THE ROLE OF TYPE III EFFECTOR PROTEIN p0903 FROM *Bradyrhizobium* sp. DOA9 ON NODULATION AND NITROGEN FIXATION IN *Arachis hypogaea* L.

Beedou Aphaiso

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บทบาทของโปรตีนเอฟเฟกเตอร์ชนิดที่ 3 p0903 จาก *Bradyrhizobium* sp. DOA9 ในการเข้าสร้างปมและการตรึงไนโตรเจน ใน *Arachis hypogaea* L.

นายบิดู อภัยโส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

.....
(Prof. Dr. Arinthip Thamchaipenet)
Chairperson

.....
(Prof. Dr. Neung Teaumroong)
Member (Thesis Advisor)

.....
(Assoc. Prof. Dr. Panlada Tittabutr)
Member (Thesis Co-Advisor)

.....
(Prof. Emeritus Dr. Nantakorn Boonkerd)
Member

.....
(Asst. Prof. Dr. Pakpoom Boonchuen)
Member

.....
(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)
Vice Rector for Academic
Affairs and Quality Assurance

.....
(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural
Technology

บิดู อภัยโส : บทบาทของโปรตีนเอฟเฟกเตอร์ชนิดที่ 3 p0903 จาก *Bradyrhizobium* sp. DOA9 ในการเข้าสร้างปมและการตรึงไนโตรเจน ใน *Arachis hypogaea* L.
อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. หนึ่ง เตียอำรุง, 105 หน้า

คำสำคัญ : แบรดีไรโซเบียม DOA9/ระบบหลั่งประเภทที่ 3/เอนไซม์ SUMO/ปฏิสัมพันธ์ระหว่างพืชและจุลินทรีย์

โปรตีนเอฟเฟกเตอร์ที่หลั่งผ่านระบบหลั่งประเภทที่ 3 (type III secretion system: T3SS) ของไรโซเบียมที่ตรึงไนโตรเจนได้เป็นปัจจัยสำคัญในการกำหนดความเหมาะสมเพื่อการอยู่ร่วมกันพืชตระกูลถั่ว รายงานก่อนหน้านี้พบว่า T3SS ของ *Bradyrhizobium* sp. DOA9 มีผลเชิงลบต่อการอยู่ร่วมกันกับถั่วลิสง (*Arachris hypogaea* L.) ในการศึกษานี้ ได้ศึกษาบทบาทของโปรตีนเอฟเฟกเตอร์ 4 ชนิด (p0490, p0871, SkP48, และ p0903) ที่มีโดเมนโปรตีเอสแบบ small ubiquitin-like modifier (SUMO) ซึ่งพบใน DOA9 โปรตีเอสแบบ SUMO ทั้ง 4 ชนิดของ DOA9 ถูกจัดกลุ่มภายใน clade 1 ซึ่งแบ่งย่อยเป็น 2 subclades โดยโปรตีน p0490 และ p0871 อยู่ใน subclade 1-1 ขณะที่ p0903 และ SkP48 อยู่ใน subclade 1-2 โปรตีเอสเหล่านี้มีความคล้ายคลึงกันของลำดับเบสน้อย โดยมีความเหมือนกันเพียง 57% ระหว่าง p0490 และ p0871 และ 35% ระหว่าง p0903 และ SkP48 ถึงแม้ว่าจะมีโดเมน Ubiquitin-like protease 1 (ULP1) แบบเดียวกันที่ C-terminal แต่บริเวณ N-terminal ไม่มีการทำงานของโดเมน การทำงานนั้นมีความแตกต่างกันมากระหว่างโปรตีเอสเหล่านี้ โดย p0903 และ SkP48 มีโดเมนซ้ำ (repeat domains: RDs) ที่แตกต่างกัน 2 โดเมน ขณะที่ p0490 และ p0871 ไม่มี RDs ขณะที่เชื้อสายพันธุ์ดั้งเดิม *Bradyrhizobium* sp. DOA9 (WT) และ การกลายพันธุ์ของเอนไซม์ SUMO โปรตีเอส p0490 และ p0871 กระตุ้นการเกิดปมรากใน *A. hypogea* อย่างไม่มีประสิทธิภาพ การกลายพันธุ์ของเอนไซม์ SUMO โปรตีเอส SkP48 หรือ p0903 ทำให้เกิดการอยู่ร่วมกันอย่างมีประสิทธิภาพเทียบเท่ากับสายพันธุ์ดั้งเดิม *B. arachidis* CCBAU051107 นอกจากนี้ การลบยีน *p0903* ออกทั้งหมดทำให้การเกิดปมรากและการเจริญเติบโตของพืชฟื้นคืนกลับมาใน *A. hypogea* 4 สายพันธุ์ ขอนแก่น 5 (KK5), ไทยนาน 9 (TN9), กาฬสินธุ์ 2 (KS2), และสุโขทัย 38 (SK38) การศึกษาการนำกลับเข้าไปของยีน *p0903* ไปยังสายพันธุ์ DOA9-Δ*p0903* ที่กลายพันธุ์ทำให้เห็นความสำคัญของโดเมนโปรตีน ULP1 ในการยับยั้งการเกิดปมรากใน *A. hypogaea* นอกจากนี้ การชักนำด้วยสารฟลาโวนอยด์ กลุ่ม naringenin ยังเพิ่มการแสดงออกของยีน *p0903* อย่างมีนัยสำคัญเมื่อเทียบกับสภาวะที่ไม่ได้ชักนำด้วยฟลาโวนอยด์ของเชื้อ WT และสภาวะที่ถูกชักนำด้วยฟลาโวนอยด์ กลุ่ม genistein ที่ 3 วันหลังการปลูกเชื้อ ตรวจพบการสะสมของกรดจัสโมนิก (jasmonic acid: JA) ในรากถั่วลิสงเป็น 2.32, 1.27 และ 0.64 เท่า ในเชื้อสายพันธุ์ WT, DOA9-Δ*p0903* และ Δ*p0903-p0903*-ULP-lack ตามลำดับ เมื่อเปรียบเทียบกับพืชที่ไม่ได้รับการปลูกเชื้อ

ที่มีความเข้มข้นของ JA 49.56 ไมโครกรัมต่อกรัมน้ำหนักสดของราก การแสดงออกเพิ่มขึ้นของยีนที่เกี่ยวข้องกับภูมิคุ้มกันของพืช (defense-related genes) รวมถึง transcription factor (*Myc2),* โปรตีน pathogenesis-related protein 4 (*PR4*) และ defensin (*Def2-2*) ในการส่งสัญญาณ JA/ethylene (ET) ในระยะเริ่มต้นของเชื้อรากที่ได้รับการปลูกเชื้อด้วยเชื้อสายพันธุ์ WT เมื่อเทียบกับที่ได้รับการปลูกเชื้อด้วยเชื้อกลายพันธุ์ DOA9-Δ*p0903* นอกจากนี้ p0903 ยังถูกระบุว่าเป็นโปรตีนควบคุมสำคัญของการส่งสัญญาณการอยู่ร่วมกันในระยะเริ่มต้นในถั่วลิสงสายพันธุ์ KK5 โดยผลต่อยีนที่เกี่ยวข้องกับระยะต่างๆ ของการส่งสัญญาณในระยะเริ่มต้นของการอยู่อาศัยร่วมกัน เช่น cyclic nucleotide-gated (*CNGC*), symbiotic remorins (*SYMREM*), และ E3 ubiquitin-protein ligase LIN-1 (*CERBERUS*) ข้อมูลเหล่านี้แสดงให้เห็นถึงความสำคัญของโปรตีเอส SUMO ในระหว่างการมีปฏิสัมพันธ์ร่วมกันระหว่างแบรดีไรโซเบียมและถั่วลิสง ซึ่งอาจมีประโยชน์ในการพัฒนาการผลิตหัวเชื้อที่ประสิทธิภาพสูงเพื่อเพิ่มผลผลิตถั่วลิสง

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2567

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

BEEDOU APHAISO : THE ROLE OF TYPE III EFFECTOR PROTEIN p0903 FROM *Bradyrhizobium* sp. DOA9 ON NODULATION AND NITROGEN FIXATION IN *Arachis hypogaea* L. THESIS ADVISOR : NEUNG TEAUMROONG, Ph.D. rer. nat., 105 PP.

Keyword : *Bradyrhizobium* sp. DOA9/Type III Secretion System/SUMO proteases/ Plant- microbe Interaction.

Effector proteins secreted via the type III secretion system (T3SS) of nitrogen-fixing rhizobia are key determinants of symbiotic compatibility in legumes. However, a previous report revealed that the T3SS of *Bradyrhizobium* sp. DOA9 has negative effects on *Arachris hypogaea* symbiosis. In this study, it was found that the symbiotic role of 4 effector proteins (p0490, p0871, SkP48, and p0903), which contain the small ubiquitin-like modifier (SUMO) protease domain identified in DOA9 during symbiosis, were characterized. The four DOA9 SUMO proteases are clustered within clade 1, which is further divided into two subclades. Proteins p0490 and p0871 are grouped in subclade 1-1, while p0903 and SkP48 fall within subclade 1-2. These proteases exhibit low sequence similarity, with only 57% identity between p0490 and p0871, and 35% identity between p0903 and SkP48. Despite sharing a common Ubiquitin-like protease 1 (ULP1) domain typically located at the C-terminal, the N-terminal regions, which lack functional domains, are highly divergent among these proteases. Notably, p0903 and SkP48 each contain two distinct repeat domains (RDs), whereas p0490 and p0871 lack RDs. While the *Bradyrhizobium* sp. DOA9 wild type (WT) strain and mutantion of SUMO-proteases p0490 and p0871 induced inefficient nodulation in *A. hypogea*, mutation of the SUMO-proteases SKP48 or p0903 restored an efficient symbiosis comparable to that of the type of strain *B. arachidis* CCBAU051107. Additionally, the complete deletion of p0903 restored nodulation and plant growth in four peanut cultivars (KK5, TN9, KS2, and SK38). A complementation study of DOA9-Δ*p0903* with various mutated forms of p0903 highlighted the importance of the ULP1 domain in the restriction of nodulation in *A. hypogaea*. Furthermore, naringenin significantly induced the expression of the *p0903* gene compared to both the WT non-induced and genistein-induced conditions. At 3 dpi, the accumulation of jasmonic acid (JA) in peanut roots

was 2.32-, 1.27-, and 0.64-fold higher in the WT, DOA9-Δ*p0903*, and Δ*p0903-p0903*-ULP-lack strains, respectively, compared to non-inoculated plants, which had a JA concentration of 49.56 µg/g root fresh weight. The upregulation of several defense-related genes, including the transcription factor (*Myc2*), pathogenesis-related protein 4 (*PR4*), and defensin (*Def2-2*), was observed in the JA/ethylene (ET) signaling pathway at the early stages of infection in roots inoculated with the WT strain, compared to those inoculated with the DOA9-Δ*p0903* strain. Additionally, p0903 is identified as a key regulator of early symbiosis signaling in peanuts (KK5), influencing genes involved in various stages of early signaling, such as cyclic nucleotide-gated channels (*CNGC*), symbiotic remorins (*SYMREM*), and E3 ubiquitin-protein ligase LIN-1 (*CERBERUS*). These data highlight the importance of SUMO-protease effectors during the symbiotic interaction between bradyrhizobia and peanuts, which could be useful for the development of a high-quality inoculant in increasing yield of peanut.

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Student's Signature _____
Advisor's Signature _____
Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

^{a} C	=	degree Celsius
%	=	percentage sign
*	=	Asterisks
µF	=	microfarad
µg	=	microgram
µL	=	microliter
µm	=	micrometer
µM	=	micromolar
µmol/m^2	=	micromole per square meter measured in a second
Δ	=	deletion mutant symbol
Ω	=	insertional mutant symbol or ohms
aa	=	amino acid
AG medium	=	Arabinose-Gluconate Medium
ANOVA	=	Analysis of Variance
ARA	=	Acetylene Reduction Assay
BNF	=	Biological nitrogen fixation
cefo^{r}	=	cefotaxime resistant
Ct	=	Threshold cycles
Ct ($^{-\Delta\Delta CT}$)	=	2^(–delta delta Ct) method
DNA	=	Deoxyribonucleic Acid
dpi	=	days post inoculation
Et al.	=	Et alia (and other)
ETI	=	Effector-Triggered Immunity
h	=	hour
ha	=	hectare
H/D/C	=	Histidine/Aspartic acid/Cysteine
IRLC	=	Inverted Repeat-Lacking Clade

LIST OF ABBREVIATIONS (Continued)

ISR	=	Iinduced Systemic Resistance
IT	=	Infection Threads
JA	=	Jasmonic Acid
Kg	=	Kilogram
KK5	=	Khon Kaen 5
Km^r	=	Kanamycin resistant
KS2	=	Kalasin 2
kv/cm	=	Kilovolt/centimeter
LB	=	Luria-Bertani
LRR	=	leucine-Rich Repeat
M	=	Molarity
m	=	minute
MAMPs	=	Microbe-Associated Molecular Patterns
MAPKs	=	Mitogen-Activated Protein Kinases
mg	=	milligram
ml	=	milliliter
mm	=	millimeter
N_2	=	Nitrogen gas
NBS	=	Nucleotide-Binding Site
NEL	=	new E3 ubiquitin ligase
NF	=	Nod Factor
ng	=	nanogram
NS	=	Not Significant
OD	=	Optical Density
ORFs	=	Open Reading Frames
OROG	=	One Research One Graduate
PCR	=	Polymerase Chain Reaction
pDOA9	=	plasmid of DOA9
ptr	=	presumed transcriptional regulator

LIST OF ABBREVIATIONS (Continued)

qRT-PCR	=	quantitative Reverse Transcription Polymerase Chain Reaction
RD	=	Repeat Domain

CHAPTER I

INTRODUCTION

1.1 Introduction

The symbiotic relationship between rhizobia and legumes represents a fascinating and complex biological process with significant agronomical and ecological importance. This interaction begins when nitrogen-fixing rhizobia release signalling molecules called Nod factors (NFs), which trigger the formation of root nodules and facilitate bacterial infection. Within these nodules, rhizobia convert atmospheric nitrogen into a plant-accessible form in exchange for photosynthates and other nutrients from the host plant. Among rhizobia, *Bradyrhizobium* strains are particularly valuable in agriculture because of their ability to form symbioses with economically important legume crops such as soybeans, peanuts, and cowpeas (Ayalew et al., 2021; Prakamhang et al., 2015; Vicario et al., 2016). This mutually beneficial partnership has attracted considerable scientific interest, as it plays a crucial role in sustainable agriculture and global nitrogen cycling.

In several strains of *Bradyrhizobium*, a type III secretion system (T3SS) plays a crucial role during symbiosis, significantly influencing the interaction with host plants (Okazaki et al ., 2016; Teulet et al., 2019). The T3SS serves as a nanosyringe structure found in Gram-negative bacteria. Its function is to deliver type III effectors (T3Es) into eukaryotic host cells during interaction with the host (Büttner, 2016). As observed in pathogenic bacteria, the T3Es identified in rhizobia, often referred to as nodulation outer proteins (Nops), can act as double-edged swords, depending on the host plant (Staehelin & Krishnan, 2015). They can promote symbiosis by suppressing the plant's immune system; however, if recognized by plant resistance proteins, they can trigger an immune response known as effector-triggered immunity (ETI), which can block rhizobial infection. This dual functionality highlights the complex interplay between rhizobia and their host plants, whereby the same effector proteins can either facilitate or hinder symbiosis based on the plant's immune recognition mechanisms.

Moreover, the diverse array of T3Es identified in rhizobial strains contributes to host genotype-specific compatibility or incompatibility across various legume species highlighting the intricate nature of rhizobia-legume interactions (Camuel et al., 2023; Ge et al., 2016).

Our knowledge concerning the impact of T3Es on the symbiotic interaction of rhizobia with *Arachis hypogaea* L. remains extremely limited, despite this species being the second most important legume crop after soybean. Moreover, this legume plays a crucial role in sustainable agriculture and food security worldwide, particularly in developing countries, because of its nutritional value and the importance of its symbiosis with rhizobia (Neves et al., 2023). Compared with the case of no inoculation, *Bradyrhizobium* inoculation increased the pod yield by 40%, reducing the need for nitrogen chemical fertilizers. The symbiosis between peanuts and rhizobia not only reduces the need for chemical fertilizers but also contributes to improved soil health, making peanuts a uniquely sustainable crop in the face of growing agricultural challenges (Asante et al., 2020; Valetti et al., 2016). Thus, as the global demand for peanuts continues to rise, understanding and optimizing this symbiosis will be crucial for ensuring sustainable production and food security.

Bradyrhizobium sp. DOA9 was originally isolated from an *Aeschynomene americana* nodule in paddy soil and possesses a unique megasymbiotic plasmid of 7.36 kb, a rare feature among *Bradyrhizobium* strains (Noisangiam et al., 2012; Okazaki et al., 2015). DOA9 has the ability to nodulate a wide range of legume plants, with its T3SS playing a crucial role in host interactions. The T3SS has a positive effect on symbiosis with *Stylosanthes hamata* but negatively impacts interactions with *A. hypogaea*, *Crotalaria juncea*, *Macroptilium atropurpureum* and *Vigna radiata* (Songwattana et al., 2017). *In silico* analysis revealed fourteen putative effectors in the DOA9 genome, including the novel SUMO-like type III effector SkP48, the longest T3E found to date. SkP48 has been shown to negatively influence the symbiotic interactions of DOA9 with *A. hypogaea*, *C. juncea* and *Vigna* species (Piromyou, et al., 2021). The SUMO protease represents one of the most abundant classes of T3E identified in *Bradyrhizobium* strains. For example, the SUMO-protease Bel2-5 in *B. elkanii* USDA61 can completely inhibit nodulation in *Rj4/Rj4* soybeans (Ratu et al., 2021a). Conversely, the SUMO-protease Sup3 from *Bradyrhizobium* sp. WSM1744,

Bradyrhizobium sp. ORS86, and *Bradyrhizobium* sp. NAS96.2 has been found to directly activate nodulation in *A. indica* (Camuel et al., 2023). Additionally, Bel2-5 of USDA61 triggered nodulation in the Nod factor receptor 1 soybean mutant (*nfr1*) (Ratu et al., 2021a). SUMO proteases, also known as desumoylating enzymes, play a critical role in regulating protein function by removing small ubiquitin-like modifier (SUMO) moieties from target proteins. These enzymes are essential components of the dynamic sumoylation system, which allows for rapid and specific modulation of protein activity in response to cellular needs and environmental conditions (Wilkinson & Henley, 2010; Yadav et al., 2024). NopD, a SUMO protease-containing effector from *Bradyrhizobium* sp. XS1150, processes plant SUMO proteins and cleaves SUMO-conjugated substrates, confirming the functionality of its Ubiquitin-like protease 1 (ULP1) domain. These findings support the idea that NopD homologues function similarly to their counterparts in plant pathogens, specifically by deSUMOylating targeted plant proteins (Xiang et al., 2020). In the genome of *Bradyrhizobium* sp. DOA9, three additional SUMO proteases (p0490, p0871, and p0903) were identified, although their functions remain unstudied. Therefore, this study investigated their roles in the symbiotic interaction between DOA9 and *A. hypogaea*. Notably, mutating the SUMO protease p0903 restored nodulation in peanuts. The function of the ULP1 domain in p0903 and the molecular mechanisms underlying the symbiotic interaction mediated by p0903 were also elucidated in this study.

1.2 Objectives

1.2.1 To investigate the role of the putative SUMO-T3Es of DOA9 in *A. hypogaea* KK5 symbiosis.

1.2.2 To find the function of the SUMO-p0903 of DOA9 in *A. hypogaea* cultivars symbiosis.

1.2.3 To know the role of the ULP1 domain of SUMO-p0903 in *A. hypogaea* KK5 symbiosis.

1.2.4 To understand the function of SUMO-p0903 involved in the *A. hypogaea* symbiosis related genes and plant defense response genes.

CHAPTER II

LITERATURE REVIEWS

2.1 Biological nitrogen fixation

Biological nitrogen fixation (BNF) was first reported in 1901 (Beijerinck, 1901), regarding groups of prokaryotes can fix atmospheric nitrogen. The nitrogenase enzyme is used in nitrogen fixation reaction to convert nitrogen from the atmosphere to ammonium. Plants assimilated the ammonium from the bacteria and gave shelter and carbon sources to bacteria (Mousavi et al., 2020). These bacteria include living soil bacteria, aquatic microorganism, and symbiotic bacteria (Postgate, 1982; Coale et al., 2024). Nitrogenase contains of two protein components: dinitrogenase and dinitrogenase reductase. The nitrogenases share the same structural ability but differ in the heterometallic element present in the active site of the dinitrogenase unit, and they are sensitive to oxygen (López-Torrejón et al., 2016). The *nif* gene family encodes nitrogenase with iron-molybdenum cofactors at the active site. The iron-vanadium cofactor, encoded by the *vnf* genes, is another cofactor of nitrogenase. The alternative nitrogenase cofactor, which uses iron as the sole cofactor (FeFe-co), is encoded by the *anf* genes (Willing et al., 1989). Therefore, nitrogenases are complex metalloenzymes that convert N_2 to NH_4 by reductase enzymes. The nitrogen fixation process needs 16 molecules of ATP to fix 1 mole of nitrogen as following equation.

$$N_2 + 8H^+ + 16ATP + 8e^- \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

Two components of the nitrogenase enzyme, which are the catalytic and the reductase units, with the size of proteins between 220-250 and 50-60 kD depending on the organism (**Fig. 2.1**) (Halbleib & Ludden, 2000).

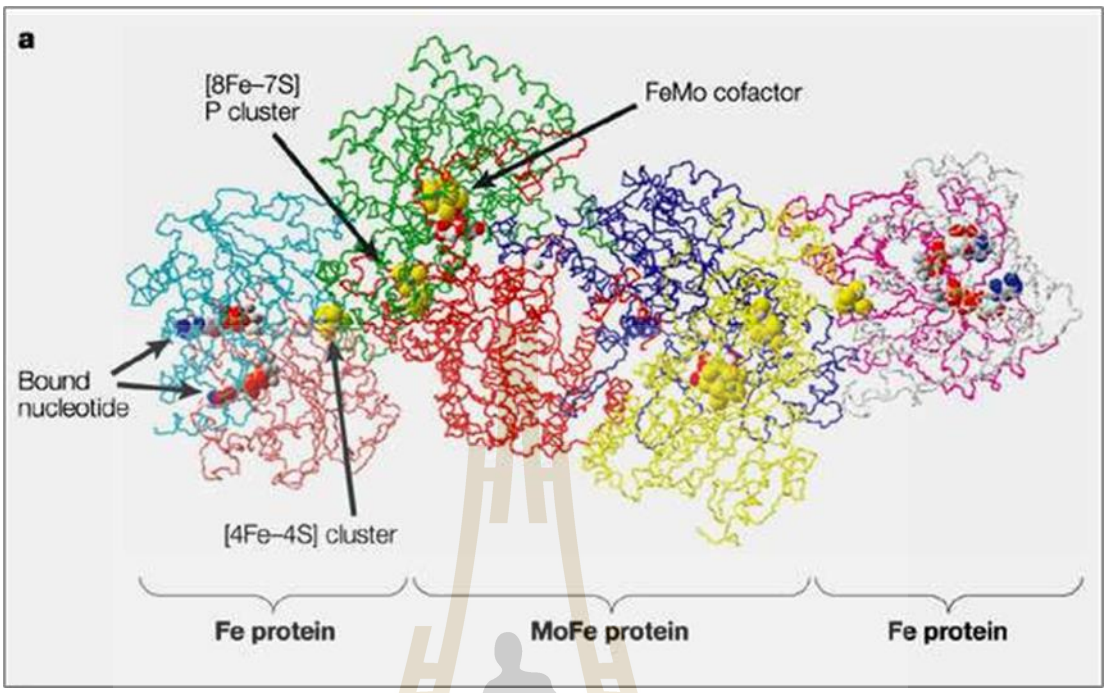

Figure 2.1 Nitrogenase structure (Dixon & Kahn, 2004).

2.2 Nodulation mechanisms in *Rhizobium* and leguminous plant symbiosis

The nodule formation process uses the symbiosis signaling pathway through the production and secretion of Nod Factors (NFs) (D'haeze & Holsters, 2002) . The symbiosis starts with the leguminous plants secretion of flavonoids that encourage the reading of the Nod factor gene to transcribe NFs. Then NFs are secreted outside the cells of rhizobia and accepted by Nod factor receptors in plant root cell to generate nodule organogenesis (Chaintreuil et al., 2016). The symbiosis between *Rhizobium* and legumes requires a process that exchanges nitrogen from bacteria to the legumes, and dicarboxylic acids from the legumes provide to bacteria (Patra & Mandal, 2022).

2.2.1 Rhizobial entries plant root

The Rhizobia have entering near of the root hair-entry and crack-entry. The intracellular plant tissue infection is the most invaded pathway of rhizobia to enter plant root cells, which is called infection thread (IT) (Oldroyd et al., 2011). The root hair-entry is determined predominantly in legume species, such as Pisum sp, Trifolium sp, Vicia sp, Lotus japonicus, Medicago truncatula, and Glycine max (Bonaldi et al., 2011). Another pathway of the infection process, known as crack-entry, occurs

in approximately 25% of cases in tropical and warm temperate areas, symbiosis with plants such as Arachis hypogaea, Neptunia natans, Chamaecrista fasciculata, Mimosa pudica, Sesbania rostrata, Stylosanthes guianensis, and some species in Aeschynomene (Arrighi et al., 2012; Guha et al., 2016; Chaintreuil et al., 2018; Sharma et al., 2020).

2.2.2 Host specificity for nodule formation

In leguminous symbiosis with rhizobia, there exists a precise recognition mechanism between the host and *Rhizobium*. Depending on the plant species, for example, *Bradyrhizobium* employs either a nod factor-dependent (NF-dependent) or nod factor-independent (NF-independent) pathway for nodulation. The symbiotic interaction between bradyrhizobia and *Aeschynomene* has led to several novel characteristics, including nodulation that is independent of nod factors, as well as nodulation occurring on stems and roots. However, the symbiotic relationship among three groups of *Aeschynomene-Bradyrhizobium*, whether they occur with nod factor-dependent or nod factor-independent mechanisms, remains unclear (Miché et al., 2010). The first group, consisting of *A. elaphroxylon* and *A. americana*, can develop nodules on their roots through association with non-photosynthetic bradyrhizobia (ORS301). The second group, comprising of *A. nilotica* and *A. afraspera*, can develop nodules on both stems and roots. These nodules develop through associations with photosynthetic bradyrhizobia on stems and with both photosynthetic and non-photosynthetic bradyrhizobia on roots. The third group, including *A. indica* and *A. sensitiva*, generated nodules exclusively on roots and stems with photosynthetic bradyrhizobia such as *Bradyrhizobium* sp. strains BTAi1 and ORS278 (Giraud et al., 2007). Other *Bradyrhizobium* species can cross-inoculate with multiple host plant groups. The photosynthetic *Bradyrhizobium* sp. ORS285 possesses a unique set of canonical *nodABC* genes used exclusively for interaction with the specific host *A. afraspera* in an NF-dependent manner. However, neither *A. indica* nor *A. sensitiva* utilize additional genes to support alternative mechanisms for host nodule organogenesis (Bonaldi et al., 2011).

