Basic studies for characterizing *Phytophthora* mating hormone receptor

（疫病菌交配ホルモン受容体の解析に向けた基盤的研究）

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March 2016

Dedication

This thesis is dedicated to my parents Baorong ZHANG, Huimin SUN and my husband Shiwei ZHU who have always stood by me and encouraged me when I need help.

List of Abbreviation

Acp: Aminocaproic acid

CBB: Coomassie Brilliant Blue

CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

DMSO: Dimethyl Sulphoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic Acid

FG beads: Ferrite Glycidyl methacrylate beads

HEPES: 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonicacid

HPLC: High Performance Liquid Chromatography

MBP: Maltose-Binding Protein

MES: 4-Morpholineethanesulfonic acid hydrate

PAP: Photo-affinity Probe

PEG: Polyethylene Glycol

PMSF: Phenylmethanesulfonyl fluoride

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

TRIS: tris (hydroxymethyl) aminomethane

UV: Ultra Violet

Contents

Chapter 1

Introduction

1.1. Origin and Classification of *Phytophthora*

The genus *Phytophthora* is well known as a plant pathogen. The first report recorded as a new breaking out potato epidemic is from an East coast port of North America in 1843. Then it rapidly spread in the USA and Canada,[1] even in Europe.[2] The worst devastating destroy was well known as " Great Irish famine" happened in Ireland between 1845 and 1852,[3] which caused the starvation death of approximately one million people and forced another similar number people to emigrate from Ireland.[4] Until now, the Irish Potato Famine continues to be one of the most important crop diseases causing significant losses worldwide.

When the potato epidemic first appeared, it was not clear what caused this new disease. J. E. Teshemacker had suggested that this disease was of a fungus origin. J. P. F. C. Montagne in France described the potato crop pathogen and gave it the name *Botrytis infestans*.[5] In the United Kingdom, M. Berkeley described the fungal infection as the cause of potato murrain famine and chose to classify this fungus in the genus *Botrytis*.[6] During study, the life cycle of the potato blight pathogen was uncovered by a famous German mycologist Anton de Bary who finally renamed the fungus to *Phytophthora* that derived from the Greek and means "the plant-destroyer".[6]

According to the present finding, the genus *Phytophthora* had been classified as Domain Eukarya; Kingdom Stramenopila; Phylum Oomycota; Class Oomycetes; Order Perenosporales and Family Pythiaceae.[7] Until now, after Anton de Bary first coined the genus name *phytophthora* to potato blight pathogen, over 100 species was classified in the genus *phytophthora*, and the number is increasing.[8]

1.2. Pathogenicity

Phytophthora species are all devastating pathogens, causing highly destructive diseases in a variety of agricultural crops, and natural ecosystems worldwide. For example, *P. infestans*, the pathogen of late blight, originated from America triggered the "Great Irish Famine" in Europe and remains to be a very difficult disease in Asia.[9,10] *P. ramorum* caused the Sudden Oak Death and severely damaged woodlands in North America and Europe.[11] *P. cinnamomi* is a major pathogen threating the natural and wild-lands vegetation in Australia.[12]

Phytophthora can cause massive fruit and tubers rot; leaf and stem blight; root and crown canker in a huge range of plants (Fig 1.1). In which, some species, such as *P. cinnamomi*, *P. nicotianae* (syn. *P. parasitica*), and *P. cactorum*, each can attack hundreds of different plant host species. While others species only have narrow host ranges, infecting just a few host plant species.[13] For example, *P. infestans* as a pathogen is capable of infecting potato and tomato; *P. sojae* infects soybean (*Glycine max*) only.

Figure 1.1. Infected location caused by *Phytophthora* species.

The successful infection to plant is usually initiated by the spore dispersing. Five potential mechanisms of dispersal of *Phytophthora* species in the field were summarized[14] and it can be separated to two main aspect, airborne and soilborne phase.

Airborne *Phytophthora* species are spread by wind and rain splish aplash via sporangia. And the highest concentration peaks of airborne sporangia that were recorded are the season of autumn and spring.[15] Like *P. infestans*, which produces asexual sporangia that are local dispersed through waterborne spores and most distant transport due to the truly airborne inoculum.[16]

While the major dispersal mechanisms in soil are via root, surface water, rain splash, human and insect activity. Soil-borne *Phytophthora* species using the motive zoospores chemotactically attracted to the nearby roots to initiate the infection. Soil-borne species can cause root rots and damping-off diseases of seedlings and tubers of many important crop plants and threaten natural ecosystems. The invasive soil-borne pathogen *P. cinnamomi*, a major threaten in the Western Australia, which can survive adverse conditions and spread in soil by root to root contacting to infect the susceptible hosts.[17] Most *Phytophthora* species contain a soil-borne phase. The asexual spores or sexual oospores, which survive in the soil or root debris, can provide the primary inoculation for subsequent epidemics.

1.3. Life history of *Phytophthora*

Instead of life cycle, a term called the life history was recommended by Dick for the lower eukaryotes.[18] The life history of *Phytophthora* is dual phase, which can be separated into an

asexual reproduction and a sexual reproduction stage. Each reproduction stage can produce relevant spores of the infective structures.

1.3.1. Asexual reproduction and asexual spores

The asexual life history is the driving force behind rapid polycyclic epidemics to plant crops. Under favorable weather conditions, suitable moisture and temperature, plant crops can be destroyed by *Phytophthora* within days.

The thallus of the *phytophthora* is called mycelium. It consists of a large number of branched, thread-like structures named as hyphae which normally with extension of hyaline and aseptate feature. *Phytophthora* can be cultured continually as hyphae for infection, but in nature spores must be required continually. The most common and characteristic asexual spore in *Phytophthora* is called sporangia. *Phytophthora* species produce sporangia at special termini of the hyphae with variable size and shapes on the plant surfaces, including leaves and roots.[19] In some species, like airborne *P. infestans*, sporangia can easily detach from hyphae and be blown or splashed with water to nearby healthy plants or travel several kilometers. While other species, such as *P. parastica* or *P. sojae*, sporangia are difficult to be released from the hyphae.

The sporangia are vegetative structures that under the higher temperature (*P. infestans* >14 °C) can directly germinate to produces new mycelium. However, at cooler temperature, they usually convert their structures by indirectly germination into swimming spores called zoospores. Zoospores are short-lived, biflagellate spores (Fig. 1.2) that can swim on plant leaf surfaces or underground soil pores through water.

They can attract by nonspecific chemoattractants (such as amino acids) and other chemicals given off that excluded by plant roots or succulent plant parts. Once zoospores landed on the suitable plant part, they stop swimming, drop their flagella, and form a cyst (encysted zoospores).[20] A cyst is a short-lived resting structure that immediately germinates to form hyphae to grow into plant cells for the further infection and starting secondary asexual life history.

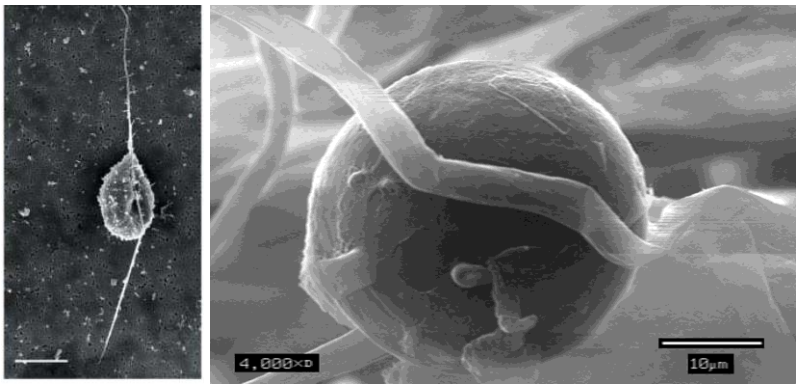


Figure 1.2. Images of asexual spores: zoospore (left) and chlamydospore (right).

Chlamydospores (Fig. 1.2), another kind of asexual spores, produced by some *Phytophthtora* species with round and thick walls for protecting them for long-term survival. For example, species *P. cinnamomi*, *P. ramorum*, and *P. parasitica* can germinate thick-walled chlamydospores, however *P. infestans* does not produce chlamydospores.[21,22] *Phytophthora* chlamydospores are separated from the hyphae by a septum. In the presence of water or during wet weather, chlamydospores can germinate to form sporangia.

1.3.2. Sexual reproduction and sexual spore

Sexual reproduction, a very important feature in the life history of *Phytophtora*, can increase the genetic variability within a species and lead to faster development of resistance to fungicides. It can produce sexual reproductive spore, so called oospore. Based on the ability to form sex organ and sexual spore, *Phytophthora* species can be divided to two groups: homothallic and heterothallic.[23] Some species such as *P. parasitica*, *P. cactorum*, and *P. citricola* are homothallic (self-fertile) and capable of forming sex organ and abundant oospores by single isolates to complete their life cycle.[24] While other species including *P. capsici*, *P. infestans*, and *P.palmivora* are heterothallic (self-sterile) which require pairing with two complementary strain of the opposite mating type (design as A1 and A2) to compete the sexual recombination.[25,26] Some species such as *P. megasperma* and *P. cinnamomi* even contain both homothallic and heterothallic isolations.[27, 28]

The sexual structures of *Phytophthora* are combined of an oogonium (female component) and an antherdium (male component). When mated, antheridium introduces gametes into oogonia, either by the oogonium passing through the antheridium (amphigyny) (*P. ilicis*, Fig. 1.3) or by the antheridium attaching to the proximal (lower) half of the oogonium (paragyny) (*P. cactorum*, Fig. 1.3), to produce diploid oospores.[19]

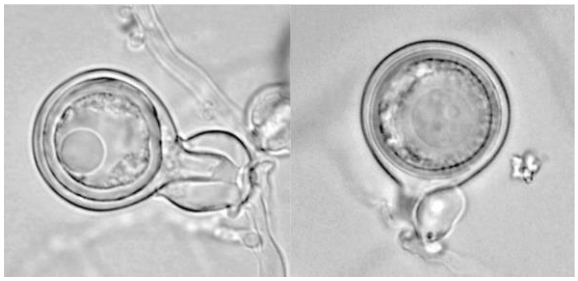


Figure 1.3. Images of sexual spores: amphigyny (left) and paragyny (right)

Oospores are thick-walled, globes structures serve as a *Phytophthora* resting phase that enable long-term survival in plant tissue or soil under severe conditions.[29] When the environments are suitable, oospore can germinate to develop into single or multiple germ tubes or sporangia. And the cycle will continue as is would asexually.

1.4. Mating hormones

The sexual reproduction of *Phytophthora* rarely occurs in the natural field. Researchers had limited knowledge of sexual reproduction at the early stage of study. Until 1911, Clinton first reported the oospore formation of *P. infestans* in the pure culture.[30] Before 1922, it was believed that *P. palmivora* did not produce sexual organs for the sexual reproduction.[31] Leonian in 1931 even suggested that *P. infestans* was homothallic due to the production of oospores.[32] However, in 1957 two sexually compatible A1 and A2 mating types were first found in *P. infestans*.[33] Oospores were formed in great abundance when isolation of these two mating type were paired.[34] After that many other species in mating were demonstrated. 350 isolates of *Phytophthora* that representing 30 species of this genus (homothallic or heterothallic) were further studied to observe the sexual phenomena. Oospores formation occurred when pairing A1 mating type and A2 mating type intra- and inter-specifically; however no oospores formed in A1 × A1 or A2 × A2 pairings.[35] The possible explanation about this an unusual phenomenon is that the development of oospores may be due to biochemical stimulation during pairing.[25]

To confirm this hypothesis, a cellophane membrane technique was performed. A1 mating

type and A2 mating type were paired on the opposite sides of a cellophane disk placed on V-8 agar Juice medium. After incubation 7 days at 24℃, both mating type produced oospores.[23] However cellophane method was not very successful, because both A1 and A2 mating type can penetrate through the cellophane membrane.[24] The problem was subsequently solved by Ko, who replaced the cellophane membrane with polycarbonate membrane that resists penetration of the mycelial growth and successfully observed oospore formation on each A1 and A2 mating types.[36] The hyphae cannot penetrate the polycarbonate membrane, then something produced by one mating type of the stain must diffuse through the membrane to induce the sexual reproduction in the other strain to form oospores. The formed oospores unequivocal proved the production of diffusible hormonal or pheromone-like substances were found in *Phytophthora*. Ko also postulated that the sex hormone produced by A1 mating type of *Phytophthora*, designated as hormone α1, only induce sexual reproduction on A2 mating type. Similarly, sexual reproduction of A1 mating type can only be induced by hormone α2 produced by A2 mating type (Fig. 1.4).[24, 37] Polycarbonate membrane method for hormonal regulation of sexual reproduction has been detected in many other species,[38-40] even in the interspecific mating.[41]

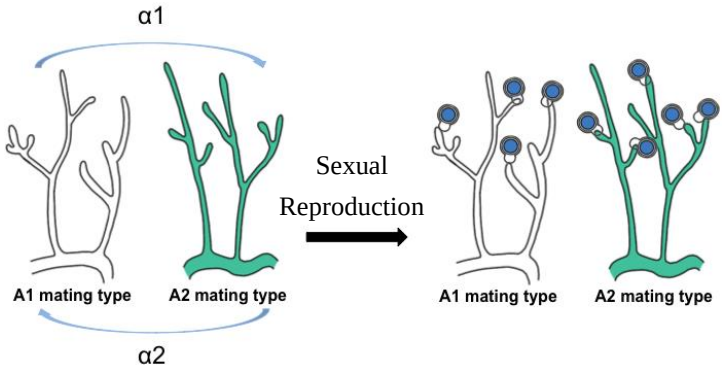


Figure 1.4. Illustration of mode of sexual reproduction in heterothallic species of *Phytophthora*.

Polycarbonate membrane method was also able to detect hormonal regulation of sexual reproduction by pairing homothallic species of *Phytophthora* with A1 and / or A2 mating type of heterothallic species of *Phytophthora* in the opposite side of the membrane.[37] The phenomenon of homothallic species of *Phytophthora* were also induced to form oospores, after pairing with heterothallic spices, suggested that sexual reproduction in homothallic species of *Phytophthora* may also be regulated by α hormones.

The α hormone, produced by one mating type, can be absorbed by cellulose nitrate and cellulose acetate filters and further induces oospore formation on the opposite mating type.[42] Both α1 and α2 hormone can be extracted by 95% ethanol from the Millipore filter, and α2 is more polar than α1 hormone.[43] Even though the physical and chemical properties of α hormones were characterized by large-scale extraction from *P. parasitica*, however their chemical structural elucidation remained unsettled.[44]

A breakthrough came in the year 2005. The structure of α□ (Fig. 1.5) was uncovered by using 1.2 mg of pure hormone□ □which was isolated from 1,830 L of culture broth of A1 mating type of *P. nicotianae*.[45] The absolute configuration of two asymmetric centers of α1 hormone were then successfully determined.[46] Later the total synthesis and absolute configuration of α□ hormone of *Phytophthora* were described.[47] In the year 2011, the second mating hormone of *Phytophthora*, hormone α2 was finally structurally elucidated by spectroscopic analysis and total synthesis.[48] In the mean time, the possible biosynthesized pathway of α hormones in the mating type strains was also uncovered (Fig. 1.5).

