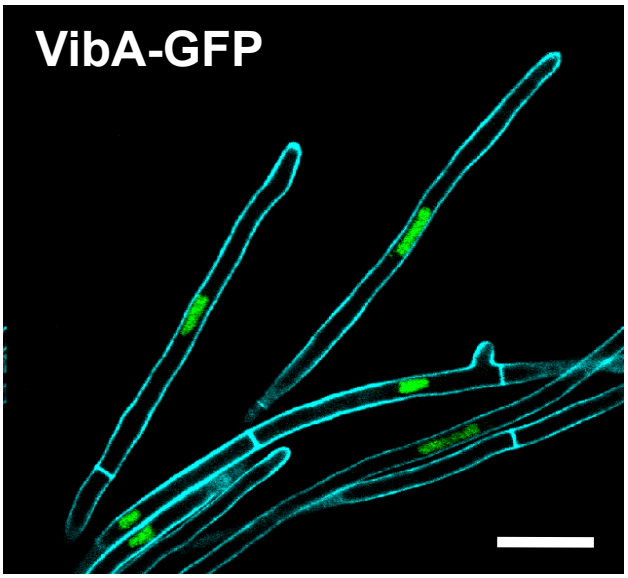



**Fig. 7** Localization of VibA-GFP in hyphae of *E. festucae.* VibA-GFP was expressed under the control of a TEF promoter in *E. festucae* E437 strain and monitored by confocal-laser microscopy. Bar = 10 μm.

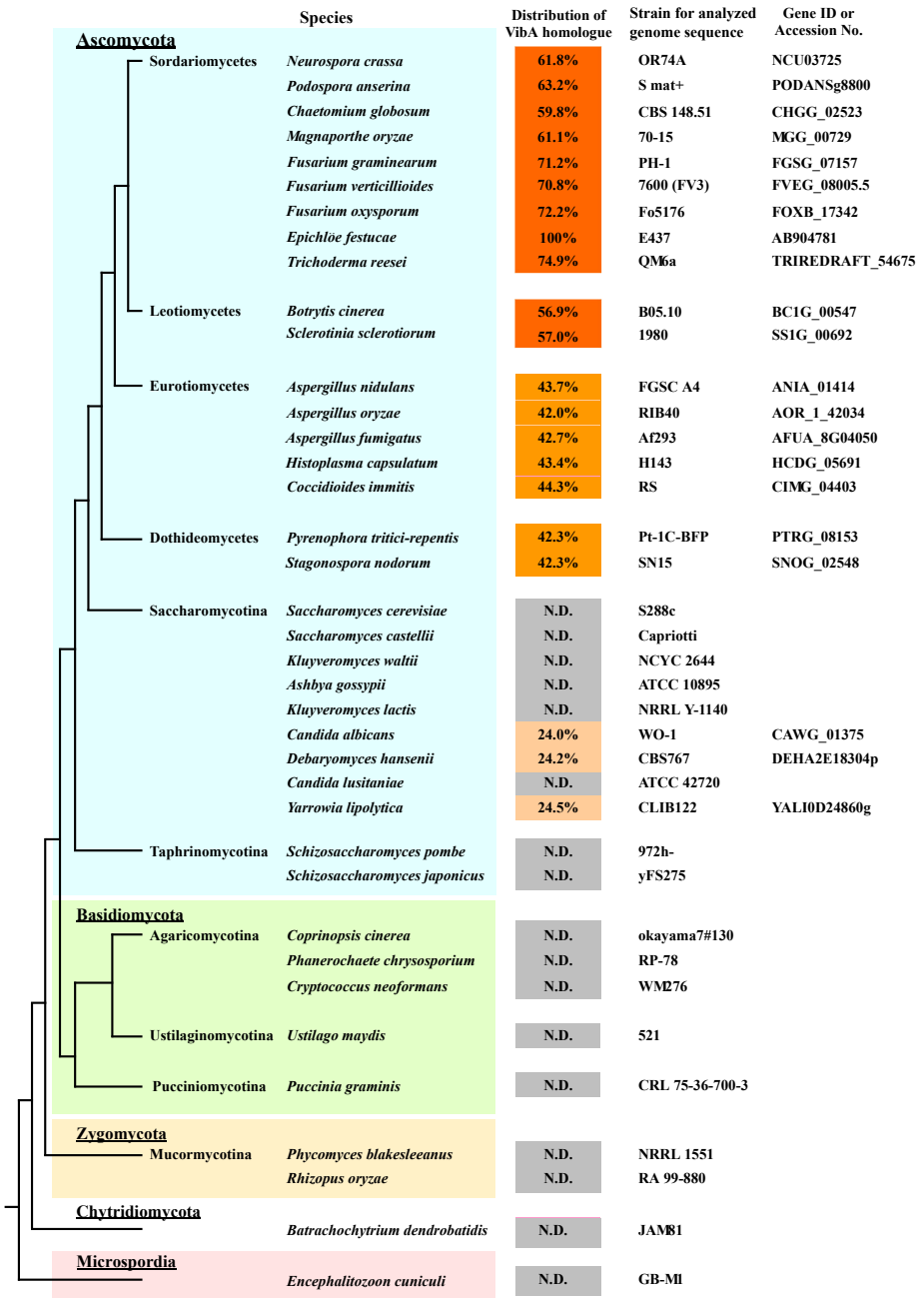



**Fig. 8** Distribution of Vib1- like genes in the fungi kingdom. Percentage similarity of deduced amino acid sequence with EfVibA was calculated with MacVector program (ver. 11) with default setting. N.D. means not detected. Adapted from Takemoto et al., (2007).

the *vibA* locus without any extra integration of the vector (Fig. 3d). Both *E. festucae* E437 and *vibA* mutant have three *Xho*l restriction sites, located within the *vibA* coding sequence and its regulatory regions but they differ on the locations of *Xho*l recognition sites. This explains the difference in generated band sizes in DNA gel blot (Fig. 3a and d). Deletion of *vibA* did not have any significant effect on mycelial growth (Fig. 9 and 10), but like the mutant 830, the *vibA* mutants could not inhibit the mycelial growth of *D. erythrospila* (Fig. 9). Likewise, *vibA* deletion mutants also could not inhibit the mycelial growth of *D. siccans*, *D. dictyoides*, *C. graminicola* and *B. sorokiniana*. Moreover, the culture filtrate of *vibA* mutant could not inhibit the conidial germination of *D. erythrospila* (Fig. 11).

To further confirm that *E. festucae vibA* is essential for the antifungal activity of isolate E437, plasmid pNPP152 containing the full-length *vibA* gene along with its regulatory regions, was transformed into protoplasts of the Δ*vibA* knockout mutant. The candidate complement transformants were then subjected to dual culture assay against *D. erythrospila.* Geneticin-resistant complement transformants inhibited the mycelial colony growth and conidial germination of *D. erythrospila* (Figs. 9 and 11).

**Mutation in VibA affects extracellular protease production.**

Previous report indicated that *N. crassa* VIB-1 is required for the production of downstream effectors associated with heterokaryon incompatibility, including the production of extracellular proteases (Dementhon et al., 2006). To investigate the role of *E. festucae* VibA in the production of proteases, the ability of the *E. festucae* strains to produce extracellular proteases was detected on PDA medium with gelatin as substrate. Extracellular

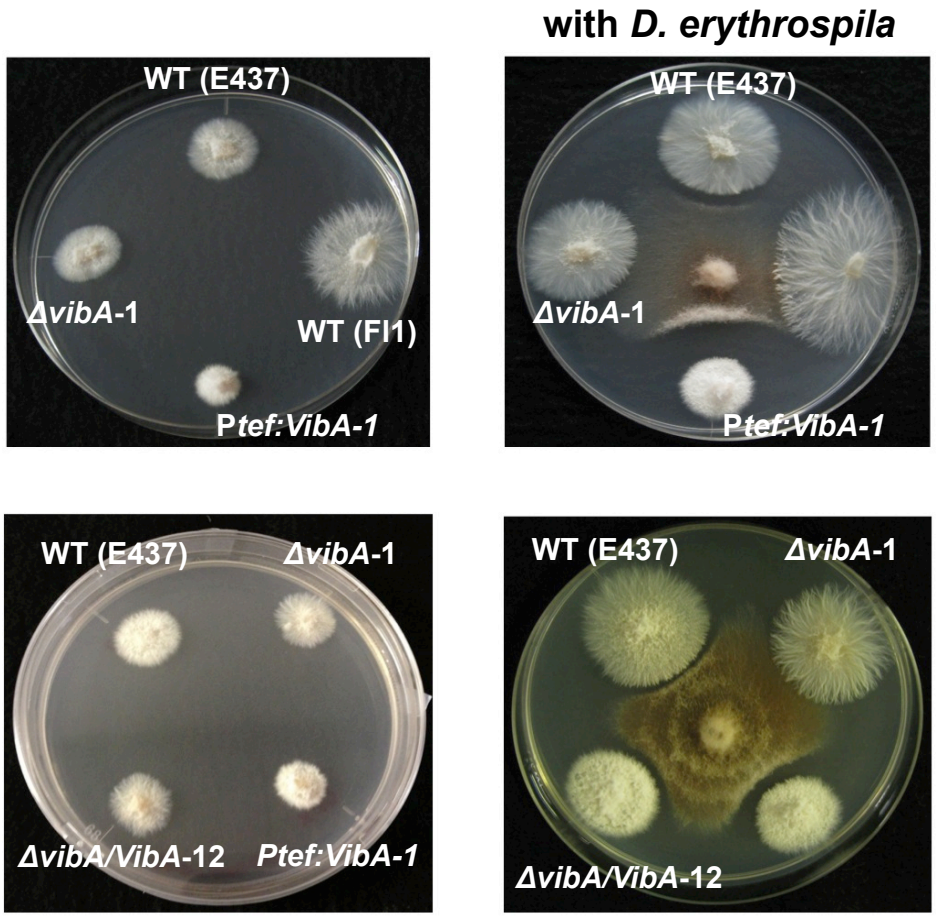



**Fig. 9** Effect of *vibA* gene deletion and overexpression on colony morphology, radial growth and antimicrobial activity against *D. erythrospila*. Colony morphology of *E. festucae* wild type strain E437 (WT E437), wild type strain Fl1 (WT Fl1), *vibA* deletion mutant (*ΔvibA*-1), *vibA* complement transformant (*ΔvibA/VibA*-12) and *vibA*-overexpressing strain (P*tef*:*VibA-1*) on PDA before (left panels) and after (right panels) pathogen inoculation. The *E. festucae* strains were allowed to grow on PD agar for 7 days before mycelial block of the pathogen is placed on the culture plate.

