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十字花科黑斑病菌生合成 tenuazonic acid 之 PKS-NRPS 基因鑑定

Identification of a PKS-NRPS gene involved in the biosynthesis of tenuazonic acid in *Alternaria brassicicola*

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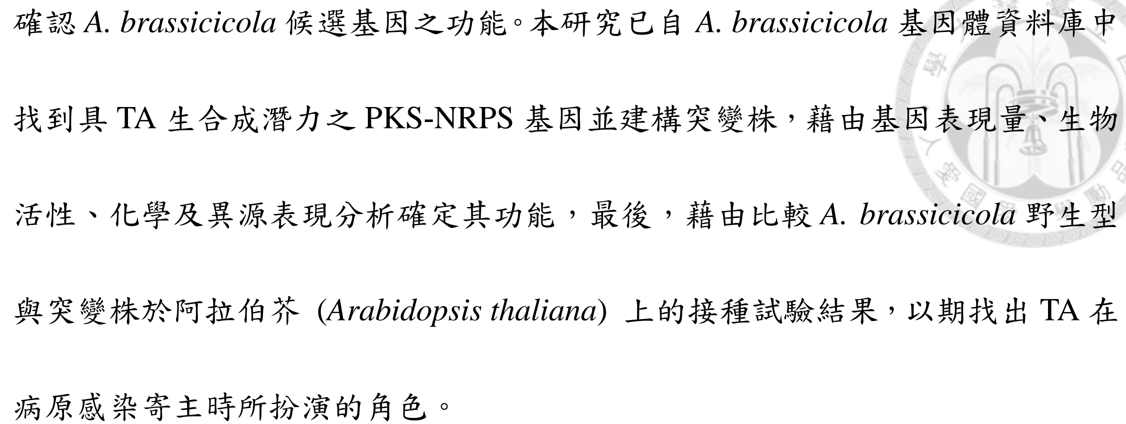
中文摘要

十字花科黑斑病菌 *Alternaria brassicicola* 為一重要的植物病原真菌，於 *in vivo* 及 *in vitro* 情況皆會生成大量具有生物活性之二級代謝物，其中研究歷史最長的為三種真菌毒素，包括：alternariol (AOH)、alternariol-9-methyl ether (AME)及 tenuazonic acid (TA)，但至目前為止，對於這些毒素的生合成路徑及在病原性中所扮演的角色仍未有詳細的研究，本研究目標即為鑑定 *A. brassicicola* TA 生合成基因。根據 TA 放射性標定分析及結構研究，可知其前驅物為 acetoacetate 及 isoleucine，而其生合成路徑中可能同時有 polyketide synthase (PKS) 及 non-ribosomal peptide synthetase (NRPS)的參與。本實驗首先利用 TA 對蜜蜂疫病病原細菌 *Paenibacillus larvae* subsp. *larvae* 之抗生活性作為篩選方式，檢測實驗室之 *A. brassicicola* 菌株是否生合成 TA。並於 *A. brassicicola* 基因體資料庫中搜尋可能為 TA 生合成之候選基因，分析候選基因與已知的 PKS/NRPS 基因之親緣關係，並從胺基酸序列預測其功能性結構 (functional domain)。進一步地於 TA 生合成誘導實驗中，以 qPCR 分析候選基因之表現量，選定具有生合成 TA 潛力之候選基因，以基因槍轉殖方法針對該候選基因進行突變。轉殖突變株經篩選、純化後，與野生型菌株同時進行 TA 生合成之誘導，進行生物活性檢測及化學分析，觀察 TA 生合成情形。此外，本研究同時建構異源表現系統 (heterologous expression system)，將 *A. brassicicola* 之候選基因轉殖入 *Aspergillus nidulans* LO2026 菌株，進行基因表現誘導，純化其培養液並分析誘導之產物，以進一步



確認 *A. brassicicola* 候選基因之功能。本研究已自 *A. brassicicola* 基因體資料庫中找到具 TA 生合成潛力之 PKS-NRPS 基因並建構突變株，藉由基因表現量、生物活性、化學及異源表現分析確定其功能，最後，藉由比較 *A. brassicicola* 野生型與突變株於阿拉伯芥 (*Arabidopsis thaliana*) 上的接種試驗結果，以期找出 TA 在病原感染寄主時所扮演的角色。

關鍵詞：十字花科黑斑病菌 (*Alternaria brassicicola*)、二級代謝 (secondary metabolites)、tenuazonic acid、異源表現 (heterologous expression)

Abstract

Brassica black spot disease fungus, *Alternaria brassicicola* is an important plant pathogenic fungus that produces plenty of secondary metabolites under both *in vitro* and *in vivo* conditions, including 3 most well-known mycotoxins: alternariol (AOH), alternariol-9-methyl ether (AME) and tenuazonic acid (TA). Thus far, very limited information is available concerning their biosynthesis and roles in pathogenesis. In this study, we aim to identify the genes involved in the biosynthesis of TA in *A. brassicicola*. With the knowledge of structural discovery and isotope feeding researches on TA, the activities of both polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) are speculated to be essential in the biosynthesis of TA while isoleucine and acetoacetate were identified to be its precursors. We first examined the TA production in *A. brassicicola* wild type strain by agar diffusion assay on honey foulbrood disease pathogen, *Paenibacillus larvae* subsp. *larvae*. Candidate genes were identified from the genome database of *A. brassicicola* and applied to bioinformatical analysis on evolutionary relationship and functional domain prediction with known PKS/NRPS genes. Further, AB04556.1 gene was chosen from candidate genes for constructing a gene-disrupted mutant on basis of gene expression level examined by qPCR. The transformants which constructed via biolistic transformation were purified and compared gene expression as well as TA production

with wild type. Additionally, we construct a heterologous expression system on *Aspergillus nidulans* LO2026 strain in order to find out the role that AB04556.1 gene played in the biosynthesis of TA. In this study, we found a potentially candidate gene from the genomic database of *A. brassicicola* and generated a gene-disrupted mutant for analyzing of the biosynthesis of TA. Ultimately, inoculation was held to understand how TA affects to the pathogenesis during infection.

Keywords: *Alternaria brassicicola*, secondary metabolites, tenuazonic acid, heterologous expression

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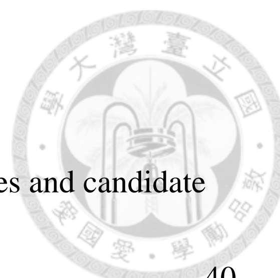

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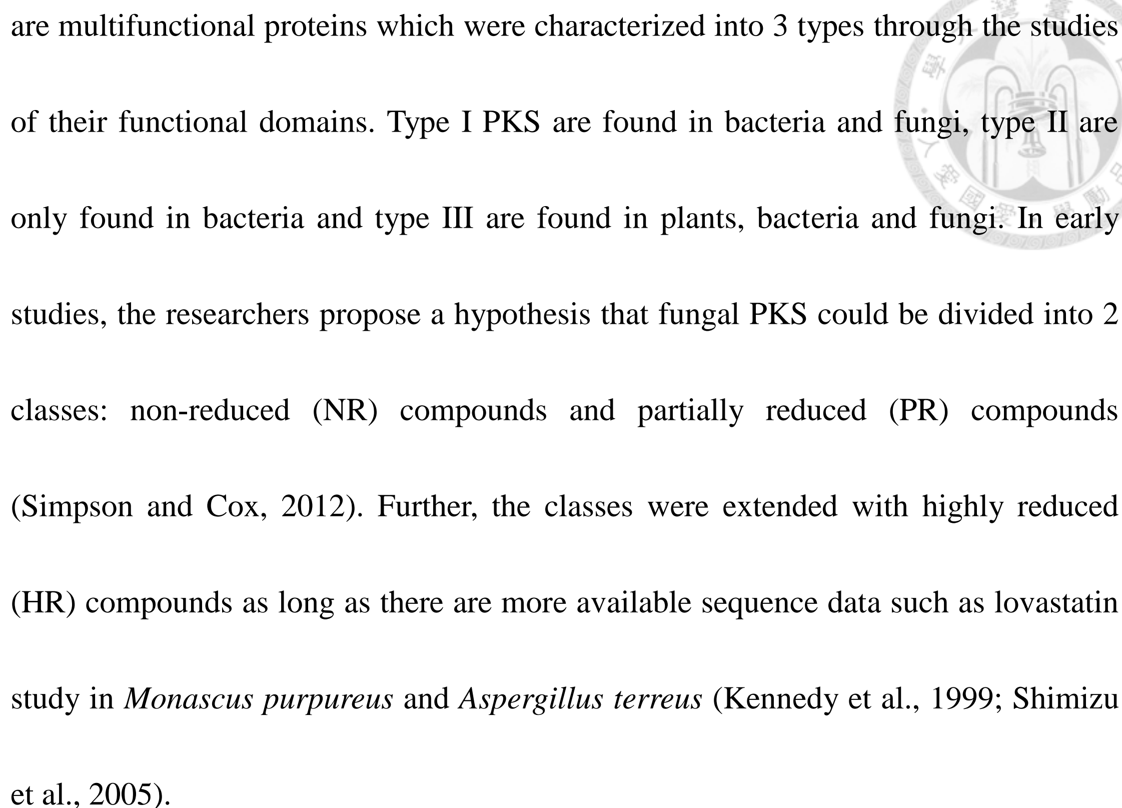
Chapter I Introduction

1.1 Fungal secondary metabolites (polyketides, non-ribosomal peptides, terpenes, and alkaloids)

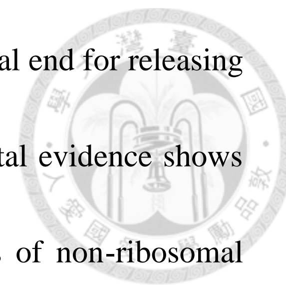
Fungal species are known to produce great natural compounds through different secondary metabolic pathways. These fungal secondary metabolites perform a broad spectrum of useful bioactivities and play tremendous roles in nature. Some secondary metabolites have been developed into pharmaceuticals such as penicillin and statins; others could be toxic to animals and humans, such as aflatoxin, fumonisins and few could be both toxic and benefic that can be applied on clinical therapy, for instance, ergot alkaloids which identified from wheat pathogen, *Claviceps purpurea*. The fungal secondary metabolism is complex and normally consists of multiple synthetic developments. As well we can identify four classes of fungal secondary metabolites, comprising polyketides, non-ribosomal peptides, terpenes and alkaloids (Keller, 2005; Yu and Keller, 2005; Misiek and Hoffmeister, 2007; Fox, 2008).

Polyketides are the most abundant fungal secondary metabolites and exemplified best by aflatoxin which is a carcinogen and lovastatin which is a notable compound exercised for lowering cholesterol (Kennedy et al., 1999; Yu, 2004; Georgianna, 2009). Polyketides are derived from short chain carboxylic acids such as acetate, malonate as well as propionate and synthesized via polyketide synthases (PKS). PKS



are multifunctional proteins which were characterized into 3 types through the studies of their functional domains. Type I PKS are found in bacteria and fungi, type II are only found in bacteria and type III are found in plants, bacteria and fungi. In early studies, the researchers propose a hypothesis that fungal PKS could be divided into 2 classes: non-reduced (NR) compounds and partially reduced (PR) compounds (Simpson and Cox, 2012). Further, the classes were extended with highly reduced (HR) compounds as long as there are more available sequence data such as lovastatin study in *Monascus purpureus* and *Aspergillus terreus* (Kennedy et al., 1999; Shimizu et al., 2005).

Non-ribosomal peptides are derived from amino acids and synthesized with multimodular enzymes which called non-ribosomal peptide synthetase (NRPS). NRPS contains several functional domains that are used for recognizing, activating, and covalent binding of specific amino acids. The finalized peptide will be released subsequently after the formation of peptide bonds. The β-lactam antibiotics such as penicillin are belonged to this class of secondary metabolites. In NRPS, each amino acid is recognized and activated with adenylation domain (A domain), attached to one another through peptidyl carrier domain (P domain) and the peptide bonds are formed by condensation domain (C domain). These 3 domains are typical consists of NRPS whereas some NRPSs lack complete domain; have an addition of methylation domain



or have a thioesterase (TE) domain that usually located at C-terminal end for releasing the resulting peptides. Unlike polyketide synthases, it has no crystal evidence shows how the functional domains are used iteratively in the synthesis of non-ribosomal peptides (Smith et al., 1990; Finking and Marahiel, 2004).

Terpenes are known to exist in plant such as camphor while fungi also have some relevant terpenes, including carotenoids, gibberellins and mycotoxin trichothecenes. Terpenes are consisted of isoprene units and can be modified in diversified ways to become cyclic or linear form, saturated or unsaturated. According to previous studies, terpene cyclase is essential for the production of terpenes and following by various modifications (Keller, 2005).

Alkaloids are usually derived from tryptophan and dimethylallyl pyrophosphate which is associated to the synthetic pathway of terpenes. Besides tryptophan, the synthesis of alkaloids occasionally involves other amino acids as precursor. Alkaloids are synthesized with conjoint functions of several enzymes such as dimethylallyl tryptophan synthetase, methyltransferase and NRPS. The most well-known alkaloids are ergot alkaloids which are found in *C. purpurea* and related species and have been identified 3 main groups: the clavine type, the lysergic-acid type and the peptide alkaloids. Ergot alkaloids are found as a mycotoxin that damage central nervous system or cause gangrene on extremities, but also can be used for therapeutic

application such as obstetric and neurologic treatment (Tudzynski et al., 1999; von Nussbaum, 2003; Keller, 2005).

1.2 PKS-NRPS hybrids

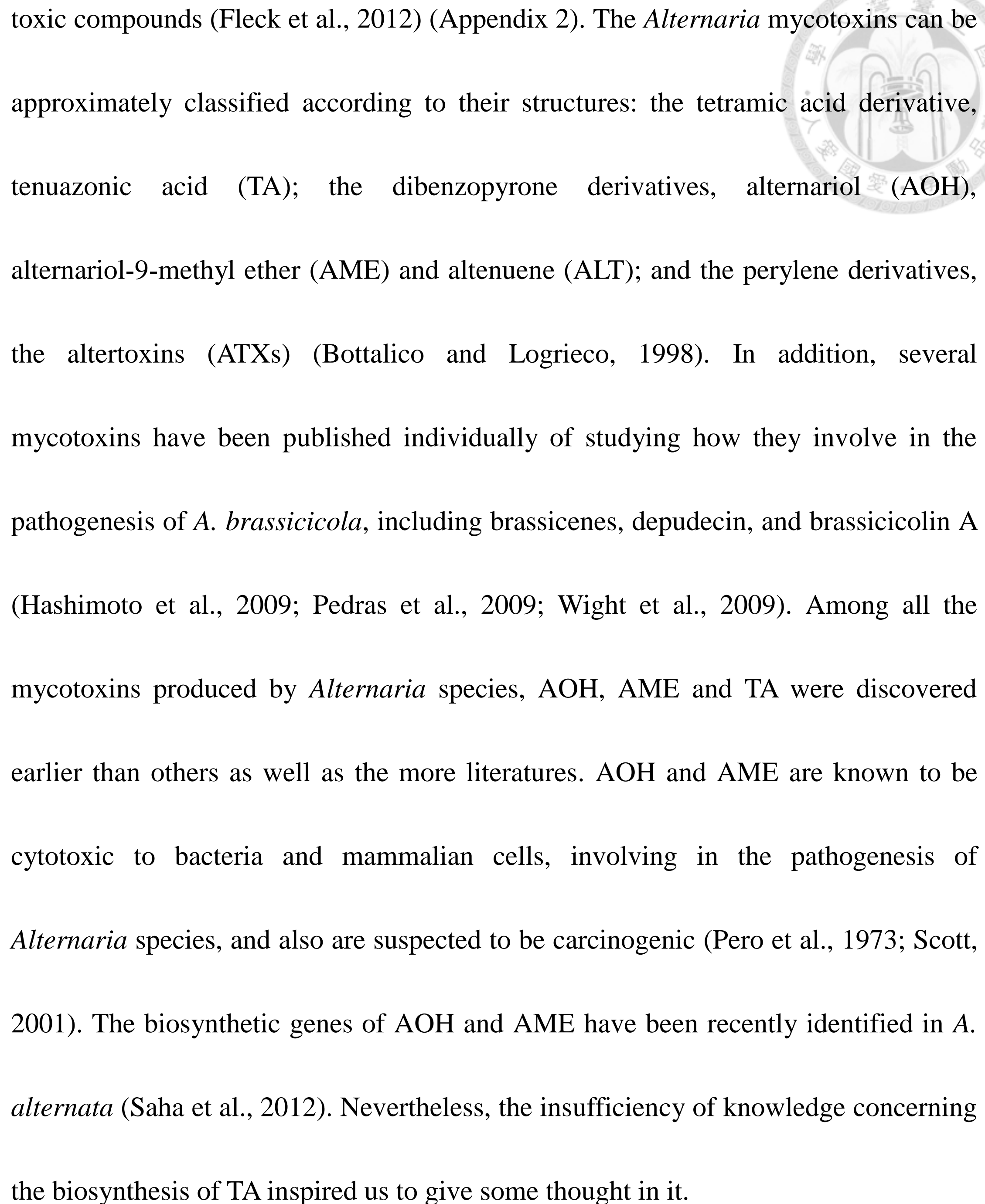
Lately, an exceeding number of PKS-NRPS hybrid enzymes of fungi have been studied. These enzymes consist of fungal type I PKS combined with a NRPS that is occasionally truncated and show distinct relation by analyzing the phylogenetic tree of PKS domains KS and AT with other PKS genes (Collemare, 2008a). The metabolites of PKS-NRPS hybrids are usually tetramic acids (Royles, 1995), except for lovastatin and compactin. The LNKS gene of *Aspergillus terrus* was published as PKS but soon after been identified as a PKS-NPRS hybrid but has truncated NRPS module. LNKS synthesizes lovastatin which is a cholesterol-lowering compound and used in pharmaceutical therapy (Campbell, 2010). MlcA of *Penicillium citrinum* is an orthologue of LNKS and synthesizes compactin, an analogue of lovastatin (Abe, 2002).

In addition to pharmaceuticals, the metabolites of PKS-NRPS hybrids have many different bioactivities. For instance, Avirulence Conferring Enzyme1 (Ace1) of *Magnaporthe oryzae* is an avirulence (AVR) gene. Ace1 expresses at the initiation of penetration by appressorium and *M. oryzae* which carries Ace1 gene is not able to infect rice cultivars that have corresponding R gene, Pi33 (Böhnert et al., 2004). *M.*

oryzae has the largest number of PKS-NRPS hybrids among all the other ascomycetes. According to the bioinformatics analysis, 9 PKS-NRPS hybrids were revealed in the genome of *M. oryzae*. In these 9 genes, Ace1 and Syn2 are belongs to an infection-specific gene cluster that regulates the early stage of infection (Fudal, 2007) and only express *in vivo* (Collemare, 2008a). In addition, there were many mycotoxins or other bioactive compounds which synthesized by PKS-NRPS hybrids were identified, such as an antiviral compound, equisetin which synthesized by EqiS of *Fusarium heterosporum*; fusarin C that requires FusS of *Fusarium verticillioides*; TenS which involves in the biosynthesis of tenellin in *Beauveria bassiana* as well as ApdA and PsoA which were currently identified in *Aspergillus nidulans* and *Aspergillus fumigatus* that are responsible for the biosynthesis of aspyridone and pseurotin (Song et al., 2004; Sims et al., 2005; Bergmann et al., 2007; Eley et al., 2007; Maiya et al., 2007; Halo et al., 2008).