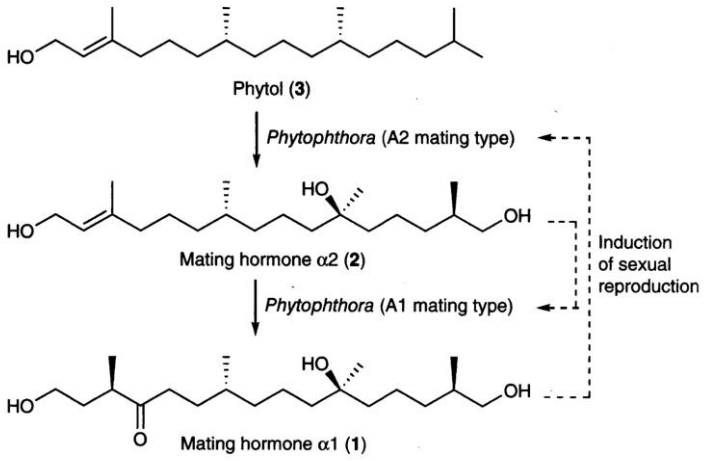


Figure 1.5. The biosynthetic route of α hormones.

In the A2 mating type of *P. nicotianae*, phytol as a precursor was converted to α2 (solid arrowed line), which promotes sexual reproduction in the A1 mating type (dotted arrow line). The A1 mating type then modifies α2 to α1 (solid arrowed line), which can induce oospore formation (dotted arrow line) on the A2 mating type. However the A1 mating type cannot convert phytol to α1. For now, despite the structural similarity of α hormones, the biosynthetic pathway and signaling pathway (hormone-receptor binding) of α hormones in *Phytophthora* remain unclear.

1.5. Previous researches on microorganisms signal molecules

Recent studies showed that α hormones are lipophilic hormones that regulate the sexual reproduction in the Oomycetes fungus-like organisms, *Phytophthora*. The hormones or pheromones regulating sexual reproduction and cell differerciation had been studied in

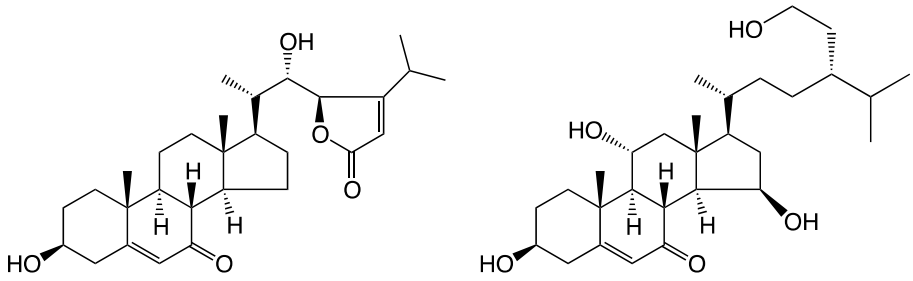
various kinds of microorganisms. Among them, lipophilic chemical signals like the *Phytophthora* α hormones are summarized in Table 1.1.

Table 1.1. References on lipophilic hormones and pheromones of microorganisms

Chemical signals	Source and function	No. of references containing compound name*	No. of references containing "receptor"*	Receptor identified
Antheridiol	Antheridia inducer from female strain of water mold *Achlya* sp.	79	12	No: only binding proteins (HSPs) identified
24(28)-dehydrooogoniol	Oogonia inducer from male strain of water mold *Achlya* sp.	28	1	No
Ectocarpene	Sperm attractant of brown algae *Ectocarpus* sp.	52	7	No
Sirenin	Sex attractant from female organ of water mold *Allomyces* sp.	56	1	No
Trisporic acids	Gametes inducer of Zygomycete *Mucor mucedo*	139	0	No
A-factor	Differentiation inducer of Actinomycete *Streptomyces griseus*	Too many	5025	Yes: ArpA

* Based on SciFinder search

The genus *Achlya* belonged to the class Oomycetes was most phylogenetically related to the genus *Phytophthora* among microorganisms. *Achlya* is also the most primitive eukaryote that is known to secret and respond to steroid hormones.[49] A series of experiments using *A. bisexualis* and *A. ambisexualis* were performed and revealed that the secretion from female hyphae (called as hormone A) could initiate the sexual reaction by inducing the formation of antheridial branches, then the sexually activated male strain produced a substance (called as hormone B) which could induce the oogonial in the female.[50-52] In 1965, the active compound hormone A was isolated from *A. bisexualis* strain T5 and renamed as antherdiol (Fig. 1.6). Few years later, the structure of antherdiol, as well as synthesis pathway of this compound was proposed.[53]



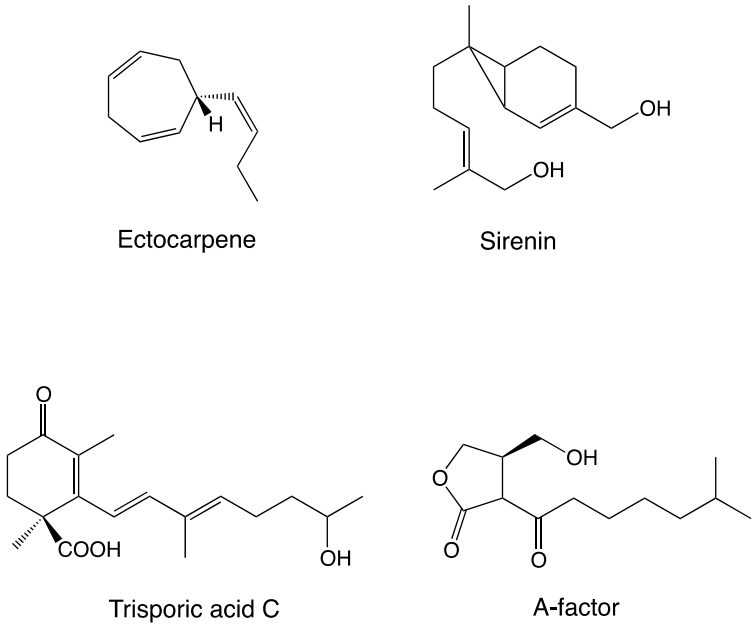
Antheridiol 24(28)-Dehydro-oogoniol

Figure 1.6. Chemical structures of the sexual hormones in other Oomycete

Unlike antherdiol, hormone B as the second hormone secreted by male *Achlya* was slower to be elucidated due to its low available quantities. The progress was improved when the hermaphroditic strain *A. heterosexualis* was found to secrete hormone B without pre-stimulation by antheridiol. Several compounds named oogoniol were obtained, and the 24(28)-dehydro-oogoniol (Fig. 1.6) that had actually biological activity was found to be the

true hormone.[54] After determining the *Achlya* hormones, a few researches were conducted to identify the hormone receptor. Unfortunately, the receptors for both steroid hormones had not been identified yet. Only one antheridiol-induced protein that very similar to the 85 kDa heat shock protein (HSP) of *Achlya* was detected.[55]

Ectocarpene (Fig. 1.7) was reported to be a volatile attractant that secreted by the brown alga *Ectocarpus* sp..[56] When gametes were getting surrounded, the female gametes could produce this substance to attract the male gametes. The chemical structure of ectocarpene had been described in 1971. However, no hormone receptor had been found yet.[57]



Ectocarpene Sirenin

Trisporic acid C A-factor

Figure 1.7. Chemical structures of the sexual hormones in microorganisms

Sirenin (Fig. 1.7) is an oxygenated sesquiterpene that is secreted by the female gametes of fungus *Allomyces* sp. and attracts the male gametes.[58] The structure of sirenin, 9-[(*E*)-4-hydroxy-3-methyl-2-butenyl]-10-hydroxy-2-carene, was determined in 1968 and no

receptor had been identified yet.[59]

Trisporic acids (trisporic acid A, B and C) were terpenoid C_{18} carboxylic acids that are secreted by the fungus *Blakeslea trispora*. And in the culture of *B. trispora*, trisporic acid C (Fig. 1.7) was produced in a large amount. It had been reported that trisporic acid could induce the formation of progametangia in both the plus and minus strains of *Mucor mucedo*.[58]

A-factor (Fig. 1.7) was a microbial hormone that induces secondary metabolites production and morphological development in *Streptomyces griseus*.[60, 61] The isolation and structure of A-factor hormone was described by Yamada in 1987.[62] Then the A-factor receptor protein, ArpA, was detected in the cytoplasmic fraction by radioactive labeling experiment[63] and proved to have the transcriptional repressor function by genetic studies.[64]

Although A-factor and its receptor had been well studied, these are the system of a prokaryote, *Streptomyces griseus*, which is phylogeretically far away from *Phytophthora*, suggesting the completely different biological system between Bacteria and *Pytophthora*. On the other hand, the most similar *Achlya* and other eukaryote organisms have not been successful in the receptor identification (Table 1.1).

Based on these information, the identification of the receptor of *Phytophthora* mating hormones is expected to be a highly difficult job. Therefore, I also looked into the information on sex-related genes of *Phytophthora*.

1.6. Genes related to *Phytophthora* sexual reproduction

In the heterothallic species of the genus *Phytophthora*, mating happens when the two

opposite mating types (A1 and A2) are co-cultured together. Although the sexual reproduction in *Phytophthora* had been well described in cytological and physiological aspects, little was known at the molecular level. Analyses of mating in other oomycetes are rare;[65] the true fungi had little relevance to *Phytophthora* because they were taxonomically unrelated.[66] Even the molecular studies in *Phytophthora* had been limited. Until now, the genome sequence drafts of only few *Phytophthora* species, such as *P. infestans*, *P. ramorum*, *P. sojae* and *P. capsici*, had been completed.[67-69] The knowledges of genes involved in sexual development and oospore formation are very important. If such genes were identified, it might be possible to identify these hormone receptor-related genes and reveal a novel strategy to control this plant pathogen by manipulating the relative genes instead of environmentally harmful chemicals.

In the year 2002, eight genes (M-12, M-24, M-25, M-81, M-82, M-90, M-96 and M-97) up-regulated in the *P. infestans* during sexual development were identified by using the suppression subtractive hybridization technique.[70] Two genes (M-82 and M-97) induced about 42- and >100-fold in the early stage of mating, before gametangial initiation appeared, which means these genes might encode proteins involved in initial interaction between two mating types. Another notable information was that M-82 and M-97 genes were expressed at very low but detectable level. This phenomenon resembled the mating pheromone-receptor gene system upregulated in the basidiomycete *Ustilago maydis*.[71] The sequence analysis of the gene M-82 revealed that the predicted M-82 protein had very low similarity ($E = 10^{-5}$) to the croquemort receptor of *Drosophila melanogaster*.[72] Other six genes which were detected at a later stage of mating with the induction levels from 64- to >100-fold, might function in forming gametangia and oospores. Sequence analysis of the later genes revealed that the

production of M-25 and M-81 resembled the elicitor proteins, M-24 and M-90 interacting with RNA and stabilizing RNA. M-12 and M-96 were not identified to any protein. More information about stage-specific gene expression had been reported later[73-76].

In the year 2007, a large-scale screen for genes induced during sexual development was performed in *P. infestans* using Affymetrix Gene-Chips.[77] By targeting 15,644 unigenes in *P. infestans*, 87 genes upregulated by more than 10-fold during mating were identified, in which 28 genes were induced more than 100-fold. In all the 87 mating induced genes, 44 had been significantly matched to proteins in Genbank. And only four genes related or matched with gene previously identified. Pi000192 and Pi000193 might relate to gene M-25, Pi004848 was the same gene as M-90, and Pi014092 was related to gene M-96. Other 40 genes were recognized as regulators such as zinc finger transcription factors, protein kinases and phosphatase or enzymes, respectively, and none of them were related to the potential receptor proteins. Notably, a half of the genes that induced more than 100-fold were not matched the protein sequences in Genbank. These genes might provide us a direction to focus for uncovering the potential receptor proteins.

1.7. Objectives of this study

The sexual reproduction of *Phytophthora* was triggered by the mating hormones α1 and α2

produced by A1 and A2 mating types, respectively, to induce the sexual reproduction of the counter mating type. Although the structures of α hormones were solved in 2005 and 2011, respectively,[45,48] chemical biological aspects such as identification of their receptors and biosynthetic systems are to be elucidated. In this thesis, efforts were focused on analyzing the α1 receptor.

The first objective of this study is focus on the specificity of α hormones. Although the function of α2 was thought to be only inducted the sexual reproduction of the A1 mating type, α2 was found to interfere with the α1-induced sexual reproduction of the A2 mating type. Detail analysis was carried out by using 10 α hormone derivatives, and a plausible mechanism for this phenomenon was proposed from the aspect of the α1 receptor (Chapter 2).

The second objective is to identify the α1 receptor by several prepared probes. Fluorescent probes were used to detect the specific expression of the receptor in different mating types and organelle in mycelia and in protoplasts. A magnetic probe was used to purify the receptor from protein extracts of mycelia. A photoaffinity probe was used to isolate the receptor. The details of these probes will be discussed in Chapter 3.

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Chapter 2

Specificity of α hormones: Interference of α1 activity

2.1. Introduction

The heterothallic species of *Phytophthora* consist of two mating types A1 and A2, which, when paired, produce sexual spores called oospores. Oospores are a persistent form of progeny that can survive in extreme environments for a prolonged period[1] and is responsible for genetic diversity[2] and global migration.[3] This feature of the sexual reproduction makes it difficult to control this plant pathogen in agricultural fields. The ability that heterothallic *Phytophthora* species produce oospores was regarded to be due to the chemical stimulation during pairing.[4] This hypothesis was first proposed by Ashby, and after that, lots of effort had been made to try to obtain evidences to support this hypothesis. Although early attempts were not so successful, a direct evidence was finally provided by Ko, who used polycarbonate membrane that only allowed the penetration of water soluble chemicals and successfully observed oospore formation on both the opposite mating types.[5] This observation suggested that unknown diffusible hormonal substances were produced and secreted by each mating type of *Phytophthora*. A further proposal by Ko was that the mating hormone produced by the A1 mating type, designated as α1, can induce the sexual reproduction of the counter A2 mating type, whereas the sexual reproduction of the A1 mating type can only be induced by hormone α2 produced by the A2 mating type.[6]

The structure of hormone α1 was solved by using 1.2 mg of pure hormone that was isolated from the A1 mating type culture broth of *P. nicotianae*.[7] Hormone α1 was determined as a novel linear diterpene with 4 stereogenic centers. The absolute configuration of two asymmetric centers of α1 hormone were then successfully determined.[8] Later, the total synthesis of 4 possible stereo isomers revealed the absolute configuration of α□ (Fig. 2.1).[9] Six years later, our group determined the structure of the second mating hormone of

Phytophthora α2 (Fig. 2.1) by spectroscopic analysis and total synthesis, as well as their biosynthetic pathway.[10]

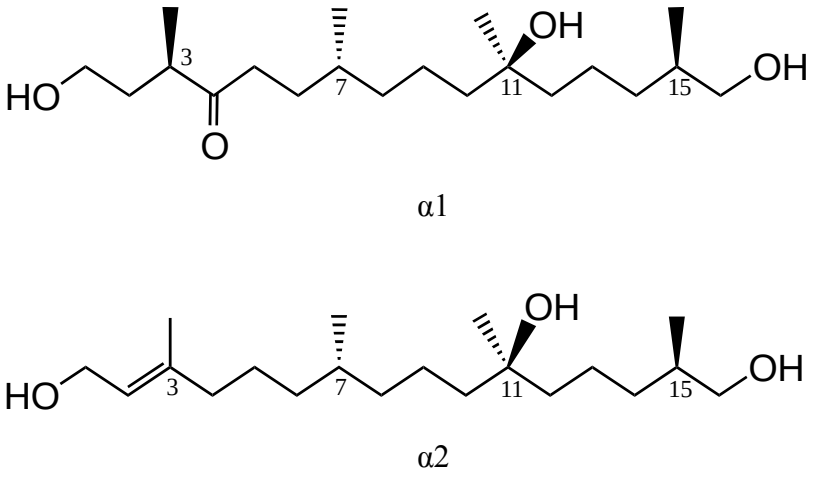


Figure 2.1. Structures of *Phytophthora* mating hormones α1 and α2

The sexual reproduction of *Phytophthora* rarely occurs in the field but is easily observed in the laboratory. When the A1 and A2 mating types are co-cultured across a membrane filter (to avoid direct contact), both types produce oospores within a few days. Based on the theory proposed by Ko, the recognition between α hormones and their mating types is exclusive[6] (Fig. 2.2A). Therefore, I thought that there was no interference (potentiation or inhibition) between two mating hormones. However, in the course of mating experiments, I observed that the α1-induced oospore formation of the A2 type was suppressed in the presence of α2, which is originally a product of the A2 type. This phenomenon was also observed when the A1 type was replaced with α1, so it appears that α2 is an antagonist of α1 (Fig. 2.2B). I herein report a detailed analysis of this unexpected phenomenon using a paper disk hormone assay.

