Analysis of an antimicrobial metabolite and its biosynthetic gene of a fungal endophyte

(真菌エンドファイトの生産する抗生物質およびその生合成遺伝子の解析)

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CHAPTER 1

General Introduction

1.1. Introduction

Living organisms are high reservoir of the 1 million different bioactive compounds [1]. Microorganisms are a particular rich source of more than 20,000 biologically active compounds, effecting for their survival and performances [2]. To date, microorganisms associating with plants provide bioactive natural products more than their host plant themselves [3]. Endophytes encompass a ubiquitous biodiversity including archaea, bacteria, actinobacteria, fungi, and unicellular eukaryotes, such as algae and amoebae which live as obligate or facultative ways in plant tissues without causing any damage [4]. In 1809, the German botanist Heinrich Friedrich Link firstly described about endophytes [5]. Endophytes synthesize potential number of bioactive compounds (enzyme and pharmaceutical drugs), small molecules, and phytohormones, some of which benefit to their host plants and served as excellent sources of drugs against various diseases [6]. A famous blockbuster drug in the pharmaceutical industry, taxol (paclitaxol), is a well-known diterpene alkaloid produced by endophyte *Metarhizium anisopliae* found in the bark of a *Taxus* yew tree [7].

Globally, one of the major problems facing the future of endophyte biology and natural product discovery is the rapidly diminishing rainforests that acquire a largely unknown and rare microorganisms and their products [8]. Bills *et al* analyzed that the ratio of bioactive compounds from endophytes between tropical and temperate region to the number of isolated endophytes [9], and demonstrated that higher number of bioactive compounds were found in tropical endophytes than temperate endophytes thus explains the host plants and ecosystems specifically influencing on the metabolism of endophyte [9]. Relatively unstudied endophytic bioactive compounds may open a new avenue to solve human problems including drug

resistance and orphan diseases. In addition, to discover new compounds with novel function from endophytes, biosynthetic pathways of the metabolites, modification of genetic and culturing method, and chemical synthesis are needed to be studied in advance.

1.2. The discovery of bioactive compounds from endophytes

Endophytes are an understudied group of microorganisms capable to synthesize bioactive compounds which can cure various kinds of diseases, pathogens and important alternative sources for the overcoming drug resistance. The International Union for Conservation of Nature and Natural Resources assesses about 300,000 plant species on the earth and found that most of them possess one or more endophytes, meaning that it is hard to find a endophyte free plants [10,11]. Plants lack immune response to pathogens, but endophytes synthesize chemicals in their host plants to propagate the immune response of the plants to fight against pervading pathogens and unfavorable conditions [12]. The natural products from heterotrophic group of endophytic microorganisms mainly from bacteria, fungi, and actinomycetes were recently classified into various functional alkaloids, tannins, phenols and phenolic acids, terpenes, steroids, and aliphatic compounds, which act as antibacterial, antifungal, anticancer, antioxidant, antiparasites, immunosuppressants, and agrochemicals (Figure 1.1) [13]. However, the archaebacteria found in coffee cherries, rice and maize roots, the arctic tundra rush *Juncus trifidus,* and the mycoplasmas in the red algae *Bryopsis pennata* and *B. hypnoides* [14] exist as endophytes, but there is no clear proof of their metabolites and their biological activities [15]. Metabolic potential of the endophytes depends on their lifestyle, specific living environment, and ecological problems. The facultative endophytes, due to resource rich environment of the rhizosphere, are highly competitive and microbial population produces antibiotic and anti-nematode compounds in order to survive. Contrary, obligate endophytes depend on metabolic activity of the host plants for the survival, and they likely

produce specific metabolites supporting the interaction with the host [1]. Fungi is the most ubiquitous group in endophytes and an important bioactive compounds reservoir waiting to be exploited. There are more than 1 million species of endophytic fungi remaining to be discovered, yet only about 100,000 species have been described [16]. This statistic suggests that undescribed endophytic fungi can acquire various bioactive compounds with novel functions. In addition to concept for novel endophytes, their genome sequences facilitate a mining of the novel bioactive compounds. Wang *et al.* reported that nine core genes of the secondary metabolism have been revealed from nineteen endophytic fungi using genomic and transcriptomic analysis [17].

This section describes that discovery and functions of bioactive compounds from the bacteria and fungi as main representative of heterotrophic endophytic groups.

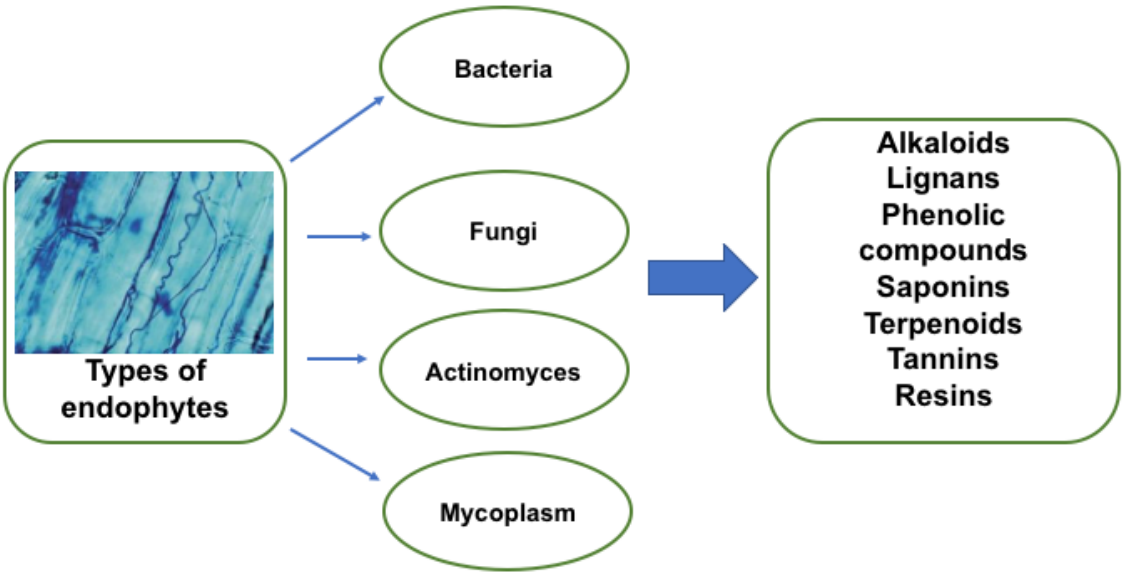

Figure 1.1. Heterotypes of the endophytes and their bioactive compounds. The image was adopted from [13].

1.2.1. Bioactive compounds from endophytic bacteria

Based on the 16S rRNA gene sequence data, 21 phyla of bacterial species have been reported to associated to the endophyte and most of the species belong to the Proteobacteria

including β and γ-Proteobacteria subgroups, Actinobacteria, and Firmicutes [12]. Diversity of the endophytic bacteria varies from Gram-positive to Gram-negative. More than 300 actinobacteria and bacterial genus have been determined as endophytes [18]. Endophytic actinomycetes are known to produce bioactive compounds with unique structures of medical importance.

Not only antimicrobial compounds but also insecticidal and anticancer compounds have been discovered from *Actinomyces*, *Actinoplanes*, *Amycolatopsis*, *Micromonospora*, *Saccharopolyspora*, and *Streptomyces* genus in actinomycetes [19]. *Bacillus* and *Streptomyces* are the most abundant bioactive metabolite producing Gram-positive endophytes found within diverse environments [10]. The *Streptomyces* is the most dominant genus in endophytic actinomycetes, which alone provides 75% of all natural antibiotics with the various bioactivities [20]. There are number of bioactive compounds with antimicrobial activities discovered from endophytic *Streptomyces* such as kakudamycins, xiamycins, clethramycin, cedarmycin A and B, saadamycin (Table 1.1) [21-25]. In addition, antimalarial (coronamycin, munumbicin D), and antifungal (munumbicin D) activities were discovered [26-27]. *Bacillus* and *Streptomyces* endophytic species proposed as main antimicrobial metabolites in endophytic bacteria. Moreover, metabolites from endophytic *Bacillus* spp. are responsible for the crop management. For example, endophytic *B. amyloliquerfaciens* and *B. subtilis* produced a lipopeptide which inhibited *Sclerotinia sclerotiorum* fungus growth [28]. Similarly, an antifungal peptide was produced by *B. amyloliquefaciens* strain Blu-v2 [29]. Hydrolytic enzymes (surfactin, fengycin, and iturin A) produced by four *B. subtilis* strains showed biocontrol effects against *Fusarium oxysporum* f. sp. *radices-lycopersici* and *Rosellinia necatrix* [30].

Besides antimicrobial and antifungal activities of endophytic bacteria, they play a role for the promotion of host plant's growth. Seed-borne endophytic *Bacillus amyloliquefaciens* RWL-1

promotes rice plant (*Oryza sativa*) growth and increases biomass improving gibberellins [31]. Indole acetic acid and siderophores produced by *B. subtilis* strain NA-108 promoted plant growth and biomass improvement of the strawberry plant [32].

Cancer is one of the leading disease in worldwide. Because of new types of cancer cell mutations and adaptation to the traditional cancer treatments lead to search cancer-specific drugs without side effects. Endophytic actinobacteria are not only main reservoir of antimicrobial drugs but also anticancer such as exopolysaccharides, lupinacidins A and B (anthraquinones), alnumycin, maytansine, biphenyls, 4-arylcoumarins, baflomycin D, 3'-hydroxy-daidzein, and 1-monolinolein, pterocidin, salaceyins A and B [33-41]. Interestingly, many plant growth promoter compounds exhibited cytotoxic effects against cancer cells. Anthraquinones produced by *Micromonospora* sp. retard the invasion of murine colon 26-L5 carcinoma cells [34]. A biphenyl produced by endophytic *Streptomyces* sp. BO-07 strain retarded human lung adenocarcinoma EGFR-TKI-resistant cells [37]. Bioactive compounds and their activities from endophytic bacteria are summarized in Table 1.1.

Table 1.1. Bioactive compounds from endophytic bacteria

Bioactive compounds	Producer endophyte species	Activity	References
Antimicrobial			
Kakudamycin A	*Streptomyces* sp. NRRL 30566	Against *B. anthracis*	[21]
Xiamycins	*Streptomyces* sp. strain HKI0595	Against *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*	[22]
Clethramycin	*Streptomyces hygroscopicus* TP-A0623	Against *Candida albicans* and *C. glabrata*	[23]
Cedarmycin A and B	*Streptomyces* sp. TP-A0456	Against Candida glabrata IFO 0622	[24]

Saadamycin	*Streptomyces* sp. Hedaya48	Against dermatophytes and other clinical fungi	[25]
Coronamycin	*Streptomyces* sp. MSU-2110	Against *Pythium ultimum*	[26]
Munumbicin D	*Streptomyces* sp. NRRL 30562	Against *Staphylococcus aureus*	[27]
Anti- plant pathogens			
Lipopeptides	*Bacillus amyloliquerfaciens* and *B. subtilis*	*Sclerotinia sclerotiorum* fungus growth	[28]
Lipopeptides	*Bacillus amyloliquefaciens* strain Blu-v2	Antifungal, caterpillar-feeding deterrant	[29]
Anti- cancer			
Exopoly-saccharides	*Bacillus amyloliquefaciens sp.*	Against human gastric carcinoma cell lines, (MC-4 and SGC-7901)	[33]
Lupinacidins A and B (anthraquinones)	*Micromonospora lupine* sp,	Against murine colon 26-L5 carcinoma cells	[34]
Alnumycin	*Streptomyces* sp.	Against K562 human leukemia cells	[35]
Maytansine	*Streptomyces* sp.	Against A-549 lung tumor cell	[36]
Biphenyls	*Streptomyces* sp. strain BO-07	HeLa, HepG2 and Huh7 cancer cell lines	[37]
4-arylcoumarins	*Streptomyces aureofaciens* strain CMUAc130	Murine Lewis lung carcinoma	[38]
Baflomycin D, 3'-hydroxy-daidzein, and 1-monolinolein	*Streptomyces cavourensis* strain YBQ59	Human adenocarcinoma EGFR-TKI	[39]
Pterocidin	*Streptomyces hygroscopicus* strain TP-A0451	Cancer cell lines NCI-H522, OVCAR-3, SF539, and LOX-IMVI	[40]
Salaceyins A and B	*Streptomyces laceyi* strain MS53	Human breast cancer (cell line SKBR3)	[41]

Plant growth promoters			
Hydrolytic enzymes; surfactin, fengycin, and Iturin A	*Bacillus subtilis*	*Against Fusarium oxysporum* f. sp. *radices - lycopersici* and *Rosellinia necatrix*	[30]
Gibberellins	*Bacillus amyloliquefaciens* RWL-1	Plant growth and biomass improvement	[31]
Indole acetic acid and siderophores	*Bacillus subtilis* NA-108		[32]

1.2.2. Bioactive compounds from endophytic fungi

According to the oldest known fossil records, endophytic fungi have affiliated with plants for over 460 million years, originating from the Ordovician period [42,43].

An enhanced host plant tolerance against pests is the greatest advantage for the plants because of bioactive secondary metabolites produced by endophytic fungi [44]. Endophytic fungi are an abundant store of secondary metabolites such as anticancer drugs and antibiotics than that of other endophytic domains [45,13] rather than endophytic bacteria, which likely to produce enzymes, peptides, hormones, and polysaccharides (Table 1.1). Various endophytic fungi produce pharmaceutically significant bioactive compounds such as camptothecin, diosgenin, hypericin, paclitaxel, podophyllotoxin, and vinblastine (Figure 1.2 and Table 1.2) [45-47]. Many endophytic fungi capable to produce antioxidant compounds which could protect their host plants by enhancing resistance to abiotic stresses [48]. By the aid of symbiosis of endophytic fungi, plants enhance the production of their flavonoids and phenolic antioxidants [49]. The endophytic fungi *Pestalotiopsis microspore* in the combretaceaous plant *Terminalia morobensis* produces pestacin and isopestacin, both of which exhibit antioxidant and antifungal activities [50].

Medicinal plants and their endophytes are the main contributor for the bioactive compounds and secondary metabolites on the market [51]. Based on the analysis of 96

medicinal plant species hosting endophytic fungi, the accumulation of secondary metabolites is increased by the endophytic fungi than promoting growth and increase the stress resistance [45]. Production of these secondary metabolites is undetectable or low when fungus is grown *in vitro*, indicating that host plant's signaling is needed [52,53]. Some endophytic fungi can promote to biosynthesize diverse classes of phytochemicals. The well-known anticancer drug, paclitaxel (taxol) was found in the taxol-producing endophytic fungi *Taxomyces andreanae* isolated from the yew tree *Taxus brevifolia* [54]. In addition, many other endophytic fungi including the well-known *Fusarium solani* in *Taxus celebica* [55], *Alternaria* sp. in *Gingko biloba* [56], and *Aspergillus fumigatus* in *Podocarpus* sp. [57], were also found to synthesize taxol. By fermentation of the abovementioned endophytic fungi, taxol can be produced economically and saving natural resources. On the other hand, endophytic fungi are found to produced secondary metabolite only in the plants; e. g., the anti-malarial drug artemisinin was produced by the endophyte *Coetotrichum gloesporioides* only in the Sweet wormwood *Artemisia annua* [58]. Figure 1.3 shows percentages of bioactive compounds produced by endophytic fungi with antimicrobial activities in different host plants [59].

Table 1.2. Pharmaceutically significant bioactive compounds produced by some endophytic fungi.

Bioactive compounds	Producer endophyte species	Activity	References
Camptothecin	*Fusarium solani*	Antitumor	[45-47]
Diosgenin	*Cephalosporium* sp.,	Antitumor, anti-inflammatory, and cardiovascular protection	
Podophyllotoxin	*Fusarium oxysporum*, *Alternaria* sp.	Antitumor	
Paclitaxel (taxol)	*Taxomyces andreanae, F. solani, Pithoyces* sp.	Antitumor	
Hypericin	*Chaetomium globosum*	Anti-depressant	
Vinblastine	*Alternaria* sp., *F. oxysporum*	Antitumor	

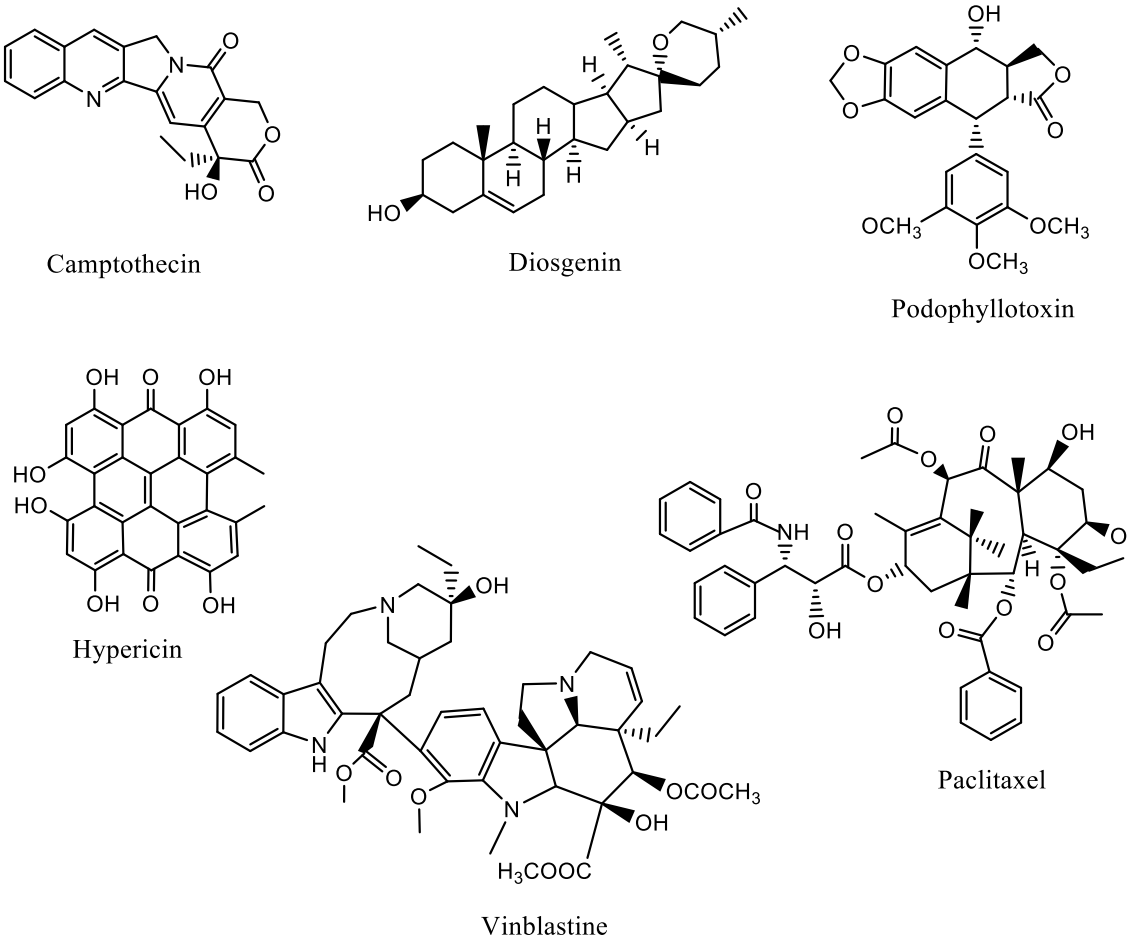

Figure 1.2. Bioactive compounds produced by some endophytic fungi.