1.3 Mycotoxin studies of *Alternaria brassicicola*

Alternaria brassicicola is a plant pathogen that infects many species of Brassica which has been used as a model system for studying the interaction between necrotrophic pathogen with *Arabidopsis thaliana* (van Wees et al., 2003; Mukherjee et al., 2010; Pochon et al., 2012). *Alternaria* species produce a large amount of secondary metabolites which were proved to have diverse activities including many



toxic compounds (Fleck et al., 2012) (Appendix 2). The *Alternaria* mycotoxins can be approximately classified according to their structures: the tetramic acid derivative, tenuazonic acid (TA); the dibenzopyrone derivatives, alternariol (AOH), alternariol-9-methyl ether (AME) and altenuene (ALT); and the perylene derivatives, the altertoxins (ATXs) (Bottalico and Logrieco, 1998). In addition, several mycotoxins have been published individually of studying how they involve in the pathogenesis of *A. brassicicola*, including brassicenes, depudecin, and brassicicolin A (Hashimoto et al., 2009; Pedras et al., 2009; Wight et al., 2009). Among all the mycotoxins produced by *Alternaria* species, AOH, AME and TA were discovered earlier than others as well as the more literatures. AOH and AME are known to be cytotoxic to bacteria and mammalian cells, involving in the pathogenesis of *Alternaria* species, and also are suspected to be carcinogenic (Pero et al., 1973; Scott, 2001). The biosynthetic genes of AOH and AME have been recently identified in *A. alternata* (Saha et al., 2012). Nevertheless, the insufficiency of knowledge concerning the biosynthesis of TA inspired us to give some thought in it.

1.4 Tenuazonic acid

Tenuazonic acid (TA) was first isolated from culture filtrate of *Alternaria tenuis* (syn. *A. alternata*) in 1957 (Rosett, 1957). It was further discovered in other *Alternaria* species, *Magnaporthe oryzae*, *Phoma sorghina* and *Aspergillus*

bertholletius. (Iwasaki, 1972; Meronuck, 1972; Steyn, 1976; Taniwaki et al., 2012). TA is a non-host specific toxin (non-host selective toxin) and was found having antitumor, antibacterial and cytotoxic activities (Gitterman, 1965; Griffin, 1983; Gallardo, 2004). The toxicity of TA has been reviewed in plants, in chicken embryos, mice and several other animals (Shigeura, 1963; Griffin, 1983; Bennett, 2003; Aver'yanov, 2007; Zhou, 2008; Zhao, 2011). Additionally, on the basis of study in *Chlamydomonas reinhardtii*, TA has also been characterized as a novel type photosystem II inhibitor which can block the electro transferring chain between Q_A and Q_B site by inducing chloroplastic oxidative burst (Chen, 2007, 2008, 2010). Results obtained from the isotope feeding test, TA has been proven as a tetramic acid which composed of *L*-isoleucine and acetoacetate (Harris, 1965; Ebbole, 2007) (Appendix 1). Based on the literatures, TA is light sensitive compound which becomes stable while incorporating with metal ion such as iron, copper, or magnesium (Joshi et al., 1984; Lebrun and Boucly, 1985). Moreover, TA has tautomerization which increases the difficulty for analyzing (Lebrun and Boucly, 1985; Shephard et al., 1991). Although TA has been isolated for decades, our knowledge of TA is currently limited, especially to the genetic regulation of its biosynthesis. Therefore, in this study, we aimed to identify the biosynthetic gene of TA in *A. brassicicola* to improve the learning of TA.

Chapter II Materials and methods

2.1 Fungal strain and growth conditions

Alternaria brassicicola wild type strain and *A. alternata* wild type strain were used in this study. These strains are routinely maintained on potato dextrose agar (PDA) plate at 26°C and subcultured weekly.

2.2 Bioinformatics

2.2.1 Characterization of PKS/NRPS genes in *A. brassicicola*

To characterize PKS/NRPS genes in *A. brassicicola*, we used published PKS/NRPS genes of *Magnaporthe oryzae*, *Aspergillus flavus*, *A. nidulans*, *Alternaria alternata*, *Fusarium heterosporum* and *Penicillium expansum* as query to search and to identify possible candidate genes in *Alternaria brassicicola* database (DOE Joint Genome Institute, JGI). These candidate genes were applied into NCBI, SMART, NRPSpredictor2 and PKS/NRPS analysis database to predict their functional domains (Roettig et al., 2011).

2.2.2 Alignment

Mega version 5.20 (Tamura et al., 2011) was used to conduct amino acid sequence alignment of known PKS/NRPS genes and candidate genes of *A. brassicicola*, and then to distinguish genes with the potential in using the precursor of tenuazonic acid from all candidates following the analysis of phylogenetic tree.

2.3 Induction of toxin production in *A. brassicicola*

Conidia were harvested from 1-week-old culture with sterile water, filtered through two-layer Miracloth then inoculated into flask with 100 ml modified Czapek-Dox liquid medium (KH_2PO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, NaCl 0.25 g/L, KCl 0.25 g/L, yeast extract 1 g/L, NH_4Cl 0.06 g/L, $NaNO_3$ 0.25 g/L, $FeSO_4 \cdot 7H_2O$ or $CuSO_4 \cdot 7H_2O$ 0.01 g/L, $ZnSO_4 \cdot 7H_2O$ 0.01 g/L, glucose 10 g/L or NaOAc anhydrous 13.7 g/L as carbon source, pH 5.5, autoclave 20 min at 121°C, 15 psi) in a concentration of 850 conidia per ml; and were kept incubating at static and dark condition at 28°C for 7-14 days (Brzonkalik, 2011).

2.4 RNA extraction and reverse transcription

To monitor the change of the target gene expression level between induction and non-induction culture, the cultures were made a collection of different time buckets; then fungal hypha was preserved in -80°C before RNA extraction and culture filtrate was kept in 4°C before tenuazonic acid assay. RNA was prepared by Trizol total RNA isolation reagent (Invitrogen). To remove DNA contamination, RNA was treated with TURBO DNA-free™ kit (Ambion). RNA was applied to reverse transcription by SuperScript™ III Reverse Transcriptase (Invitrogen) with oligo-dT primers in order to get full length cDNA for heterologous expression and the cDNA that used in real-time PCR analysis were prepared by High Capacity cDNA Reverse Transcription Kit (Invitrogen) with random primers.

2.5 Real-time PCR

Real-time PCR was performed using ABI Step One machine and KAPA SYBR FAST qPCR kit. The total 25 μl PCR mixture contains 12.5 μl 2x SYBR Green, 5.5 μl ddH_2O, 1 μl forward primer (2.5 μM), 1 μl reverse primer (2.5 μM) and 5 μl cDNA (1 ng/μl). The primer pairs used in this study are listed in Table 2.

2.6 Tenuazonic acid assay

2.6.1 Chemical analysis (TLC/HPLC)

To conduct chemical analysis, the culture filtrate was first acidified with thousandth volume of 10 M HCl and extracted twice with an equal volume of ethyl acetate (EtOAc). The mixture was vortexed vigorously, then centrifuged at 4600 g for 5 min. EtOAc supernatants from both extraction steps were collected, combined together then evaporated by rotary evaporator at 40°C water bath. The dried pellet was measured and re-dissolved in methanol (Merck, HPLC grade) (Brzonkalik, 2011). For each sample, original 0.01 g extracts will be re-dissolved in 0.2 ml methanol. On TLC analysis, 10 μl samples were applied on TLC plate which coating with silica gel (Merck) and the plate was developed with chloroform: methanol (9:1) or hexane: acetone (3:1). After extension, TLC plate was evaporated in an iodine vapour chamber for detection. The result was further distinguished in comparison of its Rf value with standard compound (tenuazonic acid-copper salt, Apollo). For HPLC

analysis, TA standard solution of 200 ppm in methanol was used for calibration. The analysis was performed with HPLC device (Hitachi L-7100, Tokyo, Japan) equipped with a reverse phase column (Mightysil, RP-18, 5 μ, 4.6×250 mm, KANTO Chemical Co., Inc.). Mobile phase solution was 0.3% $ZnSO_4$ in 10% MeOH at a flow rate of 1 ml/min. TA was monitored with an UV detector (Hitachi L-7400, Tokyo, Japan) at 277 nm. For each sample, a 2.5 μl of solution was injected and the running time was 30 min.

2.6.2 Bioassay

Paenibacillus larvae subsp. *larvae* type strain which obtained from FIRDI (BCRC number 14187) was used in this assay. This bacterial strain is maintained on brain-heart infusion agar (BHIA) medium at 37°C. The antibiotic activity of tenuazonic acid was observed by agar diffusion assay. First, 18 hr liquid-cultured bacteria were spread on BHIA plate through cotton swab to create a bacterial lawn. Next, 1 ml culture filtrate was condensed by vacuum evaporator, re-dissolved in 10 μl sterile ddH_2O, and applied on circular filter paper (0.6 cm in diameter). The filter paper was putted onto the bacterial lawn after air-dried completely. The restriction ring will be determined at the following day (Gallardo, 2004).

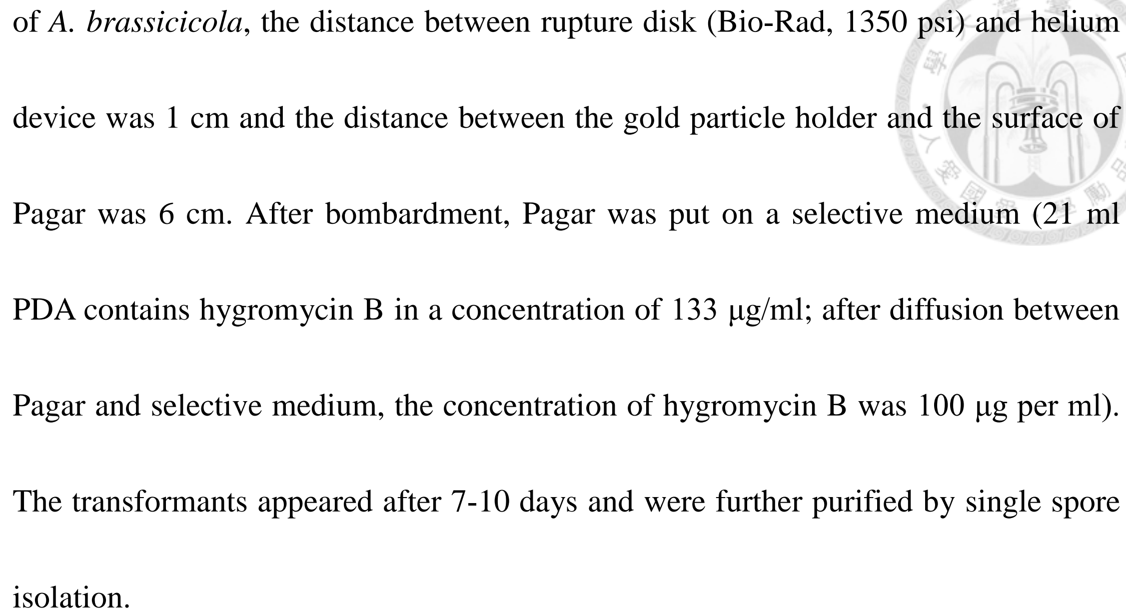
2.7 Construction of gene disruption mutant

2.7.1 Generation of a disruption plasmid pBSAB1

To generate disruption plasmid pBSAB1, pBlueScript SK+ (pBSK+) was used for further modification. The pBSK+ was modified by adding a 1.4 kb hygromycin resistant cassette which derived from pCB1004 to generate pBS-HYG. Then pBS-HYG was excised by double digestion with ClaI and SacI, and pBSAB1 was ultimately accomplished by the insertion of a 1.3 kb partial homologous fragment of AB04556.1 gene from *A. brassicicola*. M13F and M13R primer pairs were used to amplify linear minimal element (LME) for following transformation. (Cho et al., 2006)

2.7.2 Biolistic transformation

Biolistic transformation of conidia of *A. brassicicola* was performed with a helium-driven device (Trail and Köller, 1993; Yao, 1995). *A. brassicicola* conidia were harvested from 1-week-old culture on PDA plate. The conidia suspension was adjusted to a concentration of $1.4*10^7$ conidia per ml. Then 0.2 ml conidia suspension was spread on "Pagar" (a 9 cm Whatman No.4 filter paper coating with 7 ml PDA) 15 min before used by sterile glass spreader. To manipulate biolistic transformation, 0.6 micron gold particles coated with DNA were prepared for use (1 μg DNA for each shot, and 0.6 mg gold particle for each μg of DNA). To deliver DNA into the conidia

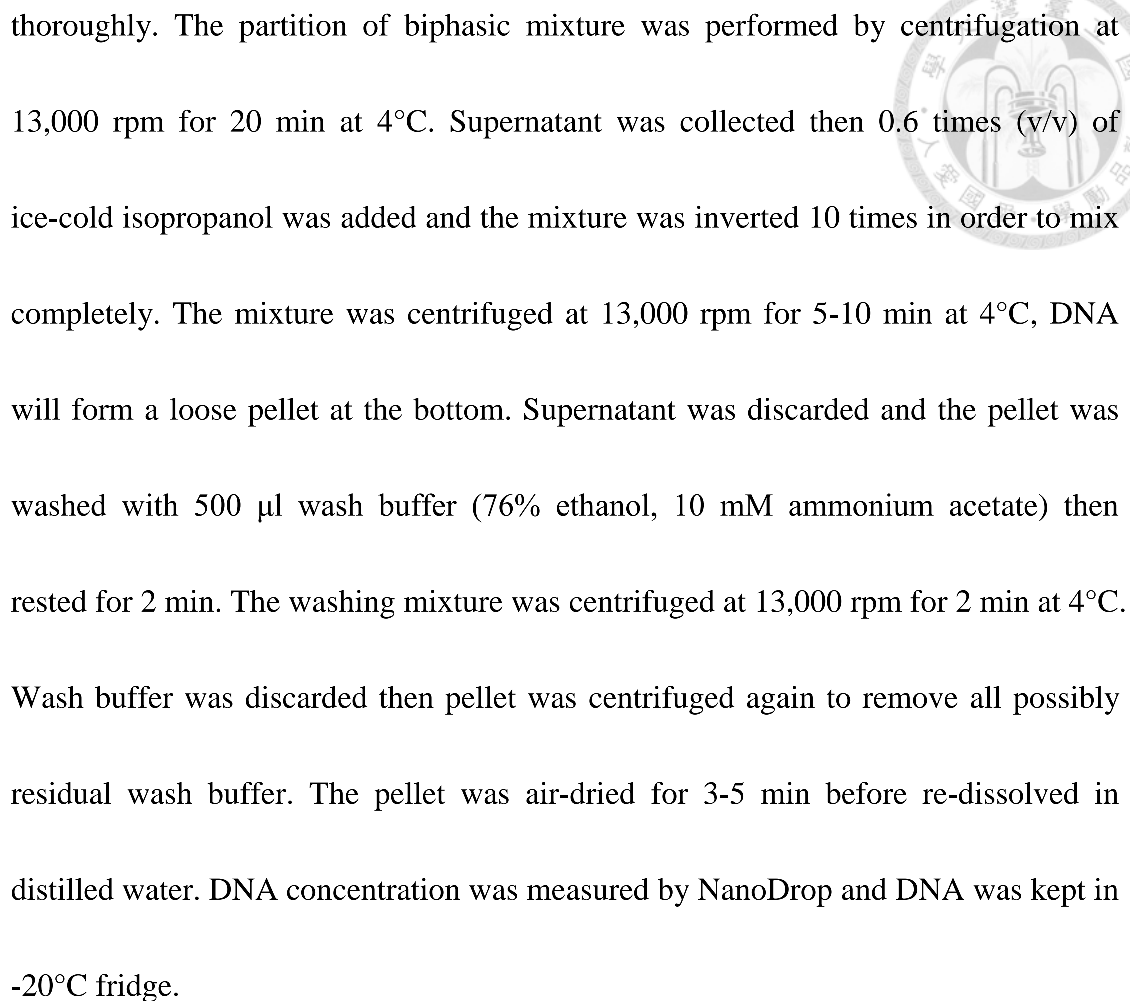


of *A. brassicicola*, the distance between rupture disk (Bio-Rad, 1350 psi) and helium device was 1 cm and the distance between the gold particle holder and the surface of Pagar was 6 cm. After bombardment, Pagar was put on a selective medium (21 ml PDA contains hygromycin B in a concentration of 133 μg/ml; after diffusion between Pagar and selective medium, the concentration of hygromycin B was 100 μg per ml). The transformants appeared after 7-10 days and were further purified by single spore isolation.

2.8 Southern blot

2.8.1 Genomic DNA preparation

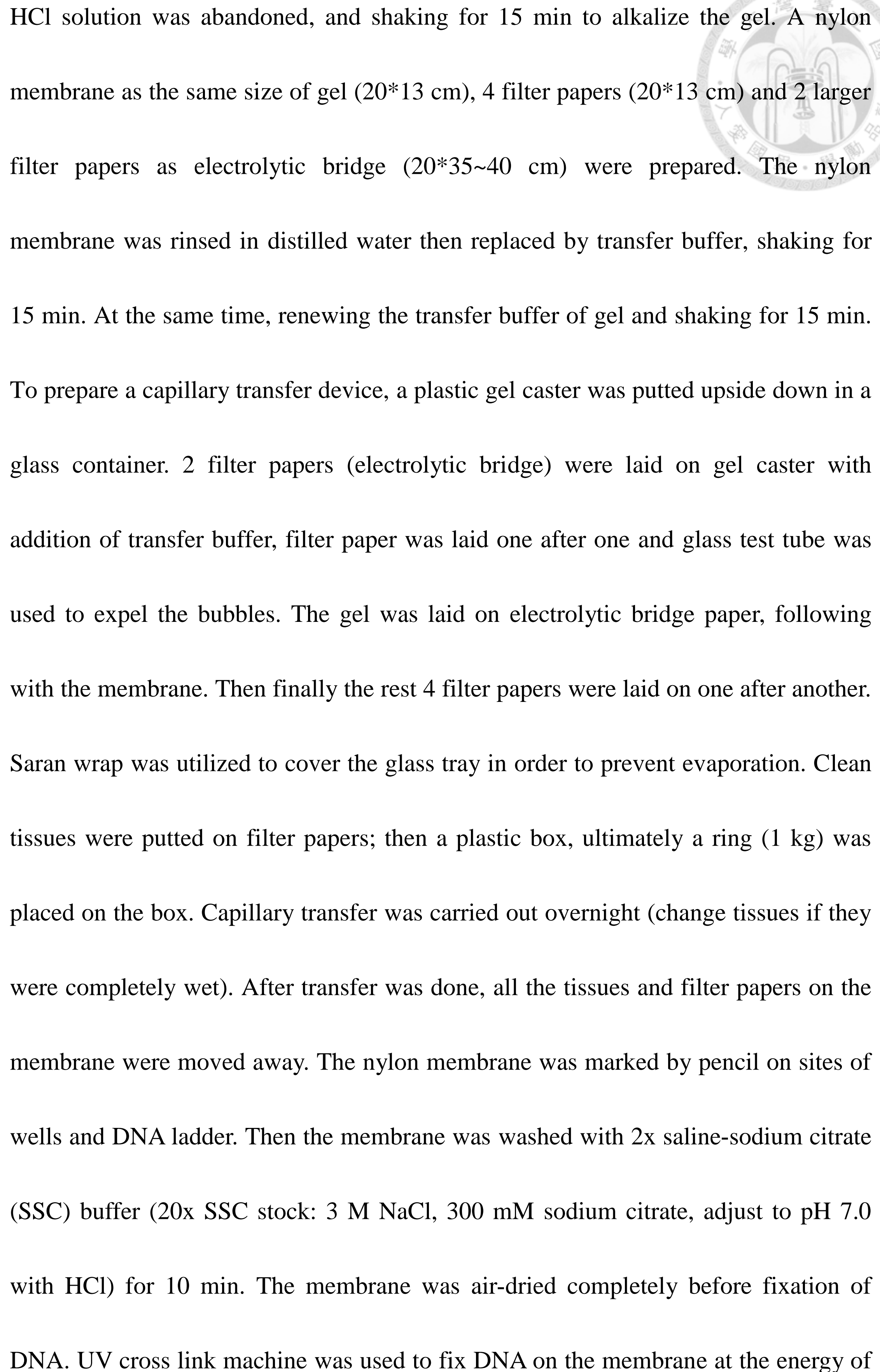
The hyphal tissues were inoculated in 125 ml flask with 50 ml PDB (potato dextrose broth); incubated at 28°C, 125 rpm shaking for 3 days. The hyphal tissues were harvested then kept it in -80°C fridge for temporary preservation. To extract genomic DNA, 100-200 mg hyphal tissue was homogenized by liquid nitrogen with mortar and pestle. 500 μl 65°C pre-heated CTAB buffer was added (2% CTAB, 1.4 M sodium chloride, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 2% PVP-40) immediately into homogenized hyphal tissue, then β-mercaptoethanol was added to a final concentration of 2%. The mixture was incubated in a 65°C water bath for 30 min and inverted several times at each 10 min. After incubation at 65°C, the mixture was inverted 10 times following by adding 500 μl phenol: chloroform (1:1) till mixed