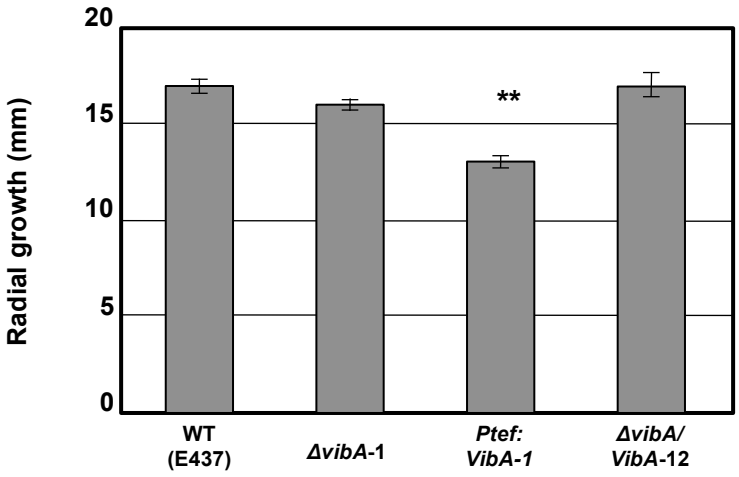



**Fig. 10** Radial growth of *E. festucae* strains (E437 (WT E437), *vibA* deletion mutant (*ΔvibA*-1), *vibA*-overexpressing strain (P*tef*:*VibA-1*) and *vibA* complement transformant (*ΔvibA/VibA*-12)) 7 days after inoculation on PDA. Error bars indicate the standard deviation. Data marked with asterisks are significantly different from the control (WT E437) as assessed by the two-tailed Student's *t* test: (**$p < 0.01$).

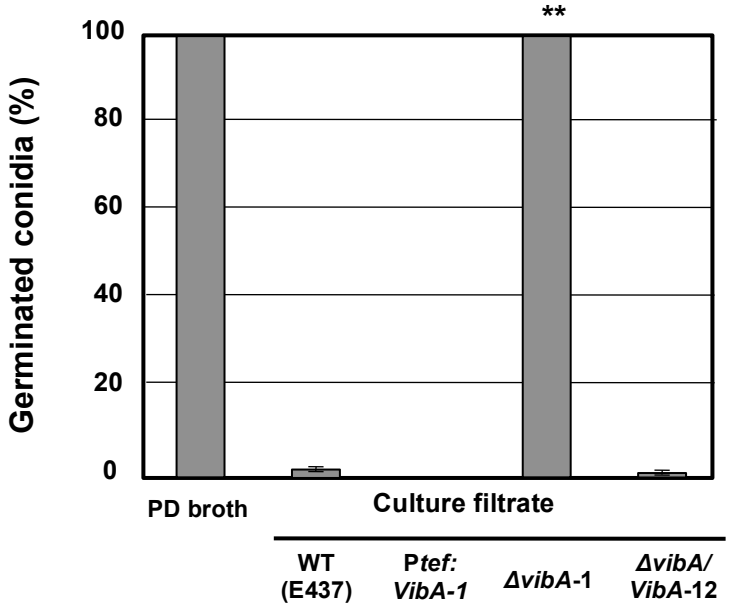



**Fig. 11** Inhibitory activity of culture filtrate of *E. festucae* wild type E437 (WT E437), *vibA*-overexpressed (P*tef:VibA*-1), *vibA* deletion mutant (Δ*vibA*) and *vibA* complemented transformant (*ΔvibA/VibA*-12) on conidial germination of *D. erythrospila*. Percentage of germinated conidia after 12 h incubation in PD broth or culture filtrate of *E. festucae* strains. The frequency of germinated conidia was calculated from at least 150 spores. Data are means ± standard error from 3 biological replicates (>50 spores/experiment). Data marked with asterisks are significantly different from control (WT E437) as assessed by the two-tailed Student's *t* test (**$p < 0.01$).

protease activity was detected as a visible halo around the colony of endophyte isolates (Vermelho et al., 1996). Within 10 days after the inoculation, visible halo (cleared area) around the colony was obvious for wild type E437, while no halo was detected around the colony of the *vibA* mutant (Fig. 12). A relatively small halo was observed around the colony of mutant 830. These results indicated that *E. festucae* VibA is involved in the production of extracellular proteases.

**Expression analysis of *vibA* gene in mutant 830 and non-antifungal wild type Fl1 isolate.**

Expression levels of *vibA* gene in *E. festucae* isolate E437, 830 mutant, Δ*vibA* mutant and non-antifungal wild type isolate Fl1 in axenic culture was investigated. The relative expression level of *vibA* gene in mutant 830 was significantly decreased compared with wild type E437 (approx. 20% of wild type) indicating that the loss of antifungal activity in mutant 830 was a consequence of the compromised function of *vibA* promoter by vector insertion (Fig. 13). The relative *vibA* expression level in Fl1, an *E. festucae* isolate with no inhibitory activity against any of the test pathogens, is lower (approx. 60%) compared to isolate E437. As expected, no expression of *vibA* gene was detected in *vibA* deletion mutant (Fig. 13).

***vibA* expression is enhanced when challenged by the pathogen.**

To determine whether *vibA* in *E. festucae* is differentially expressed in the absence of or when co-cultured with grass pathogen, transformants expressing GFP under the control of *vibA* promoter were subjected to inhibition assay with *D. erythrospila*. Confocal microscopy of transformants showed that the expression of GFP is enhanced when the mycelia of the

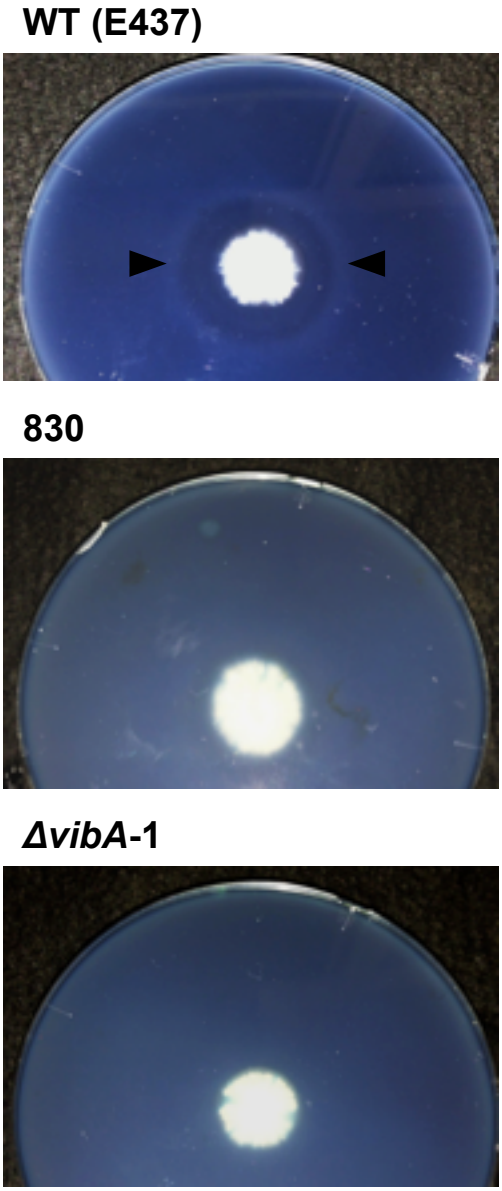



**Fig. 12** Extracellular protease production activity of *E. festucae* wild type strain E437 (WT E437), *vibA* deletion mutant (*ΔvibA*-1) and mutant 830. *E. festucae* strains were grown for 10 days on PDA containing 1 % (w/v) gelatin as substrate. Activity of extracellular proteases was detected as visible halo (indicated by arrowheads) around the colony of *E. festucae*.

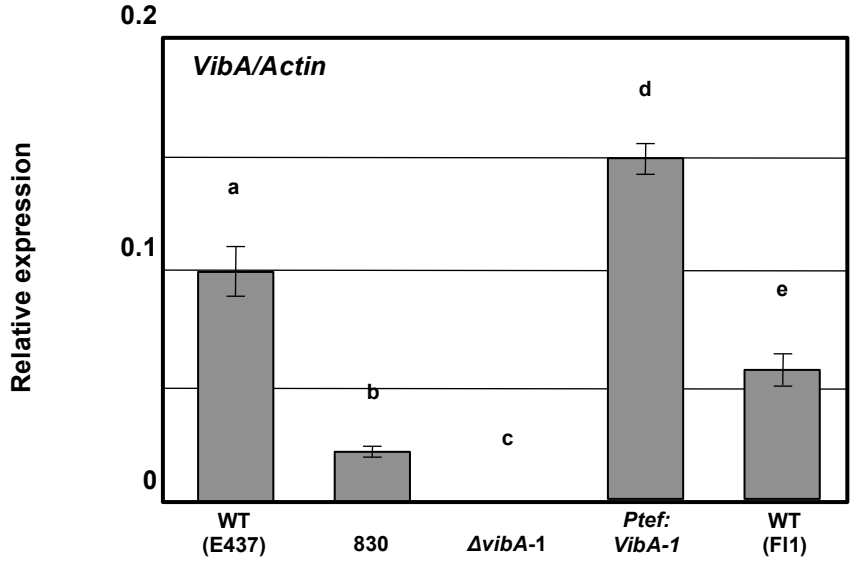



**Fig. 13** Relative expression of *vibA* in *E. festucae* strains (E437 (WT E437), wild type strain Fl1 (WT Fl1), REMI 830 mutant, *vibA* deletion mutant (*ΔvibA*-1), and *vibA*-overexpressing strain (P*tef*:*VibA-1*) in axenic culture. Total RNA was isolated from mycelia of *E. festucae* strains grown in PDB for 7 days and relative expression levels of *vibA* in endophyte strains were normalized against *actin* gene. Different letters indicate the significant difference as assessed by the two-tailed Student's *t* test ($p < 0.05$).

endophyte is challenged by a pathogen. Enhanced expression of GFP was obvious despite the 5 mm average distance between the two fungal species. On the other hand, mycelia of the transformant that were not confronted by the pathogen showed low GFP fluorescence signal (Fig. 14), suggesting that expression of endophyte *vibA* is enhanced by the encounter with other fungal species.

**Overexpression of *E. festucae vibA* enhances its inhibitory activity against grass pathogens.**

*vibA* gene under the control of TEF promoter was introduced in isolate E437 to investigate the effect of enhanced *vibA* expression on anti-fungal activity. Transformation vector pNPP154 containing P*tef:vibA* cassette was introduced in E437 protoplast. Ten *vibA*-overexpressing transformants were then subjected to *in vitro* inhibitory assay against grass pathogens. Overexpression of the *vibA* reduced radial growth of colony and enhanced production of aerial hyphae (Fig. 9 and 10). All the tested *vibA*-overexpressing transformants showed enhanced inhibitory activity against *D. erythrospila, D. siccans, D. dictyoides* and *C. graminicola.* Enhanced inhibitory activity was manifested as a wider clear zone between colonies of *vibA*-overexpressing transformants and the grass pathogens (Figs. 9 and 15). Moreover, transformants overexpressing *vibA* could inhibit the mycelial growth of *Rhizoctonia solani, Magnaporthe grisea* and *Sclerotinia homoeocarpa*, but the wild type E437 could not (Fig. 15).

