國立臺灣大學生命科學院植物科學研究所

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利用 Tsukuba System 於菸草中實現瞬時蛋白質表達

Transient Protein Expression in Tobacco Using the Tsukuba System

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MASTER'S THESIS ACCEPTANCE CERTIFICATE
NATIONAL TAIWAN UNIVERSITY

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本論文係岡佳穗君（學號 R13B42025）在國立臺灣大學植物科學研究所完成之碩士學位論文，於 2025 年 12 月 23 日承下列考試委員審查通過及口試及格，特此證明

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摘要

本研究以本實驗室於日本開發之瞬時蛋白質表現系統「Tsukuba System」為核心進行。透過雙聯學位計畫，本人分別於日本與臺灣兩地進行研究。在日本，嘗試生產並進行流感抗原特異性 MHC 之四聚體化；在臺灣，則嘗試生產與植物免疫相關之蛋白質。目標基因分別插入源自 Tsukuba System 骨幹載體 pTKB3 之內質網（ER）滯留訊號載體（pTKB3-SSExt-HKD）或質外體運輸訊號載體（pTKB3-SSExt-CSPH）中。上述載體轉形至 *Agrobacterium tumefaciens* GV3101，並透過農桿菌浸潤法（agroinfiltration）於 *Nicotiana benthamiana* 中進行瞬時表現。隨後將葉片組織均質化並過濾以萃取目標蛋白質，並以 SDS-PAGE 分析確認所得蛋白之存在。日本之研究內容詳述於「Chapter 2」，臺灣之研究內容則說明於「Chapter 3」。

【Chapter 2】

重組蛋白廣泛應用於功能分析與診斷試劑開發等領域。一般而言，重組蛋白多利用 *Escherichia coli* 等異源表現系統進行生產。然而，部分蛋白質，尤其是結構複雜之蛋白，在 *E. coli* 系統中表現困難；此外，植物來源蛋白亦常面臨表現效率不佳之問題。為克服上述限制，本實驗室建立了一套植物表現系統—「Tsukuba System」。此系統結合源自雙生病毒（geminivirus）之滾環複製機制（rolling circle replication）與雙終止子（double terminator）設計。相較於既有之 magnICON 植物瞬時表現系統，Tsukuba System 可達成更快速且高產量之蛋白質瞬時表現。

本研究旨在利用 Tsukuba System 表現並分析 MHC class II 蛋白與植物免疫相關蛋白之功能，兩者皆屬於難以在 *E. coli* 中大量生產之蛋白質。

MHC 蛋白為評估免疫反應之關鍵分子，並具疫苗開發潛力。由於抗原具高度多樣性，需製備對應不同抗原之 MHC 蛋白。雖然 MHC class I 蛋白於 *E. coli* 表現系統中已有成熟技術，然而 MHC class II 蛋白之製備仍具挑戰性，目前僅能生產有限類型。此外，MHC 分子於體內可與 T 細胞受體結合，但此結合易迅速解

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離。若應用於診斷，則需更穩定之結合形式。形成 MHC 四聚體為提升結合穩定性之一種策略，可藉由增加結合位點數目以降低快速解離之機率。

MHC 單體由 α 鏈與 β 鏈組成。於 *N. benthamiana* 中共表現 α 與 β 鏈後，透過與 α 鏈融合之 His tag 進行純化，確認 β 鏈可共同被純化，顯示 MHC 單體複合體成功形成。隨後將所得 MHC 單體進行生物素化(biotinylation)，並與 avidin 結合形成四聚體。四聚體化後之 SDS-PAGE 分析顯示約 100–200 kDa 之條帶，證實多聚體之形成。此階段之蛋白產量為每 100 g 新鮮葉片約 50 ng。

Tsukuba System 成功於 *N. benthamiana* 中高效率生產 MHC 單體，然而四聚體化仍具挑戰性。雖觀察到多聚體條帶，但未明確檢測到預期之四聚體條帶，顯示組裝可能不完全或不正確。此現象可能源於生物素化 MHC 單體僅與 streptavidin 之兩至三個結合位點結合，或游離生物素佔據 streptavidin 結合位點，導致無法有效結合生物素化 MHC 單體。

為提升 MHC 四聚體形成效率，可於與 streptavidin 結合前，先依分子量分離生物素化單體，以利更有效率地分離與純化四聚體。

【Chapter 3】

植物免疫蛋白在植物抵禦病原菌過程中扮演關鍵角色，其功能鑑定對於理解植物防禦機制至關重要。然而，部分植物來源蛋白難以於 *E. coli* 中有效表現。本研究聚焦於三種番茄來源之免疫相關蛋白：SlPNGase(peptide:N-glycanase)、SlWfi1，以及功能尚未明確之 12g520。上述基因先前已被鑑定為參與番茄病原菌反應之相關基因。

SlWfi1 為定位於細胞膜之 NADPH oxidase，於病原入侵時可誘導活性氧（ROS）生成及細胞死亡反應，在防禦訊號傳遞中扮演核心角色。SlPNGase 屬於 PNGase 酵素家族，可去除醣蛋白之 N-連結型醣鏈，推測在病原反應過程中參與蛋白質品質管制。12g520 為一 LRR receptor-like 基因，係透過 Bwr12 抗性數量性狀基因座（QTL）區域之比較基因體分析所鑑定，推測參與病原相關分子模式（PAMP）辨識及啟動 PAMP-triggered immunity（PTI）反應。上述基因係本研究室於國立臺灣大學進行研究時，自萃取 RNA 合成 cDNA 後所擴增取得。本研究

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之目的在於利用 Tsukuba System 表現上述蛋白，並進行功能分析。

於植物免疫蛋白之製備方面，目標基因被克隆至 pTKB3-SSExt-HKD 與 pTKB3-SSExt-CSPH 載體，並轉形至 *E. coli*。雖成功萃取質體 DNA，然而其濃度未超過 10.0 ng/μl，不足以進行後續農桿菌轉形與 agroinfiltration 實驗。

在植物免疫相關蛋白表現方面，質體 DNA 產量偏低導致轉形效率受限。儘管參考臺灣之實驗流程進行多項條件優化，包括改變培養條件與增加通氣量，仍未顯著改善結果。*E. coli* 菌株差異及培養條件不同可能為影響因素。

此外，由於植物免疫相關蛋白之目標基因序列較大，需反覆確認載體建構之正確性。先前於臺灣之研究指出，除 pTKB3 之外之其他載體無法於 *N. benthamiana* 中成功表現上述蛋白。因此，未來將持續以 pTKB3 載體系統為核心，建立穩定且可靠之蛋白表現條件。

關鍵字：透過農桿菌浸潤法、植物免疫蛋白、MHC、MHC 四聚體、Tsukuba system、

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ABSTRACT

This research focuses on the transient protein expression system, the "Tsukuba System," developed in our laboratory in Japan. Through a double degree program, I conducted research in both Japan and Taiwan. In Japan, I produced and tetramerized influenza antigen-specific MHC, while in Taiwan, I produced plant immunity-related proteins.

Target genes were inserted into either the ER retention signal vector (pTKB3-SSExt-HKD) or the apoplast transport signal vector (pTKB3-SSExt-CSPH), both derived from the pTKB3 backbone of the Tsukuba System. The vectors were introduced into *Agrobacterium tumefaciens* GV3101 and transiently expressed in *Nicotiana benthamiana* via agroinfiltration. Leaf tissue was homogenized and filtered to extract proteins, which were confirmed by SDS-PAGE. Research conducted in Japan is described in Chapter 2, and research in Taiwan in Chapter 3.

【Chapter 2】

Recombinant proteins are widely used in applications. Typically, heterologous protein expression systems like *Escherichia coli* are employed for recombinant protein production. However, certain proteins, particularly complex ones, are difficult to express in systems such as *E. coli*. Additionally, plant-derived proteins can also pose challenges in expression. To overcome these limitations, our laboratory has developed a plant-based protein expression system called as "Tsukuba System".

MHC proteins are essential for evaluating immune responses and have potential applications in vaccine development. Because of antigen diversity, various antigen-

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specific MHC proteins are required. Although *E. coli*-based expression systems for MHC class I are well established, production of MHC class II remains limited. In addition, MHC molecules dissociate rapidly from T cell receptors in vitro, which limits diagnostic use. To improve stability, MHC tetramers can be formed to increase binding sites and reduce dissociation.

MHC monomers consist of α and β chains. Co-expression of both chains in *N. benthamiana* followed by His tag purification of the α chain confirmed co-purification of the β chain, indicating successful complex formation. The monomers were biotinylated and conjugated to avidin to generate tetramers. SDS-PAGE showed bands at approximately 100–200 kDa, suggesting multimer formation. The protein yield was 50 ng per 100 g of fresh leaf tissue.

The Tsukuba System enabled efficient production of MHC monomers in *N. benthamiana*, but tetramerization remains challenging. Although multimer bands were observed, a clear tetramer band was not detected, suggesting incomplete assembly. Possible causes include partial binding of biotinylated monomers to streptavidin or occupation of streptavidin binding sites by free biotin.

To improve tetramer formation, separating biotinylated monomers by molecular weight before streptavidin conjugation may enhance tetramer isolation.