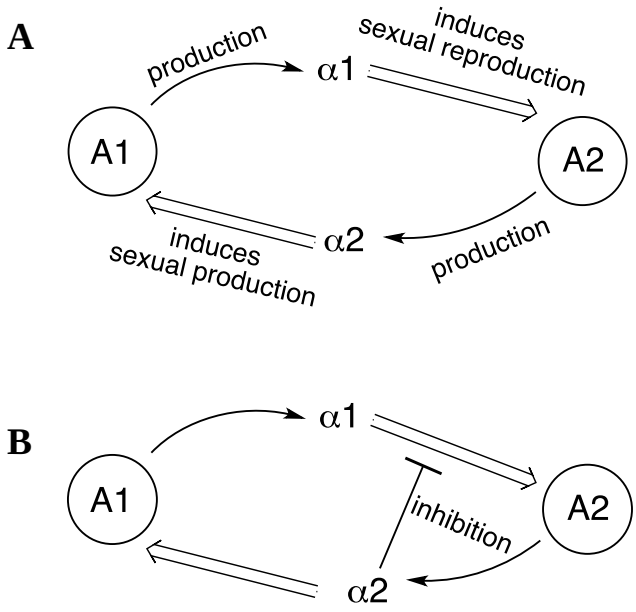


Figure 2.2. Function of *Phytophthora* mating hormones α1 and α2

A: Exclusive recognition between mating types and their hormones

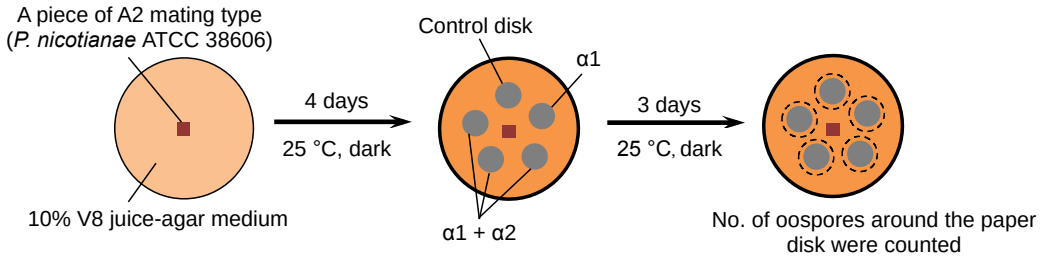
B: A new finding. α2 interferes with the α1 function, which means that A2 type blocks its sexual reproduction by own producing hormone α2.

2.2. Results and discussion

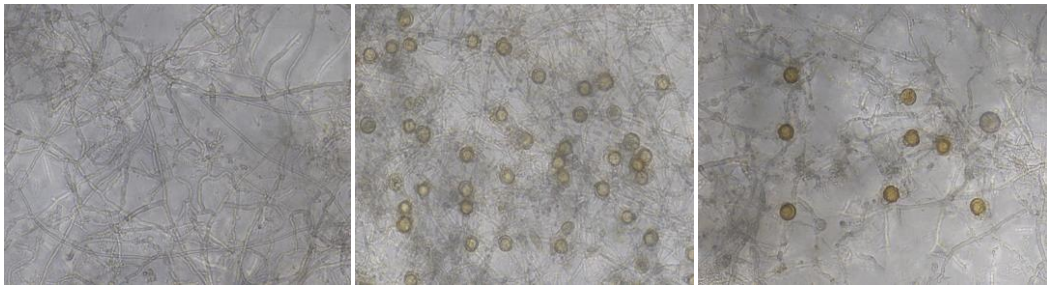
2.2.1. Interference of α1 activity by α2

To investigate the interfering effect of α2 to α1, the hormonal activity α1 in the present of α2 was tested using *P. nicotianae* ATCC 38606 (A2 mating type). Briefly, a piece of *P. nicotianae* pre-cultured on 20% V8 juice-agar medium was inoculated on the petridish containing 10 % V8 juice-agar medium. After 4 days incubation at 25℃, α1 or premixed α1 and α2 was applied to a paper disk and put on the colony. After incubation for additional 3 days, the number of oospores formed around the paper disk was counted under a microscope (Fig. 2.3A). Without α1 (control), no oospores formed on A2 mycelia. The α1-induced oospore formation was found to be suppressed by the simultaneous administration of 100 ng/disk of α2 (Fig. 2.3B). Furthermore, hormone α2 dose-dependently suppressed the α1-induced oospore formation of *P. nicotianae* (Fig. 2.3C).

A



B



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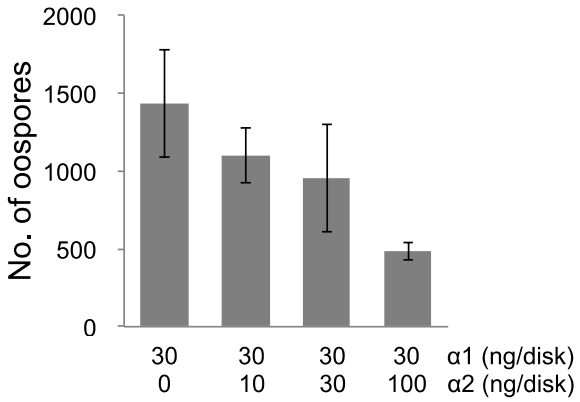

Figure 2.3. Interference activity of hormone α1 by α2.

A: Evaluation of the hormone α1 interference by α2 using *P. nicotianae* ATCC 38606 (A2).

B: Oospore formation of *P. nicotianae*. From left, control (no α1), 30 ng/disk of α1, and 30 ng/disk of α1+ 100 ng/disk of α2.

C: Dose-dependent interference of α1-induced sexual reproduction by α2.

2.2.2. Interference of α1 activity by α derivatives

To obtain further information on this unexpected phenomenon, structure-activity relationship (SAR) was examined by using α hormone derivatives **3**–**12** (Fig. 2.4), which were prepared previously.[11]

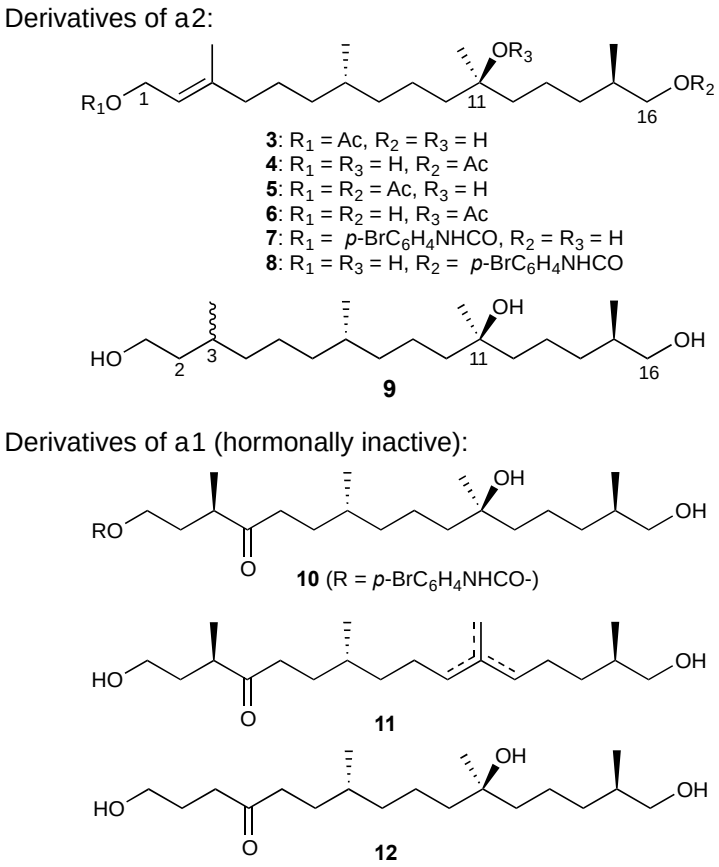

Figure 2.4. Structures of α hormone derivatives used for SAR analysis

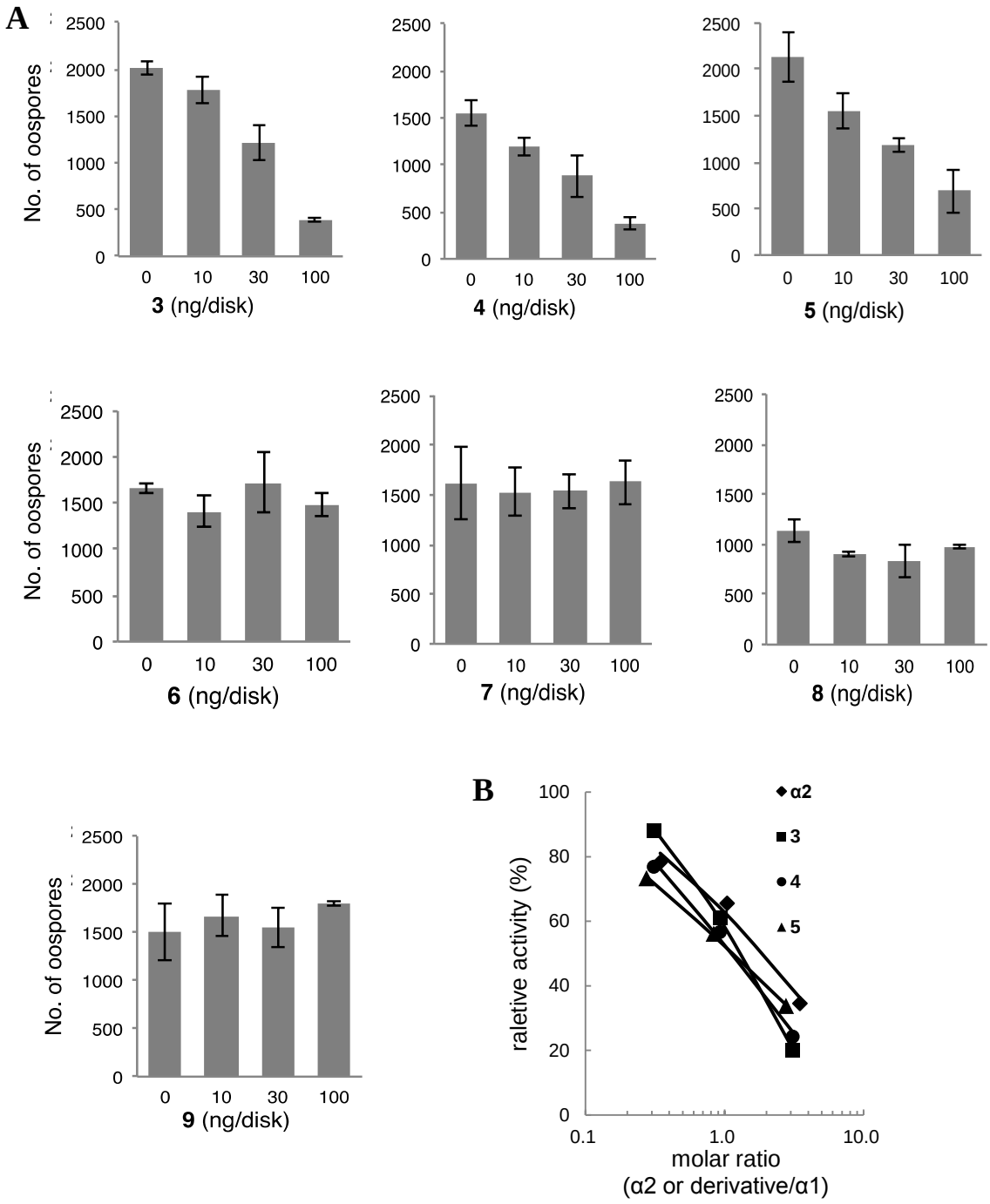

Figure 2.5. Interference of α1 activity by α derivatives.

A: The effect of α2 derivatives (**3-9**) on α1-induced oospore formation.

B: Data for α2 and compounds **3-5** are plotted (sigmoid fitting) using the relative oospore numbers as hormonal activity with the molar ratio of α2 or α2 derivatives to α1 as the horizontal axis.

Three acetates, 1-*O*-acetyl α2 (**3**), 16-*O*-acetyl α2 (**4**), and 1,16-di-*O*-acetyl α2 (**5**), showed inhibitory activity against the α1 activity (Fig. 2.5A). These activities were compared by plotting the relative activity against the molar ratio of α2 (or a derivative) to α1 (Fig. 2.5B), which indicated that the three acetate derivatives showed comparable activity to natural α2 and an approximately equivalent amount of these molecules can inhibit 50% of the α1 activity. Interestingly, no inhibitory activity was observed for 11-*O*-acetyl α2 (**6**), suggesting that of the three hydroxy groups of α2, the 11-OH group is essential for this antagonistic activity (Fig. 2.6). Since no significant inhibitory activity was observed for monocarbamates of α2 (**7** and **8**) unlike the monoacetates **3** and **4**, we hypothesized that this group might be too large to fit the ligand-binding pocket of the α1 receptor to interfere with α1 function. Another possibility, the biotransformation of the acetates to active α2, could be excluded because no significant difference in activity was observed between α2 and the acetates. The C2-C3 double bond of α2 was found to be another essential substructure because the dihydro derivative **9** showed no inhibitory effect despite the presence of the 11-OH group and the absence of the huge carbamoyl group (Fig. 2.6).

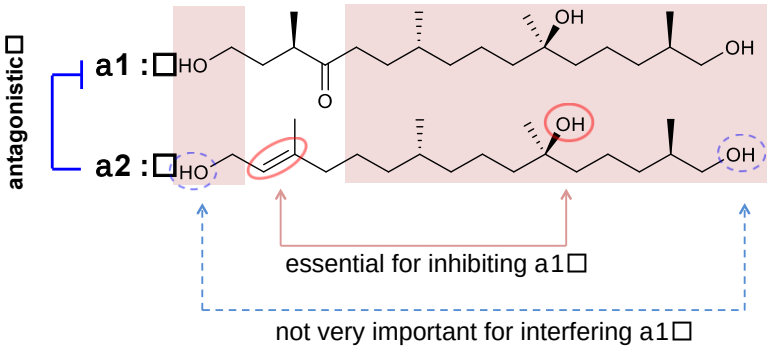

Figure 2.6. Structure-activity relationship of the α hormones.

Important structural elements are emphasized by circles.

Besides the α2 derivatives, three α1 derivatives (**10**-**12**) were tested and no-inhibitory activity was observed (Fig. 2.7), supporting the importance of the double bond of α2.