To compare the inhibitory activity of the culture filtrate of *vibA*-overexpressed mutant with that of the wild type E437, conidia of *D. erythrospila* was incubated in serially diluted

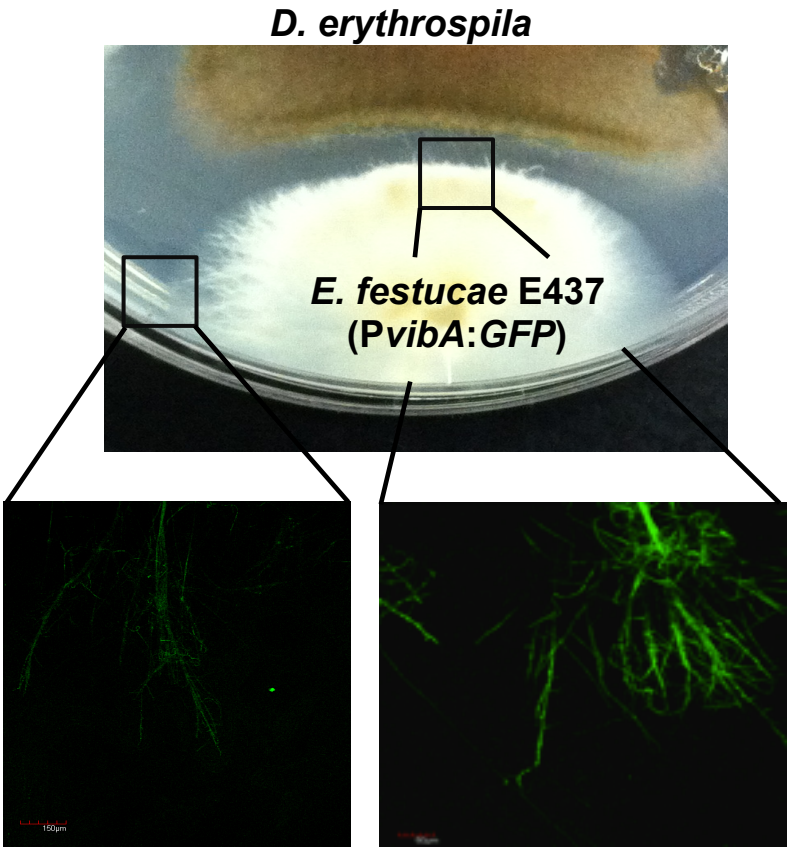



**Fig. 14** *vibA* expression is enhanced when challenged by the pathogen. Transformant expressing GFP under the control of *vibA* promoter (P*vibA:GFP*) was subjected to inhibition assay with *D. erythrospila*. Micrograph showing GFP fluorescence of endophyte hyphae not confronted (left panel) and challenged (right panel) by the pathogen.

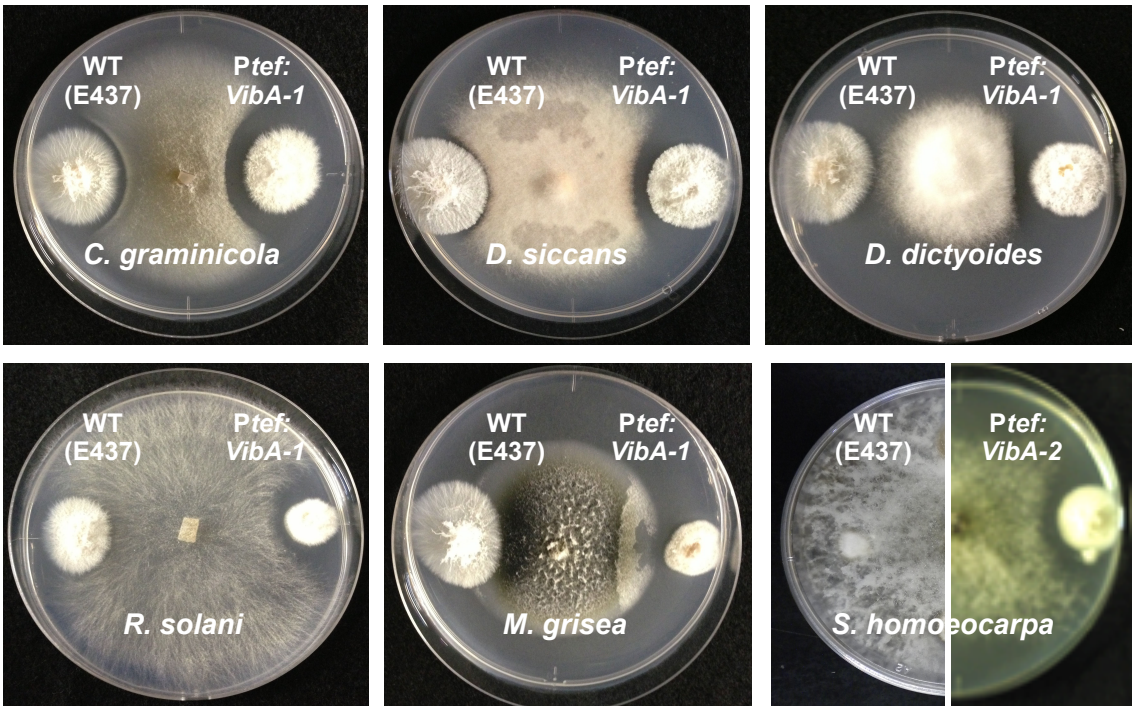



**Fig. 15** Inhibitory activity of *vibA*-overexpressing transformant (P*tef:VibA-1*) on mycelial growth of grass pathogens. Colonies of *E. festucae* wild type E437 (left side) and *vibA*-overexpressing transformant (right side) were grown for 7 days before a mycelial plug of grass pathogens was inoculated. The culture was incubated at 23°C for 7-14 days until a clear zone of inhibition was observed.

culture filtrate of *vibA*-overexpressed transformant and wild type E437. Culture filtrate of E437 diluted to 75% did not inhibit the conidial germination of *D. erythrospila*. In contrast, conidial germination of the pathogen was not observed even in the culture filtrate of *vibA*-overexpressed transformant diluted to 25% and short germ tubes of pathogen can be seen in 12% culture filtrate (Fig. 16). These results showed that the *vibA*-overexpressed transformant produces approximately 10 times more inhibitory compound than the wild type E437.

**Non-antifungal wild type Fl1 isolate gained inhibitory activity by overexpression of *vibA*.**

Transformation vector pNPP154 containing P*tef:vibA* cassette was introduced in wild type Fl1 isolate, which has no antifungal activity. Obtained transformants expressing *vibA* showed reduced radial growth, same as that of E437 overexpressing *vibA* (Figs. 9 and 17). The *vibA*-overexpressed Fl1 transformants showed inhibitory activity against *D. erythrospila* (Fig. 17). The culture filtrate of the wild type Fl1 has no inhibitory activity on conidial germination of *D. erythrospila*, but culture filtrate of Fl1 overexpressing *vibA* significantly inhibited the germination of pathogen's conidia (Fig. 18). These results indicated that enhanced expression of *vibA* is sufficient for a non-antifungal isolate to obtain antifungal activity.

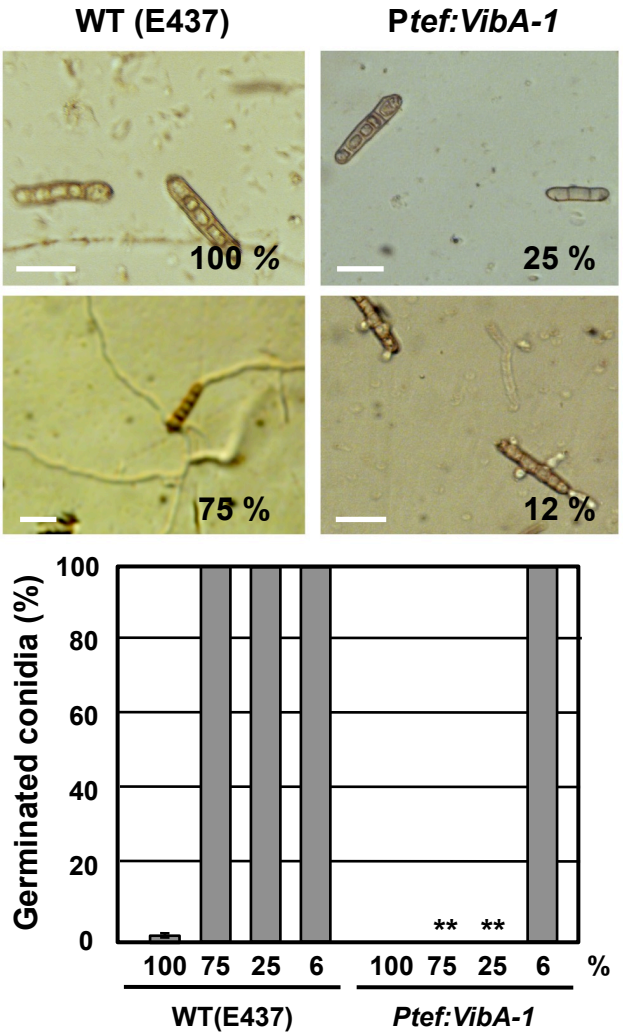



**Fig. 16** Comparison of inhibitory activity of culture filtrate of *E. festucae* wild type E437 and *vibA*-overexpressed transformant on conidial germination of *D. erythrospila.* Morphology of conidia of *D. erythrospila* after 12 h incubation in 100% or diluted culture filtrate of WT E437 or *vibA*-overexpressing transformant. Values at the image panels refer to the concentration of the endophyte culture filtrate. Percentage of germinated conidia after 12 h incubation in different concentration of culture filtrate from *E. festucae* strains. The frequency of germinated conidia was calculated from at least 150 spores. Data are means ± standard error from three biological replicates (>50 spores/experiment). Data marked with asterisks are significantly different from control (WT E437) as assessed by the two-tailed Student's *t* test (**$p < 0.01$).

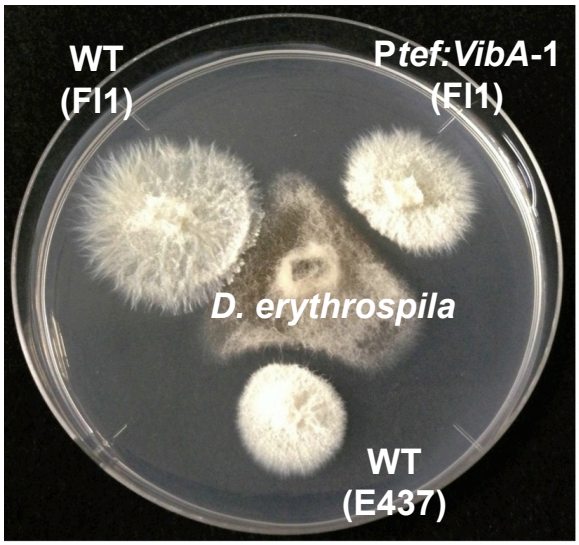



**Fig. 17** Gain of function in a non-antifungal isolate Fl1 by expression of *vibA*. Inhibitory activity of mycelial colony of *E. festucae* wild type Fl1 (WT Fl1), wild type E437 (WT E437) and *vibA*-expressing Fl1 transformant (P*tef:VibA*-1 Fl1), against *D. erythrospila* .