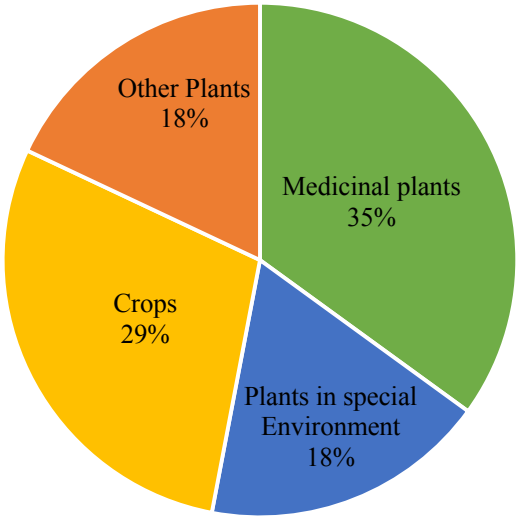

Figure 1.3. Percentages of bioactive compounds produced by endophytic fungi with antimicrobial activities in different host plants [59].

1.3. Bioactive compounds from *Epichloë* endophytic fungi

Epichloë genus taxonomically belongs to the *Sordariomycetes* subclass, *Ascomycota* division, mostly found in inter and intracellular spaces of above ground parts of cool season perennial grasses and tribes of the subfamily of Pooideae [12]. Currently, 43 taxa with 34 species as well as subspecies and varieties have been designated to the *Epichloë* genus [60]. Their mutual interaction benefits the host plants by protecting them from insects and diseases, and by increasing their tolerance to environmental stresses such as drought [61-65]. This protective mechanism is regulated by bioactive compounds in obligate symbiont *Epichloë* endophytes with plants. To date, several bioactive secondary metabolites have been isolated and characterized from *Epichloë* endophytes. Bioactive compounds search from *Epichloë* endophytes was extensively studied since alkaloids were identified as an insecticide in 1980 [66]. To date, four major classes of bioactive alkaloids including ergot alkaloids (ergovaline and chanoclavine), indole diterpenes (lolitrem B and epoxy-janthitrem), pyrrolopyrazidines (lolines), and pyrrolopyrazine (peramine) were identified [67-70]. The lolines and peramine are used as an insecticide [69,70]. The ergot alkaloids and indole-diterpenes are known as the fescue poisons causing grazing livestock and ryegrass staggers [69]. Indole-3-acetic acid, indole-3-ethanol, methylindole-3-carboxylate, indole-3-carbox-aldehyde, *N*,*N'*-diacetamide, and cyclonerodiol were isolated from cultures of *E. festucae* isolates as antifungal agent against grass pathogens [71]. A cyclic peptide, epichlicin, produced by *E. typhina* inhibits the spore germination of *Clasdosporium phlei* [72]. Seventeen antifungal metabolites including a new product, 3-(2'-(4"-hydroxyphenyl)acetoxy)-2*S*-methylpropanioc acid, were isolated from *E. bromicola* [73]. Four fungitoxic compounds including novel C-11 epoxy fatty acid, ethyl *trans*-9,10-epoxy-11-oxoundecanoate were isolated from endophytic fungus *E. typhina* species on *Phleum pretense* L. [74] (Figure 1.4 and Table 1.3). In the *Epichloë* genus, *E. festucae* deposits total 42 secondary metabolite genes, of which 21 genes assigned for the NPRS [75]. This

finding suggests that *E. festucae* is reservoir of unknown bioactive compounds with novel activities and may improve the plant tolerance against biotic and abiotic stresses. Bioactive compounds produced by *Epichloë* species summarized in Table 1.3.

Table 1.3. Bioactive compounds produced by *Epichloë* species.

Bioactive compounds	Producer endophyte species	Activity	References
Indole-3-acetic acid	*E. festucae*	Antifungal	[71]
Indole-3-ethanol			
Methylindole-3-carboxylate			
Indole-3-carbox-aldehyde			
N,*N'*-diacetamide			
Cyclonerodiol			
Epichlicin	*E. typhina*	Against germination of *C. phlei*	[72]
3-(2'-(4"-hydroxyphenyl)acetoxy)-2*S*-methylpropanioc acid	*E. bromicola*	Antifungal, Phytotoxic, and cytotoxic	[73]
Lolitrem B	*E. festucae* var. *lolii*	Ryegrass straggers	[69,70]
Peramine (pyrrolopyrazine)	*E. festucae* var. *lolii*	Weakly toxic in livestock, toxic for crop pests	
Ergovaline	*E.coenophiala*	Toxicosis in livestock	[67]
Chanoclavine	*E. typhina*	Toxicosis in livestock	[68]
N-formyllolline	*E. typhina*	Insecticide	[69]
Ethyl *trans*-9,10-epoxy-11-oxoundecanoate	*E. typhina*	*Cladosporium herbarum*	[74]
9-oxononanoate			
Ethyl azelate			
5-hydro-3,4-dimethy-5-pentyl-2 (5*H*)-furanone)			

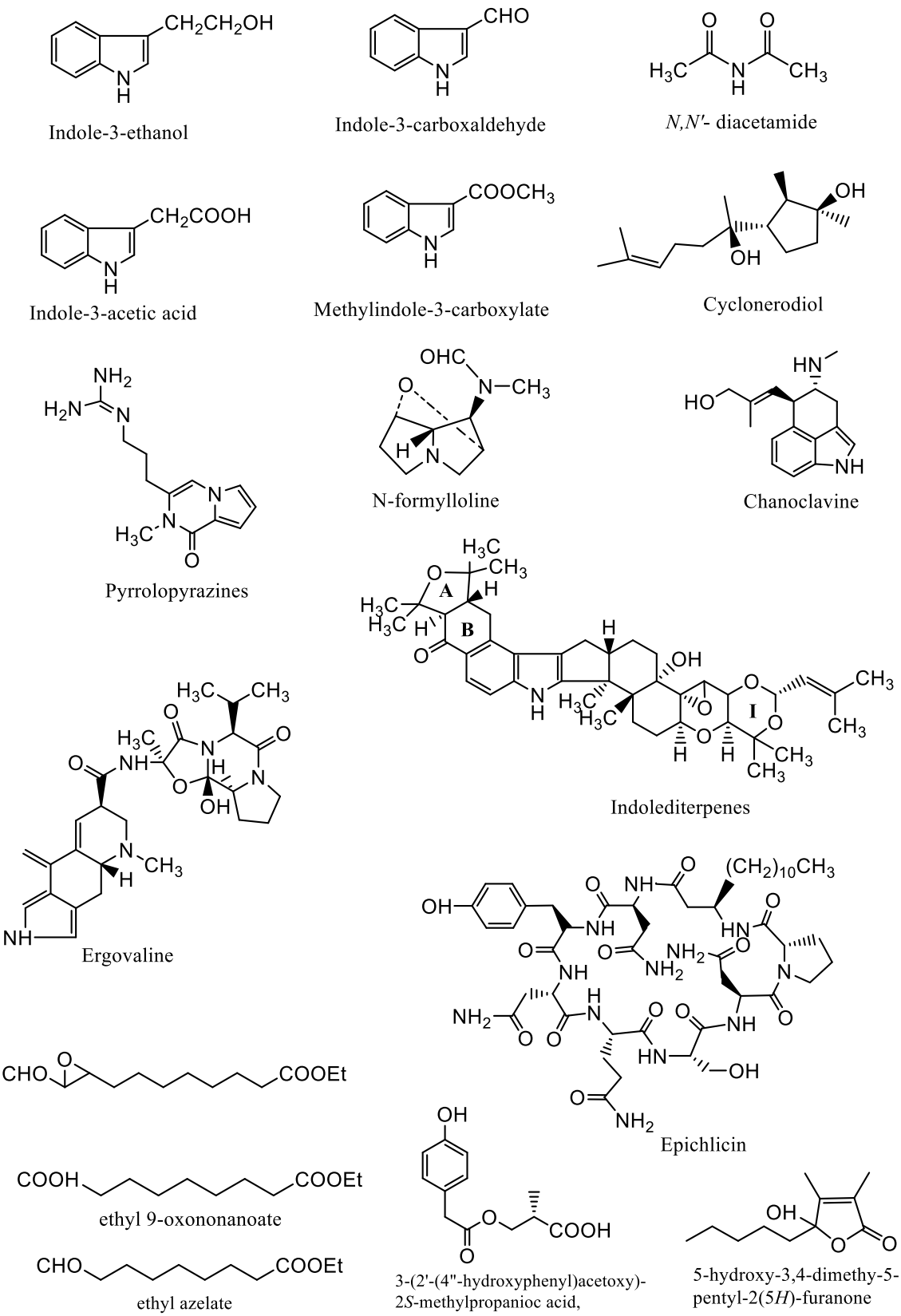

Figure 1.4. Bioactive compounds produced by *Epichloë* species.

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CHAPTER 2

Isolation of an antifungal substance from *vibA*-overexpressing *E. festucae* strains

2.1. Introduction

Endophytic fungi are a widely distributed domain found in plants, resides intercellular and intracellular spaces of tissue. Endophytic fungi have been attracted an attention due to its toxicity to livestock in beginning of 20th century [1]. Endophytic fungi have an ability to protect their own hosts synthesizing diverse types of bioprotective chemicals such as alkaloids, steroids, terpenoids, phenyl propanoids, and aliphatic metabolites [2]. Number of secondary metabolites produced by endophytic fungi is higher than that of any other endophytic classes [2]. *Epichloë festucae* is a frequent symbiont of in *Festuca*, *Lollium*, and *Koeleria* spp. plants and attractive model for genetic analysis because of acquiring the haploid genome [3]. To date *E. festucae* have been characterized by:

1. Production of insecticide such as lolines and peramine.
2. Production of the anti-vertebrate alkaloids.
3. Vertical transmission by host seeds.
4. Enhancing the survival of their host plants.
5. All *Epichloë* including *E. festucae* sp. secretes an enzyme, subtilisin-like proteinase in order to breakdown plant cell walls [4].

Epichloë, a genus of the endophytic fungi of the family Clavicipitaceae, produces insecticide alkaloids, antifungal cyclic peptide, and indole diterpenes (Table 1.3, Chapter 1).

Key genes for the ergot alkaloid biosynthesis in *Epichloë* have been well-documented due to abundance of this class of compounds [5-7]. In the *Epichloë* genus, *E. festucae* deposits total

42 secondary metabolite genes, of which 21 genes assigned as NPRSs [8]. This finding suggests that *E. festucae* is reservoir of unknown natural products encoded by NPRSs with diverse biological capabilities. At present, it is reported that genes responsible for the antifungal activities of *E. festucae* are limited. It has been demonstrated that deletion of the genes encoding complex NADPH oxidase enzymes in *E. festucae* are involved in a disordered fungal growth of grass tissues and even death of the grass plant [9]. Similarly, it was revealed that deletion of *vibA* transcription factor gene had lost antifungal activity. Therefore, the transcription factor VibA was identified as an essential factor for the induction of the antifungal activity [10].

VibA is a conserved p53-like transcription factor involved in cell death and nonself recognition leading to heterokaryon incompatibility in ascomycete fungi [11]. VibA also plays a role for the negative regulation of conidiation, formation of aerial hyphae, production of extracellular protease in response to both carbon and nitrogen starvation, and glucose sensing/metabolism and regulation of carbon catabolite repression [12-14].

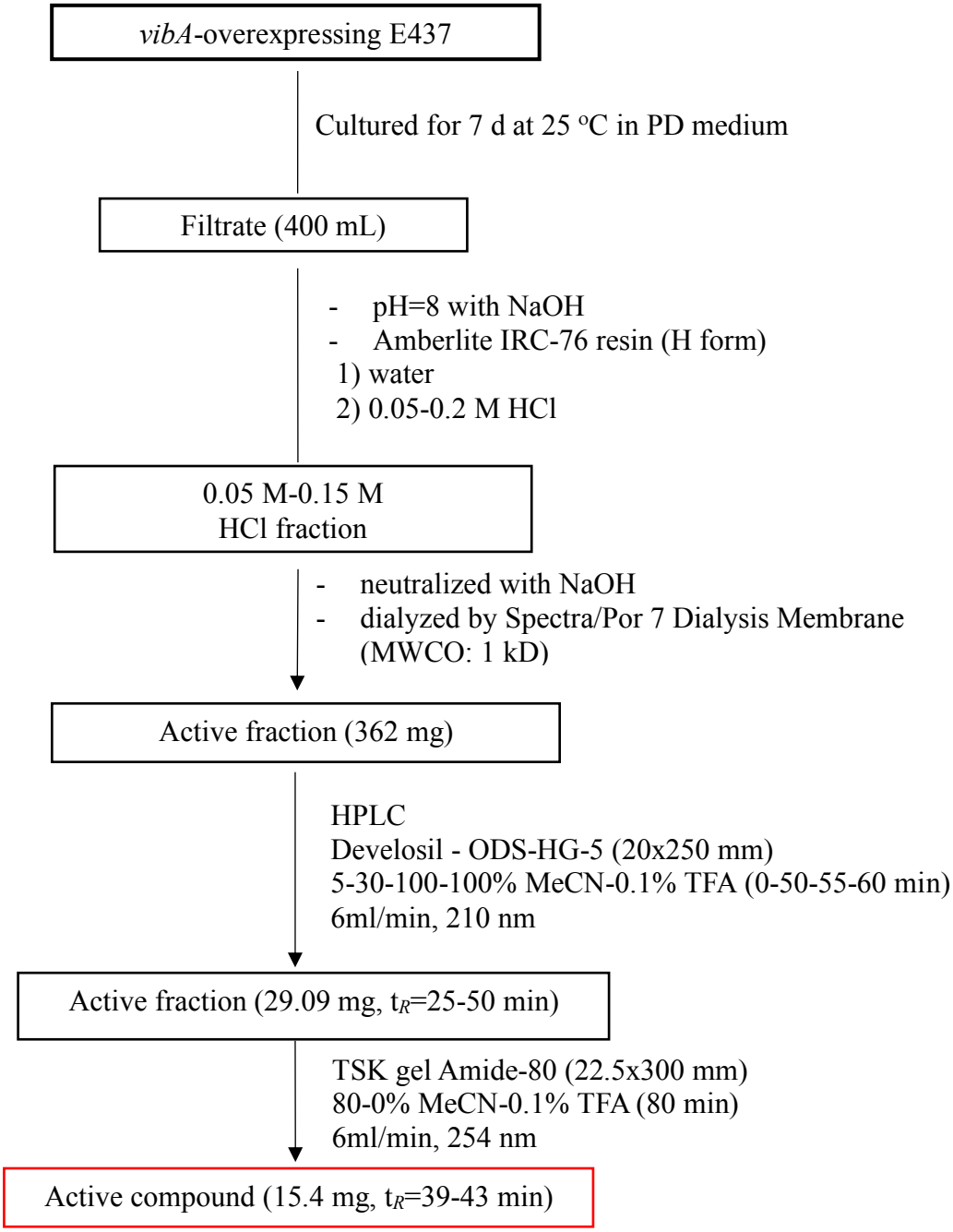
In this chapter, purification and chemical characterization of the unknown antifungal substance produced by the *vibA* gene-overexpressing transformants P*tef::VibA* (E437) and P*tef::VibA* (Fl1) derived from two strains of *E. festucae* will be presented.

2.2. Results and Discussion

2.2.1. Chemical identification of an antifungal substance produced by a *vibA*-overexpressing strain of *E. festucae* E437

The *vibA* gene-overexpressing transformant P*tef::VibA* (E437) derived from *E. festucae* strain E437 (wild type) was cultured in a liquid PD medium. A culture filtrate was found to inhibit conidial germination of the plant pathogen *D. erythrospila*. It was previously shown

that the activity was 10-times as high as that of the original E437 strain [10]. The active filtrates were subjected to three-steps purification: (1) cation exchange resin (Amberlite-IRC 76), (2) reversed-phase HPLC (ODS column) (Figure 2.1A), and (3) normal-phase HPLC (HILIC column) (Figure 2.1B), affording an active substance in the yield of 38.6 mg/L (Scheme 2.1).