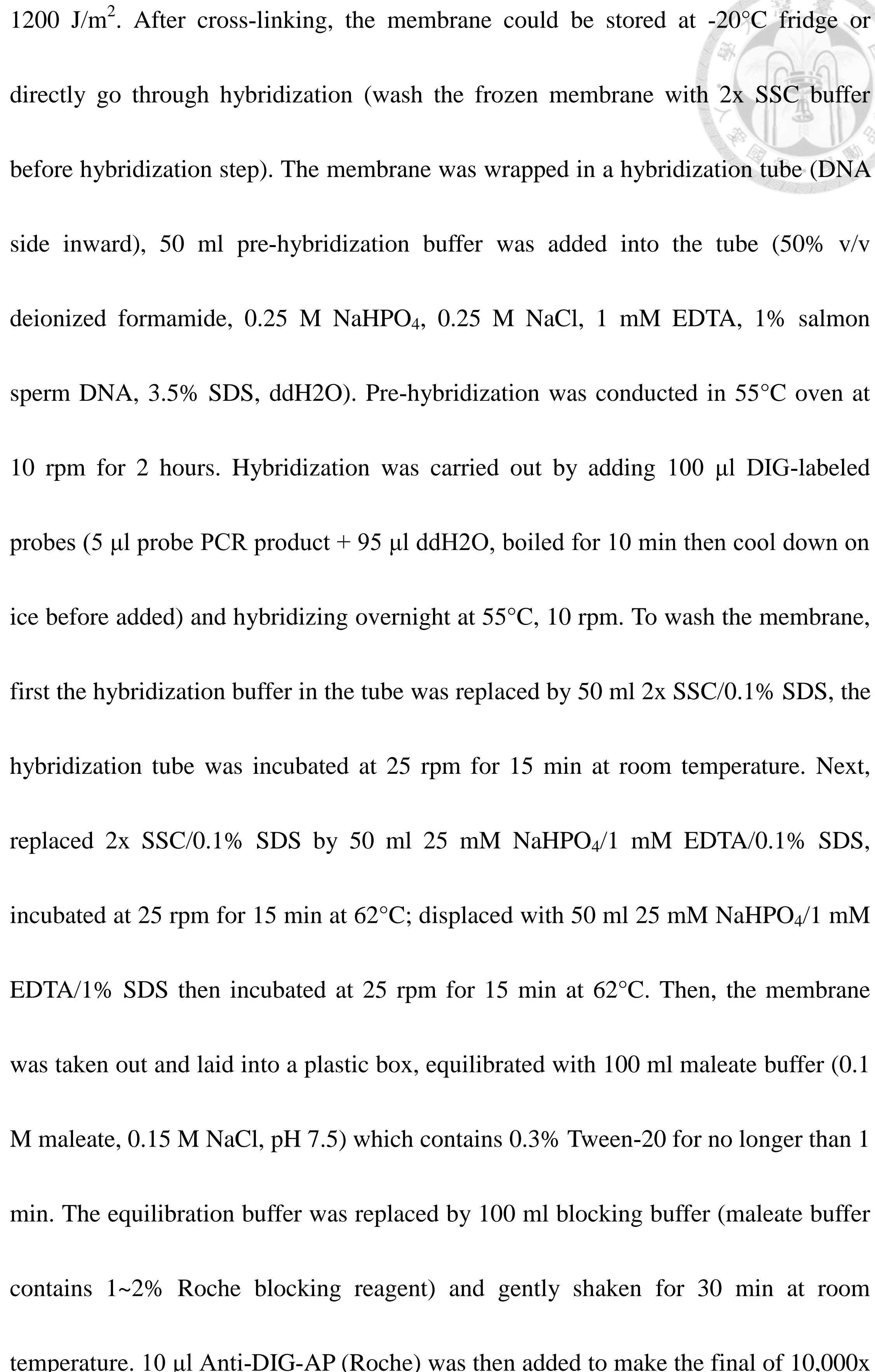
thoroughly. The partition of biphasic mixture was performed by centrifugation at 13,000 rpm for 20 min at 4°C. Supernatant was collected then 0.6 times (v/v) of ice-cold isopropanol was added and the mixture was inverted 10 times in order to mix completely. The mixture was centrifuged at 13,000 rpm for 5-10 min at 4°C, DNA will form a loose pellet at the bottom. Supernatant was discarded and the pellet was washed with 500 μl wash buffer (76% ethanol, 10 mM ammonium acetate) then rested for 2 min. The washing mixture was centrifuged at 13,000 rpm for 2 min at 4°C. Wash buffer was discarded then pellet was centrifuged again to remove all possibly residual wash buffer. The pellet was air-dried for 3-5 min before re-dissolved in distilled water. DNA concentration was measured by NanoDrop and DNA was kept in -20°C fridge.

2.8.2 Southern blot

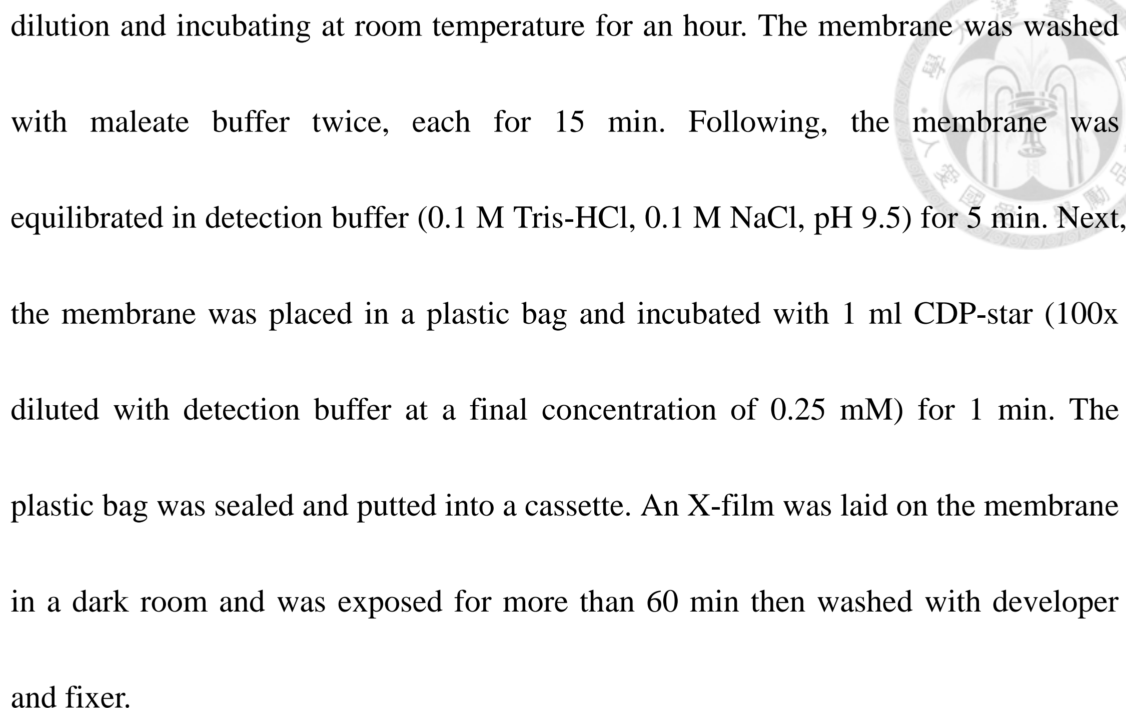
15-20 μg gDNA was digested with different restriction enzymes in a total volume of 30 μl for 16-20 hr. Electrophoresis was performed by running 0.8% agarose gel (20*13 cm) in 0.5x TBE buffer at 40 V for 17 hr. After electrophoresis, the gel was stained by fresh-prepared ethidium bromide (EtBr) for 30 min. The stained gel was checked on UV box and marked on DNA ladder. The gel was transferred into a clean plastic box and 0.25 N HCl was added to cover the gel, gently shaking for 10 min. Transfer buffer (0.4 M NaOH, 0.6 M NaCl) was added right after



HCl solution was abandoned, and shaking for 15 min to alkalize the gel. A nylon membrane as the same size of gel (20*13 cm), 4 filter papers (20*13 cm) and 2 larger filter papers as electrolytic bridge (20*35~40 cm) were prepared. The nylon membrane was rinsed in distilled water then replaced by transfer buffer, shaking for 15 min. At the same time, renewing the transfer buffer of gel and shaking for 15 min. To prepare a capillary transfer device, a plastic gel caster was putted upside down in a glass container. 2 filter papers (electrolytic bridge) were laid on gel caster with addition of transfer buffer, filter paper was laid one after one and glass test tube was used to expel the bubbles. The gel was laid on electrolytic bridge paper, following with the membrane. Then finally the rest 4 filter papers were laid on one after another. Saran wrap was utilized to cover the glass tray in order to prevent evaporation. Clean tissues were putted on filter papers; then a plastic box, ultimately a ring (1 kg) was placed on the box. Capillary transfer was carried out overnight (change tissues if they were completely wet). After transfer was done, all the tissues and filter papers on the membrane were moved away. The nylon membrane was marked by pencil on sites of wells and DNA ladder. Then the membrane was washed with 2x saline-sodium citrate (SSC) buffer (20x SSC stock: 3 M NaCl, 300 mM sodium citrate, adjust to pH 7.0 with HCl) for 10 min. The membrane was air-dried completely before fixation of DNA. UV cross link machine was used to fix DNA on the membrane at the energy of



1200 J/m^2. After cross-linking, the membrane could be stored at -20°C fridge or directly go through hybridization (wash the frozen membrane with 2x SSC buffer before hybridization step). The membrane was wrapped in a hybridization tube (DNA side inward), 50 ml pre-hybridization buffer was added into the tube (50% v/v deionized formamide, 0.25 M $NaHPO_4$, 0.25 M NaCl, 1 mM EDTA, 1% salmon sperm DNA, 3.5% SDS, ddH2O). Pre-hybridization was conducted in 55°C oven at 10 rpm for 2 hours. Hybridization was carried out by adding 100 μl DIG-labeled probes (5 μl probe PCR product + 95 μl ddH2O, boiled for 10 min then cool down on ice before added) and hybridizing overnight at 55°C, 10 rpm. To wash the membrane, first the hybridization buffer in the tube was replaced by 50 ml 2x SSC/0.1% SDS, the hybridization tube was incubated at 25 rpm for 15 min at room temperature. Next, replaced 2x SSC/0.1% SDS by 50 ml 25 mM $NaHPO_4$/1 mM EDTA/0.1% SDS, incubated at 25 rpm for 15 min at 62°C; displaced with 50 ml 25 mM $NaHPO_4$/1 mM EDTA/1% SDS then incubated at 25 rpm for 15 min at 62°C. Then, the membrane was taken out and laid into a plastic box, equilibrated with 100 ml maleate buffer (0.1 M maleate, 0.15 M NaCl, pH 7.5) which contains 0.3% Tween-20 for no longer than 1 min. The equilibration buffer was replaced by 100 ml blocking buffer (maleate buffer contains 1~2% Roche blocking reagent) and gently shaken for 30 min at room temperature. 10 μl Anti-DIG-AP (Roche) was then added to make the final of 10,000x



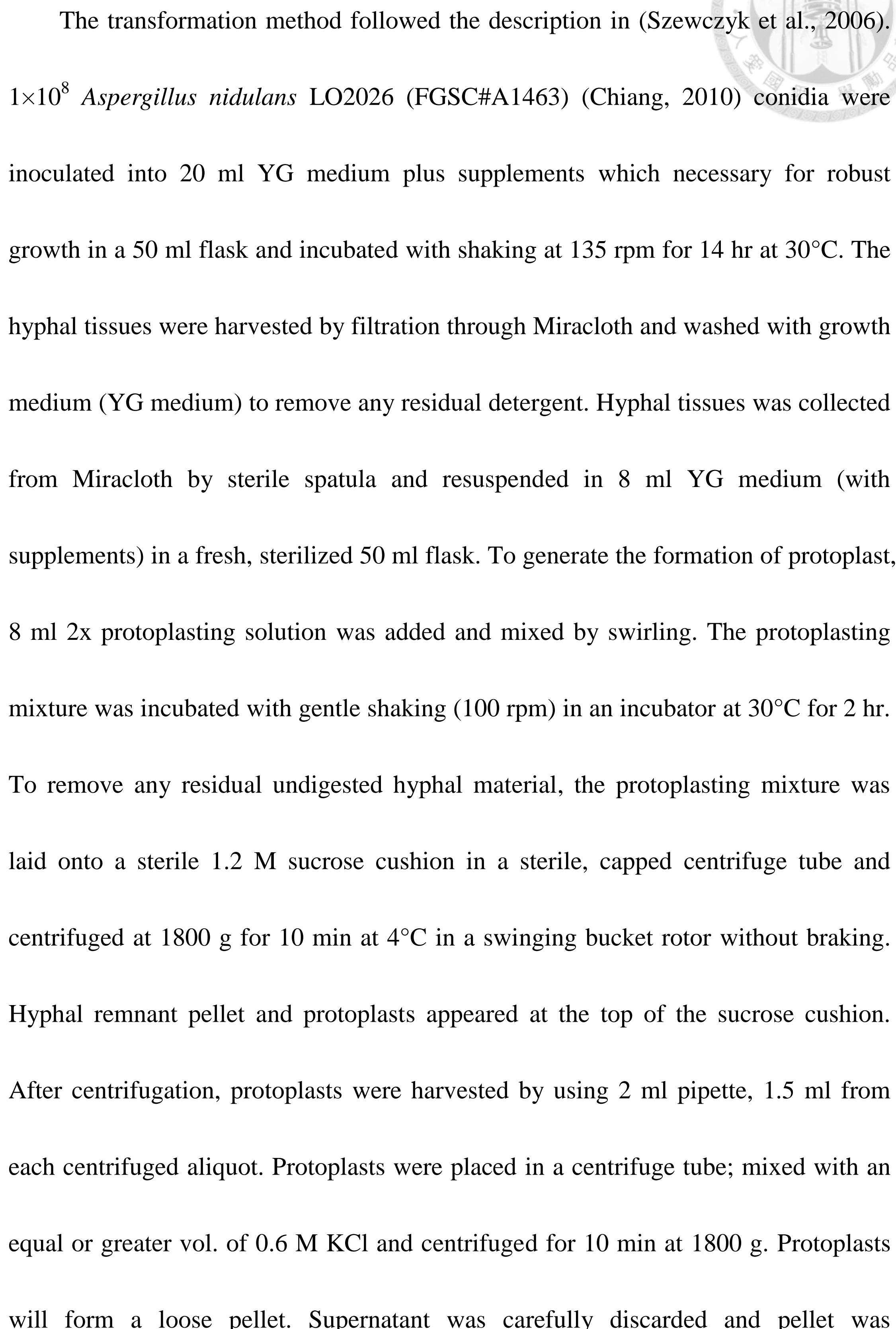
dilution and incubating at room temperature for an hour. The membrane was washed with maleate buffer twice, each for 15 min. Following, the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. Next, the membrane was placed in a plastic bag and incubated with 1 ml CDP-star (100x diluted with detection buffer at a final concentration of 0.25 mM) for 1 min. The plastic bag was sealed and putted into a cassette. An X-film was laid on the membrane in a dark room and was exposed for more than 60 min then washed with developer and fixer.

2.9 Heterologous expression

2.9.1 Construction of expression plasmid

Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) was used in this construct. First, AfpyroA-alcA fragment was cut from pGEMT-AfpyroA-alcA plasmid which was obtained from Wang's group with NotI (New England Biolabs, NEB). This fragment was treated with Antarctic phosphatase (AP, NEB) to dephosphorylate. Then AfpyroA-alcA fragment was cloned into TOPO plasmid. After the construction of pTOPO-AfpyroA-alcA, the direction of alcA promoter was checked before addition of the gene which is going to be expressed. pTOPO-AfpyroA-alcA was digested with SacI (NEB), inserted with AB04556 cDNA full length fragment which contains SacI site at both 5' and 3' end. Last, this finalized construct was used for heterologous expression.

2.9.2 Protoplast transformation

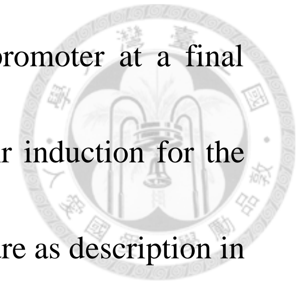


The transformation method followed the description in (Szewczyk et al., 2006). 1×10^8 *Aspergillus nidulans* LO2026 (FGSC#A1463) (Chiang, 2010) conidia were inoculated into 20 ml YG medium plus supplements which necessary for robust growth in a 50 ml flask and incubated with shaking at 135 rpm for 14 hr at 30°C. The hyphal tissues were harvested by filtration through Miracloth and washed with growth medium (YG medium) to remove any residual detergent. Hyphal tissues was collected from Miracloth by sterile spatula and resuspended in 8 ml YG medium (with supplements) in a fresh, sterilized 50 ml flask. To generate the formation of protoplast, 8 ml 2x protoplasting solution was added and mixed by swirling. The protoplasting mixture was incubated with gentle shaking (100 rpm) in an incubator at 30°C for 2 hr. To remove any residual undigested hyphal material, the protoplasting mixture was laid onto a sterile 1.2 M sucrose cushion in a sterile, capped centrifuge tube and centrifuged at 1800 g for 10 min at 4°C in a swinging bucket rotor without braking. Hyphal remnant pellet and protoplasts appeared at the top of the sucrose cushion. After centrifugation, protoplasts were harvested by using 2 ml pipette, 1.5 ml from each centrifuged aliquot. Protoplasts were placed in a centrifuge tube; mixed with an equal or greater vol. of 0.6 M KCl and centrifuged for 10 min at 1800 g. Protoplasts will form a loose pellet. Supernatant was carefully discarded and pellet was

resuspended in 2 ml 0.6 M KCl and transferred into two sterile 1.5 ml microcentrifuge tubes. To pellet the protoplasts, centrifuging at 2400 g for 3 min. Supernatant was removed and pellet was resuspended in 1 ml 0.6 M KCl, repeat 2 additional times. Pellet from each tube was resuspended in 0.5 ml 0.6 M KCl, 50 mM $CaCl_2$ and combined together in one tube. The protoplasts were pelleted by centrifuging at 2400 g for 3 min and resuspended in a vol. of 0.6 M KCl, 50 mM $CaCl_2$ appropriate for subsequent transformation (2×10^7 protoplasts can be divided into 10×100 μl aliquots). To 100 μl protoplast suspension, linearized expression plasmid in less than 15 μl TE buffer (better less than 10 μl) was added. The mixture was vortexed 6-8 times for 1 s at max speed following by adding 50 μl of RT, freshly filtered PEG solution. Then the mixture was vortexed 4-5 times for 1 s at max speed. Next, the mixture was placed in an ice water bath for 25 min. After ice water bath incubation, 1 ml RT filtered PEG solution was added and mixed by gently pipetting at least 10 times. The mixture was left at RT for 25 min follows was spread one tenth volume or greater on both selective plates and non-selective plates.

2.9.3 Induction of heterologous expression

To induce the heterologous expression, follow the procedures described in (Chiang, 2009). Conidia of expression transformant were inoculated into LMM liquid (lactose minimum medium) and incubated for 18 hr at 37°C, 200 rpm. After 18 hr



incubation, cyclopentanone was added as an inducer for alcA promoter at a final concentration of 30 mM. Culture filtrate was collected after 48 hr induction for the following chemical analysis. The procedures of chemical analysis are as description in tenuazonic acid assay (section 2.6).

2.10 Inoculation

The conidia of both *Alternaria brassicicola* wild type and mutants were harvested from 1-week-old PDA culture, suspended in distill water and adjusted to a concentration of $5*10^5$ conidia/ml. *Arabidopsis thaliana* Col-0 3-week-old cultivars were used for inoculation. Standard tenuazonic acid which dissolved in methanol, methanol and distill water were applied as control. Each treatment was using 5-10 μl suspensions dropped on leave. The treated leaves were further kept in moist chamber at 22°C. The symptoms appeared after 2 to 3 days and the results were evaluated 1 to 5 days post inoculation.

