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Comparative analysis of the pathogenesis-related protein 10 gene family and their role during legume symbiosis

Prepared and presented by:

Toufik Yahia Takhi

Evaluation jury:

President	**:** Dr. Benaceur Farouk	MCA.	Amar Telidji University
Examiner	**:** Dr. Rezzoug Asma	MCB.	Amar Telidji University

Under the supervision of:

Supervisor	**:** Dr. Fathi Berrabah	Amar Telidji University
Co-supervisor	**:** Prof. Véronique Gruber	Paris Cité University

Sustained on **03/07/2024**

Abstract

Pathogenesis-related class 10 (PR10) proteins play various roles during plants' growth and resistance to biotic and abiotic stress. However, little is known about their role in the context of legume-rhizobia symbiosis, particularly in nodules. This present study aims to bridge that gap by characterizing the various roles of PR10s in the nodules of *Medicago truncatula* (Medtr). Initially, phylogenetic analysis of *PR10*s revealed two distinct *PR10* groups, PBI and PBII. The expression analysis performed on nodules of two Medtr mutants, *symbiotic Cysteine-rich Kinase-Like* (*symCRK*) and *defective in nitrogen fixation 2* (*dnf2*), showed an upregulation of *PR10s* in *symCRK* and *dnf2* mutant nodules compared to the WT, with PBI showing a higher expression than PBII. Collinearity analysis revealed three collinear *PR10* gene pairs that all belong to PBI, suggesting that PR10s were possibly subjected to tandem gene duplication. The multiple sequence alignments of 500 nucleotides up-stream *PR10* genes show a high conservation of 250 nucleotides in that site between PBI *PR10* genes and a loss of this conservation in PBII *PR10* genes. The conserved 250 up-stream nucleotides display a high density of putative binding sites for bZIP, NAC, and Myb transcription factors. Interestingly, molecular docking findings highlight that PBI PR10s bind phenolic compounds more efficiently than PBII. Moreover, multiple sequence alignments revealed that, unlike PBII PR10 proteins, PBI proteins have a high conservation of RNase motifs, which are important for the activation of programmed cell death during plant defenses. Finally, protein enrichment data revealed that PR10s can additionally accumulate in the different cellular compartments of nodules, including the cytoplasm, microsomes, and symbiosome. Altogether, our data show that PBI PR10s of *M. truncatula* are more conserved and highly functional during nodule defense responses compared to PBII PR10s. Thus, this study provides a valuable understanding of PR10s and their role during legume-rhizobia symbiosis.

Keywords: PR10, Medicago, nodule defense response, nodule immunity, symbiosis.

Résumé

Les protéines de la classe 10 liées à la pathogénèse (PR10) jouent divers rôles durant la croissance des plantes et leur résistance aux stress. Cependant, leur rôle dans le contexte de la symbiose légumineuse-rhizobium, en particulier dans les nodules, est encore mal connu. Pour cela, cette étude vise à caractériser les divers rôles des PR10 dans les nodules de *Medicago truncatula* (Medtr). Initialement, l'analyse phylogénétique des protéines PR10 a révélé l'existence de deux groupes de PR10, PBI et PBII. L'analyse de l'expression réalisée sur les nodules de deux mutants de Medtr, *symbiotic Cysteine-rich Kinase-Like* (*symCRK*) et *defective in nitrogen fixation 2* (*dnf2*), a montré une régulation positive des PR10 dans les nodules du *symCRK* et *dnf2* par rapport au type sauvage (WT), avec une expression plus élevée de PBI par rapport à PBII. L'analyse de colinéarité a révélé trois paires de gènes PR10 colinéaires appartenant tous à PBI, suggérant que les PR10 ont probablement été soumis à une duplication locale. L'alignement des séquences de 500 nucléotides en amont des gènes PR10 montrent une haute conservation de 250 nucléotides dans cette région entre les différents membres de PBI et une perte de cette conservation chez les membres de PBII. Les 250 nucléotides conservés en amont présentent une densité élevée de sites de liaison putatifs pour les facteurs de transcription bZIP, NAC et Myb. En revanche, les résultats de l'amarrage moléculaire montrent que les protéines PR10 de PBI se lient aux composés phénoliques plus efficacement que celles de PBII. De plus, l'alignement de séquence d'acides aminés a révélé que, contrairement aux protéines PR10 de PBII, les protéines de PBI ont une forte conservation des motifs RNase, importants pour l'activation de la mort cellulaire programmée lors des réactions de défenses des plantes. Finalement, les données d'enrichissement protéique ont révélé que les PR10 peuvent également s'accumuler dans différents compartiments cellulaires des nodules, y compris le cytoplasme, les microsomes et le symbiosome. En résumé, nos résultats montrent que les PR10 de PBI de *M. truncatula* sont plus conservés et plus fonctionnels lors des réactions de défense dans les nodules par rapport aux PR10 de PBII. Ainsi, cette étude fournit une compréhension précieuse des PR10 et de leur rôle lors de la symbiose des légumineuses avec les rhizobiums.