【Chapter 3】

P Plant immune proteins play a vital role in protecting plants from pathogens, making their functional characterization crucial. However, some plant-derived proteins are difficult to express in *E. coli*. In this study, I focus on three tomato-derived immune proteins, SlPNGase (peptide:N-glycanase), SlWfi1, and the functionally uncharacterized 12g520, previously identified as pathogen-responsive genes. SlWfi1 is a membrane-

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localized NADPH oxidase that induces reactive oxygen species (ROS) production and cell death upon pathogen invasion. SlPNGase belongs to the PNGase family, which removes N-linked glycans from glycoproteins and contributes to protein quality control during pathogen response. 12g520 is an LRR receptor-like gene identified in the Bwr12 resistance QTL region and is thought to be involved in pathogen-associated molecular pattern (PAMP) recognition and activation of PAMP-triggered immunity (PTI). The genes were previously amplified from tomato cDNA synthesized from RNA in research conducted at National Taiwan University. The aim of this study is to perform functional analysis by expressing these proteins using the Tsukuba System.

The target genes were cloned into pTKB3-SSExt-HKD and pTKB3-SSExt-CSPH and transformed into *E. coli*. Although plasmid extraction was successful, DNA concentration did not exceed 10.0 ng/μl, which was insufficient for Agrobacterium-mediated agroinfiltration. Low plasmid yield hindered successful transformation. Despite modified culture conditions and increased aeration based on Taiwanese protocols, no significant improvement was observed, possibly due to differences in *E. coli* strains and growth conditions.

Because the target genes are large, repeated verification of vector construction is required. Previous research in Taiwan showed that vectors other than pTKB3 failed to express these proteins in *Nicotiana benthamiana*. Therefore, future efforts will focus on achieving reliable expression using the pTKB3 vector system.

Keywords: Agrobacterial infiltration, MHC, MHC tetramer, plant immune proteins, Tsukuba system

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List of abbreviations

Abbreviation	Definition
Amp	Ampicillin
AS	Acetosyringone
Avi	Avidin
bp	base pairs
DNA	deoxyribonucleic acid
DSB	DNA double strand break
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
FT	flow through
FW	Fresh weight
Gen	Gentamycin
GC/MS	gas chromatography / mass chromatography
kDa	kilo Dalton
km	Kanamycin
LB	Luria-Bertani
LC / MS	liquid chromatography / mass chromatography
LIR	long intergenic region
mL	milliliter
MQ	Milli-Q
MHC	Major Histocompatibility Complex
OD	optical density
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
Rif	Rifampicin
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TALEN	transcription activator-like effector nuclease

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List of abbreviations (continued)

Abbreviation	Definition
TBS	tris buffered saline
TTBS	TBS-tween20
Tris	tris (hydroxymethyl) aminomethane
WT	wild type
His	Histidin

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Chapter 1 General Introduction

1.1 Transient expression

Transient expression is a method that temporarily modifies genetic material to produce a target protein for a limited time. One such method is agrobacterial infiltration. This technique utilizes the property of *Agrobacterium* to deliver a portion of its T1 plasmid's T-DNA region into the nucleus of infected plants. By infecting plants with *Agrobacterium* carrying a gene encoding the target protein inserted into the T-DNA region, the target protein can be expressed in the plant.

1.2 Tsukuba System

The Tsukuba System is a transient protein expression system that incorporates a Gemini virus-derived replication system and a double terminator. Developed in our laboratory, it is characterized by achieving significantly higher protein yields compared to previous systems[1,2].

Geminivirus is a plant-infecting virus with circular single-stranded DNA, named for its viral particles having an octahedral shape. This virus possesses a rolling circle replication system, where the circular DNA is replicated extensively by the DNA replication enzymes Rep/RepA. This mechanism is incorporated into the Tsukuba System vector. The target gene, 35S promoter, Rep/RepA genes, etc., are introduced into the region flanked by LIRs within the vector. Once this sequence enters the plant, the LIRs are recognized, leading to circularization. Rep/RepA then replicates large amounts of single-stranded DNA along the circularized DNA. The replicated DNA incorporates a 35S promoter: target gene: terminator set. The 35S promoter enables transcription of the

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target gene mRNA, which is then translated to produce the target protein. However, even when using this vector for transient expression in *Nicotiana benthamina*, yields were only 1-2 mg/g fresh weight, failing to surpass expression levels achieved with the magnICON system.

Insufficient expression was attributed to transcription inhibition via read-through and RNA interference. Read-through refers to the phenomenon where transcription continues beyond the terminator into the sequence following it (Ryojie et al., 1983). Therefore, this vector incorporates a double terminator consisting of a heat shock protein terminator linked to an extensin terminator. This suppressed expression reduction due to read-through, enabling yields exceeding the magnICON system. This vector can express proteins in various plants. Experiments were conducted in multiple plants, including Solanaceae, Cucurbitaceae, and Asteraceae, but the highest expression levels were observed in *Nicotiana benthamiana* [1,2].

1.3 Extensin secretion signal (SSExtSSExt) and HypGP signal (SP10SP10)

Secretion signals (signal peptides) are peptide sequences attached to proteins and are known to be involved in protein transport and localization. The extensin secretion signal (SSExt) is a signal peptide derived from extensin, a major structural protein constituting plant cell walls. In heterologous protein production, adding this SSExt has been shown to increase the accumulation of the target protein in the apoplast (Jiang et al., 2020). Furthermore, the HypGP signal, a different signal peptide, consists of a sequence composed of repeating "serine-proline" units (SP10). This SP10 is known as a molecular carrier; adding it enhances the secretion of the target protein into the apoplast (Zhang et al., 2016). Secretion into the apoplast allows the protein to evade degradation associated with intracellular accumulation, potentially increasing yield. Indeed, previous studies

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have demonstrated that adding SSExt and SP10 to interferon, a type of cytokine, improved its solubility and increased yield.[2].

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Chapter 2 Production of antigen-specific MHC

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Chapter 2.1 Introduction

The MHC (Major Histocompatibility Complex) is a molecule involved in antigen presentation and essential for initiating immune responses. In particular, MHC class II is known to be difficult to produce because it forms an antigen presentation groove as a dimer of α and β chains, requiring proper folding and subunit binding.

Traditionally, *E. coli* and mammalian cells have been primarily used for recombinant protein production. However, post-translational modifications are limited in *E. coli*, and mammalian cells are costly to culture. In contrast, transient expression using plants is attracting attention as a production platform for pharmaceutical and research proteins because it is low-cost, highly scalable, and possesses the modification mechanisms of eukaryotes.[3,4]

2.1.1 MHC (Major Histocompatibility Complex)

MHC is a group of cell surface proteins widely present in vertebrates. These molecules play a central role in the adaptive immune response by presenting antigenic peptides to T cells. MHC class I molecules are expressed on nearly all nucleated cells. They present self and non-self peptides, derived from intracellular degradation, on the cell surface, thereby inducing recognition and elimination of target cells by $CD8^+$ cytotoxic T cells. Conversely, MHC class II molecules are primarily expressed on antigen-presenting cells (dendritic cells, macrophages, B cells, etc.). They present internalized extracellular antigens as peptides, activating $CD4^+$ helper T cells and thereby contributing to the regulation of the overall immune response through antibody production and cytokine secretion. MHC exhibits high polymorphism, and the diverse

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alleles present among individuals are deeply related to susceptibility to transplant-associated immunity and autoimmune diseases.

2.1.2. Antigen presentation

Antigen presentation refers to the process by which antigen-derived peptide fragments are presented on the cell surface bound to MHC molecules, enabling recognition by the T cell receptor (TCR). Intracellular proteins are degraded by proteasomes and other mechanisms; the resulting peptides are loaded onto MHC class I molecules within the endoplasmic reticulum and presented on the cell surface. Conversely, proteins taken up from the extracellular environment are degraded within the endosome-lysosome system and presented bound to MHC class II molecules. Antigen presentation is an essential step for T cell activation, and its efficiency and quality significantly influence the intensity and specificity of the immune response.

2.1.3. MHC Class I / Class II

MHC Class I specializes in eliminating virus-infected cells and tumor cells by presenting antigens to $CD8^+$ T cells, while Class II specializes in inducing helper functions such as antibody production and macrophage activation by presenting antigens to $CD4^+$ T cells. The major structural difference lies in their antigen-presenting sites. MHC Class I has an antigen-presenting site composed solely of the α chain, whereas MHC Class II has an antigen-presenting site composed of both the α and β chains. This study targeted the production of MHC Class II molecules, which are

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considered structurally more complex and difficult to express.Figure 1 shows a schematic diagram of the MHC monomer.(Figure 1).

2.1.4 MHC tetramer

MHC tetramer is a reagent that multivalentizes the same MHC–peptide complex by binding four molecules together, serving as a tool for labeling and detecting specific T cells with high affinity. It is generally prepared by binding biotinylated MHC monomers to streptavidin, which possesses four biotin-binding sites. While monomeric MHC–TCR interactions are low-affinity and transient, tetramerization enhances apparent affinity, enabling highly sensitive detection of specific antigen-specific T cell populations via flow cytometry. In this study, we attempted tetramerization of MHC class II produced in plants.

Research objectives

This study utilized the "Tsukuba System," a transient expression system that incorporates a replication mechanism derived from geminivirus and a double terminator. First, MHC class II (influenza antigen-specific MHC), a complex structural protein composed of multiple subunits, was produced and purified in *Nicotiana benthamiana*, and its structure and folding were confirmed. Furthermore, we attempted biotinylation using the biotinylating enzyme BirA and tetramerization by binding with streptavidin, with the aim of investigating the possibility of constructing an MHC class II tetramer production system using plants.