Chapter III Results

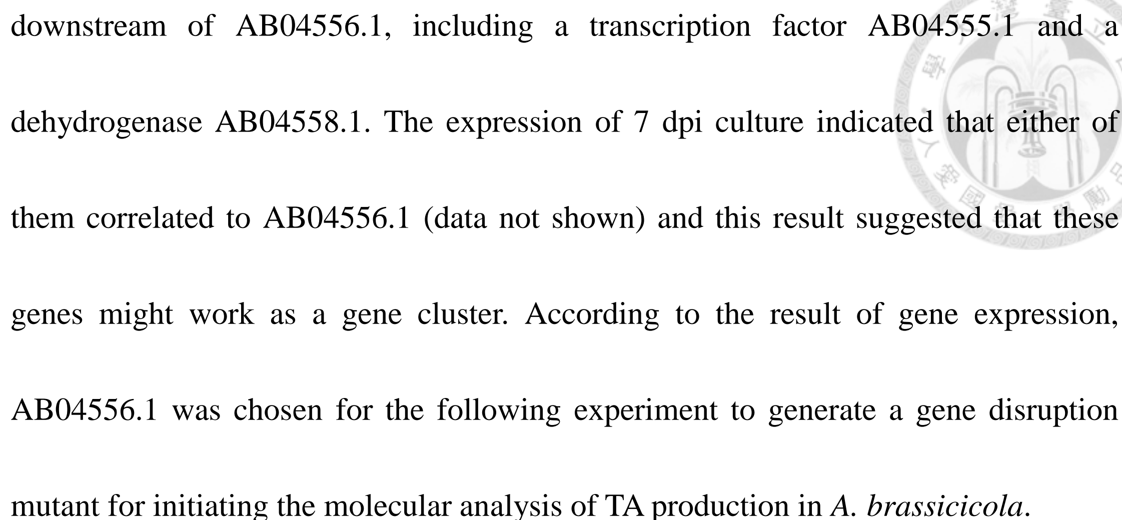
3.1 Bioinformatical analysis

Analysis of the genome revealed that *A. brassicicola* contains many secondary metabolic genes that including 11 PKSs, 6 NRPSs and 2 putative PKS-NRPS hybrids. Some of the PKSs differ from the well-known architectures that comprising only KS domain, partial KS domain, starting with DH or Chalon- and Stilben-Synthase domain instead of KS. To the NRPSs, 3 of them encode large repeated NRPS which consists of ca 5000-6000 amino acids. In this study we identified 2 putative PKS-NRPS hybrids from PKSs which are AB04556.1 gene and AB07780.1 gene. These 2 genes have been characterized as PKS in the genome database of *A. brassicicola* but after we applied these amino acid sequences into NCBI, SMART and NRPSpredictor2, we presumed that they have functional domains which can be found in NRPS but truncated. Both these 2 genes have no condensation domain and only AB04556.1 has incomplete adenylation domain. By virtue of phylogenic analysis with other published PKS-NRPS hybrids, we found that AB04556.1 was linked to known PKS-NRPS hybrids while AB07780.1 was more related to 2 PKSs of *A. brassicicola* (Fig. 1). We considered these two genes could be the candidate genes responding for the biosynthesis of TA whereas we presumed that AB04556.1 has higher potentiality. We conducted the analysis of gene expression level via real-time PCR to distinguish how

these two genes react under TA-induced and non-induced conditions.

3.2 TA induction and gene expression (real-time PCR)

According to reference, there are two cultural conditions for inducing and non-inducing TA production in *A. brassicicola* which is modified Czapek-Dox medium along with different carbon source, glucose and sodium acetate. We primarily used these two cultural conditions for testing TA-producing ability of the *A. brassicicola* strain in our lab. We also followed the reference to use *A. alternata* as control. After 3-7 days post inoculation, we collected the culture filtrate and then applied condensed filtrate for agar diffusion assay with *P. larvae* subsp. *larvae*. By measuring the restriction ring, both *A. brassicicola* and *A. alternata* produced TA during incubation with glucose but not with sodium acetate (Table 1). Throughout the agar diffusion assay, we discovered *A. brassicicola* presented higher TA-producing ability than *A. alternata* (data not shown). We harvested hyphal tissues from the culture of glucose and sodium acetate and the tissues were used for RNA extraction. We designed real-time PCR primer pairs at both PKS and NRPS domains to detect how 2 candidate genes behaved under TA-induced and non-induced conditions. As revealed by relative gene expression analysis, only AB04556.1 showed significant differentiation (Fig. 3) but AB07780.1 has not been induced while culturing with glucose (data not shown). Additionally, we examined two genes upstream and

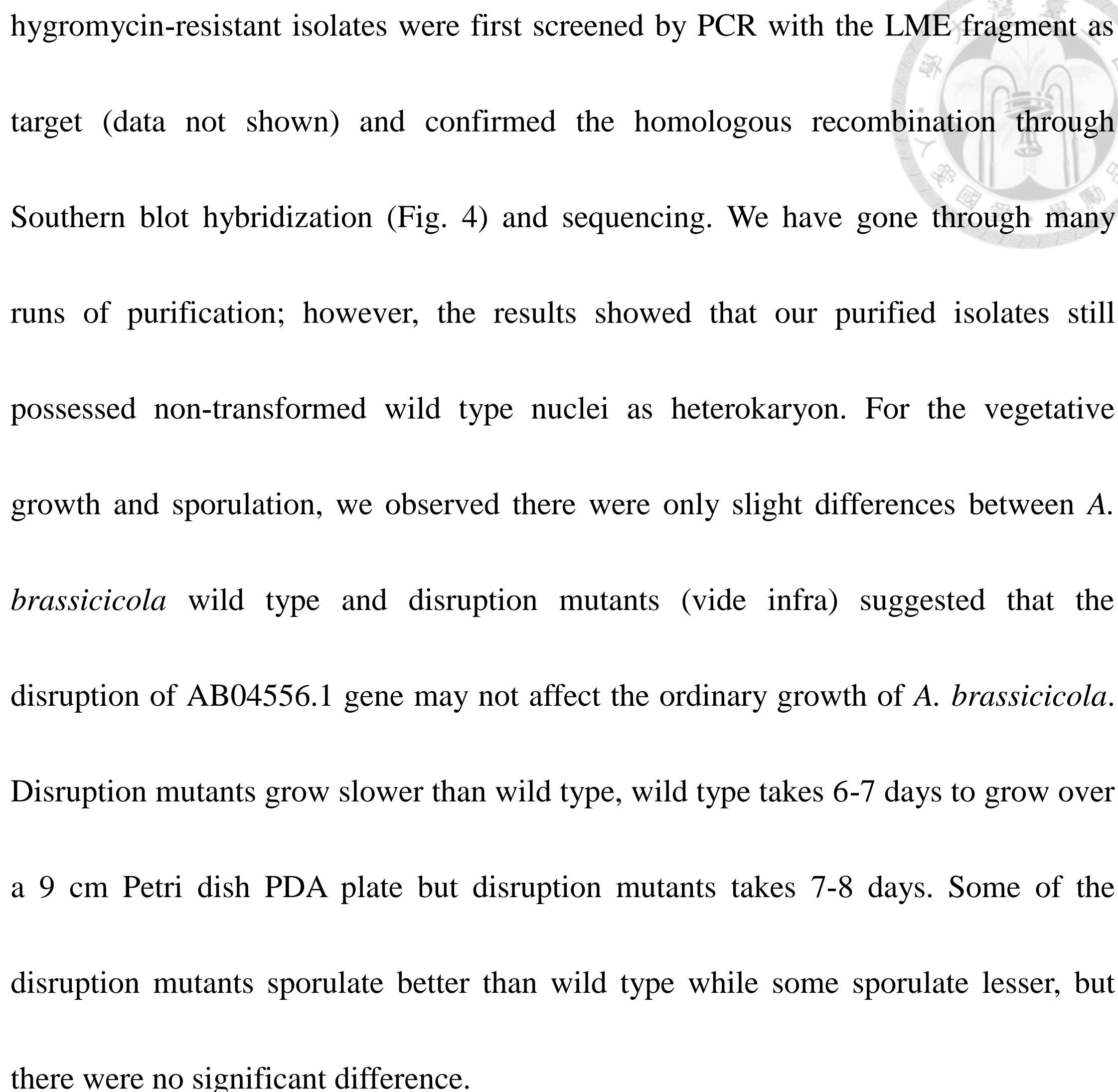


downstream of AB04556.1, including a transcription factor AB04555.1 and a dehydrogenase AB04558.1. The expression of 7 dpi culture indicated that either of them correlated to AB04556.1 (data not shown) and this result suggested that these genes might work as a gene cluster. According to the result of gene expression, AB04556.1 was chosen for the following experiment to generate a gene disruption mutant for initiating the molecular analysis of TA production in *A. brassicicola*.

3.3 Gene disruption mutants

3.3.1 Generation of mutants

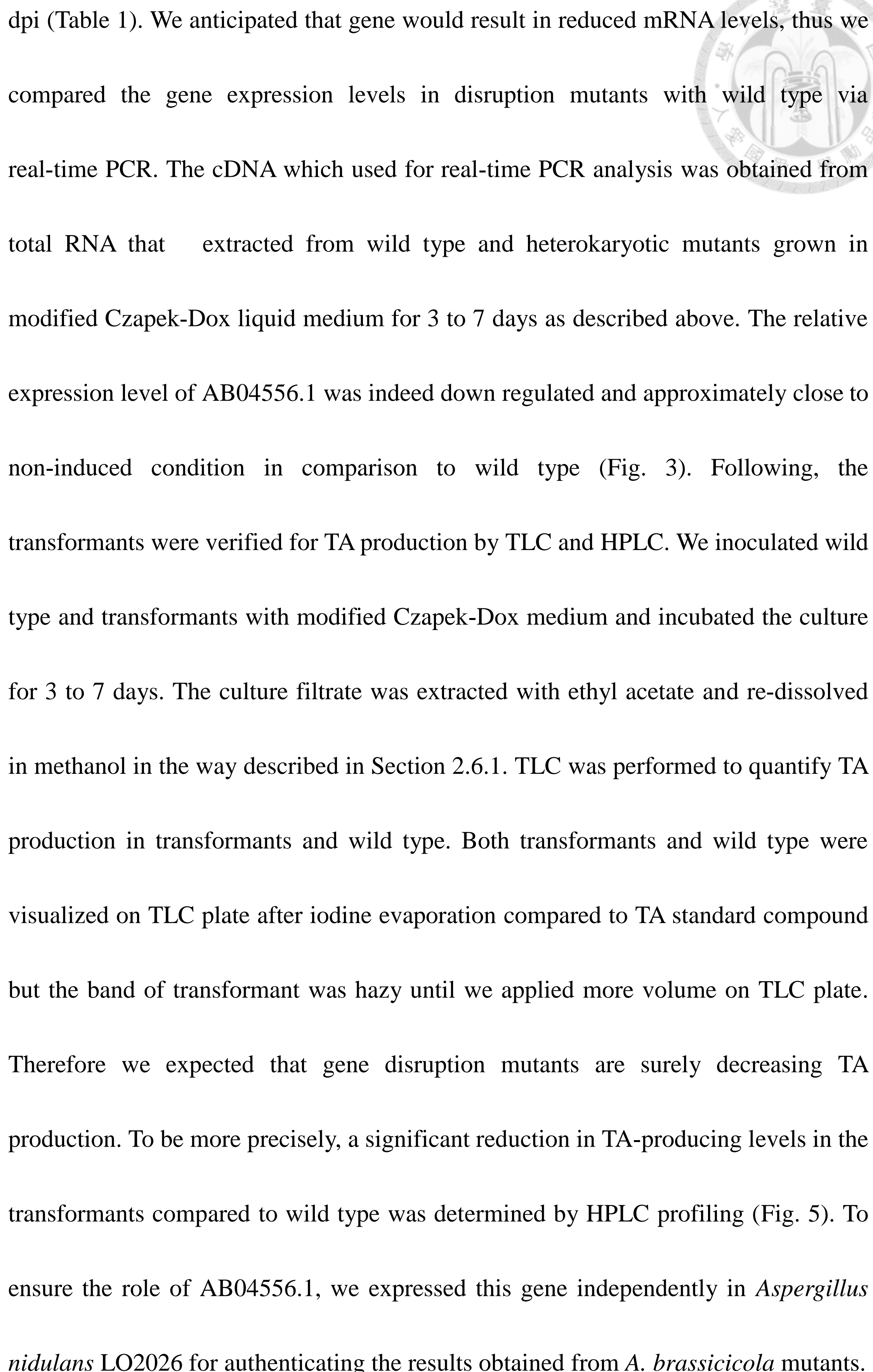
A 1.3 kb fragment located in 5’ of the coding region of AB04556.1 adjoined with a 1.4 kb hygromycin B resistant cassette was constructed in pBSK+ to build a gene disruption vector pBSAB1. Disruption of AB04556.1 in *A. brassicicola* genome employed the single crossing over strategy (Cho et al., 2006). In an attempt to transform protoplasts of *A. brassicicola* with PEG-mediated transformation was failed (Cary and Stovall, 1992; Chung et al., 2002; Cho et al., 2006). The biolistic-mediated transformation operates high velocity micro-particles to deliver nucleic acid into target cells and was used in many filamentous fungi (Armaleo et al., 1990; Lorito et al., 1993; Hilber et al., 1994). The transformation frequency of *A. brassicicola* in this study was 1 transformant per 5 μg of DNA per 10^7 conidia. We further purified the transformant by single spore isolation and hyphal tip isolation for multiple runs. All



hygromycin-resistant isolates were first screened by PCR with the LME fragment as target (data not shown) and confirmed the homologous recombination through Southern blot hybridization (Fig. 4) and sequencing. We have gone through many runs of purification; however, the results showed that our purified isolates still possessed non-transformed wild type nuclei as heterokaryon. For the vegetative growth and sporulation, we observed there were only slight differences between *A. brassicicola* wild type and disruption mutants (vide infra) suggested that the disruption of AB04556.1 gene may not affect the ordinary growth of *A. brassicicola*. Disruption mutants grow slower than wild type, wild type takes 6-7 days to grow over a 9 cm Petri dish PDA plate but disruption mutants takes 7-8 days. Some of the disruption mutants sporulate better than wild type while some sporulate lesser, but there were no significant difference.

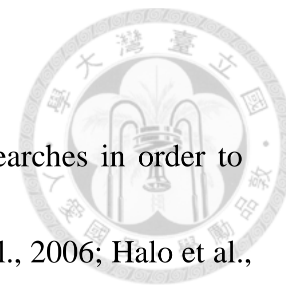
3.3.2 Analysis of TA biosynthesis through down-regulation

We tested many isolates of TA-producing ability by agar diffusion assay on *P. larvae* subsp. *larvae* and disruption mutants 2-3 and 6-3 which showed reduced production of TA during purification were chosen for further analyzing the TA biosynthesis in this study. We examined the antibiotic activity of the culture filtrate of these 2 purified isolates by measuring the restriction ring. For 3 dpi culture, both 2-3 and 6-3 showed meaningful loss of TA but accumulated TA nearly like wild type at 7

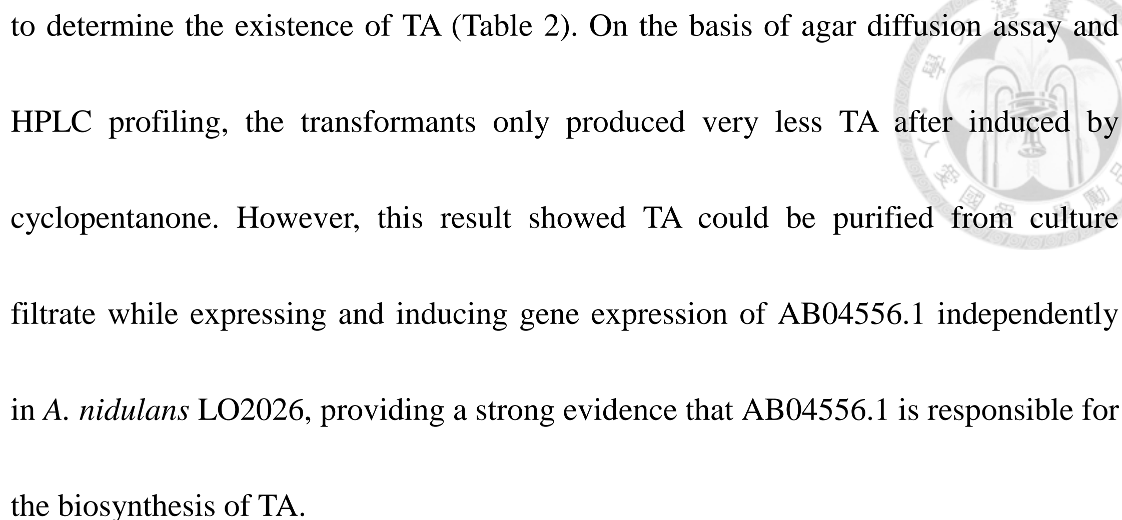


dpi (Table 1). We anticipated that gene would result in reduced mRNA levels, thus we compared the gene expression levels in disruption mutants with wild type via real-time PCR. The cDNA which used for real-time PCR analysis was obtained from total RNA that extracted from wild type and heterokaryotic mutants grown in modified Czapek-Dox liquid medium for 3 to 7 days as described above. The relative expression level of AB04556.1 was indeed down regulated and approximately close to non-induced condition in comparison to wild type (Fig. 3). Following, the transformants were verified for TA production by TLC and HPLC. We inoculated wild type and transformants with modified Czapek-Dox medium and incubated the culture for 3 to 7 days. The culture filtrate was extracted with ethyl acetate and re-dissolved in methanol in the way described in Section 2.6.1. TLC was performed to quantify TA production in transformants and wild type. Both transformants and wild type were visualized on TLC plate after iodine evaporation compared to TA standard compound but the band of transformant was hazy until we applied more volume on TLC plate. Therefore we expected that gene disruption mutants are surely decreasing TA production. To be more precisely, a significant reduction in TA-producing levels in the transformants compared to wild type was determined by HPLC profiling (Fig. 5). To ensure the role of AB04556.1, we expressed this gene independently in *Aspergillus nidulans* LO2026 for authenticating the results obtained from *A. brassicicola* mutants.