Mots-clés: PR10, réactions de défense nodulaire, immunité des nodules, symbiose.

الملخص

تلعب بروتينات الفئة 10 المتعلقة بالدفاع عن الأمراض(PR10)أدوارًا مختلفة أثناء نمو النباتات ومقاومتها لمختلف الإجهادات البيئية. ومع ذلك، لا يُعرف الكثير عن دورهم في سياق التعايش بين البقوليات والمستجذريات، خاصة في العقد الجذرية. لهذا تهدف هذه الدراسة الحالية إلى كشف مختلف الأدوار التي تقوم بها هذه البروتينات في العقد الجذرية لنبتة الفصة البرميلية *Medicago truncatula* (Medtr). كخطوة أولية, أظهرت تحاليل الوراثة العرقية لبروتينات PR10 وجود مجموعتين مختلفتين من هذه البروتينات PBI و PBII . أظهرت تحاليل تعبير جينات *PR10* التي أجريت على العقد الجذرية لنباتات الفصة المهجنة و البرية أنه هناك مستوى تعبير عالي للمجموعة الأولى PBI في عقد النباتات المهجنة مقارنة مع المجموعة الثانية PBII في كلا عقد النباتات, المهجنة و البرية. تحاليل التوازي الجيني كشفت وجود ثلاث أزواج جينية *PR10* متوازية تنتمي جميعها ل PBI مما يشير أن بعض أعضاء PR10 قد تعرضت الى تكرار الجينات الترادفي. أظهرت محاذاة التسلسل المتعدد لـ 500 نيوكليوتيدة في الموقع العلوي لجينات *PR10* 250 نيوكليوتيدة محفوظة بين غالبية جينات المجموعة الأولى PBI وفقدان هذا الحفظ في المجموعة الثانية PBII . النيكلويدات 250 المحفوظة في الموقع العلوي تحتوي على كثافة عالية من مواقع الربط المحتملة مع عوامل النسخ NAC, bZIP , و Myb . من جهة أخرى, نتائج الالتحام الجزيئي تسلط الضوء على أن بروتينات المجموعة الأولىPBI ترتبط بالمركبات الفينولية بكفاءة أكبر مقارنة مع المجموعة الثانيةPBIIمن بروتينات PR10. إضافة على ذلك, كشفت محاذاة التسلسل المتعدد للنيوكليوتيدات أن على عكس بروتينات PBII, بروتينات PBI لديها حفظ عالي لمواقع RNase التي تشكل واحدة من أهم وظائف البروتينات PR10 , والتي تساهم بطريقة مباشرة في تفعيل موت الخلايا المبرمج أثناء دفاعات النبات. أخيرًا، كشف تحليل بيانات إثراء البروتينات أن بروتينات PR10 يمكن أن تتراكم أيضًا في مقصورات خلوية مختلفة في خلايا العقد الجذرية، بما في ذلك السيتوبلازم، الميكروسومات، والسيمبيوسوم. كخلاصة تُظهر بياناتنا أن بروتينات PBI أكثر حفظًا و أكثر وظيفية أثناء استجابات الدفاع في العقد الجذرية مقارنة ببروتينات PBII . وبالتالي، توفر هذه الدراسة فهمًا قيمًا لبروتينات PR10 ودورها أثناء التعايش بين البقوليات والمستجذريات.

الكلمات المفتاحية: PR10, استجابات دفاع العقد الجذرية, مناعة العقد الجذرية, التعايش.