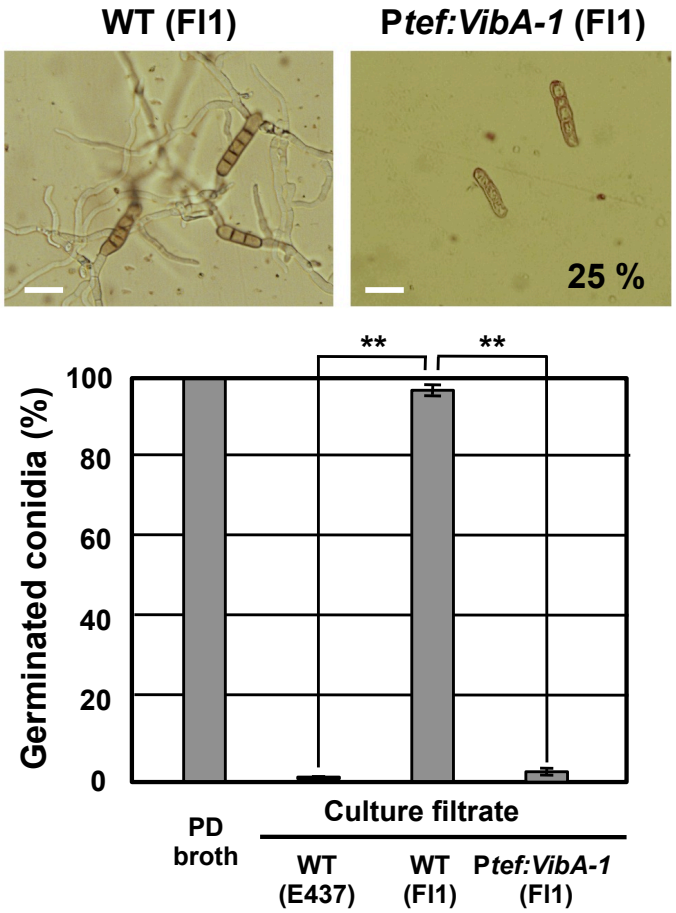



**Fig. 18** Percentage of germinated conidia after 12 h incubation in culture filtrate of *E. festucae* wild type Fl1 (WT Fl1), wild type E437 (WT E437) and *vibA*-expressing Fl1 transformant (P*tef:VibA*-1 Fl1). The frequency of germinated conidia was calculated from at least 150 spores. Data are means ± standard error from three biological replicates (>50 spores/experiment). Data marked with asterisks indicate significant difference as assessed by the two-tailed Student's *t* test (**$p < 0.01$).

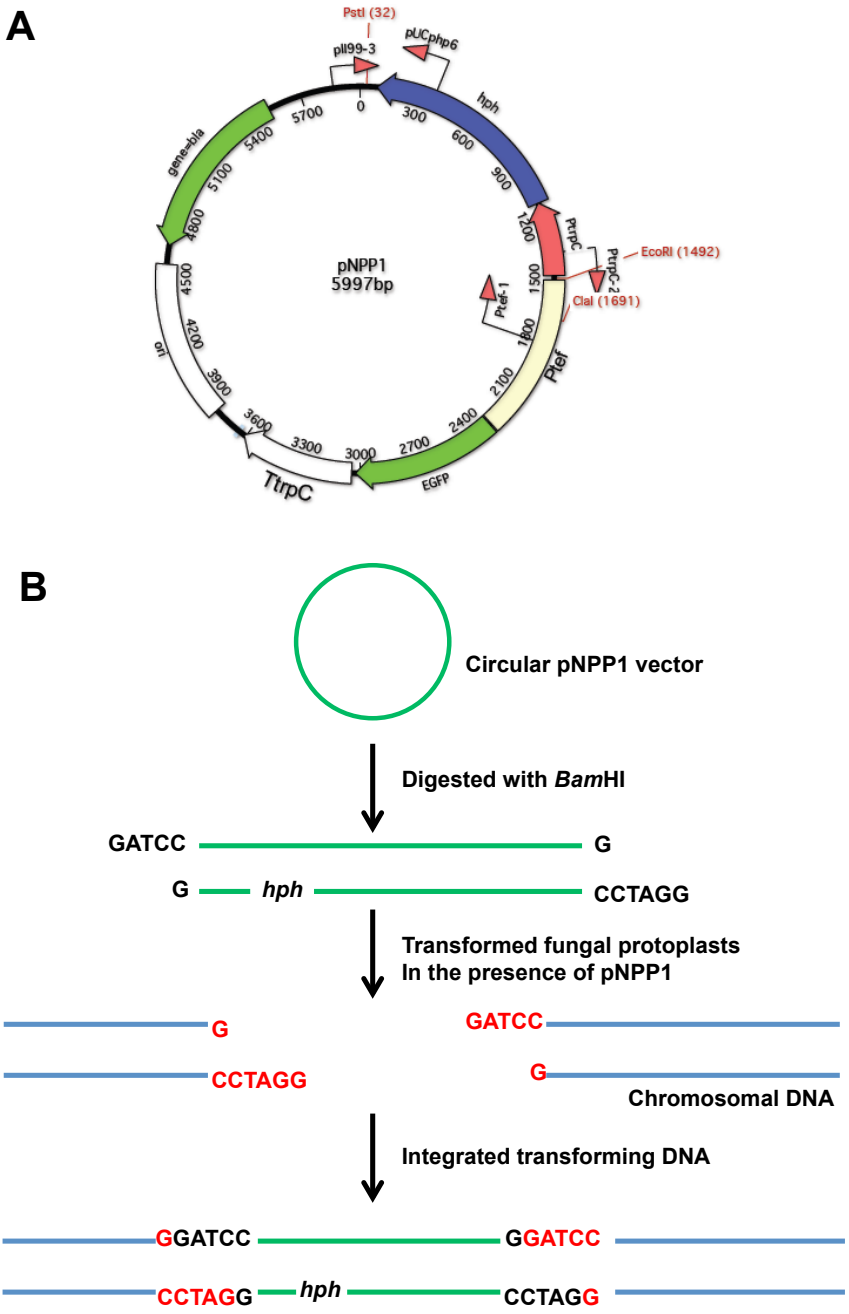



**Fig. 19 (a)** Physical map of transformation vector pNPP1 used in this study. Ptef, Tef promoter; TtrpC, trpC terminator; PtrpC, trpC promoter; EGFP, enhanced green fluorescence protein; hph, hygromycin B phosphotransferase; ori, replication origin; gene=bla, ampicillin resistance gene *bla*. **(b)** Schematic representation of restriction enzyme-mediated integration (REMI) of transforming DNA into fungal chromosome DNA.

## DISCUSSION

Using the bioprotective isolate *E. festucae* E437, this chapter presents the involvement of a vegetative incompatibility gene in the biosynthesis and regulation of inhibitory substances produced by the endophyte against grass pathogens.

An *E. festucae* E437 mutant, designated as 830 that lost its antifungal activity against the test pathogen *D. erythrospila* was isolated. Mutant 830 had plasmid insertion in the promoter region of *vibA* gene that encodes a putative transcription factor homologous to *N. crassa* VIB-1. In *N. crassa*, VIB-1 is required for the expression of genes involved in non-self recognition leading to heterokaryon incompatibility and cell death (Dementhon et al., 2006). In filamentous fungi, heterokaryon incompatibility as a consequence of non-self recognition is manifested by the rejection of heterokaryon formation among genetically different isolates of the same fungal species (Glass and Kaneko, 2003). While the hyphal cell fusion and formation of vegetative heterokaryon between different individuals can be beneficial for fungi as functional diploidy and formation of hyphal network for nutrient transport and resource utilization (Fleissner et al., 2008), heterokaryon incompatibility can also be advantageous to organisms as a mechanism to reduce the risk of transmission of infectious factors in cytoplasm and avoid exploitation by aggressive strains (Glass and Kaneko, 2003). In *N. crassa*, heterokaryon incompatibility is genetically regulated by *het* loci wherein the genetic differences in *het* loci can constrain heterokaryon formation (Glass and Kaneko, 2003). A loss of function mutation in *N. crassa vib-1* suppressed the *het-c*- and *mat*-associated heterokaryon incompatibility, hence heterokaryosis by hyphal fusion occurs with strains with which they were formerly incompatible (Xiang and Glass, 2002; Xiang and Glass, 2004). In addition to vegetative incompatibility, *N. crassa* VIB-1 is also involved in the negative regulation of conidiation, formation of aerial hyphae, development of protoperithecia (female reproductive

structure) and production of extracellular protease (Hutchison and Glass, 2010; Xiang and Glass, 2002; Xiang and Glass, 2004).

VibA (VIB-1) contains an NDT80/PhoG DNA binding domain. Several studies have shown that transcription factors with NDT80/PhoG DNA binding domain have diverse functions in different Ascomycota species. In *Saccharomyces cerevisiae*, Ndt80p is a transcriptional regulator of meiosis and sporulation. *S. cerevisiae* Ndt80p binds to a 9 bp regulatory sequence in the promoter region of target genes called middle sporulation element (MSE) and is expected to directly activate ~150 genes including those related to multiple processes for meiotic commitment and sporulation (Chu et al., 1998; Chu and Herskowitz, 1998; Winter, 2012). On the other hand, in *Candida albicans*, Ndt80p was isolated as a regulator of the gene for drug efflux pump, *CDR1*. Further analyses indicated that *C. albicans* Ndt80p is also involved in the regulation of large number of genes for diverse biological functions including sterol metabolism and drug resistance (Chen et al., 2004; Sellam et al., 2009). *N. crassa* genome has three genes encoding putative transcription factor with NDT80/PhoG DNA binding domain (Hutchison and Glass, 2010). None of them is required for meiosis while VIB-1 and FSD-1 (closest homologue of yeast Ndt80p) are both involved in the formation of protoperithecia (Hutchison and Glass, 2010).

Among the limited reports on the functional analysis of *vib-1* homologue in filamentous Ascomycota, *vib-1* orthologue in *Aspergillus nidulans*, *xprG* (*phoG*) was shown to be involved in protease production in response to nutrient limitation. Deletion mutants of *xpr*G cannot utilize proteins as carbon or nitrogen source (Katz et al., 2006). *E. festucae vibA* mutant, like in the cases of *N. crassa vib-1* and *A. nidulans xprG* mutants (Dementhon et al., 2006; Katz et al., 2006), is also defective in the production of extracellular protease. It is

therefore likely that the positive regulation of protease production is a conserved role of VIB-1-like transcription factors in filamentous Ascomycota fungi.