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Chapter 2.2 Materials and methods

2.2.1 Plant material

Nicotiana benthamiana was used as a transient expression host. Plants aged 4–6 weeks after sowing were used for agroinfiltration. Cultivation was performed in a growth chamber maintained at 24 ℃± 2 ℃.

2.2.2 Vector construction

In the Japanese study, pTKB3-SSExt-HKD-DR3 and pTKB3-SSExt-HKD-DR8 were used as vectors to produce MHCs named DR3 and DR8.

In the Taiwanese study, pTKB3-SSExt-HKD-12g520, pTKB3-SSExt-HKD-Wfi1, pTKB3-SSExt-HKD-SlPNGase, pTKB3-SSExt-CSPH-12g520, pTKB3-SSExt-CSPH-Wfi1, and pTKB3-SSExt-CSPH-SlPNGase.

These vector constructs are shown in Figure 2.

2.2.2.1 Culture of *E. coli* colonies

Colonies of *E. coli* (HST08 in Japan, DH5α in Taiwan) obtained by transformation were touched with the tip of a yellow pipette tip and transferred into test tubes containing 10 mL of liquid LB medium (kanamycin 50 μg/ mL) (Table 1). These were then shaken overnight at 37 ℃.

2.2.2.2 Plasmid extraction from *E. coli*

To extract plasmids from *E. coli,* the FavorPrep Plasmid Extraction Mini Kit (Chida Science) was used. 10 mL of *E. coli* culture was transferred to a 15 mL Falcon tube, centrifuged (3700 rpm, 5 minutes, room temperature), and the supernatant was discarded.

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The *E. coli* pellet was dissolved in 250 μL of FAPD1 Buffer and transferred to a 1.5 mL tube. Add 250 μL of FAPD2 Buffer, mix by inverting several times, wait until clear, then let stand at room temperature for 2 minutes. Add an additional 350 μL of FAPD3 Buffer and repeat inverting until a white precipitate forms. Centrifuge (15,000 rpm, 5 min, room temperature), transfer the supernatant to the FAPD column set in the Collection tube, centrifuge again (15,000 rpm, 30 sec, room temperature), and discard the liquid accumulated in the tube after passing through the column. Add 400 μL of WF Buffer to the FAPD column, centrifuge (15,000 rpm, 30 seconds, room temperature), and discard the liquid accumulated in the tube. Add 700 μL of Wash Buffer to the FAPD column, centrifuge (15,000 rpm, 30 sec, room temperature), discard the liquid accumulated in the tube, and centrifuge again (15,000 rpm, 3 min, room temperature) to remove water from the column. Transfer the FAPD column to a 1.5 mL tube. Add 100 μL of sterile water (70 ℃) to the FAPD column. Allow to stand for 1 minute, then centrifuge (15,000 rpm, 1 min, room temperature) to recover the plasmid. Store the recovered plasmid in the 1.5 mL tube at -20 ℃.

2.2.2.3 Restriction enzyme digestion

Add any restriction enzyme and the appropriate r-cut smart(NEB) for each restriction enzyme to the plasmid to be cut, treat at 37 ℃for 1 hour, and obtain linearized plasmid.

2.2.2.4 PCR of the target gene

The In-Fusion HD Cloning kit (Clontech) was used to prepare constructs via homologous recombination of gene products designed to have 15-base homology with the vector ends. Primers were custom-synthesized (Eurofins Genomics) as

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oligonucleotides fusing the 15-base vector terminus with 24 to 28 bases of gene-specific sequence. PCR was performed using each gene's cDNA as a template, yielding PCR products where the 15-base sequence homologous to the vector terminus was fused to the CDS of each gene. KOD-plus-neo (Yohbo Bio) was used as the PCR reagent. Reaction conditions were 98 ℃ for 10 seconds, 50 ℃ for 30 seconds, and 68 ℃ for 90 seconds for 30 cycles, followed by 68 ℃ for 2 minutes. The PCR products were separated by electrophoresis in a 0.8% agarose gel containing ethidium bromide. Bands were visualized using a Super LED Viewer (BioSpeed Co., Ltd.) and excised from the gel with a cutter knife. The templates and primers used for preparing each insert are shown in the table. (Table 3).

2.2.2.5 Purification of DNA fragments from agarose gel

PCR products and vectors recovered from agarose gels were purified using the QIAquick Gel Extraction Kit (QIAGEN). The gel block containing the desired DNA fragment was transferred to a 1.5 mL tube. Three times the volume of QG buffer was added, and the gel was dissolved by heating at 50 ℃ for 10 minutes in a dry bath incubator. An equal volume of isopropanol was added to the gel, mixed, and then loaded onto the column. Centrifuge (8000 rpm, 1 min), discard the supernatant that passed through the column. Return the column, add 750 μL of PE buffer, centrifuge again (8000 rpm, 1 min), and discard the supernatant. Return the column, spin empty (8000 rpm, 3 min, 4 ℃), and place the column in a new 1.5 ml tube. Add 40 μL of sterile water warmed to 70 ℃, let stand for 1 minute, then centrifuge (8000 rpm, 1 minute, 4 ℃). Store the liquid extracted from the column as purified PCR product at -30 ℃.

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2.2.2.6 In fusion response

Add 1 μL of PCR product, 1 μL of restriction enzyme-treated vector plasmid, and 0.5 μL of 5× In Fusion HD Enzyme premix (Clontech) to a 1.5 mL tube. Mix thoroughly using a pipette. Incubate at 50 ℃ for 15 minutes, then transfer to ice.

2.2.2.7 *E. coli* transformation using the heat shock method

DH5α Competent cells, ChampionTMDH5α high (Cosmo Bio), stored at -80 ℃, were thawed on ice. To 25 μL of competent cells, 2.5 μL of the In-fusion reaction mixture was added. The mixture was incubated on ice for 30 minutes, then heated at 42 ℃ for 45 seconds, followed by incubation on ice for 2 minutes. Add 100 μL of SOC medium to the post-heat shock solution, transfer to a 1.5 mL tube, and shake at 37 ℃for 1 hour. Plate 100 μL onto LB medium containing antibiotics (Table 1) and incubate overnight at 37 ℃ in an incubator MIR-262 (SANYO).

2.2.2.8 Colony PCR

PCR was performed on *E. coli* colonies transformed with vectors post-fusion reaction. AmpliTaq Gold 360 Master Mix (Applied Biosystems) was used as the PCR reagent. Reaction conditions were 95 ℃ for 30 seconds, 55 ℃ for 30 seconds, and 72 ℃ for 2 minutes, repeated for 40 cycles. PCR products were separated by electrophoresis on a 0.8% agarose gel containing ethidium bromide, and bands were detected using a Printgraph (Ato Co., Ltd.).

2.2.2.9 Sequence analysis

Sequence analysis was outsourced to Fasmac Co., Ltd. For this, extracted plasmid samples, primers, and MQ were prepared. The resulting sequences were aligned using

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NCBI-Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), confirming the correctness of the introduced gene.

2.2.2.10 Transformation of *Agrobacterium* using the electroporation methods

Agrobacterium (GV3101) stored at -80 ℃ was thawed on ice and diluted fivefold with 10% glycerol. The transformation vector was pipetted into a 1 μL microtube on ice, and 100 μL of the *Agrobacterium* solution was added. This mixture was transferred to a 2 mm cuvette, and electroporation was performed using the Gene Pulser Xcell (Bio-Rad). Conditions were set at 2.5 kV, 25 μF, and 200 Ω. A 100 μL inoculum was transferred to antibiotic-supplemented LB medium (Table 1) and cultured at 28 ℃ for 2 days to obtain colonies. To confirm construct introduction into *Agrobacterium*, PCR was performed using AmpliTag Gold 360 (Applied Biosystems). Reaction conditions followed the protocol recommendations. PCR products were separated by electrophoresis on a 0.8% agarose gel containing ethidium bromide, and bands were detected using a Printgraph (Atto Corporation).

2.2.3 Transient expression using tobacco

2.2.3.1 Preparation of the infection solution

Agrobacterium carrying a plasmid with the target gene was pre-cultured for two days in 10 mL of LB medium (Table 1) (kanamycin 50 μg/mL, gentamycin 30 μg/mL, rifampicin 30 μg/mL) for two days. This culture was then subcultured into 1000 mL of LB medium (kanamycin 50 μg/mL, gentamycin 30 μg/mL, rifampicin 30 μg/mL, 20 μM acetosyringone, 10 mM MES (pH 5.6). The culture was confirmed to have reached an OD600 of approximately 1 using a spectrophotometer. The pellet was then centrifuged (4500 rpm, 15 min, room temperature), resuspended in 1000 mL infiltration buffer (Table

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2), and allowed to stand for 2 hours to form the inoculum solution. For syringe infiltration, the volumes used were: 5 mL pre-culture medium, 200 mL main culture medium, and 100 mL infiltration buffer.

2.2.3.2 Syringe infiltration

Using a toothpick, small holes were made in the tobacco leaves. The leaves were then held between fingers and a syringe, and the infectious solution was infiltrated from the underside. Cultivation was performed at 25 ℃ for 4 days under 16-hour light exposure conditions.

2.2.3.3 Vacuum Infiltration

Place the infected solution in a beaker, set it in a vacuum desiccator, immerse all tobacco leaves in the infected solution, and apply vacuum for 5 minutes. After vacuuming, culture at 25 ℃ for 4 days under 16-hour light exposure conditions.