2.2.2.1 Nodule formation by nod-factors

Generally, rhizobial nodulation is driven by *nod* genes responsible for nodulation organogenesis. Plant flavonoids, including flavone, chalcone, isoflavone, and flavanone, induce the expression of *nod* genes. This

induction serves as the initial step in plant-bacterial communication. The expression of these *nod* genes depends on a chemical signal from the legume, which subsequently activates the NodD transcriptional activator. The NodD protein interact with the plant biochemical pathways (**Fig. 2.2**). Several rhizobacterial species use an NFs-dependent to from nodule. Nodule signaling involves the binding of Nod factor (NFs) to specific receptors on the plasma membrane of plant cells. This binding trigger calcium spiking, which is essential for activating the expression of nodule organogenesis genes, thus initiating the nodulation process.

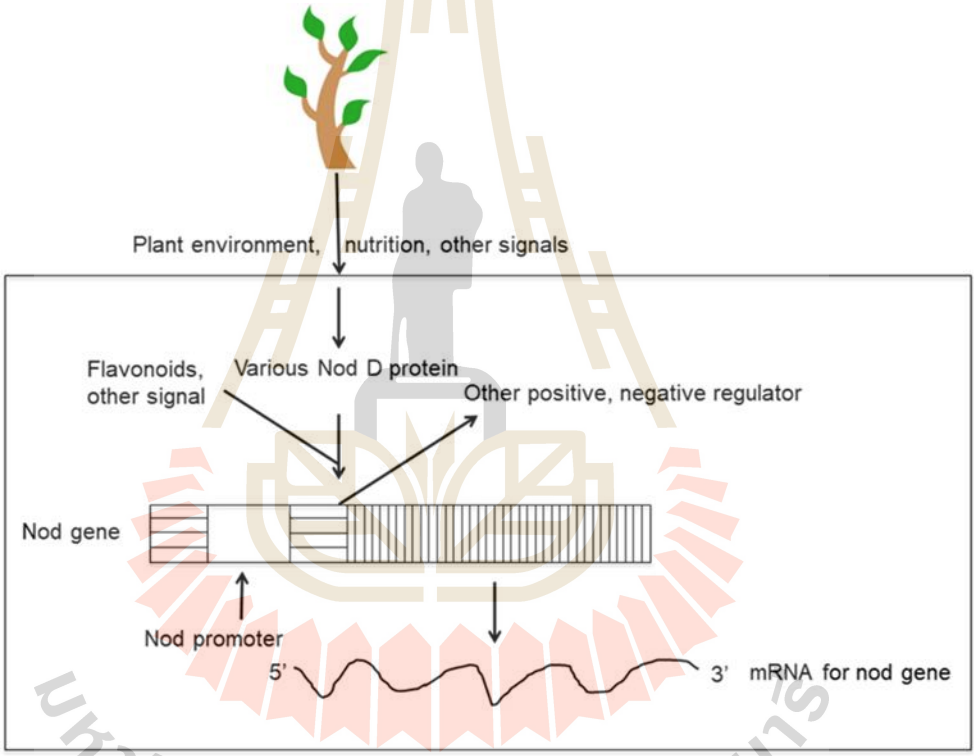

Figure 2.2 The NodD function and their structure (Patra & Mandal, 2022).

Following the induction of calcium spikes, cytokinin receptors are activated, leading to increased cytokinin production, which in turn promotes nodule organogenesis (Madsen et al., 2010; Tirichine et al., 2007). The cytokinin hormone is associated with cortical cell differentiation and the initiation of nodule organogenesis. The cytokinin receptors Lotus Histidine Kinase 1 (*LHK1*) in *L. japonicus* and Cytokinin Response 1 (*MtCRE1*) in *M. truncatula* were well studied model. The cytokinin

receptors Lotus Histidine Kinase 1 (*LHK1*) in *L. japonicus* and Cytokinin Response 1 (*MtCRE1*) in *M. truncatula* were well studied model (**Fig. 2.3**).

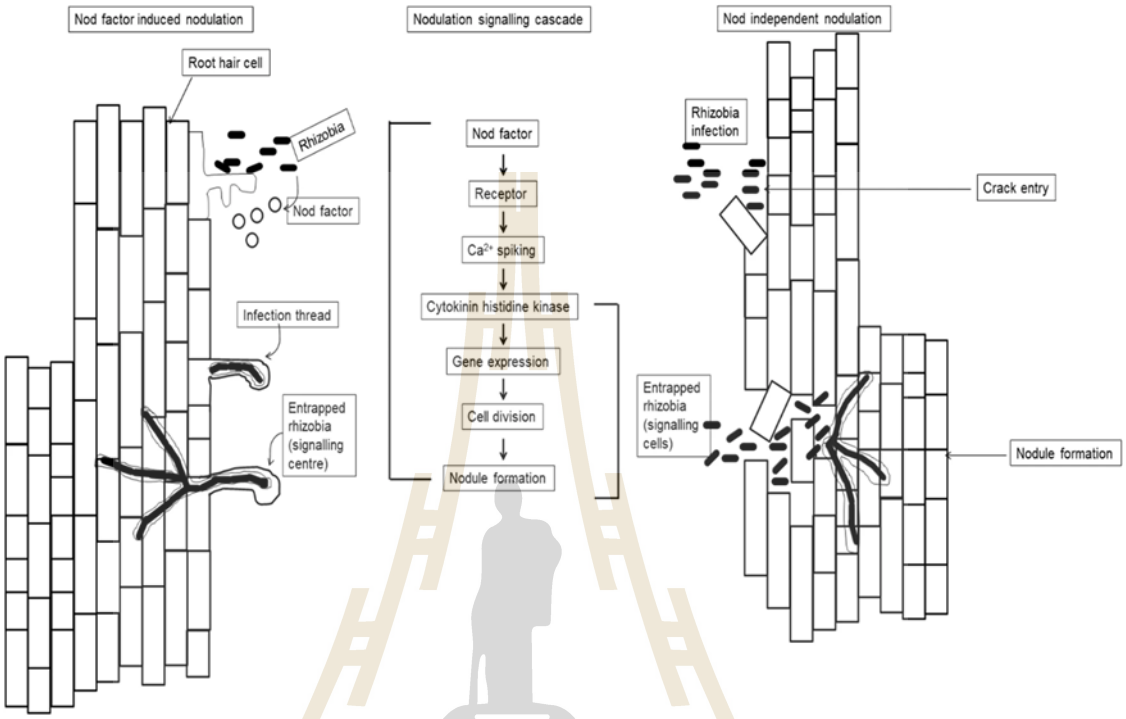

Figure 2.3 The nod-dependent and nod-independent nodulation process (Patra & Mandal, 2022).

The LHK1 mutant exhibits a reduced number of nodules but does not completely inhibit nodulation. The other two cytokinin receptors, LHK1A and LHK3, along with LHK1, play a similar role in regulating nodule formation. The triple mutant of *lhk1*, *lhk1a*, and *lhk3* genes restricts nodule organogenesis. The advanced function of the LHK1 mutant constitutively expresses nodule organogenesis with the help of spontaneous nodule formation 2 (*snf2*) (Held et al., 2014).

2.2.2.2 Nodulation without nod-factors

Although NF-independent nodule formation is less well-known than NF-dependent nodule organogenesis. In the NF-independent pathway, rhizobia enter the cell through crack-entry and initiate organogenesis, though the mechanism for this process is unclear. While NF is not required, downstream mechanisms may still be involved (**Fig. 2.4**). In the absence of NF-dependent signaling, the genes

involved in some mechanisms such as type 3 secretion system (T3SS) are involved with NF-independent pathways for nodulation. For instance, *Bradyrhizobium* sp. strain ORS278 lacks nod factors but associates with CCaMK in *A. evenia* for nodulation. Additionally, homologous factors *LjSYMRK* and *LjHK1* have been found to performance upstream and downstream of CCaMK, respectively (Fabre et al., 2015).

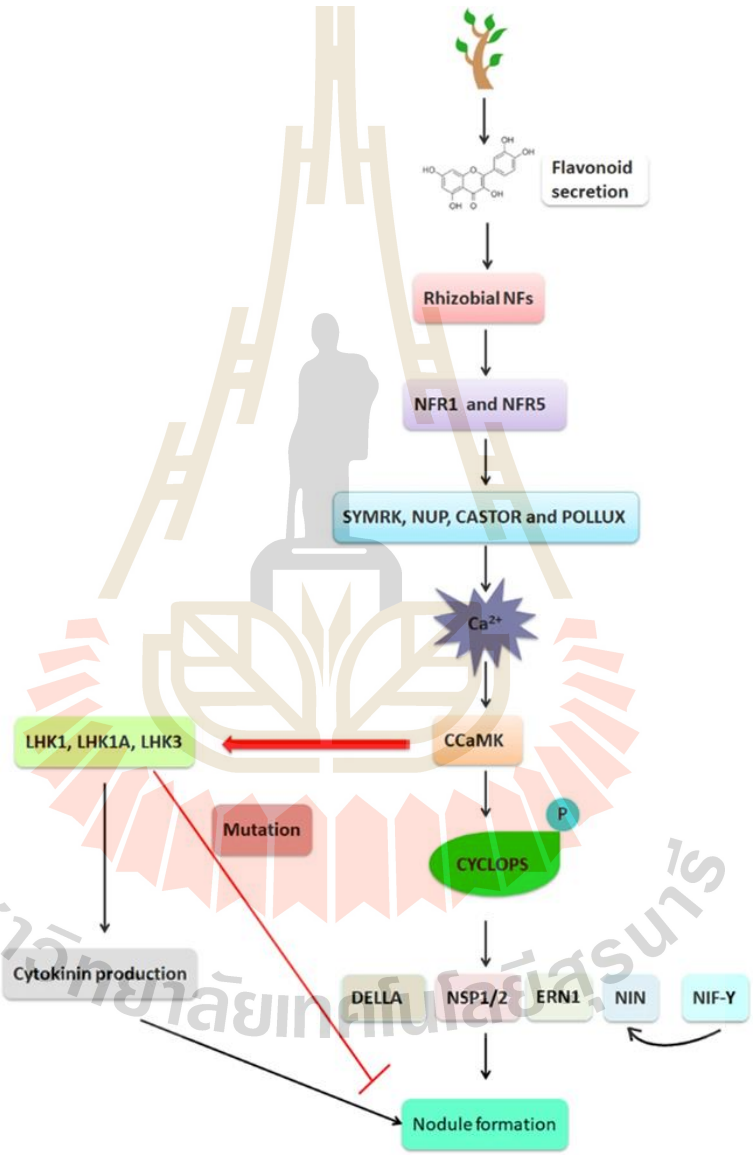

Figure 2.4 The Nod-factor nodule pathway (Patra & Mandal, 2022).

2.3 Type of secretion systems in rhizobia

Table 2.1 Rhizobia utilize various protein secretion systems, categorized into six type secretion systems (T1SS-T6SS) based on their secretion mechanisms (Costa et al., 2015).

Rhizobium strains	Type secretion systems	Effector proteins	References
R. leguminosarum bv. viciae	T1SS	841 and 14	(Costa et al., 2015)
Rhizobium sp. NGR234	T2SS	-	(Schmeisser et al., 2009)
Sinorhizobium fredii USDA257	T3SS	NopA, NopB, NopC, NopL, and NopP	(Krishnan et al., 2011)
S. fredii HH103	T3SS	NopC	(Jiménez-Guerrero et al., 2020)
Rhizobium sp. NGR234	T3SS	NopT	(Dai et al., 2008)
B. diazoefficiens USDA110	T3SS	NopE	(Piromyou, Nguyen, et al., 2021)
Mesorhizobium loti MAFF303099	T3SS	-	(Sánchez et al., 2009)
R. etli CFN42 and *M. loti* R7A	T4SS	-	(Hubber et al., 2004; Lacroix & Citovsky, 2016)
Bradyrhizobium sp. SUTN9-2	T4SS	-	(Wangthaisong et al., 2023)
Gram-negative bacteria	T5SS	-	(van Ulsen et al., 2014)
R. etli Mim1	T6SS	-	(Salinero-Lanzarote et al., 2019)

2.4 Type III secretion system (T3SS) and type III effectors (T3Es)

The secretory machinery of the T3SS, recognized as the injectisome, comprises approximately 20 distinct proteins (Tampakaki, 2014; Teulet et al., 2022). These proteins assemble into a basal body spanning the bacterial envelope and an extracellular needle

that links the bacterium to the targeted eukaryotic cell for the secretion of T3Es (**Fig. 2.5**). Though the architecture of T3SSs in various human pathogens like *Salmonella*, *Yersinia*, and *Escherichia* species has been widely elucidated, the corresponding secretion systems in rhizobia remain relatively understudied (Büttner, 2012; Deng et al., 2017; Notti & Stebbins, 2016). Despite this, given the notable conservation among proteins forming the basal bodies of both rhizobia and pathogenic T3SS, it is anticipated that their secretion machineries share similar ultrastructural features and functionalities. However, rhizobial T3SS exhibit distinctive characteristics such as a split secretin, comprising RhcC1 and RhcC2 components.

B. elkanii T3SS mutant triggers nodulation signaling in *G. max*. The soybean En1282 containing mutated *NFR1* gene, which boosted the expression of the *ENOD40* and *NIN* genes, but not when inoculated with the *B. elkanii* T3SS mutant (Okazaki et al., 2013). T3SS was able to create a different pathway in this instance that can replace the conventional NFs-dependent nodulation development. After *B. diazoefficiens* symbiotic genes are sequenced, a gene cluster (*tts*) that encodes T3SS was found similar to those in *M. loti* MAFF303099 and *Rhizobium* strain NGR234. There are multiple open reading frames (ORFs) in the T3SS structural core component. Any of these clusters that are altered can made different changes in nodulation capacity. A nod box promoter was found upstream of the two-component regulatory family *ttsI*. Additionally, the NodW transcriptional activator protein and the *NodD1*, *NodD2*, and *NolA* gene regions are required for this activation. Through the T3SS, rhizobia secrete some secretary proteins into the media. *NopX* and *nopL* (formerly known as *nolX* and *y4xL*) from *Rhizobium* strain NGR234 are the only known genes that are known to be secreted proteins. Various deconjugating activities targeting SUMO, ubiquitin, and Nedd8 exemplify an optimized protease designed to disrupt specific ubiquitin-like post-translational modifications during host cell infection (Y. Li et al., 2023).

Many rhizobia species, particularly *Bradyrhizobium*, has the T3SS (Tampakaki, 2014). The T3SS in *B. diazoefficiens*, on the other hand, is unable to produce the nodule in *A. indica*. Despite having T3SS apparatus, the strain formed nodules in *A. indica*, suggesting that it contains unique T3Es for nodule organogenesis. When *Bradyrhizobium* sp. ORS285 used with several NF-dependent and NF-independent

Aeschynomene species, the T3SS played a distinct role. When interacting with *Aeschynomene* species in an NF-dependent manner, the symbiotic features of an ORS285 T3SS mutant (*rhcN*) play a positive or negative function. In *A. uniflora*, a positive effect of mutant T3SS was discovered, with the mutant indicating a higher number of nodules. In *A. afraspera*, the negative effect demonstrated that fewer nodules are generated compared to the wild type. The identical mutation, on the other hand, has no effect on the interaction with any of the NF-independent species *A. evenia, A. indica*, and *A. sensitiva*. An additional non-photosynthetic *Bradyrhizobium* strain, STM6978, can form nodules in *A. indica* through an NF-independent mechanism; however, it is completely reliant on T3SS for nodule formation. As a result, two NF-independent symbiotic activities have been identified: one that is T3SS-dependent and one that is T3SS-independent (Okazaki, et al., 2016). In various rhizobia strains, the value of T3SS has been thoroughly proven. T3SS codes for two types of proteins: pilus forming and T3Es. Pilus can pass through the cell wall and plasma membrane created by NopX, NopA, and NopB in plants. Symbiosis nodulation needs effector proteins like NopL, NopM, and NopT (Tampakaki, 2014; Teulet et al., 2019).

B. diazoefficiens putative T3Es, NopT1 and NopT2, are predicted to be cysteine proteases that are a member of the YopT/AvrPphB effector family (Fotiadis et al., 2012). *B. diazoefficiens* Is-34 restricted nodulation incompatibility *Rj4* soybeans identifying the putative T3E (MA20_12780 protein) as a virulence factor (Tsurumaru et al., 2015). T3SS is found not only in legume symbiotic associations, but also in rice endophytic associations such as *Bradyrhizobium* sp. SUTN9–2 (Piromyou et al., 2015; Songwattana et al., 2017). InnB enhanced nodulation of at least one *V. mungo* cultivar, according to inoculation assays utilizing different *Vigna* species. These findings suggested that the unique T3E encoded by innB regulates symbiosis with *Vigna* species (Nguyen et al., 2018). The symbiotic activities of rhizobial T3Es play separate and complementary roles in suppressing host immune function, and it is hypothesized that ErnA initiates organ development in plants through an as-yet-unidentified mechanism (Teulet et al., 2019). NopD, a potential T3E of *Bradyrhizobium* sp. XS1150, has been characterized the function. A functional N-terminal secretion signal sequence for NopD was discovered, which might take the place of the NopL effector released by *Sinorhizobium* sp. NGR234 (Xiang et al., 2020). *Lotus* accessions have three or more

checkpoints to eradicate undesirable symbionts, including those present during the post-infection stage, each of which recognizes a different T3SS effector protein (Kusakabe et al., 2020). The plant pathogen *Xanthomonas campestris* XopD effector is similar to the rhizobial effector Bel2-5, which has the ability to cause nitrogen-fixing nodules on soybean *nfr* mutants. Although the effector combinations that promoted nodulation in *V. unguiculata* and *V. mungo* were different, both species involved NopT and NopAB, suggesting that they are important determinants for nodulation, and to a lesser extent, NopM1 and NopP1, which are additionally necessary for optimal symbiosis with *V. mungo* (Songwattana et al., 2021).

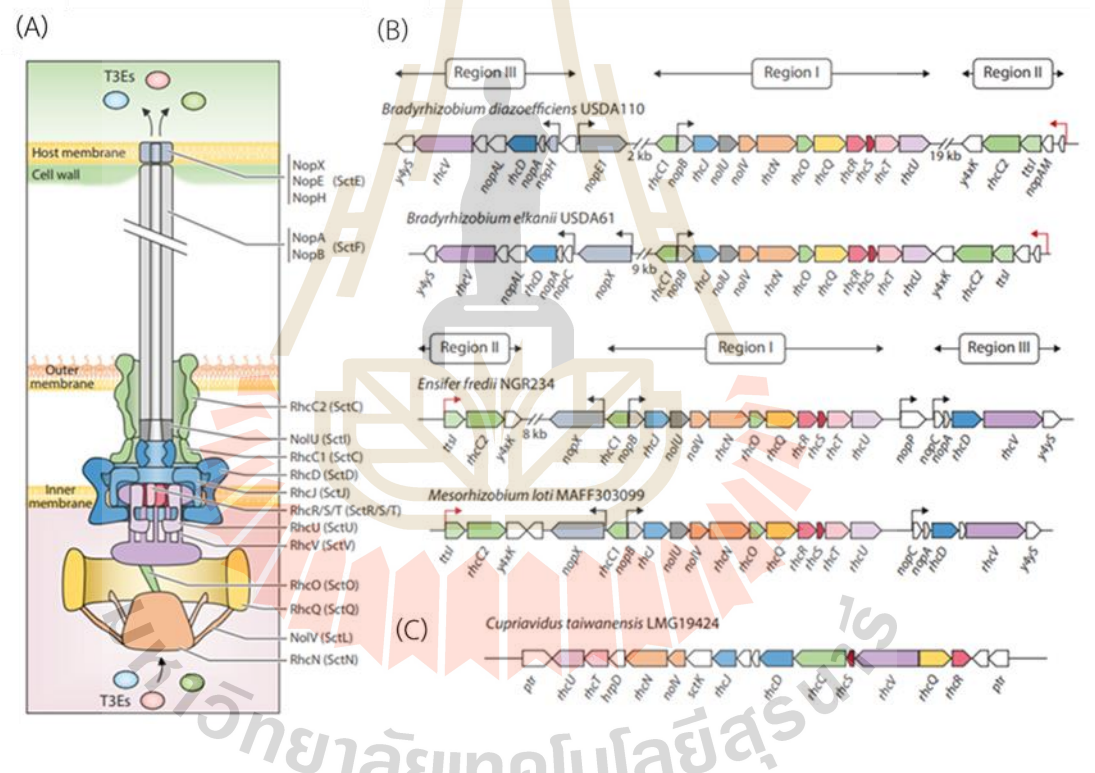

Figure 2.5 Diagram illustrating the T3SS machinery and the genetic arrangement of T3SS gene clusters found in rhizobia. (A) The proteins constituting the T3SS apparatus are denoted Sct (secretion and cellular translocation). (B) Depiction of the genetic componant of T3SS clusters discovered in numerous a-rhizobium, featuring three predominantly present region I to III. (C) Depiction of an unusual T3SS gene cluster identified in the ß-rhizobium *Cupriavidus taiwanensis* LMG19424, including its presumed transcriptional regulator (*ptr*) (Teulet et al., 2022).

2.4.1 Co-regulation betweenT3SSs and nodulation genes

In rhizobia, genes responsible for encoding the structural components of T3SSs are typically clustered and often located in close proximity to symbiotic genes like the *nod* genes required for the synthesis of NFs. In rhizobia, the genes encoding the structural components of T3SS are usually grouped together and frequently positioned near symbiotic genes, such as *nod* genes, which are essential for the synthesis of Nod factors (NFs). In alpha-rhizobia, with the exception of the *rhcC1*, *nopE*, and *nopX* genes, which are monocistronic, the T3SS genes are organized into three main genetic elements believed to constitute dissimilar operons (Tampakaki, 2014; Teulet et al., 2020). Among these, one primary operon housing the majority of T3SS structural genes remains extremely preserved, while other two operons frequently exhibit strain specific variations in gene structure and gene arrangement. In beta-rhizobia, such as the *Cupriavidus taiwanensis* strain LMG19424, the genes encoding components of a symbiotic T3SS are organized into two divergently transcribed elements. These elements exhibit a genetic organization more similar to the T3SS loci of the humanoid opportunistic disease *Burkholderia cenocepacia* (Saad et al., 2012).

One notable aspect of alpha-rhizobial T3SS is their coordinated regulation by key *nod* genes, whose transcription depends on flavonoid and NodD-dependent mechanisms (**Fig. 2.6**) (Viprey et al., 1998). Subsequently, the transcriptional activator *TtsI* binds to conserved promoter sequences known as *tts* boxes, which are located upstream of genes and operons encoding components of the T3SS and T3Es (Bolzan De Campos et al., 2011; Krause et al., 2002). Conversely, in *C. taiwanensis*, the activation of T3SS functions has been reported to be unlinked to flavonoids and *TtsI*, instead being triggered by glutamate, akin to the mechanism observed in plant diseases like *Ralstonia solanacearum* and *Pseudomonas aeruginosa* (Saad et al., 2012).

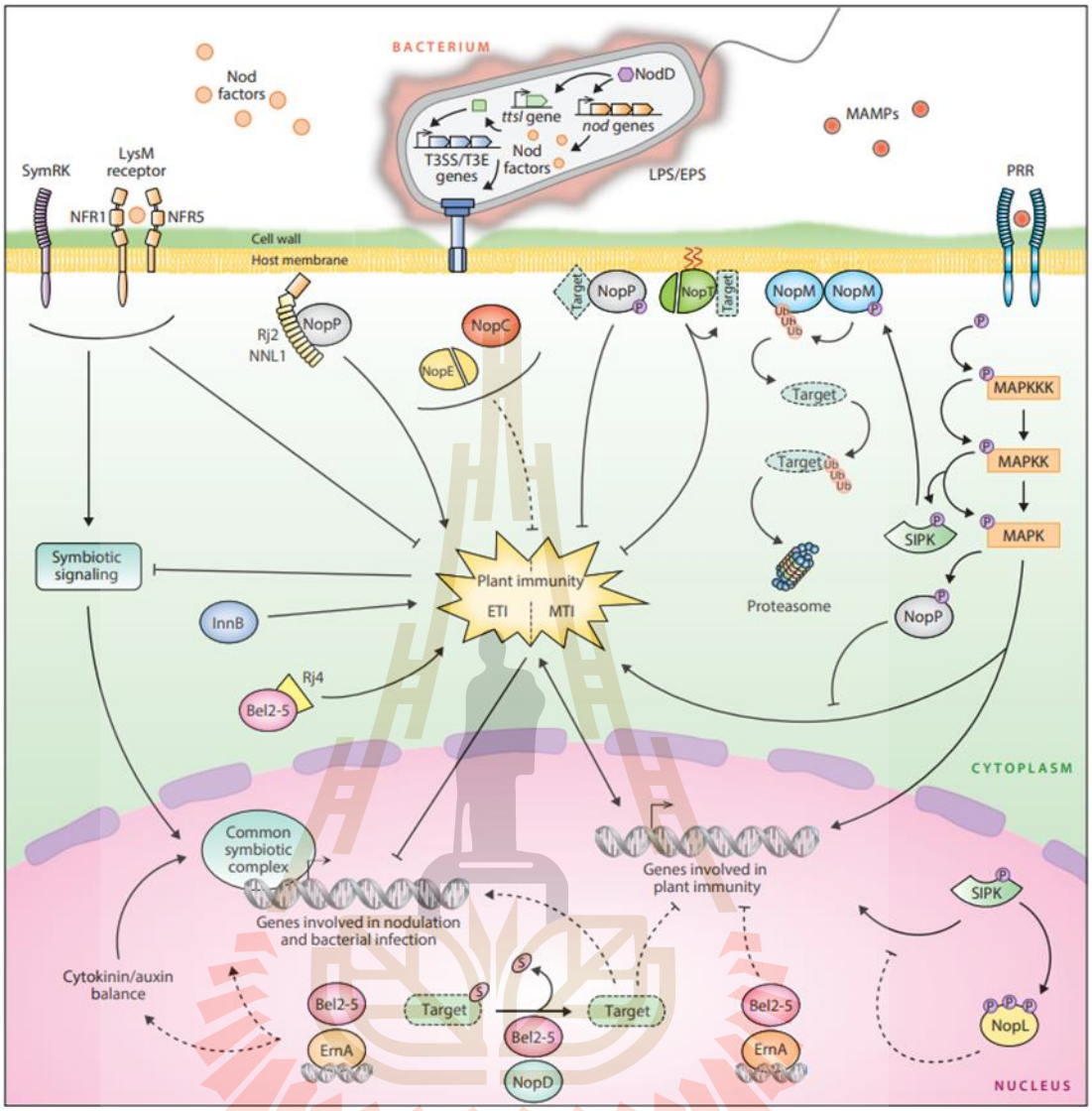

Figure 2.6 The diverse functions of rhizobial T3SS in symbiosis with leguminous plants, highlighting the complex molecular interactions between factors secreted by rhizobia and various plant components involved in microbe perception (Teulet et al., 2022).