List of Abbreviations

PRR	: Pattern recognition receptor
PAMP	: Pathogen-associated molecular pattern
MAMP	: Microbe-associated molecular pattern
DAMP	: Damage-associated molecular pattern
Flg	: Bacterial flagellin
EF-Tu	: Elongation factor Tu
PGN	: Peptidoglycan
LPS	: Lipopolysaccharides
CDPK	: Calcium-dependent protein kinases
ROS	: Reactive oxygen species
NOS	: Nitrogen-reactive species
PTI	: Pattern-triggered immunity
T3SS	: Type III secretion system
ETS	: Effector-triggered susceptibility
NLR	: Nucleotide-binding leucine-rich repeat and domains
NBS	: Nucleotide-binding site
NBS-CC	: NBS-Coiled Coiled
ETI	: Effector-triggered immunity
PCD	: Programmed cell death
SAR	: Systemic acquired resistance
SA	: Salicylic acid
JA	: Jasmonic acid
NPR1	: NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN GENES 1
PR	: Pathogenesis-related
HR	: Hypersensitive response
AM	: Arbuscular mycorrhiza
AMF	: Arbuscular mycorrhizal fungi
EcM	: Ectomycorrhiza
OrM	: Orchidaceous mycorrhiza

ErM : Ericoid mycorrhiza

PGPM : Plant growth-promoting microbes

PGPF : Plant growth-promoting fungi

PGPR : Plant growth-promoting rhizobia

SNF : Symbiotic nitrogen fixation

Nod : Nodulation

ISR : Induced systemic resistance

IPR : Intracellular pathogenesis-related

CSBP : Cytokinin-specific binding protein

MLP : Major latex protein

Medtr : *Medicago truncatula*

WT : Wild-type

DNF2 : DEFECTIVE IN NITROGEN FIXATION 2

SymCRK : Symbiotic CYSTEINE-RICH KINASE-LIKE

RSD : REGULATOR OF SYMBIOSOME DIFFERENTIATION

TF : Transcription factor

NAD1 : NODULES WITH ACTIVATED DEFENSE 1

NIN : NODULE INCEPTION

TDB : Terminal differentiation of bacteroids

RT : Reverse transcription

MEGA : Molecular Evolutionary Genetics Analysis

NWK : Newick

BNM : Buffered nodulation medium

gff : General feature format

PB : Phylogenetic branch

dpi : days post inoculation

RNase : Ribonuclease

NTPase : Nucleoside-triphosphatase

GFP : Green fluorescent protein

NMR : Nuclear magnetic resonance

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Part I. Literature Review

Chapter 1. Plants immunity

1.1. Plants interact with a wide range of microbes

Plant roots harbor myriad communities of microbes. These microbes colonize different regions of plants and can be categorized into different types based on their location. Microbes found on plant surfaces are termed epiphytes, while endophytes inhabit the insides of plant tissues; phyllospheric microbes are located on leaf surfaces; and rhizospheric ones thrive in the soil surrounding plant roots [1]. The rhizosphere and the phyllosphere are, respectively, the zones connecting the terrestrial and aerial parts of plants to the surrounding environment. The rhizosphere sustains numerous eukaryotic and prokaryotic organisms, ranging from macroscopic animals, such as nematodes and microarthropods, to small, microscopic beings, like bacteria, fungi, archaea, and protists. The dense microbial populations found in the rhizosphere can be considered "the second genome of plants" [2].

Plants constantly interact and form complex trophic exchange networks with symbiotic microbes. Based on the effect of microorganisms on plants, they can be classified into three categories: (i) positive interactions (beneficial for promoting the growth and development of plants), (ii) negative interactions (harmful, affect plant growth, and sometimes lead to their death), or (iii) neutral interactions (have no effects on the plant's physiology) [2].

Regardless of the outcome for the symbiotic individual, such interactions are essential for life, and the rhizosphere plays a pivotal role in hosting and sustaining these partnerships, maintaining, as a result, the overall health and stability of ecosystems [3].

Plants and pathogens, during the course of evolution, have evolved sophisticated defensive and offensive mechanisms to distinguish between harmful and beneficial microbes and to respond accordingly.