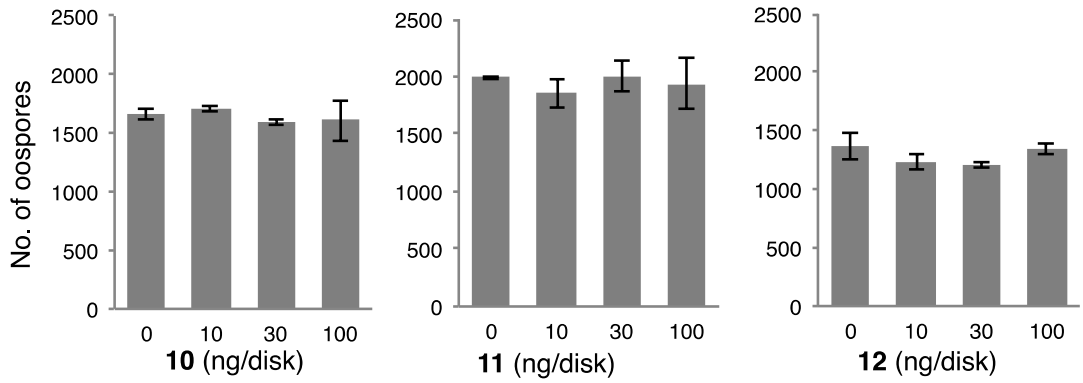

Figure 2.7. Graphical representation indicating the effect of α1 hormone derivatives **10**-**12** on the α1-induced oospore formation.

2.2.3. A putative model for the interference of α1 by α2

In the *Phytophthora* sexual reproduction system (Fig. 2.2), α1 and α2 originated from phytol exclusively induce the sexual reproduction of A2 and A1 mating types, respectively. Since this suggests the presence of the hormone receptors that strictly recognize the corresponding hormone, the result described above is surprising for us. The hormonal activity of α1 is likely to be triggered by its recognition by the α1 receptor ($R^{\alpha 1}$) expressed in the A2 mating type, and several structural features essential for the α1 function have been reported.[8)] We propose a model to explain the above-mentioned interference of the α1-mediated sexual reproduction by α2 (Fig. 2.8). The middle part (including two essential stereo centers of C7 and C11) of α1 is recognized by the recognition site of the receptor $R^{\alpha 1}$, and the left portion (including another essential α-methyl-branching ketone at C3-C4) of **1** binds to the active site

of $R^{\alpha 1}$, leading to the sexual reproduction of the A2 mating type. When α2 is present in the same time, the middle part of α2 is also recognized by the recognition site of $R^{\alpha 1}$, because this part is structurally shared with α1. On the other hand, the left part of α2 is not capable of activating the active site of $R^{\alpha 1}$ probably due to the absence of the ketone functionality, although this part retains the ability to bind to the active site. This antagonistic binding of α2 results in blocking α1 to induce the sexual reproduction of the A2 mating type. This may be a unique system inherent in the A2 mating type to avoid unprofitable sexual transformation by masking its receptor with its own mating hormone α2.

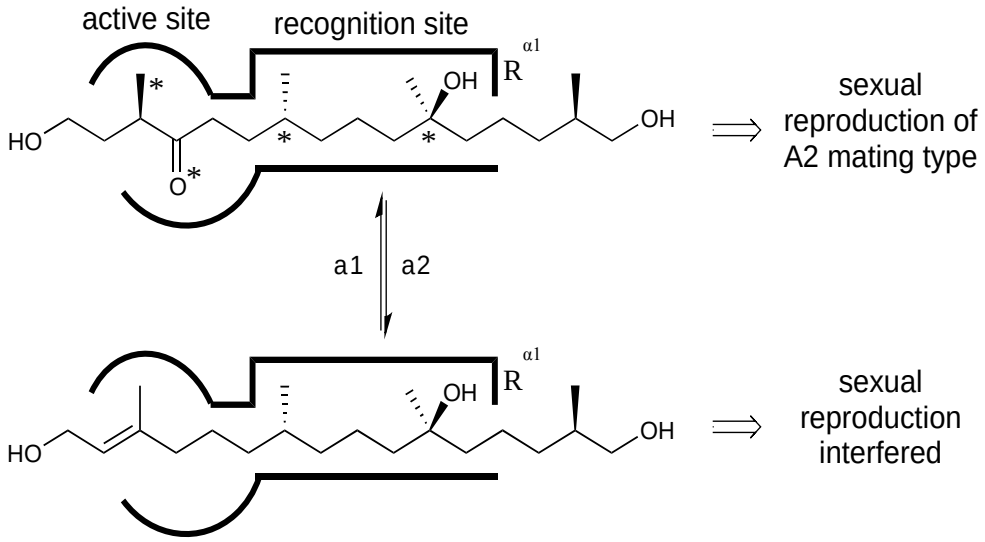

Figure 2.8. A schematic model for interference of α1-mediated hormonal activity by α2. α1 binds to the receptor ($R^{\alpha 1}$) expressed in the A2 mating type to promote its sexual reproduction; the substituents and asymmetric centers indicated with asterisks are essential for the hormonal activity [8] (upper chart). The ligand α1 in the complex is replaced by α2, in which the double bond may tightly bind to and block the active unit of the receptor $R^{\alpha 1}$, interfering with the α1-induced sexual reproduction (lower chart).

2.3. Materials and Methods

2.3.1. *Phytophthora* strains and hormone samples

The A2 mating type (ATCC 38606) of *P. nicotianae* was purchased from the American Type Culture Collection (ATCC, YA, USA). All the stock strains were cultured on 20% V8-juice medium with 2% agar and 0.3% $CaCO_3$. Each 10 mL medium was injected to the test tube, autoclaved and cultured at 25 °C, 60% humidity for 2 weeks in a KCL-2000A incubator (Tokyo RIKAKIKAI, Co., LTD, Japan), then preserved at 15 °C and renewed within 3 months.

Hormones α1, α2 and all the derivatives were synthesized by Dr. A. Yajima.[11]

2.3.2. Evaluation of hormonal activity

To evaluate α1 activity, a block (3 × 3 × 3 mm) of A2 mating type *P. nicotianae* (ATCC 38606) was inoculated onto a 9 cm Petri plate containing 20% V8-agar medium, which consists of distilled water, 20% V-8 juice (Campbell Soup Company, Camden, NJ, USA), 2% agar, and 0.3% $CaCO_3$. The plates were pre-cultured at 25 °C, 60% humidity for 10 days in the dark. A piece (5 × 5 × 3 mm) from plate edge area of the pre-cultured colony was then incubated for 4 days on a Petri plate (ϕ 9 cm) containing 10% V8-juice, 2% agar, and 0.02% $CaCO_3$. A solution (total 30 μL) of a test sample mixed with 30 ng of α1 was applied to a paper disc (8 mm diameter, 7 mm thickness, Advantec®, Tokyo, Japan). Sample were dried for 30 min in vacuo and then placed on the above mentioned culture disk at a distance of 1.5

cm from the colony center. The test plates were incubated at 25 °C, 60% humidity in the dark.

2.3.3. Microscopy observation

After 3d post inoculation (dpi), the medium around the paper disk (ϕ 1.8 cm) was cut out, and the total number of oospores formed in the entire area was counted under an Olympus CK40-F100 Inverted Phase Contrast Microscope.

2.3.4. Data collection and statistics analysis

Three of five duplicate data for each dose (0, 10, 30, and 100 ng/disc) were used for evaluating the hormonal activity of α1.

All the compounds were independently detected twice to check the repeatability.

2.4. References

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Chapter 3

Preliminary studies on α1 receptor in *Phytophthora*

3.1. Introduction

3.1.1. Mechanism of *Phytophthora* sexual reproduction

Heterothallic species of the plant pathogen *Phytophthora*, consist A1 and A2 mating types, which when paired, perform sexual reproduction to produce sexual spores called oospores. The *Phytophthora* uses mating hormones to regulate its sexual reproduction. Hormone α1 secreted by A1 mating type induces oospores in the A2 mating type，while hormone α2 produced by A2 mating type induces sexual reproduction in the counter mating type A1.[1] The structure of hormone α1 was solved by Qi *et al* in 2005.[2] Later, the total synthesis and absolute configuration of this hormone was described by Yajima *et al*.[3] In 2011, the structure of hormone α2 was uncovered, as well as biosynthetic pathway of the hormones as described in Chapter 1.[4]

Based on the previous researches, the mechanism of *Phytophthora* sexual reproduction was proposed as follows (Fig. 3.1).

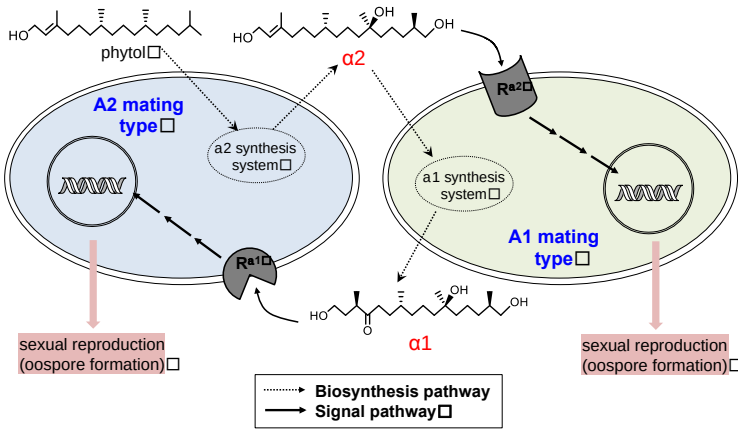


Figure 3.1. Putative mechanism for *Phytophthora* sexual reproduction (receptor location is temporary)

The A2 mating type of *Phytophthtora* was found to convert phytol to α2, which is then accepted by the counter mating type A1 to induce the sexual reproduction of the A1 mating type. The A1 mating type then converts α2 to α1, which is then accepted by the A2 mating type to induce oospores on A2 mating type. In the *Phytophthtora* species, there must be hormone biosynthesis systems and α hormone receptors to recognize the fine structure of the hormones. In this chapter, I focus on several chemical probes used to identify the hormone receptors, especially α1 receptor, $R^{\alpha 1}$.

3.1.2. Typical methods for receptor identification

The receptor screening methodologies can be mainly based on the detection of a second messenger, a functional response, or the interaction of a ligand with its receptor.[5] In which, the ligand-receptor interaction plays a very important role in biological system and their classic determination method by affinity resins (Fig. 3.2) was widely and successfully used for protein targeting. The affinity resins preparation requires structure-activity relationship studies of a bioactive small molecular ligand to determine the appropriate sites for linker modification.[6] Most conventional and convenient way for ligand modifying is radioactive labeling. Ligands labeled with radio-isotopic labels such as ^{3}H, ^{125}I and ^{32}P that did not affect the affinity between the ligand and receptor for binding. However, the radioactive receptor-ligand binding assay has many disadvantages. They are producing radioactive waste, requiring special laboratory technology and conditions, expending expensive cost, and so on. Worst of all, they are hazardous to human health.[7] Then, the non-radioactive labeled new

technologies must be developed.

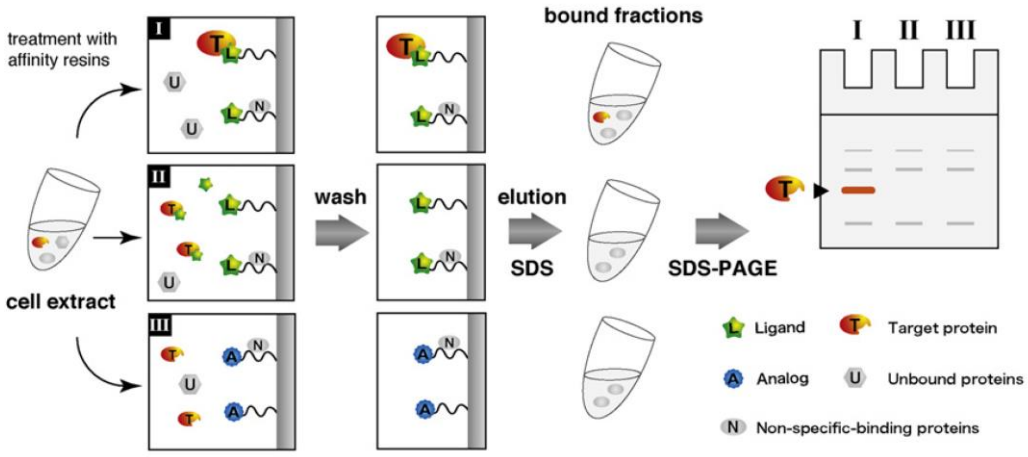


Figure 3.2. Identification of the protein target of a bioactive small molecule

3.1.2.1. Fluorescence labeling

Fluorescence become increasingly popular as a multifaceted detection toll in life science due to the greater photostability, enhanced brightness, and improved physical properties of the dyes.[8, 9] Especially for the Alexa Fluor family, which is insensitive to pH, photostable, and high solubility in water. Most importantly, it consists with a broad range of excitation and emission wavelengths that can be suitable for the most of the detection techniques. Fluorescent molecules binding to receptors is a good strategy due to the visualization of receptor locations.[10] Morishima *et al.* developed a high-affinity fluorescent probe for selecting the α_{1L}-adrenoceptor.[11] Although the fluorescent probe displayed a 10-fold reduction in binding affinity with receptor, it was still successfully visualized the α1L-adrenoceptor localization which is in the muscle layer of the human prostate. Hern *et al.* reported a muscarinic probe, synthesized by the reaction of a telenzepine amino congener to an Alexa

Fluor 488 fluorophore which have a nanomolar affinity and very slow dissociation kinetics to visualize and monitor receptor–ligand complexes in living cells.[12] Irani *et al.* developed a bioactive Alexa Fluor 647 brassinosteroid (BR) analogue, first visualized the receptor-ligand complexes in living *Arabidopsis thaliana* cells.[13]

However the choice of the fluorescence to label ligands is critical in experimental development, such as selection of the correct fluorescent dye, the molecular size and the use of a spacer.[14] In most cases, a spacer between ligand and fluorophore is needed to reduce steric hindrance caused by the attachment of a bulky fluorophore. However sometimes the length of the spacer can also bring a negative effect on the affinity, for this reason a range of labeled ligands need to be examined to find the optimal ones. Moreover, an appropriate fluorophore should also be non-toxic and have excitation and emission profiles in the visible region.[15]

3.1.2.2. Affinity beads

Isolation and purification the potential proteins without reducing their activity is a very difficult work. Especially when the proteins must be purified from crude cell extracts with the retaining of their inherent activity. Affinity chromatography matrixes bearing with bioactive molecules play an important role in the discovery of target proteins and the elucidation of bioactive interactions. The successful isolation of target proteins depends on the maximum binding efficiency between synthesized matrices and cellular extractions.[16] Magnetic adsorbent particles as matrix possess a uniquely attractive property that can be derivative with any of the ligands to purify target proteins.[17] Magnetic separation is relatively fast, easy,

gentle and highly efficient.[18] Many protein purification articles treated with magnetic particles or magnetic beads were performed. However, instability and low dispersibility of conventional matrices sometimes leads to low efficiency in affinity purification. Most importantly, inefficiency of conventional matrices, in particular, nonspecific binding of irrelevant proteins to the affinity matrices is a significant limitation to this biochemical approach.[19]