3.4 Heterologous expression in *Aspergillus nidulans* LO2026



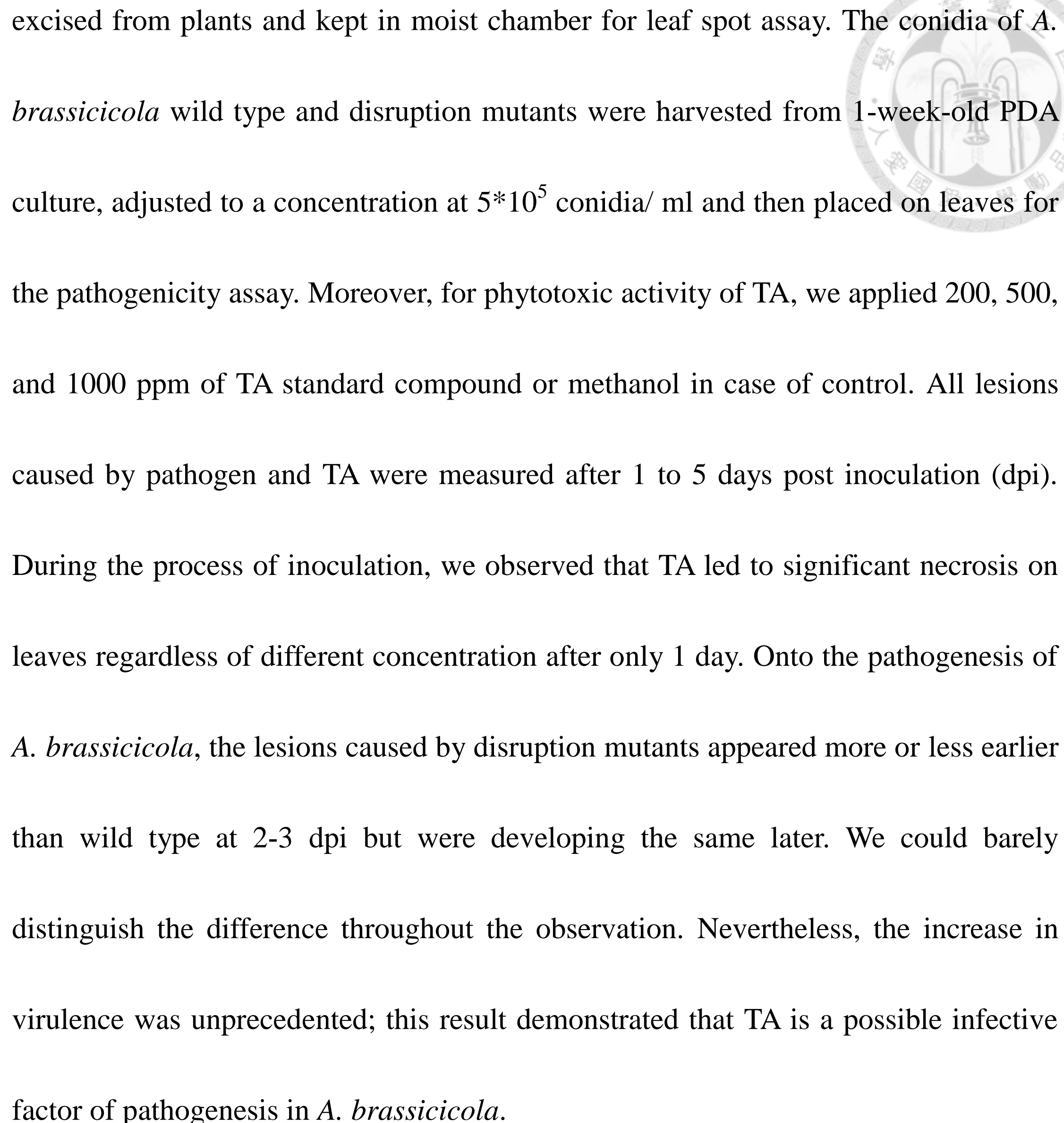
Heterologous expression system has been used in many researches in order to study gene function independently (Felenbok, 1991; Szewczyk et al., 2006; Halo et al., 2008; Liu, 2009a, b; Seshime, 2009; Chiang, 2010; Xu, 2010). The *A. nidulans* expression system has been used for studying the biosynthesis of asperfuranone (Chiang, 2009). In this study, *A. nidulans* LO2026 strain (pyrG89; pyroA4, nkuA::argB; riboB2, stcJ::AfriboB) was employed for the heterologous expression of AB04556.1 gene. We first amplified the cDNA full length from AB04556.1 and then transferred it to a derivative of TOPO vector constructed with selectable marker (AfpyroA) and inducible promoter (alcA) like described in Chapter II, section 2.9.1. Protoplasts of *A. nidulans* LO2026 were transformed through PEG-mediated transformation with the expression plasmid for expression trials; transformation with an empty vector which consists of only selectable marker and inducible promoter provided as control (Szewczyk et al., 2006). Selection of transformants was carried out on glucose minimal medium (GMM) lacking pyridoxine for several rounds, allowing the differentiation of transformants carry AfpyroA gene, 4 transformants were isolated and confirmed by PCR screening (data not shown). We then compared the metabolite profile of our transformants under normal and inducing conditions with HPLC (Fig. 6) and the culture filtrate was also applied to agar diffusion assay in order



to determine the existence of TA (Table 2). On the basis of agar diffusion assay and HPLC profiling, the transformants only produced very less TA after induced by cyclopentanone. However, this result showed TA could be purified from culture filtrate while expressing and inducing gene expression of AB04556.1 independently in *A. nidulans* LO2026, providing a strong evidence that AB04556.1 is responsible for the biosynthesis of TA.

3.5 Inoculation

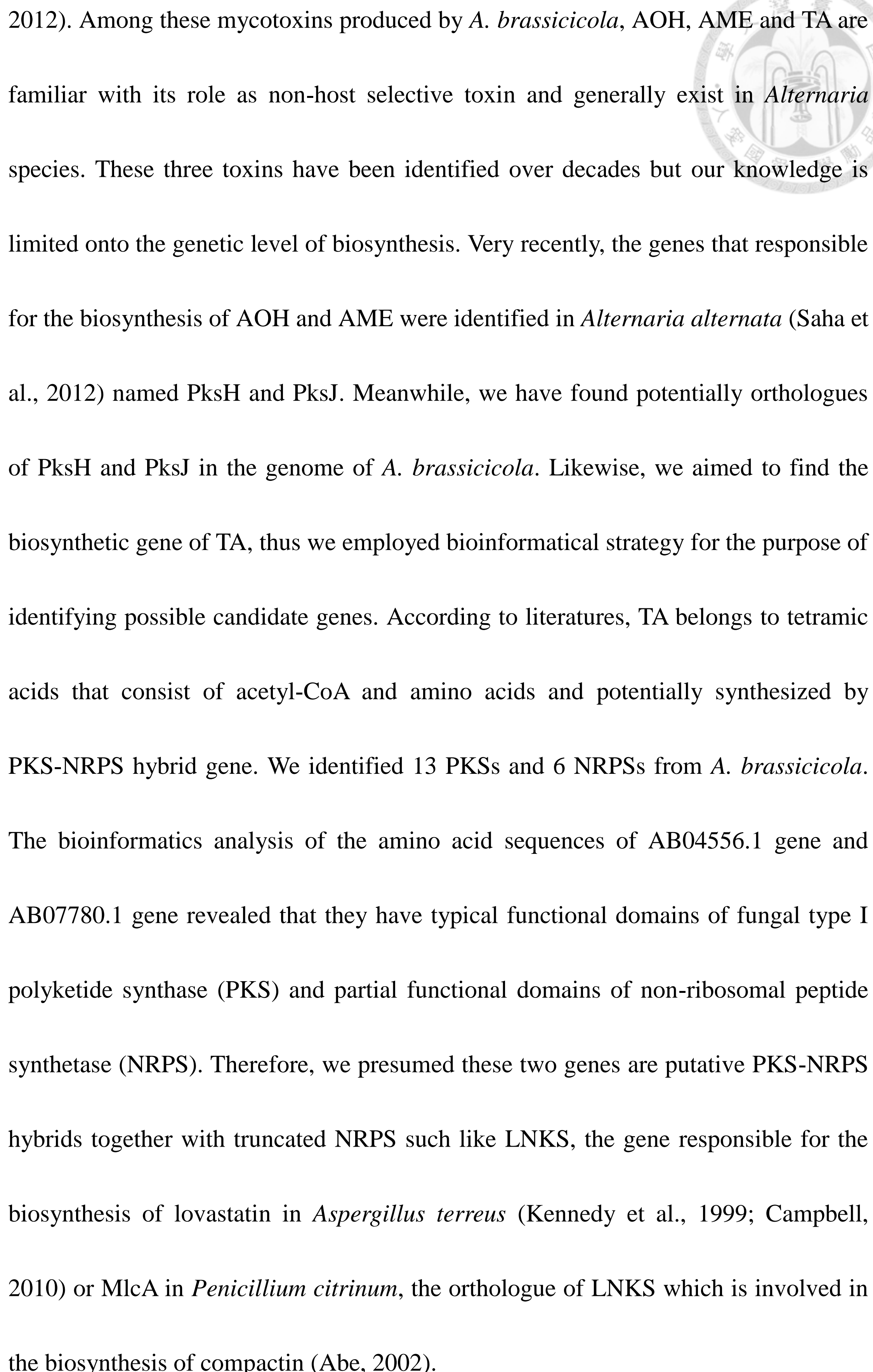
TA was found in many fungal species, especially in plant pathogenic fungi like *A. alternata, A. brassicicola and M. oryzae* (Iwasaki, 1972; Meronuck, 1972; Gallardo, 2004). In accordance with references, TA is a non-host selective toxin which targets to electron transferring chain between Q_A to Q_B site of the photosystem II (Chen, 2007, 2008, 2010; Zhao, 2011). To date, there was limited information concerning the role of TA during infection. In the pathogenesis study of hemi-biotrophic pathogen *M. oryzae*, TA has thought to be a elicitor-like mycotoxin that induced local disease resistance while inoculated with *M. oryzae* on susceptible rice cultivars (Aver'yanov, 2007). We performed pathogenicity assays with *A. brassicicola* wild type, disruption mutants along with TA standard compound in the interest of understanding the role of TA in necrotrophic plant pathogen. Towards the pathogenicity assays, the inoculation was carried out on 3-week-old *A. thaliana* Col-0 cultivars. The lower leaves were



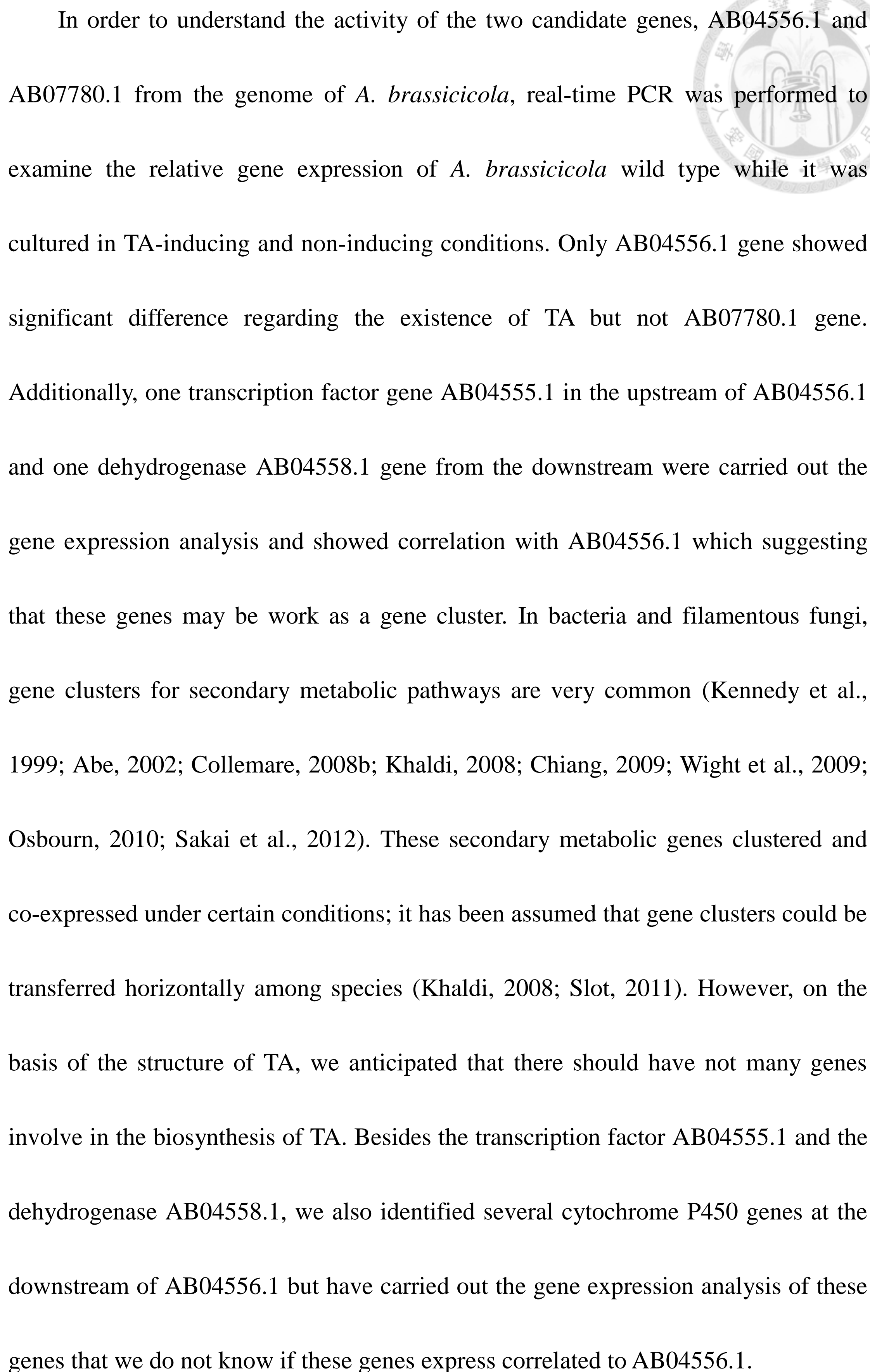
excised from plants and kept in moist chamber for leaf spot assay. The conidia of *A. brassicicola* wild type and disruption mutants were harvested from 1-week-old PDA culture, adjusted to a concentration at $5*10^5$ conidia/ ml and then placed on leaves for the pathogenicity assay. Moreover, for phytotoxic activity of TA, we applied 200, 500, and 1000 ppm of TA standard compound or methanol in case of control. All lesions caused by pathogen and TA were measured after 1 to 5 days post inoculation (dpi). During the process of inoculation, we observed that TA led to significant necrosis on leaves regardless of different concentration after only 1 day. Onto the pathogenesis of *A. brassicicola*, the lesions caused by disruption mutants appeared more or less earlier than wild type at 2-3 dpi but were developing the same later. We could barely distinguish the difference throughout the observation. Nevertheless, the increase in virulence was unprecedented; this result demonstrated that TA is a possible infective factor of pathogenesis in *A. brassicicola*.

Chapter IV Discussion

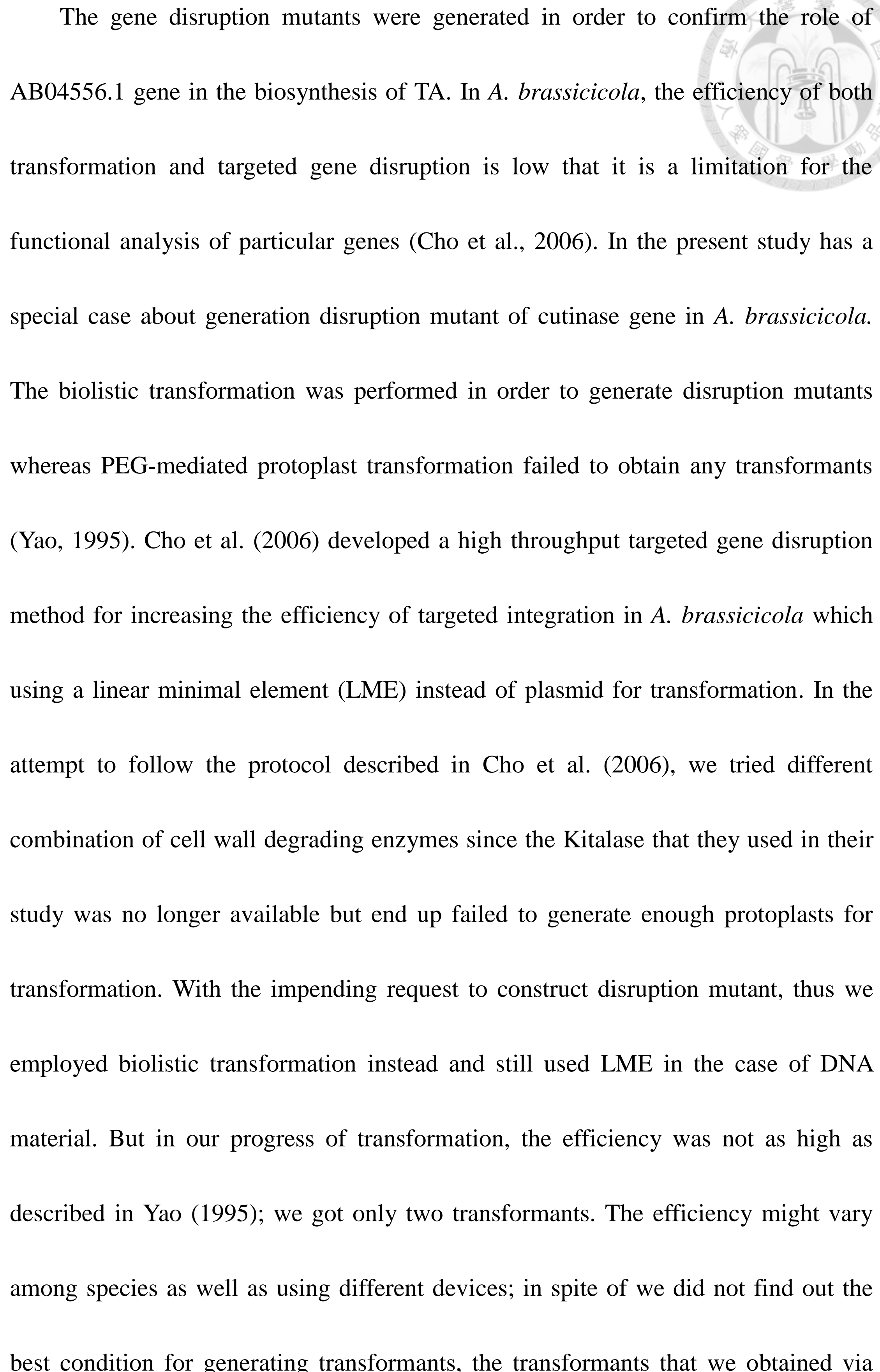
A. brassicicola is a competitive saprophyte and also a strong pathogenic fungus that responsible for black spot disease on Brassica species, causing great yield losses worldwide (Rotem, 1994). It is now the genomic tools are available for both *A. brassicicola* (Grigoriev et al., 2012) and corresponding hosts such as *Arabidopsis thaliana* (Cao et al., 2011), which provided a new foresight toward the research of the interaction between pathogen and host. The *in silico* analysis of the *A. brassicicola* genome has indicated a large number of genes that potentially involved in pathogenicity and some were further demonstrated their role in pathogenesis, for instance, transcription factors (Cho et al., 2009; Srivastava et al., 2012), phytotoxic metabolites (Hashimoto et al., 2009; Wight et al., 2009) and many other factors which are involved in such as hydrolytic enzyme, signal transduction, cell wall integrity and hyphal fusion (Cho et al., 2007; Kim et al., 2007; Craven et al., 2008; Cho et al., 2009; Kim et al., 2009). In addition to the pathogenicity factors described above, *A. brassicicola* produces various mycotoxins which are known to involve in pathogenesis but lack of molecular researches, including AB-toxin, altertoxins, alternariol (AOH), alternariol-9-methyl-ether (AME), tenuazonic acid (TA), altenuene (ALT), brassicenes, depudecin, and brassicicolin A (Oka et al., 2005; Hashimoto et al., 2009; Pedras et al., 2009; Wight et al., 2009; Fleck et al., 2012; Schwarz et al.,