1.2. Pathogenesis. A harmful plant-microbe interaction

The sessile nature of plants renders them vulnerable to constant attacks from microbial pathogens. A microbe is said to be a pathogen of plants (phytopathogen) if it causes disease or sometimes the death of their plant hosts [4]. To ward off such pathogens, plants acquired countless mechanisms to withstand their evasion. Performed defenses [5] are physical barriers that constitute the first line of defense for plants, such as rigid cell walls [6], waxy cuticles, and antimicrobial metabolites that protect plant cells from pathogen attacks [7].

Plants can also perceive pathogens through the detection of specific molecules produced by microbes. The detection of such stimuli can lead to the activation of complex molecular events, leading to the stimulation of innate immune responses [5].

1.2.1. Pattern-triggered immunity

The first level of pathogen perception involves pattern recognition receptors (PRRs), which are receptors found on the surface of host plant cells that recognize the pathogen- or microbe-associated molecular pattern (PAMPs or MAMPs, e.g., bacterial flagellin (Flg); elongation factor Tu (EF-Tu); peptidoglycan (PGN); lipopolysaccharides (LPS); fungal chitin; and β-glucans from oomycetes) [5, 7–9], or host-derived, damage-associated molecular patterns (DAMPs, e.g., like cutin and apoplastic peptide fragments), that can be perceived through the extracellular leucine-rich repeat domains making up PRRs [9]. Once PRRs recognize these patterns, they induce a set of defense signaling cascades and modules that include the phosphorylation of mitogen-activated protein kinases (MAPKs), the influx of extracellular calcium to the host cell, which is perceived by calcium-dependent protein kinases (CDPKs), and the eventual depolarization of the cell membrane, activation of ROS-mediated signaling that leads to the enzymatic production of reactive oxygen species (ROS) [4, 7, 8, 10] (e.g., the conversion of oxygen superoxide into hydrogen peroxide (H2O2) by superoxide dismutase), and the biosynthesis of nitrogen-reactive species (NOS) such as nitric oxide [8]. These modules further trigger the activation of specific transcription factors that lead to the induction of multiple defense responses. Such defense responses include stomatal closure in leaves and the eventual induction and secretion of ROS and antimicrobial compounds [5]. All these reactions and cascades make up the first layer of immunity, pattern-triggered immunity (PTI).

1.2.2. Effector-triggered immunity

Some pathogens have evolved mechanisms to evade PTI, where they deliver specific effector proteins that can act either outside (termed apoplastic effectors) or inside (cytoplasmic/symplastic effectors) host plant cells to bypass PTI signaling [6, 8, 11, 12]. Bacterial pathogens use the type III secretion system (T3SS), which corresponds to a membrane-embedded molecular syringe that translocates effector proteins from the bacteria's cytoplasm directly to the cytoplasm of host plant cells [13, 14]. Alternatively, fungi and oomycetes secrete their effectors via the haustorium into and around host cells [8, 15, 16].

Inside host cells, pathogen effectors block key defense signaling proteins that lead to the suppression of PTI, resulting in an effector-triggered susceptibility (ETS) [4, 10]. In response

to such effectors, plants have also evolved defensive strategies to counteract ETS by inducing the resistance gene (R gene), which encodes resistance intracellular receptors capable of specifically recognizing pathogen effectors via their nucleotide-binding site (NBS)-leucine-rich repeat (LLR) and NBS-Coiled Coiled (CC) domains [17]. By recognizing pathogen effectors, NBS-LRR/CC activate the second layer of defense called Effector-Triggered Immunity (ETI) [5, 6, 8, 9, 18], which is a more robust and faster defense response compared to PTI [19], leading to programmed cell death (PCD) of infected host cells. Cells surrounding the PCD site secrete, as a response to pathogen invasion, antimicrobial compounds and reinforce their cell wall through the deposition and accumulation of callose [20, 21].

1.2.3. Systemic acquired resistance

The stimulation and the onset of PTI and ETI trigger an induced resistance across whole plant tissues [8]. This onset spreads a broad-spectrum disease resistance against secondary infections to distal undamaged plant tissues that are far from the infection site [19, 22]. The spread of resistance across plant tissues is known as systemic acquired resistance (SAR). SAR aims to increase the basal immunity level in all host cells to avoid the spread of disease to non-infected tissues [8, 19]. SAR increases via the synthesis and secretion of long-distance signals that include salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling, which are plant hormones that propagate resistance to distant undamaged plant tissues [5, 8]. SA stimulates and binds to the key regulatory protein NONEXPRESSOR OF PATHOGENESIS-RELATED (PR) PROTEIN GENES 1 (NPR1) that, when activated, induces the expression and upregulation of defense-related protein-coding genes, often referred to as the pathogenesis-related (PR) protein genes [5, 23, 24].