To resolve these issues, a high-performance affinity magnetic beads, ferriteglycidyl methacrylate (FG beads) that have high stability and dispersibility in both organic solvents and aqueous solution were developed to enable one-step affinity purification with target proteins.[20] The FG beads carried with a structure consisting of several 40 nm diameter MPs/poly(styrene-co-GMA)/polyGMA, and showed extremely high performance without non-specific binding of proteins compared with other commercial beads. To purify thalidomide-binding proteins, Ito *et al.* performed affinity purification using FG beads. The carboxylic thalidomide derivative was covalently conjugated to the beads and incubated with HeLa cell extracts to identify thalidomide-binding protein cereblon (CRBN).[21] Umeda *et al.* using FG beads successfully purified the arginine target factor complex, so called cofactor(s).[22] More recently, with the fixation of apigenin and genistein (two major flavonoids) onto magnetic FG beads, Oishi *et al*. discovered the direct target of flavonoids was apigenin, but not genistein.[23]

3.1.2.3. Photoaffinity labeling

The photoaffinity labeling (PAL), introduced by Westheimer in 1960s,[24] is a special type of

chemical modifications, which is often applied to study the interaction between ligands and their receptors in biological filed.[25] In the process of the photoaffinity labeling, under the activation by UV irradiating, a ligand is covalently modified with a photoactive group (PG). The modified complex then can be used to investigate the ligand receptor interactions; to isolate the unknown receptors; or to identify the location of receptors. There are three major photoactive groups that are used in photoaffinity labeling (Fig. 3.3): aryl azide; benzophenone; and diazirine.[26]

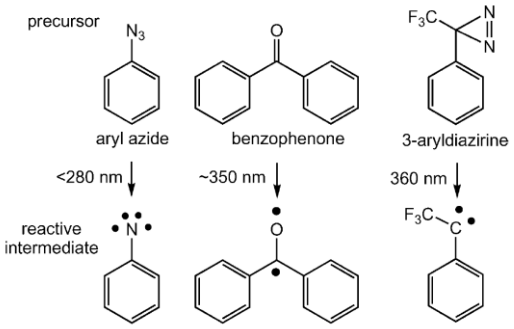


Figure 3.3. Photochemical reactions of major photophores for photoaffinity labeling

Although aryl azides are easily prepared, they are photoactived below 280nm wavelength at which cause damage to biological molecules, and produce nitrenes.[27] azides can also cause the undesired side production: keteimines.[28] Benzophenones are reusable for photolabeling, however it sometimes needs long period of irradiation which is a risk to yield nonspecific binding.[29] Diazirine are widely used to study ligand-receptor interaction and identify unknown receptors. It can rapidly form cross-link to biomolecules with short irradiation time that even can be used on living cells. Although nonspecific labeling is a major problem in photoaffinity labeling, this can be avoid by adding negative control reagent and using more

purified protein extractions.[30]

Photoaffinity labeling often associate with other techniques for rapid detection or isolation the target proteins, such as radioactive labeling or click chemistry reaction (Fig. 3.4).[31]

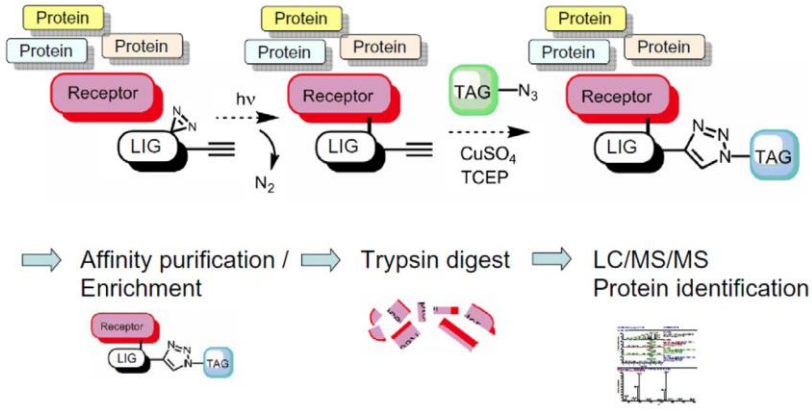


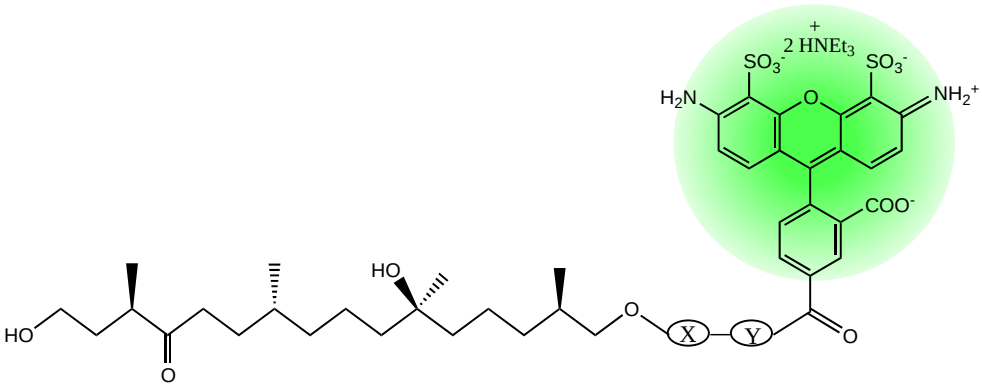
Figure 3.4. Labeling of unknown receptors with use of activatable bifunctional probes.

Some plant hormones brassinosteroids (BRs), jasmonate (JA) associate with photoaffinity labeling strategy has benn successfully detected the hormone-binding domain.[32-34]

3.2. Results and discussion

3.2.1. Fluorescent probe: Mating type specificity and location of the receptor

Three fluorescent probes α1-probes 1-3, which consist of ligand, linker and fluorescent parts were designed by our team and synthesized by our co-worker Dr. A. Yajima at Tokyo University of Agriculture. Two linkers, aminocaproic acid (Acp) and polyethylene glycol (PEG), were used (Fig. 3.5).



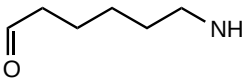
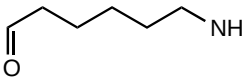
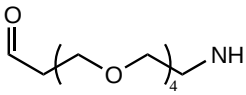
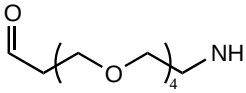
probe name	linker X	linker Y
a1- probe 1 (a1- Acp-Alexa 488)		none
a1- probe 2 (a1- Acp-PEG-Alexa 488)		
a1- probe 3 (a1- PEG-Alexa 488)	none	

Figure 3.5. Chemical structures of fluorescent probes.

3.2.2. Hormonal activity of fluorescent probes

The hormone activity of α1-fluorescent probes was tested by using *P. nicotianae* ATCC 38606 (A2 mating type) by the method described in Chapter 2. The results are summarized in Fig. 3.6.

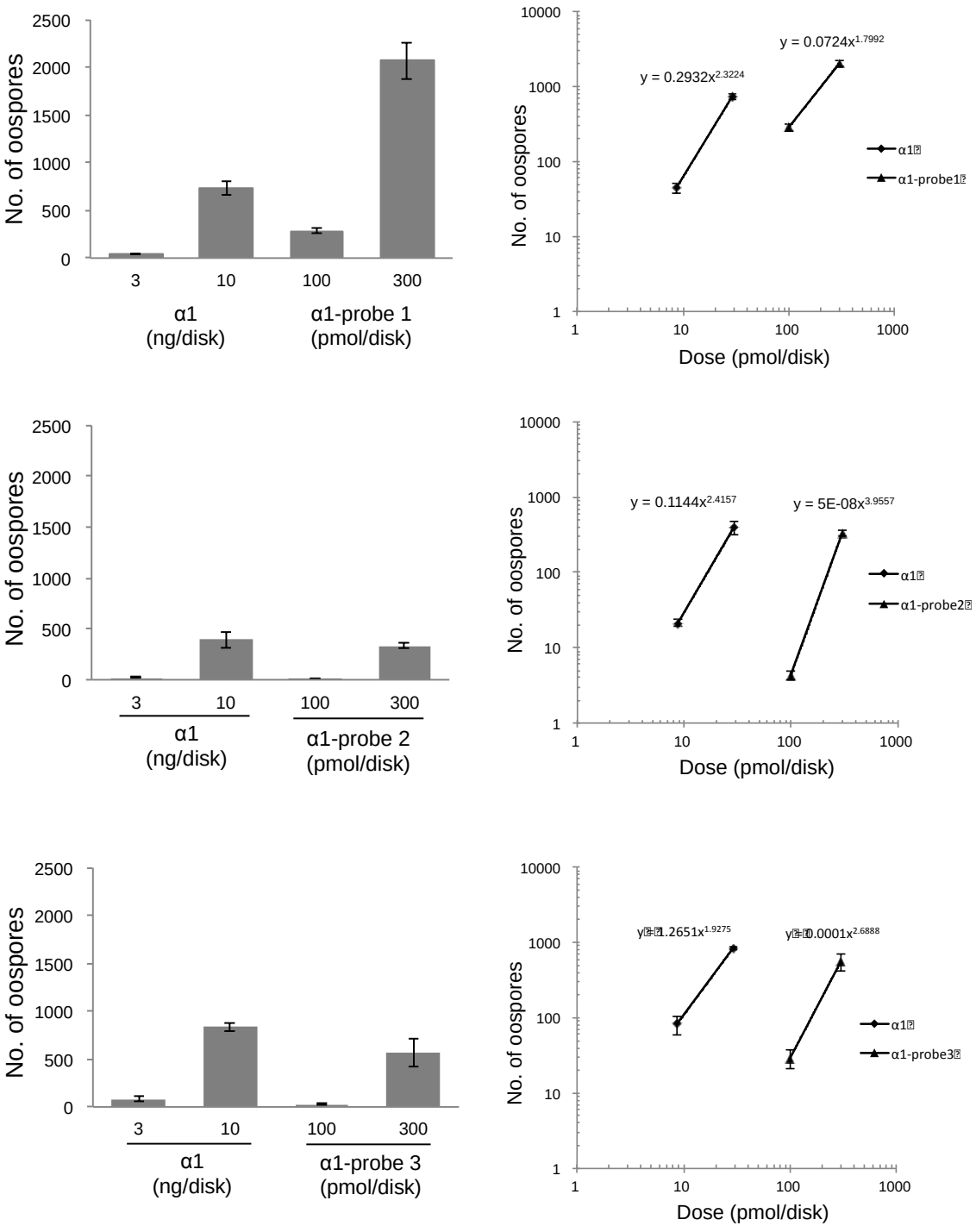


Figure 3.6. Hormonal activity of fluorescent probes α1-probe 1-3.

Left: Original data.

Right: Comparison graphs of probes and α1.

The hormonal activity of α1 fluorescent probes was evaluated based on their oospores-inducing ability to the A2 mating type strain of *P. nicotianae*. To compare the hormonal activity of α1 and the probes, I calculated the dose (in pmol) required for inducing 400 oospores based on Fig. 3.6. Namely, the hormonal activity = 100 × (dose of α1 required for inducing 400 oospores) / (dose of α1 fluorescent probes required for inducing 400 oospores). The data are summarized in Table 3.1. After calculating, the hormonal activities of α1 fluorescent probes were 18%, 7.4% and 7.2% respectively.

Table 3.1. Relative hormonal activity of α1-fluorescent probe 1-3.

Probe	Dose of probe for 400 oospores (pmol)	Dose of α1 for 400 oospores (pmol)	Relative activety (%)
1	136.10	24.64	18
2	224.54	16.51	7.4
3	310.05	22.25	7.2

3.2.3. Staining of mycelia with fluorescent probes

To evaluate specific staining ability of α1 fluorescent probes, the *Phytophthora* mycelia were treated with α1-probe 1 (as an example) or Alexa Fluor 488 dye (as control). Mycelia were stained by α1-probe 1 but not by fluorescent dye without α1 part, indicating that this fluorescent probe could be used for detecting the receptor (Fig. 3.4).

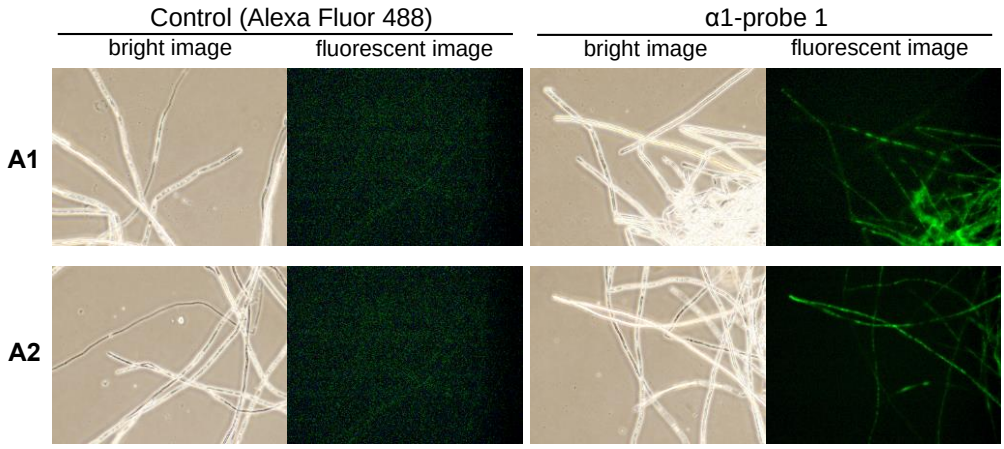


Figure 3.7. *Phytophthora* mycelia stained with α1-probe 1 and Alexa Fluor 488 dye. Mycelia were stained with 4 μM Alexa Flour 488 or α1-probe 1 for 60 min. The images were detected at 1 sec. exposure time.

To investigate the location of receptors in *Phytophthora*, 7 days old mycelia of A2 mating type was used to incubate with 4 μM of α1-probes, without or with free α1 to compete with each probe. The mycelia of A1 mating type was also incubated with probes to compare (Fig. 3.8). From the images, A2 mating type can be stained by all α1-probes. Hormone, adding free α1 did not reduce the intensity of the fluorescent light, and both A1 and A2 mating types were equally stained, which inferred that these results might be due to the non-specific staining of cell organelle (for example cell wall).