Scheme 2.1. Purification steps of the antifungal ε-PL from P*tef::VibA* (E437)

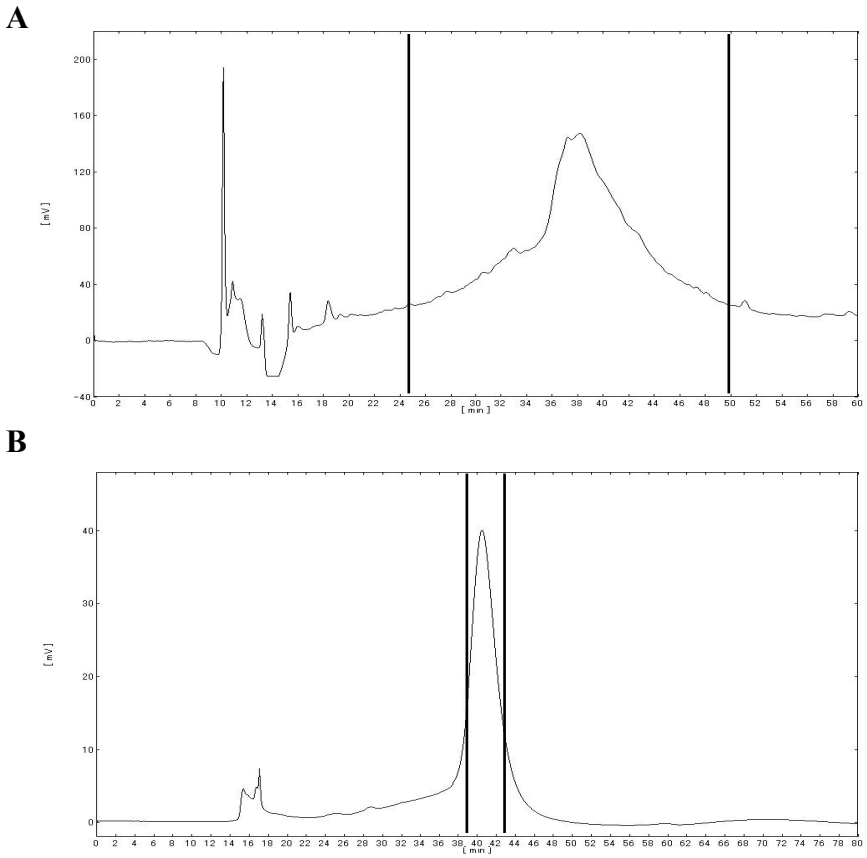


Figure 2.1. Antifungal substance purification after cation exchange resin by preparative HPLC. (**A**) the second step of the scheme 2.1, ODS column purification of P*tef::VibA* (E437), (**B**) the third step of the scheme 2.1, TSK gel amide-80 column purification of P*tef::VibA* (E437).

The chemical structure of the active substance was analyzed by NMR spectroscopy in D_2O (Figure 2.2-2.6). A heteronuclear multiple quantum correlation (HSQC) spectrum suggested the presence of one CH and four CH_2 groups (Figure 2.5). One quaternary carbon atom observed at 169.6 ppm suggested the presence of one carboxyl group. Double quantum filtered correlation spectroscopy (DQF-COSY) revealed the important partial structure of >CH-CH_2-CH_2-CH_2-CH_2- (Figure 2.4). A hetero-nuclear multiple-bond connectivity (HMBC) experiment was next performed to connect the partial structure and the carboxyl group. The HMBC correlations were found between C=O and the protons of Hα, Hβ, and Hε (Figure 2.6 and Table 2.1), indicating that the structure was a cyclic form of lysine (α-amino-ε-caprolactam) or ε-polylysine (ε-PL) (Figure 2.7).

Table 2.1. NMR data of ε-PL (400 MHz, deuterium oxide)

Position	δ_C (ppm), type	δ_H (ppm), mult. (*J*, Hz)	HMBC correlations (to C)
α	53.3, CH	3.90, t (6.8)	γ, β, C=O
β	30.5, CH_2	1.85, m	α, C=O
γ	21.7, CH_2	1.37, quint (7.3)	β, ε
δ	28.0, CH_2	1.55, quint (7.3)	β, ε
ε	39.1, CH_2	3.24, m	C=O
C=O	169.6, C	-	

The absolute configuration of the lysine unit was determined as L by the advanced Marfey's method [15] using L- and D-1-fluoro-2,4-dinitrophenyl-5-leucinamides (FDLA). Therefore, it was concluded that the antifungal substance produced by P*tef::VibA* (E437) strain was ε-poly-L-lysine (ε-PL) (Figure 2.8). The second possible structure, α-amino-ε-caprolactam was denied because of the ^{13}C-NMR difference [16] and MALDI-TOF MS analysis of the ε-PL (Figure 2.10). Figure 2.8 shows the FDLA analysis of hydrolyzed product. In the case of α-amino-ε-caprolactam, its hydrolyzed product is L-lysine, being identical to that of ε-PL. Thus, FDLA analysis cannot decide which structures are correct.

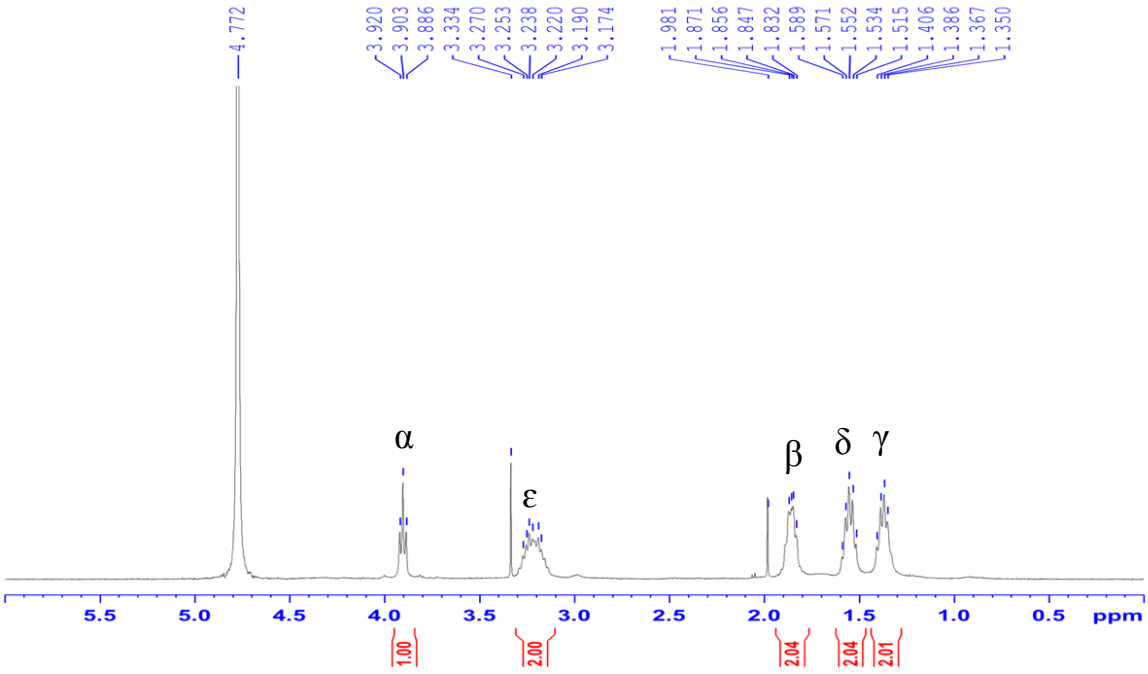


Figure 2.2. ^{1}H-NMR spectrum of ε-PL from P*tef::VibA* (E437) (400 Hz, D_2O)

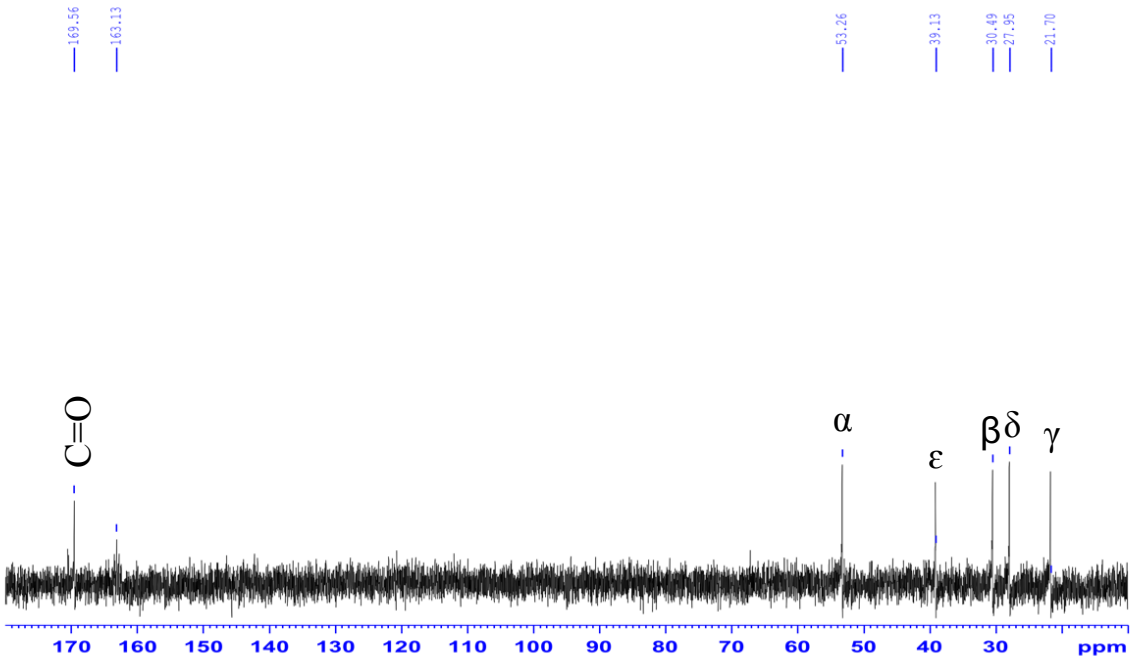


Figure 2.3. ^{13}C-NMR spectrum of ε-PL from P*tef::VibA* (E437) (100 Hz, D_2O)

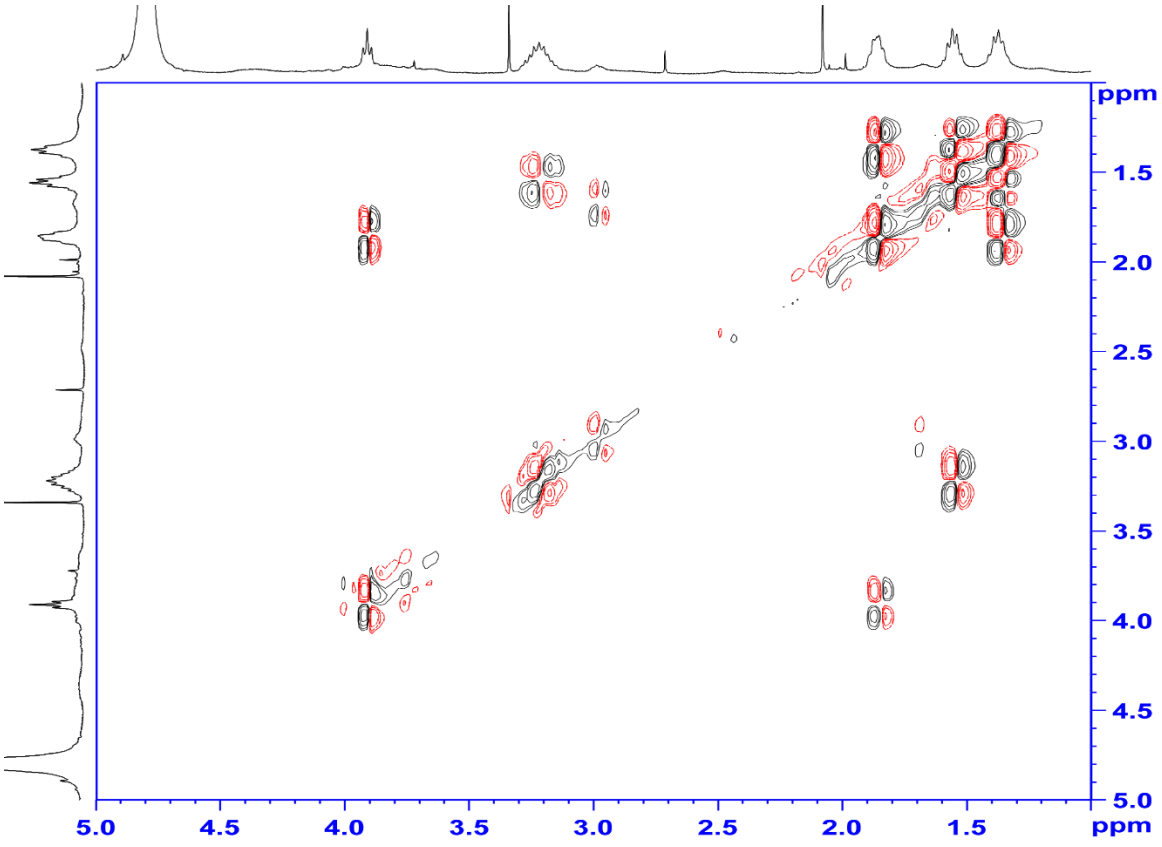


Figure 2.4. DQF-COSY spectrum of ε-PL from P*tef::VibA* (E437) (400 Hz, D_2O)

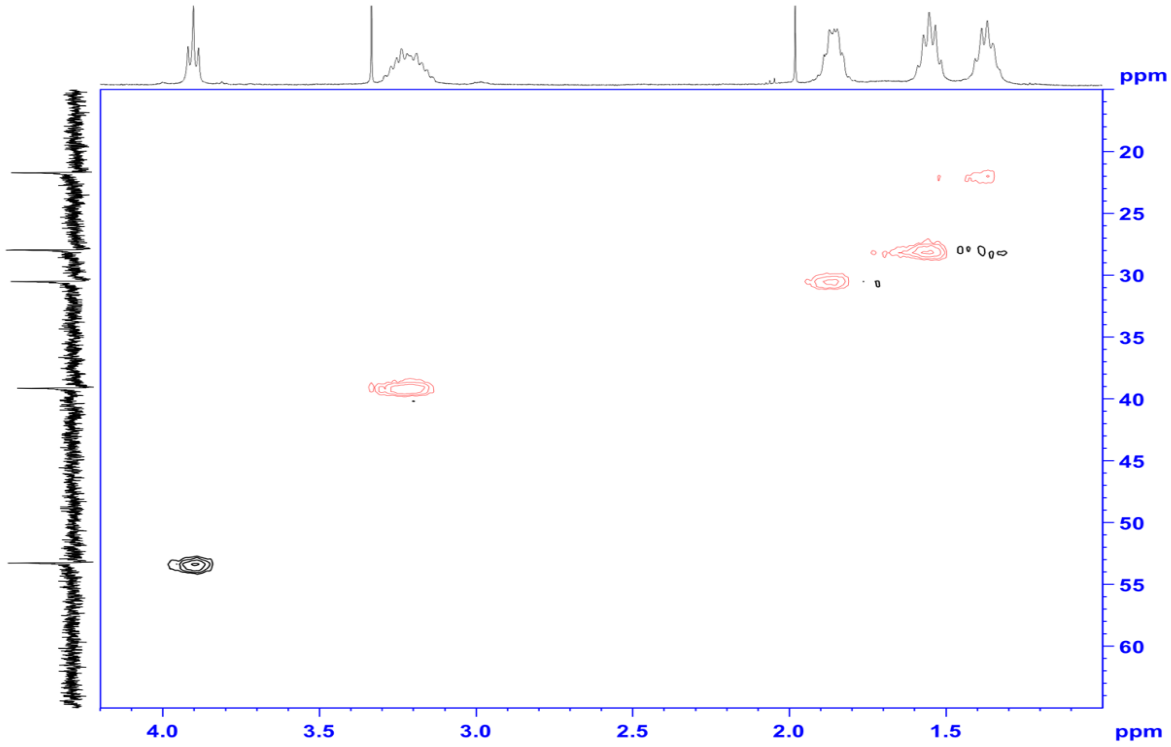


Figure 2.5. HSQC spectrum of ε-PL *from* P*tef::VibA* (E437) (400 MHz, D_2O)

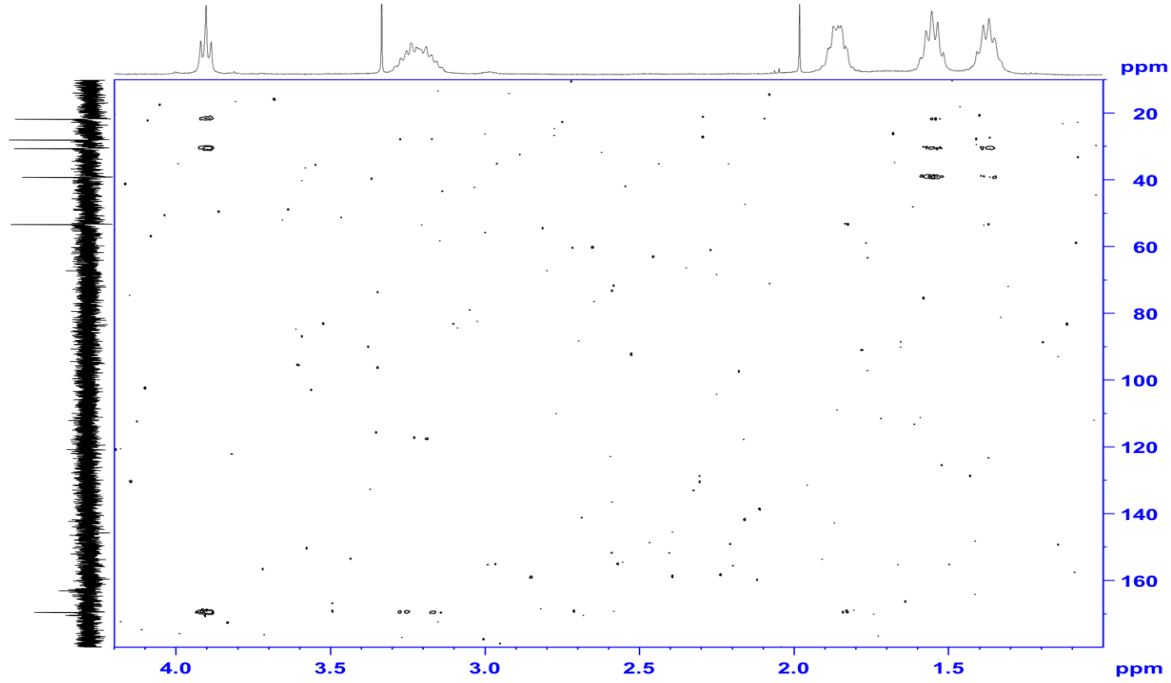


Figure 2.6. HMBC spectrum of ε-PL from P*tef::VibA* (E437) (400 Hz, D_2O)

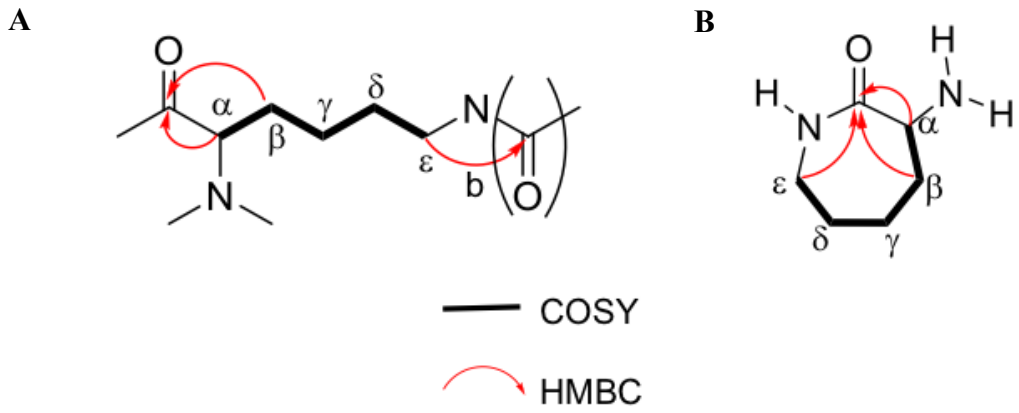


Figure 2.7. Two-dimensional NMR correlations for the active substance from P*tef::VibA* (E437) transformant. (**A**) ε-polylysine (ε-PL), (**B**) cyclic form of lysine (α-amino-ε-caprolactam).