1.3. Pathogenesis-related proteins

Pathogenesis-related proteins (PRs) are small proteins that are synthesized by plants during the defense against a wide range of biotic and abiotic stresses [24, 25]. They are localized in various cellular compartments, including the vacuole, the cytoplasm, cell walls and they can accumulate in other intracellular and extracellular spaces [26, 27]. PRs are thermostable, protease-resistant proteins with a low molecular weight that are found in numerous plants and are often employed in various biochemical reactions, particularly during plant defense responses [24]. In addition to that, PRs can also promote the growth and development of plants [24, 25]. Even though PRs are expressed during both biotic and abiotic stresses, they are still termed “pathogenesis-related” proteins.

1.3.1. The different PR classes

PRs are currently classified into 19 different families (PR-1 to PR-19) that differ in structure, enzymatic activity, and target specificity (Table 1). They include, but are not limited to, CAP-domain proteins (pfam00188; PR1); hydrolytic β-1,3-glucanases (PR-2); chitinases (PR-3, 4, 8, 11); proteinase inhibitors (PR6); peroxidases (PR-9); plant defensins (PR-12); oxalate oxidase and oxalate-oxidase-like proteins (PR-15, 16); secretory proteins (PR17); carbohydrate oxidases (PR-18); and a novel potential antimicrobial protein isolated from the pine tree species *Pinus sylvestris*, which became the 19th PR class (PR-19) [24, 27–30].

Table 1. The different family classes of pathogenesis-related proteins, their functions, and the plant species where they were first discovered.

PR Family	Properties/Functions	Plant source	References
PR-1	• Inhibit pathogens • Antifungal and antiviral activities	*Nicotiana. tabacum*	[27, 31, 32]
PR-2	• β-1,3-glucanases • Antimicrobial activities	*N. tabacum*	[27, 31, 32]
PR-3; PR-4; PR-8; PR-11	• Chitin hydrolysis (Chitinases) • Antiviral and antifungal activities	*N. tabacum* *Cucumis sativus* *N. tabacum* respectively	[27, 32–35]
PR-5	• Thaumatin-like proteins • Antifungal activity	*N. tabacum*	[27, 32, 33]
PR-6	• Protease inhibitors	*Solanum lycopersicum*	[27, 32, 36]
PR-7	• Endoproteases • Potential antimicrobial activities	*S. lycopersicum*	[27, 32, 37]
PR-9	• Peroxidases	*N. tabacum*	[27, 32, 38]
PR-10	• Ribonuclease-like proteins • Antimicrobial and antinematode activities • Cold and salt stress tolerance • Initiation of programmed cell death during hypersensitivity reaction	*Petroselinum crispum*	[10, 27, 32, 39]
PR-12	• Defensin • Abundance in different plant parts	*Raphanus raphanistrum*	[27, 32, 40]

Table 1. (Continued)

PR Family	Properties/Functions	Plant source	References
PR-13	• Thionine • Defense proteins	*Arabidopsis thaliana*	[27, 32, 41]
PR-14	• Lipid-transfer protein • Defense against pathogens • Abundant in plant cell walls	*Hordeum vulgare*	[27, 32, 42]
PR-15; PR-16	• Oxalate oxidase and oxalate-oxidase-like proteins • ROS generation	*H. vulgare*	[27, 32, 43, 44]
PR-17	• Antifungal and antiviral • Secretory protein • Proteolysis	*N. tabacum*	[27, 32, 45]
PR-18	• Antibacterial activities • Carbohydrate oxidase properties	*Helianthus annuns*	[27, 30, 32, 46]
PR-19	• Potential antimicrobial • Yet to be discovered	*Pinus Sylvestris*	[27, 30, 32, 47]

1.3.2. The pathogenesis-related protein 10

The family 10 of plant pathogenesis-related proteins (PR10) is primarily a multigenic group of proteins that can be found predominantly in the cytosol and the nucleus [48] but also on the cell membrane or forming complexes with mitochondrial and plant cells' apoplast proteins [24]. They are expressed in several plant tissues, such as roots, stems, flower compartments, fruits, and, in some plant species, pollen grains [49]. Members of the *PR10* gene family are multifunctional, often induced during the defense against a wide range of biotic and abiotic stresses [26, 50], ranging from the defense against invading pathogens and limiting abiotic conditions (salinity, drought, cold, etc.) to the development of plants.