While no particular report has implicated the protease gene as essential in the execution of cell death during heterokaryon incompatibility, a strong increase in the cellular proteolytic activity has been observed during the incompatibility reaction in *Podospora anserina* (Bégueret, 1972; Bégueret and Bernet, 1973). After fusion of incompatible individuals, rapid cytological changes of hyphal cells including the vacuolization of the cytoplasm happened. This was followed by the destruction of cytosolic compartments, further implicating the involvement of cell lytic enzymes in the induction of cell death in heterokaryon incompatibility. In contrast, the *E. festucae* E437 isolate, as fungal antagonist did not cause hyphal cell lysis of the confronted pathogen but affected its active apical hyphal growth and differentiation activity (Niones and Takemoto, 2014). Additionally, the extracellular protease production ability of *E. festucae* wild type isolates was not necessarily associated with its antimicrobial activity in culture (Niones and Takemoto, 2014). Further supporting this deduction are the two *E. festucae* E437 non-antifungal mutants isolated recently, T547 and T692 showing extracellular protease activity comparable with the wild type E437 (Hashikawa T. and Takemoto D., unpublished data). Altogether, given that NDT80/PhoG type transcription factor of *S. cerevisiae* and *C. albicans* has a large number of direct target genes (Chu et al., 1998; Sellam et al., 2009), it is likely that the genes for the production of inhibitory metabolite in *E. festucae* rather than the cell lytic enzymes, are critical target of VibA for the antifungal activity of endophyte.

Recognition of the pathogen is not essential for the isolate E437 to produce inhibitory compound in culture, but pathogen recognition is probably an important cue for an enhanced synthesis of inhibitory compound. Under our experimental condition, despite not having

quantified the intensity of GFP fluorescence, an elevated fluorescence intensity of GPF under the control of *vibA* promoter was obvious when the endophyte colony was co-cultured with *D. erythrospila.* The increased expression of GFP was observed despite the 5 mm gap between colonies of the pathogen and the endophyte. Relative to the hyphal diameter of the two interacting fungal species, a 5 mm zone of inhibition can be an extremely long distance for the endophyte to directly recognize the presence of the pathogen. Diffusible secretory product from pathogen might be recognized by the endophyte as extraneous substances. Alternatively, a few directly confronted endophytic hyphae might induce the response of the remaining cells via intracolony communication. As for the case of fungal heterokaryon incompatibility, induction of cell death occurred when cells of incompatible individuals fused and products of *het* loci directly interacted in continuous cytosol (Glass and Kaneko, 2003). Collectively, the results presented imply that while the phenomena of heterokaryon incompatibility and the antagonistic effect of endophyte against pathogen are both consequences of non-self recognition, factors affecting the induction of responses are apparently distinct from each other.

Overexpressing *vibA* gene in a genome of an antifungal epichloae isolate increased the amount of inhibitory compound produced by the transformant. The antifungal activity of the culture filtrate of *vibA*-overexpressed transformant was about 10 times stronger compared with the wild type. Consequently, compared with the wild type isolate, a wider zone of inhibition was observed in the dual culture assay involving the *vibA*-overexpressed transformants. Moreover, the ability of the *vibA*-overexpressing transformant to inhibit test pathogens that were not inhibited by the wild type isolate indicates that the test pathogens have different levels of sensitivity to the inhibitory compound produced by the endophyte. *R. solani and S. homoeocarpa* are fast- growing fungi, thus it seems likely that a higher

concentration of inhibitory endophyte product is needed to inhibit the mycelial growth of these two pathogens. Furthermore, overexpressing *vibA* in a non-antifungal *E. festucae* wild type Fl1 seemingly increases the quantity of the synthesized inhibitory compound at a certain concentration that is effective to inhibit the mycelial growth and conidial germination of *D. erythrospila*. This apparent effective antifungal concentration threshold was also evident when the wild type E437 culture filtrate diluted to 75% did not exhibit antifungal activity against *D. erythrospila*.

The production of antifungal compound is not proportional with the expression activity of VibA as a transcription factor. Compared with the *vibA* expression level in the antifungal wild type isolate, *vibA* expression in mutant 830 is about 20% of the wild type E437. However, the culture filtrate of mutant 830 has no inhibitory activity. Conversely, the expression level of *vibA* in P*tef:vibA* transformant is only 1.5 times more than the wild type, but the inhibitory activity of the culture filtrate of the same transformant was about 10 times stronger compared with the wild type. Nonetheless, the expression level of *vibA* in P*tef:vibA* transformant is surprisingly low considering it is under the control of a highly expressive TEF promoter gene. One possible explanation for this is that increasing the expression of *vibA* incurs developmental cost to the endophyte, as demonstrated by the reduced hyphal growth of the *vibA*-overexpressing transformants. On the other hand, *vibA* expression level in a non-antifungal wild type isolate Fl1 was about 60% of the E437 wild type isolate, but introduction of P*tef:vibA* cassette conferred antifungal activity to Fl1. This observed difference maybe due to the two amino acid substitutions between VibA of the antifungal (E437) and the non-antifungal (Fl1) *E. festucae* isolates. This amino acid difference could possibly affect the activity of VibA as the transcription factor to induce target genes required for the production of antifungal compound.

# CHAPTER 3

## Identification and characterization of inhibitory compound produced by *E. festucae* E437 isolate

## INTRODUCTION

*Epichloë festucae* belongs to the Clavicipitaceous class of endophytes which members are considered defensive mutualists of host grasses (Clay, 1988). This fungal class lineage is noted to produce bioactive compounds, of which the impact may either positively or negatively affect plant host fitness, depending on the plant species and epichloae species interaction.

In a symbiotic association of epichloae endophytes with grasses of subfamily Pooideae, peramine, indole diterpenes (i.e. lolitrem B), loline alkaloids (i. e. N-formylloline and ergot alkaloids (i.e. ergovaline) are the biologically active metabolites identified in endophyte-infected grasses (Lane et al., 2000; Rowan, 1993). Peramine is a potent feeding deterrent of adult Argentine stem weevil (ASW) (Rowan and Gaynor, 1986; Rowan et al., 1990). ASW is an economically important pest of perennial ryegrass in New Zealand. Another class of compound, the janthitrems contribute to insect toxicity manifested by feeding deterrence, reduced survival and reduced development (Tapper and Lane, 2004). On the other hand, while lolitrem B and ergovaline have anti-insect activities, they are also known as mammalian toxins. Lolitrem B is known as the causative agent of 'ryegrass staggers', a neuromuscular disorder among sheep grazing ryegrass dominant pastures during

and after periods of water stress. Ergovaline, on the other hand, has been implicated in fescue toxicosis.

Similar to the production of anti-insect metabolites by the mutualistic endophyte that protects the host from insect herbivory, the mechanism of antibiosis is also proposed for the increased fungal disease resistance in endophyte-infected plants (Siegel and Latch, 1991; Yue et. al., 2000). The production of fungal growth suppressors by the endophytes was demonstrated in the dual culture assay between epichloae endophytes and cultures of known grass pathogens. Some epichloae isolates inhibited the growth of fungal grass pathogens, *in vitro* (Siegel and Latch, 1991; White and Cole, 1985). Moreover, the antifungal activity of epichloae endophyte in culture varies with the pathogen and endophytes isolate combination (Christensen et al. 1991; Li et al., 2007; Ren et al., 2009).

Few studies have so far attempted to identify and characterize the fungal inhibitors produced by *Epichloë* species in culture. Yue et al. (2000) identified indole-3-acetic acid and indole-3-ethanol, a sesquiterpene and a diacetamide from cultures of epichloae isolates as antifungal metabolites against grass pathogens. Seto et al. (2007) reported that cyclic peptide, epichlicin, produced by *E. typhina* inhibits the spore germination of *Clasdosporium phlei*. However, the association of disease resistance to these chemical components has not been established *in planta*.

Results in Chapter 1 and 2 have so far provided that an antifungal-producing *E. festucae* E437 strain can suppress grass leaf spot (*Dreschslera erythrospila*) disease development in perennial ryegrass (*Lolium perenne*). In addition, the involvement of a vegetative incompatibility blocked gene *vibA*, in the biosynthesis and regulation of antifungal compound by *E. festucae* E437 strain has also been demonstrated. The objective of this

chapter is to identify and isolate the potential inhibitory compound produced by the bioprotective isolate of *E. festucae.*

## MATERIALS AND METHODS

### Biological materials.

*Epichloë festucae* strain E437 was from the collection of Prof. Christopher L. Schardl (University of Kentucky, USA). The *vibA*-overexpressing isolate of *E. festucae* strain 437 was obtained through the fungal transformation process described in Chapter 2. Cultures of *E. festucae* were grown on 2.4% potato dextrose agar and maintained at 23˚C or kept in 4˚C until use. The test fungal grass pathogen *Drechslera erythrospila* (isolate 638, MAFF No. 305378), from the collection of the National Institute of Agrobiological Sciences (NIAS, Japan) was grown on PDA at 23˚C and maintained at 4˚C until use.

### Fungal assay.

#### a. Preparation of *E. festucae* culture filtrate.

A mycelial block (1 x 1 cm) of *Epichloë* isolate grown on PDA was finely chopped and then inoculated in 100 ml flasks containing 50 ml of 2.4% potato dextrose (PD) broth. The flasks were kept in an orbital shaker (100 rpm) at 23°C. Culture filtrates were collected at seven days after endophyte inoculation. The culture filtrates were then sterilized using a filter membrane unit (Millex-HA filter unit, 45 μm pore diameter, Millipore, Billerica, MA, USA) and kept in 4˚C until use.

**b. Sporulation and preparation of conidial suspension of *Drechslera erythrospila.***

Mycelial plug of *D. erythrospila* grown on PDA was inoculated on sterile ryegrass leaf pieces placed on the surface of 1.2% tap water agar plates. Plates were incubated at 25˚C with a cycle of twelve hours white light (cool white fluorescent tube) supplemented with near ultraviolet light followed by twelve hours of dark condition. One to two weeks after inoculation, sporulating leaf pieces were transferred in 2 ml tubes and suspended in 1 ml of sterile distilled water. The tubes were agitated with a mechanical vibrator to dislodge the conidia and then leaf pieces were removed. The collected spore suspension was centrifuged at 8,000 rpm for 1 min, and resuspended in distilled water to give a final concentration of 1 x $10^4$ conidia/ml. Spore suspensions were made up just prior to use.

**c. Spore germination assay.**

Twenty µl of culture filtrate fraction was added into a sterile biconcave microscope slide and mixed with 5 µl of conidial suspension of *D. erythrospila*. In the control test, conidial suspension was mixed with sterile PD broth. Unless stated, 100% concentration of each extraction fraction was mixed with conidial suspension. All treatments were replicated three times. The slides were placed in Petri dishes and kept at 25°C. After twelve hours, conidial germination was determined and scored from at least 150 conidia per treatment. Germination was defined, as having occurred when a germ tube produced is at least half in length of the spore. The replicated experiment was repeated three times.