2.4.2 The dispersion and evolutionary trajectory of T3SS in rhizobia

T3SSs of rhizobia are not universal, but found in many genera. In most important genera such as *Rhizobium*, *Ensifer*, and *Mesorhizobium*, the co-occurrence of *nod* and T3SS genes is not a common occurrence (Sugawara et al., 2013; Tong et al., 2020). For instance, among 48 *Ensifer* strains examined, a third were found to possess

both T3SS and T4SS. Similar to T3SS, T4SS facilitate the delivery of effector molecules into the cytoplasm of host cells, with several studies confirming their positive or negative modulation of symbiosis, depending on the legume host (Cascales & Christie, 2003; Wangthaisong et al., 2023). In contrast, over 90% of the *Bradyrhizobium* genus contain *nod* and T3SS genes on symbiotic islands. Additionally, symbiotic genes share an evolutionary lineage within *Bradyrhizobium* (Teulet et al., 2020). Nod factors would facilitate nodule organogenesis, while T3Es would aid in subverting host immune responses to ensure intracellular infection (Hungria et al., 2015; Parker, 2015). In the process of coevolving with host plants, rhizobia might have also evolved alternative mechanisms to evade plant immunity, like altering cell surface components or employing substitute protein secretion systems (Gibson et al., 2008; Gourion et al., 2015). Among the seven families of T3SS gene clusters identified in gram negative bacteria (Gazi et al., 2012), rhizobia predominantly employ the **a**-RhcI category to interact with plant hosts. Some strains may have two different T3SS clusters; however, their functions remain largely unexplored (Tampakaki, 2014). In the case of the *E. fredii* strain NGR234, the T3SS-II locus showed no effectiveness in symbiosis (Schmeisser et al., 2009). Conversely, while the function of the atypical T3SS in *C. taiwanensis* strain LMG19424 did not affect its host *M. pudica* (Saad et al., 2012).

2.4.3 No standardized formula for cocktails of rhizobial effectors

The advent of secretome investigation methods facilitated the initial discoveries of rhizobia T3Es in *Bradyrhizobium* and *Ensifer* strains (Hempel et al., 2009; Viprey et al., 1998). Originally termed nodulation outer proteins (Nops) to align with *Yersinia* outer proteins (Yops), it is worth noting that not all Nops are considered bona fide effectors (Marie et al., 2001). For instance, NopX, NopA, and NopB function as pilus machineries, whereas recently discovered T3Es such as InnB, Bel2-5, and ErnA were not classified as Nops. The identification of T3Es that are symbiotic with the legume host is not yet complete. Genome analyses, utilizing searches *tts* boxes and homologies of T3Es in symbiotic or disease bacteria, have assisted in identifying the putative effectomes of rhizobia (Busset et al., 2021; Ratu et al., 2021b). The numbers of T3E repertoires differ between *Ensifer* and bradyrhizobia (Kimbrel et al., 2013; Ratu et al., 2021). Although no core effectome was not identified, homologs of NopC, NopM, NopP, and NopT are normally shared among *Ensifer* and *Bradyrhizobium*, suggesting the presence of an early

effectome core (Teulet et al., 2020). Moreover, several T3Es were specific within rhizobia (Deakin & Broughton, 2009; Jimenez-Guerrero et al., 2015; Jiménez-Guerrero et al., 2017; Teulet et al., 2019). These differences in the components of T3Es and their rapid evolution in rhizobia potentially aid in improved adaptation to specific hosts.

2.4.4 Symbiotic functions of rhizobial T3Es

Numerous publications have indicated that deleting T3Es can lead to improvements, impairments, or no effect on symbiosis with host plants. For instance, in *E. fredii* NGR234, a nopT knockdown formed fewer number of nodules on *Tephrosia vogelii* but exhibited better nodulation on *Crotalaria juncea*, while maintaining proficiency similar to the wild type on *Lablab purpureus* (Dai et al., 2008; Kambara et al., 2009). Although the symbiotic efficiency of T3E mutants is host-dependent, various putative effector genes such as *bel2-5*, *ernA*, *nopC*, *nopD*, *nopP*, and *innB* have shown both negative and positive effects on symbiosis (Jimenez-Guerrero et al., 2015; Nguyen et al., 2018; Ratu et al., 2021a; Schechter et al., 2010; Teulet et al., 2019; Xiang et al., 2020). There were cumulative effects on symbiosis by deleting numerous effectors in some strains. For example, in *E. fredii* NGR234, double deletion of *nopL* and *nopP* restricted nodulation of *Flemingia congesta* more than single deletion strain (Skorpil et al., 2005). Also, in *B. vignae* ORS3257, double deletion of *nopP1* and *nopM1* suppressed nodulation of *A. indica* and *V. mungo* over single mutations (Songwattana et al., 2021; Teulet et al., 2019). The function of rhizobial T3SSs in plant host symbiosis involves not only T3Es, but also a combined cocktail of T3Es that can act together to support or suppress, depending on host specificity (Deakin & Broughton, 2009; Songwattana et al., 2021).

2.4.5 Functional analysis of T3Es

Like T3Es found in pathogenic plant bacteria, rhizobial T3Es often demonstrate a modular structure, consisting of various functional domains and motifs for subcellular localization within host cells (Bastedo et al., 2019; Dowen et al., 2009; Staehelin & Krishnan, 2015). Functional characterization of rhizobial effectors has primarily been conducted using transgenic or transfected non-legume hosts such as *Nicotiana benthamiana*, *N. tabacum*, and *A. thaliana*, which are commonly employed models for T3E analysis. These investigational methods have revealed useful similarities among rhizobial T3Es and those of plant pathogens, particularly regarding

domains required for manipulating the posttranslational modification within legume hosts, such as SUMOylation, ubiquitinylation, or phosphorylation (**Fig 2.6**).

2.4.5.1 Manipulating host Ubiquitin-SUMO pathways

Ubiquitin and small ubiquitin-like modifier (SUMO) are small proteins utilized by plant cells to precisely modify various substrate proteins. These posttranslational modifications, ubiquitinylation and SUMOylation, affect many aspects of protein biology, including activity, stability, location, and interactions with other proteins (Berndsen & Wolberger, 2014; Morrell & Sadanandom, 2019; Sharma et al., 2016). Although both ubiquitin and SUMO are attached to target proteins through three-step enzymatic cascades, different enzymes participate in each step: E1 ligases, E2 conjugating enzymes, and E3 ligases (**Fig. 2.7**) (K. et al., 2021). Several of the NopM proteins in many rhizobia contain a new E3 ubiquitin ligase (NEL) domain in the C-terminal region, along with a conserved leucine-rich repeat (LRR) domain in the N-terminal region, believed to confer specificity with substrate proteins (Bastedo et al., 2019; Rohde et al., 2007; Xu et al., 2018) When expressed in *N. benthamiana*, NopM from *Rhizobium* sp. strain NGR234 suppresses the production of reactive oxygen species induced by flagellin (Xin et al., 2012). In contrast to NopM, another rhizobial effector contains a ubiquitin-like protease (ULP)-like domain in the C-terminal region for deSUMOylation protease (Ratu et al., 2021a; Xiang et al., 2020). Several plant pathogens possess conserved ULP1 domain in effector protein and function in infecting the host plant (Kim et al., 2011). Remarkably, both Bel2-5 of *B. elkanii* USDA61 and NopD of *Bradyrhizobium* sp. XS1150 harbor a ULP1 domain that works similarly to XopD of *X. campestis*. Both proteases have been confirmed to goal the legumes cell nucleus and carry the catalytic residues H/D/C within their respective ULP1 domains, which are essential for activity (Ratu et al., 2021a; Xiang et al., 2020). Interestingly, an examination of NEL domains and ULP1 domains in the predicted proteomes of sequenced *Bradyrhizobium* genus members revealed that NopM and SUMO proteases are prevalent in symbiotic strains equipped with a T3SS, constituting the two most abundant families of T3Es (Teulet et al., 2020). Consequently, it suggests that T3Es of bradyrhizobia may primarily target plant cellular functions related to ubiquitin and SUMO modification pathways during symbiotic interactions.

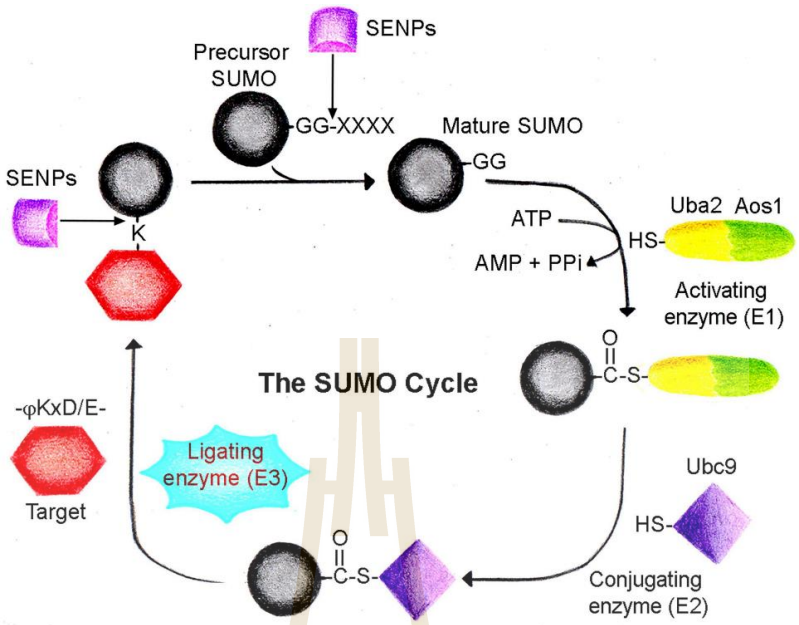

Figure 2.7 The SUMO cycle comprises a series of enzyme-mediated reactions involving the SUMO target protein. Initially, the precursor SUMO is activated by sentrin proteases (SENPs) to produce its mature form. The heterodimeric SUMO-activating enzyme (Uba2/Aos1, E1) utilizes ATP to form a thioester bond with the mature SUMO. This activated SUMO is then transferred to the conjugating enzyme (Ubc9, E2), creating another thioester bond. Subsequently, SUMO is conjugated to a target lysine residue, typically found in a –ψKxD/E- motif (where ψ is a large hydrophobic amino acid, K is the target lysine, X is any amino acid and D/E is aspartate or glutamate), with the optional involvement of a ligating enzyme (E3). The cycle is completed when SENP family proteases recycle the conjugated SUMO) (K. et al., 2021).

2.4.5.2 Regulation of MAPK signalling

Recognition of Microbe-Associated Molecular Patterns (MAMPs) by plant plasma membrane receptors initiates plant immune responses through the stimulation of mitogen-activated protein kinases (MAPKs) (Wang, et al., 2020). Many T3Es from pathogens disrupt the phosphorelays required for Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI) activation by mimicking substrates for MAPK activity (Büttner, 2012; Wang, et al., 2020). Effectors from both pathogenic

and symbiotic microorganisms can be delivered into the host cell via secretion systems, allowing them to interfere with the immune system and disrupt cellular processes (**Fig. 2.8**) (J. Wang et al., 2022). NopL, NopM, and NopP in *E. fredii* NGR234 have been identified as substrates for plant kinases (Bartsev et al., 2004; Ge et al., 2016; Skorpil et al., 2005; Xu et al., 2018). NopM and NopL also undergo phosphorylation in planta by the MAPK salicylic acid–induced protein kinase (SIPK) of *N. tabacum* (NtSIPK) (Ge et al., 2016; Xin et al., 2012; Xu et al., 2018; Zhang et al., 2011). The interactions between NopL and NtSIPK in the nucleus of plant cell have been observed (Ge et al., 2016). NopL likely disrupts MAPK signaling in legume cells, as not activation of its multiple phosphorylation sites led to a nodulation phenotype. The nodule phenotype, similar to that of the nopL mutant, showed nodule senescence in *Phaseolus vulgaris* (Ge et al., 2016). In contrast, NopM contains a single serine residue that has been identified as phosphorylated *in vitro* and *in vivo* (Ge et al., 2016). Given that NopM also functions as an E3 ubiquitin ligase, it may inhibit both protein degradation processes and the MAPK pathway.

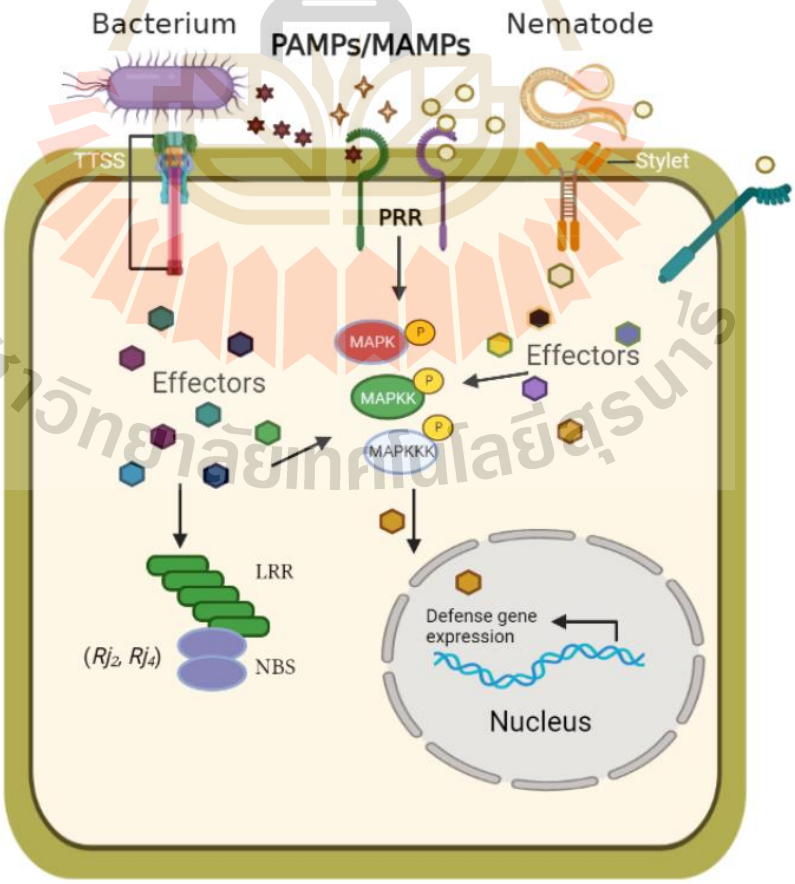

Figure 2.8 Pathogens and rhizobia, representing various lifestyle classes (color-coded and labeled), activate PAMPs (pathogen-associated molecular patterns) and MAMPs (microbe-associated molecular patterns) as they colonize soybean. The soybean responds by expressing extracellular PRRs (pattern recognition receptors) to initiate PRR-mediated immunity (PTI). However, both pathogens and rhizobia produce effectors that interfere with PAMP/MAMP perception in soybean. These effectors target specific subcellular locations to inhibit PTI, thereby promoting virulence and symbiosis. (J. Wang et al., 2022).

2.4.5.3 The enzymatic degradation capabilities of rhizobial T3Es

Numerous effectors from plant diseases have been observed to enzymatically cleave proteins upon entering plant cells (Büttner, 2016; Dean, 2011). The NopT effectors of rhizobia, by homology, are part of the extensive AvrPphB/YopT family of Type T3Es. Many reviews indicate that within a host cell, NopT undergoes proteolytic self-cleavage, exposing residues essential for lipid acylation modification, which is necessary for targeting the plant plasma membrane (**Fig. 2.9**) (Dowen et al., 2009; Fotiadis et al., 2012). The conserved amino acid catalytic residues C/H/D of cysteine proteases recognized in NopT effectors are essential for optimal self-cleavage and induction of a hypersensitive response in altered tobacco cells (Dai et al., 2008; Dowen et al., 2009; Fotiadis et al., 2012; Kambara et al., 2009). Additionally, NopE1 proteins from *B. diazoefficiens* USDA110 have demonstrated autoproteolytic activity, facilitated by two metal ion-inducible autocleavage (MIIA) domains when incubated with Ca^{2+} (Schirrmeister et al., 2011; Wenzel et al., 2010). The self-cleavage property of NopE1 is crucial for *V. radiata* symbiosis (Wenzel et al., 2010). The precise roles of NopE1 and NopE2 of USDA110, whether as effectors cleaving specific target proteins or facilitating the translocation of T3Es of rhizobia across the host plasma membrane, are not yet fully understood (Teulet et al., 2020).

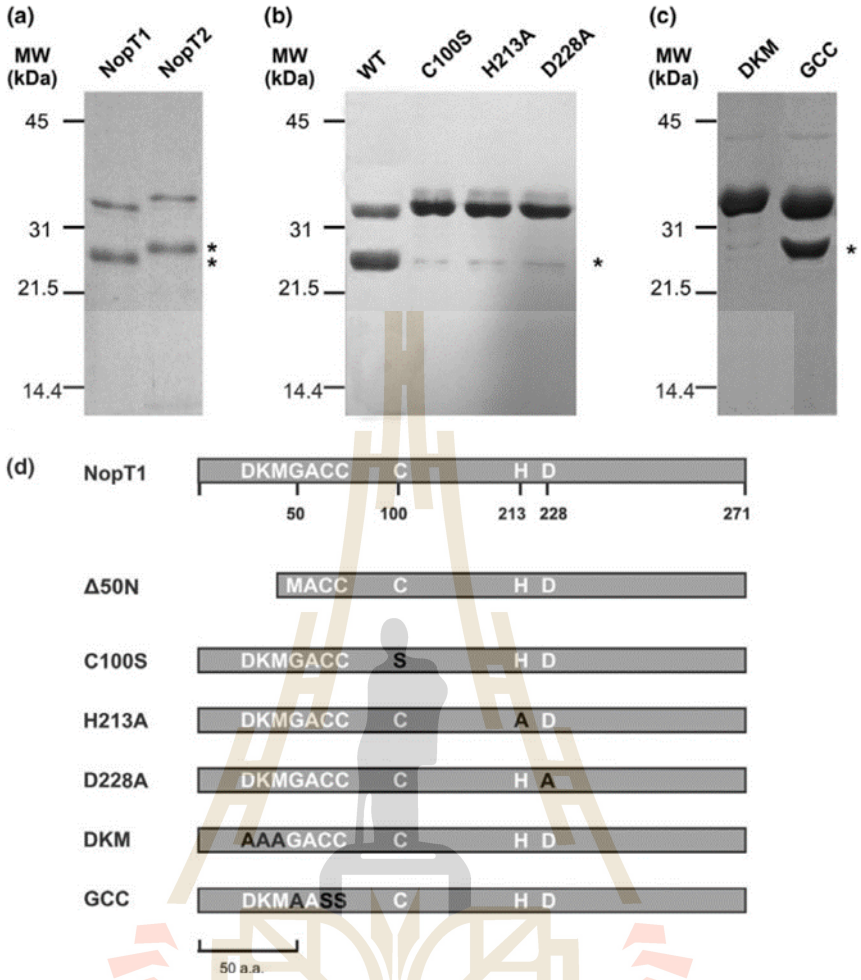

Figure 2.9 (A) The enzyme cleavage activity of NopT1 and NopT2 was analyzed by Western Blot. (B) Mutant proteins of NopT1, with alterations in the catalytic triad residues (C/H/D), showed a deficiency in enzymatic activity. (C) Enzyme cleavage was assessed for the DKM and GCC mutant proteins of NopT1. (D) Various deletion mutants of NopT1 were constructed. The amino acids C100, H213, and D228 form the catalytic core of NopT1 cysteine proteases, while G50 is identified as a potential myristoylation site, and C51 and C52 are putative palmitoylation sites (Fotiadis et al., 2012).

2.4.6 Targeting and the role of carbohydrate in cellular interactions

Rhizobial T3Es can induce Effector-Triggered Immunity (ETI) responses in legumes, thereby restricting the host range of strains and/or limiting symbiotic effectiveness to specific hosts (**Fig. 2.10**) (Miwa & Okazaki, 2017; Staehelin & Krishnan,

2015). Dominant alleles have long been identified to limit nodulation by specific of *G. max* species (Caldwell, 1966). However, recent genetic analyses have unveiled the roles of cytosolic plant receptors and their corresponding T3Es in host specificity. For instance, alleles such as *Rj2* and *Rfg1* at the same locus on *G. max* chromosome 16 encode closely identical Toll-interleukin receptor (TIR)-nucleotide-binding site (NBS)-LRR proteins, which are crucial for the contrasting specificities of *B. japonicum* USDA122 and *E. fredii* USDA257 (Yang et al., 2010). NopP from USDA122 plays a pivotal role in symbiotic incompatibility, with specific amino acids being essential for *Rj2*-mediated defense marker stimulation. Notably, even an amino acid difference among *Rj2* alleles can lead to incompatible symbiosis (Sugawara et al., 2019). While NopP from *B. diazoefficiens* USDA110 does not induce ETI in certain soybean cultivars (Sugawara et al., 2018), it impedes nodulation in soybean accessions possessing a functional *Gm*NNL1 locus, which encodes another TIR-NBS-LRR protein capable of detecting NopP variants (Zhang et al., 2021). Interestingly, disruption of *Gm*NNL1 by a short interspersed nuclear element (SINE)-like transposon enhances nodulation and nitrogen fixation by previously rejected strains, potentially explaining why most cultivated soybeans harbor an inactivated *GmNNL1* locus, inadvertently selected during breeding for enhanced N_2 fixation and plant biomass (Zhang et al., 2021). Two different effectors, NopP from *Bradyrhizobium* sp. ORS3257 and InnB from *B. elkanii* USDA61, were identified as mediators of this incompatibility in *V. radiata* (Hien et al., 2020; Songwattana et al., 2021). Certain studies aimed at identifying effector targets on a large scale have uncovered a core network of plant proteins likely targeted by various phytopathogens (Mukhtar et al., 2011; Weßling et al., 2014). It is possible that certain T3Es secreted by rhizobia may target plant proteins within this immune hub to facilitate symbiotic infections. Consequently, specific NBS-LRR proteins responsible for guarding the immune hub against pathogen effectors might recognize rhizobial T3Es and trigger a response, thereby limiting the infection by proficient rhizobia.

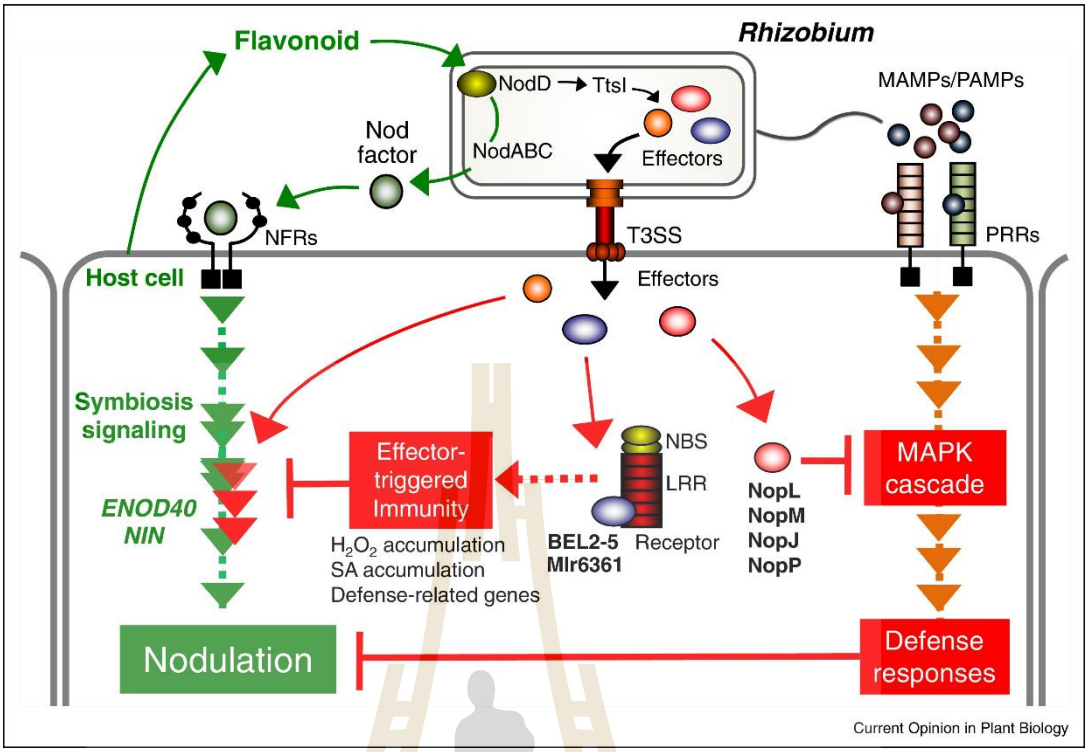

Figure 2.10 Rhizobial T3Es are involved in plant defense signaling. Flavonoids produced by host legumes induce the expression of Nod factors (NFs) in rhizobia. These NFs are recognized by NF receptors (NFRs), which initiate symbiosis signaling that leads to nodulation. Rhizobial T3Es can promote nodulation by directly activating Nod factor signaling. The plant host defense signaling is triggered by rhizobial MAMPs/PAMPs. However, rhizobial T3Es block the MAPK cascade, thereby suppressing host defenses. T3Es such as BEL2-5 and Mlr6361 activate the host defense and then blocking nodulation (Miwa & Okazaki, 2017).

2.4.7 Alternatives to Nod factor-dependent nodulation: T3SS options

The traditional understanding that nodulation solely relies on photosynthetic *Bradyrhizobium* strains, and that nodule organogenesis on roots and stems of *Aeschynomene* species lacked the typical *nod* genes essential for NF synthesis, has been challenged (Giraud et al., 2007). Initially regarded as an anomaly, the occurrence of nodulation without NFs when legume species were inoculated with rhizobia mutants to form nodules has been observed (Madsen et al., 2010; Okazaki et al., 2013). Notably, nodulation of the soybean cultivar Enrei by a *nodC*

mutant of the nonphotosynthetic *B. elkanii* USDA61 was found to be dependent on the role of T3SS (Okazaki et al., 2013). As certain symbionts of *Aeschynomene* species, such as Bradyrhizobia strains ORS278 and BTAi1, are both photosynthetic and lack NFs and T3SSs, it suggests the existence of two different NF-independent pathways supporting nodulation, one of which involves T3Es (Okazaki et al., 2016). Additionally, the NF-independent nodulation mechanism used to bypass infection threads prevails in about 25% of known legume species (Okazaki et al., 2013). The utilization of T3Es for nodulation appears widespread among bradyrhizobia, as various non-photosynthetic *Bradyrhizobium* strains can induce nodule formation on *A. indica* in a T3SS-dependent manner (Okazaki et al., 2016). Intriguingly, the outcome of nodule cell infection varies depending on the strain used. For instance, *B. elkanii* USDA61 proliferates in the intercellular space between nodule cells, while *B. vignae* ORS3257 infects nodule cells intracellularly (Okazaki, et al., 2016). Nonetheless, in the case of ORS3257, intracellular bacteria fail to fix sufficient nitrogen, possibly due to inhibited bacteroid differentiation or premature degradation. Notably, several T3Es, including ErnA, NopT, NopAB, NopM1, and NopP1, were found to play crucial roles in nodulation of *A. indica*, regulating various aspects such as nodule organogenesis, infection, and maintenance of chronic infection within nodule cells (Teulet et al., 2019). However, the extent to which these T3SS-dependent steps ensure effective symbioses in natural environments remains uncertain. Only a few strains isolated from field nodules, including *B. mercantei* SEMIA6399, *Bradyrhizobium* sp. Y36, and *B. liaoningense* CCBAU83689, have been identified to lack nod genes while carrying T3SS loci. This indicates T3Es may serve as the primary determinants for nodulation (Teulet et al., 2020).