2.2.4 Protein purification

2.2.4.1 Extraction using lysis buffer

Place frozen tobacco in a blender, add liquid nitrogen while grinding, then add lysis buffer (Table 2) at three times the tobacco volume and mix thoroughly. Place on ice and sonicate for 20 minutes, then filter the solution through Miracross (Merck). Centrifuge the solution that passed through the Mira-Cross (12,000 rpm, 30 min, 4 ℃), transfer the supernatant to a beaker, and adjust the pH to 7.0.

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2.2.4.2 Thiosulfate precipitation

Measure the volume of the resulting solution and add ammonium sulfate to achieve a final concentration of 30%. Stir overnight at 4 ℃ using a stirrer. The next day, centrifuge (12,000 rpm, 30 minutes, 4 ℃) to obtain the supernatant. Subsequently, ammonium sulfate was added to the 30% supernatant to achieve a final concentration of 80%. This mixture was stirred on ice for 1 hour using a stirrer, then centrifuged (12,000 rpm, 30 min, 4 ℃) to obtain the precipitate. This precipitate was dissolved in 50 mL of Binding buffer (Table 2).

2.2.4.3 Preparation of dialysis membranes

Cut the dialysis membrane (Fisherbrand, Fisher Scientific) used for dialysis to an appropriate length with scissors, place it in a beaker, and soak it in pure water for 15 minutes. Transfer the dialysis membrane to a 10 mM sodium bicarbonate solution and bring it to a boil using a microwave. Boil for 10 minutes while stirring with a stirrer. Next, transfer the dialysis membrane to a 10 mM EDTA solution, bring it to a boil using a microwave, and boil for 10 minutes while stirring with a stirrer. Allow the dialysis membrane to cool to room temperature while immersed in the 10 mM EDTA solution, then store it at 4 ℃.

2.2.4.4 Dialysis

Dialysis was performed using binding buffer (pH 7.4) (Table 2). The buffer volume used was 5 to 10 times that of the dialysis sample. The inside of the dialysis membrane was washed with DW, the bottom was folded twice, secured with a clip, and the dialysis sample was poured in. The top was also secured with a clip, placed in the dialysis buffer, and stirred using a stirrer (4 ℃, 1 hour). After one hour, the solution was replaced with

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an equal volume of fresh Binding buffer, stirred again (4 ℃, 1 hour), and the sample was recovered.

This sample was centrifuged again at 20,000 rpm for 30 minutes at 4 ℃. The final supernatant was stored frozen, and the pellet was resuspended in Citrate Lysis Buffer at the same weight (mL) as the leaves initially used and stored frozen.

The final supernatant adjusted in section 4.1.2 was subjected to membrane filtration. Membrane filters (Merck Millipore) were loaded into the filtration apparatus, and filtration of the supernatant was performed by applying suction using a vacuum pump (Nalgene).

Ultrafiltration was performed to separate high-molecular-weight proteins from low-molecular-weight proteins. Using Sartorius' Vivaspin 14000 MWCO, centrifugation was conducted at 8000 rpm for 1 minute. The liquid remaining inside the tube was used in the subsequent steps.

2.2.4.5 Affinity chromatography

Centrifuge the dialysed samples (20,000 rpm, 20 min, 4 ℃) to obtain the supernatant. Set up a filter (Membrane Filters, Merck Millipore) in a vacuum pump (Nalgene), and pass the supernatant through the filter under reduced pressure. Use TALON Metal Affinity Resin (Takara Bio) as the column for adsorbing the His-tag. After adding resin to the column, it was washed with Binding Buffer (pH 7.4). The sample was then slowly eluted, and the liquid eluting from the column was collected as Flow Through (FT). After sample loading, the column was washed again with Binding Buffer (pH 7.4). Elution buffer (Table 2) was then applied in five fractions to elute the protein, yielding fractions Elution1 to Elution5. The presence of the target protein in these fractions was confirmed by SDS-PAGE and Western blotting. The membrane used for Western blotting was

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subsequently stained with CBB (Coomassie Brilliant Blue) staining solution to assess the purity of the target protein.

2.2.5 Protein expression analysis

2.2.5.1 SDS-PAGE

Work was performed wearing gloves. First, the gel plates were washed with detergent, thoroughly rinsed, and then used. The gel plates were set into casting frames and secured using a casting stand. The lower layer running gel (Table 2) was prepared first. Sterile water, 30% acrylamide mix, and Tris (Table 2)were mixed, and the mixture was vacuumed for 5 minutes to remove air bubbles from the gel. Then, the remaining reagents were added and mixed quickly and carefully to avoid foaming. The mixture was poured into the gel plate to about 3/4 of its height. Using a pipettor, water-saturated butanol (leveled approximately 1 mm above the gel surface) was gently layered onto the gel surface. This was done by adding small amounts across the entire width of the gel, rather than pouring from a single point. The gel was then left undisturbed in a 50 ℃ incubator for 30 minutes to solidify. When the gel had sufficiently polymerized, a small amount of water would accumulate between the gel and the water-saturated butanol, making the gel-butanol interface clearly visible. This was used to confirm gel polymerization. The water-saturated butanol was removed, and the gel was thoroughly washed with pure water. Next, the upper stacking gel (Table 2) was prepared. Reagents were mixed similarly to the running gel and poured into the gel plate. To prevent air bubbles, the solution was poured to fill the entire gel plate without accounting for the comb insertion space. The comb was inserted diagonally from the edge, taking care to avoid introducing bubbles. With the comb fully inserted, the gel was left to solidify at 50 ℃ for 30 minutes.

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Dilute the 10× running buffer (Table 2) 10-fold with distilled water and place it in the electrophoresis chamber. After setting the gel, fill the gel chamber with running buffer. Use a pipette to remove air bubbles beneath the gel plate to ensure uniform current flow. Apply each sample to the wells and run a current of 20 mA for 90 minutes per gel.

2.2.5.2 Western blotting

Using the TransBlot Turbo system (BIO-RAD), proteins were transferred from the electrophoresis gel to a PVDF membrane (GE Healthcare). The membrane was cut to the same size as the electrophoresis gel, soaked in 100% MeOH for about 10 seconds, and then immersed in the Semidry buffer. Two semi-dry gel pads were also soaked in semi-dry buffer. Only the necessary portion of the SDS gel was cut to the minimum required size. Layering was performed from the bottom up in the following order: anode plate, semi-dry gel pad, membrane, SDS gel, semi-dry gel pad, cathode plate. A voltage of 18V was applied for 30 minutes. Care was taken to prevent air bubbles from entering during this process. After completion, the membrane was immersed in TTBS (1x TBS, 0.1% Tween 20) (Table 2) and gently washed.

Next, immunostaining was performed. Blocking buffer (Table 2) was prepared, and the membrane was immersed in it and shaken at room temperature for 1 hour. Subsequently, the primary antibody was added to the blocking buffer at a concentration of 1:20,000. The membrane was immersed in this primary antibody solution and shaken overnight in a cold room at 4 ℃. The next day, the membrane was opened and immersed in TTBS (Table 2) for 5 minutes while shaking, then the TTBS was discarded. This was repeated four times. Next, the secondary antibody was added to the secondary antibody buffer at a concentration of 1/10,000, the membrane was immersed in this, and shaken at room temperature for 1 hour. Washing with TTBS was performed again (5 minutes × 3

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times, 30 minutes × 1 time). Finally, detection was performed using an HRP color development reaction. After thorough washing, the membrane surface was uniformly immersed in 1 mL of color development solution (Luminate "Forte Western HRP Substrate, MILLIPORE) to ensure complete coverage. Following the color development reaction, the membrane was promptly imaged using a LuminoGraph III Lite (ATTO). The antibodies used are shown in each figure.

2.2.5.3 Silver staining

The samples were subjected to SDS-PAGE, and the resulting gel was silver-stained. Silver staining was performed using the MS Silver Staining Kit (Pharma Foods Co., Ltd.). The gel was immersed in the pretreatment solution for 2 minutes and agitated. It was then transferred to the fixing solution and agitated twice for 15 minutes each. Next, it was immersed in the sensitizing solution, agitated for 30 minutes, and washed with MQ (5 min × 3). After shaking in the staining solution for 20 minutes, it was washed again with MQ (1 min × 2). The gel was then immersed in the developing solution and allowed to soak until a staining pattern appeared. Once the pattern was visible, it was immersed in the stopping solution and shaken for 10 minutes. It was then washed with MQ (10 min × 2).

2.2.6 Protein yield analysis

The extracted MHC fraction was added to 1×SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, 100 mM DTT), heated at 95 ℃ for 5 minutes to ensure complete denaturation, and cooled to room temperature. A stock solution of BSA (Bovine Serum Albumin) (ThermoScientific) of known concentration was serially diluted to prepare standard solutions of 62.5 ng, 125 ng, 250

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ng, 500 ng, and 1.0 μg per lane. Samples and standard solutions were kept on ice until use.

Samples and BSA standards were loaded onto the same gel, with a molecular weight marker run concurrently. SDS-PAGE was performed using a 10–12% polyacrylamide gel under the previously described conditions (20 mA/gel, approximately 90 minutes). After electrophoresis, the gel was immersed and shaken in Coomassie Brilliant Blue (CBB) staining solution for 60 minutes. Subsequently, it was decolorized with a decolorizing solution (methanol: acetic acid: water = 3:1:6) until the target bands became clearly visible. After washing with pure water, the bands were photographed using LuminoGraph III Lite (ATTO) under exposure conditions that prevented band saturation.