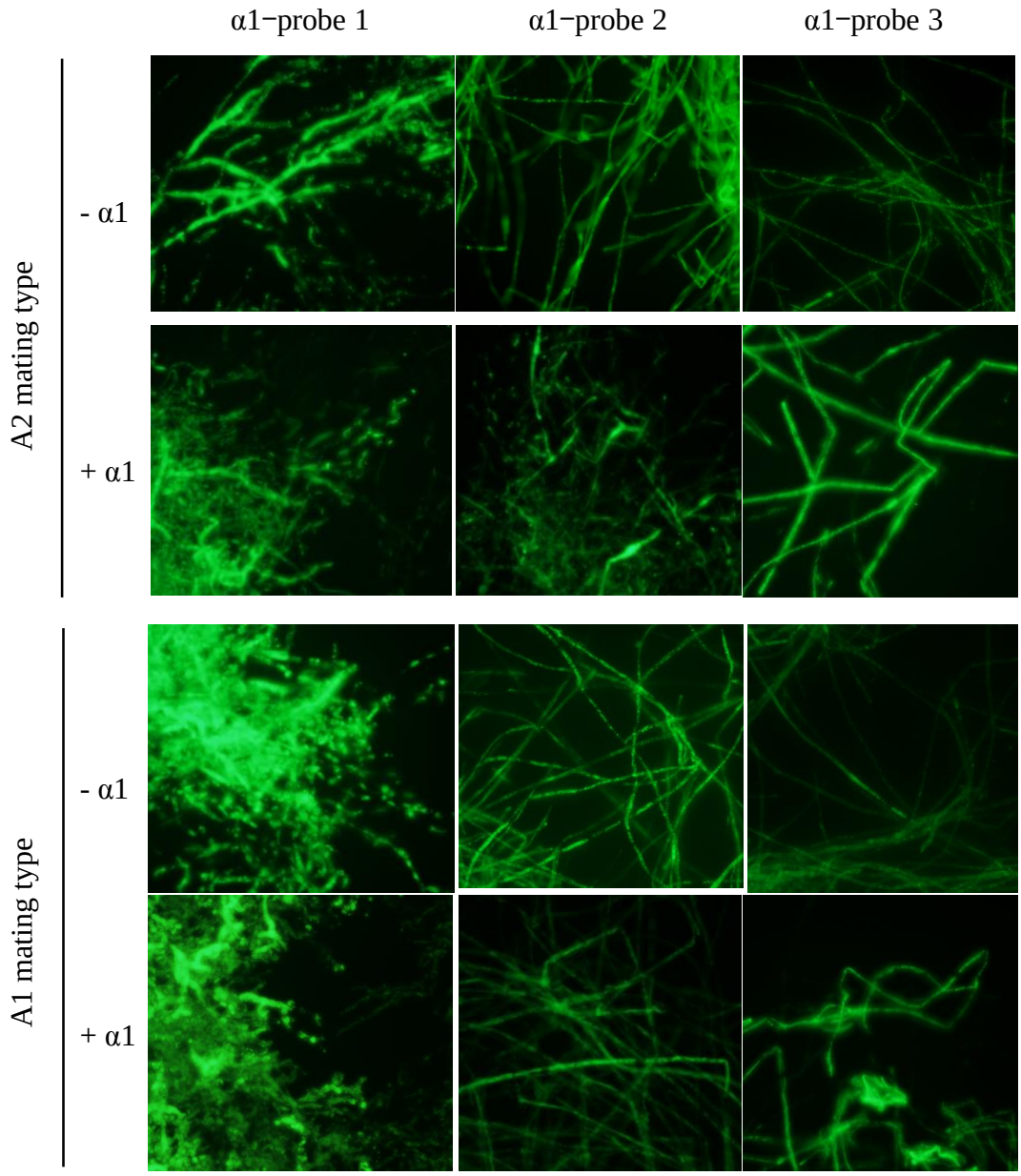
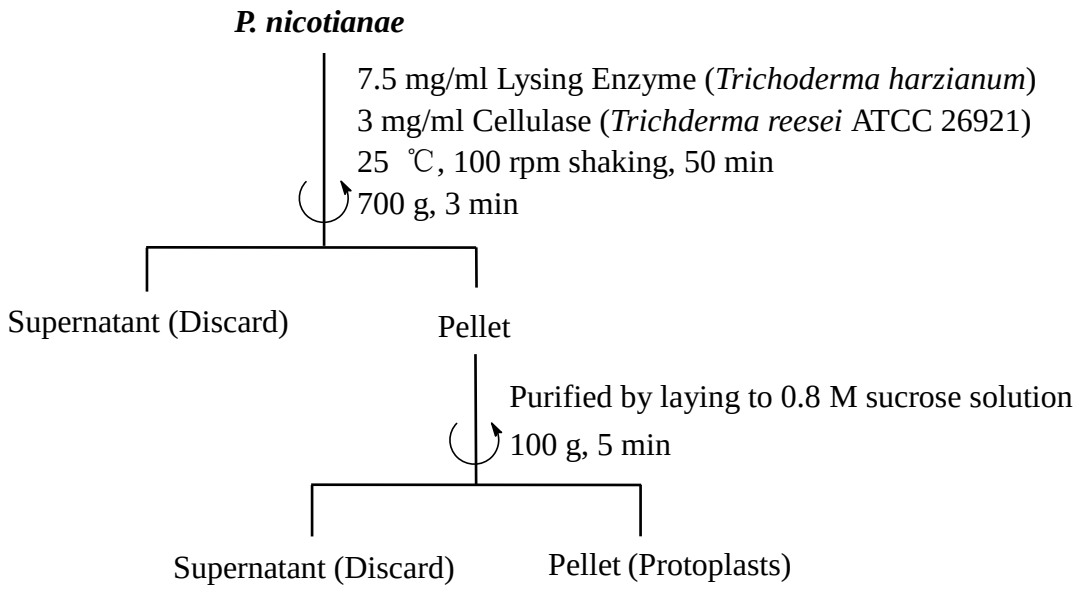


Figure 3.8. Images of A1 and A2 mating types of *Phytophthora* mycelia stained with α1 fluorescent probes. (Mycelia were incubated with 4 μM free α1 or α1-probes with 60 min. Images were detected at 1 sec. exposure time).

3.2.4. Staining of protoplasts with fluorescent probes

To avoid the non-specific cell wall staining, protoplasts (Scheme 3.1) were prepared from the *Phytophthora* mycelia (both A1 mating type and A2 mating type) and incubated with α1 fluorescent probes 1, which showed the highest hormonal activity in all α1 fluorescent probes (Fig. 3.9). The protoplasts of both A1 and A2 mating types were not stained, and the addition of free α1 showed no difference between A1 and A2 mating types.



Scheme 3.1. Protoplast preparation

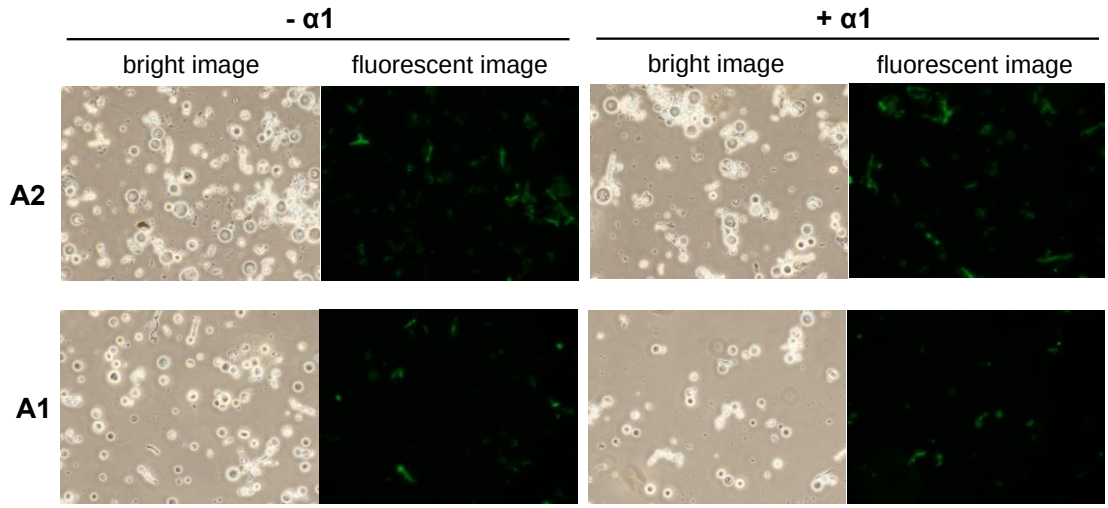


Figure 3.9. *Phytophthora* protoplast incubated with α1-probe 1.

Protoplasts were incubated with 4 μM free α1 or α1-probes with 60 min. Images were detected at 1 sec. exposure time

3.2.5. Magnetic Beads Probe: Affinity separation

In order to partially purify the α1 receptor expressed in *Phytophthora* A2 mating type, magnetic beads probe α1-FG beads probe (Fig. 3.10), in which PEG-linked α1 was coupled with FG beads, was prepared and used for the affinity purification in vitro. PEG-linked α1 was synthesized by Dr. A. Yajima at Tokyo University of Agriculture.

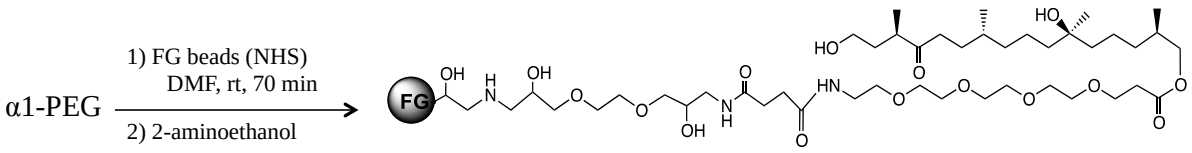
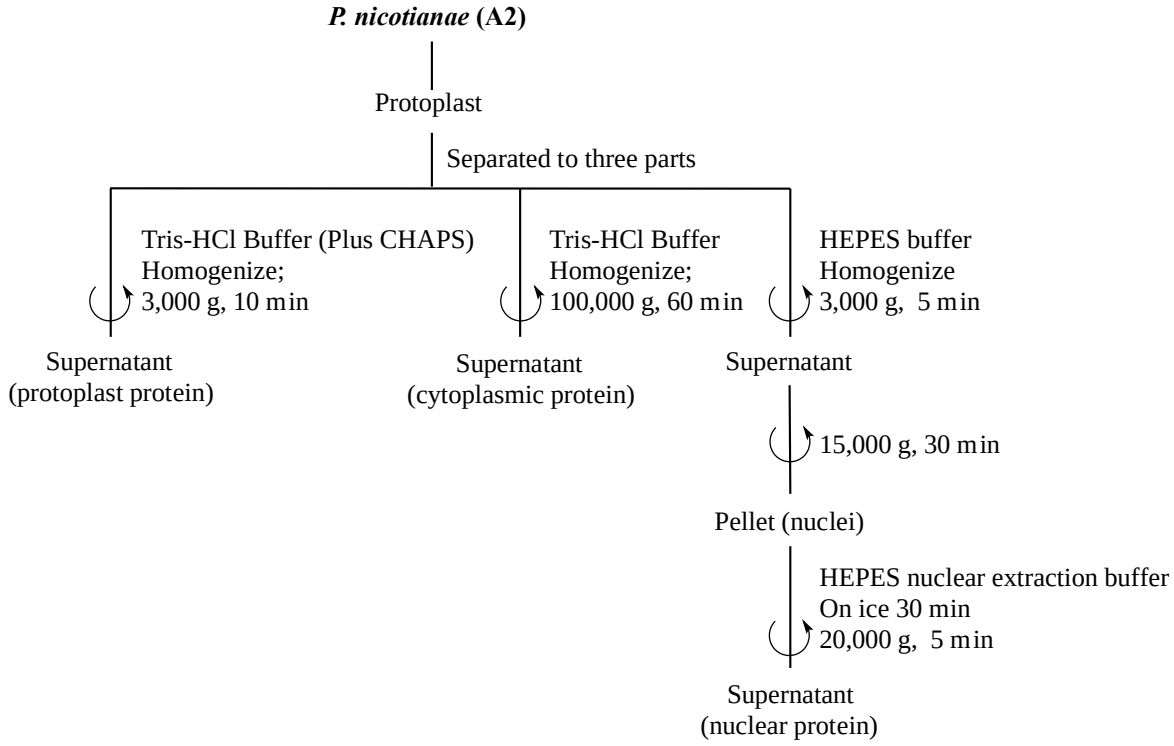


Figure 3.10. Preparation of α1-FG beads probe

Three kinds of protein were prepared from mycelia of A2 mating type of *P. nicotianae*, that

is, protoplast protein (P), cytoplasmic protein (C) and nuclear protein (N) (Scheme 3.2). These protein extracts were incubated with control FG beads (without α1 bound on the beads surface) and α1 FG beads probe, without or with free α1 to compete (Fig. 3.11).



Scheme 3.2. Preparation of protoplast, cytoplasmic and nuclear proteins from mycelia

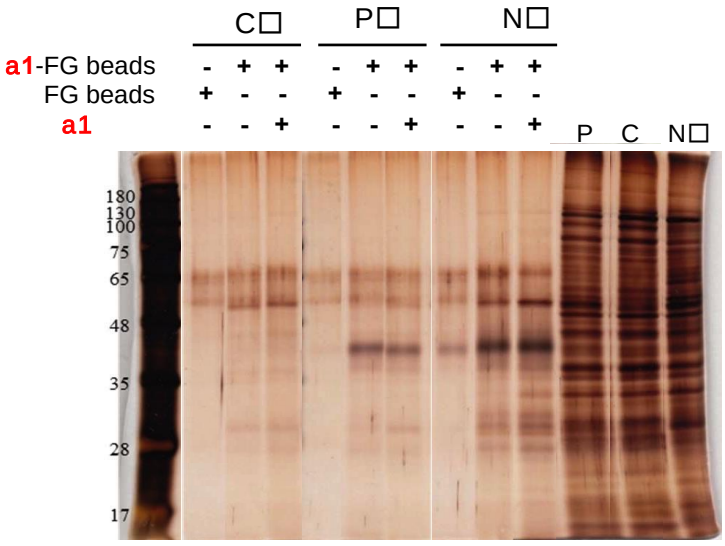


Figure 3.11. Affinity purification of α1 receptor using α1-FG beads probe.

Gels were silver stained. C: cytoplasmic protein; P: protoplast protein; N: nuclear protein.

Although, for the cytoplasmic protein (C), no clear bands were detected, a possible band was observed around 40 kDa for the protoplast protein and nuclear protein. However, because of no significant difference between without and with free α1, that band was inferred as a non-specific binding band.

3.2.6. Photoaffinity probe: α1-PAP

The photoaffinity probe, α1-PAP (Fig. 3.12), which consists of the ligand, reactive diazirine, and alkyne parts for the click chemistry reaction, was designed by our team and synthesized by Dr. Yajima at Tokyo University of Agriculture. This probe associated with two popular chemical modifications: (1) diazirine part, which is one of the most popular photoactive groups used for photoaffinity labeling and covalently binds to receptor or binding protein under UV irradiation;[26] (2) alkyne part, which performs click chemistry reaction with an azide group in the present of Cu(I).[35]

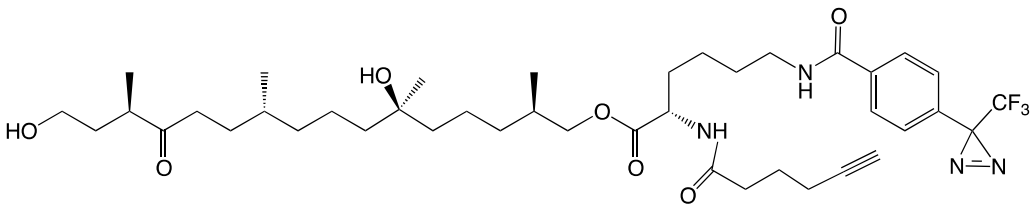


Figure 3.12. Chemical structure of photoaffinity probe α1-PAP.

First of all, the hormone activity of α1-PAP was tested using the A2 mating type of *P. nicotianae* ATCC 38606. 100 and 300 pmol/disk of α1-PAP, as well as 3 and 10 ng/disk of α1, were applied on the colony incubated for 4 days an 10% V8-juice medium. After 3 days

incubation, the medium around the paper disk was cut out, and the number of oospores formed in the entire area were counted. The results are summarized in Fig. 3.13.

To compare the hormonal activity of α1 and α1-PAP, reciprocal doses required for inducing 400 oospores were calculated. Based on the equation for both α1 and α1-PAP (Fig. 3.13 right), the relative hormonal activity of α1-PAP was determined to be 6.2%.