2.4.8 Rhizobial T3Es with nodulation inducing abilities

The significance of ErnA in legume nodulation was investigated through two methods (Teulet et al., 2019). Firstly, the *ernA* gene was transferred to a recipient bradyrhizobia and used to inoculate *A. indica*. Secondly, ernA was expressed in transgenic roots of *A. indica*, forming structures resembling nodules. ErnA, spanning 370 amino acids, lacks recognized role of domains except for a NLS necessary for plant nucleus. Supporting this notion, various T3Es from plant pathogens (Büttner, 2016), such as TAL effectors from *Xanthomonas* spp. and HsvG/HsvB from *Pantoea*

agglomerans, were revealed to influence plant gene expression (Boch et al., 2014; Boch & Bonas, 2010; Büttner, 2016; Nissan et al., 2006). Although ErnA from *Bradyrhizobium* sp. ORS3257 cannot induce nodule formation, it stands out alongside Bel2-5 of *B. elkanii* USDA61. While Bel2-5, spanning 1,328 amino acids, disrupts symbiosis with the soybean cultivar BARC-2, it is essential for nodulating the soybean Enrei *nfr1* mutant, which is unable to sense NFs. (Ratu et al., 2021). Bel2-5 likely alters gene expression by deSUMOylating plant proteins, similar to XopD of *X. campestris* (Kim et al., 2011). ErnA and Bel2-5 may target common elements in the NF-signaling pathway to regulate the expression of *CYCLOPS*, *CCaMK*, *CRE1*, *LBD16*, *NIN*, and *NF-Y*, or affect cytokinin and auxin phytohormone levels, crucial for nodule formation (Teulet et al., 2022). ErnA homologs are prevalent in the genomes of most *Bradyrhizobium* strains possessing a T3SS, unlike Bel2-5, which is found in select strains (Teulet et al., 2019, 2020). Strikingly, strains harboring Bel2-5 and ErnA also carry NF genes and originate from host plant reliant on well-matched NFs for nodule formation. This prompts questions about the roles of Bel2-5 and ErnA in NF-dependent symbioses and the environments in which they synergize with NFs to enhance nodulation or serve as adaptations to interact with NF-unresponsive legumes.

2.5 *Bradyrhizobium* sp. DOA9

Bradyrhizobium sp. DOA9 was isolated from the soil on rice field, and the host plant was *A. americana* (Noisangiam et al., 2012). This strain has a broad host range in many groups of legumes, such as Dalbergioid, Robinoid, and Milletoid, and acts as an endophytic bacterium in rice (Teamtisong et al., 2014). DOA9 has a single chromosome with 7.1 Mbp and a plasmid with 0.7 Mbp of nodulation gene (*nod*), nitrogen fixation (*nif*), type III, and type IV secretion systems (T3SS and T4SS). pDOA9 has the *tts* box which encodes nodulation outer protein, structural component machinery, cupin, and a hypothetical protein (Okazaki et al., 2015). The nodulation (*nod*) gene on the megaplasmid of *Bradyrhizobium* sp. DOA9 is extremely diverse and has a wide variety of hosts (pDOA9). The general regulator genes *nodD* (*nodD1* and *nodD2*) and *nodA* (*nodA1* and *nodA2*) were discovered to be duplicated on pDOA9. An acyltransferase, which the *nodA* gene encodes, regulates the addition of an acyl

chain to the chitooligosaccharides (COs) construction and helps to define the host plant (Wulandari et al., 2022a).

Neither nodD1 and nodD2 did not affect nodulation formation, indicating the role of these regulatory genes in DOA9. In contrast, nodA2 and nodB are crucial for Nod-Factor translation and the nodulation of plant symbiosis. These findings suggest that the strain DOA9 host range may be determined by the functional redundancy of regulatory *nodD* genes and the structure of Nod-Factors (Wulandari et al., 2022a). The plasmid pDOA9 experienced alterations throughout legume evolution, expanding host specificity and enhancing nodulation compatibility. These changes were contingent upon the chromosomal background of the recipient as well as the restrictions imposed by legume hosts (Wulandari et al., 2022b). The nodulation (*nod*) gene on the megaplasmid of *Bradyrhizobium* sp. DOA9 is extremely diverse and has a wide variety of hosts (pDOA9).

The *nifDK* and *nifA* genes of the DOA9 strain have exceptional abilities to fix nitrogen both during free-living and symbiotic growth. It has previously been demonstrated that the expression of the two *nifDK* clusters varies with the development environment, pointing to various regulatory mechanisms (Wongdee et al., 2016, 2018). The *Bradyrhizobium* sp. DOA9 strain harbors chromosomally (c) and plasmidally (p) encoded RpoN proteins. Both the chromosomal and plasmid-encoded RpoN proteins in strain DOA9 exhibit pleiotropic functions during both free-living and symbiotic phases (Wongdee et al., 2023).

A functioning T3SS in DOA9 prevents the strain from interacting symbiotically with rice, but not with legumes (Songwattana et al., 2017). The functions of SkP48 effector protein from *Bradyrhizobium* sp. DOA9 is reducing the effectiveness of nodulation in *A. hypogea* and preventing nodulation in a number of *Vigna* species and *C. juncea* (Piromyou et al., 2021). DOA9 compared the genes organization of T3SS with other rhizobia (**Fig. 2.11**). With the exception of *V. radiata* cv. SUT4 and *C. juncea*, which nodulate, the T3SS mutation (*rhcN*) had a minor impact on nodule formation. The DOA9-Ω*rhcN* strain enhanced nodule phenotype and nodulation efficiency. Overall, the findings showed that DOA9 very wide range of hosts may be attributed to the existence of two divergent *nodA* genes, while T3SS may induce plant immunity depending on the species of plant (Songwattana et al., 2016).

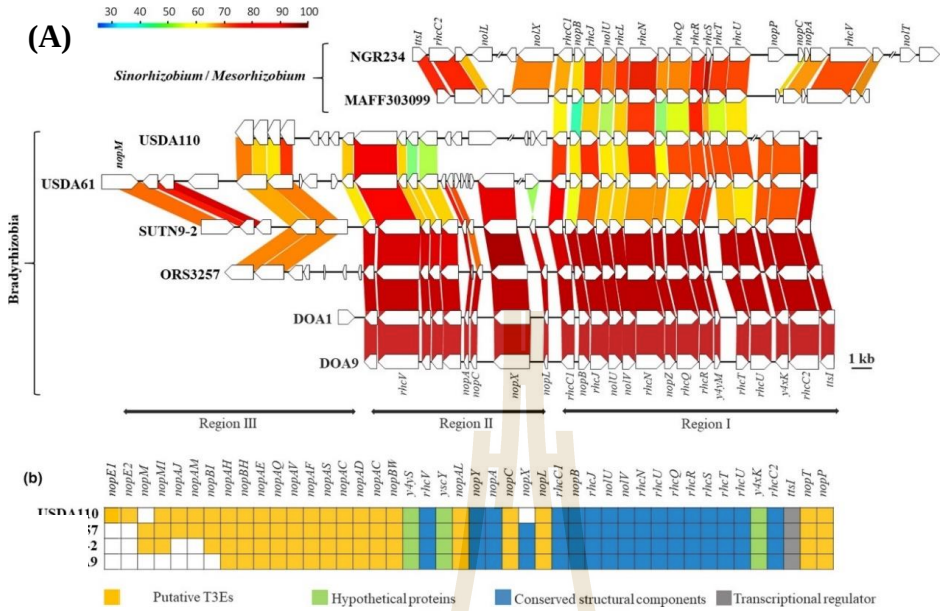

Figure 2.11 (A) *Bradyrhizobium* strains were used in the comparison of the T3SS gene organizations. Open arrows show the locations and length of the expected ORFs. DNA sections that are not visible are indicated by double slash marks. The conserved gene areas between the examined strains are represented by colored strips, and the colors denote the degree of similarity. (B) Distribution of probable T3E families and preserved structural elements in bradyrhizobia. The bradyrhizobial strains are mentioned at the top with the structural elements and T3E family names. The color-coded boxes are shown in the key; Yellow boxes indicate probable T3Es, green boxes indicate hypothetical proteins, blue boxes indicate conserved structural elements, and gray boxes indicate transcriptional regulators (Piromyou et al., 2019).

2.6 *Arachis hypogaea* and Bradyrhizobia symbiosis

Groundnuts (*Arachis hypogaea*) have been cultivated since ancient times in regions such as Brazil and Peru. They were widely cultivated in the West Indies during the pre-Columbian era. It is believed that groundnuts were dispersed globally during the 16th and 17th centuries (Boogerd & van Rossum, 1997). Taxonomically, groundnuts belong to the Leguminosae family, subfamily Papilionoideae, and tribe Aeschynomeneae. They are

closely related to genera such as *Aeschynomene*, *Stylosanthes*, and *Discolobium*, among others. Within the *Arachis* genus, there are 22 species, of which 9 are known to nodulation (Allen & Allen, 1981; De Faria et al., 1989). *A. hypogaea*, the most commonly cultivated species, is not found in the wild (Allen & Allen, 1981). The nodulation of *A. hypogaea* was first reported by Poiteau in 1853, while nodules of *A. glabrata* were depicted by Bentham in Martius' Flora Brasiliensis in the mid-19th century. Groundnuts exhibit a high capacity for symbiotic nitrogen fixation. Compared to other tropical legumes, groundnuts accumulate a significant amount of nitrogen (van Rossum, 1994). Peanuts rank as the fourth largest oil crop globally. In 2018, the worldwide harvest area for peanuts expanded to 28.5 million hectares (He et al., 2021). Over 100 countries cultivate peanuts, contributing to a global production of approximately 44 million metric tons and an average yield of 1,655 kg/ha (FAOSTAT, 2017). About 90% of the world production was in Asia (58.3%) and Africa (31.6%), with the top three producers being China (16.6 M tons), India (6.6 M tons), and Nigeria (3.4 M tons) (FAOSTAT, 2017). Peanut is the second legume crop in the world after soybean. The ideal growing conditions for peanuts include an average rainfall ranging from 500 to 1200 mm, with sandy loam soils being particularly suitable. Additionally, peanut pod yields typically range from 780 to 2900 kg per hectare (McDonald, 1985).

2.6.1 Crack entry on *Arachis*

Root nodule symbiosis (RNS) occurring in legumes facilitates the creation of specialized nodules that accommodate nitrogen-fixing diazotrophs known as rhizobia (Oldroyd, 2013). RNS encompasses two primary developmental pathways: rhizobial invasion and nodule organogenesis (Oldroyd et al., 2011). The predominant mode of rhizobial invasion involves "root hair entry," where rhizobia infiltrate via intracellular infection threads (IT), observed notably in legumes such as *Medicago* spp., *G. max*, and *L. japonicus* (Okazaki et al., 2013; Oldroyd et al., 2011). However, in *S. rostrata* and *Neptunia*, a variation occurs wherein rhizobia enter through epidermal cracks. (Goormachtig et al., 2004). In contrast, approximately 25% of legumes, particularly those of subtropical genera like *Aeschynomene, Arachis*, and *Stylosanthes*, exhibit a simpler "crack-entry" mode, where rhizobia penetrate through epidermal cracks at the lateral root bases (LRBs) intercellularly, without the formation of IT (Sprent, 2007). Some *Bradyrhizobium* sp. can nodulate in the groundnut and fix the nitrogen to groundnut needs at 55% (Hardarson & Atkins, 2003). The

nodulation process of *Brdyrhizobium* sp. infects groundnut plants by an intercellular (crack entry) pathway. Crack entry infection is when the root hair has the space between the root hair wall and adjoining epidermal and cortical cells. They colonize intercellular spaces via an endocytosis-like process through sub-epidermal cortical cells (**Fig. 2.12**) (Okubo, Fukushima, & Minamisawa, 2012). *Arachis*, a legume employing the "crack-entry" mode, shares a close phylogenetic relationship with the *Aeschynomene* genus (Lavin et al., 2001). Specifically, *Bradyrhizobium* sp. SEMIA6144 uses NF-dependent symbiosis with *Arachis* (Ibáñez & Fabra, 2011). Interestingly, the *Bradyrhizobium* sp. DOA9 used ND for nodulation on peanut (Muñoz et al., 2015), but it less efficiency of nitrogen fixation (Songwattana et al., 2021). However, *Arachis* has been detected to induce suboptimal yet persistent nodule formation with NF-deficient strains like BTAi1 (Guha et al., 2022). Nevertheless, questions remain regarding nodulation by potential NF-producing rhizobia. Although *Arachis* primarily uses NF-dependent nodulation, it can also use NF-independent symbiosis, which is less efficient for nodulation (Guha et al., 2022).

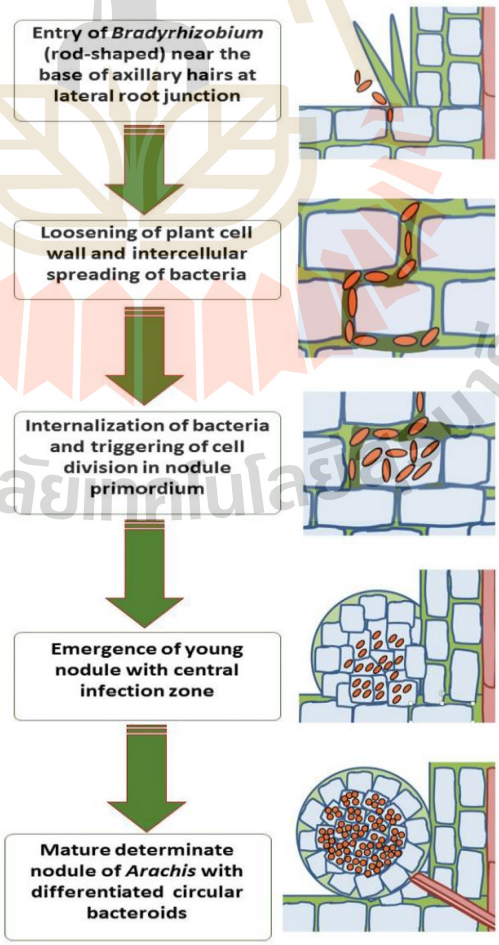

Figure 2.12 *Bradyrhizobium* symbiosis with groundnut occurs via the 'crack-entry' pathway, with nodules developing through various stages of this entry mode (Sharma et al., 2020).

2.6.2 *Rhizobium* isolated form *Arachis*

Phenotypic and genotypic characterization was performed on sixty-two rhizobial isolates obtained from nodules of *A. hypogaea* in north-western Morocco (El-Akhal et al., 2009). All 22 strains of *rhizobium* isolated from peanuts showed rapid growth, and most of them acidified the culture medium (Lyra et al., 2013). Enzyme-linked immunosorbent assay and antibody adsorption tests were used to determine the minimal somatic antigen composition of 243 strains of *Bradyrhizobium* sp. isolated from *Arachis* using 12 antisera (Kishinevsky et al., 1993). Interestingly, *R. pakistanensis* sp. nov. BN-19T is a novel species in the genus *Rhizobium*, isolated from peanuts (Khalid et al., 2015). In addition, based on genotypic and phenotypic features, strains CCBAU 53390^{T}, CCBAU 51670^{T}, and CCBAU 51778^{T} are designated as the type strains of three novel species, for which the names *B. nanningense* sp. nov., *B. guangzhouense* sp. nov., and *B. zhanjiangense* sp. nov. are proposed, respectively (Li et al., 2019). The isolation strains of *rhizobium* were characterized for their plant growth-promoting abilities. Several strains demonstrated effectiveness in solubilizing inorganic phosphate, synthesizing indole acetic acid (IAA), and amplifying the nitrogen-fixing *nifH* gene (Jain et al., 2020; Khalid et al., 2020). Furthermore, the peanut rhizobia isolated and identified in this study were effective at fixing N_2 and adapted well to acidic soils, demonstrating significant application potential (P. Liu et al., 2019). *Bradyrhizobium* sp. SMVTL-02, closely related to *B. zhanjiangense*, could be considered a potential biofertilizer inoculum for *A. hypogaea* production in Laos (Phimphong et al., 2023).

2.6.3 The role of T3SS nodulation on *Arachis* symbiosis

With the symbiosis in groundnut, the T3SS was discovered to be significant. Using PCR-based sequencing, it was possible to determine where the *rhcRST* and *rhcJ-C1* fragments were distributed in the T3SS gene cluster in the peanut-nodulating bradyrhizobia isolated from Guangdong Province, China. A third of the bradyrhizobial strains from peanuts and the T3SS-bearing strains from other *Bradyrhizobium* genomic species were found to be T3SS-positive (Ruan et al., 2018).

The *Bradyrhizobium* sp. strain Lb8, isolated from peanut root nodules, underwent sequencing using PacBio long reads. Strain Lb8 was found to harbor both Type III and Type IV protein secretion systems (Paudel et al., 2020). *B. guangxiense* CCBAU53363 mutant lacking *nolA* and *nodD1* was downregulated on T3SS, and regulated T3E *nopP* gene indirect way (Shang et al., 2022b). In the case of the strains isolated from *Arachis*, *Bradyrhizobium sp.* XS1150, the T3SS mutant (*rhcST*) resulted in negative nodulation on *T. vogelli* (Xiang et al., 2020). *Bradyrhizobium* sp. strain DOA9, isolated from *A. americana*, restricted nodulation on *Arachis*, as indicated by the absence of the T3SS (*rhcN*) (Songwattana et al., 2017). Interestingly, the deletion of the plasmid (pDOA9) containing T3SS suppressed nodulation on *Arachis* (Wulandari et al., 2022b). The *Bradyrhizobium* isolated from *Arachis* demonstrated the presence of T3SS and utilized it to positively induce nodulation on the host plant. However, in the case of DOA9 isolated from *A. americana*, T3SS suppressed nodulation on *Arachis*. The genetic makeup of the original host may be more suitable for symbiosis with *Arachis*.

2.6.4 T3Es and SUMO proteases for *Arachis* model

Effectors secreted through the type III protein secretion system (T3SS) of rhizobia play pivotal roles as host-specific determinants in the symbiosis within nodules. NopD, in addition to its C-terminal domain in isolation, demonstrates the ability to process small ubiquitin-related modifier (SUMO) proteins and cleave SUMO-conjugated proteins. Importantly, this functionality was rendered inactive in a variant of NopD carrying a cysteine-to-alanine substitution within the catalytic core, known as NopD-C972A. Functionality assays demonstrated that while NopD induces cell death upon expression in *N. tabacum*, its catalytically inactive variant, NopD-C972A, fails to do so. Moreover, inoculation experiments employing mutant strains of XS1150 indicated that the protease activity of NopD negatively impacts nodulation of *T. vogelli* (Xiang et al., 2020). Through mutational analysis, the majority of the putative domains/motifs within Bel2-5 were crucial for both NF-independent nodulation and nodulation restriction in *Rj4* soybean (Ratu et al., 2021b). The SkP48 Type 3 Effector (T3E) from DOA9, containing the SK and ULP1 domains, inhibits nodulation in *Vigna* species, *C. juncea*, and *Arachis*. SkP48 serves as a critical factor in suppressing nodulation and nodule organogenesis across various legumes by inducing effector-

triggered immunity through the activation of salicylic acid biosynthesis. This induction proves detrimental to rhizobial infection (**Fig. 2.13**) (Pongdet et al., 2021).

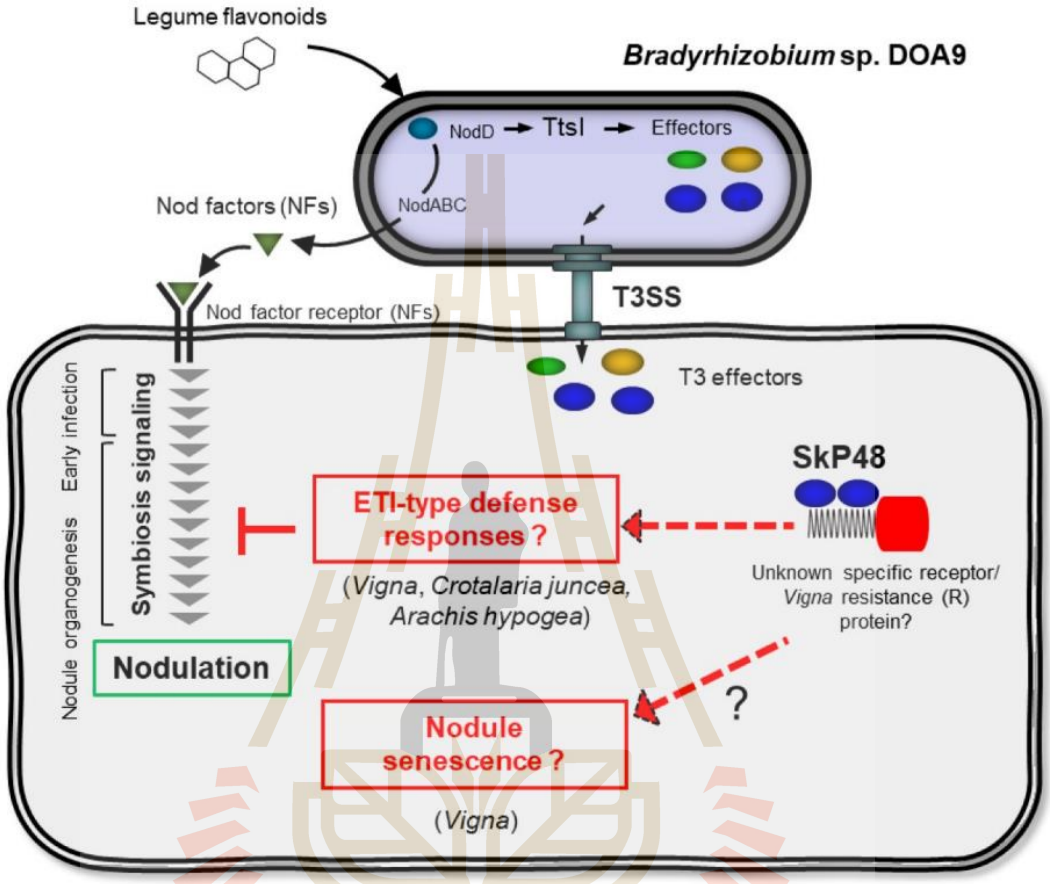

Figure 2.13 A new putative T3E, SkP48, in *Bradyrhizobium* sp. DOA9 is specific to symbiosis with legumes. Hypothetically, SkP48 is a factor that restricts the nodulation of *Vigna* varieties, *Arachis*, and, *Crotalaria* possibly by encouraging phytohormone-mediated effector-triggered immunity (PmETI-type) in the host. SkP48 might be recognized by unknown receptor(s) or a specific resistance (R) protein in the legumes, consequently triggering an R protein-mediated ETI-type defense response (RmETI-type) to restrict nodulation. The dotted line indicates unclear symbiotic mechanisms (Pongdet et al., 2021).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Phylogenetic tree and *in silico* protein analysis

The MicroScope and NCBI platforms were used to search for the amino acid sequence SUMO T3Es of all strains, which are accessible at http://www.genoscope.cns.fr/agc/microscope (Vallenet et al., 2013) and https://www.ncbi.nlm.nih.gov/protein/. A phylogenetic tree was subsequently constructed via MEGA11, with 1,000 bootstrap replications. The protein domain analysis was conducted via SMART, a platform for analysing protein domains available at http://smart.embl-heidelberg.de/smart/change_mode.pl and a protein domain diagram. Additionally, repeat domain analysis of SUMO-proteases p0903 and SkP48 from DOA9 was conducted via WebLogo and Microsoft Excel 2010. WebLogo (accessible at https://weblogo.berkeley.edu/logo.cgi) was utilized to generate graphical representations of the repeat sequences (RSs) within the repeat domain alignments of SUMO-proteases p0903 and SkP48. A radar graph created via Microsoft Excel 2010 was used to construct a spider map of the RSs of SUMO-proteases p0903 and SkP48.

3.2 Bacterial strains and growth conditions

The bacterial mutants of *Bradyrhizobium* sp. DOA9 used in this study are summarized in **Table 3.1**. DOA9 and the derivative mutants were subsequently grown in yeast mannitol (YM) media or arabinose-gluconate (AG) media at 28°C (Sadowsky et al., 1987; Vincent, 1970). *Escherichia coli* strains XL2-Blue (Agilent) and S17-1 were cultured in Luria–Bertani (LB) media at 37°C (Sambrook et al., 1989). When needed, the media were supplemented with the following antibiotic concentrations: 20 µg/mL nalidixic acid (Amresco); 20 µg/mL cefotaxime, Cefo (GoldBIO); 50 µg/mL kanamycin, Km (BIO BASIS); and 200 µg/mL spectinomycin, Sm (Glentham).

Table 3.1 The bacterial strains were used in this investigation.

Strains	Characteristics	Reference
DOA9	Wild-type strain *Bradyrhizobium* sp.	(Noisangiam et al., 2012)
DOA9-Ω*rhcN*	Insertional *rhcN* of pDOA9 by integration of pVO155-*Sp/Sm-npt2-gfp*; Sp/Sm^r at *Xba*I/*Sal*I	(Songwattana et al., 2017)
LMG26795	Wild-type strain *Bradyrhizobium arachidis*	(Wang et al., 2013)
LMG26795-Ω*rhcN*	Insertional *rhcN* of LMG26795 by integration of pVO155-*cefo-npt2-gfp*; cefo^r at *Xba*I/*Sal*I	Provided from Lab UMR PHIM
DOA9-Δ*p0490*	Deletion of *p0490* of pDOA9 by double crossed recombination with up/down *p0490*.pK18mob-*sacB-cefo*^{r}	This study
DOA9-Δ*p0871*	Deletion of *p0871* of pDOA9 by double crossed recombination with up/down *p0871*.pK18mob-*sacB-cefo*^{r}	This study
DOA9-Δ*p0903*	Deletion of *p0903* of pDOA9 by double crossed recombination with up/down *p0903*.pK18mob-*sacB-cefo*^{r}	This study
DOA9-Δ*SkP48*	Deletion of *Skp48* of pDOA9 by double crossed recombination with up/down *Skp48*.pK18mob-*sacB-cefo*^{r}	(Piromyou, Songwattana, et al., 2021)
Δ*p0903+p0903*	Complementation of *p0903* gene by introducing pMG103-*p0903-8xHis-npt2-cefo*^{r} into DOA9-Δ*p0903*	This study
Δ*p0903+p0903*-D728A	Complementation of *p0903* with the mutagenesis site direct at C728A, resulting pMG103-*p0903-8xHis-D728A-npt2-cefo*^{r} into DOA9-Δ*p0903*	This study

Table 3.1 Continue.

Strains	Characteristics	Reference
Δ*p0903+p0903*-C763A	Complementation of *p0903* with the mutagenesis site direct at C728A, resulting pMG103-*p0903-8xHis-D763A-npt2-cefo*^{r} into DOA9-Δ*p0903*	This study
Δ*p0903+p0903*-ULP-lack	Complementation of *p0903-ULP1* lacking by introducing pMG103-*ULP-lack-npt2-cefo*^{r}*-gfp* into DOA9-Δ*p0903*	This study

Δ, deletion mutants; Ω, insertional mutants. *cefo*^{r}, cefotaxime resistant; *Km*^{r}, kanamycin resistant; *Sp/Sm*^{r}, spectinomycin and Streptomycin resistant genes cassette.