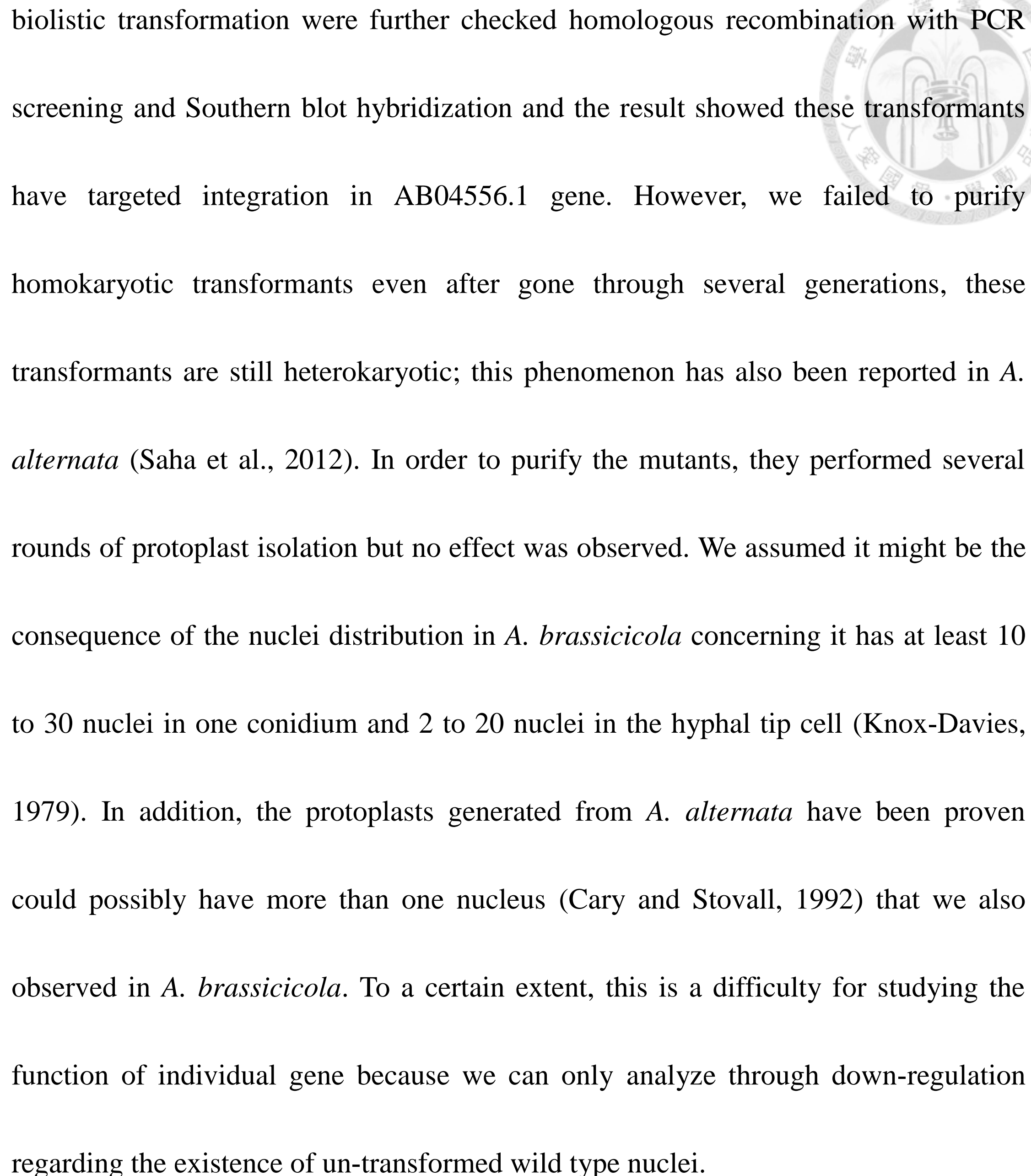
2012). Among these mycotoxins produced by *A. brassicicola*, AOH, AME and TA are familiar with its role as non-host selective toxin and generally exist in *Alternaria* species. These three toxins have been identified over decades but our knowledge is limited onto the genetic level of biosynthesis. Very recently, the genes that responsible for the biosynthesis of AOH and AME were identified in *Alternaria alternata* (Saha et al., 2012) named PksH and PksJ. Meanwhile, we have found potentially orthologues of PksH and PksJ in the genome of *A. brassicicola*. Likewise, we aimed to find the biosynthetic gene of TA, thus we employed bioinformatical strategy for the purpose of identifying possible candidate genes. According to literatures, TA belongs to tetramic acids that consist of acetyl-CoA and amino acids and potentially synthesized by PKS-NRPS hybrid gene. We identified 13 PKSs and 6 NRPSs from *A. brassicicola*. The bioinformatics analysis of the amino acid sequences of AB04556.1 gene and AB07780.1 gene revealed that they have typical functional domains of fungal type I polyketide synthase (PKS) and partial functional domains of non-ribosomal peptide synthetase (NRPS). Therefore, we presumed these two genes are putative PKS-NRPS hybrids together with truncated NRPS such like LNKS, the gene responsible for the biosynthesis of lovastatin in *Aspergillus terreus* (Kennedy et al., 1999; Campbell, 2010) or MlcA in *Penicillium citrinum*, the orthologue of LNKS which is involved in the biosynthesis of compactin (Abe, 2002).



In order to understand the activity of the two candidate genes, AB04556.1 and AB07780.1 from the genome of *A. brassicicola*, real-time PCR was performed to examine the relative gene expression of *A. brassicicola* wild type while it was cultured in TA-inducing and non-inducing conditions. Only AB04556.1 gene showed significant difference regarding the existence of TA but not AB07780.1 gene. Additionally, one transcription factor gene AB04555.1 in the upstream of AB04556.1 and one dehydrogenase AB04558.1 gene from the downstream were carried out the gene expression analysis and showed correlation with AB04556.1 which suggesting that these genes may be work as a gene cluster. In bacteria and filamentous fungi, gene clusters for secondary metabolic pathways are very common (Kennedy et al., 1999; Abe, 2002; Collemare, 2008b; Khaldi, 2008; Chiang, 2009; Wight et al., 2009; Osbourn, 2010; Sakai et al., 2012). These secondary metabolic genes clustered and co-expressed under certain conditions; it has been assumed that gene clusters could be transferred horizontally among species (Khaldi, 2008; Slot, 2011). However, on the basis of the structure of TA, we anticipated that there should have not many genes involve in the biosynthesis of TA. Besides the transcription factor AB04555.1 and the dehydrogenase AB04558.1, we also identified several cytochrome P450 genes at the downstream of AB04556.1 but have carried out the gene expression analysis of these genes that we do not know if these genes express correlated to AB04556.1.

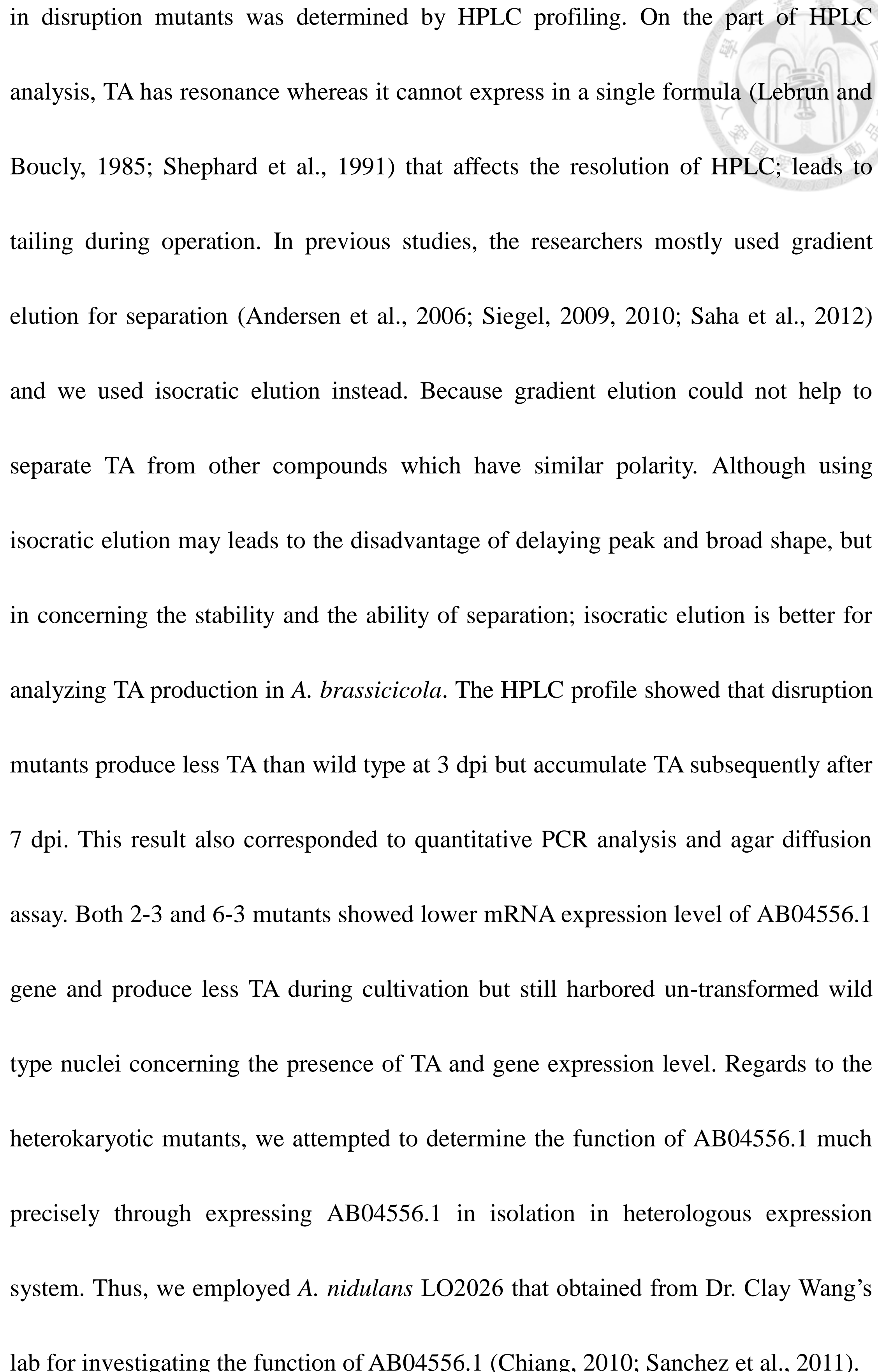


The gene disruption mutants were generated in order to confirm the role of AB04556.1 gene in the biosynthesis of TA. In *A. brassicicola*, the efficiency of both transformation and targeted gene disruption is low that it is a limitation for the functional analysis of particular genes (Cho et al., 2006). In the present study has a special case about generation disruption mutant of cutinase gene in *A. brassicicola.* The biolistic transformation was performed in order to generate disruption mutants whereas PEG-mediated protoplast transformation failed to obtain any transformants (Yao, 1995). Cho et al. (2006) developed a high throughput targeted gene disruption method for increasing the efficiency of targeted integration in *A. brassicicola* which using a linear minimal element (LME) instead of plasmid for transformation. In the attempt to follow the protocol described in Cho et al. (2006), we tried different combination of cell wall degrading enzymes since the Kitalase that they used in their study was no longer available but end up failed to generate enough protoplasts for transformation. With the impending request to construct disruption mutant, thus we employed biolistic transformation instead and still used LME in the case of DNA material. But in our progress of transformation, the efficiency was not as high as described in Yao (1995); we got only two transformants. The efficiency might vary among species as well as using different devices; in spite of we did not find out the best condition for generating transformants, the transformants that we obtained via

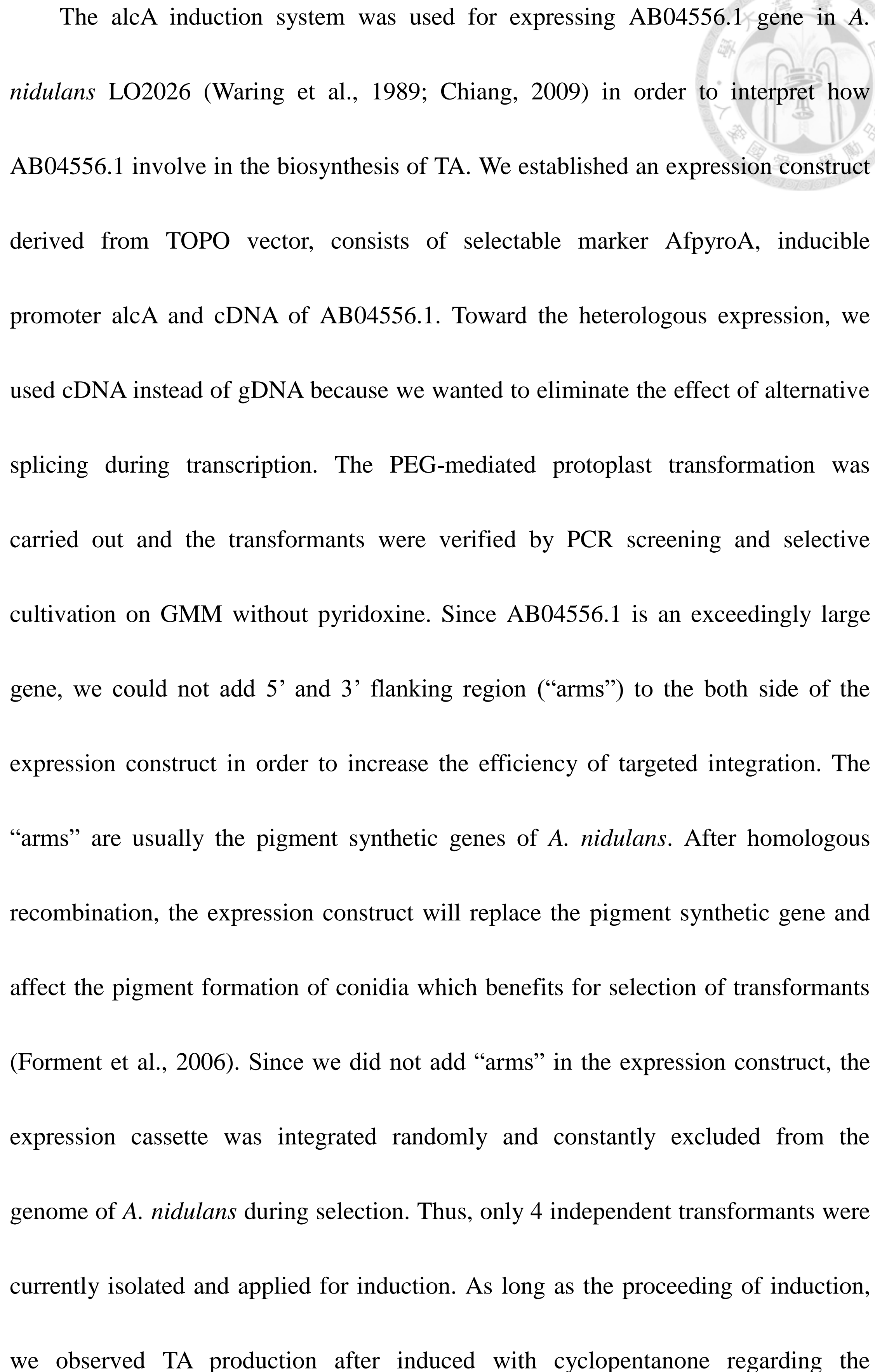


biolistic transformation were further checked homologous recombination with PCR screening and Southern blot hybridization and the result showed these transformants have targeted integration in AB04556.1 gene. However, we failed to purify homokaryotic transformants even after gone through several generations, these transformants are still heterokaryotic; this phenomenon has also been reported in *A. alternata* (Saha et al., 2012). In order to purify the mutants, they performed several rounds of protoplast isolation but no effect was observed. We assumed it might be the consequence of the nuclei distribution in *A. brassicicola* concerning it has at least 10 to 30 nuclei in one conidium and 2 to 20 nuclei in the hyphal tip cell (Knox-Davies, 1979). In addition, the protoplasts generated from *A. alternata* have been proven could possibly have more than one nucleus (Cary and Stovall, 1992) that we also observed in *A. brassicicola*. To a certain extent, this is a difficulty for studying the function of individual gene because we can only analyze through down-regulation regarding the existence of un-transformed wild type nuclei.

With regard to heterokaryotic mutants, we compared the gene expression level between wild type and mutants; the disruption mutants showed relatively less gene expression level than wild type. Next, we analyzed the TA-producing ability through agar diffusion assay, TLC and HPLC. The result also indicated that disruption mutants can still produce TA in comparison of wild type. However, a certain reduction of TA



in disruption mutants was determined by HPLC profiling. On the part of HPLC analysis, TA has resonance whereas it cannot express in a single formula (Lebrun and Boucly, 1985; Shephard et al., 1991) that affects the resolution of HPLC; leads to tailing during operation. In previous studies, the researchers mostly used gradient elution for separation (Andersen et al., 2006; Siegel, 2009, 2010; Saha et al., 2012) and we used isocratic elution instead. Because gradient elution could not help to separate TA from other compounds which have similar polarity. Although using isocratic elution may leads to the disadvantage of delaying peak and broad shape, but in concerning the stability and the ability of separation; isocratic elution is better for analyzing TA production in *A. brassicicola*. The HPLC profile showed that disruption mutants produce less TA than wild type at 3 dpi but accumulate TA subsequently after 7 dpi. This result also corresponded to quantitative PCR analysis and agar diffusion assay. Both 2-3 and 6-3 mutants showed lower mRNA expression level of AB04556.1 gene and produce less TA during cultivation but still harbored un-transformed wild type nuclei concerning the presence of TA and gene expression level. Regards to the heterokaryotic mutants, we attempted to determine the function of AB04556.1 much precisely through expressing AB04556.1 in isolation in heterologous expression system. Thus, we employed *A. nidulans* LO2026 that obtained from Dr. Clay Wang's lab for investigating the function of AB04556.1 (Chiang, 2010; Sanchez et al., 2011).



The alcA induction system was used for expressing AB04556.1 gene in *A. nidulans* LO2026 (Waring et al., 1989; Chiang, 2009) in order to interpret how AB04556.1 involve in the biosynthesis of TA. We established an expression construct derived from TOPO vector, consists of selectable marker AfpyroA, inducible promoter alcA and cDNA of AB04556.1. Toward the heterologous expression, we used cDNA instead of gDNA because we wanted to eliminate the effect of alternative splicing during transcription. The PEG-mediated protoplast transformation was carried out and the transformants were verified by PCR screening and selective cultivation on GMM without pyridoxine. Since AB04556.1 is an exceedingly large gene, we could not add 5' and 3' flanking region ("arms") to the both side of the expression construct in order to increase the efficiency of targeted integration. The "arms" are usually the pigment synthetic genes of *A. nidulans*. After homologous recombination, the expression construct will replace the pigment synthetic gene and affect the pigment formation of conidia which benefits for selection of transformants (Forment et al., 2006). Since we did not add "arms" in the expression construct, the expression cassette was integrated randomly and constantly excluded from the genome of *A. nidulans* during selection. Thus, only 4 independent transformants were currently isolated and applied for induction. As long as the proceeding of induction, we observed TA production after induced with cyclopentanone regarding the

antibiotic activity and HPLC metabolic profile in comparison of the non-induced culture. This suggested that AB04556.1 gene is responsible for the biosynthesis of TA. Still, the other genes from the upstream and the downstream of AB04556.1 could be expressed in *A. nidulans* as well with the attempt to understand if these genes function as a gene cluster.

At the final point, we aimed to know how TA involves in the pathogenesis of *A. brassicicola*. Hence, the conidia of wild type and disruption mutants were applied on the leaves excised from 3-week-old *A. thaliana* Col-0 cultivars and the leaves were then kept in a moist chamber as well as the treatment of TA standard compound (Qui et al., 2009). The treatment of TA caused severe damage on leaves concerning the presence of hypersensitive response after only one day which has also been observed in the interaction between *M. oryzae* and corresponding rice cultivars (Aver'yanov, 2007). Meanwhile, the inoculation of wild type and disruption mutants showed no prominent difference. We observed the lesions of mutants appeared earlier to a small extent but we cannot jump to the conclusion on the slight evidence. In addition, since we used detached leaves for the leaf spot assay, the relative pathogenic symptoms between wild type and disruption mutants may be different between whole plants and excised leaves. Therefore, the approaching inoculation will be conducted on living plants and the disruption mutants also will be co-inoculated in company with TA

standard compound to assure the role of TA in pathogenesis of *A. brassicicola*.