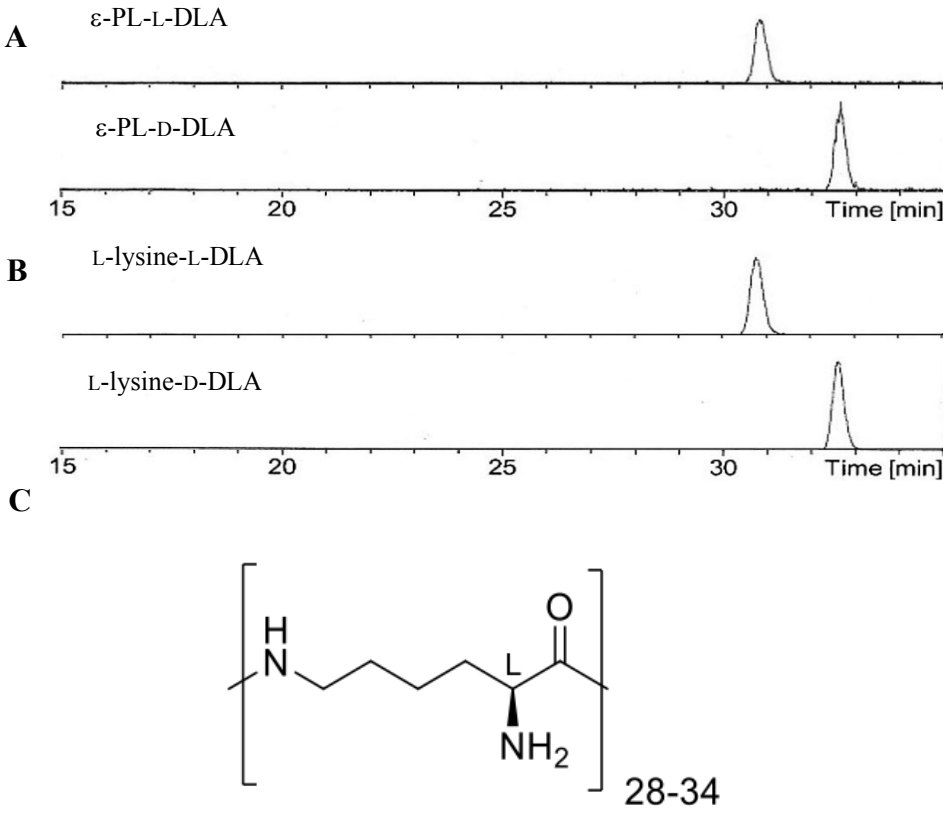


Figure 2.8. Determination of absolute configuration of ε-PL by the advanced Marfey's method. Extracted ion chromatograms obtained by LC/MS analysis of L- and D-DLA derivatives of acid hydrolysate of ε-PL from (**A**) P*tef::VibA* (E437) and (**B**) L-lysine; (**C**) absolute configuration of the ε-PL.

The structure of the active substance was finally determined as ε-PL by Matrix Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectroscopy (MALDI-TOF MS) and the degree of polymerization was found to be 28-34, which was the same as that of standard ε-PL

(Figure 2.10 and Table 2.2). The calculated mass of the major peak of DP=32 (m/z 4120.4) is m/z 4118.06 and formula are as follows.

128.095 (calculated mass of the L-Lysine monomer) *32 (DP)+19.0178 (H_2O+ H^+).

The ^{1}H NMR was superimposable to that of a standard ε-PL (bacterial origin, TFA salt) (Figure 2.9).

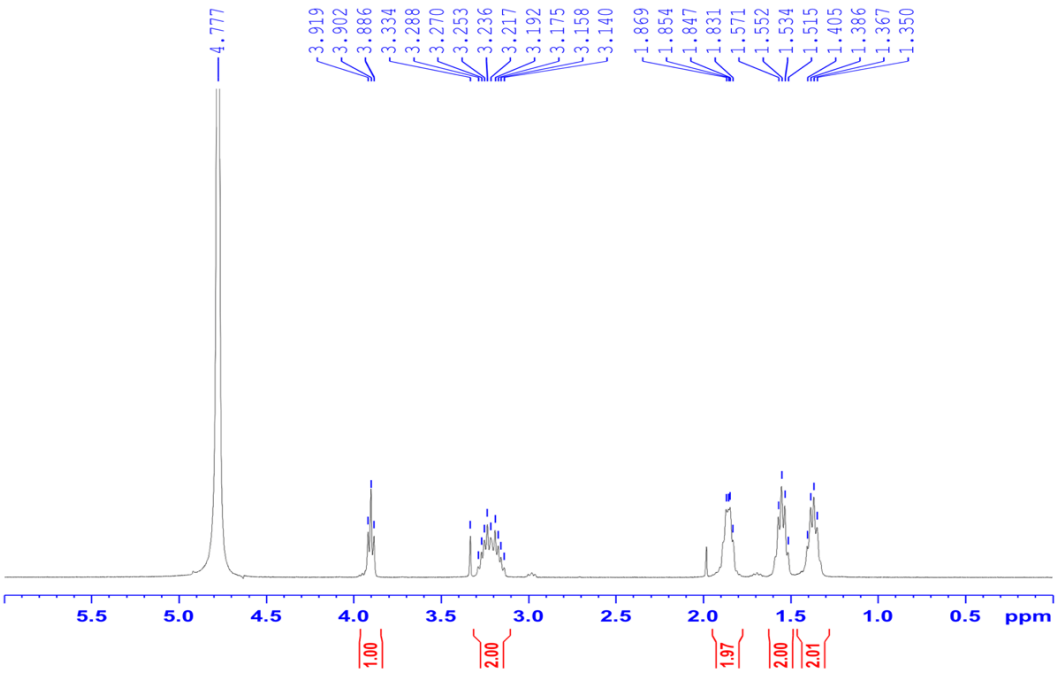


Figure 2.9. ^{1}H-NMR spectrum of standard ε-PL (TFA salt) (400 Hz, D_2O)

The production of ε-PL was enhanced more than 3.7-fold in P*tef::VibA* (E437) strain and infinite in P*tef::VibA* (Fl1) than their wild type strains (Table 2.2). Interestingly, the degree of polymerization (DP) of ε-PL is drastically depend by host strains; (1) the Fl1 strain with overexpressed *vibA* gene produced lower DP product (DP = 8−18, DP of largest population = 12) (Figure 2.13), (2) E437 strain with overexpressed *vibA* gene and E437 (wild type) exclusively produced higher DP product (DP = 28−34, DP of largest population = 32) (Figure 2.10 and Table 2.2).

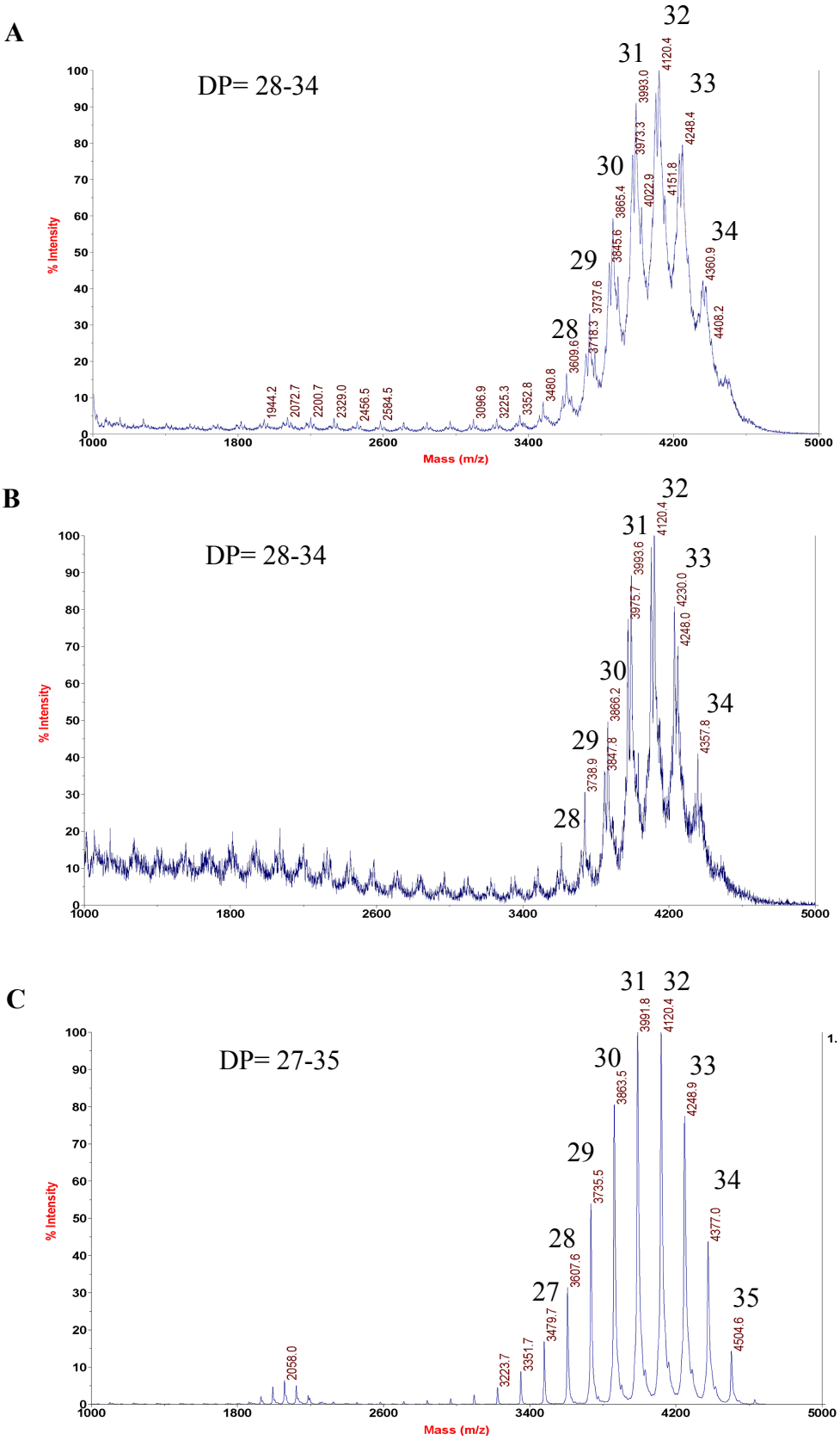


Figure 2.10. MALDI-TOF MS of ε-PL. (**A**) ε-PLs from the transformants P*tef::VibA* (E437), (**B**) wildtype (E437) and (**C**) Standard ε-PL. Number on the each peak indicate the degree of polymerization (DP).

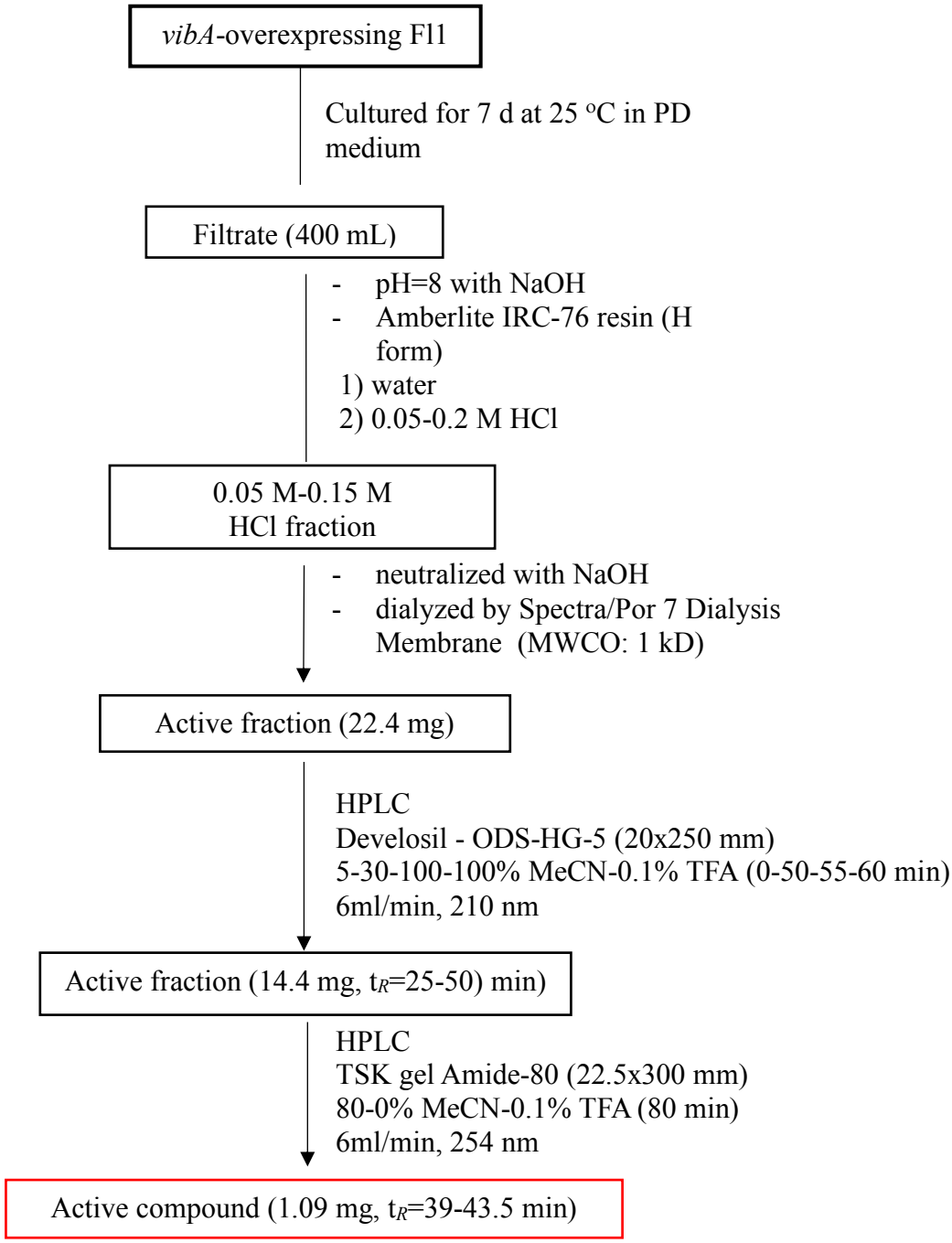
Table 2.2. Production and length of ε-PL produced by wild type and P*tef::VibA* (E437) and P*tef::VibA* (Fl1).

Strains	Production (mg/L)[a]	Chain length, DP[b]
E437 (wild type)	10.4	28−34 (32)
P*tef::VibA* (E437)	38.6	28−34 (32)
Fl1 (wildtype)	trace	-
P*tef::VibA* (Fl1)	2.7	8−18 (12)

[a]The amounts are as TFA salts after HILIC HPLC purification.
[b]The degree of polymerization (DP) was expressed for the ion peaks higher than 10% of the parent ion peak in MALDI-TOF MS (Figure 2.10). The DP for the highest ion peak (DP of largest population) within the lower or higher DP product is indicated in parenthesis.

2.2.2. Chemical identification of an antifungal substance produced by a *vibA*-overexpressing strain of *E. festucae* Fl1

The *vibA* gene-overexpressing transformant P*tef::VibA* (Fl1) derived from *E. festucae* strain Fl1 (wild type) was cultured in a liquid PD medium same as transformant P*tef::VibA* (E437). The active filtrates were subjected to three-steps purification: (1) cation exchange resin (Amberlite-IRC 76), (2) reversed-phase HPLC (ODS column) (Figure 2.11A), and (3) normal-phase HPLC (HILIC column) (Scheme 2.2), affording an active substance in the yield of 2.7 mg/L (Figure 2.11B).



Scheme 2.2. Purification steps of the antifungal ε-PL from P*tef::VibA* (Fl1).

The chemical structure of the active substance from P*tef::VibA* (Fl1) was showed an identical spectrum with the ε-PL from P*tef::VibA* (E437) transformant by ^{1}H-NMR spectroscopy in D_2O (Figure 2.12). The degree of polymerization of the ε-PL from P*tef::VibA* (Fl1) was found to be 8-18 by Matrix Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectroscopy (MALDI-TOF MS) (Figure 2.13 and Table 2.2).