The acquired gel image was loaded into ImageJ (NIH). Each band was enclosed within an area of identical size using the rectangle tool. The Area value obtained via "Analyze → Measure" was used as an indicator of band intensity. The Area value of the background region adjacent to each band was subtracted to calculate the background-corrected Area value. Next, the area ratio was calculated from the corrected Area values of Bovine Serum Albumin (BSA) loaded on the same gel and the corrected Area values of the target protein (MHC α-chain or β-chain).

The obtained values were corrected for loading volume, total extraction buffer volume, and plant fresh weight used for extraction, and converted to yield per 100 g fresh weight (μg/100 g fresh weight; μg).

2.2.7 Protein tetramerization

2.2.7.1 Protein biotinylation

To 14 μg of protein obtained in 4.5, add 14 μL of BirA (Cosmo Bio), 0.3 μL of 1 M $MgCl_2$, 0.3 μL of 50 μM biotin, and 1.5 μL of 100 mM ATP. Incubate at 37 ℃ for 1 hour.

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Subsequently, the steps from 5.1 to 5.3 were performed, and the presence of biotinylated protein was similarly confirmed.

2.2.7.2 Affinity chromatography

Centrifuge the sample obtained in 7.1 (20,000 rpm, 20 min, 4 ℃) to obtain the supernatant. Set a filter (Membrane Filters, Merck Millipore) in a vacuum pump (Nalgene), and pass the supernatant through the filter under vacuum. Use TALON® Metal Affinity Resin (Takara Bio) as the column to adsorb the His tag. After adding the resin to the column, it was washed with Binding Buffer (pH 7.4). The sample was then slowly eluted, and the liquid eluting from the column was collected as Flow Through (FT). After sample loading, the column was washed again with Binding Buffer (pH 7.4). Elution buffer (Table 2) was then applied in five fractions to elute the protein, yielding fractions Elution1 to Elution5. The presence of the target protein in these fractions was confirmed by SDS-PAGE and Western blotting. The membrane used for Western blotting was subsequently stained with CBB (Coomassie Brilliant Blue) staining solution to assess the purity of the target protein.

2.2.7.3 Making MHC tetramer

Add 10 μL of the protein obtained in 7.2 to a 3000 kDa pore size column and centrifuge at 4 ℃ and 4000 g for 10 minutes. The FT accumulated in the tube was discarded in multiple steps after adding 1 μg of streptavidin (Invitrogen), and the supernatant was pipetted after each centrifugation. Subsequently, the process from 5.1 to 5.3 was followed, and the presence of the protein was similarly confirmed.

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Chapter 2.3 Results

MHC (CR3 and DR8) expression and purification using the Tsukuba System

Vectors incorporating DR3 and DR8 into the Tsukuba system (pTKB3-SSExt-HKD-DR3, pTKB3-SSExt-HKD-DR8) were transformed into *Agrobacterium* GV3101. This transformed *Agrobacterium* was cultured, and an infection solution was prepared. This solution was used to infect *Nicotiana benthamiana* plants approximately 6 weeks after sowing using syringes. Four days after infection, leaves were sampled for subsequent purification. The sampled leaves were ground and extracted.

2.3.1. Ammonium sulfate precipitation

Leaves were lysed in lysis buffer, and ammonium sulfate was added to achieve a final concentration of 30%. Fractions consisting of 30% supernatant and 30% precipitate were obtained. After dialysis of the supernatant and the precipitate dissolved in lysis buffer, purification was performed using affinity chromatography with TALON resin.

2.3.2. Purification of MHC (DR3, DR8)

Ultrafiltration was performed to separate high-molecular-weight proteins from low-molecular-weight proteins for further extraction. The high molecular weight protein solution and low molecular weight protein solution obtained by ultrafiltration were analyzed by Western blotting. As a result, bands for DRA and DRB were confirmed in the high molecular weight protein solution. Therefore, affinity chromatography using TALON resin was performed, followed by treatment with elution buffer. The solution obtained from this procedure was subjected to Western blotting analysis. For this analysis,

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Anti-poly His antibody was used to detect the α-chain, and Anti-poly Avi antibody was used to detect the β-chain. Bands were confirmed. (Figure 3)

2.3.3 Measurement of yield per fresh weight of MHC monomer

Transformed *Agrobacterium* was used to infect tobacco plants, and 100 mg of tobacco leaves were sampled 4 days post-infection. These leaves were disrupted using a Cell Destroyer, 500 μL of Lysis Buffer was added, and soluble proteins were extracted. This sample was subjected to SDS-PAGE. At the same time, fractions containing commercially available BSA of known concentrations at varying amounts were prepared and run simultaneously. The band intensity was measured using ImageJ, and a calibration curve was constructed. Based on this curve, the sample concentration was determined, and the yields of DR3 and DR8 were calculated (Figure 4).

2.3.4. Biotinylation of MHC monomers

Biotinylation of the monomers obtained in Section 1.2 was performed. The experiment confirmed that bands were only observed when BirA and biotin were added (Figure 5).

2.3.5 MHC tetramer preparation

In section 1.4, streptavidin was added to biotinylated MHC monomers to attempt tetramer formation. The solution obtained from this procedure was subjected to Western blotting analysis. For detection, anti-poly Avi antibodies were used in each case. As a result, bands were confirmed (Figure 6).

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Chapter 2.4 Discussion

2.4.1 Vectors

Vectors incorporating DR3 and DR8 into the Tsukuba System (pTKB3-SSExt-HKD-DR3, pTKB3-SSExt-HKD-DR8) were transformed into *Agrobacterium* GV3101. This transformed *Agrobacterium* was cultured, and an infection solution was prepared. This solution was used to infect *Nicotiana benthamiana* plants approximately 6 weeks after sowing using syringes. Four days after infection, leaves were sampled for subsequent purification. The sampled leaves were ground and extracted.

2.4.2 MHC monomer

During purification of MHC (DR3, DR8), analysis was performed using Anti-poly His antibody for detecting the α-chain and Anti-poly Avi antibody for detecting the β-chain. As a result, bands for DRA (the α-chain common to DR3 and DR8), DRB3 (the β-chain of DR3), and DRB8 (the β-chain of DR8) were confirmed (Figure 3). This suggests that the purified proteins are DR3 and DR8 monomers. Bands were observed at approximately 40 kDa for DRA and approximately 45 kDa for both DRB3 and DRB8. Subsequently, MHC monomers were analyzed using ImageJ after electrophoresis alongside BSA of known concentration. Sample concentrations were determined based on the calibration curve, and the yield of MHC monomers was calculated (Figure 4). Calculations from these results indicated yields of 50 μg/100 g FW for both DR3 and DR8.

Biotinylation was confirmed to occur only when BirA and biotin were added, demonstrating successful biotinylation of the MHC monomers.

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In electrophoresis after attempting tetramer formation, a broad band was observed at positions above 100 kDa. While bands at approximately 40 kDa for DRA and approximately 45 kDa for DRB3 and DRB8 were confirmed during monomer verification, this band cannot be definitively identified as a tetramer. However, it indicates the presence of monomers or tetramers, confirming the presence of proteins across a broad molecular weight range.

However, it can be stated that not all MHC exists in the tetrameric state. This is thought to be due to factors such as biotin monomers binding to the biotin-binding site of streptavidin, causing the biotin-binding site to be occupied by non-biotinylated MHC monomers. Therefore, it is considered necessary to ensure the complete removal of biotin monomers and the removal of BirA. Specifically, we aim to prevent non-target MHC monomers from binding to streptavidin's biotin-binding site by performing size exclusion chromatography to purify only the high-molecular-weight MHC monomers again before tetramerization.

2.4.3 MHC tetramer

MHC tetramers are designed to enhance binding with T cells for diagnostic and therapeutic applications. Ultimately, we aim to confirm the presence of MHC tetramers to evaluate their binding with T cells, though we currently lack the capability to perform tests using T cells. To assess binding with specific T cells, our future goal is to reliably produce tetramers.

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Chapter 3 Expression of protein involved in plant immunity

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Chapter 3.1 Introduction

Plant immunity-related proteins

Plant immune proteins play a central role in protecting plants from pathogens, and elucidating their functions is important. However, some plant-derived proteins are difficult to express and fold properly in *Escherichia coli (E. coli)*. Therefore, in this study, we focused on SlPNGase, SlWfi1, and the functionally uncharacterized protein 12g520, which have been reported to be involved in the tomato immune response, and attempted to produce them using a plant expression system. These genes were cloned after cDNA synthesis in previous research at National Taiwan University and are stored as vector inserts.[5,6]

3.1.1 SlWfi1

SlWfi1 is a membrane-localized NADPH oxidase known to play a central role in defense signaling by inducing reactive oxygen species (ROS) production and hypersensitive cell death during pathogen invasion.

3.1.2 SlPNGase

SlPNGase belongs to the PNGase family and is an enzyme that removes N-linked glycans from glycoproteins. During pathogen response, it is thought to be involved in **quality control (ERAD-related)** of misfolded proteins.

3.1.3 12g520

12g520 is a gene identified through comparative genomic analysis focusing on the Bwr12 resistance QTL region in tomatoes. Its sequence suggests it is an LRR-type

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receptor-like protein, potentially involved in PAMP recognition and pattern-triggered immunity (PTI) activation. However, the actual ligands and downstream signaling remain unresolved, making the production of recombinant proteins essential for functional analysis.

Research objectives

This study utilized the "Tsukuba System," a transient expression system that incorporates a replication mechanism derived from geminivirus and a double terminator.