1.3.2.1. Sequence properties of PR10s

Most *PR10* genes have an open reading frame (ORF) of 456–489 bp [51, 52], with some exceptions for the (S)-norcoclaurine synthases (NCS) group of PR10s that have a longer ORF from 633 to 696 bp, where the two groups share a 28–38% sequence identity and putative structural homology [51]. The ORF encodes for PR10 proteins that are relatively small and acidic with multiple conserved features and functions.

1.3.2.2. Structural and functional features of PR10s

The three-dimensional (3D) structure of PR10 proteins has been extensively studied using X-ray crystallography and/or solution nuclear magnetic resonance (NMR) spectroscopy [53]. Most PR10 proteins are considered as small acidic molecules with a molecular weight of 15–18 kDa [54] and 154–163 amino acids that form their structure [49]. These proteins and the Bet v 1 pollen allergens share similar 3D structures, which is the reason behind the nomenclature "Bet v 1-like" of PR10s [53, 55]. The 3D structure of PR10s consists mostly of a seven-stranded anti-parallel β-sheet (β1 to β7) that surrounds an amphipathic C-terminal α-helix (α3) and another two short N-terminal α-helixes (α1, α2) that are positioned orthogonally to embrace α3 by forming a V-shaped conformation [26, 49]. This conformation allows for a large internal hydrophobic, solvent-accessible cavity, which can be considered a general feature of PR10s [49] that plays a crucial role in the biological function of PR10s and serves as a binding site for a wide variety of ligands like steroids, cytokinin, flavonoids and fatty acids, phytoprostanes, phytomelatonin, gibberellic acid, etc. [26, 27, 49]. The ligand-binding activity of PR10s highlights, furthermore, their role in hormone-mediated signaling pathways.

Additionally, PR10s contain another functional domain, a phosphate-binding loop motif called P-loop (consensus sequence: AA 46–54, GXGGXGX), which forms the nucleotide binding site and contains a glycine-rich motif that is highly conserved among nucleotide binding proteins [26, 48, 56]. This glycine-rich motif is primordial for the ribonuclease (RNase) activity of PR10s [48, 57], which directly influences the hypersensitive response (HR) in plants by contributing to PCD and the overall resistance of plants to diseases [48].

Moreover, the diverse abundance of PR10 proteins across the plant kingdom renders them structurally and functionally variable, and no unique structure or biological function has been assigned to PR10 proteins. Among these functions, PR10 proteins can play a role in the development and growth of plants, their defense against pathogens, UV protection, antioxidation, and other abiotic stress resistance functions. Some PR10 proteins have been reported to possess papain inhibitory activities against *Meloidogyne incognita*, the root-knot nematode, illustrating their nematostatic and nematicide effects [58]. In addition to that, the silencing of PR10s in the plant *Malus domestica* during the infection by the fungus *Valsa mali* increases the plant's susceptibility to the fungus, while the overexpression of these genes reduces the infection [59], indicating their role in plant defense signaling pathways and the overall resistance to pathogens.