3.3 Plasmid construction, mutagenesis, and complementation

The clean deletion mutants of DOA9-Δ*p0490*, DOA9-Δ*p0871*, and DOA9-Δ*p0903* were constructed via a double homologous recombination method. To achieve this goal, PCR fragments of approximately 750-1,000 base pairs corresponding to the regions flanking each gene were combined via overlap extension PCR (primer **Table 3.2** and construction map **Fig. 3.1**). The cloning sites of the *p0490* and *p0871* genes were *Eco*RI/*Bam*HI (Enzyme: Thermo Scientific), whereas the *p0903* gene was *Xba*I (Enzyme: Thermo Scientific) and *Bgl*II (Enzyme: NEB). After overlapping the PCR products, they were ligated into the pK18mob-*sacB-cefo* plasmid (Tsai & Alley, 2000). In addition, the recombinant plasmids were introduced into *E. coli* S17-1 and then transferred into DOA9 via conjugation, followed by biparental mating. The selection of these recombinants was carried out following previously established protocols (Giraud et al., 2010).

For the complementation Δ*p0903+p0903* strain, a DNA fragment of 2,532 bp containing the *p0903* gene along with its promoter sequence and an 8xHis tag before the stop codon was used. PCR was performed via specific primers, as indicated in **Table 3.2**, and the resulting map is shown in **Fig. 3.1**. The restriction site was

*EcoR*I/*Sal*I (Enzyme: Thermo Scientific), which was subsequently ligated into the pMG103-*npt2-cefo* plasmid (Wongdee et al., 2018). The recombinant plasmid (pMG103-*p0903-8xHis-npt2-cefo*) was introduced into the DOA9-Δ*p0903* strain through electroporation (17.5 kv/cm, 100 Ω, and 25 μF) (Wongdee et al., 2016). The specific primers were used to verify the cloning of the complementation mutant, as shown in **Table 3.2** (Wongdee et al., 2016).

To complement the Δ*p0903+p0903*-ULP-lack domain, a 1,778-bp fragment of the *p0903* gene lacking the ULP domain was PCR-amplified via the specific primers detailed in **Table 3.2**. The construction map is shown in **Figure 3.1**. The *Sal*I (Enzyme:Promega) restriction site was used for cloning the PCR product. The inserted gene was subsequently integrated into pMG103-*npt2-cefo-npt2-gfp*. The complementation construct plasmid was then introduced into the DOA9-Δ*p0903* strain via electroporation. The mutant was selected on YM media supplemented with antibiotics, confirmed via PCR, and chosen as previously described (Wongdee et al., 2016).

For the site-directed mutagenesis of Δ*p0903+p0903*-D728A and Δ*p0903+ p0903*-C763A, the plasmid pMG103-*p0903-8xHis-npt2-cefo* was used for construction, as indicated by the construction map in **Fig. 3.1**. This process was performed with the Q5 Site-Directed Mutagenesis Kit (NEB) along with the primers listed in **Table 3.2** for PCR amplification (Camuel et al., 2023). PCR was conducted to introduce changes in the amino acids D728A and C763A, substituting them with alanine residues (Xiang et al., 2020b). The PCR product was subsequently ligated for construction of the plasmid. The recombinant plasmid was then electroporated into the DOA9-Δ*p0903* strain. Mutants were selected on YM media supplemented with antibiotics and verified via PCR as described previously (Wongdee et al., 2016b).

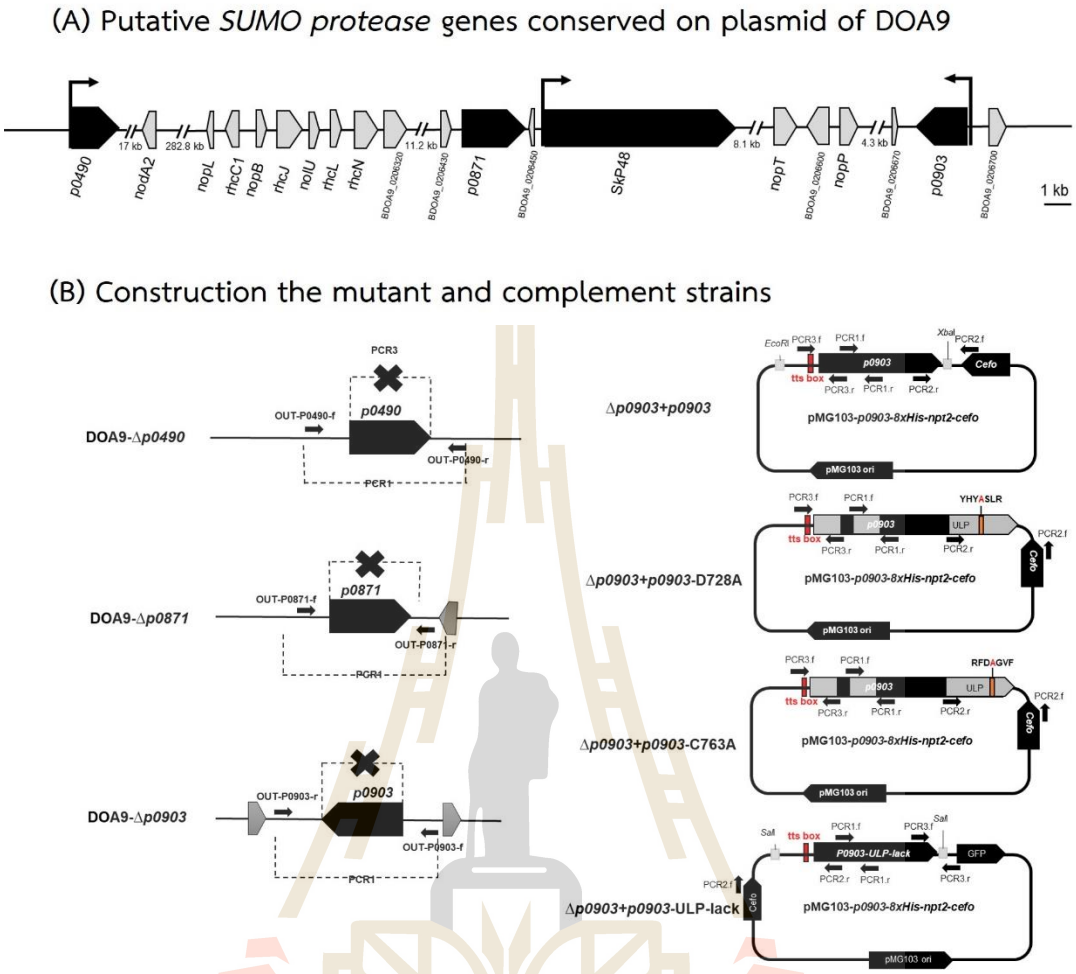

Figure 3.1 (A) Genetic arrangement of the putative *SUMO protease* genes located in the symbiotic region on the plasmid of DOA9. Arrows denote the orientations and sizes of the ORFs; grey arrows represent other genes, while black arrows signify putative *SUMO protease* genes T3Es, and tts box motifs are illustrated by black rectangular arrows. (B) The diagram also elucidates the construction map of the SUMO-protease mutants and complementation strains, detailing the binding sites of specific primers for verification as described in **Table 3.2**.

Table 3.2 The detail of primers used in this study for the construction and verification of derivative mutant and complementation strains.

Construction			
Name	**Name of primer**	**Sequence 5′ to 3′**	**Relevant characteristics**
DOA9-Δ*p0490*	UP-P0490-F	CAGGAATTCTCTGATCTTCGCCCTATCCTCG	Amplification of the upstream and downstream flanking DNA of the *p0490*. The PCR fragments were merged by overlap extension PCR and cloned into pK18mob-*sacB-cefo* at the *Eco*RI/*Bam*HI sites. This construction was used to obtain the DOA9-Δ*p0490* mutant by double crossing over.
	UP-P0490-R	ATCGCCACGCGCTAGCCTCACGTCGATAGATGAAC	
	DW-P0490-F	GTAGGCTAGCGCGTGGCGATCTCTACGATTTC	
	DW-P0490-R	GATGGATCCGATCATCTCGGAATCCTGGACAAC	
DOA9-Δ*p0871*	UP-P0871-F	GCTCGAATTCCGATACCTGTCATTGGCTGAAC	Amplification of the upstream and downstream flanking DNA of the *p0871*. The PCR fragments were merged by overlap extension PCR and cloned into pK18mob-*sacB-cefo* at the *Eco*RI/*Bam*HI sites. This construction was used to obtain the DOA9-Δ*p0871* mutant by double crossing over.
	UP-P0871-R	ATCGTGGTCAACCACTCTCCCTAGATGACATGAC	
	DW-P0871-F	CCTCTCACCATGACCACGATCCGGCAAGGAC	
	DW-P0871-R	GCAGGATCCGGAAGCTCCTGAGTCGTGATAC	
DOA9-Δ*p0903*	UP-P0903-F	ATGTCTAGAGAAAGACCGGAGGCAATC	Amplification of the upstream and downstream flanking DNA of the *p0903*. The PCR fragments were merged by overlap extension PCR and cloned into pK18mob-*sacB-cefo* at the *Xba*I/*Bgl*II sites. This construction was used to obtain the DOA9-Δ*p0903* mutant by double crossing over.
	UP-P0903-R	CCGCTCAGAAACACATAGCGACCTCGACTCGACTTTAG	
	Dw-P0903-F	CGAGGTCGCTATGTGTTTCTGAGCGGCACATCTCAATC	
	Dw-P0903-R	CGATCTAGACGGTGCCCCTAAACAATC	

Table 3.2 Continue.

Construction			
Name	**Name of primer**	**Sequence 5′ to 3′**	**Relevant characteristics**
Δ*p0903+p0903*	P0903-Com-F	GAACTGAATTCTCGTCAGCCTGTCGTCAGGC	The PCR was amplified 2,532-bp of the *p0903* gene
	P0903-Com-R	ATGGTCGACTCAATGATGATGATGATGATGATGATGG AAA	including *p0903* gene, its promoter sequence, an 8xHis tag. The PCR fragment was cloned into
		CAGCCCCTGAGTCGATTTTG	pMG103-*npt2-cefo* at the *EcoRI*/*Xba*I sites. This construction was used to obtain the Δ*p0903+p0903* mutant by electroporation into DOA9-Δ*p0903*.
Δ*p0903+ p0903*-D728A	*SUMO-p0903D728A.f*	GCGTCACTCCGAAAACGAAACGGT	The PCR was amplified 8,898-bp of
	SUMO-p0903D728A.r	GTAATGATAGGCGACCGGGCTTTC	pMG103-*p0903-8xHis-npt2-cefo* plasmid sequence. The PCR fragment was ligated. This construction was used to obtain the Δ*p0903+p0903*-D728A mutant by electroporation into DOA9-Δ*p0903*.
Δ*p0903+ p0903*-C763A	SUMO-p0903C763A.f	GCGGGCGTCTTCTTGGTGGACGCC	The PCR was amplified 8,898-bp of
	SUMO-p0903C763A.r	ATCAAACCTGTTCTGCTGCTGGGC	pMG103-*p0903-8xHis-npt2-cefo* plasmid sequence. The PCR fragment was ligated. This construction was used to obtain the Δ*p0903+p0903*-D763A mutant by electroporation into DOA9-Δ*p0903*.

Table 3.2 Continue.

Construction				
Name	**Name of primer**	**Sequence 5′ to 3′**		**Relevant characteristics**
Δ*p0903+p0903*-ULP-lack	SUMO-P0903-DOA9-pm-f	CACCCGCTGTCGACCGGCGGCGAAGCGGCTTGGACATTTG		The PCR was amplified of 1,778-bp of the *p0903* gene including *p0903* gene, its promotor and 6xHis tag. The PCR fragment was cloned into pMG103-*npt2-cefo-gfp* at only *Sal*I site. This construction was used to obtain the Δ*p0903+p0903* mutant by electroporation to DOA9-Δ*p0903*.
	SUMO-p0903-ULP-Lack-r	AGCATCGTCGACTCAATGATGATGATGATGATGGGTCGGAGACGCCCGCACAGAATG		
Verification				
DOA9-Δ*p0490*	PCR1	OUT-P0490-f	GGCGAGCGGTCATATGTTGC	The positive amplification of PCR1 was detected on DOA9-Δ*p0490*, while DNA fragment performed by PCR2 and PCR3 amplification was undetected.
		OUT-P0490-r	CAACTAGCCGTTCCTGCTCATC	
	PCR2	M13-R	GCGGATAACAATTTCACACAGGA	
		OUT-P0490-r	CAACTAGCCGTTCCTGCTCATC	
	PCR3	OUT-P0490-f	GGCGAGCGGTCATATGTTGC	
		Cefo-R	CCTCCCGACTGCCGCTCTA	
DOA9-Δ*p0871*	PCR1	OUT-P0871-f	GGCGAGCGGTCATATGTTGC	The positive amplification of PCR1 was detected on DOA9-Δ*p0871*, while DNA fragment performed by PCR2 and PCR3 amplification was undetected.
		OUT-P0871-r	ATTGCAAGCTTTCCGGCCTAC	
	PCR2	M13-R	CGACCATGCGGGGAGAACAAAG	
		OUT-P0871-r	ATTGCAAGCTTTCCGGCCTAC	
	PCR3	OUT-P0871-f	GGCGAGCGGTCATATGTTGC	
		Cefo-R	GTGTGGCAGGCAATGTGGGATAG	

Table 3.2 Continue.

Construction				
Name	**Name of primer**	**Sequence 5′ to 3′**		**Relevant characteristics**
DOA9-Δ*p0903*	PCR1	OUT-P0903-f	CAACTTTGCCTCTGGTCCC	The positive amplification of PCR1 was
		OUT-P0903-r	CTCAAGCCAAAAGGCACGC	detected on DOA9-Δ*p0903*, while DNA
	PCR2	M13-R	CGACCATGCGGGGAGAACAAAG	fragment performed by PCR2 and PCR3
		OUT-P0903-r	CTCAAGCCAAAAGGCACGC	amplification was undetected.
	PCR3	OUT-P0903-r	CAACTTTGCCTCTGGTCCC	
		Cefo-r	GTGTGGCAGGCAATGTGGGATAG	
Δ*p0903+ p0903*	PCR1	SUMO-P0903-DOA9-pm-in.f	GCTAGTGGTTTGCGCAGGAAAATAC	The positive amplification of three PCR was
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC	detected on Δ*p0903+p0903* strain.
	PCR2	Cefo-Fin-f	GCTATGGCACCACCAACGATATC	
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC	
	PCR3	SUMO-P0903-DOA9-pm-fin.f	ATGGCCCAGCAGCAGAACAGGTTTG	
		M13-R	GTCATAGCTGTTTCCTG	
Δ*p0903+ p0903*-D728A	PCR1	SUMO-P0903-DOA9-pm-in.f	GCTAGTGGTTTGCGCAGGAAAATAC	The positive amplification of three PCR was
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC	detected on Δ*p0903+p0903*-D728A strain.
	PCR2	Cefo-Fin-F	GCTATGGCACCACCAACGATATC	
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC	
	PCR3	SUMO-P0903-DOA9-pm-fin.f	ATGGCCCAGCAGCAGAACAGGTTTG	
		M13-R	GTCATAGCTGTTTCCTG	

Table 3.2 Continue.

Construction			
Name	**Name of primer**	**Sequence 5′ to 3′**	**Relevant characteristics**
Δ*p0903+p0903*-C763A	PCR1	SUMO-P0903-DOA9-pm-in.f	GCTAGTGGTTTGCGCAGGAAAATAC The positive amplification of three PCR was detected on Δ*p0903+p0903*-D763A strain.
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC
	PCR2	Cefo-Fin-F	GCTATGGCACCACCAACGATATC
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC
	PCR3	SUMO-P0903-DOA9-pm-fin.f	ATGGCCCAGCAGCAGAACAGGTTTG
		M13-R	GTCATAGCTGTTTCCTG
Δ*p0903+p0903*-ULP-lack	PCR1	SUMO-P0903-DOA9-pm-in.f	GCTAGTGGTTTGCGCAGGAAAATAC The positive amplification of three PCR was detected on Δ*p0903+p0903*-ULP-lack strain.
		SUMO-P0903-DOA9-pm-in.r	ATGCGACAAATCGGTATAGTGTAAC
	PCR2	Cefo-Fin-F	GCTATGGCACCACCAACGATATC
		SUMO-P0903-DOA9-pm-deb.r	TGGCAAGTGCCATACCTCCGCAATTG
	PCR3	SUMO-P0903-DOA9-pm-fin.f	ATGGCCCAGCAGCAGAACAGGTTTG
		M13-R	GTCATAGCTGTTTCCTG

The primer base sequences in (GCG) were displayed a substitution of amino acids with alanine, indicated by the red mark.

3.4 Peanut nodulation and symbiosis analysis

The *A. hypogaea* KK5 cultivar tested is a Thai-published variety that is normally cultivated by Thai farmers (**Table 3.3**). This study complies with local and national regulations in Thailand. The seeds of the peanut cultivars were washed in 70% ethanol for 30 s and then rinsed once with sterile water. The samples were subsequently subjected to surface sterilization with a 3% (v/v) sodium hypochlorite solution for 30 s, followed by five washes in sterilized water. After being germinated on vermiculite for 3 days, the seedlings were transplanted into Leonard jars filled with vermiculite and watered with BNM (Ehrhardt et al., 1992). The plants were cultivated under controlled environmental conditions: 28°C with a 16-hour light and 8-hour dark cycle at light intensities of 300 μmol/m^2 s and 70% humidity. DOA9 and its derivatives were grown in YM media at 30°C for five days. At 5 days, each seedling was inoculated with 1 mL of a 5-day-old inoculum, adjusted to an optical density at 600 nm of 1 (approximately 10^9 cells/mL), and washed with sterilized water before use. At 30 dpi, the leaf chlorophyll content was measured via a Minolta SPAD-502 chlorophyll meter, the number of nodules was counted, and the dry weight of the plants was noted. Additionally, living and dead bacteroid cells were observed via confocal microscopy. Finally, an acetylene reduction assay (ARA) was performed as described previously (Renier et al., 2011).

3.5 Analysis of the function of SUMO-protease p0903 in peanut cultivar symbiosis

3.5.1 Nodulation test

The 4 genotypes of *A. hypogaea* that were tested are Thai published varieties that are normally cultivated by Thai farmers (KK5, TN9, KS2, and SK38) (**Table 3.3**). The nodulation test followed the same procedure as described above for growth conditions and data collection. Each treatment consisted of six replicates, including the wild type (DOA9), *rhcN* insertion mutant strain (DOA9-Ω*rhcN*), *p0903* deletion mutant strain (DOA9-Δ*p0903*), and *p0903* complementation mutant strain (Δ*p0903+p0903*).

Table 3.3 The genotype of *A. hypogaea* cultivars.

Name of peanut cultivars	Groupe of peanut cultivars	Reference
Khon Khean 5 (KK5)	Spanish group, large-seeded peanut breeding, commercial cultivar in Thailand, and resistance necrosis disease	(Puttha & Jogloy, 2019; Riabroy et al., 2022)
Tainan 9 (TN9)	Spanish group, erect plant type and semi-early maturity and moderate resistance necrosis disease	(Kesmala et al., 2006; Puttha & Jogloy, 2019)
Kalasin 2 (KS2)	Valencia group commonly grown in Thailand and low yield in drought condition	(Boontang et al., 2010)
Soukhothai 38 (SK38)	Valencia group and is commercial cultivar in Thailand	(Sattayarak, 1997)

3.5.2 Bacteroid cells size measurement

The cultivation of the peanut KK5 cultivar followed the same procedure as described above for growth conditions. Each treatment consisted of 3 replicates, including the wild type (DOA9), the *rhcN* insertion mutant strain (DOA9-Ω*rhcN*), the *p0903* deletion mutant strain (DOA9-Δ*p0903*), and the *p0903* complementation mutant strain (Δ*p0903+p0903*). The diameter of the bacteroid cells was measured at 3 weeks. Three nodules were collected from each plant, then each nodule was broken by a loop in a tube. Next, the liquid from the nodules was collected and stained with lactophenol trypan blue (0.4%). Finally, the diameter of the bacteroid cells was measured under the microscope.

3.5.3 Bacterial RNA extraction and expression analysis

The mid log phase cultures of DOA9 and LMG26795 were washed, and their OD_{600} was adjusted to approximately 0.4 using AG medium supplemented with purified flavonoids (20 μM genistein and naringenin dissolved in DMSO) from Sigma. DMSO alone served as the negative control. The bacterial cells were subsequently cultured at 28°C for 24 hours and harvested via centrifugation (4,000 rpm for 10

minutes at 4°C). Total bacterial RNA was extracted from the induced cells via the previously described manure method (Babst et al., 1996). The extracted RNA was treated with RNase-free DNaseI (NEB) for 30 minutes at 37°C. The purity and concentration of RNA were measured via a NanoDrop, resulting in A260/280 and A260/230 ratios of 2.1 and 2.1 to 2.4, respectively. cDNA was subsequently synthesized from 500 ng of total RNA via the High-Capacity cDNA Reverse Transcription Kit (iScript, Bio-Rad) according to the manufacturer's protocol. Next, the synthesized cDNA (50 ng) was used for PCR amplification with gene-specific primers (**Table 3.4**) via the CFX Opus 96 Real-Time PCR System (Bio-Rad, USA). The PCR program consisted of an initial denaturation step at 95°C for 2 m, followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 30s. To analyse gene expression, the comparative Ct ($^{-\Delta\Delta_{CT}}$) method was used (Livak & Schmittgen, 2001), and the data were normalized to the expression of the endogenous housekeeping gene 16S rRNA (primer: PBA338F/PRUN518R). The analysis was based on data obtained from three biological replicates, and at least three PCR amplifications were performed for each sample.

3.6 Analysis of the effects of the ULP domain on peanut K55 cultivar symbiosis

The peanut cultivar KK5 was used for the symbiotic test. The treatments included DOA9, DOA9-Ω*rhcN*, DOA9-Δ*p0903*, Δ*p0903+p0903*, ULP domain deletion mutants of *p0903* (Δ*p0903+p0903*-ULP-lack), D728A point mutation of *p0903* (Δ*p0903+p0903*-D728A), and C763A point mutation of *p0903* (Δ*p0903+p0903*-C763A) strains. The plant conditions and data collection followed the same protocol used for the nodulation test described earlier. The data collection consisted of ten replications, with the exception of five replications for the ARA measurement.

3.7 Microscopy

An Olympus Fluoview FV1000 confocal laser scanning microscope (Japan) was used to examine the bacteroid morphology. Nodule sections, approximately 33–40 µm thick, were prepared using a Leica vibratome (VT1000S; Nanterre, France). The

bacteria were treated with a live/dead staining solution comprising 5 μM SYTO9 (Invitrogen) and 30 μM propidium iodide (Invitrogen) in 50 mM Tris-HCl (pH 7.0) and incubated for 15 minutes. Following staining, the sections were washed with 10 mM phosphate saline buffer. For an additional 5 min, the plant cell walls were stained with calcofluor white M2R (Sigma) at a final concentration of 0.01% (w/v). The chemicals present in the nodule stain were excited to detect the presence of live bacteroids via a previously described method (Wangthaisong et al., 2023). The Nikon Inverted Eclipse Ti-E Confocal Laser Scanning Microscope was used to study nodule development and analyze live and dead bacteroids within symbiosomes.

3.8 Gene expression analysis and measurement of jasmonic acid content in peanut root

3.8.1 Peanut gene expression analysis

The peanut seeds were surface sterilized, germinated, and grown under the same conditions as those described above for the nodulation test. The seedlings were inoculated with DOA9 and DOA9-Δ*p0903*. At 3 dpi, the peanut roots were frozen in liquid nitrogen and ground into a fine powder, and 100 mg of the powder was used for total RNA extraction via the FavorPrep Plant Total RNA Mini Kit (Favorgen). The RNA purification followed the same procedure as described above. The RNA samples were then converted into cDNA from 1,000 ng of total RNA via the High-Capacity cDNA Reverse Transcription Kit (iScript, Bio-Rad) according to the manufacturer's protocol. Then, a 10-fold dilution of cDNA was used with specific primers (Table 3.4) for amplification of each gene. The qRT-PCR conditions were the same as those described above. Relative gene expression was calculated via the Ct ($^{-\Delta\Delta CT}$) method (Livak & Schmittgen, 2001). To normalize the transcript levels of the sample genes, the expression of the β-actin gene was measured in the same samples.

3.8.2 Measurement of jasmonic acid content in peanut root

At 3 dpi, the peanut roots were harvested and ground to a fine powder in liquid nitrogen. Subsequently, 100 mg of the powder was mixed with 1 mL of 90% methanol, homogenized for 2 min at 6,000 rpm, and then incubated overnight at 4°C.

The mixture was centrifuged at 1,200×g for 10 min at 4°C, and the supernatant was collected, freeze-dried, and resuspended in 400 µL of 1 M trichloroacetic acid. This suspension underwent three partitioning steps against hexane:ethyl acetate 200 µL (1:1), with the organic phase collected and freeze-dried. The sample extract, standard jamonic acid (Glentham), was dissolved in 1000 µL of methanol, filtered through a 0.22-µm PTFE membrane, and prepared for analysis via triple quadrupole LC-MS/MS (AB Sciex QTRAP 6500 LC-MS/MS platform). The separations were performed on a Thermo Scientific ™ Acclaim ™ 120 C18 column (100 mm × 2.1 mm, 2.2 µm). The HPLC conditions and gradient were used for all analyses, which took 13.6 min. The injection sample volume was 5 µL and consisted of mobile phases A (formic acid in water with 0.1% (v/v)) and B (acetonitrile). The gradient time frame was as follows: 0 min 10% A; 1 min 10% A; 2 min 40% A; 7 min 95% A; 9 min 95% A; 9.1 min 10% A; and 13.6 min 10% A. The column compartment was heated to 35°C.

3.9 Statistical analysis

Data analysis was conducted using SPSS software (version 26.0 for Windows; SPSS Inc., Chicago, IL) to verify the statistical significance of the ANOVA results. Tukey's HSD test was employed as a post hoc test for the plant test analysis and jasmonic acid analysis, with significance levels set at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$. qRT-PCR analysis was performed, and a two-tailed Student's t-test was applied for pairwise comparisons. Statistical significance was set at $P \leq 0.05$. Detailed information on sample size and replicates is provided in the figure and table legends.

Table 3.4 The list of primers used in qRT-PCR to analyze gene expression.