Research in Taiwan is attempting to produce proteins involved in plant immunity, such as 12g520, SlWfi1, and SlPNGase, using a transient expression system in tobacco. By establishing technology for the efficient production of these proteins in plants, we aim to lay the foundation for future functional analysis in planta and elucidation of interaction mechanisms.

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Chapter 3.2 Materials and methods

3.2.1 Plant material

Nicotiana benthamiana was used as a transient expression host. Plants aged 4–6 weeks after sowing were used for agroinfiltration. Cultivation was performed in a growth chamber maintained at 24 ℃ ± 2 ℃.

3.2.2 Vector construction

pTKB3-SSExt-HKD-12g520, pTKB3-SSExt-HKD-Wfi1, pTKB3-SSExt-HKD-SlPNGase, pTKB3-SSExt-CSPH-12g520, pTKB3-SSExt-CSPH-Wfi1, and pTKB3-SSExt-CSPH-SlPNGase. These vector constructs are shown in Figure 7.

3.2.2.1 Culture of *E. coli* colonies

Colonies of *E. coli* (HST08 in Japan, DH5α in Taiwan) obtained by transformation were touched with the tip of a yellow pipette tip and transferred into test tubes containing 10 mL of liquid LB medium (kanamycin 50 μg/mL) (Table 4). These were then shaken overnight at 37 ℃.

3.2.2.2 Plasmid extraction from *E. coli*

To extract plasmids from *E. coli*, the FavorPrep Plasmid Extraction Mini Kit (Chida Science) was used. 10 mL of *E. coli* culture was transferred to a 15 mL Falcon tube, centrifuged (3700 rpm, 5 minutes, room temperature), and the supernatant was discarded. The *E. coli* pellet was dissolved in 250 μL of FAPD1 Buffer and transferred to a 1.5 mL tube. Add 250 μL of FAPD2 Buffer, mix by inverting several times, wait until clear, then let stand at room temperature for 2 minutes. Add an additional 350 μL of FAPD3 Buffer

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and repeat inverting until a white precipitate forms. Centrifuge (15,000 rpm, 5 min, room temperature), transfer the supernatant to the FAPD column set in the Collection tube, centrifuge again (15,000 rpm, 30 sec, room temperature), and discard the liquid accumulated in the tube after passing through the column. Add 400 μL of WF Buffer to the FAPD column, centrifuge (15,000 rpm, 30 seconds, room temperature), and discard the liquid accumulated in the tube. Add 700 μL of Wash Buffer to the FAPD column, centrifuge (15,000 rpm, 30 sec, room temperature), discard the liquid accumulated in the tube, and centrifuge again (15,000 rpm, 3 min, room temperature) to remove water from the column. Transfer the FAPD column to a 1.5 mL tube. Add 100 μL of sterile water (70 ℃) to the FAPD column. Allow to stand for 1 minute, then centrifuge (15,000 rpm, 1 min, room temperature) to recover the plasmid. Store the recovered plasmid in the 1.5 mL tube at -20 ℃.

3.2.2.3 Restriction enzyme digestion

Add any restriction enzyme and the appropriate r-cut smart(NEB) for each restriction enzyme to the plasmid to be cut, treat at 37 ℃ for 1 hour, and obtain linearized plasmid.

3.2.2.4 PCR of the target gene

The In-Fusion HD Cloning kit (Clontech) was used to prepare constructs via homologous recombination of gene products designed to have 15-base homology with the vector ends. Primers were custom-synthesized (Eurofins Genomics) as oligonucleotides fusing the 15-base vector terminus with 24-28 bases of gene-specific sequence. PCR was performed using each gene's cDNA as a template, yielding PCR

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products where the 15-base sequence homologous to the vector terminus was fused to the CDS of each gene. KOD-plus-neo (Yohbo Bio) was used as the PCR reagent. Reaction conditions were 98 ℃ for 10 seconds, 50 ℃ for 30 seconds, and 68 ℃ for 90 seconds for 30 cycles, followed by 68 ℃ for 2 minutes. The PCR products were separated by electrophoresis in a 0.8% agarose gel containing ethidium bromide. Bands were visualized using a Super LED Viewer (BioSpeed Co., Ltd.) and excised from the gel with a cutter knife. The templates and primers used for preparing each insert are shown in the table. (Table 6)

3.2.2.5 Purification of DNA fragments from agarose gel

PCR products and vectors recovered from agarose gels were purified using the QIAquick Gel Extraction Kit (QIAGEN). The gel block containing the desired DNA fragment was transferred to a 1.5 mL tube. Three times the volume of QG buffer was added, and the gel was dissolved by heating at 50 ℃ for 10 minutes in a dry bath incubator. An equal volume of isopropanol was added to the gel, mixed, and then loaded onto the column. Centrifuge (8000 rpm, 1 min), discard the supernatant that passed through the column. Return the column, add 750 μL of PE buffer, centrifuge again (8000 rpm, 1 min), and discard the supernatant. Return the column, spin empty (8000 rpm, 3 min, 4 ℃), and place the column in a new 1.5 mL tube. Add 40 μL of sterile water warmed to 70 ℃, let stand for 1 minute, then centrifuge (8000 rpm, 1 minute, 4 ℃). Store the liquid extracted from the column as purified PCR product at -30 ℃.

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Chapter 3.3 Results

Vector construction for purification of plant immune-related proteins using the Tsukuba System.I aimed to express plant immune-related proteins using the Tsukuba System. I constructed the vector necessary for this expression. Currently, the vector is incomplete.

Initially, I found it difficult to obtain colonies on solid medium. I introduced the target vectors (HKD, CSPH) into *E. coli* and cultured them on solid medium. However, I could only obtain 1 to 2 colonies. Therefore, I added a step of culturing on SOC medium for one hour before culturing on solid LB medium. As a result, I was able to consistently obtain 10 to 20 colonies on solid medium.

Next, I failed to extract the plasmid. I attempted it multiple times, but the concentration remained low. Therefore, during the plasmid extraction kit process, I decided to centrifuge multiple tubes of liquid medium together in the same tube before adding the culture medium to the column, and then perform the extraction using a single column. As a result, I consistently obtained a concentration of 200 ng/μl or higher. These results allowed me to obtain HKD and CSPH that did not contain the target DNA. (Figure 8)

There are challenges in obtaining the target DNA in Japan. I brought back the vector used previously from Taiwan, with the target gene already inserted into it. That vector is adsorbed onto paper. To extract DNA from this paper, I performed PCR. Figure 9 shows the result of the subsequent electrophoresis. The electrophoresis results showed that the DNA could not be detected.

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Chapter 3.4 Discussion

Vector construction for purification of plant immune-related proteins using the Tsukuba System

I aimed to express plant immune-related proteins using the Tsukuba System. We constructed vectors necessary for their expression. This study documents the vector construction process. Note that this research was conducted in Taiwan, with some work continued in Japan after returning.

3.4.1 HKD(pTKB3-SSExt-HKD) and CSPH(pTKB3-SSExt-CSPH) plasmid extract

This study utilized two Tsukuba System vectors. Therefore, we attempted to purify these vectors from stock solutions. In Taiwan, during the plasmid extraction process, extracting plasmids from multiple liquid culture bottles before column use successfully increased the plasmid concentration itself, enabling stable acquisition at 200 ng/μL. Although these plasmid extracts were successful, the DNA concentration was very low, reaching a maximum of 19.1 ng/μL. Therefore, upon returning to Japan, during the vector construction process, a one-hour pre-culture step in SOC medium was performed before culturing *E. coli* on solid LB medium. As a result, the number of colonies on the solid medium increased from one to twenty. DNA was extracted from these colonies, and the plasmid was confirmed by electrophoresis.

3.4.2 Target DNA

Obtained the target DNA from Taiwan, inserted into a vector and absorbed onto paper. This was placed in purified water, and PCR was performed using primers for pTKB3 (Table 6) from the centrifuged supernatant. However, the DNA could not be

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confirmed by electrophoresis. It is necessary to attempt DNA extraction by changing the primers used to those for the currently inserted vector.

If production of plant immune-related proteins can be achieved using the Tsukuba system, it is expected to aid in elucidating protein function in planta and understanding interaction mechanisms. Therefore, once this vector is completed, infection of tobacco plants must be performed, followed by protein extraction and purification, similar to the MHC approach.

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Chapter 4 General discussion

4.1 Production of antigen-specific MHC

I successfully produced the complex MHC class II structure in *Nicotiana benthamiana*. Although it is a difficult structure requiring the precise assembly of multiple proteins, I succeeded in producing the monomer in tobacco.

While producing the tetramer remains a challenge, I believe that by advancing this research, I can produce MHC tetramers—currently used in treatments and diagnostics—more cheaply and in larger quantities.

4.2 Expression of proteins involved in plant immunity

The creation of the target vector was not successful. However, since protein monomer production was achieved in Chapter 1, I believe protein production is possible if a similar method can be employed.

Based on these results, I believe that protein production using the Tsukuba System can affordably produce complex proteins that are difficult to produce, thereby contributing to research advancement. Although protein expression was not achieved in Chapter 2 this time, if protein extraction can be performed as in Chapter 1, it could also aid in elucidating the functions of currently functionally unknown proteins. Currently, complex proteins are produced using animal cells, which is extremely costly. If protein production using the Tsukuba System can be made more efficient, it will be possible to obtain proteins at a lower cost.