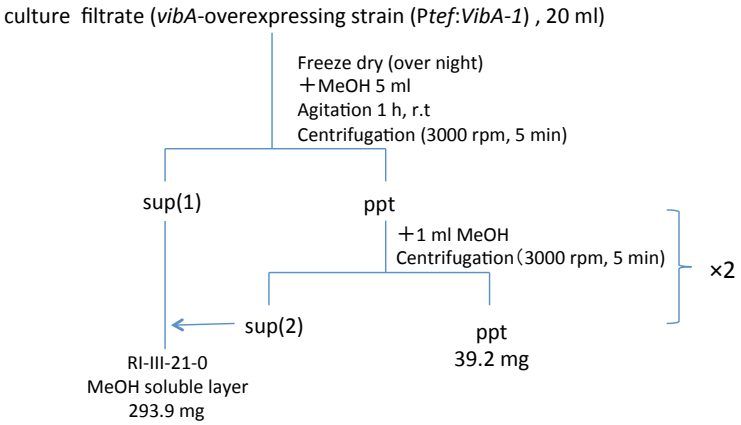
**Extraction and isolation of antifungal compound.**

Methanol, ethanol and isopropyl were evaluated as possible organic extraction solvents. *E. festucae* culture filtrate was freeze-dried overnight prior to addition of the

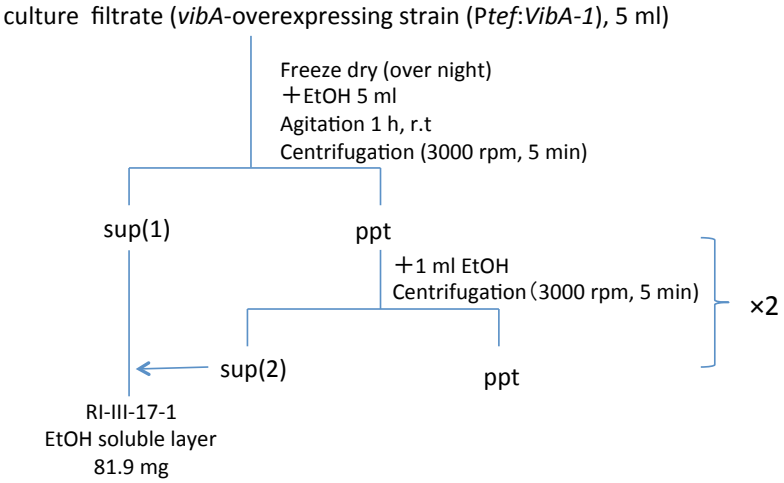
extraction solvent. The resulting suspension was placed in a shaker for 1 hr at 30ºC, and then centrifuged at 3000 rpm for 5 min. The precipitate (organic solvent -insoluble layer) was washed with the organic solvent and then again centrifuged at 3000 rpm for 5 min. The resulting precipitate (solvent -insoluble layer) and as well as the supernatant (solvent - soluble layer) were then subjected to conidial germination assay. Extraction scheme is illustrated in Figure 1.

For further purification process, the crude fraction with active inhibitory activity was then subjected to different purification columns such as open tubular columns with surface-bonded octadecylsilane (ODS) and high performance liquid chromatography (HPLC) as illustrated in Figures 2 to 4.

## Methanol soluble-insoluble




## Ethanol soluble-insoluble




## Isopropyl alcohol soluble-insoluble

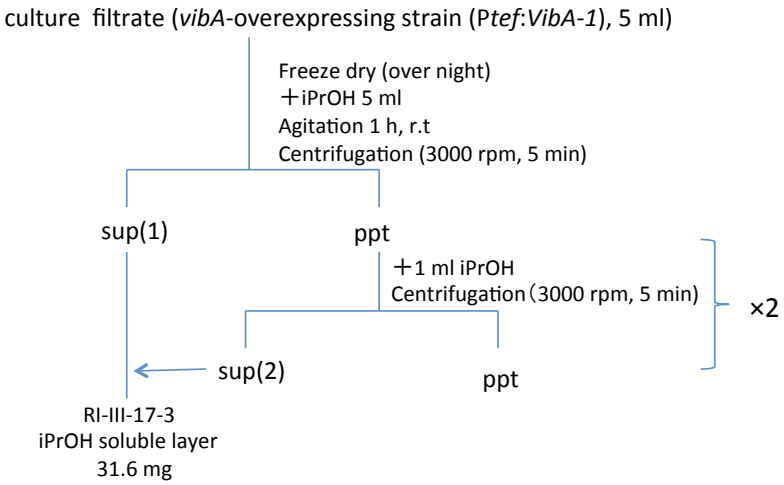



**Fig. 1** Extraction scheme using different organic extraction solvents.

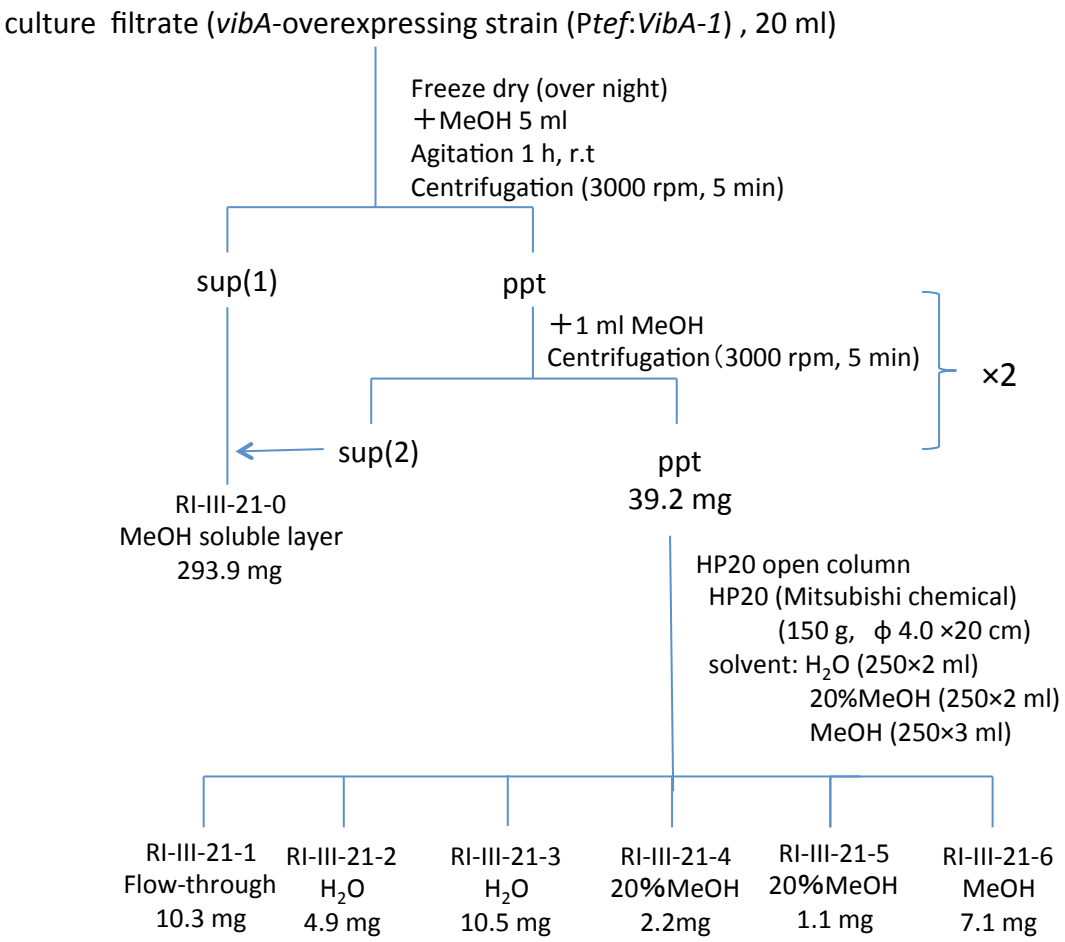



**Fig. 2** Active compound separation scheme of methanol-insoluble layer using HP20 open column.

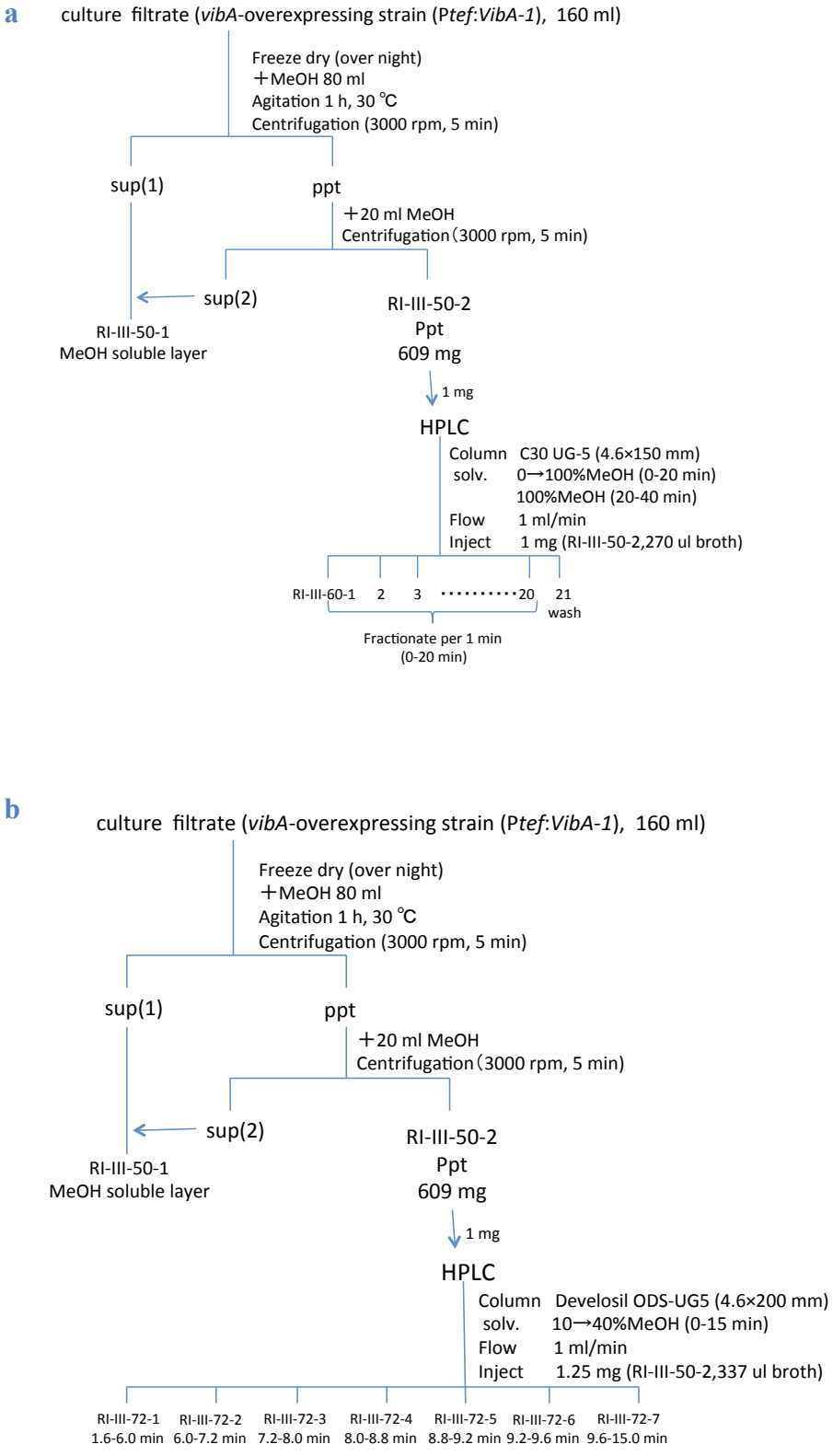



**Fig. 3** Active compound separation scheme of methanol-insoluble layer through high performance liquid chromatography (HPLC) using (a) C30 UG-5 column and (b) Devosil ODS UGS column.