Group of genes	Name of primers	Gene description	Sequences 5'-3'	Tm (°C)	References
Internal control	Actin-F	β-actin	TGGCATACAAAGACAGGACAGCCT	64	(Jogi et al., 2016)
	Actin-R		ACTCAATCCCAAGGCCAACAGAGA	63	
ISR-related genes	Myc2.F	Transcriptional factor Myc2	CCCCCAAACTCAACAACCTACT	60	This study
	Myc2.R		CAGAGATTCATCGTCGGCGT	60	
	PR4-F	Pathogenesis-related protein 4	TCGTTGATCAGTGCAGCAATGGAG	63	(Jogi et al., 2016)
	PR4-R		AGATGGCCCTGAGCATTCCCATTA	63	
	Def2.2-F	Defensin	ATAATGGCTCGCTCTCTTCCTTT	59	(Zhao et al., 2022)
	Def2.2-R		TCATTAAGCACAATGCTTCGTGC	60	
Symbiosis-related genes	SYMREM-F	Symbiotic remorins	GACTAAAATAGACAACAGGGC	54	(Karmakar et al., 2019)
	SYMREM-R		GCAGTAAAGCATGAAAGAATTTTC	55	
	CNGC-F	Cyclic nucleotide-gated channels	ATTCGGCTATGCTTGCTCTTCG	61	This study
	CNGC-R		ACCACGTAAAGCCTCGTCAATG	61	
	CERBERUS-F	E3 ubiquitin-protein ligase LIN-1	ACCCTTCCACAGCCCTTTTA	58	This study
	CERBERUS-R		AGGTTGTAGCAAGTCCAGCC	59	
SUMO-p0903	P0903-qRT.F1	SUMO-p0903	ATACAACCCGTCTTCGTCGG	59	This study
	P0903-qRT.R1		CGGGTCAAGATACTCCGCAA	59	
T3SS (*rhcN*)	rhcN.RT.D9.f	Type 3 secretion system ATPase	CATTGGCGATATGGTAGGCT	57	(Songwattana et al., 2017)
	rhcN.RT.LMG.r		CGTCAGAAGTCCATCCAGAAC	58	This study
Internal control	PRBA338F	16S ribosomal RNA	ACTCCTACGGGAGGCAGCAG	63	(Ovreås et al., 1997)
	PRUN518R		ATTACCGCGGCTGCTGG	60	

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CHAPTER IV
RESULTS AND DISCUSSION

4.1 DOA9 strain displays 4 distinct putative SUMO-protease T3Es

To investigate the relationships among four putative SUMO-proteases T3Es from DOA9 and other SUMO-proteases T3Es from members of the *Bradyrhizobiaceae* family isolated from legume crops, a phylogenetic tree analysis was conducted. The phylogenetic tree classified the putative SUMO-proteases into three clades (**Fig. 4.1**). The four DOA9 SUMO-proteases clustered within clade 1, further dividing into two subclades. Proteases p0490 and p0871 were grouped with two SUMO-proteases (NFUH01 4330016 and NopD) from *Bradyrhizobium* sp. XS1150 (isolated from *A. hypogaea*) in subclades 1-1. However, SUMO-proteases p0903 and SkP48 fell within subclades 1-2, alongside protease BE61 p0680 from *B. elkanii* USDA61 and HAP48 0027480 and HAP48 0000990 from *B. septentrionale* 1S1, the type strains isolated from *Glycine max*. Notably, these four DOA9 SUMO-proteases exhibited low sequence similarity, with only 50% identity between p0490 and p0871 and 35% identity between p0903 and SkP48. Clade 2 was divided into two subclades: subclade 2-1, which contained five SUMO-proteases from *Bradyrhizobium* species isolated from *A. hypogaea* and *G. max*, sharing only 49% similarity with the ten SUMO-proteases from *Bradyrhizobium* species isolated from *G. max* in subclade 2-2. However, the SUMO-proteases in this clade exhibited a low identity (21%) when compared to those in clade 1. In contrast, clade 3 comprised seventeen SUMO-proteases exclusively from *Bradyrhizobium* species isolated from *G. max*, which were divided into two subclades. Additionally, the similarity of these SUMO-proteases with those in clades 1 and 2 was only 9%. This finding indicated that the DOA9 SUMO-proteases possess unique characteristics and that their functions might differ significantly from those of SUMO-proteases in other *Bradyrhizobium* species due to evolutionary divergence.

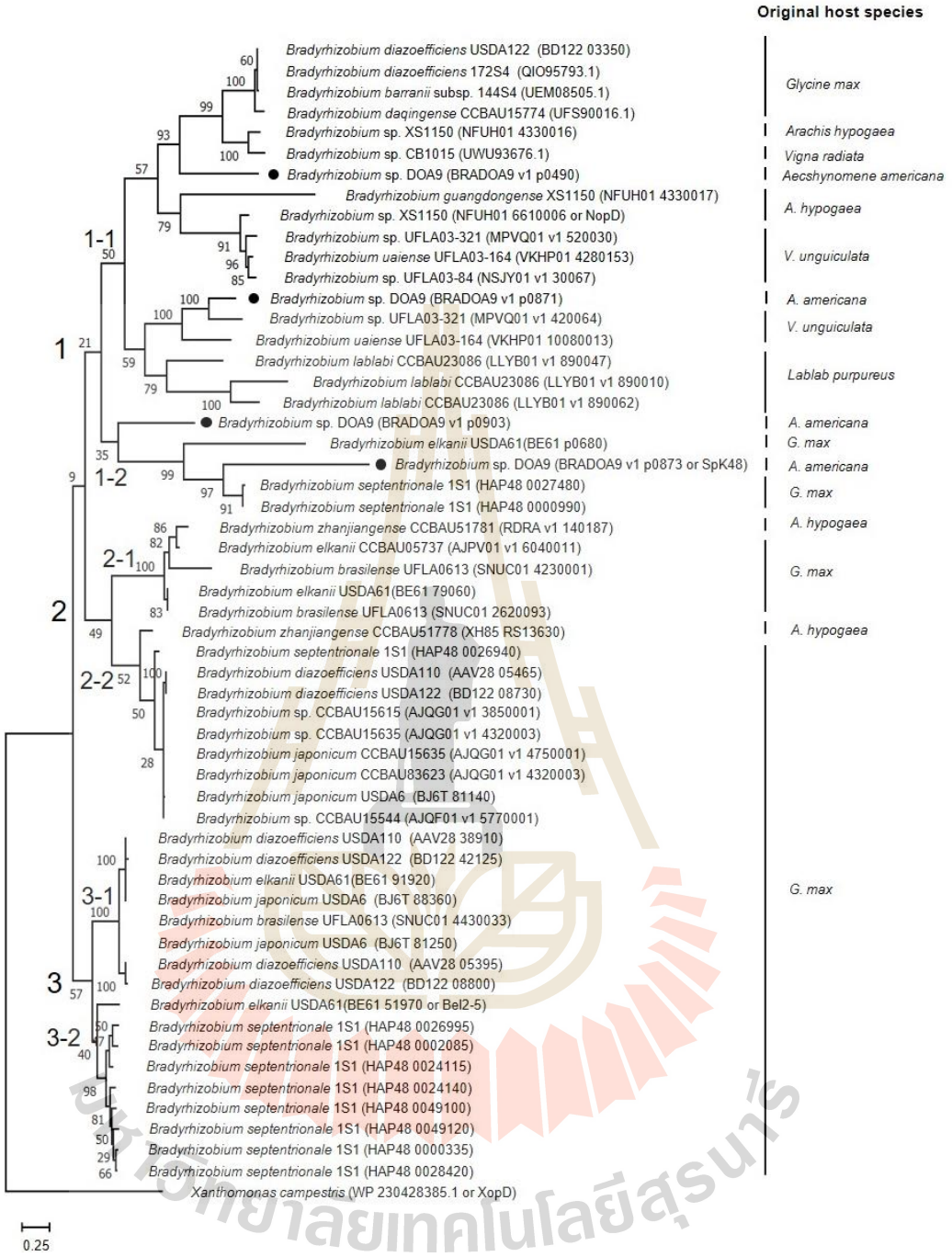

Figure 4.1 Phylogenetic tree analysis of four SUMO protease T3Es in *Bradyrhizobium* sp. DOA9 and other SUMO-proteases from *Bradyrhizobium* species isolated from legume crops and XopD from *Xanthomonas campestris*. The GenomeScope and NCBI-derived amino acid sequences of SUMO-proteases were subjected to phylogenetic analyses using MEGA11. The phylogeny was inferred using the maximum-likelihood method, and bootstrap analysis with

1,000 replications was used to determine the percentages of replicate trees in which the associated taxa clustered together. The SUMO-proteases from bradyrhizobia isolated from legume crops were classified into clades 1 to 3, with black spots marking the SUMO-proteases in DOA9.

Moreover, the domains and structural organization of the four putative SUMO protease T3Es from DOA9 were analysed and compared with those of Bel2-5 from USDA61, NopD from XS1150, and XopD from the pathogenic bacterium *Xanthomonas campestris*. All SUMO-proteases share a common ULP-like domain, typically located at the C-terminal (**Fig. 4.2** and **Fig. 4.3**). In contrast, the N-terminal regions, which lack functional domains, are highly divergent among proteases. Notably, p0903 and SkP48 each contain two distinct repeat domains (RDs). Although these RDs differ in length and exhibit low amino acid sequence identity, both proteins have unique features. SkP48 includes a shikimate kinase domain and 10 repeat sequences in each RD, whereas p0903 contains RD1 and RD2 sequences without tandem repeat sequences within the RDs. Interestingly, the repeat sequences within RDs from p0903 and SkP48 were significantly different in length and similarity. RD1 and RD2 of p0903 share 94 amino acids, whereas each RD on SkP48 contains 10 repeat sequences that share 41 amino acid conserved sequences (**Fig. 4.4A-D**). Additionally, the amino acid sequences of the two RDs of p0903 and the 20 repeat sequences in the two RDs of SkP48 showed complete divergence. This result was confirmed by the RADAR graph, which demonstrated that the amino acid composition of each repeat sequence was conserved within the group (**Fig. 4.4E**). Taken together, the strong divergence observed among the four SUMO-proteases identified in the DOA9 strain, which share only the ULP domain while having RDs on p0903 and SkP48 with differing identities, suggests that these putative T3Es might play distinct roles during the symbiotic interaction of DOA9 with its various legume hosts.

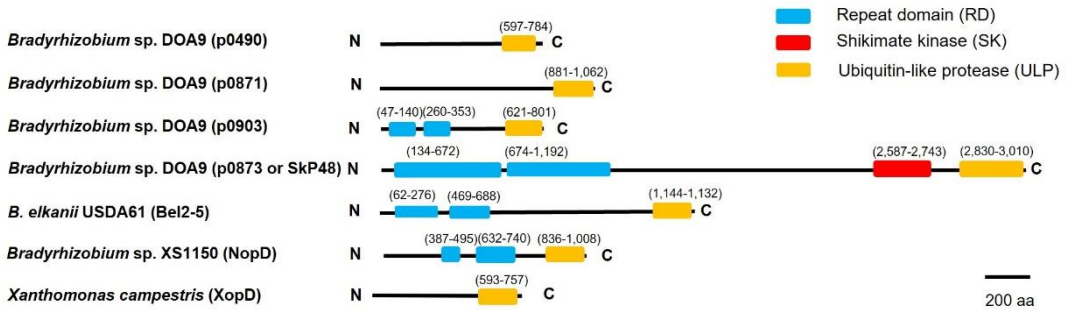

Figure 4.2 The structure organization of four-effector proteases containing the small ubiquitin-like modifier (SUMO) protease domain in DOA9 compared with other putative SUMO-proteases among *Bradyrhizobium* species and XopD from pathogenic bacteria *Xanthomonas campestris*. The domain organization includes the repeat domain (blue box), shikimate kinase domain (red box), and ubiquitin-like protease domain (orange box) are shown.

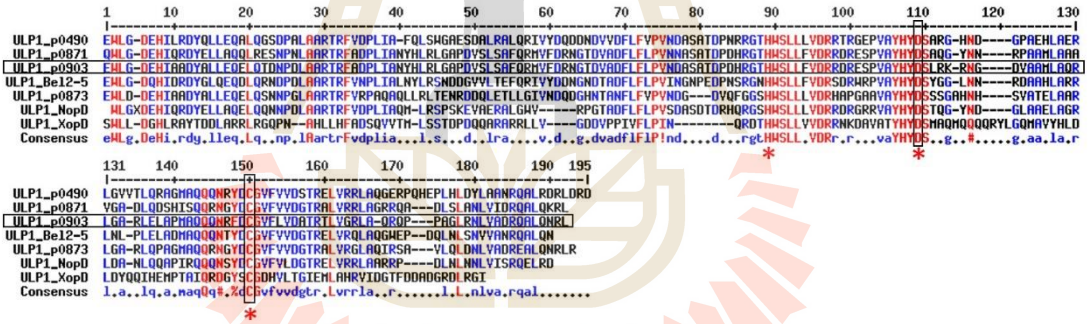

Figure 4.3 Alignment of ULP domains of four putative SUMO-protease T3Es from DOA9, Bel-2 from USDA61, NopD from XS1150, and XopD from *Xanthomonas campestris*. The catalytic core residues histidine (H), aspartic acid (D), and cysteine (C) are marked with red stars. The ULP domain and the two catalytic core amino acids, aspartic acid (D) and cysteine (C), highlighted in black blocks, were used to construct the mutants Δ*p0903+p0903*-ULP-lack, Δ*p0903+p0903*-D728A, and Δ*p0903+p0903*-C763A of SUMO-protease p0903, respectively.

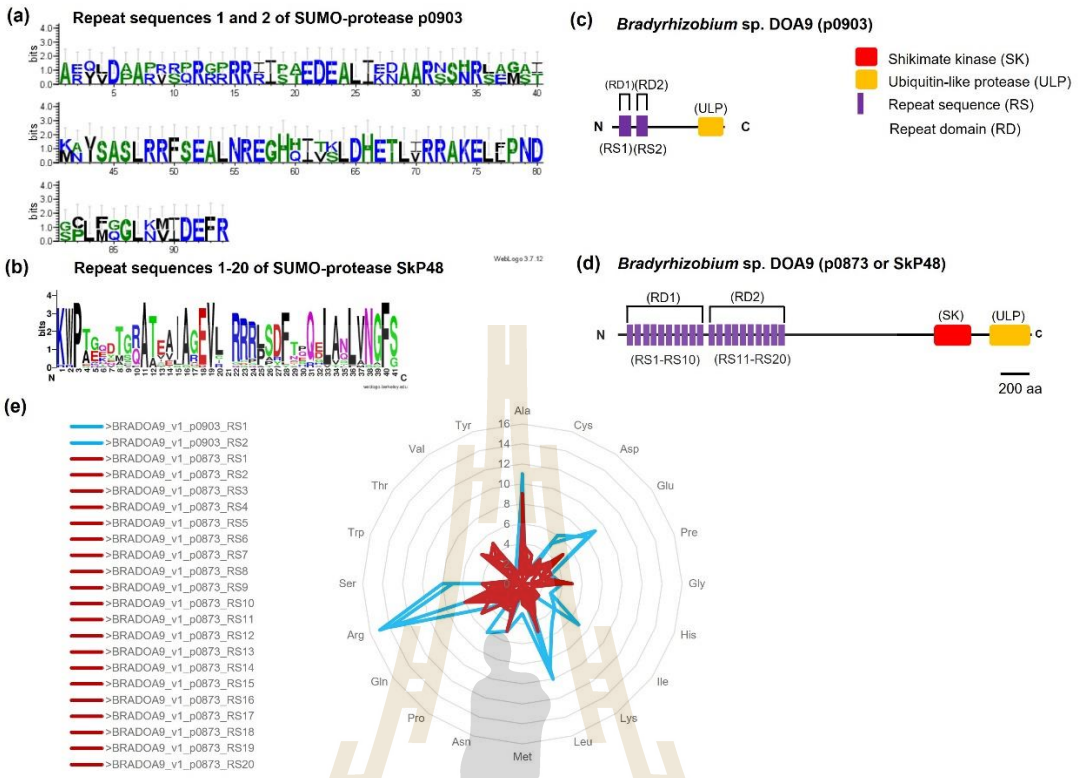

Figure 4.4 The analysis of repeat sequence (RSs) in repeat domains (RDs) and the domain structure organization of SUMO-protease p0903 and SUMO-protease (SkP48 or p0873) from DOA9. Weblogo plots illustrating the information content, measured in bits, for each position in the amino acid sequences of the alignment: (A) the plots show 2 RSs in two RDs of SUMO-protease p0903, and (B) 20 RSs in two RDs of SUMO-protease (p0873 or SkP48) from DOA9.The information content represents deviation from randomness. High bit scores, indicating conserved amino acids, are shown in large letters, while low bit scores, indicating less conserved amino acids, are shown in small letters. (C, D) The structural organization of the SUMO-protease p0903 and SUMO-protease (p0873 or SkP48) domains in DOA9. The domain organization includes the repeat domain (RD), shikimate kinase (red box), ULP domain (orange box), and repeat sequence (purple box). (E) Radar graph depicting the free amino acid profiles of 2 RSs in repeat domain 1 (RD1) and repeat domain 2 (RD2) of p0903, as well as the 20 RSs in RD1 and RD2 of p0873 or SkP48

from DOA9. The blue lines represent RS1 and RS2 of SUMO-protease p0903, while the red lines show the 20 RSs of SUMO-protease SkP48.

SUMOs constitute a class of small proteins used by plant cells to selectively modify various substrate proteins (Berndsen & Wolberger, 2014; Morrell & Sadanandom, 2019). Furthermore, SUMO proteases, such as the XopD effector in *X. campestris* and other plant pathogens, are universally conserved (Kim et al., 2011). Notably, the T3E proteases of *Bradyrhizobium* had more abundant SUMO proteases (ULP domain) in the C-terminus domain, along with greater diversity of SUMO-proteases in *Bradyrhizobium* strains isolated from various hosts (Camuel et al., 2023; Ratu, Teulet, et al., 2021). Therefore, the SUMO proteases of *Bradyrhizobium* evolved alongside the SUMO proteases of plant pathogens, which try to colonize the plant host. NopD specifically recognizes plant SUMO proteins in *G. max* and *P. vulgaris*. Furthermore, the NopD protease from strain XS1150 functions as a symbiosis-related protein, with the ability to process specific SUMO proteins and desumoylate SUMO-conjugated proteins (Xiang et al., 2020). Our results demonstrate that the SUMO-proteases in *Rhizobium* isolated from legume crops are diversified into three distinct clades (**Fig. 4.1**). Clades one and two include SUMO-proteases from *Rhizobium* isolated from multiple hosts, while clade three consists solely of isolates from *G. max*. These findings suggest that the specificity of SUMO-proteases might play a role in determining host specificity among legume species.

In DOA9, four putative SUMO-protease T3Es, p0490, p0871, SkP48, and p0903, were identified. Two of them were separated into distinct subclades of clade 1 and presented low similarity (**Fig. 4.1**). Among these proteases, four T3E-SUMO proteases share only a ULP domain in their C-terminal (**Fig. 4.2** and **Fig. 4.3**). Interestingly, SkP48 and p0903 contain two distinct repeat domains (RDs) in their N-terminal regions (**Fig. 4.4**). Although this region lacks a functional domain, it modulates cellular function exclusively in the nucleus, likely by binding to nuclear proteins via RDs (Ratu et al., 2021a). These findings suggest that the different RDs of p0903 and SkP48 might bind to different positions on the same nuclear substrate proteins or to different nuclear substrate proteins. Moreover, the ULP domain might facilitate interaction with the host target through its deSUMOylation activity. Additionally, determining the specific

RDs of p0903 and SkP48 that bind with nuclear substrate proteins in peanuts is a promising area for future research.

4.2 The SUMO-protease plays a role in peanut symbiosis efficiency

To gain a better understanding of the role of these four putative SUMO proteases during the symbiosis between DOA9 and peanuts, four deletion mutants were constructed via complete gene deletion. All derivative mutant strains were tested on the peanut KK5 cultivar (a commercial shelled peanut that is resistant to necrosis disease (Riabroy et al., 2022)) compared with the DOA9 strain and a mutant affected by T3SS machinery secretion (-Ω*rhcN*). Interestingly, It was observed that two mutants, DOA9-Δ*p0490* and DOA9-Δ*p0871*, behaved as the DOA9 strain at 30 dpi. They induced a small number of nodules that displayed low nitrogenase activity, and no plant growth benefit was observed (**Fig. 4.5A-C**). Furthermore, cytological analysis of the nodules revealed that the infection zone derived from the DOA9, DOA9-Δ*p0490* and DOA9-Δ*p0871* nodules was enriched with dead bacteria (coloured red with PI) (**Fig. 5D**). In contrast, the other two mutants, DOA9-Δ*SkP48* and DOA9-Δ*p0903*, behave as DOA9-Ω*rhcN*, which increases the nodule number, ARA activity, and plant dry weight (**Fig. 4.5A-C**). Furthermore, cytological analysis revealed that the infection zone of the nodules is predominantly occupied by living bacteria, which are stained green with SYTO9 (**Fig. 4.5D**).

To investigate whether the T3SS in a *Bradyrhizobium* strain originally isolated from *A. hypogaea* plays a role during symbiosis, a T3SS mutant (-Ω*rhcN*) of the type strain *B. arachidis* CCBAU 051107 (also named LMG26795) was also constructed and tested on the peanut KK5 cultivar. Observations at 30 dpi revealed that, contrary to what was observed with DOA9, WT-LMG26795 induced efficient nitrogen-fixing nodules, with no apparent effect of the T3SS mutation (**Fig. 4.5A-D**). This result suggests that the T3SS of DOA9 negatively affects nodulation in peanut KK5, while the T3SS of LMG26795 plays little to no role in its interaction with peanut KK5. Interestingly, while LMG26795 lacks putative T3Es encoding the SUMO-protease, it still promotes nodulation in peanut. In contrast, DOA9 could potentially be used as an inoculant for peanut crops if at least one of its incompatible T3Es (SkP48 or p0903) is deleted. These findings highlight the complex and strain-specific roles of

SUMO-protease T3Es in peanut nodulation, with SkP48 and p0903 potentially acting as key modulators of the symbiotic process in certain *Bradyrhizobium* strains.

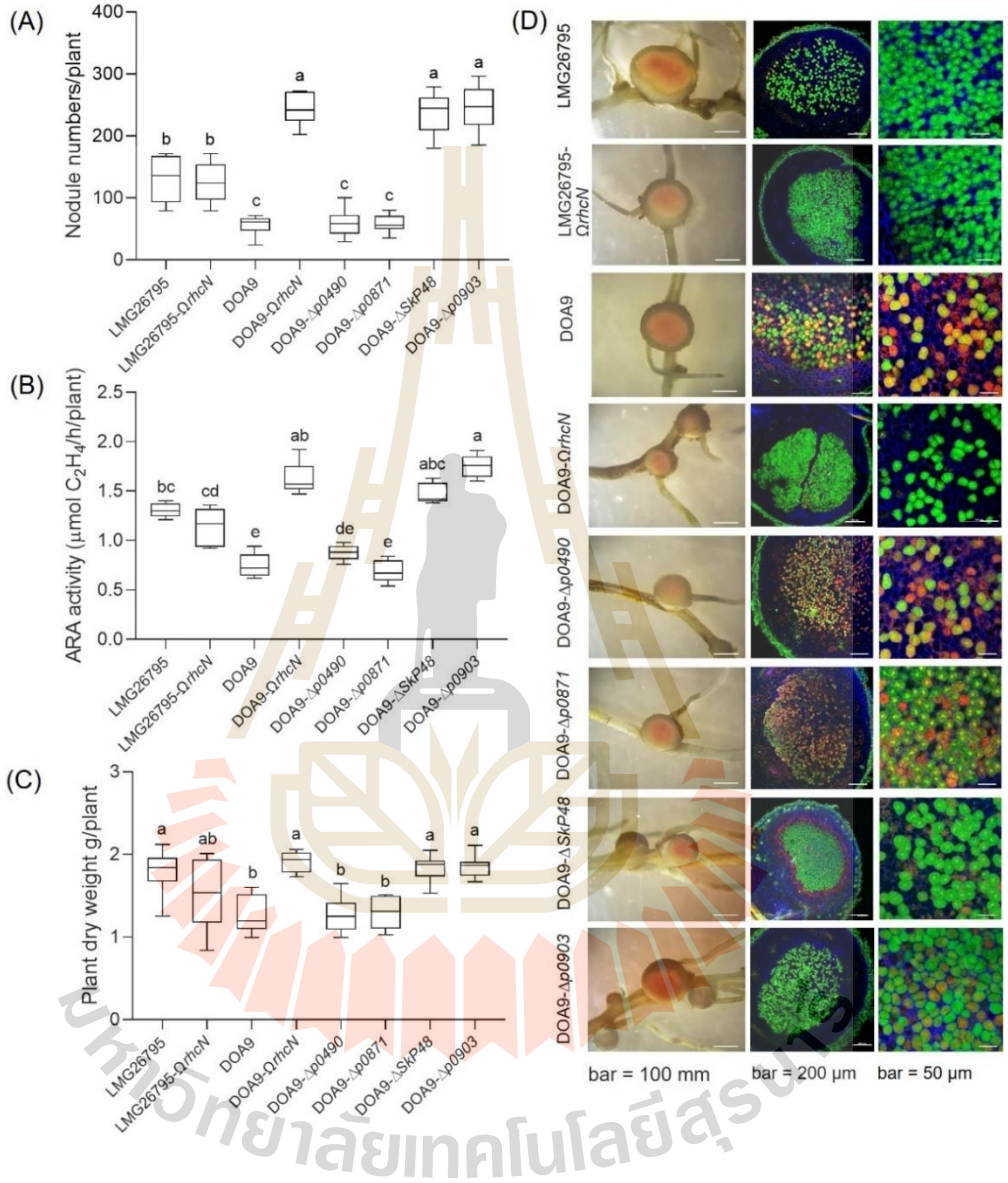

Figure 4.5 The symbiotic nodulation of *Bradyrhizobium* sp. DOA9 and its derivative mutants (DOA9-Ω*rhcN*, DOA9-Δ*p0490*, DOA9-Δ*p0871*, DOA9-Δ*p0903*, and DOA9-Δ*SkP48*), as well as *Bradyrhizobium* sp. LMG26795 and its T3SS mutant (LMG26795-Ω*rhcN*) derivative in peanut (KK5). The assessment at 30 dpi included (A) nodule number per plant, (B) ARA activity, and (C) plant dry weight. The means followed by different letters are significantly different at the 0.1% level ($P \leq 0.001$, according to Tukey's test), with a

sample size of n = 10, except for ARA activity, where n = 5. (D) Nodulation in peanut (KK5) manifested as nodules morphology (scale bar: 100 mm), and the nodule sections were stained with SYTO9 (living bacteroid cells: green), propidium iodide (dead bacteroid cells: red), and calcofluor (plant cell wall: blue); scale bar for confocal image: 200 and 50 μm.

In the process of nodulation, peanuts utilize NF-dependent mechanisms for nodulation (Guha et al., 2022). The T3SSs of *Bradyrhizobium* sp. DOA9 and *Bradyrhizobium* sp. MM6 play a negative role in symbiosis with peanuts (Lai et al., 2020; Songwattana et al., 2017). Our results also demonstrated that the T3SS of DOA9 suppresses nodulation in the peanut KK5 cultivar (**Fig. 4.5A-D**). In contrast, the T3SS of *B. arachidis* LMG26795, a peanut-nodulating strain, does not significantly impact nodulation in the peanut KK5 cultivar. Similarly, for *B. zhanjiangense* CCBAU51778, a peanut-nodulating strain, the T3SS is not important for nodulation (Shang et al., 2022a). The results suggest that the T3SS of bradyrhizobia might play either a negative role or has no effect on peanut symbiosis with the KK5 peanut cultivar, depending on the host specificity of the bradyrhizobia strains.