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Advanced this research will lead to cost reductions in studies requiring large quantities of protein. Furthermore, even when large quantities of the protein itself are needed, it can be said that low-cost and low-risk production will become possible.

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Table 1

LB medium	/ L
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agarose	12 g

Table 2

Infiltration Buffer	
1M $MgCl_2$	10 mL
1M MES	10 mL
100 mM acetosyringone	1 mL
DW	to 1000 mL

Lysis Buffer	
1M Tris (pH 8.0)	50 mL
2M NaCl	50 mL
0.1 M PMSF	10 mL
DW	to 1000 mL

15 ％ SDS-PAGE running gel	
Milli-Q	2.3 mL
30 ％ arylamide mix	5.0 mL
1.5 M Tris (pH 8.8)	2.5 mL

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10 ％ SDS	0.10 mL
10 ％ ammonium persulfate	0.10 mL
TEMED	0.0040 mL

15 ％ SDS-PAGE stacking gel

Milli-Q	4.1 mL
30 ％ acrylamide mix	1.0 mL
1.5 M Tris (pH 8.8)	0.750 mL
10 ％ SDS	0.060 mL
10 ％ ammonium persulfate	0.060 mL
TEMED	0.0060 mL

10× runnning Buffer

Tris	30.3 g
Glycine	144 g
10 ％ SDS	100 mL
DW	To 1000 mL

Semidry Buffer

Tris	3.03 g
Glycine	14.41 g
methanol	200 mL
DW	To 1000 mL

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10× TBS

NaCl	80 g
KCl	2 g
Tris	30 g
DW	To 1000 mL

TTBS

10×TBS (pH 7.4)	100 mL
20 %Tween	10 mL
DW	To 1000 mL

Blocking Buffer

Skim milk	0.5 g
Polyvinylpyrrolidone K30	0.2 g
1× TTBS	10 mL

Secondary antibody Buffer

Skim milk	0.5 g
1× TTBS	10 mL

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Binding Buffer

1M Tris-HCl (pH 7.4)	3.03 g
NaCl	14.41 g
0.1 M PMSF	10 mL
2 M imidazole	10 mL
DW	To 1000mL

Elution Buffer

1 M Tris-HCl (pH 7.4)	10 mL
NaCl	29.25 g
0.1 M PMSF	10 mL
Imidazole	17 g
DW	To 1000 mL

Table 3

Primer name	
HKD-DR3-fwd	AGCGAGAGTAGCGCCagtcgaCCAGGGCCGCCTCCGG
HKD-DR3-rev	aaataaaacttccagagtcgaCAAACGGGTTTTTAAT
HKD-DR8-fwd	AGCGAGAGTAGCGCCagtcgaGGAATTACTATTCCTAG
HKD-DR8-rev	aaataaaacttccagagtcgaGGCGACATGATGGACGA

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Table 4

LB medium	/ L
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agarose	12 g

Table 5

Infiltration Buffer	
1M $MgCl_2$	10 ml
1M MES	10 ml
100 mM acetosyringone	1 ml
DW	to 1000 ml

10× runnning Buffer	
Tris	30.3 g
Glycine	144 g
10 ％ SDS	100 ml
DW	To 1000 ml

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Table 6

Primer name	
HKD-SlWfi1_fwd	cgccagcgagagtagcgccg*tcgac*atgcaaaattcggaaaatc
HKD-SlWfi1_rev	cctgaaataaaacttccaggtcgacaaaattttctttatggaaatcaaac
HKD-SlPNGase_fwd	cgccagcgagagtagcgccgtcgacatggcgattctcatcttc
HKD-SlPNGase_rev	cctgaaataaaacttccaggtcgaccaaagcagaaaatgaacc
HKD-12g520-H1706_fwd	cgccagcgagagtagcgccgtcgacatgtggcctctccttttc
HKD-12g520-H1706_rev	cctgaaataaaacttccaggtcgacagtccgtcgtctcagagc
CSPH-SlPNGase_fwd	cgccagcgagagtagcgccg*tcgac*atggcgattctcatcttc
CSPH-SlPNGase_rev	cttgaaataacacttcaaggtcgaccaaagcagaaaatgaacc
CSPH-SlWfi1_fwd	cgccagcgagagtagcgccgtcgacatgcaaaattcggaaaatc
CSPH-SlWfi1_rev	cttgaaataacacttcaaggtcgacaaaattttctttatggaaatcaaac
CSPH-12g520_fwd	cgccagcgagagtagcgccgtcgacatgtggcctctccttttc
CSPH-12g520-rev	tggaactgacagaaccgcaacgttagtccgtcgtctcagagc

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Figure 1

Red indicates the α chain, blue indicates the β chain. The antigen-presenting site is enlarged and shown below. The antigen-presenting site of MHC class I is composed solely of the α chain, whereas the antigen-presenting site of MHC class II is composed of both the α chain and the β chain.

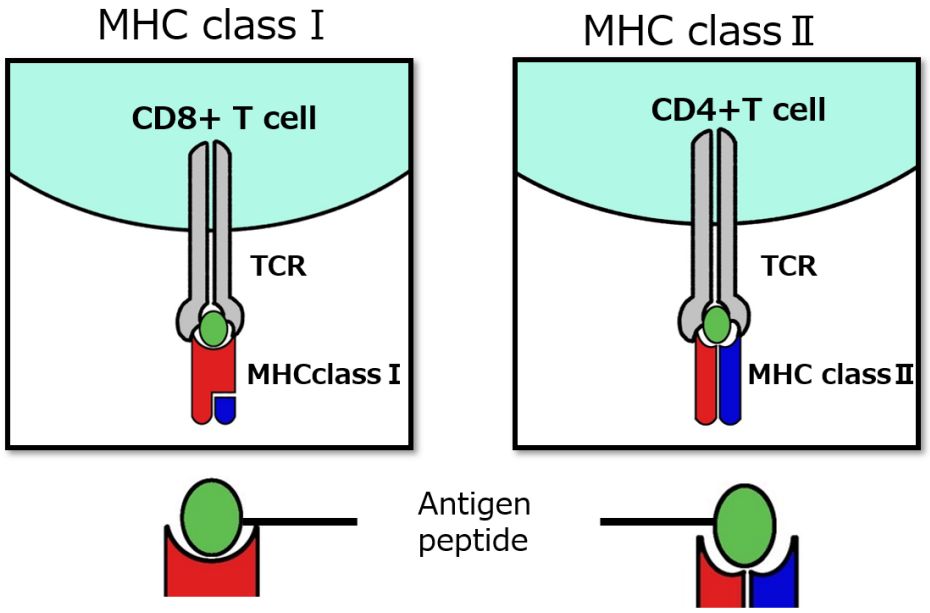

Figure 2

(A)pTKB3-SSExt-HKD

MHC production was carried out using this vector.

Additionally, for the production of plant immunity-related proteins, we aimed to construct vectors by incorporating the target gene into this vector.

(B) pTKB3-SSExt-CSPH

This vector was used exclusively for plant immunity-related proteins. We aimed to construct vectors by incorporating the target gene into this vector.

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(A)

(B)

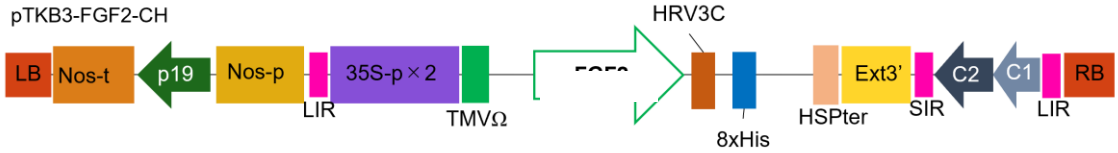

Figure 3

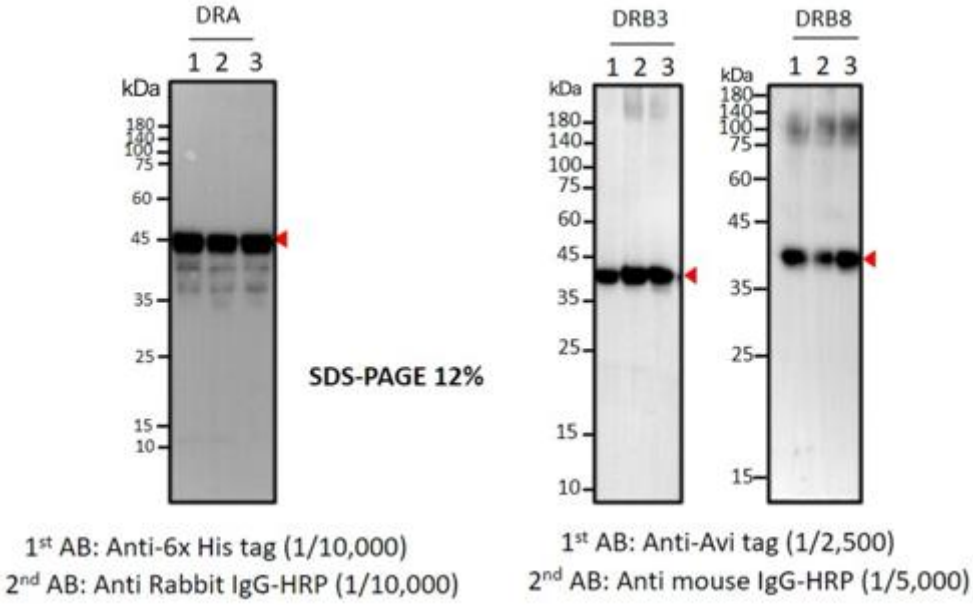

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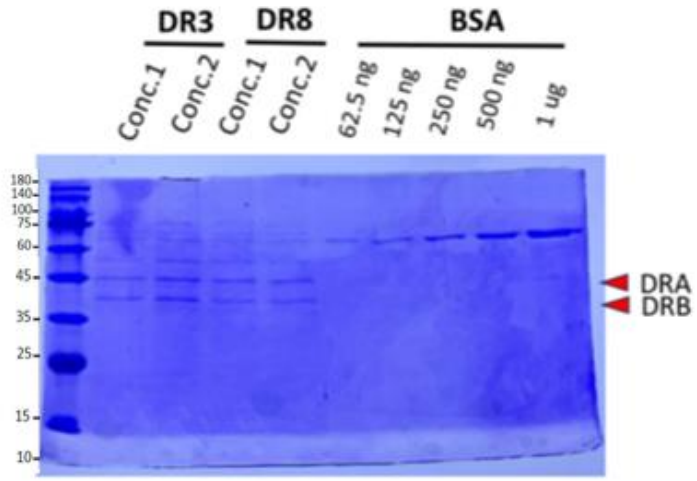
Figure 4