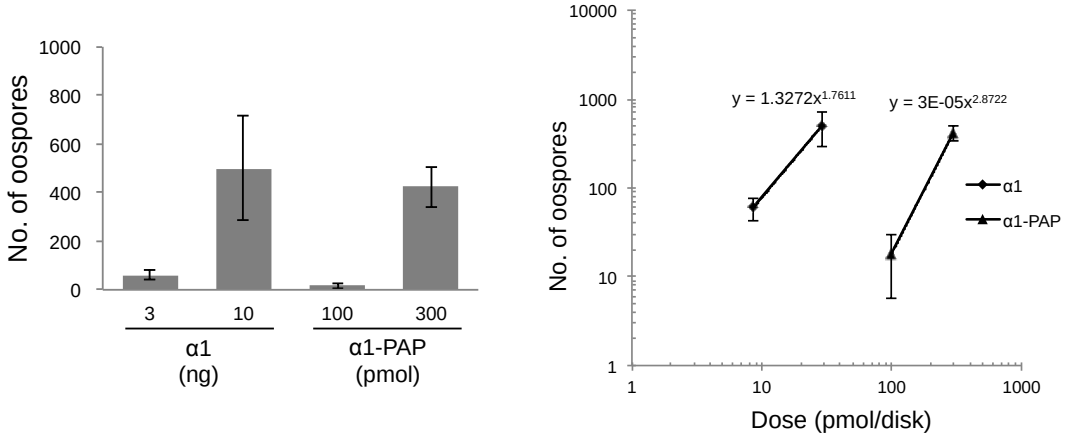


Fig. 3.13. Hormonal activity of α1-PAP.

The total oospores induced by α1 and α1-PAP (left). A graph in pmol unit (right).

To check the photo reactivity, α1-PAP was analyzed through HPLC before and after UV irradiation. Since the probe was insoluble in the protein extraction buffer (Tris-HCl buffer pH 7.5), 1% Tween 80 was added during the experiments (Fig. 3.14).

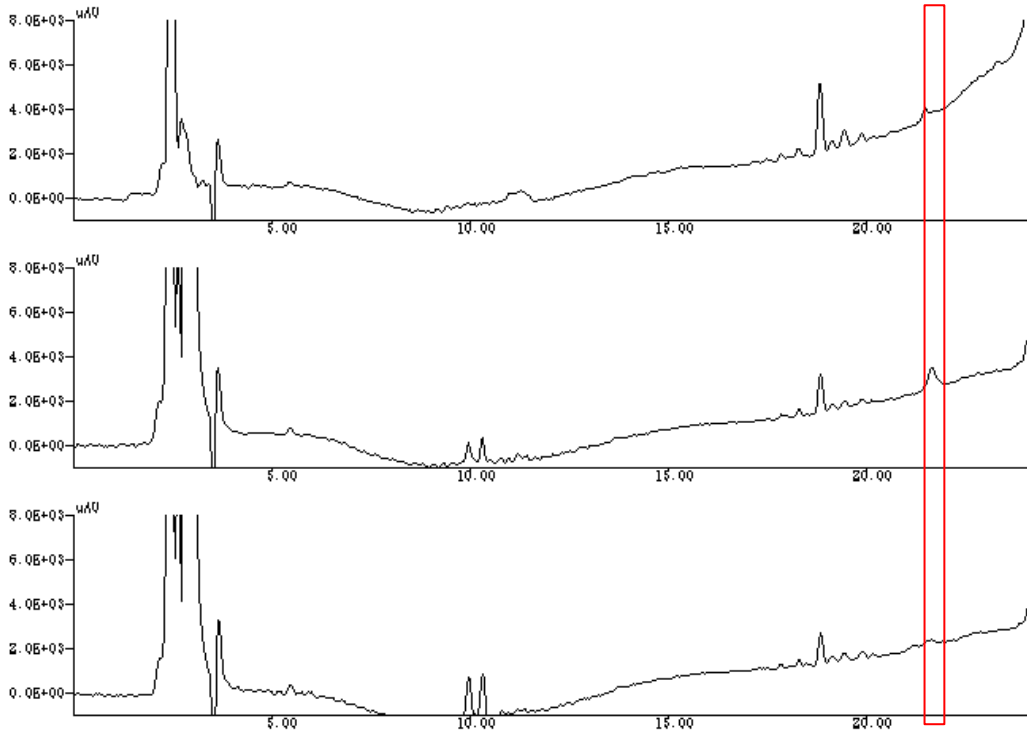


Figure 3.14. Photo reactivity of α1-PAP.

HPLC chart of 20 μl 1% tween 80 (top); 2 μM α1-PAP dissolved in Tris-HCl buffer with 1% Tween (middle); and dissolved α1-PAP after 15 min UV irradiation at 365 nm (bottom). Red square indicated the α1-PAP peak appearing time. All the samples were injected 20 μl. HPLC condition: Develosil ODS-UG-5 column; 20%-100% MeCN 20min; 100% MeCN 5 min; 1 ml/min detection at 225 nm.

The disappeared α1-PAP peak at 22 min in the HPLC chart (Fig. 3.14 bottom) indicated that this photoaffinity probe could react under the 365 nm UV irradiation in 15 min.

3.2.7. Photoaffinity labeling with α1-PAP followed by fluorescent labeling

Under the optimal conditions determined, cytoplasmic and nuclear proteins from A2

mating type mycelia of *Phytophthora* (Scheme 3.2) were extracted and incubated with α1-PAP, followed by UV irradiation, and click reaction with Alexa Fluor488 azide (Fig. 3.15). The reaction mixtures were applied to SDS-PAGE and analyzed by α fluorescent scanner.

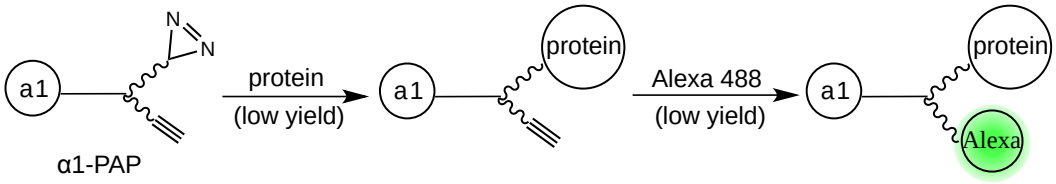


Figure 3.15. Experimental reaction of α1-PAP photoaffinity labeling

However no specific fluorescent bands were observed in the α1-protein-Alexa complex lanes (protein, α1-PAP, UV, Alexa are +) in both cytoplasmic and nuclear proteins. This unsuccessful result may be due to quite low yields of the two-step reaction, UV-promoted photoaffinity reaction followed by click reaction.

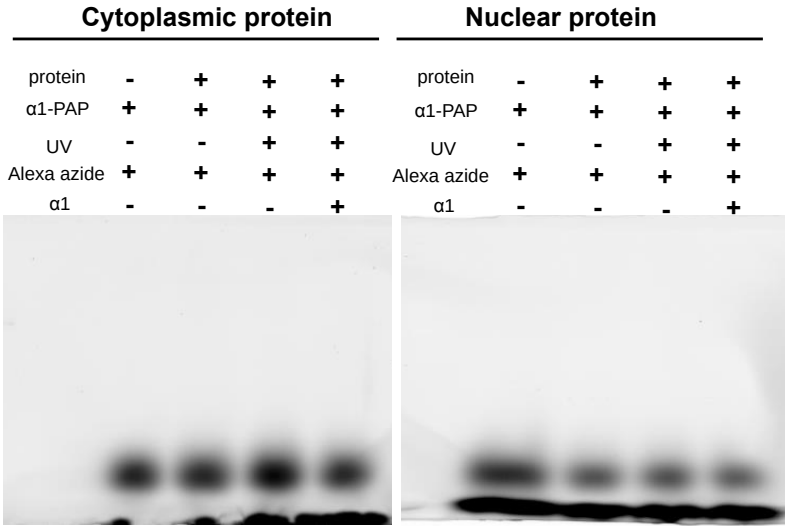


Figure 3.16. Fluoro images of α1-protein-Alexa complex.

So, a one step reaction method was next designed to perform (Fig. 3.17). Briefly, the

α1-PAP was first reacted with Alexa488 azide to prepare Alexa488-linked α1. This new photoaffinity probe, α1-PAP-Alexa 488 was then used for photoreaction with proteins.

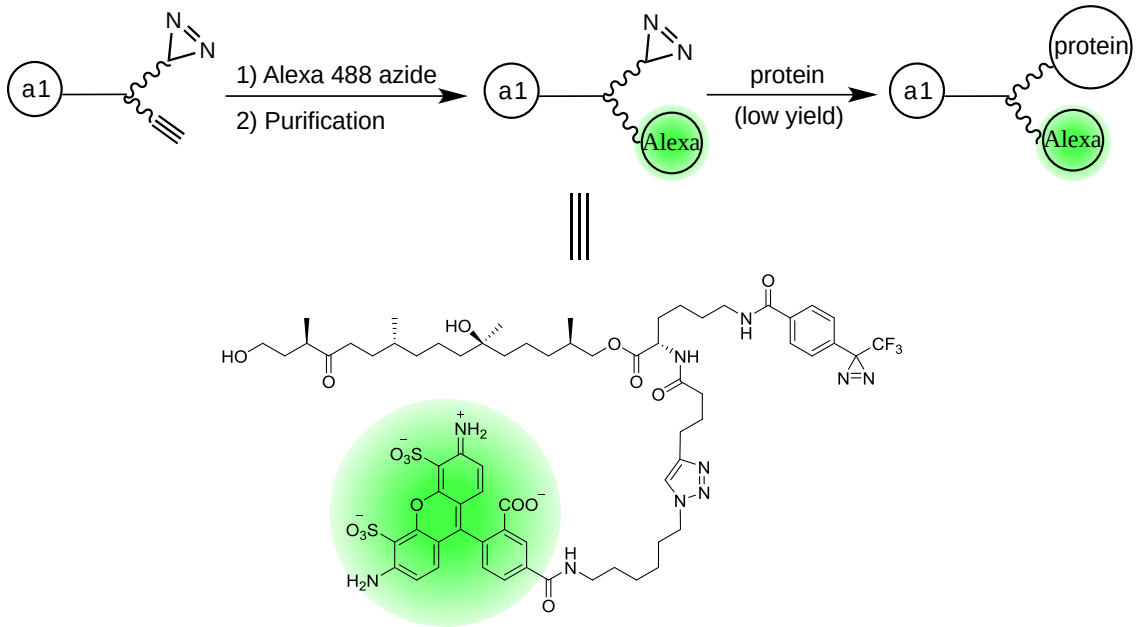


Figure 3.17. Experimental scheme of one-step photoaffinity labeling

The new probe, α1-PAP-Alexa488, was incubated with cytoplasmic or nuclear protein extracted from A2 mating type of the *P. nicotianae*, and then irradiated with UV at 365 nm for 15 min. From the SDS-PAGE gel imaging result (Fig. 3.18), the labeling efficiency was much better than the previous two-step reaction method (Fig. 3.15, 3.16). However, after adding free α1, no specific band reduced or disappeared in both cytoplasmic protein and nuclear proteins. This result may be due to the low yield of the receptor protein.

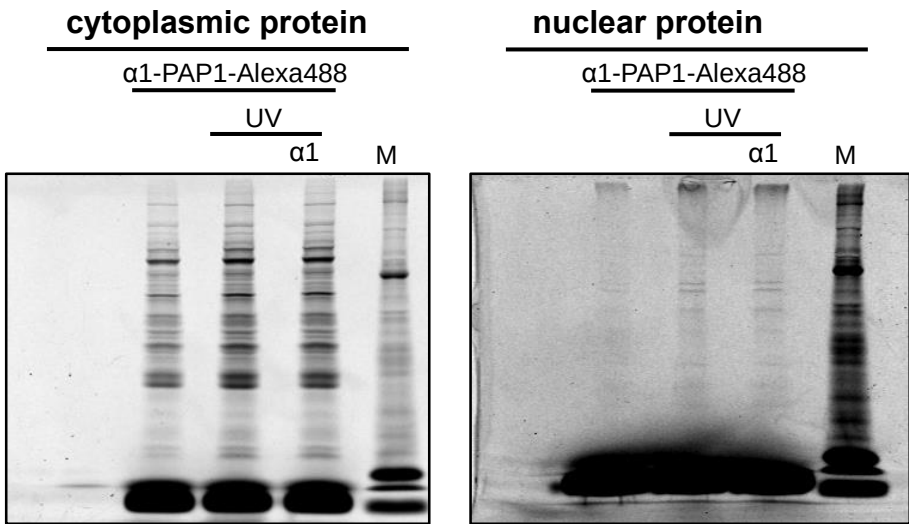


Figure 3.18. Fluorescent gel image of proteins reacted with α1-PAP-Alexa488 in one-step reaction method.

3.2.8. Hybrid probe: magnetic beads probe with α1-PAP-Alexa488

To increase the receptor concentration and to avoid non-specific binding, two methods, the affinity purification with magnetic beads and photoaffinity labeling, were combined together. The receptor in the cytoplasmic protein or nuclear protein was partially purified with the affinity FG beads, which was then subjected to equilibration with α1-PAP-Alexa488 probe, followed by UV irradiation to get photoaffinity labeled receptor (Fig. 3.19). However, after adding free α1 to affinity compete with α1 probe, the fluorescent gel image did not show the decrease of specific bands in both cytoplasmic and nuclear proteins (Fig. 3.20).

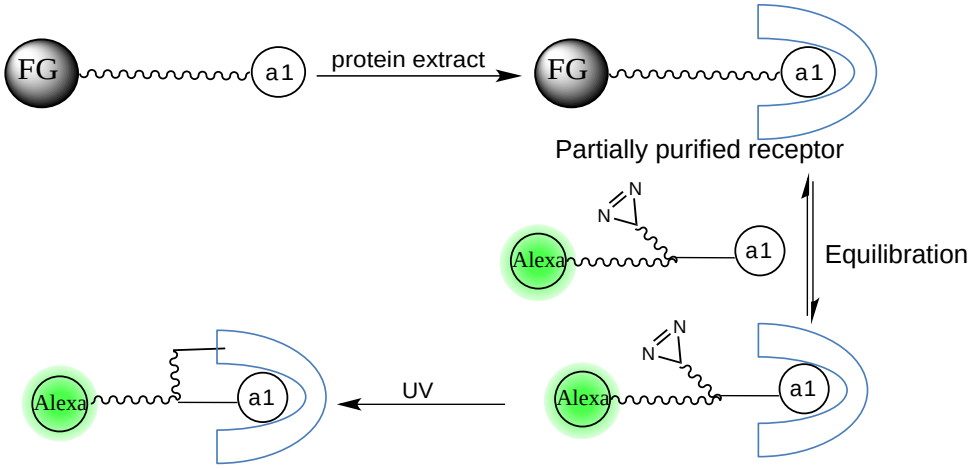


Figure 3.9. Photoaffinity labeling with α1 FG-beads and α1-PAP-Alexa488.

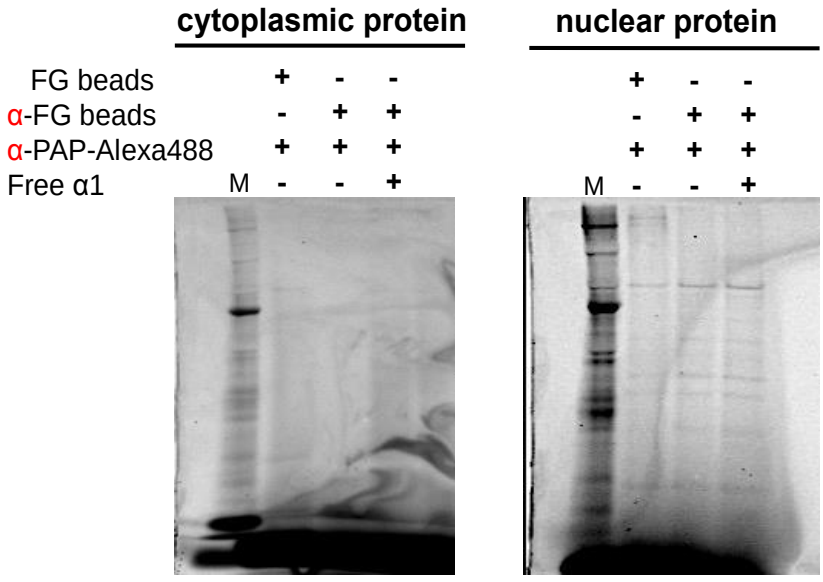


Figure 3.20 Fluorescent gel images of hybrid α1 probe.