Furthermore, SUMO-proteases SkP48 and p0903 restricted nodulation in peanuts (KK5), whereas SUMO-proteases p0490 and p0871 had no effect on peanut nodulation (**Fig. 4.5A-D**). These findings suggest that SUMO-proteases p0903 and SkP48 might trigger the plant immune response, potentially hindering symbiosis and suppressing nodulation in peanut KK5. Understanding how these two T3Es interact to interfere with the plant immune system presents an intriguing question for future research. In particular, investigating whether these two SUMO-proteases target the same molecules within the plant would be valuable. In several instances, the SUMO-protease T3Es in *Bradyrhizobium* have been shown to restrict nodulation in various legumes. For example, NopD of *Bradyrhizobium* sp. XS1150 suppressed nodulation in *T. vogelii*, and Bel2-5 of *B. elkanii* USDA61 restricted nodulation in incompatible BARC-2 (*Rj4/Rj4*) soybeans (Faruque et al., 2015; Xiang et al., 2020b). Conversely, effectors such as Sup3 from *Bradyrhizobium* sp. WSM1744, *Bradyrhizobium* sp. ORS86, and *Bradyrhizobium* sp. NAS96.2 promoted nodulation in *A. indica* (Camuel et al., 2023). Bel2-5 of USDA61 also induced nodulation in the Nod factor receptor 1

soybean mutant (*nfr1*) (Ratu, Hirata, et al., 2021a). Furthermore, several strains of bradyrhizobia isolated from peanuts (*B. zhanjiangense* CCBAU51778 and *B. zhanjiangense* CCBAU51781) contained T3Es with ULP domains (**Fig. 4.1**). In contrast, other strains (*B. arachidis* LMG26795 and *Bradyrhizobium* sp. SEMIA6144) isolated from peanuts lacked T3Es containing a ULP domain. Notably, these latter strains are considered good inoculants for increasing peanut yield (Y. H. Li et al., 2019; Vicario et al., 2016). Therefore, selecting compatible T3E SUMO-proteases in *Bradyrhizobium* for peanut inoculant production is crucial to promote plant growth.

4.3 SUMO-protease p0903 plays a role in the symbiosis of various peanut cultivars

Considering that SkP48 was previously investigated in more detail (Piromyou, Songwattana, et al., 2021), in this study, the SUMO-protease p0903 was focused. To further investigate the role of SUMO-protease p0903 T3E in regulating symbiosis among different cultivars of peanut, two mutants of p0903 were inoculated into the peanut cultivars TN9, KS2, and SK38. A nodulation test was then performed to compare the results with those of the KK5 cultivar to determine whether p0903 affects peanut cultivars. As previously observed for the KK5 cultivar, at 30 dpi, DOA9-Δ*p0903* had an increased nodule number per plant in the 3 cultivars tested, which was equivalent to that of DOA9-Ω*rhcN* (**Fig. 4.6B-4.9B**). In addition to a greater number of nodules, a gain in symbiotic efficiency (bacterial viability in the nodules, nitrogenase activity, and plant growth) was observed in the plants inoculated with the two DOA9-Δ*p0903* and DOA9-Ω*rhcN* mutants regardless of the peanut cultivars tested (**Fig. 4.6A, C, D** to **Fig. 4.9A, C, D**, respectively). Interestingly, the complementation strain Δ*p0903+p0903* exhibited nodulation patterns similar to the DOA9 strain across four peanut cultivars (**Fig. 4a-d**). Specifically, it led to a reduction in nodule number, nitrogenase activity, and bacterial viability within nodules (with the exception of TN9 cultivar), as well as overall plant growth. These effects were comparable to those observed in plants inoculated with the DOA9 strain, consistently across all tested peanut cultivars (**Fig. 4.6A, C, D** to **Fig. 4.9A, C, D**, respectively). This restoration of DOA9-like phenotypes in the complementation strain provides strong

evidence for the specific role of p0903 in regulating symbiotic interactions. Taken together, these results indicate that SUMO-protease p0903 T3E induces significant negative effects (less nodules number, weaker nitrogen fixation, and less plant growth benefit) associated with the symbiotic interaction of the DOA9 strain with the 4 peanut cultivars tested. Additionally, these results suggest that SUMO-protease p0903 might interact with homologous genes in peanut cultivars to affect symbiotic nodulation.

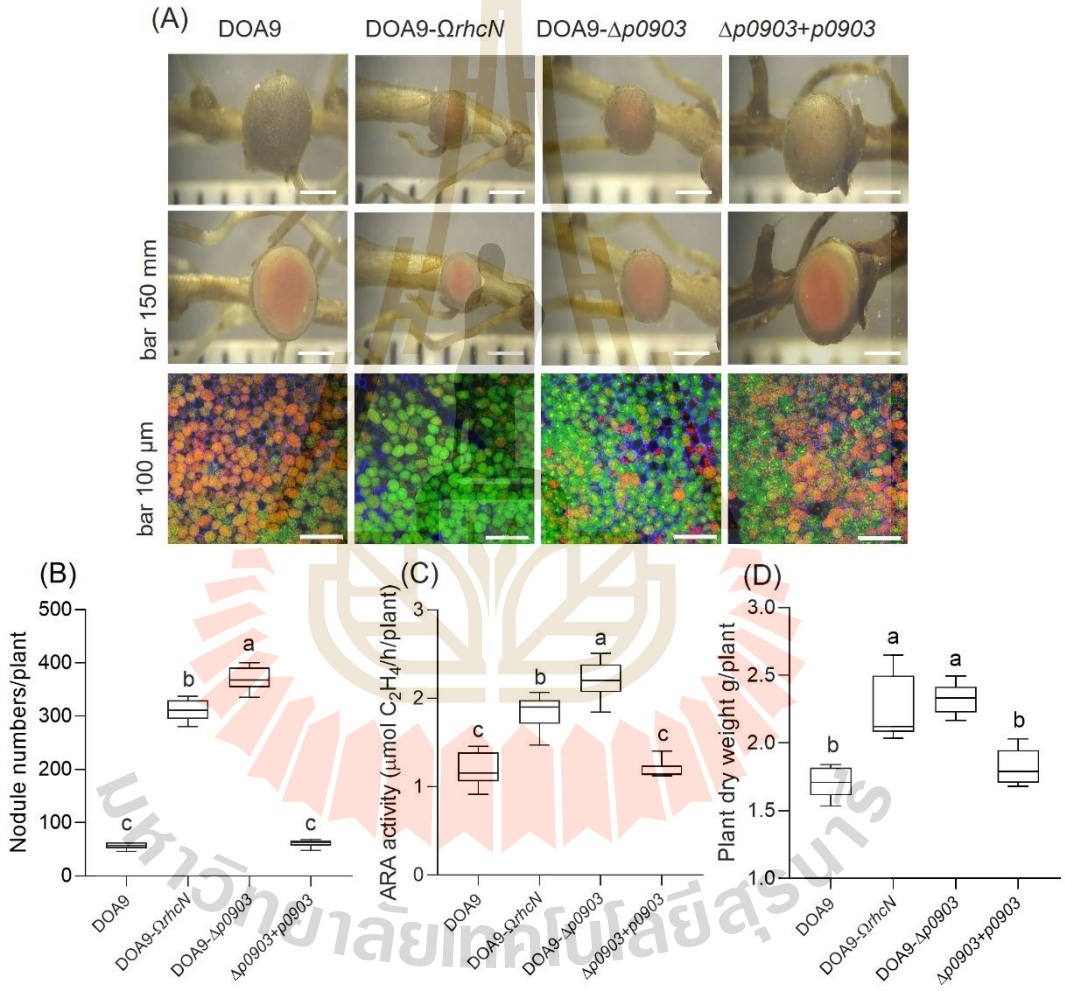

Figure 4.6 Symbiotic phenotype of *Bradyrhizobium* sp. DOA9 and its derivative mutants, including T3SS structural mutation (DOA9-Ω*rhcN*), SUMO-protease *p0903* deletion (DOA9+Δ*p0903*), and *p0903* complementation (Δ*p0903+p0903*) in *A. hypogaea* KK5 at 30 dpi. (A) Nodule phenotype and cytological analysis of nodules by confocal microscopy. The nodule sections were stained with SYTO9 (live bacteroid cells: green), propidium

iodide (dead bacteroid cells: red), and calcofluor (plant cell wall: blue); scale bar for nodule sections: 150 mm, and for confocal images: 100 µm. (B) Nodule numbers per plant were counted. (C) Acetylene reduction activity (ARA) was measured in peanut roots. (D) Plant dry weight was measured. The experiment was carried out with 6 plants per treatment. Values with different letters indicate significant differences at the 5% level ($P \leq 0.05$) as determined by Tukey's test.

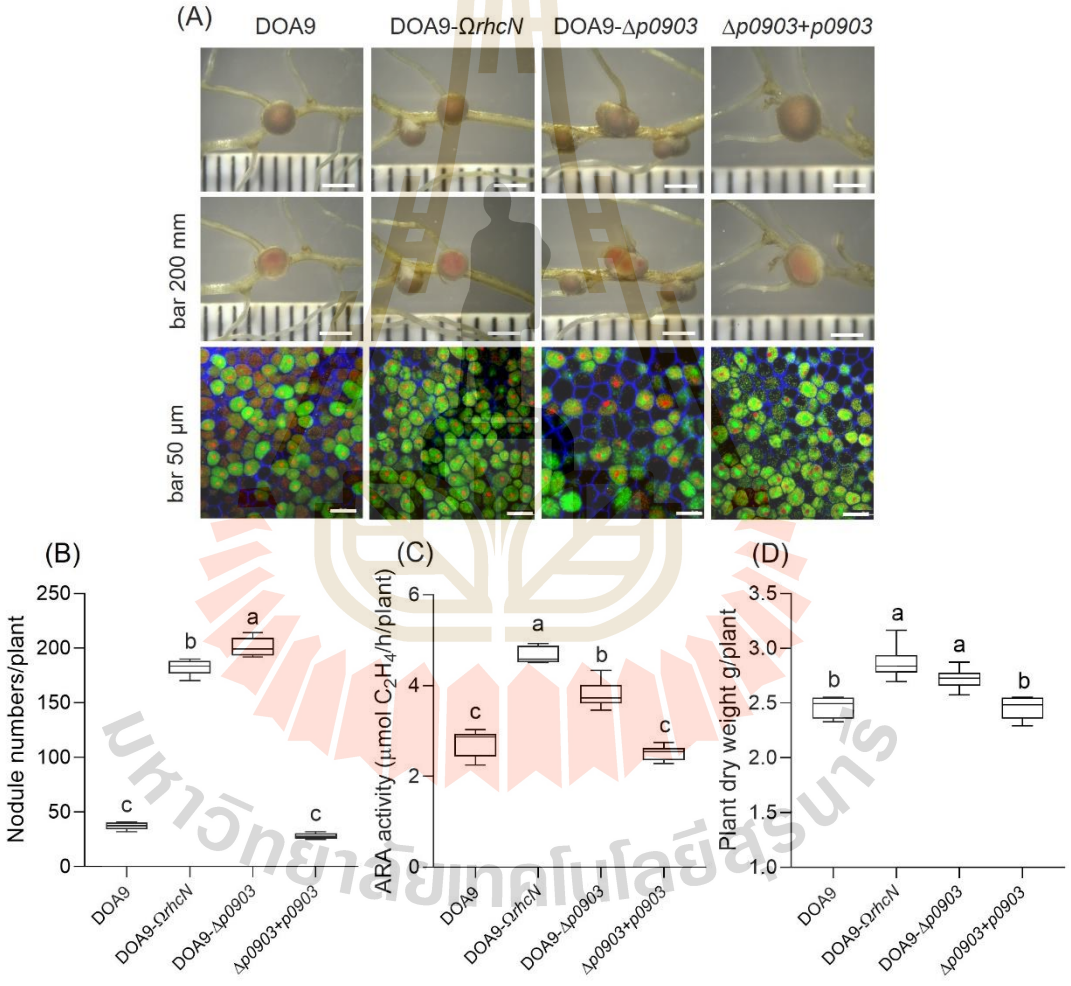

Figure 4.7 Symbiotic phenotype of *Bradyrhizobium* sp. DOA9 and its derivative mutants, including T3SS structural mutation (DOA9-Ω*rhcN*), SUMO-protease *p0903* deletion (DOA9+Δ*p0903*), and *p0903* complementation (Δ*p0903+p0903*) in *A. hypogaea* TN9 at 30 dpi. (A) Nodule phenotype and cytological analysis of nodules by confocal microscopy. The nodule

sections were stained with SYTO9 (live bacteroid cells: green), propidium iodide (dead bacteroid cells: red), and calcofluor (plant cell wall: blue); scale bar for nodule sections: 200 mm, and for confocal images: 50 µm. (B) Nodule numbers per plant were counted. (C) Acetylene reduction activity (ARA) was measured in peanut roots. (D) Plant dry weight was measured. The experiment was carried out with 6 plants per treatment. Values with different letters indicate significant differences at the 5% level ($P \leq 0.05$) as determined by Tukey's test.

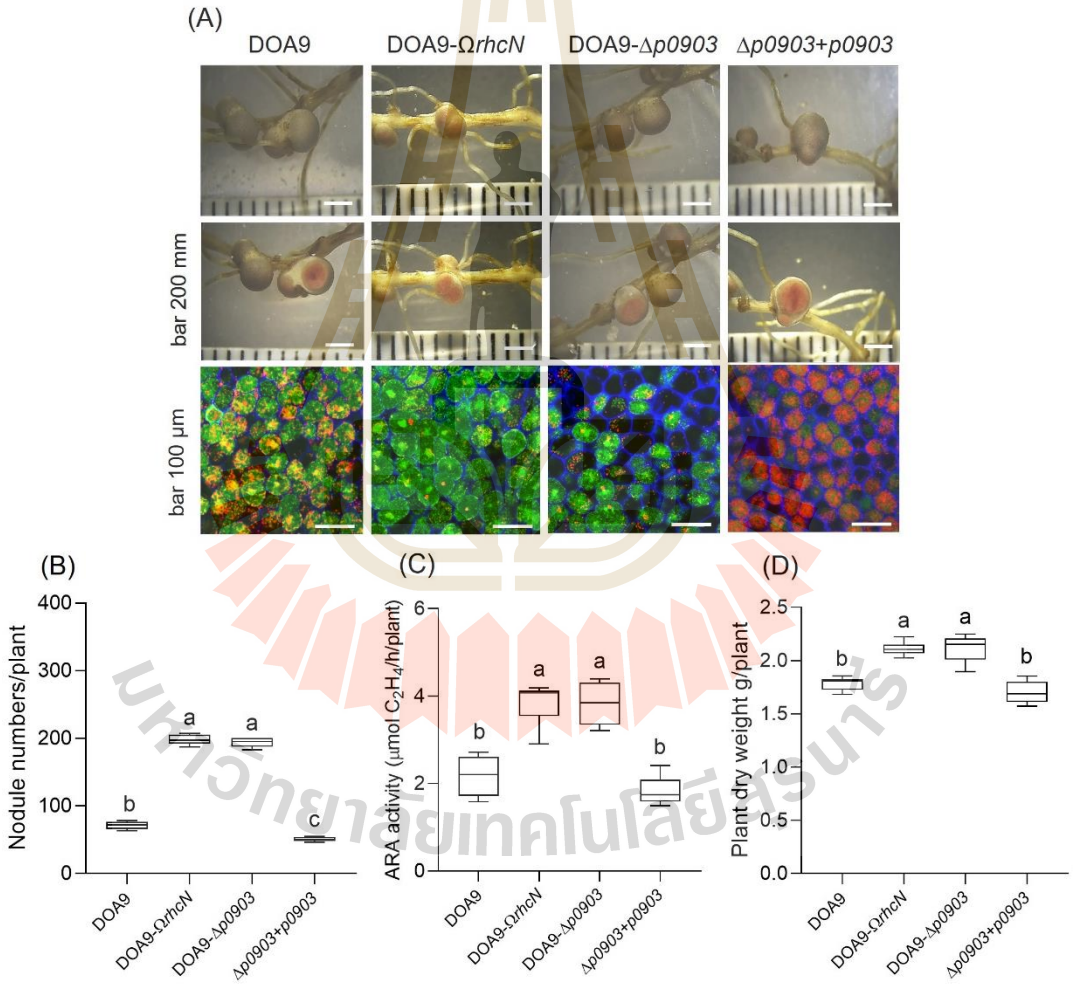

Figure 4.8 Symbiotic phenotype of *Bradyrhizobium* sp. DOA9 and its derivative mutants, including T3SS structural mutation (DOA9-Ω*rhcN*), SUMO-protease *p0903* deletion (DOA9+Δ*p0903*), and *p0903* complementation (Δ*p0903+p0903*) in *A. hypogaea* SK2 at 30 dpi. (A) Nodule phenotype

and cytological analysis of nodules by confocal microscopy. The nodule sections were stained with SYTO9 (live bacteroid cells: green), propidium iodide (dead bacteroid cells: red), and calcofluor (plant cell wall: blue); scale bar for nodule sections: 200 mm, and for confocal images: 100 µm. (B) Nodule numbers per plant were counted. (C) Acetylene reduction activity (ARA) was measured in peanut roots. (D) Plant dry weight was measured. The experiment was carried out with 6 plants per treatment. Values with different letters indicate significant differences at the 5% level ($P \leq 0.05$) as determined by Tukey's test.

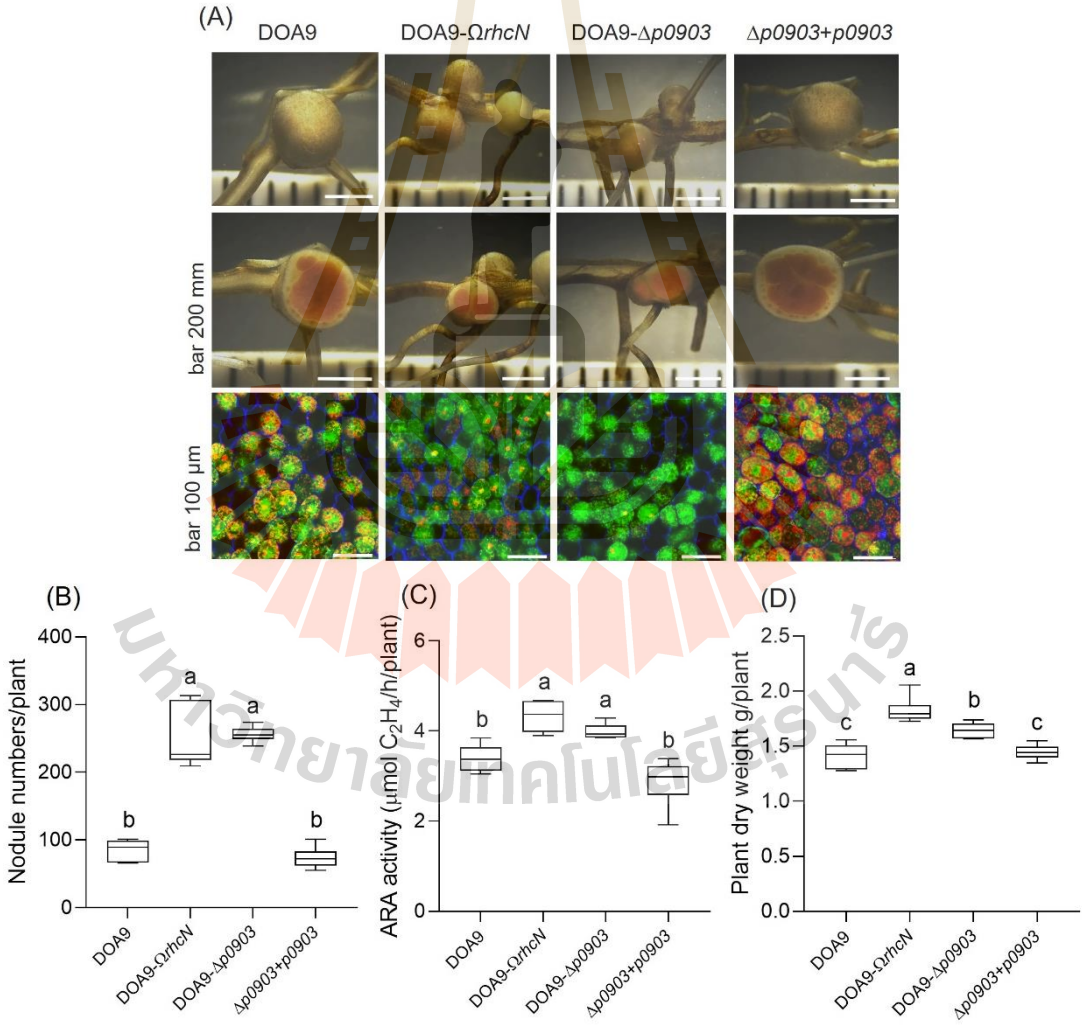

Figure 4.9 Symbiotic phenotype of *Bradyrhizobium* sp. DOA9 and its derivative mutants, including T3SS structural mutation (DOA9-Ω*rhcN*), SUMO-

protease *p0903* deletion (DOA9+Δ*p0903*), and *p0903* complementation (Δ*p0903+p0903*) in *A. hypogaea* SK38 at 30 dpi. (A) Nodule phenotype and cytological analysis of nodules by confocal microscopy. The nodule sections were stained with SYTO9 (live bacteroid cells: green), propidium iodide (dead bacteroid cells: red), and calcofluor (plant cell wall: blue); scale bar for nodule sections: 200 mm, and for confocal images: 100 µm. (B) Nodule numbers per plant were counted. (C) Acetylene reduction activity (ARA) was measured in peanut roots. (D) Plant dry weight was measured. The experiment was carried out with 6 plants per treatment. Values with different letters indicate significant differences at the 5% level ($P \leq 0.05$) as determined by Tukey's test.

In addition to Nod factors, rhizobia secrete other molecular signals, such as T3Es, in response to flavonoid compounds released by plant roots (Bolzan De Campos et al., 2011). Previous reports have indicated that root exudates from the *A. hypogaea* L. cultivar Tegua contain various flavonoids, such as genistein, daidzein, chrysin, and naringenin (Cesari et al., 2019; Taurian et al., 2008). To investigate the expression of the SUMO-*p0903* gene in DOA9 and *rhcN* gene in LMG26795 under flavonoid induction, pure flavonoids representative of those in peanut root exudates, including control (non-induced), genistein and naringenin, were used (**Fig. 4.10A**). Compared to the control, the expression of the *p0903* gene was upregulated 3.59-fold when induced with naringenin, but showed no significant difference when induced with genistein. These results indicate that naringenin stimulates the expression of the *p0903* gene, while genistein does not have this effect. The expression level of T3SS (*rhcN*) in LMG26795 was significantly higher in response to genistein and naringenin compared to the control (**Fig. 10B**). This result suggests that other T3Es in LMG26795 might also respond to the flavonoids genistein and naringenin, contributing to the upregulation of *rhcN* expression.

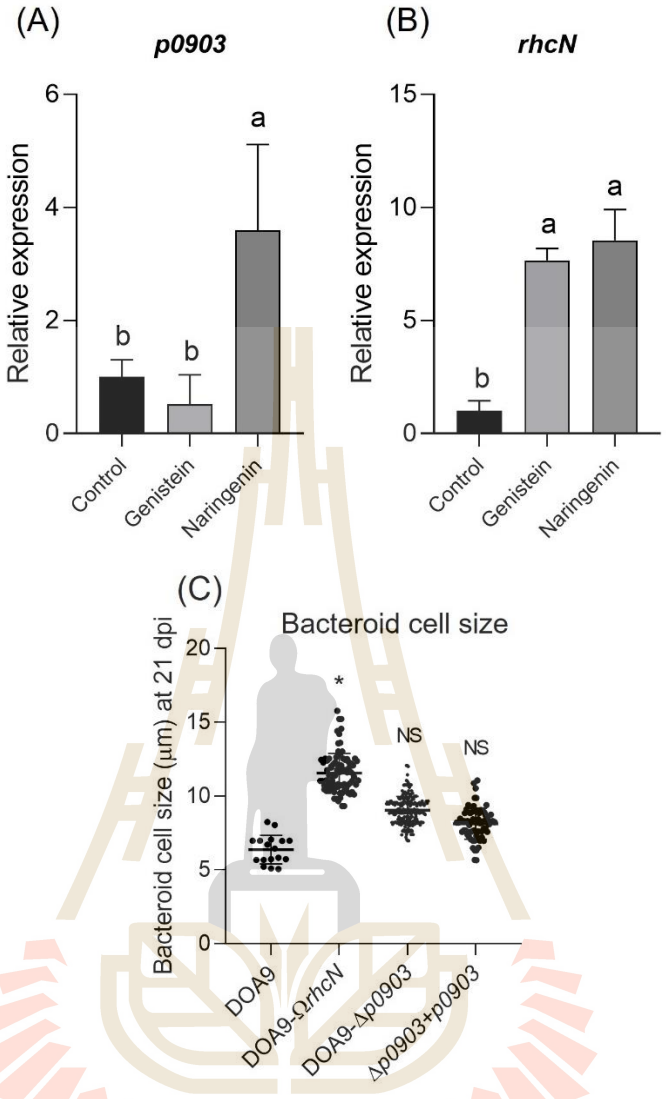

Figure 4.10 The qRT-PCR results of gene expression induced by flavonoids and the size of bacteroid cells in peanut roots (KK5). (A, B) The relative expression of the *p0903* gene in DOA9 and the *rhcN* gene in LMG26795, induced by genistein and naringenin for 24 h, compared to non-induced cells (DOA9 and LMG26795, respectively). Statistical significance was determined at the 1% level ($P \leq 0.01$) using Tukey's test (N=3), with means denoted by different letters indicating significant differences. (C) Bacteroid size in peanut (KK5) nodules after inoculation with DOA9 and its mutants (DOA9-Ω*rhcN*, DOA9-Δ*p0903*, and Δ*p0903+p0903*) at 21 dpi. Statistical significance was assessed at the 5% level ($P \leq 0.05$) using Tukey's test, where "*" indicates significant differences and "NS" indicates not significant (N=3).

Naringenin, a flavonoid found in legumes, plays a significant role in the symbiotic relationship between rhizobia and legume plants (Bosse et al., 2021). In addition, naringenin is known to activate nodulation genes in rhizobia by binding to the *NodD* transcriptional activator. This binding triggers the expression of *nod* genes, leading to the production of Nod factors that initiate the formation of root nodules where nitrogen fixation occurs (Hungria et al., 1991; Li et al., 2008; Yeh et al., 2002). Naringenin has been reported to be expressed in peanut roots (Wang et al., 2023). Furthermore, under water deficit conditions, naringenin present in the root exudates of peanuts enhances bacterial mobility, chemotaxis, and adhesion to the roots (Cesari et al., 2019). Interestingly, the expression level of the *rhcN* gene in DOA9 is induced by naringenin and peanut root exudates (Songwattana et al., 2017). Additionally, flavonoids stimulate the expression of several T3Es in *Bradyrhizobium* sp. (Diepold & Wagner, 2014; Lee et al., 2011). Our findings indicate that naringenin induces the expression of the putative T3E-*p0903* in DOA9, whereas genistein is less important (**Fig. 4.10A**). Furthermore, genistein and naringenin induced the expression of *rhcN* in LMG26795. These findings suggest that the upregulation of *rhcN* might also trigger the expression of other T3Es in this strain (**Fig. 4.10B**). LMG26795 contains several T3Es, including NopP, NopL, and NopT, but lacks of SUMO-protease T3Es. Previous studies have shown that genistein and naringenin can induce multiple T3Es in rhizobia (D. Liu et al., 2021; Ratu, Teulet, et al., 2021). Therefore, our findings suggest that while genistein and naringenin stimulate the expression of T3SS (*rhcN*) in LMG26795, they do not promote symbiosis with peanuts.