Figures and tables

LNKS_*A. terreus*
MlcA_*P. citrinum*
MGG15100_*M. oryzae*
MGG15272_*M. oryzae*
MGG14943_*M. oryzae*
MGG03810_*M. oryzae*
MGG15097_*M. oryzae*
EqiS_*F. heterosporum*
PksB_*A. alternata*
AB04556.1_*A. brassicicola*
MGG11638_*M. oryzae*
MGG12447_*M. oryzae*
FusA_*F. fujikuroi*
FusS_*F. verticillioides*
PsoA_*A. fumigatus*
CpaA_*A. flavus*
TenS_*B. bassiana*
ApdA_*A. nidulans*
CheA_*P. expansum*
MGG09589_*M. oryzae*
MGG14897_*M. oryzae*
AB07780.1_*A. brassicicola*
AB05106.1_*A. brassicicola*
AB05242.1_*A. brassicicola*
Out group

98 20 100 39 11 24 48 32 99 42 24 18 51 100 75 19 27 50 98 79 46

1

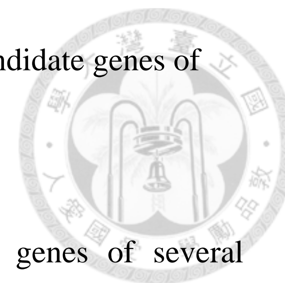


Fig. 1 Phylogenetic tree of known fungal PKS-NRPS genes and candidate genes of *Alternaria brassicicola*

Phylogenetic tree of this study consist of known PKS-NRPS genes of several filamentous fungi, including MGG_03810, MGG_09589, MGG_14943, MGG_12447 (Ace1), MGG_15097 (Syn2), MGG_15100 (Syn6), MGG_15272 (Syn7), MGG_14897 (Syn8) and MGG_11638 (Syn9) of *Magnaporthe oryzae* (*M. oryzae*); ApdA of *Aspergillus nidulans* (*A. nidulans*), CheA gene of *Penicillium expansum* (*P. expansum*), CpdA of *Aspergillus flavus* (*A. flavus*), EqiS of *Fusarium heterosporum* (*F. heterosporum*), FusA of *Fusarium fujikuroi* (*F. fujikuroi*), FusS of *Fusarium verticillioides* (*F. verticillioides*), LNKS of *Aspergillus terreus* (*A. terreus*), MlcA of *Penicillium citrinum* (*P. citrinum*), PksB of *Alternaria alternata* (*A. alternata*), PsoA of *Aspergillus fumigatus* (*A. fumigatus*), TenS of *Beauveria bassiana* (*B. bassiana*), 4 candidate genes of *Alternaria brassicicola* (AB04556.1, AB05106.1, AB05242.1 and AB07780.1) and β-tubulin synthetic gene of *A. brassicicola* (AB07628.1) was used as an out group. The amino acid sequences of these genes were aligned using ClustalW then were applied into Mega version 5.20 to conduct a boot-strapped likelihood tree for 1000 replications.

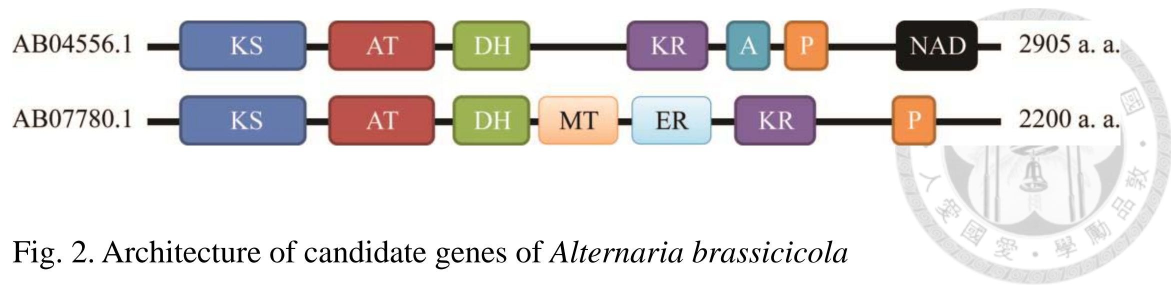


Fig. 2. Architecture of candidate genes of *Alternaria brassicicola*

The sequences of candidate genes are available in genome database of *Alternaria brassicicola* (DOE Joint Genome Institute, JGI). Functional domain abbreviations are as follows: KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; MT, methyltransferase; ER, enoyl reductase; KR, ketoreductase; A, adenylation domain; P, phosphopantetheine binding site; NAD, NAD binding domain. AB04556.1 gene contains KS, AT, DH, KR, partial A, P and NAD domain while AB07780.1 contains in additional MT and ER domain but lacks of A and NAD domain.

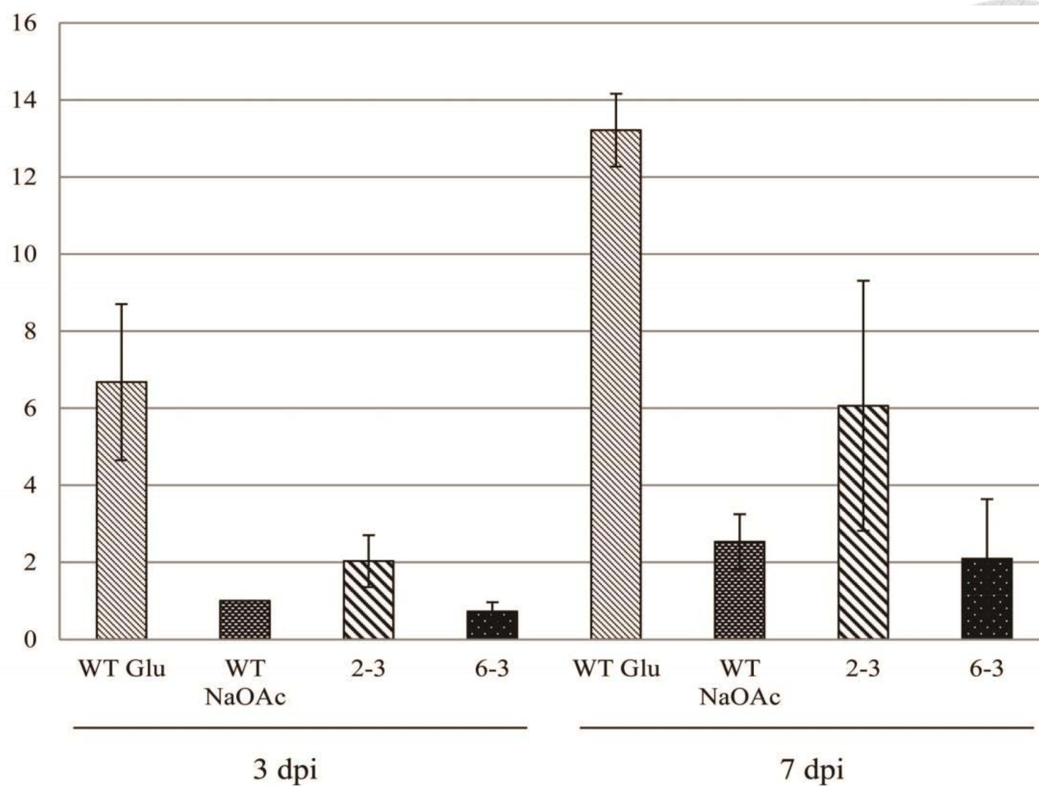
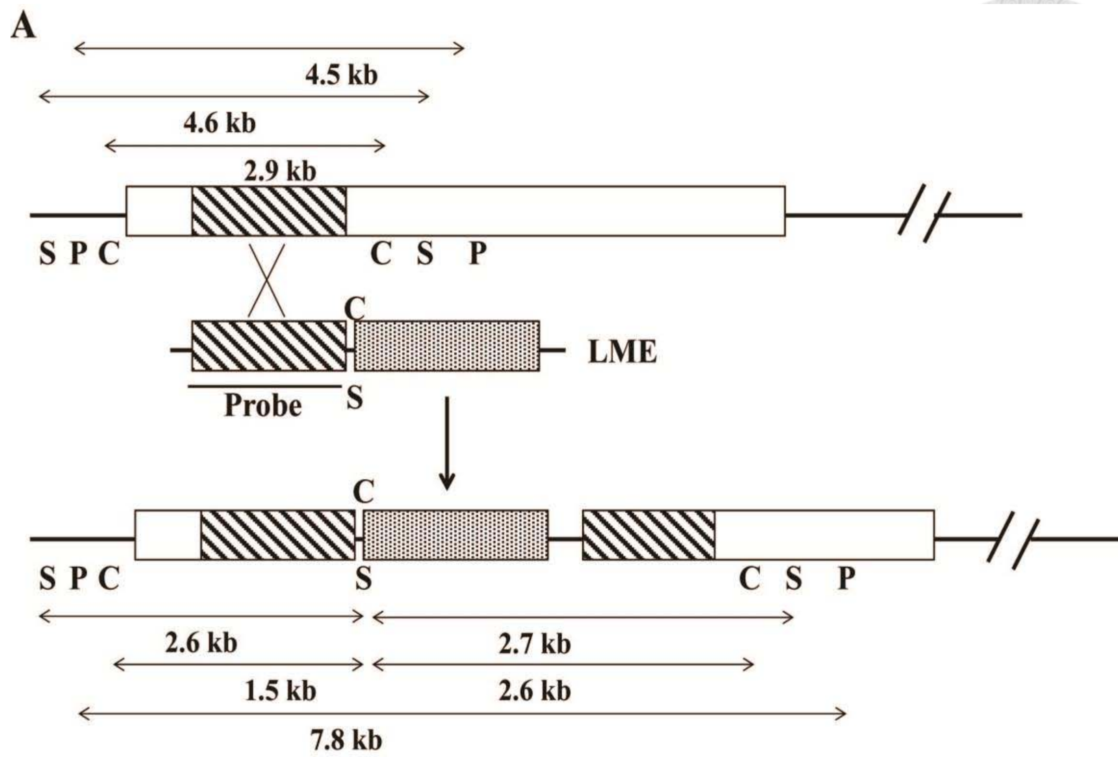


Fig. 3. Quantitative RT-PCR analysis of *A. brassicicola* wild type and gene disruption mutants

The relative expression level of AB04556 gene was examined via real-time PCR and β-tubulin gene (AB07628.1) was used as an endogenous reference. Both wild type and mutants were grown in modified Czapek-Dox medium for 3 and 7 days at 22°C in consistent darkness and static condition and wild type also grown in different carbon source includes glucose (Glu) and sodium acetate (NaOAc) while the former as positive condition for TA production and the latter as negative control. Both gene expression level of mutant 2-3 and 6-3 were reduced but mutant 2-3 still has an approximately expression level in comparison of wild type at 7 dpi.


A
4.5 kb
4.6 kb
2.9 kb
S P C
C S P
C
LME
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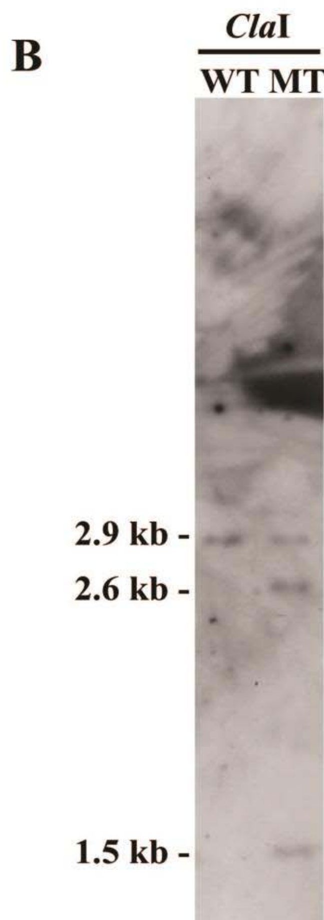
B
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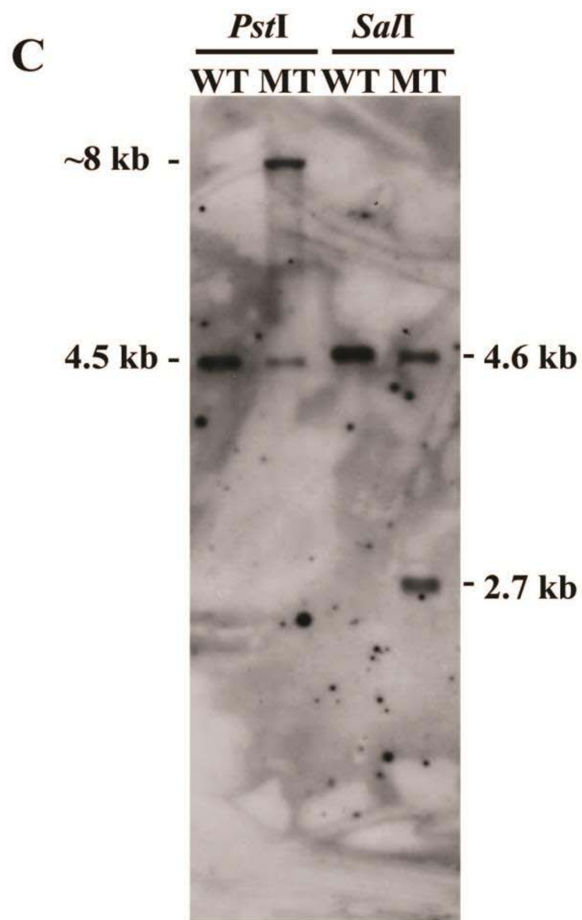
WT MT
2.9 kb -
2.6 kb -
1.5 kb -


C
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*Sal*I
WT MT WT MT
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- 4.6 kb
- 2.7 kb

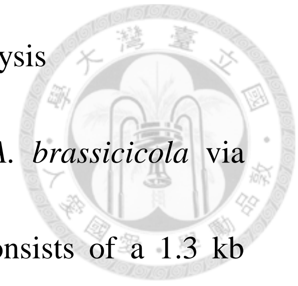
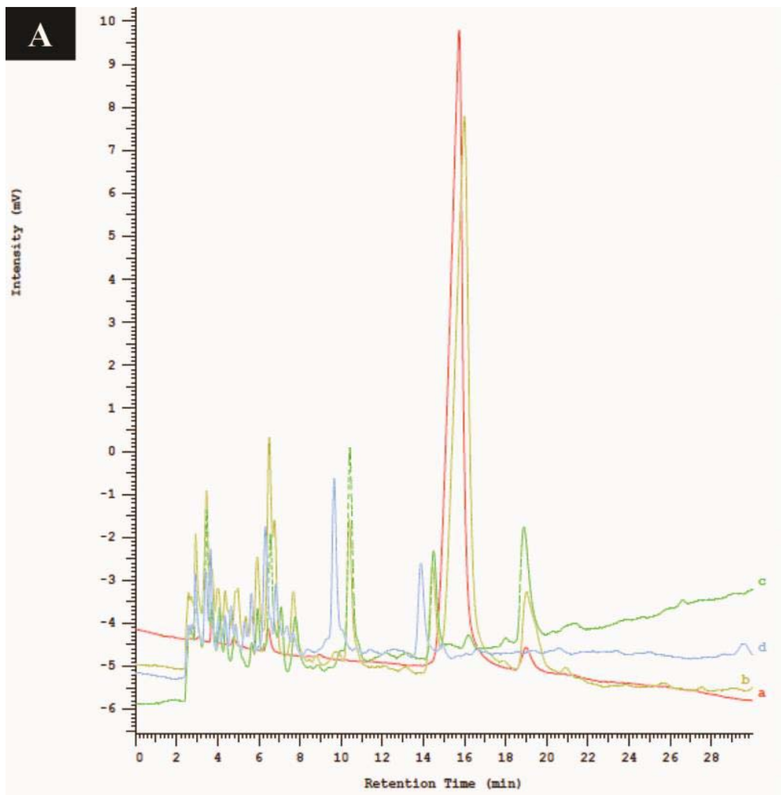
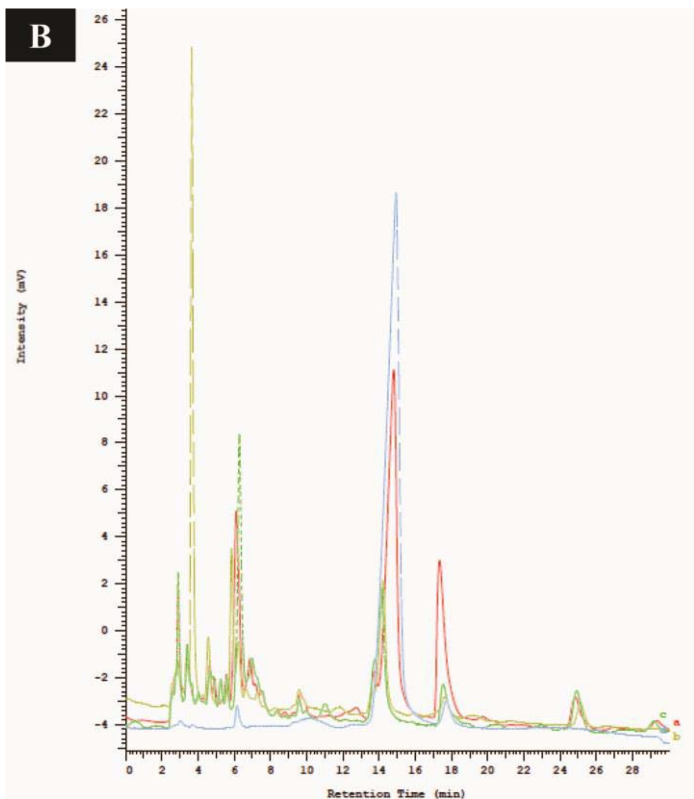


Fig. 4. Restriction map of disruption mutant and Southern blot analysis

(A) A linear minimal element (LME) was sent to wild type *A. brassicicola* via biolistic transformation to construct disruption mutants. LME consists of a 1.3 kb partial homologous fragment (hatched box) of target gene (white box) and a 1.4 kb hygromycin resistant cassette. The expected diagram was depicted by sequencing the disruption mutant. C = *Cla*I, P = *Pst*I and S = *Sal*I. **(B)** and **(C)** are Southern blot analysis of *A. brassicicola* wild type (WT) and mutant (MT). Each DNA was digested with *Cla*I **(B)**, *Pst*I or *Sal*I **(C)**. The result showed that mutant has both banding patterns of disrupted and original genome type and has a duplicate of partial homologous fragment.



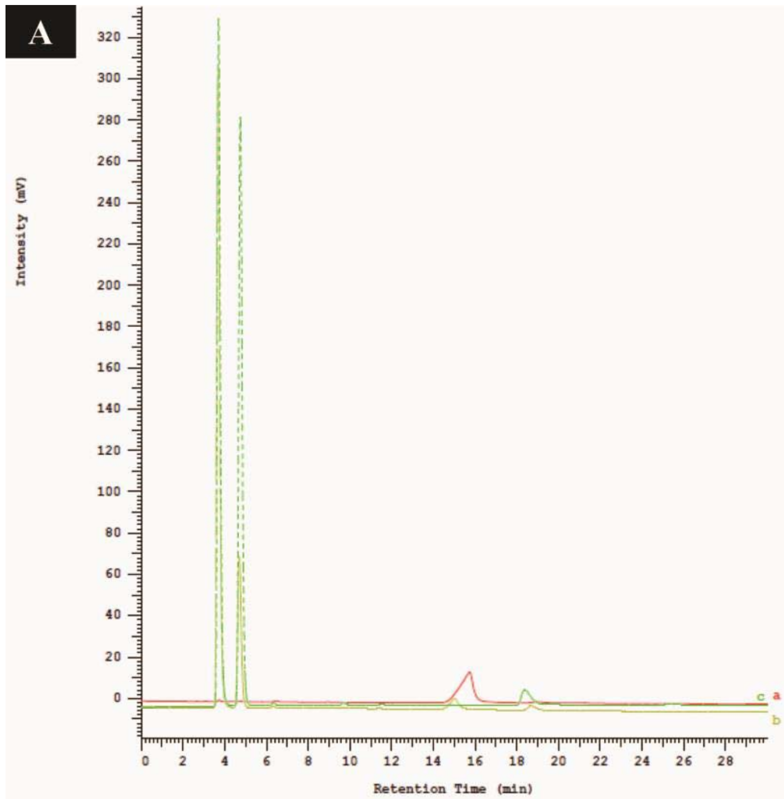
a) TA Standard
b) Wild type
c) MT 2-3
d) MT 6-3