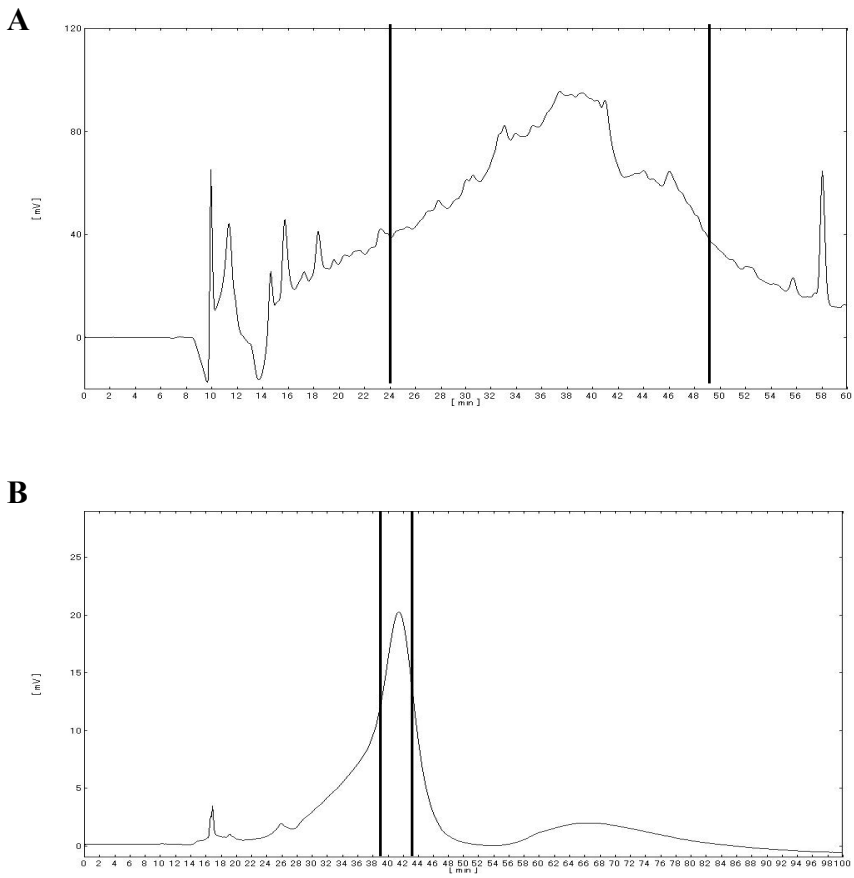


Figure 2.11. (**A**) the second step of the scheme 2.2, ODS column purification of ε-PL from P*tef::VibA* (Fl1) (**B**) the third step of the scheme 2.2, TSK gel amide-80 column purification of ε-PL from P*tef::VibA* (Fl1).

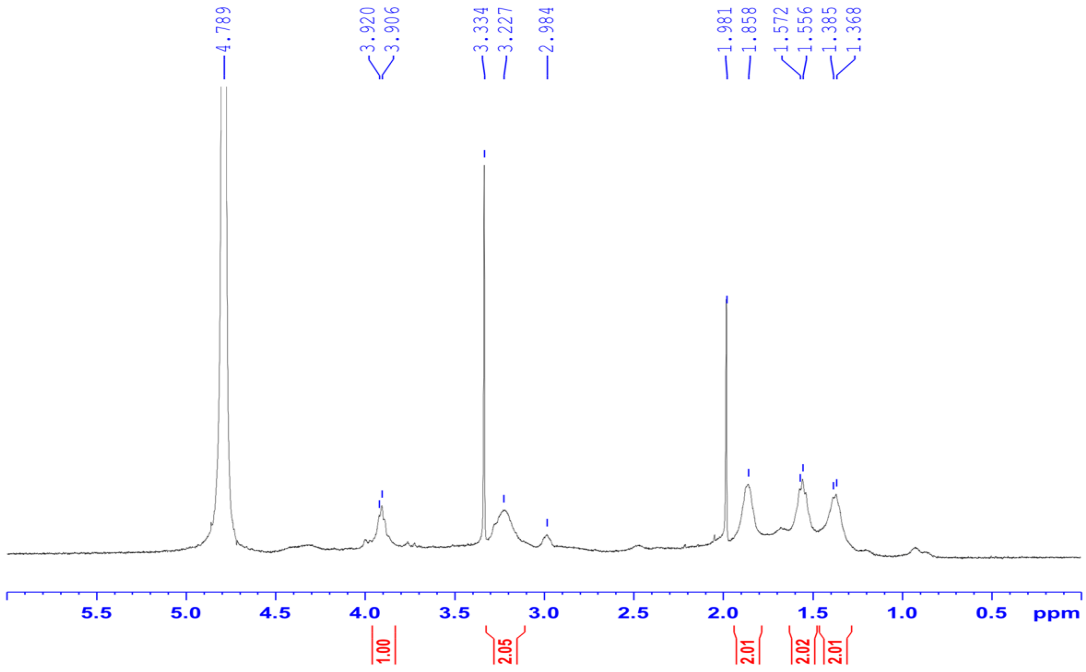


Figure 2.12. ^{1}H-NMR spectrum of ε-PL from P*tef::VibA* (Fl1) (400 Hz, D_2O)

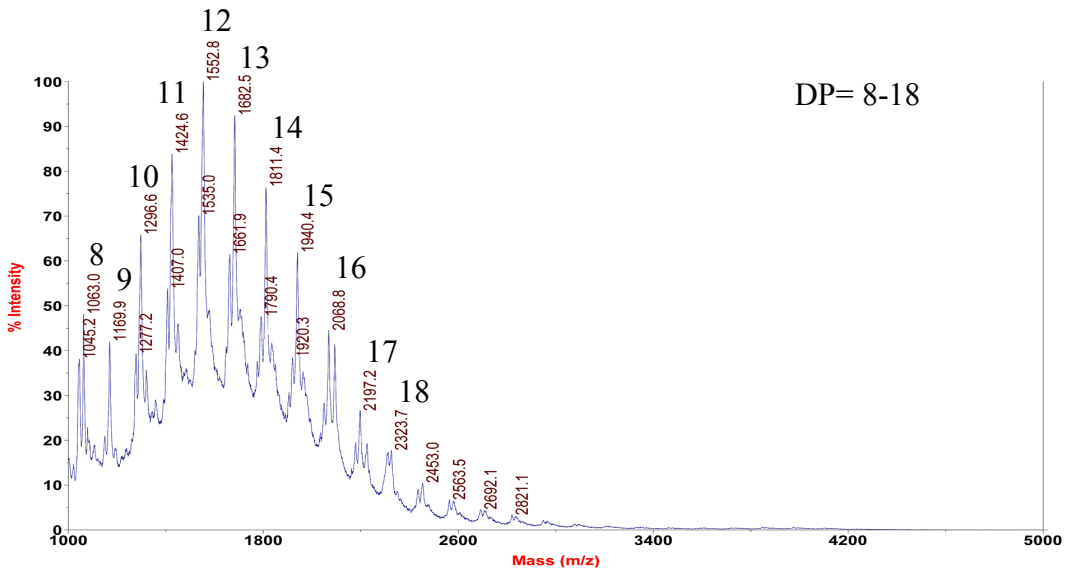


Figure 2.13. MALDI-TOF MS of ε-PL. ε-PL from the transformant P*tef::VibA* (Fl1). Number on the each peak indicate the degree of polymerization (DP).

2.3. Materials and Methods

2.3.1. Strains and culture conditions

E. festucae strain E437 isolated from the soft fescue *Festuca pulchella* was provided by Prof. Christopher L. Schardl (University of Kentucky, USA). *E. festucae* strain Fl1 isolated from the soft fescue *F. trachyphylla* was provided by Prof. Barry Scott (Massey University, New Zealand). For liquid culture, a mycelial block (1 x 1 cm) of *E. festucae* grown on a PDA medium (BD Difco, NJ, USA) at 23 °C was finely chopped and then inoculated in 50 mL of PD medium (BD Difco) in a 100 mL-Erlenmeyer flask and incubated at 23 °C, kept on an orbital shaker at 100 rpm for 5-10 days after endophyte inoculation.

2.3.2. Spectroscopic analyses

NMR spectra were recorded on an Avance ARX400 (400 MHz for ^{1}H) (Bruker Biospin, Yokohama, Japan). The chemical shifts (δ, ppm) were referenced to the internal standard (0.01% MeOH in D_2O) at δ_H 3.334 and δ_C 49.5. MALDI-TOF MS was measured by a TOF/TOF 5800 spectrometer (AB Sciex, Foster, CA, USA) with α-cyano-4-hydroxycinnamic

acid (Shimadzu Co., Tokyo, Japan) as a matrix. The calibration of MALDI-TOF MS was performed by using a mixture of PEG2000 and PEG4000 as an external calibrant. LC/MS was recorded on an HCTplus-ESI ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) in the negative ion mode.

2.3.3. Vector construction and transformation of *E. festucae* for overexpression of *VibA* gene

The *vibA* gene-overexpressing transformants P*tef::VibA* (E437) and P*tef::VibA* (Fl1) were produced as previously reported [10]. Briefly, fungal genomic DNA was isolated from mycelia grown in PD medium as previously described [17]. PCR amplifications of genomic DNA were performed using PrimeSTAR Max DNA Polymerase (Takara-Bio, Kusatsu, Japan). Plasmid pNPP195, for the constitutive expression of *epls* gene, was prepared by cloning a 1.8-kb PCR fragment of *epls* gene amplified with primers OEx-vibA-1-XbaI-F1 and OEx-vib1-EcoRI-R1, into XbaI/EcoRI sites of the pPN94 [10]. Protoplasts of *E. festucae* strains E437 and Fl1 were prepared as described previously [18] and transformed with 5 μg of the plasmid using the method previously described [19]. Transformants were selected on PDA containing 150 μg/ml hygromycin to obtain P*tef::Epls* (E437) and P*tef::Epls* (Fl1).

2.3.4. Purification and structure determination of ε-PL

Liquid cultures (400 mL in total) of the P*tef::VibA* (E437) transformant were filtered by filter membrane unit (Millex-HA filter unit, 45 μm pore diameter, Millipore, Billerica, MA, USA) and the combined filtrates were adjusted to pH 8.5 with 6 M NaOH and adsorbed on the weakly acidic cation exchange resin Amberlite IRC-76 (Organo Corp., Tokyo, Japan) (H form, 70 mL) in a column. The resin was washed with water (420 mL) and eluted with 420 mL of 0.2 M acetic acid and then each 100 mL of 0.05, 0.1, 0.15, and 0.2 M HCl at the rate of 400

mL/h. The fractions eluted with 0.05, 0.1, and 0.15 M HCl (300 mL in total) were combined, adjusted to pH 6.5 with 1M NaOH, and concentrated to approximately 30 mL, which was dialyzed with an MWCO>1 kD membrane (Spectra/Por 7 Dialysis Membrane, Spectrum laboratories Inc., Rancho Dominquez, CA, USA). The inner solution was freeze-dried to give a brown powder (362 mg), which was subjected to reversed-phase HPLC using a Develosil ODS-HG-5 column (i. d. 20 x 250 mm) (Nomura Chemical, Seto, Aichi, Japan) eluted with 5-30-100-100% MeCN (0-50-55-60 min)-0.1% triflouroacetic acid (TFA) at the flow rate of 6 mL/min and detected at 210 nm. The fractions eluted at 23–55 min were combined, concentrated, and freeze-dried to give a yellowish powder (29.09 mg), which was purified by HPLC under HILIC conditions [TSK gel Amide-80 column (i. d. 22.5 x 300 mm, Tosoh Corp., Tokyo, Japan), 80–0% MeCN (80 min)-0.1% TFA, 6 mL/min, detected at 254 nm] to obtain ε-PL TFA salt (15.4 mg, t_R = 40.5 min) as a yellowish powder (Scheme 1-2). The ε-PL samples were also purified from other transformants P*tef::VibA* (Fl1), P*tef::Epls* (E437), and P*tef::Epls* (Fl1) by the same method (Chapter-3).

2.3.5. MALDI-TOF MS analysis of ε-PL

A portion (0.1 mg) of ε-PL TFA salt in water (100 μL) was applied on a column of strong anion exchange resin Dowex-1-X2 (0.1 mL, Dow Chemical Co., Midland, MI, USA). The column was eluted with water (100 μL x10 fractions) and the fractions 3–7 were used for MALDI-TOF MS analysis to determine the degree of polymerization of ε-PL. The theoretical *m/z* value for each degree of polymerization was calculated as the centroid value of the constituent isotope peaks. A standard ε-PL (free polyamine form) originated from *Streptomyces albulus*, gifted by Yokohama Research Center, JNC Co. (Yokohama, Japan), was also analyzed for comparison.

2.3.6. Absolute Configuration

The absolute configuration of the lysine unit of ε-PL was determined by the advanced Marfey's method [15]. A portion (0.1 mg) of ε-PL TFA salt was hydrolyzed with 6 N HCl (300 μL) at 110 °C for 2 h in a sealed tube. The reaction mixture was concentrated to dryness by nitrogen flash, and the residue was dissolved in 150 μL of water. To a portion (50 μL) were added 40 μL of 1 M $NaHCO_3$ and 50 μL of a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L or D-leucinamide (L- or D-FDLA) in acetone, and the mixture was incubated at 37 °C for 1 h. The resulting mixture was treated with 20 μL of 1 M HCl and diluted with 340 μL of acetonitrile. A portion (5 μL) was applied to LC/MS analysis [Cadenza CD-C18 column (i. d. 2 x 75 mm) (Imtakt, Kyoto, Japan), 30−100% (30 min) MeCN-0.1% formic acid, flow rate 0.15 mL/min]. L-Lysine-L-DLA and L-lysine-D-DLA derivatives were eluted at 30.8 and 32.6 min, respectively.

2.4. Conclusion

An unknown antifungal substance against conidial germination of *D. erythrospila* was found to be ε-PL from *vibA* gene overexpressing transformants, DP=28−34 from *Ptef::VibA* (E437) and DP=8-18 from *Ptef::VibA* (Fl1), respectively. The production of ε-PL was enhanced more than 3.7-fold in P*tef::VibA* (E437) strain and infinite in P*tef::VibA* (Fl1) than their wild type strains. These results indicate that *vibA* gene is involved in the biosynthesis of ε-PL in *E. festucae* and that their overexpression promotes the ε-PL production.

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CHAPTER 3

Overexpression of *Epls*, a fungal ε-poly-L-lysine synthase gene, to enhance ε-PL production in *Epichloë* and evaluation of antifungal activity of ε-PLs

3.1. Introduction

A forage grass endophytic fungi, *Epichloë* belongs to the fourth dominant class Sordariomycetes in Ascomycete [1]. At least 15,000 compounds have discovered from ascomycete fungi [2]. Within them, about 160 secondary metabolites have been identified mainly from six *Epichloë* endophytic fungal species including *E. typhina*, *E. lolii*, *E. festucae*, *E. bromicola*, *E. bromicola* N1, and *Epichloë* sp [3]. All secondary metabolites obtained from *Epichloë* and more than 10 kinds of symbionts (*Epichloë gansuense*−*Achnatherum inebrians*, *Epichloë festucae* var. *lolii*−*Lolium perenne*, *Epichloë* endophyte−*Stipa robusta*, *Epichloë coenophialum*−*F. arundinacea*, *E. typhina*−*F. arundinacea*, *E. lolii*−*L. perenne*, *Epichloë uncinata*−*Festuca pratensis*, *Epichloë siegelii*−*L. perenne*, *E. bromicola*−*Elymus dahuricus*, *Epichloë typhnium*−*Poa ampla*, and some endophyte fungi not being determined, such as *Epichloë* endophyte−*Festuca argentina*, *Epichloë* sp. Lp1−*L.perenne*, *Epichloë* species−*Lolium* species, *Epichloë* species−*A. inebrians*, *Epichloë* sp.−*Achnatherum robustum*, etc.) were summarized in Figure 3.1 [3]. Key genes playing a role in biosynthesis of the bioprotective alkaloids in *Epichloë* have previously been elucidated [4-6]. The biosynthesis of alkaloids and other metabolites is generally performed by any of four core biosynthetic enzymes including:

1. Polyketide synthetase (PKS)
2. Nonribosomal peptide synthetase (NRPS)
3. Terpene synthetase (TPS)
4. Dimethylallyl tryptophan synthetase (DMATS) [2,7,8].

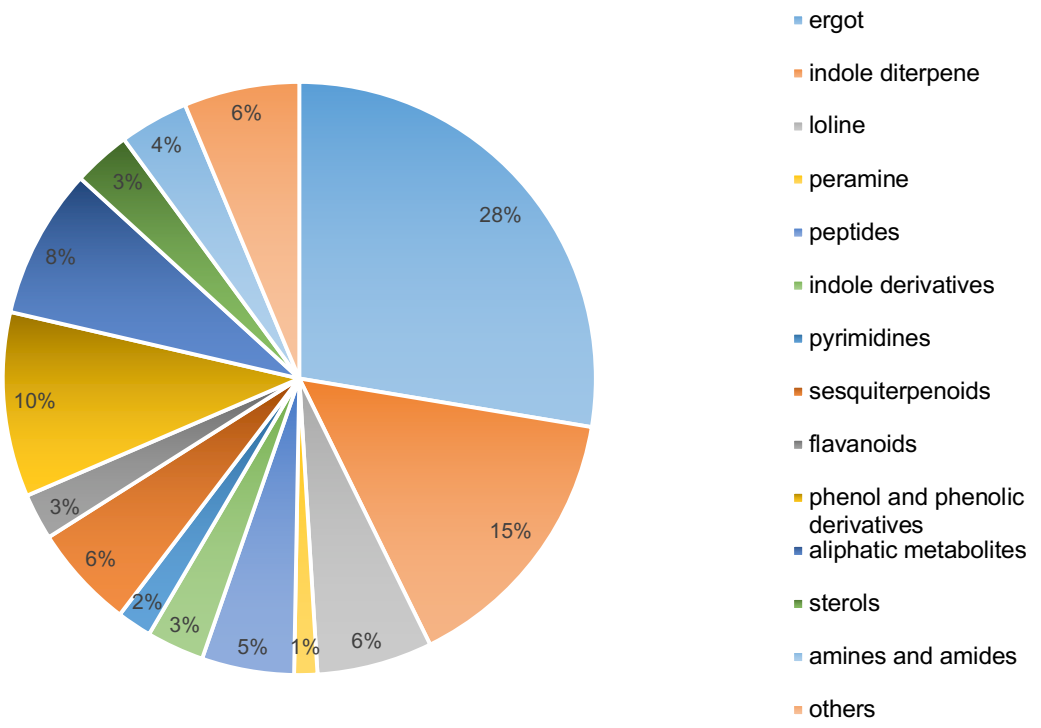

Figure 3.1. All secondary metabolites originated from *Epichloë* genus [3].

Nonribosomal peptides (NRPs) and PKS are two largest groups of secondary metabolites synthesized sequentially via condensed proteinogenic or nonproteinogenic amino acids assembling linear, cyclic or branched peptides [9,10]. ε-PL is known to be generated by NRPSs [11,12]. ε-PL has been known as an antibacterial polyamine secreted mainly by some bacteria in the Streptomycetaceae family and a few members of the Bacillaceae family [13,14]. Bacterial ε-PL is biosynthesized by the transmembrane protein ε-poly-L-lysine synthetase (Pls), which features non-ribosomal peptide synthetase with adenylation and thiolation domains, six transmembrane, and three tandem domains [12].

ε-PL is a cationic polymer with antimicrobial activity against wide spectrum of microorganisms as well as antiphage activities [15]. Because of the safety of ε-PL, ε-PL has been applied for the food preservative and medical fields.