In certain legume species within the Dalbergioids, the differentiation of rhizobia into intracellular nitrogen-fixing bacteroids is terminal and characterized by significant cell enlargement, genome endoreduplication, and a marked loss of viability (Lamouche et al., 2019). To investigate the role of SUMO-protease p0903 in relation to bacteroid cell size in peanut KK5 nodules, DOA9 and its derivative mutants (DOA9-Ω*rhcN*, DOA9-Δ*p0903*, and Δ*p0903+p0903*) were inoculated, and bacteroid cell sizes were measured. At 21 dpi, the DOA9-Ω*rhcN*, DOA9-Δ*p0903*, and Δ*p0903+p0903* mutants showed a 1.81, 1.41, and 1.27-fold increase, respectively, in bacteroid differentiation within the nodules compared to the DOA9, which had a cell size of 6.37 μm (**Fig. 4.10C**). Additionally, DOA9-Ω*rhcN* induced a significantly different

bacteroid cell size compared to the DOA9 and the *p0903*-complemented strain, while DOA9-Δ*p0903* triggered a larger bacteroid cell size, though not significantly different from the WT and the p0903-complemented strain. This suggests that other T3Es in DOA9 might work together with p0903 via the T3SS to strongly suppress bacteroid cell differentiation in peanut nodules. Bacteroid differentiation in legume root nodules is regulated by cysteine-rich (NCR) peptides produced by the host plant. Recent studies have shown that NCR-like peptides are part of the host's repertoire, playing a crucial role in determining the shape and extent of bacteroid differentiation. This terminal differentiation is associated with the inhibition of bacterial cell division machinery and significant changes in the regulation of the bacterial cell cycle (Haag & Mergaert, 2020). These results suggest that SUMO-protease p0903 might slightly suppress bacteroid cell enlargement, while other T3Es in DOA9 could be key regulators of this process in peanut nodules.

Overall, SUMO-protease p0903 appears to restrict nodulation in peanut cultivars (KK5, TN9, KS2, and SK38), affecting nodule numbers, nitrogen fixation, plant growth, and bacterial cell viability.

4.4 The negative symbiotic role of SUMO-protease p0903 is not played only by its ULP domain

The SUMO-protease activity of Bel2-5 from USDA61 and NopD from XS1150 has previously been shown to be important in compromising the symbiotic activity of these two strains on the soybean cultivar BARC-2 (*Rj4/Rj4*) and *Tephrosia vogelli*, respectively (Ratu et al., 2021a; Xiang et al., 2020). To investigate whether this is also the case for SUMO-protease p0903 during the symbiotic interaction of DOA9 with *A. hypogaea* KK5, we introduced *p0903* with its promoter into DOA9-Δ*p0903*. Moreover, the enzymatically inactive variant forms of *p0903*, including two mutants in the catalytic enzymatic core *p0903*-D728A and *p0903*-C763A and the deletion of the complete C-Ter ULP domain, were constructed and independently introduced into DOA9-Δ*p0903* (**Fig. 4.11A**). As shown in **Fig. 4.11B**, at 30 dpi, the ULP deletion mutant (Δ*p0903+p0903*-ULP-lack) and the two point mutation mutants (Δ*p0903+p0903*-D728A and Δ*p0903+p0903*-C763A) presented an intermediate phenotype in terms of the

number of nodules compared with DOA9-Δ*p0903* and DOA9-Δ*p0903* complemented with the wild-type form of *p0903* (Δ*p0903+p0903*). However, for the other symbiotic parameters examined, such as chlorophyll content, plant biomass, and ARA nitrogenase activity, no significant differences were detected between these three mutants complemented with mutated forms of ULP and either the DOA9 strain or the DOA9-Δ*p0903* mutant complemented with the wild-type form of *p0903* (**Fig. 4.11C-E**). Nonetheless, the viability of infected bacteria in nodules formed by the three mutants complemented with mutated forms of ULP was higher than that observed in nodules induced by either the DOA9 strain or the DOA9-Δ*p0903* mutant complemented with the wild-type *p0903* **(Fig. 4.11F)**. Taken together, these results suggest that the SUMO-protease activity of p0903 contributes to the negative symbiotic effect observed between strains DOA9 and *A. hypogaea* KK5 but does not explain it on its own; other domains of the protein might also play important roles in this incompatibility.

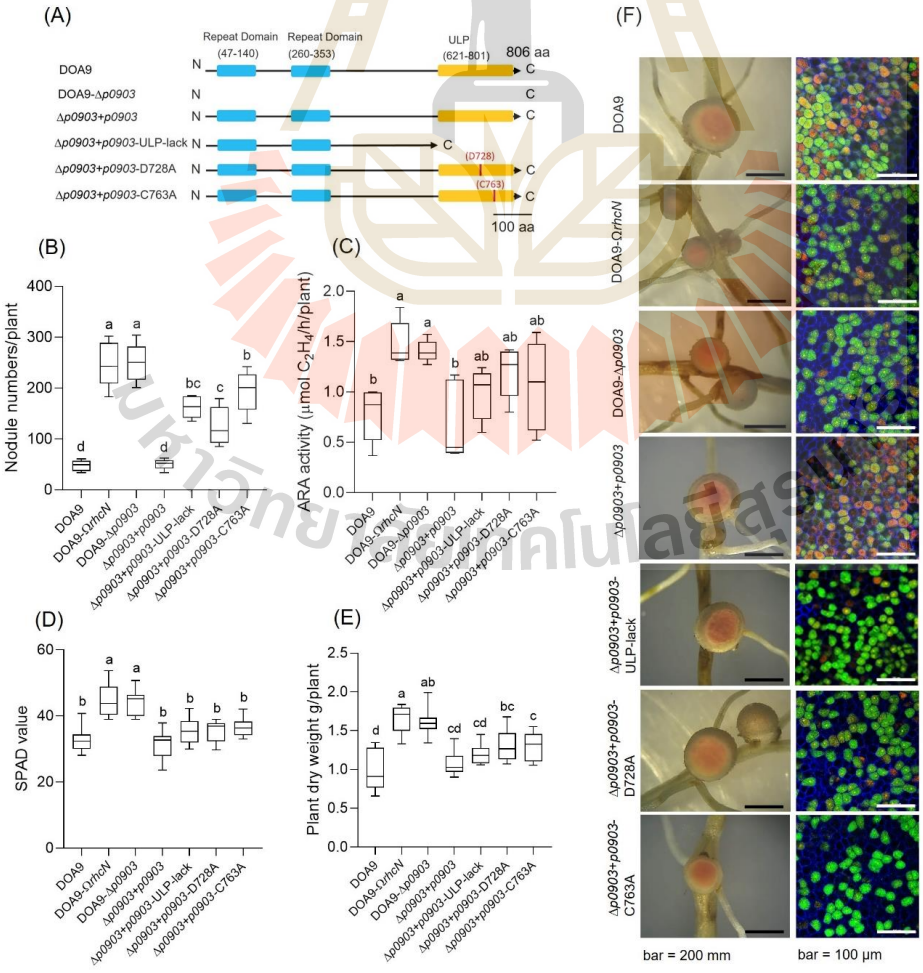

Figure 4.11 Nodulation phenotypes of *Bradyrhizobium* sp. DOA9 and its derivatives (DOA9-Ω*rhcN*, DOA9-Δ*p0903*, Δ*p0903+p0903*, Δ*p0903+p0903*-ULP-lack, Δ*p0903+p0903*-D728A, and Δ*p0903+p0903*-D763A) on the peanut cultivar (KK5). (A) Schematic representation of DOA9 and its mutant derivatives strains. p0903 possesses two internal repeat domains, a ubiquitin-like protease (ULP) domain, and catalytic core amino acids aspartic acid (D728) and cysteine (C763) in ULP domain. Evaluation at 30 dpi included (B) nodule count per plant, (C) ARA activity, (D) chlorophyll content, and (E) plant dry weight. The means indicated by different letters represent significant differences at $P \leq 0.001$ using Tukey's test, with a sample size of n = 10, except for ARA activity, where n = 5. (F) Nodulation in peanut (KK5) manifested as nodule morphology (scale bar: 200 mm). Nodule sections were stained with SYTO9 (living bacteroid cells: green), propidium iodide (dead bacteroid cells: red), and calcofluor (plant cell walls: blue); scale bar for confocal images: 100 µm.

Furthermore, in yeast cells, ULP1 plays a role in the SUMO modification pathway, with dual functions of processing the SUMO precursor into mature SUMO and removing SUMO from its substrate protein (Li & Hochstrasser, 1999). The catalytic amino acid C1268A within the ULP domain of Bel2-5 plays a critical role in suppressing nodulation in soybean BARC-2 (*Rj4/Rj4*) (Ratu et al., 2021a). In addition, NopD-C972A in XS1150 resulted in nodulation suppression similar to that of the full-length NopD gene in *T. vogelii* (Xiang et al., 2020). The SUMO-p0903 proteases on DOA9 contained the H/G/C catalytic triad residues within ULP-like domains, similar to XopD of *X. campestris* (**Fig. 4.3**). In this study, ULP, D728A, and C763A restricted nodule numbers in the peanut cultivar KK5 but not to the same level as the DOA9 (**Fig. 4.11B**). In contrast, they did not induce bacteroid cell death in nodules, similar to DOA9 **(Fig. 4.11F)**. This result suggests that the ULP domain, along with the catalytic cores D728A and C763A in the ULP domain of SUMO-protease p0903, might collaborate with the N-terminal domain to modulate the nodules on peanuts. Moreover, the pathogenic T3E HsvG features tandem repeats of 71 and 75 amino acids, serving as transcriptional activators and influencing host specificity (Nissan et al., 2006). A similar repeat sequence was identified

in *R. solanacearum* T3E RipTAL1, which is known for activating the transcription of host susceptibility genes (De Lange et al., 2013). In addition, the deletion of two RDs in Bel2-5 of USDA61 restricted nodulation to soybean BARC-2 *(Rj4/Rj4)*, similar to the deletion of Bel2-5 (Ratu et al., 2021a). These results suggest that the ULP domain of SUMO-protease p0903, together with the RD domains, might significantly suppress nodulation in peanuts. Therefore, further investigations of the interaction between SUMO-protease p0903 and peanut substrate proteins are warranted.

4.5 Gene expression analysis and jasmonic acid production on peanut root

To investigate the physiological response of plants to SUMO-protease p0903 during symbiosis, the expression of symbiosis-related genes and induced systemic resistance (ISR), which enhances defence against pathogens and microbial symbionts, including jasmonic acid (JA)-related genes, was analysed in plant roots inoculated with DOA9 and DOA9-Δ*p0903*. The expression levels of symbiosis-related genes, including cyclic nucleotide-gated channels (*CNGC*), symbiotic remorins (*SYMREM*), and the E3 ubiquitin-protein ligase LIN-1 (*CERBERUS*), at 3 dpi are depicted in **Figure 4.12A**. These three symbiosis-related genes were downregulated in the DOA9-inoculated plants compared with those in the DOA9-Δ*p0903*-inoculated plants. Furthermore, the expression levels of JA-related genes, including a transcription factor (*Myc2*), pathogenesis-related protein (*PR4*), and defensin (*Def2-2*), are presented in **Figure 4.12B**. The expression levels of *Myc2*, *PR4*, and *Def2-2* were significantly increased in the DOA9-inoculated plants than in the DOA9-Δ*p0903*-inoculated plants. These results suggest that SUMO-protease p0903 in DOA9 might suppress symbiosis-related genes while triggering JA-related genes.

To understand the role of SUMO-protease p0903 in stimulating the accumulation of JA in peanut KK5 roots, the concentration of JA was measured (**Fig. 4.12C**). The treatments included non-inoculated (NI), DOA9, DOA9-Δ*p0903*, and Δ*p0903-p0903*-ULP-lack. At 3 dpi, JA acid production in peanut roots was 2.32-, 1.27-, and 0.64-fold greater in DOA9, DOA9-Δ*p0903*, and Δ*p0903-p0903*-ULP-lack, respectively, than in non-inoculated plants, with a JA concentration of 49.56 µg/g root fresh weight. The results show that the ULP domain induces JA accumulation in peanut roots in a manner similar to that of p0903. These results indicate that SUMO-

protease p0903 plays a role in triggering the production of JA to induce JA-related genes in peanut roots, thus suppressing crucial symbiosis-related genes at the early stages of peanut nodulation with *Bradyrhizobium* sp. DOA9.

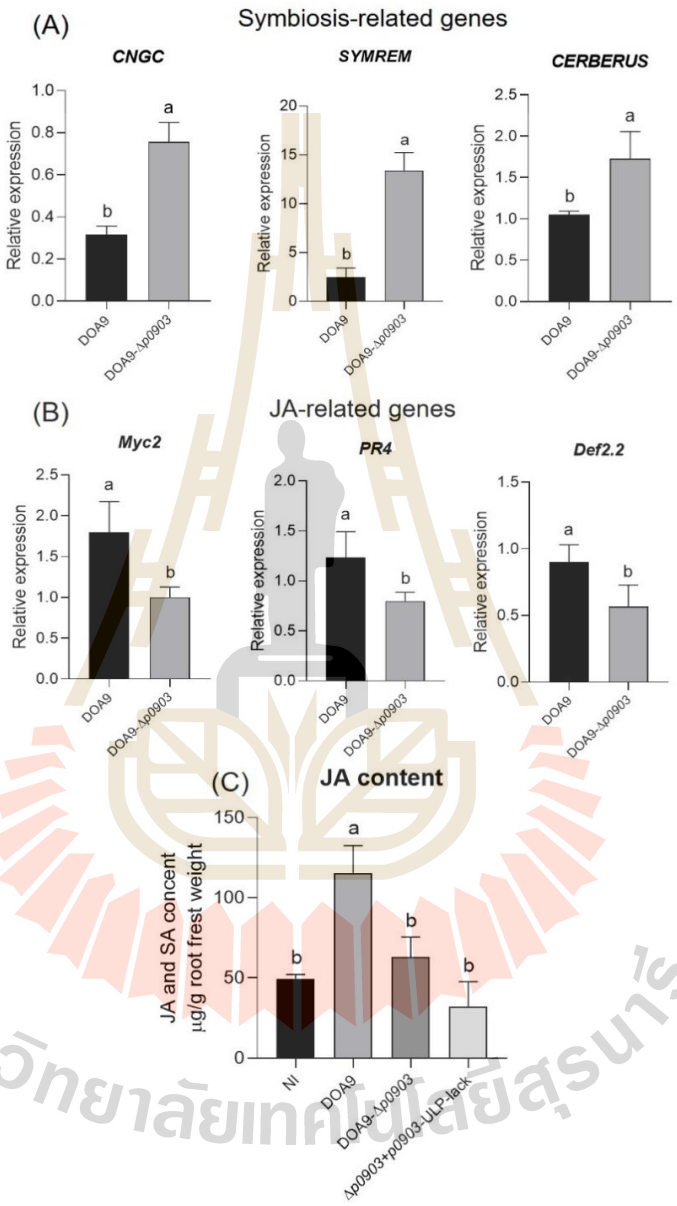

Figure 4.12 qRT-PCR results of gene expression and jasmonic acid levels in peanut roots (KK5). The relative expression levels of genes in peanut roots inoculated with DOA9 and DOA9-Δ*p0903* at 3 dpi are shown for: (A) symbiotic-related genes (cyclic nucleotide-gated channels [*CNGC*], symbiotic remorins [*SYMREM*], and E3 ubiquitin-protein ligase LIN-1

[*CERBERUS*]); (B) jasmonic acid (JA)-related genes (transcription factor [*Myc2*], pathogenesis-related protein 4 [*PR4*], and defensin [*Def2-2*]). The mean differences were considered significant at $P \leq 0.05$ using Student's t-test, with a sample size of n = 3. (C) Jasmonic acid concentrations in peanut roots for the following treatments: Non-Inoculated (NI), DOA9, DOA9-Δ*p0903*, and Δ*p0903+p0903*-ULP-lack at 3 dpi. The mean differences were considered significant at $P \leq 0.01$ using Tukey's test, with a sample size of n = 3. Means indicated by different letters represent significant differences.

Transcriptome analysis of early nodulation between peanut and *Bradyrhizobium* sp. SEMIA6144 revealed the involvement of early symbiosis signalling genes, including cyclic nucleotide-gated channels (*CNGC*), symbiotic remorins (*SYMREM*), and the E3 ubiquitin-protein ligase LIN-1 (*CERBERUS*) (Karmakar et al., 2019). Our study revealed that the expression levels of the early signalling genes *CNGC*, the interactor *SYMREM*, and infection *CERBERUS* were lower in the DOA9 than in DOA9-Δ*p0903* (**Fig. 4.12A**). *CNGC* are regulated by the calcium-bound form of calmodulin 2 (holo-CaM2) to modulate *CNGC* activity and the downstream root nodule symbiosis pathway (del Cerro et al., 2022). Furthermore, *SYMREM* 1 from *Medicago truncatula* interacts with various symbiotic receptor kinases, including *LYK3* and *SymRK*, potentially serving as a scaffold protein for assembling signalling complexes crucial for rhizobial infection (Lefebvre et al., 2010). Additionally, *LjCERBERUS* encodes a novel protein containing a U-box domain and WD-40 repeats, which produces more prenodule structures in response to *Rhizobium* infection and controls plant defence response pathways (Hervé et al., 2011; Yano et al., 2009). These results suggest that SUMO-protease p0903 might be involved in suppressing early symbiosis-related genes to reduce the number of bacterial infections.

Rhizobial invasion of plant roots can trigger an increased systemic resistance state in the host, resembling the induced systemic resistance (ISR) mechanism, which includes JA plant defence signalling (Tonelli et al., 2020). Additionally, *Myc2* orchestrates a hierarchical transcriptional cascade that regulates jasmonate-mediated plant immunity (Du MinMin et al., 2017). Pathogen-related protein 4 (*PR4*) genes are induced by activators of systemic acquired resistance and wounding (Bertini et al.,

2003). Moreover, defensins play a role in plant immune responses via the JA signalling pathway (Serrazina et al., 2021). Previous reports have shown that NopE of *B. diazoefficiens* USDA110 triggers the expression of the *defensin* gene to manage nodulation compatibility with *V. radiata* KPS2 (Piromyou et al., 2021). The results indicate that SUMO-protease p0903 can trigger the expression of JA-related genes (*Myc2*, *PR4*, and *Def2-2*) in peanut KK5 (**Fig. 4.12B**). Interestingly, SUMO-protease p0903 and its ULP domain also induce JA biosynthesis (**Fig. 4.12.C**). These findings suggest that SUMO-protease p0903 and the ULP domain might stimulate JA production, thereby inducing a defence response to inhibit early nodulation in peanut roots.

CHAPTER V
CONCLUSION

In conclusion, naringenin has been shown to play a crucial role in the symbiotic interaction between *Bradyrhizobium* sp. DOA9 and peanut plants. This compound induces the transcription of nodulation (*nod*) genes in DOA9, which leads to activation of the T3SS. The T3SS then facilitates the secretion of T3Es, such as the SUMO-protease p0903, into the root cells of the peanut cultivar KK5. The peanut KK5-mediated incompatible interaction between peanut and DOA9 involves the accumulation of JA and the induction of defence genes (*Myc2*, *PR4*, and *Def2-2*), similar to the case of effector-triggered immunity (ETI). These findings imply that SUMO-protease p0903, when recognized by the host surveillance system, triggers a phytohormone-mediated effector-triggered immunity type (PmETI type) that suppresses early symbiotic genes (*CNGC*, *SYMREM*, and *CERBERUS*) and strongly inhibits rhizobial infection (**Fig. 5.1**). To optimize the use of *Rhizobium* inoculants, it is essential to identify genetic determinants within *Rhizobium* that affect symbiotic relationships with peanut plants. One such determinant is the SUMO-protease, which plays a significant role in the regulation of symbiotic interactions. Understanding how the SUMO-protease influences symbiosis can guide the development and selection of the most effective inoculant strains. This knowledge allows the creation of tailored inoculants that maximize peanut productivity and support sustainable agricultural practices.

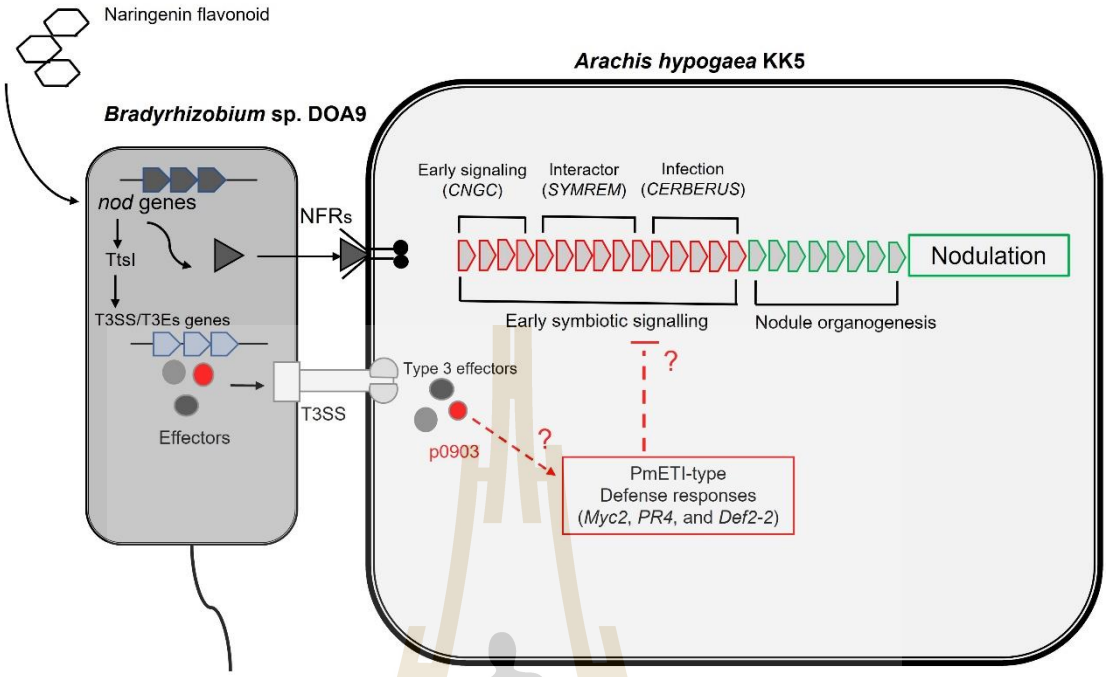

Figure 5.1 Schematic overview of the putative SUMO-protease p0903 T3E symbiotic process between *Bradyrhizobium* sp. DOA9 and peanut (KK5). Theoretically naringenin induces the expression level of the putative T3E-*p0903*. Consequently, SUMO-protease p0903 might trigger phytohormone-mediated effector-triggered immunity (PmETI-type) genes, including transcriptional factor (*Myc2*), pathogenesis-related protein 4 (*PR4*), and defensin (*Def2-2*), to block early infection. Additionally, SUMO-protease p0903 is a key regulator of early symbiosis signalling in peanuts (KK5), influencing genes involved in various stages of early signalling such as cyclic nucleotide-gated channels (*CNGC*), symbiotic remorins (*SYMREM*), and E3 ubiquitin-protein ligase LIN-1 (*CERBERUS*). The dotted line indicates the unclear symbiotic mechanism.

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APPENDIX

Appendix A.1 Luria-Betani (LB) medium (Bertani, 1951).

1.	Tryptone	10.0 g
2.	Nacl	10.0 g
3.	Yeast Extract	5.0 g
4	Agar	15.0 g
5.	Distilled Water	up to 1 liter

Adjust the pH to 7.0 with 5 N NaOH.

Appendix A.2 Yeast Extract Manitol medium (Bertani, 1970).

1.	Yeast Extract	0.5 g
2.	Mannitol	5.0 g
3.	K_2HPO_4	0.5 g
4.	Nacl	0.1 g
5.	$MgSO_4 \times H_2O$	0.2 g
6.	Agar	15.0 g
7.	Distilled Water	up to 1 liter

Adjust the pH to 6.8-7.0 with 5 N NaOH

Appendix A.3 Modified Arabinose Gluconate medium (Sadowsky, 1987).

1.	Yeast Extract	1.0 g
2.	L-Arabinose	1.0 g
3.	MES-Buffer	1.1 g
4.	Sodium Gluconate	1.0 g
5.	HEPES	1.3 g
6.	Agar	15.0 g
Stock Solutions:		
7.	$NaSO_4$ (12.5 g/100 ml)	2 ml
8.	$Cacl_2.2H_2O$ (0.65 g/100 ml)	2 ml
9.	K_2HPO_4 (6.25 g/100 ml)	2 ml
10.	$Fecl_3.6H_2O$ (0.433 g/100 ml)	2 ml
11.	$MgSO_4.7H_2O$ (9 g/100 ml)	2 ml
12.	Nacl (15 g/100 ml)	2 ml
13.	Distilled Water	up to 1 liter

Adjust the pH to 6.8-7.0 with 5 N NaOH.

BIOGRAPHY

Mr. Beedou Aphaiso was born on December 1, 1991, in Borikhamxay, Lao PDR. He completed his primary education in 2022 at Boung Khang village, Pakkading district, Borikhamxay province. He continued his education at Boung Khang village for pre-high school and completed his final high school years at Veingkham village, Pakkading district, Borikhamxay province.

Mr. Aphaiso earned a Bachelor's degree in Agronomy from the Faculty of Agriculture, University of Lao. It took him six years to complete his degree, from December 1, 2008, to October 30, 2014. During this time, he also participated in an 11-month Agrostudies training program in Israel, focusing on plant fields. His BSc thesis focused on "Nano Elicitors to Biocontrol Leaf Spot *Curvularia oryza* in Rice.

Mr. Aphaiso pursued a Master's degree in Biotechnology at Suranaree University of Technology (SUT) in Nakhon Ratchasima, Thailand. He was awarded a scholarship by the One Research One Graduate (OROG) of the Thailand Research Fund from December 23, 2017, to October 22, 2019. His master's research focused on innovative technology for producing liquid and solid inoculants of *Bradyrhizobium diazoefficiens* USDA110.

He then went on to pursue a Doctorate in Biotechnology at SUT, receiving another OROG scholarship from February 2020 to December 2024. During his doctoral studies, he conducted a three-month lab research stint from June 12 to September 7, 2023, at the Plant Health Institute of Montpellier, UMR-PHIM, IRD/ CIRAD/INRAE/Université de Montpellier/SupAgro in Montpellier, France. This was funded by the Campus France PHC-SIAM project N° 48151X. His doctoral research explored the role of type III effector protein p0903 from *Bradyrhizobium* sp. DOA9 in nodulation and nitrogen fixation in *Arachis hypogaea* L.