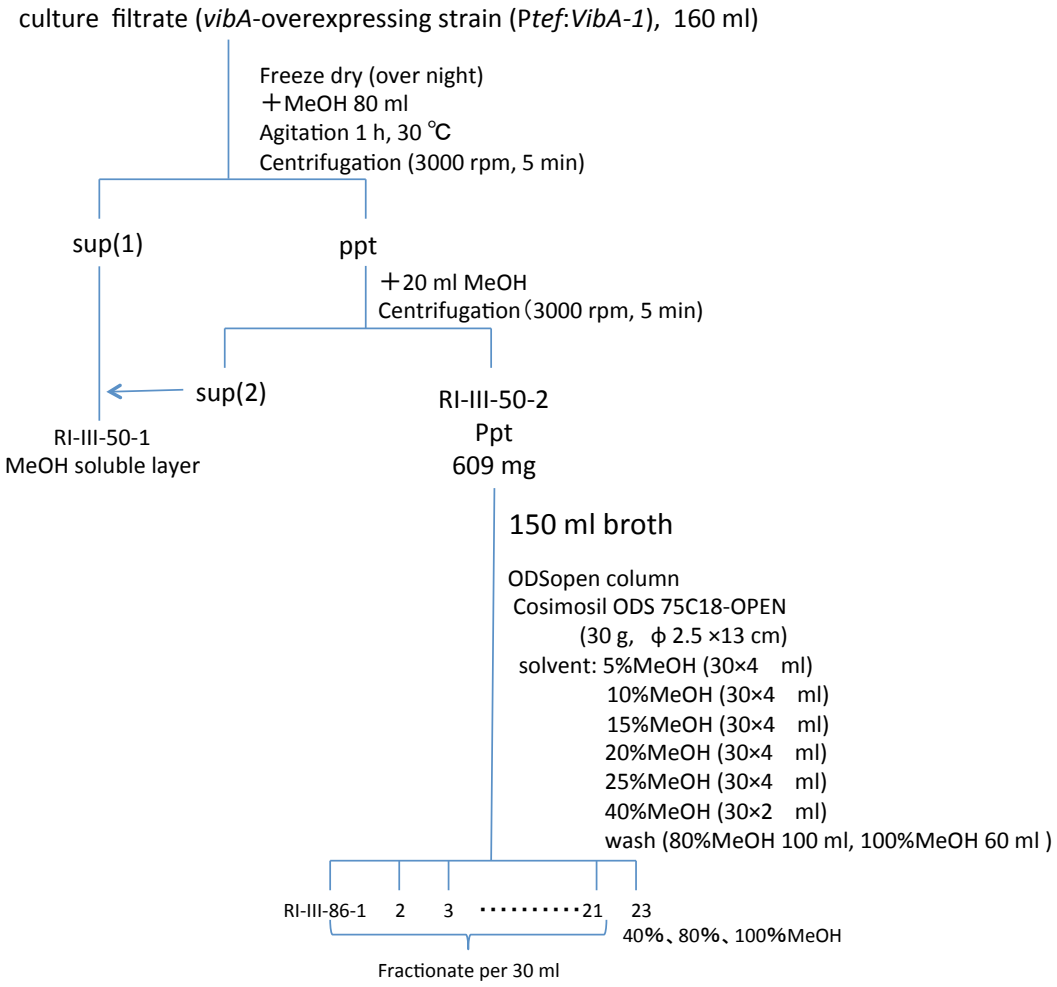



**Fig. 4** Active compound separation scheme of methanol-insoluble layer through octadecylsilane (ODS) open column.

**RESULTS**

In the preliminary characterization of the culture filtrate of *E. festucae* E437 isolate (as presented in Chapter 1), it was found out that bioprotective compound produced by endophyte in culture is a thermal stable and a low-molecular weight antifungal compound. Moreover, as this endophyte isolate did not cause hyphal cell lysis of affected pathogen, the candidate inhibitory substance is possibly not a proteinaceous cell lytic enzyme. With these results, experiments were performed to identify the active antifungal compound produced by this *E. festucae* isolate. Instead of using the wildtype strain of the antifungal-producing isolate of *E. festucae* E437, the *vibA*- overexpressing strain of *E. festucae* E437 isolate was used in the isolation and characterization of potential antifungal compound. This *vibA*-overexpressing strain is producing more antifungal compound compared with the wildtype E437, resulting in the enhanced inhibitory ability against mycelia growth and conidial germination of the test pathogen, *D. erythrospila* (as presented in Chapter 2).

The first step was the screening for an extraction organic solvent. Methanol, ethanol and 2-Propanol were evaluated as possible organic solvents, following the extraction scheme shown in Figure 1. Among the organic solvents that were used for extraction, only the methanol-insoluble fraction could inhibit the conidial germination of *D. erythrospila* (Fig. 3). Moreover, methanol was also efficient as extraction solvent, as at relatively small quantity of precipitate (600 mg), anti-fungal inhibitory activity was detected in the methanol-insoluble layer.

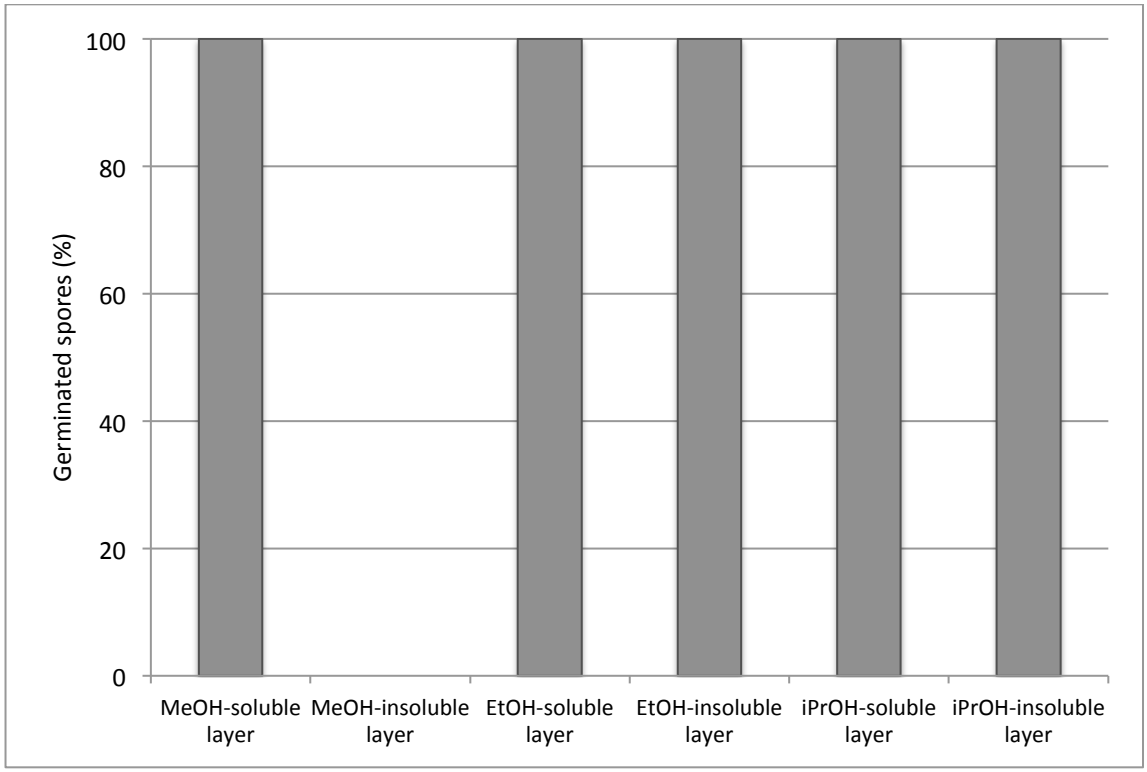



**Fig. 3** Percentage of germinated conidia after 12 h incubation in fractions of *E. festucae* culture filtrate using different organic solvents. The frequency of germinated conidia was calculated from at least 150 spores. MeOH, methanol; EtOH, ethanol; iPrOH, isopropyl alcohol.

The methanol-insoluble crude fraction was then subjected to further purification process using different separation columns as illustrated in Figures 2 to 4. Methanol-insoluble crude fraction was initially subjected to high performance liquid chromatography (HPLC) using the C30 UG-5, a highly non-polar column (Fig. 4). This was done on the premise that the antifungal compound contains highly polar component. However, this method yielded very low volume of extracted fractions. To address this, the open column ODS was used to further purify the methanol-soluble layer. Through this method larger amount of fractions were collected for spore inhibition assay. Nonetheless, none of the fractions generated through the use of different separation columns exhibited inhibitory activity against the conidial germination of the test pathogen, *D. erythrospila*.

**DISCUSSION**

Fungal endophytes are known producers of secondary metabolites, most of them demonstrating bioprotective activity against abiotic and biotic stresses. They are a rich source of novel natural products for exploitation in modern medicine, agriculture and industry. Alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids are among the well-studied compound with antimicrobial activities isolated from endophyte extracts (as reviewed in Tan and Zou, 2001). Biological and physiological activity of these fungal metabolites ranged from insecticidal and antimicrobial to initiation of physiological responses associated with protection from abiotic stresses such as drought and temperature (Kuldau and Bacon, 2008; Malinowski and Belesky, 2000; Tan and Zou, 2001).

The inhibitory activity of some strains of epichloae endophytes against important grass pathogens has been demonstrated, *in vitro* (Christensen et al., 1991; Christensen and Latch, 1991; Siegel and Latch, 1991). Siegel and Latch (1991) reported that growth of pathogens that are sensitive to fungal inhibitory strains of *Epichloë* were not inhibited by paper disks containing known insect-deterring fungal metabolites such as loline and peramine alkaloids. Such observations may imply that the antimicrobial compounds synthesized by epichloae endophytes may be different, both in structure and function, from that of anti-insect secondary metabolites. On the other hand, specific compounds such as indole derivatives, a sesquiterpene, a diacetamide, and a cyclic peptide have been identified from cultures of antimicrobial epichloae endophytes (Seto et al., 2007; Yue et al., 2000).

After showing that culture filtrate of *E. festucae* E437 isolate can inhibit the spore germination of the test pathogen, *D. erythrospila*, the physicochemical properties of antifungal substance were evaluated. As presented in Chapter I, culture filtrate of *E. festucae* E437 isoalate contains a thermostable, non-cell lytic enzyme and low-molecular weight bioactive metabolite. These results are important to the succeeding steps towards the isolation and characterization of the biologically active compounds produced by the endophyte.

Fungal inhibitors were purified from the culture broth of an antifungal isolate of *E. festucae* using a bioassay-directed extraction with *D. erythrospila* as the test organism. Organic extracts of the endophyte culture filtrate were then subjected to spore inhibition assay. Results of the extraction and isolation study so far have demonstrated that the methanol-insoluble layer of the endophyte culture broth contains the bioactive compound against conidial germination of the test pathogen. After crude extraction of the culture filtrate using methanol as extraction solvent, the succeeding compound separation methods failed to

isolate the bioactive layer. None of the separation fractions showed inhibitory activity against conidial germination *D. erythrospila*.