The objective of this chapter is to describe the isolation of the gene responsible for the biosynthesis of ε-PL and a biological evaluation of ε-PLs as inhibitory activity against not only spore germination of the grass pathogen *Drechslera erythrospila*, oomycete *Phytophthora infestans*, and the polyxenous plant pathogen *Botrytis cinerea* but also growth inhibition against 8 plant pathogens.

3.2. Results and discussion

3.2.1. Identification of ε-PL biosynthetic gene *Epls* from *E. festucae*

The genes for ε-PL biosynthetic enzyme Pls (ε-poly-L-lysine synthetase) have been identified from bacterial species, such as *Streptomyces albulus* and *Kitasatospora setae* [12, 16, 17], but the corresponding genes have not been isolated from the fungi kingdom. Using *S. albulus* Pls as query of blastp search, EfM3.024590 was identified from the genome sequence of the endophytic fungus *E. festucae* as a candidate gene for Pls. Although identity of amino acid sequence between *S. albulus* Pls and *E. festucae* EfM3.024590 is only 51%, they have a similar domain architecture including N-terminal adenylation (A) and thiolation (T) domains for non-ribosomal peptide synthetases, and C-terminal tandem C1, C2 and C3 domains, which are unique to Pls (Figure 3.2). The substrate-binding pocket of adenylation domains [18] is conserved among bacterial Pls, EfM3.024590, and the lysine-binding pocket in the first A domain of the *Bacillus licheniformis* bacitracin synthetase 2 (Figure 3.3). Therefore, EfM3.024590 was designated as Epls for the *E. festucae* ε-PL synthetase. The name "Epls" is proposed for the fungal Pls as the name Pls is widely used for a component of fungal enzymes for the production of reactive oxygen species (e. g., [19]). Homologues of Epls are found in a few Ascomycota fungi and well conserved especially in the family Clavicipitaceae, including some plant- and entomo-pathogenic fungi (Figure 3.4).

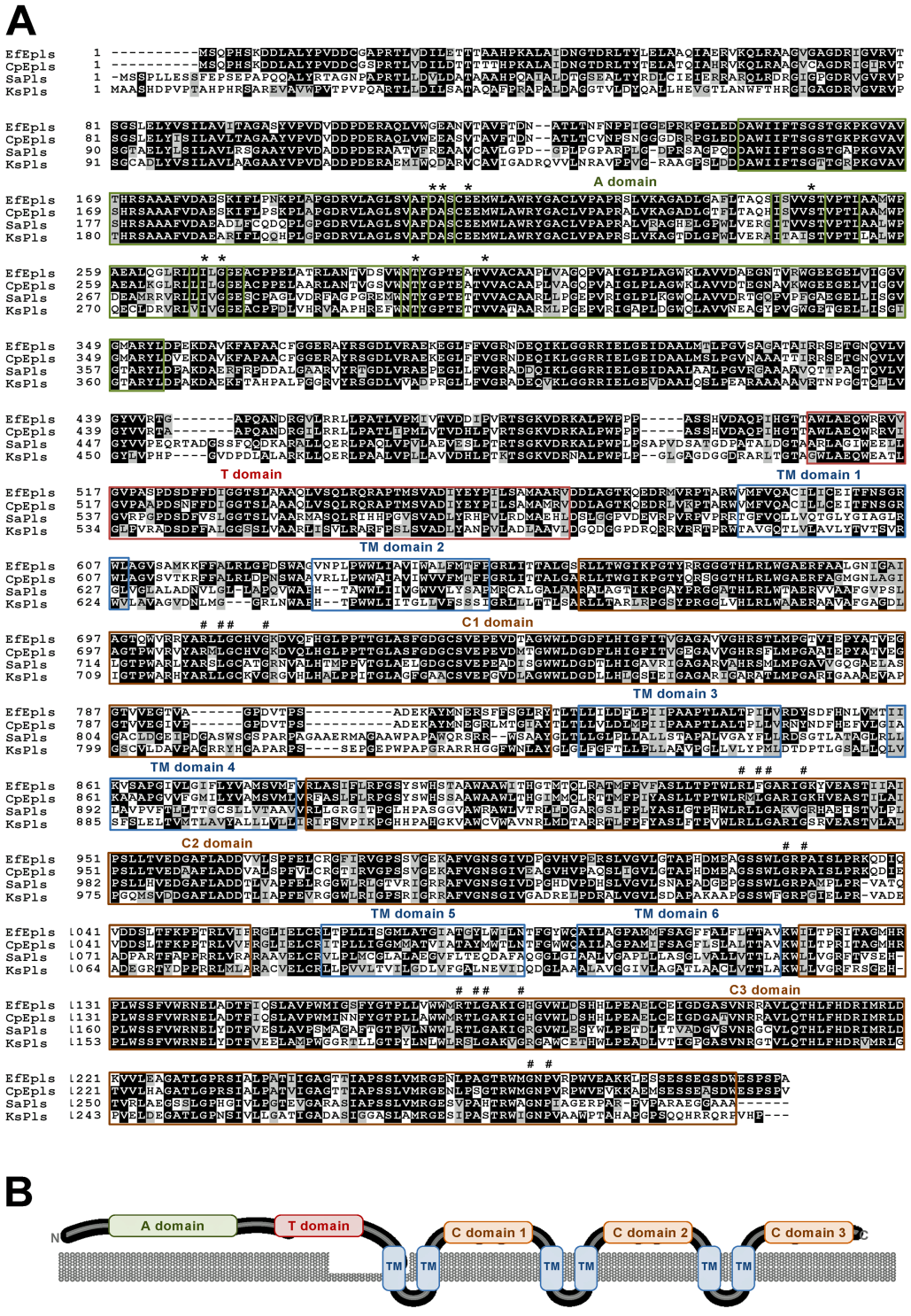

Figure 3.2. Structure of ε-poly-L-lysine synthetase of *E. festucae*. (**A**) Alignment of the deduced amino acid sequences of fungal and bacterial ε-poly-L-lysine synthetases (Epls and Pls, respectively). Non-ribosomal peptide synthetases adenylation (A) and thiolation (T) domains, transmembrane (TM) domains, and C-terminal tandem C1, C2 and C3 domains are indicated by green, red, blue and orange respectively. Amino acid residues for substrate-binding pocket in A domain [18] are indicated by asterisks, and conserved amino acids in tandem C domains for lysine polymerization [20] are indicated by hash marks. Ef, *Epichloë festucae*; Cp, *Claviceps purpurea*; Sa, *Streptomyces albulus*; Ks, *Kitasatospora setae*. (**B**) Predicted architecture of *E. festucae* Epls.

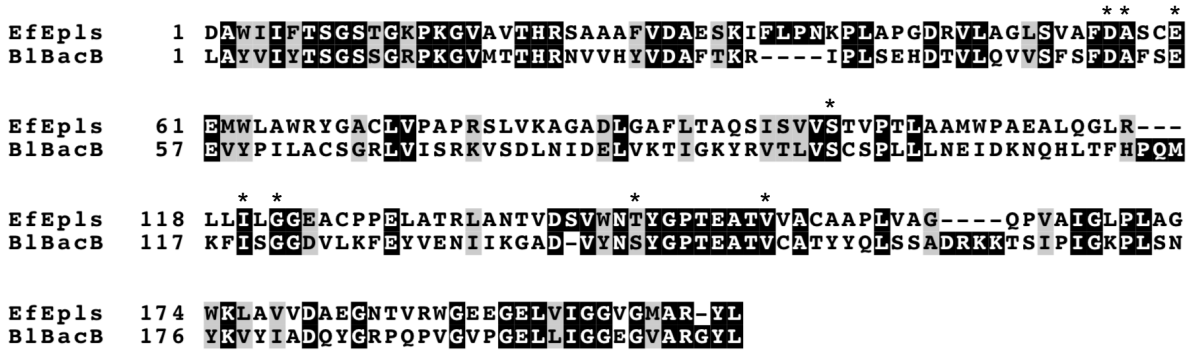

Figure 3.3. Alignment of the deduced amino acid sequences of adenylation domain of *E. festucae* ε-PL synthetase (EfEpls) and the first adenylation domain of *Bacillus licheniformis* bacitracin synthetase 2 (BlBacB, accession No. AAC06347). Amino acid residues for the lysine-binding pocket in the first A domain of BlBacB are indicated by asterisks.

3.2.2. Overexpression of *Epls* gene in *E. festucae* strains

E. festucae strain E437 produces an antifungal substance, while a culture filtrate of strain Fl1 shows no detectable antifungal activity [21]. Overexpression of *vibA* in E437, P*tef::VibA* (E437) strain, and that in Fl1, P*tef::VibA* (Fl1) strain, showed the enhancement of the antifungal activity compared with their wild type strains [22]. Although this phenomenon suggested that the transcription factor gene *vibA* is involved in the biosynthesis of ε-PL, the direct evidence of the participation of the *epls* gene in *E. festucae* was still unclear. To investigate the role of *E. festucae epls* gene, *E. festucae* strains E437 and Fl1 were transformed with a vector for the overexpression of *epls* gene under the control of TEF (Translation elongation factor) promoter [23].

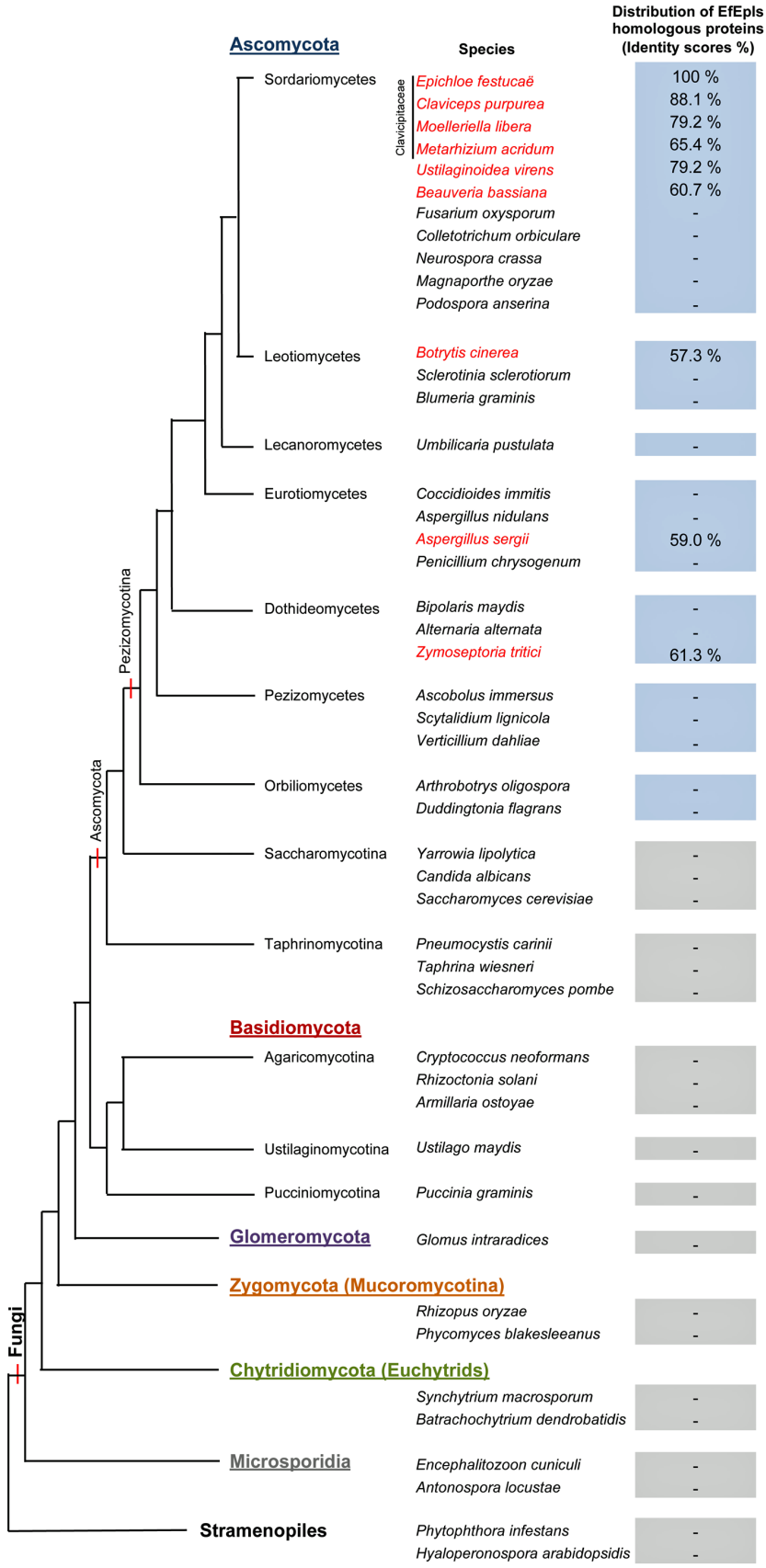

Figure 3.4. Distribution of Epls homologues in the fungi kingdom. Percentage identities of deduced amino acid sequence with *E. festucae* Epls were calculated with MacVector program (ver. 15) with default setting.

ε-PL was purified from the obtained transformants P*tef::Epls* (E437) and P*tef::Epls* (Fl1) were analyzed by MALDI-TOF MS (Table 3.1 and Figure 3.5). The production of ε-PL was enhanced by 6.7-fold in P*tef::Epls* (E437) and infinite in P*tef::Epls* (Fl1) as much as that of the wild type strains (Table 3.1).

Table 3.1. Production and length of ε-PL produced by wild type and *epls*-overexpressing strains of *E. festucae*.

Strains	**Production (mg/L)[a]**	**Chain length, DP[b]**
E437 (wild type)	10.4	28−34 (32)
P*tef::Epls* (E437)	69.9	8-25 (15) and 26-33 (31)
Fl1 (wildtype)	trace	-
P*tef::Epls* (Fl1)	13.9	8−20 (12)

[a]The amounts are as TFA salts after HILIC HPLC purification.
[b]The degree of polymerization (DP) was expressed for the ion peaks higher than 10% of the parent ion peak in MALDI-TOF MS (Figure 3.5). The DP for the highest ion peak (DP of largest population) within the lower or higher DP product is indicated in parenthesis.

These results clearly demonstrate that not only *vibA* but also *epls* genes are involved in the biosynthesis of ε-PL in *E. festucae* and that their overexpression promotes the ε-PL production. It is interesting to note that the degree of polymerization (DP) of ε-PL is drastically affected by host strains; (1) the Fl1 strains (two transformants) produced lower DP product (DP = 8−20, DP of largest population = 12), (2) the E437 strains (wild type and *epls*-overexpressing transformant) produced not only lower DP product but also higher one (DP= 26−33, DP of largest population= 31) (Figure 3.5 and Table 3.1).

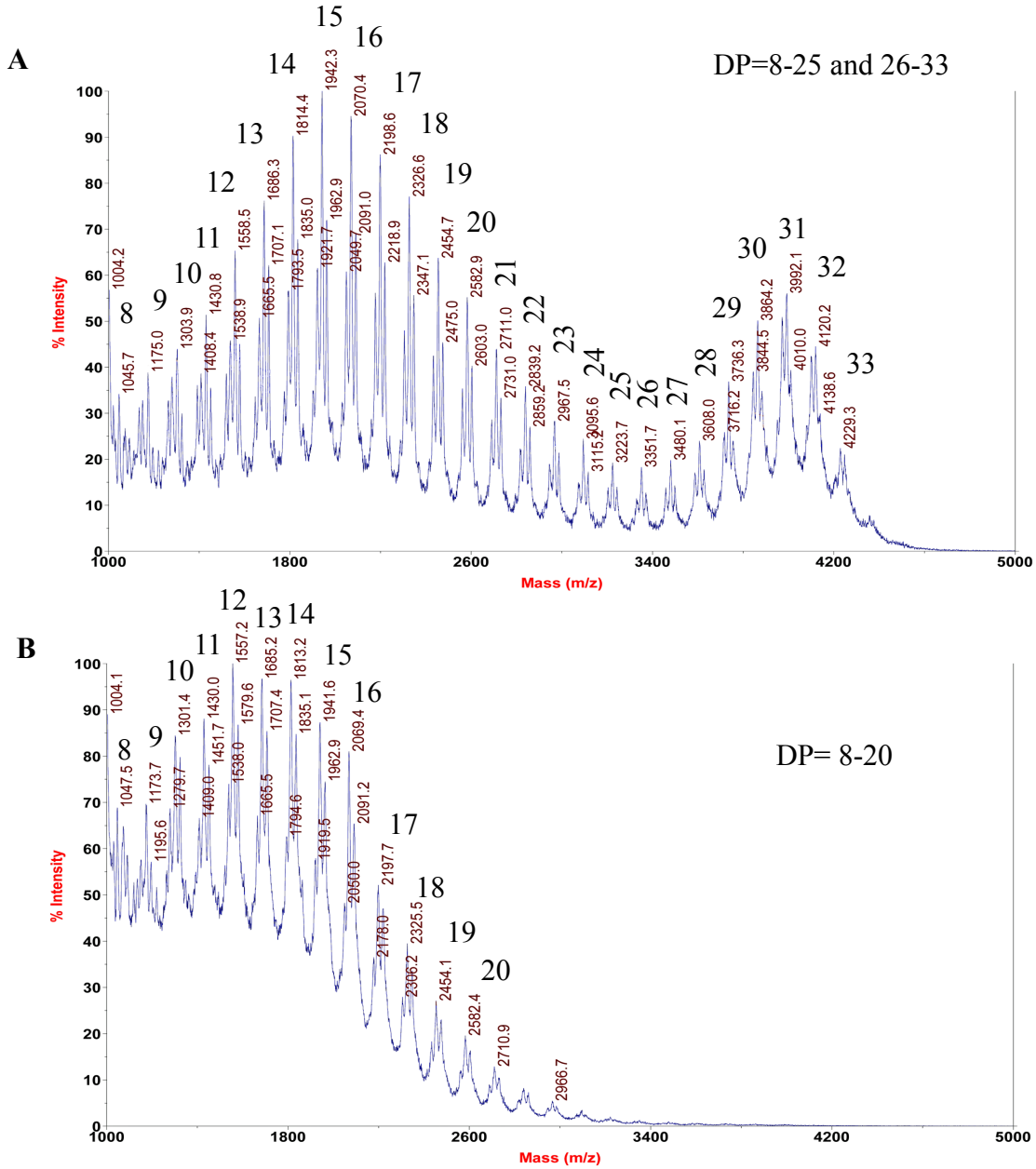

Figure 3.5. MALDI-TOF MS of ε-PL. (**A**) ε-PLs from the transformants P*tef::Epls* (E437), and (**B**) P*tef::Epls* (Fl1). Number on the each peak indicate the degree of polymerization (DP).