3.3. Materials and Methods

3.3.1. Hormonal activity of α1 probes

A block of A2 mating type *P. nicotianae* (ATCC 38606) was inoculated onto a 9 cm Petri plate containing 20 ml of distill water, 20% V-8 juice (Campbell Soup Company, Camden, NJ), 2% agar, 0.3% $CaCO_3$. The plate was incubated at 25 °C, 60% humidity for 10 days in the dark. A piece (5 × 5 × 3 mm) of the pre-cultured colony was incubated on a 9 cm Petri plate containing 10% V-8 juice, 2% agar, and 0.02% $CaCO_3$ and incubated for 4 days under the same conditions. Solutions of α1 or α1 probes were applied to paper disk (ϕ 8 mm, 7 mm thickness, Advantec®, Tokyo, Japan), which were dried for 30 min in vacuo and then placed upside down on a cultured colony at a distance of 1.5 cm from the colony center. The plates were incubated at 25 °C, 60% humidity in the dark. Relative activity was calculated as follows. Three of five duplicate data for each dose (3 and 10 ng/disk of α1; 100 and 300 pmol/disk of α1 probes) were used for calculating the relative activity as shown in Figure 3.6. and 3.13. The doses inducing 400 oospores as D_{400} were obtained from the dose-oospore graphs. Relative activity (%) was obtained by the following equation: $100 \times [D_{400}(\alpha1)] / [D_{400}(\alpha1 \text{ probes})]$.

3.3.2. Protoplast preparation

The protoplasts were prepared by the method of Wang *et al.* (2011)(36) with the following modification: 3 dyas old *P. nicotianae* mycelia (1 g wet weight), cultured in a liquid 20%

V8-juice medium were washed with 0.8 M mannitol and then placed into 10 ml of the digestion solution (0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 1 mM $CaCl_2$, 7.5 mg/ml lysing enzyme [from *Trichoderma harzianum*, Sigma, USA], and 3 mg/ml cellulase [from *Trichoderma reesei* ATCC 26921, Sigma, USA]) and incubated for 50 min at 25 °C with 100 rpm shaking. The protoplasts were filtered through Miracloth and harvested by centrifugation at 700 g for 3 min. The pellet (corresponding 1 g mycelia) was resuspended in 4 ml of 0.6 M mannitol in a 15 ml tube, further purified by laying to 4 ml of 0.6 M sucrose solution (0.6 M sucrose, 20 mM MES, pH 7, and 1 mM $CaCl_2$) in 15 ml tube and then centrifuged at 100g for 5 min. The protoplasts were obtained as a pellet.

3.3.3. Fluorescent probes staining

Seven days old fresh mycelia (1 mg) or protoplasts (corresponding 1 mg mycelia) from A2 mating type of *P. nicotianae* ATCC 38606 were incubated with 1ml of 4 μM α1 fluorescent probes for 1 h at room temperature in the dark, without or with free α1 (4 μM). A1 mating type was also incubating with probes to compare. After incubation, the mycelia or protoplasts were washed with water or digestion solution (see 3.3.2), respectively, for 3 times and then observed under a microscope.

3.3.4. Protein extraction

The protoplasts were used to extract several fractions of proteins.

For the protoplast protein, the protoplast (corresponding 0.5 g mycelia) were added to 1 ml

of lysis buffer (50 mM Tris, pH 5.7, 150 mM NaCl, 20 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM DTT, protease inhibitor cocktail [P8215-5ML, Sigma, USA], and 2% CHAPS) in a 1.5-ml tube and incubated on ice for 30 min followed by 10 min centrifugation at 3,000 g (supernatant).

For the cytoplasmic protein, the protoplasts (corresponding 0.5 g mycelia) were added to 1 ml of lysis buffer (the same as above but without CHAPS) in a 5-ml tube, mashed with a glass masher and then centrifuged at 100,000 g for 1 h (supernatant).

For the nuclear protein, the protoplasts (corresponding 0.5 g mycelia) were added to 1 ml of Medium A (20 mM HEPES, pH 7.5, 1 M sorbitol, 7% ficoll, 1 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 20 mM DTT and protease inhibitor), mashed with a glass masher, centrifuged at 3,000 g for 5 min. The supernatant was then centrifuged at 15,000 g for 30 min. The pellet (nuclei), was then added to 500 μl of nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail [P8215-5ML, Sigma, USA]), incubated on ice for 30 min and centrifuged at 20,000 g for 5 min (supernatant).

The amount of three proteins (obtained from 0.5 g mycelia) were 3.8, 1.7, 0.4 μg/μl, respectively, based on Bradford protein analysis.

3.3.5. Affinity purification with FG Beads

The α1 fixed FG beads or empty beads were prepared by reaction of 0.5 mg beads (Tamagawa Seiki Co., LTD. Nagano, Japan) contain 15 nmol/mg α1 and suspended in EtOH

as 37.5 μM solution. 0.5 mg of beads (200 μl) were washed with 1ml of 100nM KCl buffer (20 mM HEPES, 12.5% glycerol, 0.1 M KCl, 1mM $MgCl_2$, 0.2mM $CaCl_2$, 0.2 M EDTA, 0.1% NP-40, 1mMDTT and 0.2mM PMSF), and incubated with cytoplasmic or nuclear protein extracts (200 μg in 200 μl buffer) (± 180 μM α1) at 4 °C for 4 h. After washing 3 times with 100nM KCl buffer, the beads were incubated with 40 ml of 1M KCl buffer (with 1 mM DTT and 0.2 mM PMSF) for 20 min at 4 °C to remove the non-specific binding proteins. After collecting the supernatant with magnetic stand, the remained beads suspended in SDS loading buffer (50 μl) and heated at 98 °C for 5 min. Beads suspension set on a magnetic stand, and the binding proteins (15 μl each) were subjected to SDS-PAGE, and detected by silver staining.

3.3.6. Photoaffinity probe labeling

The labeling reaction was initiated by incubation of cytoplasmic or nuclear protein (both 7 μg/100 μl) with (1 μM) α1-PAP (± 50 μM α1) at 4 °C for 1 h and then exposed to UV at 365 nm (Handy-UV lamp, AS ONE, Japan) at a distance of 3 cm for 15 min. After UV irradiation, the labeling mixtures were then reacted with 2 μM Alexa Fluor 488 azide in the present of 50uM THPTA, 0.5 mM copper sulfate and 0.5 mM sodium ascorbate, shaking in 4 °C for 1 h to obtain α1-protein-Alexa complex. The complex was then applied 20 μl on SDS-PAGE, and detected by Typhon 9400 Variable Mode Imager (General Electric, USA).

3.3.7. Hybrid probe labeling

The α1 fixed FG beads (200 μl) were washed with 1ml of 100nM KCl buffer (20 mM HEPES, 12.5% glycerol, 0.1 M KCl, 1mM $MgCl_2$, 0.2mM $CaCl_2$, 0.2 M EDTA, 0.1% NP-40, 1mMDTT and 0.2mM PMSF), and incubated with cytoplasmic or nuclear protein extracts (700 μg/ml) at 4 °C for 3 h. After washing 3 times with 100nM KCl buffer, the beads were incubated with 40 ml of 1M KCl buffer (with 1 mM DTT and 0.2 mM PMSF) for 20 min at 4 °C to remove the non-specific binding proteins. Then repeat the above step to increase the binding protein amount. After that, collected the beads with magnetic stand and resuspend the beads with 200 μl of 100nM KCl buffer. 10 μM of α1- PAP-Alexa 488 (± 200 μM α1) were incubated with beads at 4 °C for 3 h, separated the supernatant with magnetic stand and then exposed the supernatant to UV at 365 nm (Handy-UV lamp, AS ONE, Japan) at a distance of 3 cm for 15 min to obtain α1-protein-Alexa complex. The complex was then applied 20 μl on SDS-PAGE, and detected by Typhon 9400 Variable Mode Imager (General Electric, USA).

3.4. References

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Chapter 4

Conclusion and future direction

The sexual reproduction of the plant pathogen *Phytophthora* was regulated by mating hormones, α1 and α2, which has been successfully characterized several years ago. In this thesis, I focus on describing the specificity of mating hormones as well as screening the α1 receptor in A2 mating type of *Phytophtora* sp. using a series of α1 probes.

The first result of this research described in Chapter 2 was that the *Phytophthora* mating hormone α2 was found to be an antagonist of the counterhormone α1. The hormonal activity of α1 for *P. nicotianae* (A2 mating type) was interfered with in the present of the counterhormone α2 and some α2 derivatives (Fig. 4.1). Structure-activity relation ship was examined, and a model to explain this unexpected phenomenon was proposed (Fig. 2.8). The hormone α2 produced by A2 mating type might bind to the own α1 receptor in the absence of the counter part of the A1 type. These results could provide hints not only to analyze the α hormone receptors but also to control the *Phytophthora* diseases.

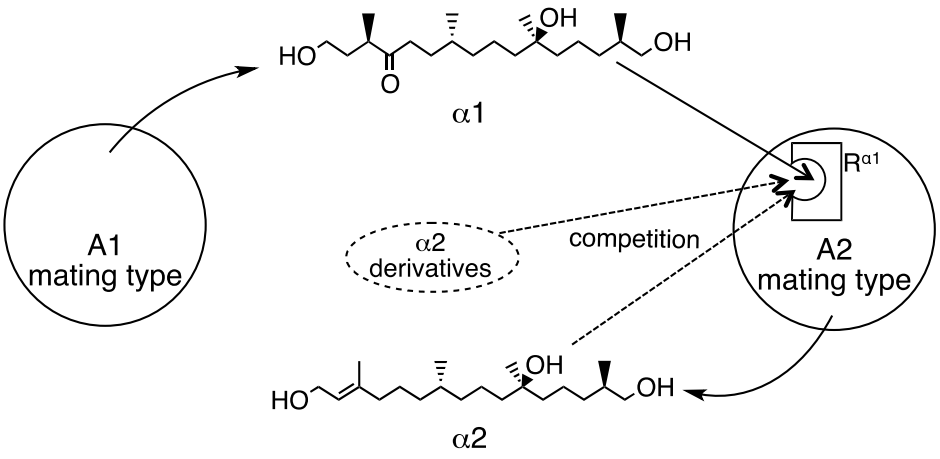

Figure 4.1. Interference of α1-induced sexual reproduction of Phytophthora A2 mating type by α2.

α2 and some α2 derivetives could bind to α1 receptor ($R^{α1}$).

Interaction between α2 and α2 receptor (in A1 mating type) is omitted.

In Chapter 3, several kinds of α1 probes, such as fluorescent, affinity beads, photoaffinity, and their hybrid probes (Fig. 4.2), were used for screening the α1 receptor harbored in the A2 mating type stain of *P. nicotianae*. Fluorescent probes were used for demonstrating mating type specificity and location of the receptor with fresh *Phytophthora* mycelia and protoplasts; a magnetic FG beads probe was used for affinity separation of binding proteins from both cytoplasmic and nuclear proteins; photoaffinity probe, α1-PAP and α1-PAP-Alexa488, were used for receptor isolation from cytoplasmic and nuclear proteins; finally; and a hybrid method consisting of the magnetic beads and α1-PAP-Alexa488 probe was also used to separate α1 binding proteins. Although specific binding proteins has not been detected yet, this work will provide with useful basic information for the future studies. Further attempts need to be conducted to characterize the α hormone receptors.

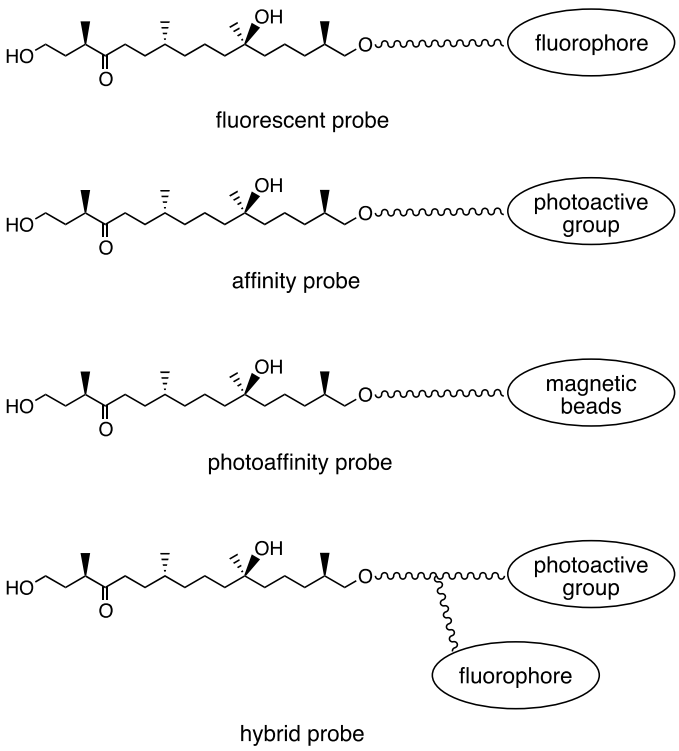

Figure 4.2. α2 probes used for α1 receptor identification.

Acknowledgment

I would like to express my greatest gratitude to my research director, Dr. Makoto Ojika, for his invaluable guidance, untiring support, enthusiastic encouragement and endless patience throughout my whole study period. His passionate and rigorous scientific attitude reshaped my viewpoint in regard to research. He always encourages me to study hard, think more and try more on my research area. And he is willing to listen and help me solving my problem in a good way. He spent endless time reviewing and modifying my paper to make it perfect. Without his guidance and persistent help, this doctoral dissertation would not have been possible.

I consider it an honor to work with Dr. Arata Yajima and his team, Tokyo University of Agriculture. They extend their support in my research by providing several kinds of synthetic α1 probes.

I cannot find words to express my gratitude to Dr. Tatsuhiko Kondo for so many discussions with his. It gives me great pleasure in acknowledging the support and help during my confusing time.

I would like to give my special thanks to technical assistant Ms. Tomoya Asano, for her effort in helping me in the researching experiment and study.

I am indebted to all the past and current members in the Ojika lab for sharing their experiences and knowledge, also their unforgettable friendship. My gratitude always goes to all the faculty and staffs in the department for their selfless help.

Finally, I owe my deepest gratitude to my beloved parents for their infinite love and support. Without them, I would not have been able to accomplish what I did. Last but not least, I want to give my heartfelt thank to my husband Shiwei ZHU. Without him, I would not have the thought to study in Japan and have this wonderful oversea experience. He always supports my study by providing scientific discussions and new ideas. And he always takes care of me in life. I could not image what my life would be without him.

List of Publications

1) Li Zhang, Arata Yajima, Makoto Ojika, The *Phytophthora* mating hormone α2 is an antagonist of the counterhormone α1, *Biosci. Biotechnol. Biochem.* in press.
2) Li Zhang, Arata Yajima, Makoto Ojika, Preparation of chemical probes for identifying *Phytophthora* mating hormone receptor, in preparation.