Having an efficient bioassay system is paramount to the successful identification of biologically active fractions. The choice of test pathogen and the strain of inhibitory compound producing *E. festucae* contributed to the efficient spore germination-mediated bioactive assay. The test pathogen, *D. erythrospila* easily produced spores in water agar culture medium supplemented with sterile leaves of perennial ryegrass. Moreover, the use of an *E. festucae* strain with increased capacity of antibiotic production (*E. festucae vibA*-overexpressing transformant) offered the advantage of a culture filtrate containing more inhibitory compound. As a result, biologically active fractions can be determined in a relatively smaller volume of a crude endophyte culture broth. However, as shown in Chapter 2, inhibition of the conidial germination of *D. erythrospila* was negatively affected even by the apparent slight reduction of the inhibitory compound concentration. This could possibly explain why none of the fractions generated through the use of different separation columns exhibited inhibitory activity against the conidial germination of *D. erythrospila*. Thus, it would be interesting to determine the level of sensitivity of other test pathogens to the bioactive metabolite produced by *E. festucae* 437 isolate.

## GENERAL DISCUSSION

Fungal endophytes colonize the inter- and/or intra-cellular healthy tissues of the host plant without causing apparent symptoms of disease. As known producers of bioprotective metabolites, they are considered a rich source of novel natural products for exploitation in modern medicine, agriculture and industry.

Epichloae endophytes (holomorphic *Epichloë* spp. and anamorphic *Neotyphodium* spp.) are clavicipitaceous fungi (Ascomycota) symbiotically colonizing the intercellular spaces of vegetative and reproductive above-ground tissues of Poaceae grass species. Epichloae endophytes are generally considered as mutualists that confer bioprotective benefits to their host plants under the conditions of biotic and abiotic stresses.

While the protection of the symbiota from insect herbivory is the best-studied benefit from this association, the role of epichloae endophytes in plant protection against grass pathogens is poorly understood. There are conflicting results on the biological control effect of epichloae on plant diseases. Depending on the epichloae isolate and grass pathogen combination, endophyte infection may improve or even reduce disease resistance. It is not helpful as well that in most reported cases of disease suppression- either positive or negative influence, as a result of epichloae-pathogen interaction, specific mechanisms for the observed results were not clarified. Nonetheless, similar to the insect deterrent activity of epichloae endophytes that varies between the insect species and the endophyte strains producing different anti-insect metabolites (Kuldau and Bacon, 2008), the inconsistent effect of

endophyte infection on host disease resistance could be explained by the diverse characteristics of endophyte strains and target pathogens used in the previous studies.

This dissertation contains three studies elucidating the antibiosis mechanism of plant disease suppression by the mutualistic endophyte *E. festucae*. Moreover, the genetic evidence of the biosynthesis and regulation of epichloae-derived antimicrobial substances is presented.

Results of the dual culture assay between the 14 *E. festucae* isolates and 8 test pathogens showed differences in the magnitude of inhibitory activity depending on the endophyte isolate - grass pathogen combination. This highlights the inherent genetic differences among the endophyte strains, supposed to produce a range of antifungal compounds. Apparently, some endophyte isolates have the potential to produce greater quantities of one or more antifungal compounds. Likewise, this also reflects the variation in sensitivity among isolates of the test pathogens on the endophyte-derived inhibitory compound.

*E. festucae* E437 isolate had the most broad spectrum of inhibitory activity against test grass pathogens. Moreover, among the endophyte isolates only the culture broth of *E. festucae* E437 can inhibit the spore germination of the test pathogen, *D. erythrospila*. Further analyses showed that *E. festucae* E437-infected perennial ryegrass exhibited reduced leaf spot disease (*D. erythrospila)*, implicating the involvement of the endophyte-derived antifungal compound in plant disease suppression.

Understanding the behavior of *E. festucae* E437 isolate as a fungal antagonist as well as the physicochemical properties of the antifungal compound it produces are imperative if were are to exploit its potential as biological control agent. As fungal antagonist, *E. festucae*

E437 isolate did not cause hyphal cell lysis of affected pathogen but reduced the pathogen's apical hyphal growth and differentiation activity. Also, physicochemical characterization and partial purification by solvent extraction of the culture broth revealed the presence of a thermostable, non-cell lytic enzyme, methanol-insoluble low- molecular weight metabolite in the bioactive compound produced by *E. festucae* 437.

Through plasmid insertion mutagenesis approach, genetic evidence for the production and function of inhibitory compound by *E. festucae* E437 isolate has been elucidated. This study has so far identified *E. festucae* VibA, a transcription factor containing NDT80/PhoG DNA binding domain, as an essential factor for the antifungal activity of endophytic fungi against grass pathogens. VibA is a homologue of a transcription factor *Neurospora crassa* VIB-I that regulates genes in vegetative incompatibility and promotion of cell death. Deletion mutant of *vibA* lost its antifungal activity against grass pathogens, whereas non-antifungal endophytes isolate acquired antifungal activity by enhanced expression of *vibA*. All together, these results implicate that VibA could be a master transcription factor for the expression of antifungal activity of *E. festucae.*

The involvement of a heterokaryon incompatibility gene in the antagonistic effect of endophyte against pathogen is particularly interesting in the sense that while both phenomena are consequences of nonself recognition, factors affecting the induction of responses are apparently distinct from each other. To date no work has been reported on the functional role of a heterokaryon incompatibility gene in interspecies antagonistic interactions. Thus, the work presented here not only demonstrated the molecular mechanisms for the production of antifungal compound by the endophytic fungus, but also the overlapping and distinct

mechanisms between intraspecies heterokaryon incompatibility and interspecies antagonistic interactions.

Altogether, the work presented here deepens our current understanding of the role of an antimicrobial-producing mutualistic grass endophyte in plant protection. While in the current experimental system, the mechanism of antibiosis plays an active role in disease resistance and thus may appear to predominate; it is most likely not an exclusive role. Other mechanisms by which endophytes improve fungal disease resistance may act in concert to bring about an increased level of plant disease suppression. Thus, it would be interesting to determine if *E. festucae* E437 isolate can also induce plant defence reactions or possibly alter host chemistry leading to enhanced plant disease resistance.

Grasses have contributed to the development of humankind, as there are several important species that serve as essential food crops, as forage of livestock, and for recreational and conservation purposes. The ecological and biological benefit in endophyte – grass symbiota hold great promise for utilization in biological control. The identification of the gene involved in the biosynthesis and regulation of epichloae-derived antimicrobial substances provides basis for future exploration, manipulation and possible genetic improvement of *E. festucae* endophytes in their practical application as biological control agent.

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## SUMMARY

Epichloae endophytes (holomorphic *Epichloë* and anamorphic *Neotyphodium*) are clavicipitaceous fungi (Ascomycota) that maintain a systemic and genetically stable, mutualistic association with a broad spectrum of cool season grasses of the subfamily Pooideae. They colonize the intercellular spaces of leaf sheaths, leaf blades, stems, young inflorescences and seeds without eliciting obvious defense responses from plant cells. All the *Neotyphodium* and most *Epichloë* species have the ability to disseminate via seeds of their host plant. The sexual state *Epichloë* causes choke disease that affects inflorescences, however, "choking" symptom is shown only on a few infected flowering tillers while the other tillers remain asymptomatic.

In mutualistic relationship between epichloae endophytes and cool-season grasses, protection of the host plant from insect and vertebrate herbivores, diseases and increase tolerance on abiotic stresses such as drought are the reported benefits that the endophyte confers to its host plant. The protective ability of some epichloae species makes them suitable agents of biological plant protection against economically important grass diseases, insect and small animal herbivore.

While the protection of the host plant from insect herbivory by the epichloae endophytes is well characterized, the mechanism of host protection against grass pathogens by endophyte is largely unknown. The three studies presented here demonstrated the production of antifungal compound by the endophytic symbiont is involved in disease suppression and thus contribute to our understanding of the role of a mutualistic endophyte in plant disease resistance.

The antifungal activity of 14 *E. festucae* isolates obtained from different temperate grass species and cultivars from different countries against 8 grass pathogens was assessed, *in vitro*. An *E. festucae* isolate exhibiting the broadest spectrum antifungal activity, E437, showed growth inhibitory activity against *Drechslera erythrospila*, *D. siccans*, *D. dictyoides*, *Colletotrichum graminicola* and *Bipolaris sorokiniana.* This endophyte isolate did not cause hyphal cell lysis of affected pathogen but reduced the pathogen's apical hyphal growth and differentiation activity. Moreover, E437 isolate produced a thermal stable and a low-molecular weight antifungal compound in culture. Plants infected with E437 isolate exhibited reduced disease symptom caused by *D. erythrospila* on perennial ryegrass, indicating that the antifungal compound produced by *E. festucae* E437 isolate could be involved in the protection of the host plant.

To elucidate the genetic basis of the production of antifungal compound by *E. festucae* E437, mutants of this endophyte isolate was generated through REMI, and then screened for mutants defective in the production of fungal inhibitory substance for antifungal activity against the test pathogen, *D. erythrospila.* An *E. festucae* E437 REMI mutant 830 which is defective in the production of fungal inhibitory substance, contains a plasmid insertion in the promoter region of *vibA* gene, encoding a homologue of a transcription factor VIB-1. VIB-1 in *Neurospora crassa* is a regulator of genes essential in vegetative incompatibility and promotion of cell death.

A deletion of *vibA* gene lost the ability of *E. festucae* isolate E437 to produce inhibitory compound leading to non-inhibition of mycelial growth and conidial germination of a leaf spot pathogen, *Dreschslera erythrospila*. Overexpression of the *vibA* enhanced the endophyte ability to inhibit the mycelial growth of the test pathogens.

Moreover, the *vibA*-overexpressed transformant inhibited the growth of test pathogens of which the wild type isolate could not. Additionally, a non-antifungal *E. festucae i*solate, Fl1, attained antifungal activity by enhanced expression of *vibA*. These results demonstrated that enhanced expression of *vibA* is sufficient for a non-antifungal isolate to obtain antifungal activity, implicating the role of VibA as a master transcription factor for the production of antifungal compounds by epichloae endophyte.

Lastly, experiments were performed to isolate and identify the chemical structure of the inhibitory compound synthesized by a bioprotective isolate *E. festucae* E437 strain. Results on the initial steps towards characterization of the inhibitory substance, showed the presence of a thermostable, non-cell lytic enzyme and methanol-soluble low-molecular weight metabolite in the bioactive compound produced by *E. festucae*.

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List of publications:

1) Niones JT and Takemoto D.
   An isolate of *Epichloë festucae*, an endophytic fungus of temperate grasses shows growth inhibitory activity to selective grass pathogens
   Journal of General Plant Pathology (in press)

2) Niones JT and Takemoto D.
   VibA, a homologue of a transcription factor for fungal heterokaryon incompatibility, is involved in the antifungal compound production in the plant symbiotic fungus *Epichloë festucae*
   Eukaryotic Cell (submitted)