3.2.3. Inhibitory activity of ε-PL against spore germination of fungal and oomycete pathogens

Three samples of ε-PL (as TFA salts), a standard bacterial ε-PL, P*tef::Epls* (E437)-derived ε-PL containing long chain one, and P*tef::Epls* (Fl1)-derived ε-PL containing only short chain one, were evaluated for the inhibition of spore germination and hyphal growth of fungal and

oomycete plant pathogens: grass pathogen *D. erythrospila*, gray mold *B. cinerea,* and potato late blight pathogen *P. infestans*. The conidia or zoosporangia of pathogens were treated with ε-PL for 20 h, and the length of germinated hyphae was measured (Figure 3.6).

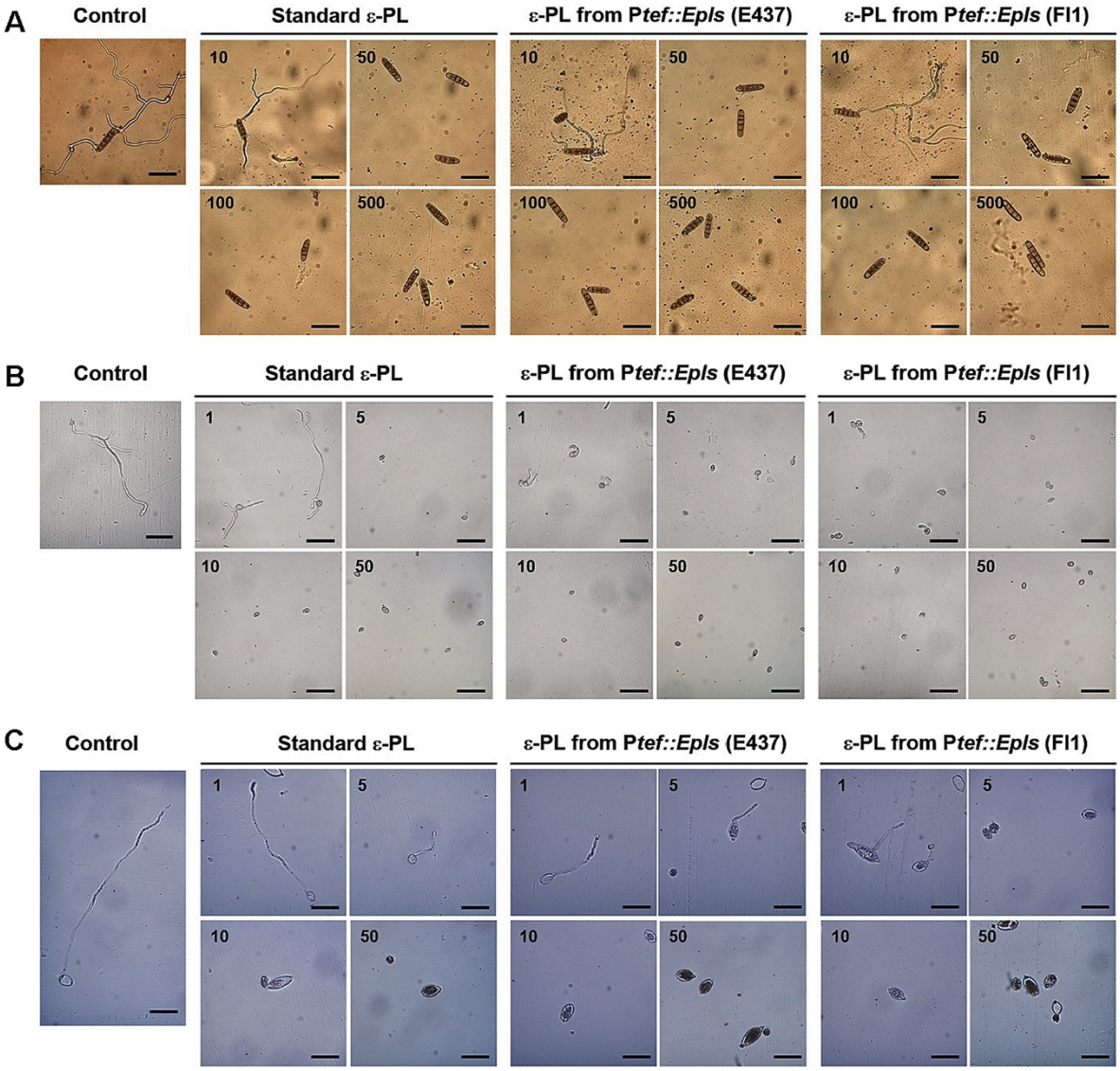

Figure 3.6. Inhibitory activity of ε-PL against spore germination of plant pathogens. (**A**) Conidia of *D. erythrospila,* (**B**) conidia of *B. cinerea*, and (**C**) zoosporangia of *P. infestans* were treated with TFA salts of standard ε-PL, and P*tef::Epls* (E437)- and P*tef::Epls* (Fl1)-derived ε-PL at the indicated concentrations (mg/mL) for 20 h. Bars = 50 µm.

The spore germination of *D. erythrospila* and other two pathogens was almost suppressed by three ε-PL samples at 50 and 5 µg/mL, respectively (Figures 3.7). *B. cinerea* was most effectively inhibited by ε-PL (Figure 3.7B). The ε-PL with only short chain lengths isolated

from P*tef::Epls* (Fl1) indicated a little higher activity than that containing long chain one from P*tef::Epls* (E437).

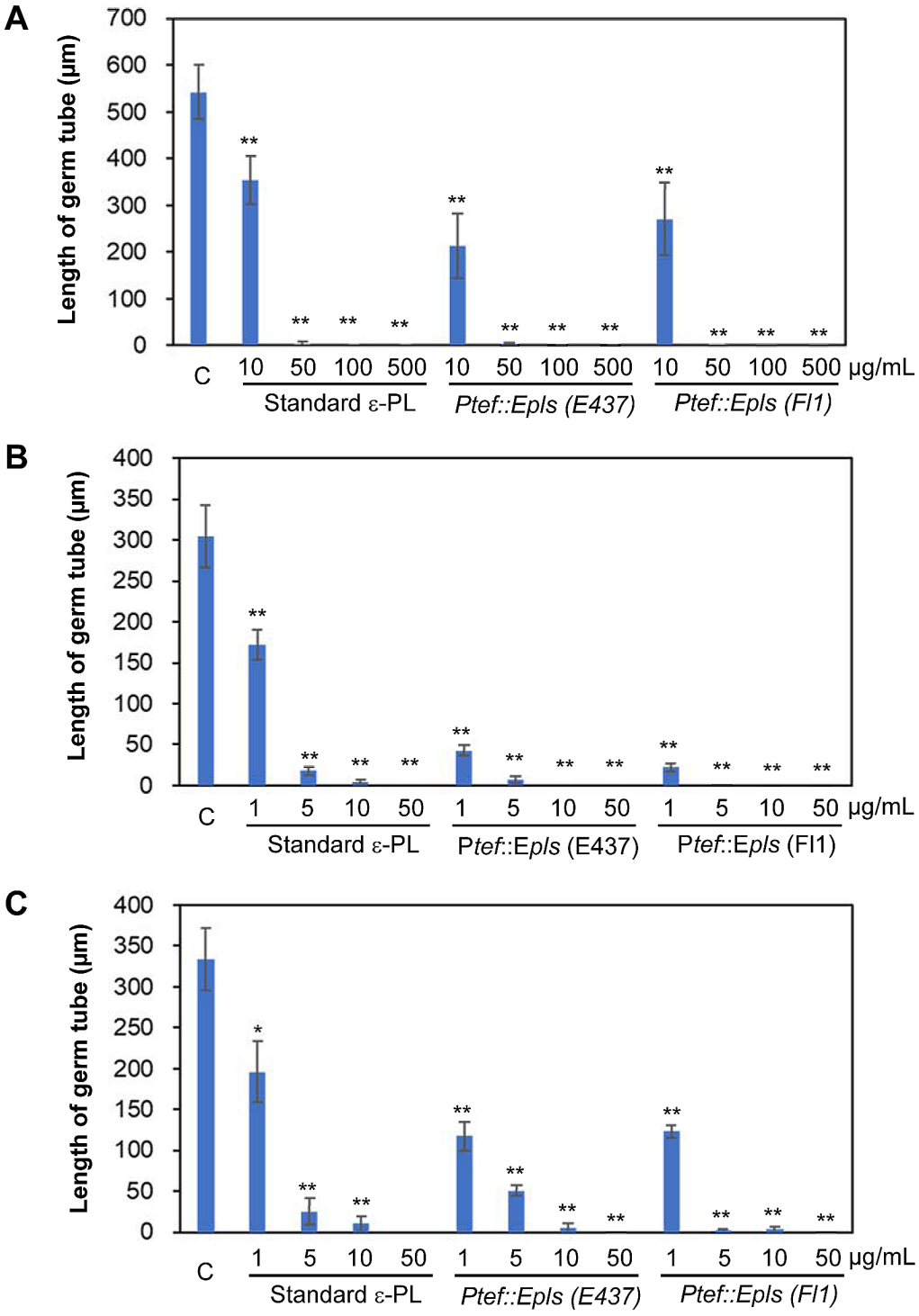

Figure 3.7. Quantitative analysis for inhibitory activity of ε-PL against spore germination and hyphal elongation of (**A**) *D. erythrospila,* (**B**) *B. cinerea*, and (**C**) *P. infestans*. Length of germinated hyphae was measured for the microscopic images shown in Figure 4. Data are

means with standard errors (n = 6). Data marked with asterisks are significantly different from control (C) as assessed by two-tailed Student's *t* tests: *P < 0.05 and **P < 0.01.

In order to investigate the target of ε-PL in the growth inhibition of pathogens, germinating hyphae of *D. erythrospila* were stained with Calcofluor White reagent, which is a fluorescent dye that binds to the polysaccharide polymers on fungal cell-wall chitin. For germinating hyphae of *D. erythrospila*, the fluorescent dye was accumulated at the hyphal tip, where the cell wall is actively synthesized (Figure 3.8A, arrowheads). On the other hand, *D. erythrospila* hyphae treated with 50 μg/mL of standard ε-PL, P*tef::Epls* (E437)- or P*tef::Epls* (Fl1)-derived ε-PL showed decreased accumulation of polysaccharide polymers at hyphae tips (Figure 3.8B-D), suggesting that ε-PL inhibited the polarized production of polysaccharide for cell wall at hyphal tip of *D. erythrospila.*

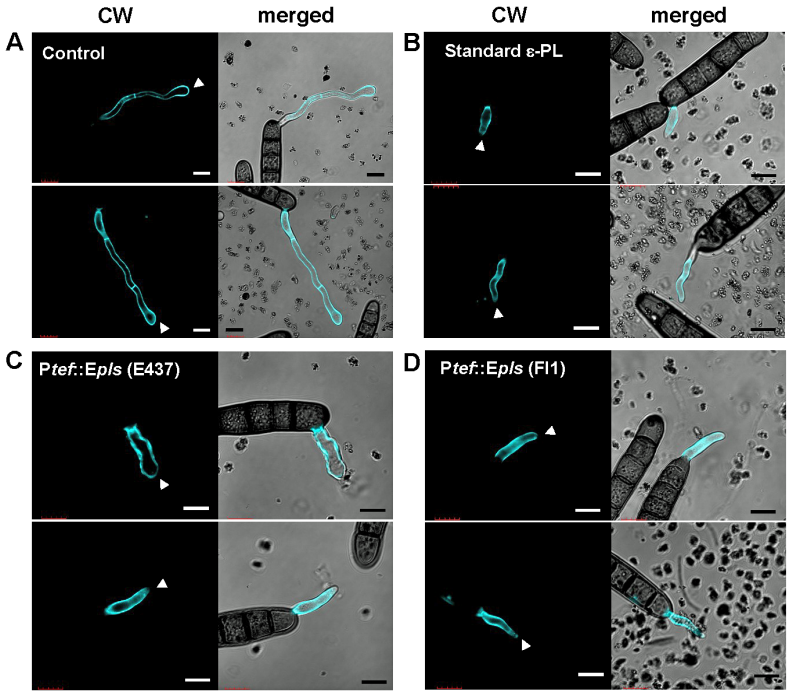

Figure 3.8. Morphology of germinated hyphae of *D. erythrospila* treated with ε-PL. Conidia of *D. erythrospila* were incubated with (**A**) water, (**B**) 50 μg/mL standard ε-PL, **(C)** 50 μg/mL ε-PL from P*tef::Epls* (E437), or (**D**) ε-PL from P*tef::Epls* (Fl1) for 20 h at 23 °C. Germinated hyphae were stained with Calcofluor White and observed under a confocal laser scanning microscope. Arrowheads indicate the tip of growing hyphae. Bars = 10 μm.

3.2.4. Antifungal activity of ε-PL

The observation of the conidial germination inhibition against *D. erythrospila*, *B. cinerea*, and *P. infestans* prompted us to evaluate antifungal activity of ε-PL by a paper disk diffusion method against 8 plant pathogens: *D. erythrospila, P. capsici, C. orbiculare, F. oxysporum*, *B. cinerea, M. oryzae, A. alternata,* and *A. niger*. The results showed that both ε-PL samples produced by P*tef::Epls* (E437) with long and short chain lengths and P*tef::Epls* (Fl1) with short one showed the antifungal and anti-oomycete activities against only *D. erythrospila* and *P. capsici* at 300 μg/disk, which was comparable to that of standard ε-PL (Figure 3.9). There was no clear polymer-size dependency in the antimicrobial activity of ε-PL (Table 3.2).

Table 3.2. Antifungal activity of ε-PL[a]

Fungi	MID (μg/disk)		
	Standard ε-PL (DP = 25−35)	**ε-PL (DP = 8−25 and 26−33)[b]**	**ε-PL (DP = 8−20)[c]**
D. erythrospila	300	300	300
P. capsici	300	300	300
C. orbiculare	>300	>300	>300
F. oxysporum	>300	>300	>300
B. cinerea	>100	>100	>100
M. oryzae	>100	>100	>100
A. alternata	>100	>100	>100
A. niger	>100	>100	>100

[a] *Expressed as minimum inhibitory dose (MID) that induces a definite inhibitory zone between paper disk and pathogen colony.*
[b] *Isolated from Ptef::Epls* (E437) *transformant. Degree of polymerization (DP) is indicated in parenthesis.*
[c] *Isolated from Ptef::Epls* (Fl1) *transformant.*

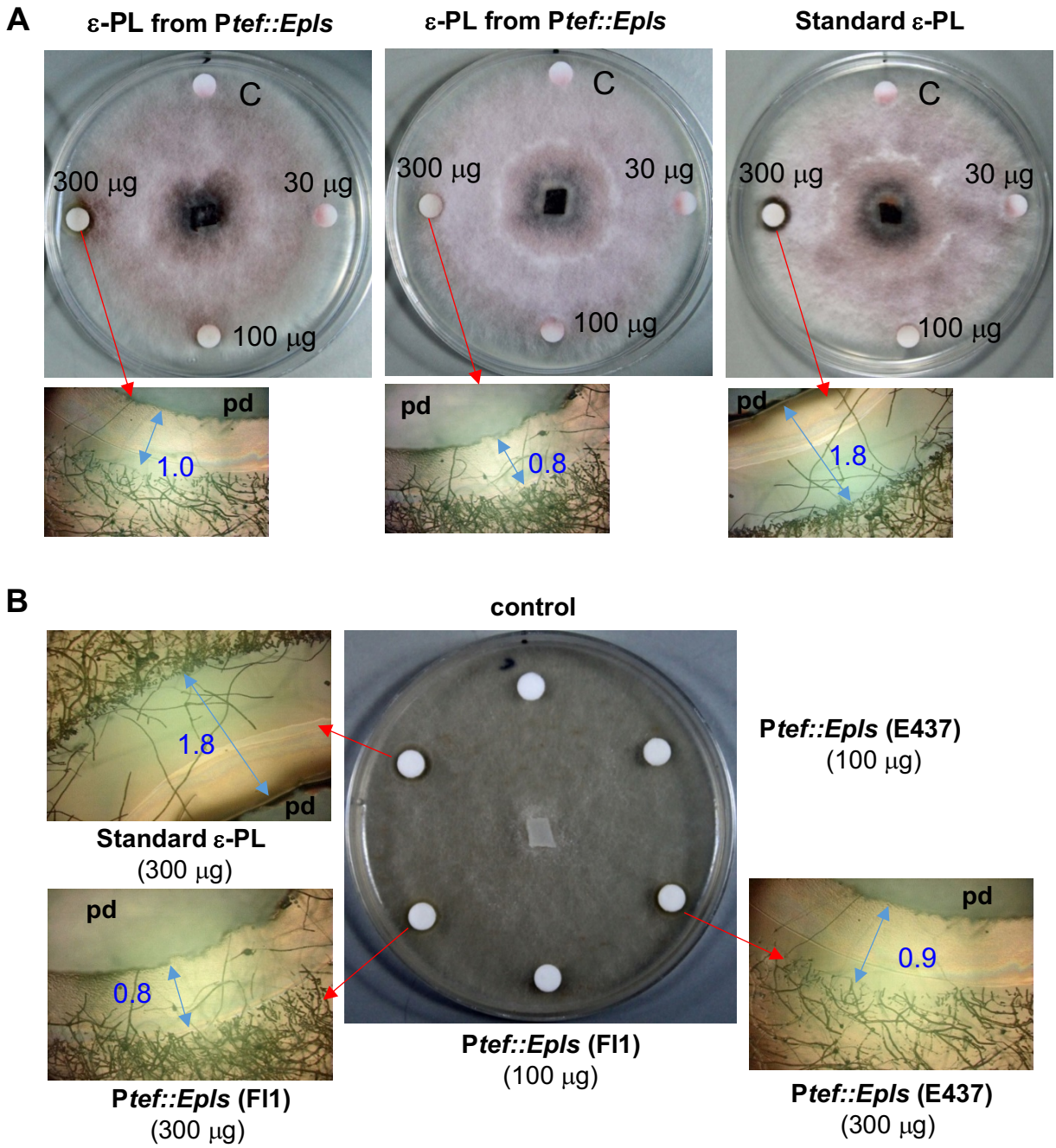

Figure 3.9. Fungal growth inhibition by ε-PL against **(A)** *Drechslera erythrospila* and **(B)** *Phytophthora capsici*. Effects of the ε-PL was observed 3 (**A**) or 5 days (**B**) after applying sample-containing disks. Inhibition zones between paper disk edge and colony front (in mm) around the paper disk ("pd") were observed through a stereo microscope.