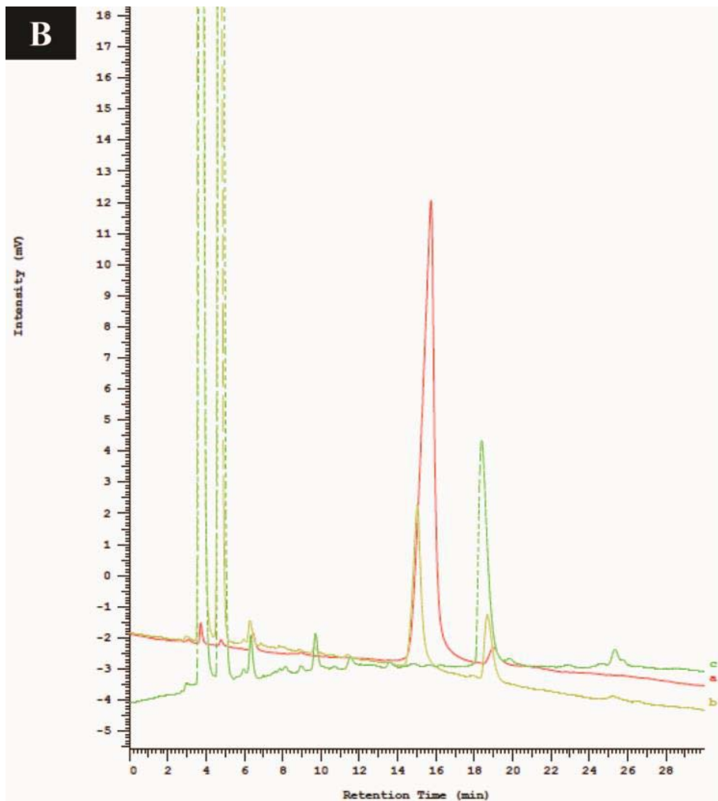


a) TA Standard
b) Wild type
c) MT 2-3
d) MT 6-3

Fig. 5. HPLC chromatograms of *A. brassicicola* wild type and disruption mutants

HPLC was performed by a device (Hitachi L-7100, Tokyo, Japan) equipped with a reverse phase column (Mightysil, RP-18, 5 μ, 4.6×250 mm, KANTO Chemical Co., Inc.). Mobile phase solution was 0.3% $ZnSO_4$ in 10% MeOH at a flow rate of 1 ml/min. TA was monitored with UV detector (Hitachi L-7400, Tokyo, Japan) at 277 nm. Retention time was 15.06 ± 0.8 min for TA. (A) HPLC profile of 3 day-post-inoculation (dpi) samples and TA standard (B) HPLC profile of 7 dpi samples and TA standard


A
Intensity (mV)
Retention Time (min)
a) TA Standard
b) Induced
c) Non-induced


B
Intensity (mV)
Retention Time (min)
a) TA Standard
b) Induced
c) Non-induced

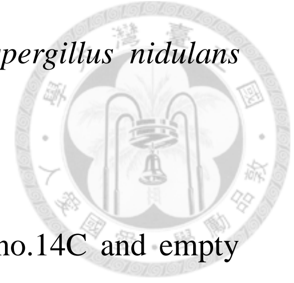
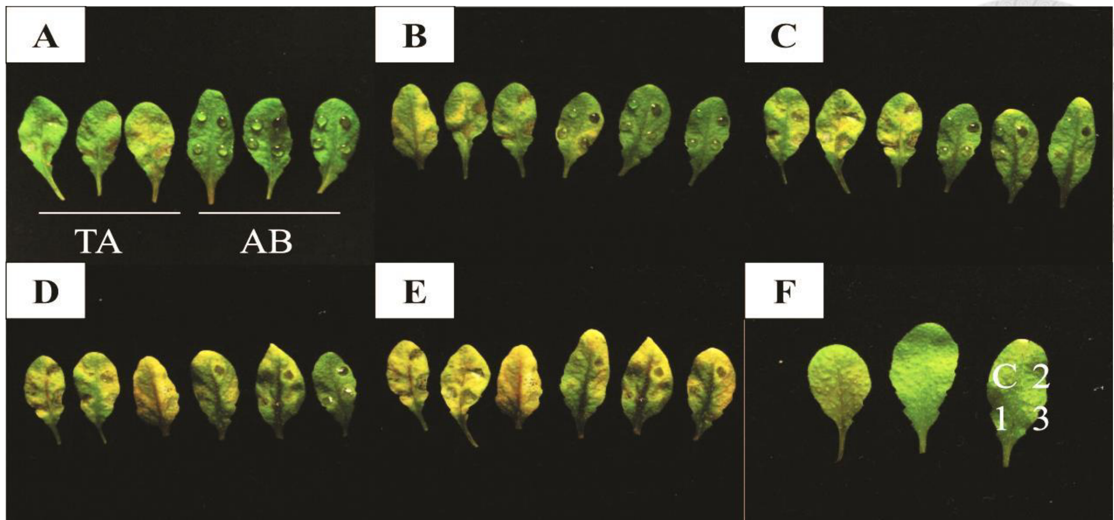


Fig. 6. HPLC chromatograms of heterologous expression in *Aspergillus nidulans* LO2026

(A) Complete HPLC profile of TA standard, induced culture of no.14C and empty vector (B), (C) Enlarged chromatograms of the performance of TA


A
B
C
TA
AB
D
E
F
C 2
1 3

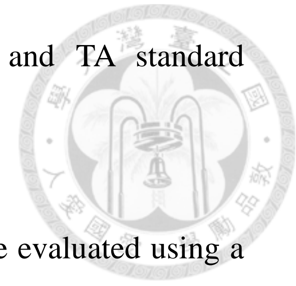


Fig. 7. Pathogenicity assay of *Alternaria brassicicola* (AB) and TA standard compound on *Arabidopsis thaliana* Col-0 cultivars

Pathogenesis of *A. brassicicola* and phytotoxic activity of TA were evaluated using a leaf spot assay on *A. thaliana* leaves. The lower leaves were excised for this assay and the treatment was like the schematic information at panel (F). For the study of pathogenesis, a 10 µl drop of conidia suspension ($5*10^5$ conidia/ ml) harvested from different *A. brassicicola* strains, or sterile distilled water in the case of the control, was added on leaf directly: C = control, 1 = wild type, 2 = mutant 2-3, and 3 = mutant 6-3. For the study of phytotoxic activity, a 5 µl drop of TA standard compound was dropped on leaf: C = methanol, 1 = 200 ppm, 2 = 500 ppm, and 3 = 1000 ppm. These leaves were kept in a moist chamber at 22°C. Three leaves, with 4 applications, were used for each treatment. The symptoms were recorded after 1 to 5 days post inoculation (dpi). All evaluations were made in triplicate. (A) 1 dpi, (B) 2 dpi, (C) 3 dpi, (D) 4 dpi, (E) 5 dpi and (F) healthy control.

Table 1 Bioassay of *A. brassicicola* wild type and mutants

Strains	**3 dpi culture filtrate**	**7 dpi culture filtrate**
Wild type Glucose	0.65 ± 0.10 cm	0.80 ± 0.05 cm
Wild type NaOAc	X	X
Mutant 2-3	0.30 ± 0.05 cm	0.55 ± 0.15 cm
Mutant 6-3	0.35 ± 0.10 cm	0.40 ± 0.10 cm

＊ The radius of restriction ring was measured after 1 day

The antibiotic activity of tenuazonic acid was evaluated by agar diffusion assay. The culture filtrate of 3 or 7 days post inoculation (dpi) was collected and 100 times condensed for each application.

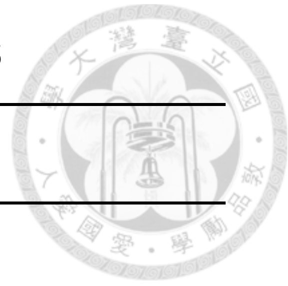


Table 2 Bioassay of heterologous expression in *A. nidulans* LO2026

Strains	**48 hpi culture filtrate**
Empty vector	X
Induced no. 3	0.15 ± 0.01 cm
Induced no. 14C	0.15 ± 0.01 cm
Induced no. 14F	0.15 ± 0.01 cm

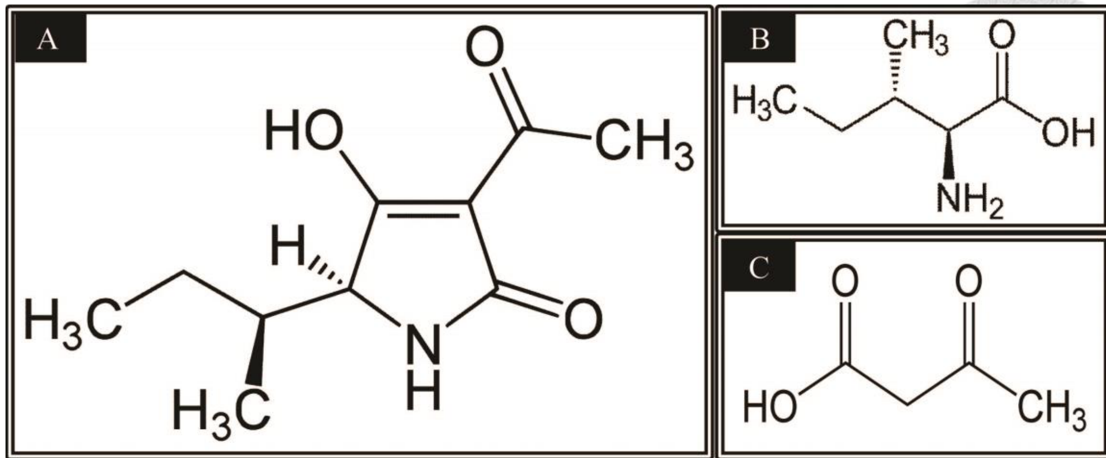
＊ The radius of restriction ring was measured after 1 day

The culture filtrate of 48 hours post induction (hpi) was collected and condensed 200x for each application. The empty vector was used as a negative control and 3 strains (no.3, no. 14C and no.14F) were used for evaluating the production of TA after 48 hpi.

Table 3 Primers used in this study

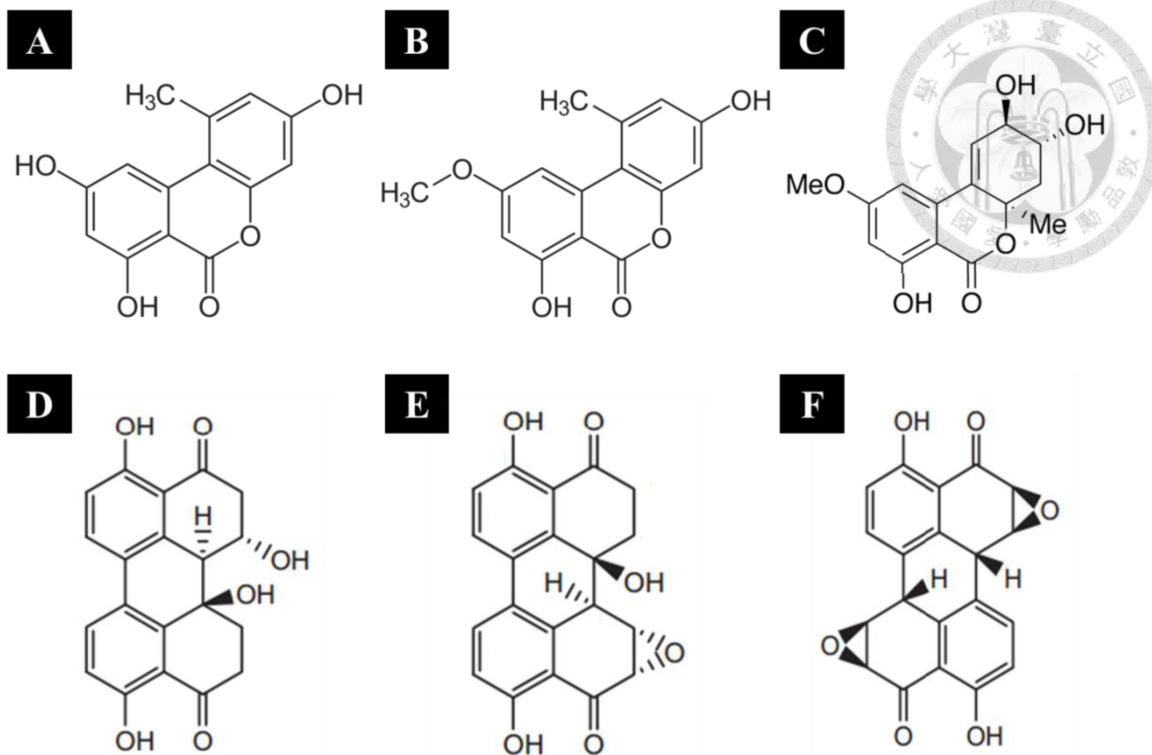
Primer number	Sequence (5'-3')	Application
WC993	CACCACCTGCCTGCGTTT	β-tubulin qPCR forward (F)
WC994	CGCAATCCGACCATGAAGA	β-tubulin qPCR reverse (R)
WC1310	AAGGTCACACAAGCCATGCA	AB04556 PCR-F
WC1311	TAGGGCGTCGCTCAGGTAGA	AB04556 PCR-R Probe-R
WC1326	CGAGCTCATGTCACCGAAGGC	cDNA-F
WC1327	CGAGCTCCTATGTACTTCCTAACTTTAGCAAAG	cDNA-R
WC1328	CGAGCTCCTTCTAAGCTATGGGATCTTCT	Disruption-F Probe-F
WC1332	CCATCGATTAGGGCGTCGCTCAGGTAGA	Disruption-R
WC1353	CGTGGTGGGAAGAGCGATAG	AB04556 PCR-R
WC1354	GGGCCAAACTTCCGACAGT	AB04556 qPCR-F
WC1355	CTTGTCGCATCCAAGAGCAA	AB04556 qPCR-R
WC1397	CAATGCTCTTCACCCTCTTCG	AfPyroA-F
WC1442	CTGTCTGAGAGGAGGCACTGATGC	AfPyroA-R
WC1480	ACTCTCACTACCAGGCTCGGC	AB04556 PCR-R

Appendix



Appendix 1 Tenuazonic acid and its precursors

(A) Tenuazonic acid (B) L-isoleucine (C) Acetoacetate



Appendix 2 Mycotoxins of *Alternaria* species

(A) Alternariol (AOH); (B) Alternariol-9-methyl-ether (AME); (C) Altenuene (ALT);

(D) Altertoxin I (ATX I); (E) Altertoxin II (ATX II); (F) Altertoxin III (ATX III)

(Fleck et al., 2012)

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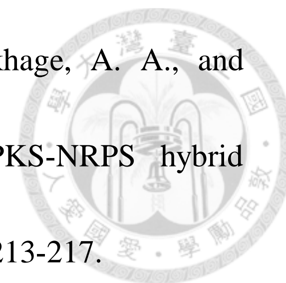

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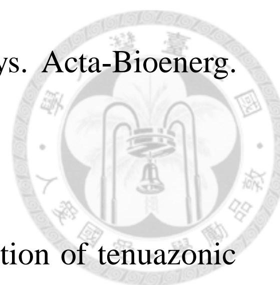

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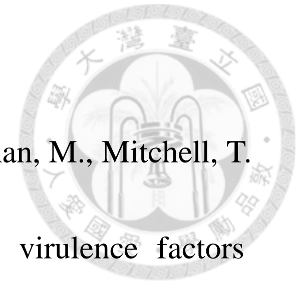

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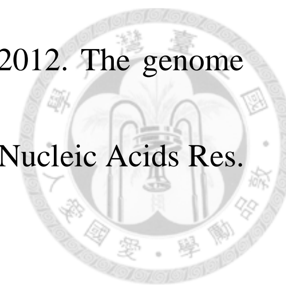

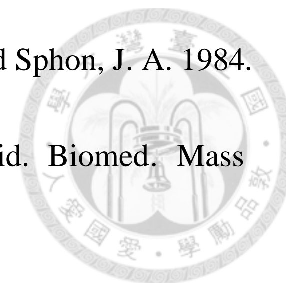

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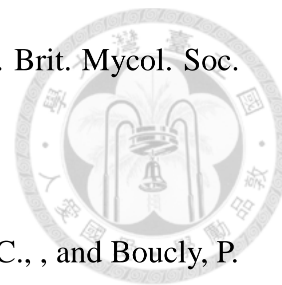

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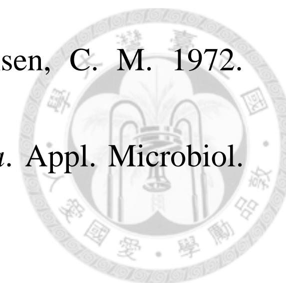

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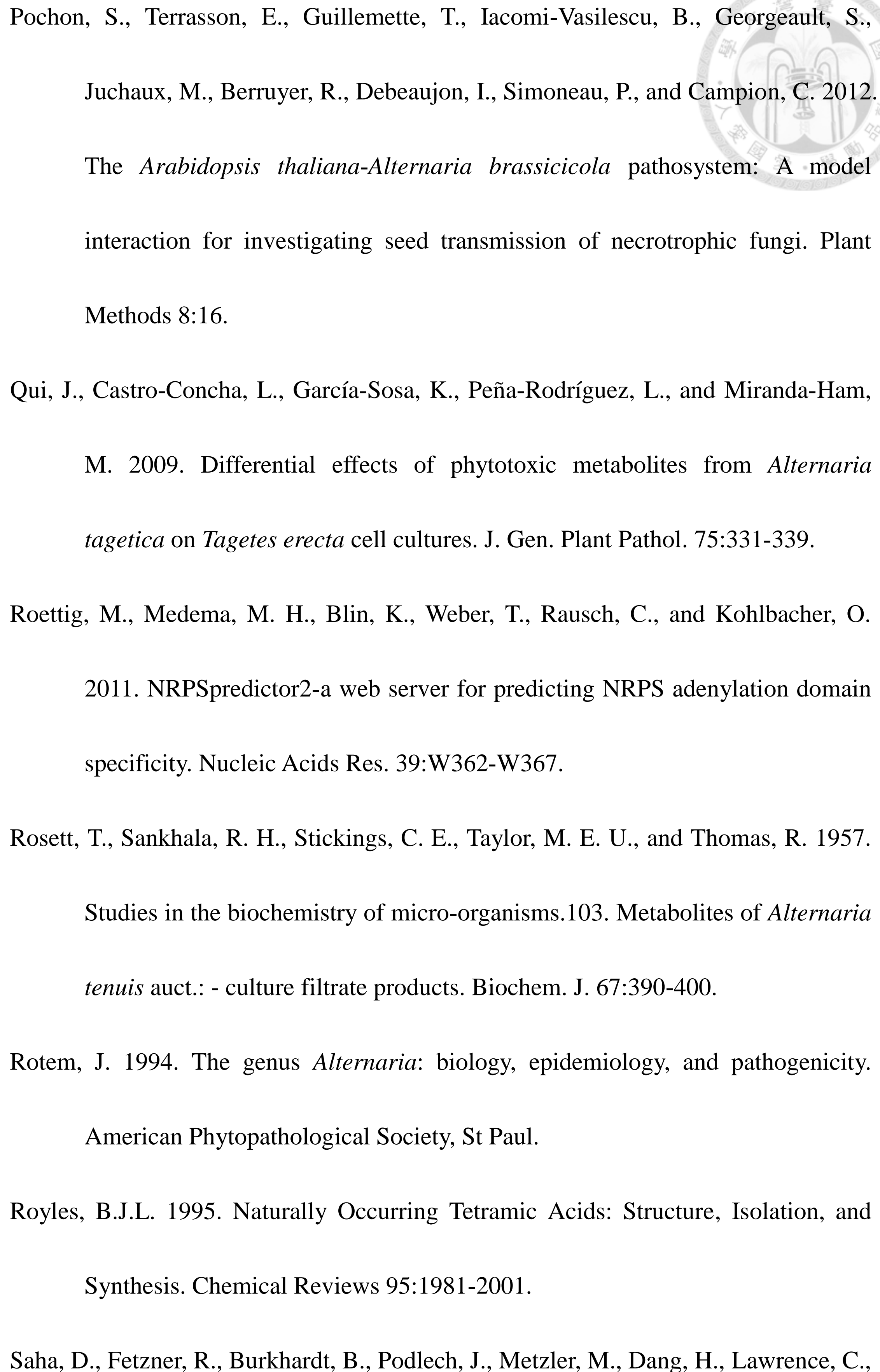

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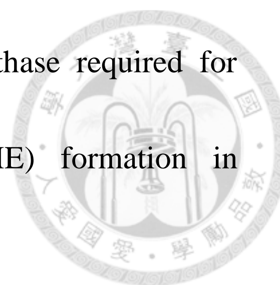

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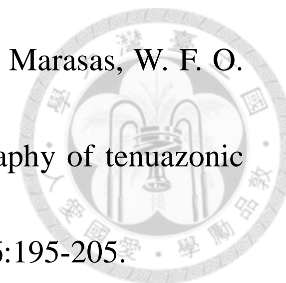

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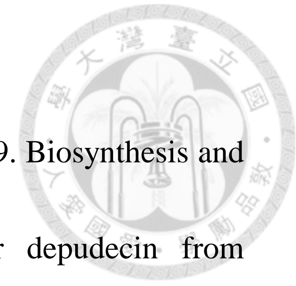

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