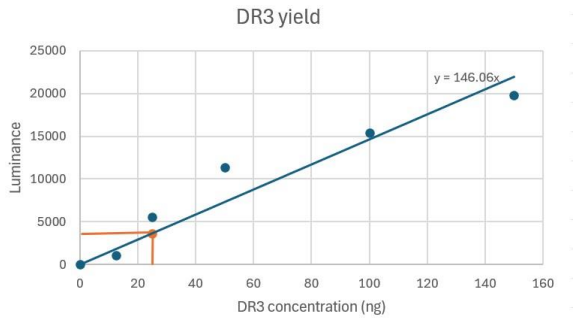
(A)Data of SDS-PAGE with using BSA

(B)(C)Calculated yield of DR3/8

(A)



(B)



(C)

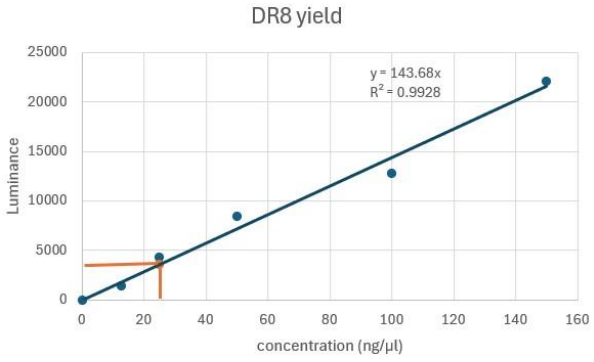


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Figure 5

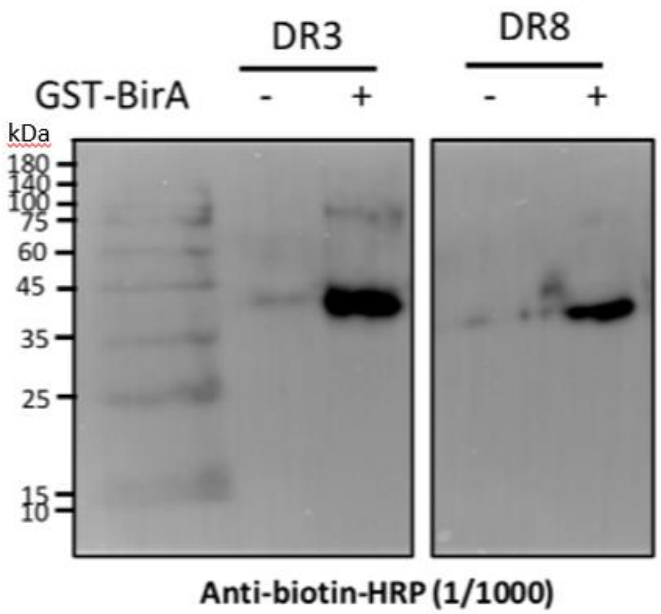
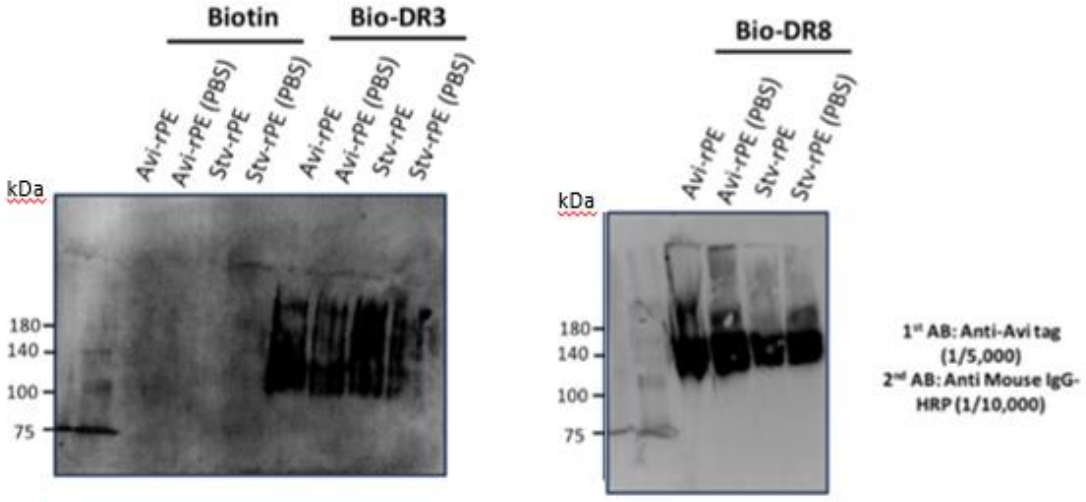


Figure 6



doi:10.6342/NTU202600779

Figure 7

(A)pTKB3-SSExt-HKD

MHC production was carried out using this vector.

Additionally, for the production of plant immunity-related proteins, we aimed to construct vectors by incorporating the target gene into this vector.

(B) pTKB3-SSExt-CSPH

This vector was used exclusively for plant immunity-related proteins. We aimed to construct vectors by incorporating the target gene into this vector.

(A)

(B)

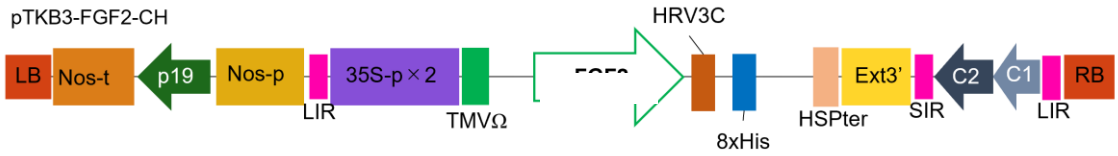


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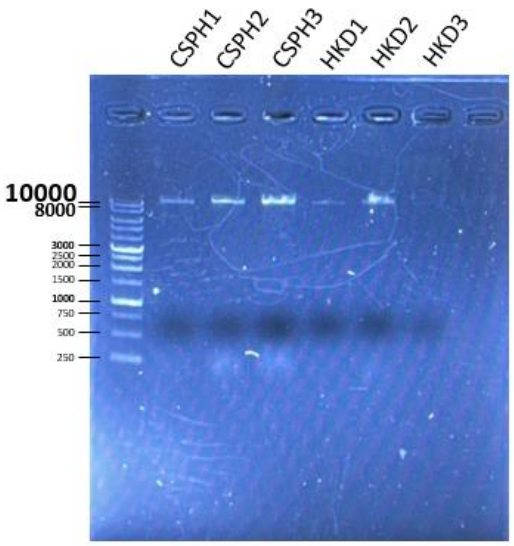
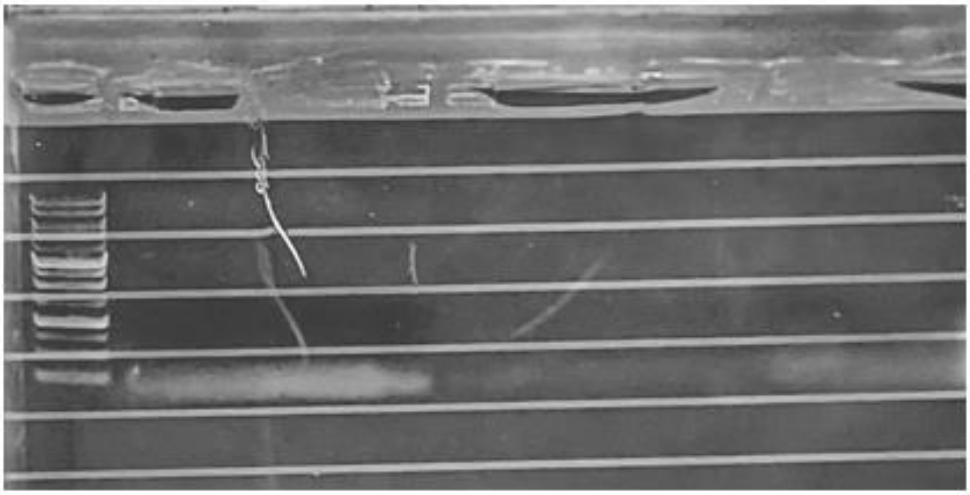
Figure 8



pTKB3-SSExt-HKD：14976 bp
pTKB3-SSExt-CSPH：15006 bp

Figure 9



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