3.3. Materials and Methods

3.3.1. Strains and culture conditions

E. festucae strain E437 isolated from the soft fescue *Festuca pulchella* was provided by Prof. Christopher L. Schardl (University of Kentucky, USA). *E. festucae* strain Fl1 isolated

from the soft fescue *F. trachyphylla* was provided by Prof. Barry Scott (Massey University, New Zealand). For liquid culture, a mycelial block (1 x 1 cm) of *E. festucae* grown on a PDA medium (BD Difco, NJ, USA) at 23 °C was finely chopped and then inoculated in 50 mL of PD medium (BD Difco) in a 100 mL-Erlenmeyer flask and incubated at 23 °C, kept on an orbital shaker at 100 rpm for 5-10 days after endophyte inoculation. Fungal pathogens *D. erythrospila* (MAFF No. 305378) was obtained from National Institute of Agrobiological Sciences (NIAS, Japan), *Colletotrichum orbiculare* 104-T was provided by Dr. Yasuyuki Kubo (Kyoto Prefectural University, Kyoto, Japan), *Fusarium oxysporum* f. sp. *lycopersici* CK3-1, *B. cinerea* NBc1, and *Alternaria alternata* M-71 were provided by Dr. Takashi Tsuge (Chubu University, Kasugai, Japan), *Magnaporthe oryzae* Ken53-35 was from Dr. Yukio Tosa (Kobe University, Kobe, Japan) and *Aspergillus niger* AJ117065 was provided by Ajinomoto Co. (Kawasaki, Japan). All fungi were grown on PDA medium (0.4% potato extract, 2% glucose, and 1.5% agar) at 25 °C and kept in 20% glycerol at -80 °C for long storage. Oomycete pathogen *P. infestans* 08YD1 was provided by Ms. Kayo Shirai (Hokkaido Central Agricultural Experiment Station, Japan) and grown on rye media at 20 °C. *P. capsici* NBRC 30696 was purchased from the Biological Resource Centre, National Institute of Technology and Evaluation (NBRC, Chiba, Japan) and cultured on 5% V8 vegetable juice-1.5% agar medium at 25 °C.

3.3.2. Vector construction and transformation of *E. festucae* for overexpression of *Epls* Gene

Fungal genomic DNA was isolated from mycelia grown in PD medium as previously described [24]. PCR amplifications of genomic DNA were performed using PrimeSTAR Max DNA Polymerase (Takara-Bio, Kusatsu, Japan). Plasmid pNPP195, for the constitutive expression of *epls* gene, was prepared by cloning a 3.9-kb PCR fragment of *epls* gene amplified

with primers pPN94-EfPLS-F AACCTCTAGAGGATCATGAGTCAACCTCACTCCAA and pPN94-EfPLS-R ACGTTAAGTGCGGCCTTACGCTGGAGAGGGAGACT into the pPN94 [25] digested with *Bam*HI/*Not*I using In-Fusion Cloning (Takara Bio) (Figure 3.10). The sequence of *epls* gene is deposited in the GenBank database under the accession number LC517046. Protoplasts of *E. festucae* strains E437 and Fl1 were prepared as described previously [26] and transformed with 5 µg of the plasmid using the method previously described [27]. Transformants were selected on PDA containing 150 µg/ml hygromycin to obtain P*tef::Epls* (E437) and P*tef::Epls* (Fl1). The *vibA* gene-overexpressing transformants P*tef::VibA* (E437) and P*tef::VibA* (Fl1) were produced as previously reported [22].

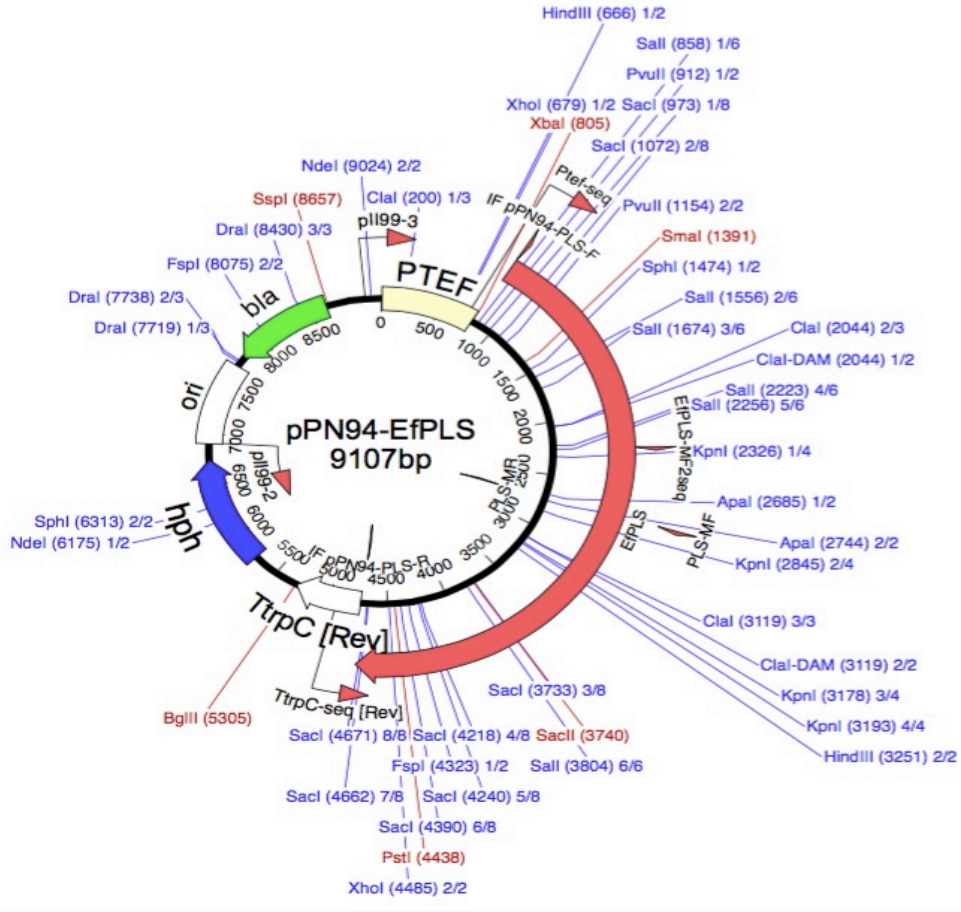

Figure 3.10. pPN94 construct for the constitutive expression of *epls* gene.

3.3.3. MALDI-TOF MS analysis of ε-PL

A portion (0.1 mg) of ε-PL TFA salt in water (100 μL) was applied on a column of strong anion exchange resin Dowex-1-X2 (0.1 mL, Dow Chemical Co., Midland, MI, USA). The column was eluted with water (100 μL x10 fractions) and the fractions 3–7 were used for MALDI-TOF MS analysis to determine the degree of polymerization of ε-PL. The theoretical *m/z* value for each degree of polymerization was calculated as the centroid value of the constituent isotope peaks. A standard ε-PL (free polyamine form) originated from *Streptomyces albulus*, gifted by Yokohama Research Center, JNC Co. (Yokohama, Japan), was also analyzed for comparison.

3.3.4. Spore germination assay

Conidial suspension of *D. erythrospila* was prepared as previously described previously [21]. Conidiation of *B. cinerea* was induced by culturing on PDA at 23 °C under near-ultraviolet light for 2 weeks, and conidia were suspended in glucose-phosphate solution (10 mM glucose, 10 mM NaH_2PO_4). Zoosporangia suspension of *P. infestans* was prepared as previously described [28]. Spore suspension of pathogens (approx. 1 x 10^3 spores/mL) are mixed with ε-PL TFA salt at an indicated concentration on a sterile concave microscope slide. The slide was placed in a humidified plastic petri dish and incubated at 23 °C for 20 h. The germination of pathogen spores is observed under a microscope BX51 (Olympus, Tokyo, Japan) and the length of germinated hyphae was analyzed by using an open source software Image J (ver 1.50i).

3.3.5. Cell wall staining

The spores of *D. erythrospila* were treated with 50 μg/mL of ε-PL TFA salt under the above-mentioned conditions. A solution of Calcofluor White Stain (0.1 mg/mL final

concentration, Fluka, Taufkirchen, Germany) was applied to the spore suspension, and stained hyphae were observed under a confocal laser scanning microscopy FV1000-D (Olympus) with excitation at 405 nm and fluorescence emission between 425 and 475 nm.

3.3.6. Antifungal assay

The fungi (*D. erythrospila, C. orbiculare, F. oxysporum, B. cinerea, M. oryzae,* and *A. alternata*) were cultured on PDA medium (0.4% potato extract, 2% glucose, and 1.5% agar) in a 9 cm Petri dish at 25 °C for 1−12 days until colonies grew to approximately 3−4 cm in diameter. *P. capsici* NBRC 30696 was cultured on 5% V8 vegetable juice-1.5% agar medium at 25 °C for 2 d. Paper disks (6 mm in diameter) soaked with 10 μL of a compound solution in 50% DMSO-water were placed 1 cm away from the colony front, and then they were incubated for an additional period until the colony front reached the control disk. The inhibition zone (distance between the paper disk edge and the colony front, mm) was measured to evaluate the activity. The minimum inhibitory dose (MID, μg/disk) is defined as the minimum dose that induced a weak but obvious inhibition zone (1−1.5 mm). For the black mold *A. niger*, a loopful conidia was cultured at 25 °C for 4 d on PDA medium. The colony surface was gently washed with 5 mL of 0.1% Tween 20, and a portion (0.5 mL, 5×10^6 conidia) was fused in 100 mL of PDA (0.4% potato extract, 2% glucose, and 1.5% agar) at 43 °C to prepare conidia-containing agar plates. Paper disks soaked with a compound solution in 50% DMSO-water were placed on the agar plates and incubated for 2 h, and the diameter of the halo was measured. For the case that growth inhibition was observed (*D. erythrospila* and *P. capsici*), the incubation was extended until the colony covered the paper disk and then the inhibition zones were observed through a stereo microscope VH-Z100 (Keyence, Osaka, Japan).

3.4. Conclusion

A homologue of the *Streptomyces* bacterial *pls* genes was found in the eukaryotic endophytic fungous *E. festucae,* so called *epls* in this study. Overexpression of the *epls* gene in *E. festucae* strains E437 enhanced the production of ε-PL by 6.7-fold (70 mg/L). Two different lengths of ε-PLs, short one (DP = 8−20) from the *epls*-overexpressing *E. festucae* Fl1 (P*tef::Epls* (Fl1)) and long one (DP=8-25 and 26-33) from *epls*-overexpressing *E. festucae* E437 strains (P*tef::Epls* (E437)), were determined. The spore germination assay showed that both the short and long ε-PLs effectively inhibited the spore germination of the grass pathogens *D. erythrospila*, the oomycete *P. infestans* and the polyxenous plant pathogen *B. cinerea*. These ε-PLs showed weak growth inhibition (MID= 300 μg/disk or higher doses) against *D. erythrospila* and *P. capsici*. In addition, both long and short ε-PLs interfered the apical growth of the germinating hyphal tubes via reducing the polarized production of polysaccharide polymers on the cell-wall chitin.

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CHAPTER 4

General discussion

Infection of the fungal endophyte *E. festucae* E437 to perennial ryegrass enhanced disease resistance of the host plant to the grass pathogen *D. erythrospila* [1], suggesting that this endophyte produces unknown bioactive metabolite(s), which is effective to suppress the infection of fungal pathogens. It was actually observed in co-culture experiments that the endophyte grown on an agar medium inhibits the growth of *D. erythrospila*. The transformant *Ptef::VibA* (E437) that possesses an overexpressed *vibA* gene showed stronger antifungal activity than the wild type strain against a wide range of plant pathogens [2]. These phenomena attracted an attention and prompted to reveal the chemical nature of the antifungal compound and its production mechanism. In this study, by culturing the transformant followed by chromatographic purification and spectroscopic analysis, the antifungal principle was identified as ε-poly-L-lysine (ε-PL) consisting of 24−35 lysine units for the first time.

ε-PL has been known as an antibacterial polyamine secreted mainly by some bacteria in the Streptomycetaceae family [3] and a few members of the Bacillaceae family [4,5]. Production of ε-PL in fungi has also been reported for Clavicipitaceae fungi, including ergot fungi *Claviceps purpurea* [6] and *Epichloë* sp. MN-9 [7]. Bacterial ε-PL is biosynthesized by the transmembrane protein ε-poly-L-lysine synthetase (Pls), which features nonribosomal peptide synthetase with adenylation and thiolation domains, six transmembrane, and three tandem domains [8]. After an in silico analysis, a gene encoding the protein with the same domain architecture was identified from the genome of *E. festucae*, which was designated as *epls*. A phylogenetic analysis revealed that homologues of the *E. festucae epls* are found mainly in Clavicipitaceae species, being consistent with the above-mentioned fungal producers of ε-PL. Overexpression of the *epls* gene in *E. festucae* strains E437 enhanced the production of ε-PL.

Moreover, strain Fl1, a nonproducer of ε-PL, also produced ε-PL by overexpression of *epls*, indicating that Epls is the biosynthetic enzyme (or at least responsible) for the production of ε-PL in this symbiotic fungus. Since the overexpression of the *vibA* gene in *E. festucae* also enhanced the production of ε-PL and VibA is known as a transcription factor with diverse functions in Ascomycota fungi [9-11], it is expected that VibA directly regulates the expression of *epls*. However, our preliminary investigations indicated that the overexpression of *vibA* did not enhance the transcription of *epls* (data not shown) (Figure 4.1). Thus, the role of *E. festucae* VibA in the ε-PL production remains to be determined. Interestingly, while the wild-type E437 strain and the P*tef::VibA* (E437) transformant produced ε-PL with long chain lengths (DP = 28−34), others also produced that with short chain lengths (DP=8−18). Especially, the transformants derived from the Fl1 strain, P*tef::VibA* (Fl1) and P*tef::Epls* (Fl1), produced exclusively short chain polymers.

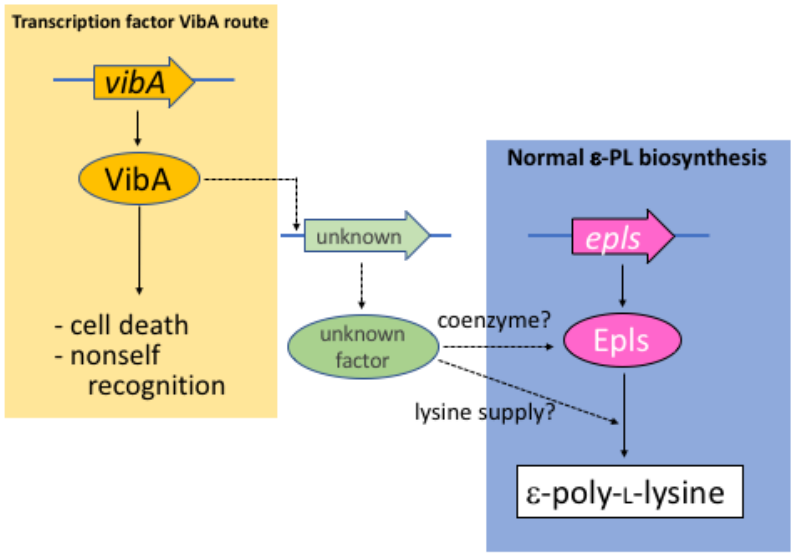

Figure 4.1. A plausible mechanism for ε-PL biosynthesis.

While most of the ε-PLs originated from *Streptomyces* species are known to possess long chains (DP = approximately 27−35), only limited numbers of microorganisms have been identified as short-chain ε-PL producers [12]. Hamano *et al.* (2014) suggested that the linker

regions connecting with the transmembrane domain of Pls were responsible for the shortening ε-PL length in *Streptomyces* species [13]. It was also reported that supplementing culture media with glycerol and glucose shortened the ε-PL chain length [14]. Among the constructed transformants, P*tef::Epls* (E437) was the best producer of ε-PL (approximately 70 mg/L). Since ε-PL has attracted attention as an antimicrobial agent with a wide inhibitory spectrum against Gram-positive and negative bacteria, fungi, yeasts, and phages [3], several strategies to improve the production of ε-PL have been conducted; for example, modifying culture conditions (supplementation with glucose or other metabolic precursors, modifying pH, etc.) [5,15], inducing double antibiotic-resistant mutations [16] and genome shuffling [17]. The present study suggests that the gene overexpression strategy under the control of the TEF (Translation elongation factor) promoter is one of the excellent strategies for the high production of ε-PL. The spore germination assay demonstrated that both the short (DP = 8−20) and long size ε- PLs (DP =26−33) effectively inhibited the spore germination of the grass pathogen *D. erythrospila* at 10 μg/mL and the oomycete *P. infestans* and the polyxenous plant pathogen *B. cinerea* at 1 μg/mL. To elucidate the target of ε-PL in the spore germination inhibition, a fluorescent staining of the germinating tubes of *D. erythrospila* with the polysaccharide-binding fluorophore Calcofluor White was performed. The result suggested that ε-PL interfered the apical growth of the germinating hyphal tubes via reducing the polarized production of polysaccharide polymers on the cell-wall chitin. A similar staining pattern was also observed in the hyphal tips of *D. erythrospila* co-cultured with *E. festucae* [1]. Isolated ε-PL samples showed weak growth inhibition (MID= 300 μg/disk or higher doses) against the tested fungi, unlike bacteria that are known to be susceptible to ε-PL. This could be due to the relatively high lipid and polysaccharide contents in the fungal cell wall [18,19] and/or the biodegradation by the fungal protease [20]. In summary, this studies demonstrated the effectiveness of the gene-overexpression strategy, not only for an efficient

supply of the safe and potential antifungal, antibacterial, and antioomycete agent ε-PL, but also for breeding pest-tolerant pastures by infecting with genetically modified endophytes like *Epichloë*.

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