1.3.2.3. Regulation of PR10s

Given the diversity and importance of PR10 proteins in plant cells, the mechanisms by which they are regulated involve complex molecular mechanisms and signaling pathways. *PR10* genes have been shown to be induced through the action of signaling compounds such as SA, JA, and ET, which modulate their expression levels during stress responses [27, 51]. The expression of PR10s in rice was found to be influenced by light signals, where these proteins were induced in the presence of light, while no expression was seen in dark conditions. Alternatively, the expression of PR10s in some plants was found to be organ- and tissue-specific. For instance, PR10s from maize were induced mainly in roots [51, 60]. Saffron *PR10* genes were highly expressed in anther and tapetal tissues, with low expression in stigmas and roots and no expression in leaves [51, 61]. *PR10* genes possess cis-regulatory elements in their upstream regions that are recognized by various transcription factors, such as the TFs WRKY, AP2/ERF, bZIP, RAV2, GATA1, and MYB [24, 51], which demonstrate the importance of PR10s in diverse defense response pathways. Additionally, post-translational modifications, like phosphorylation, play a crucial role in regulating the function of PR10 proteins. A study highlighted that the RNase activity of PR10 to cleave invading viral RNAs was enhanced due to subsequent phosphorylation [62]. Moreover, epigenetic regulation (e.g., DNA methylation and histone modifications) and RNA interference like microRNAs can also influence the expression of PR-10 genes [24]. PR10 proteins have been found to regulate other PR10 proteins, where the knockdown of a *Medicago truncatula* PR10 leads to an antagonistic induction of other PR proteins (e.g., PR5), resulting in increased tolerance to the infection by *Aphanomyces euteiches* [63].

1.3.2.4. Diversity of PR10s

PR10 proteins/Bet v 1-like proteins are present as a multigene family that are wildly distributed across plants. Based on amino acid sequence similarities, subcellular location, and function, they can be divided into intracellular pathogenesis-related (IPR) proteins (also called classic PR-10 proteins), cytokinin-specific binding proteins (CSBPs), and major latex proteins (MLPs) [48, 49, 51]. PR10s can be found in many seed plants, such as parsley, pea, potato, bean, soybean, celery, alfalfa, etc. They can also be found in many monodicots, like asparagus, rice, lily, sorghum, and others. In addition to that, they were also reported in multiple tree species, like *Pinus lambertiana*, *Pinus strobus, Pinus monticola*, *Pinus pinaster*, *Betula verrucosa*, *Jatropha curcas*, *Fagus sylvatica*, etc. [51–53, 56].

Chapter 2. Plant-microbe symbiosis

2.1. Symbiotic plant-microbe interactions

Symbiosis is a beneficial interaction between two or more partnering organisms. In this association, both partners obtain benefits from each other. Plants often engage in symbiotic relationships with bacteria and fungi, where they provide plants with nutrients, promote their growth, and offer an overall beneficial effect on plants [64, 65]. In return, plants provide a preferential ecological niche for microbes containing nutrients like sugars, amino acids, organic acids, and other compounds [66]. Examples of symbiotic plant-microbe associations include mycorrhizae, rhizobia, and plant growth-promoting microbes [67]. This part will explore some of these interactions to further highlight the importance of beneficial plant-microbe partnerships.

2.1.1. Mycorrhizae

Mycorrhizae is an association between fungal hyphae (mycobiont) and plant roots (photobiont) [68], in which both partners exchange various benefits. Fungi offer plants an enhanced uptake of water and nutrients, such as phosphorus, nitrogen, and others. Due to the dense network of mycelium that extends the surface area of plant root systems [68, 69], mycorrhizal fungus can help with stress tolerance by improving the overall plant's resilience to salinity, drought, and other extreme environmental conditions. Mycorrhizal associations also help in disease resistance by producing antimicrobial compounds and stimulating the plant's defense system. Plants, in return, provide fungi with organic compounds [68–70].

Mycorrhiza can be divided into four major types that differ by the locations and structure of their hyphae [71]: arbuscular mycorrhiza (AM), ectomycorrhiza (EcM), orchidaceous mycorrhiza (OrM), and ericoid mycorrhiza (ErM). These variations depend on the cellular interactions that happen between partners as well as the partner's taxonomy [72].

2.1.2. Plant growth-promoting microbes

The rhizosphere contains a dense inhabitation of a variety of plant growth-promoting microbes (PGPM). These microbes can range from plant growth-promoting fungi (PGPF) to plant growth-promoting rhizobacteria (PGPR) that provide positive direct and indirect interactions with plants [64]. By improving seed germination and regulating the production of phytohormones such as cytokinins, ACC deaminase, auxin, and gibberellin [64, 73], they can also increase the availability and uptake of soil nutrients, such as siderophore-producing

rhizobacteria that can facilitate iron absorption by plant roots [74]. PGPM can produce various types of molecules that benefit plants, such as antioxidants against abiotic stresses; exopolysaccharides that facilitate water retention; osmoregulation, which helps regulate homeostasis; and increased resistance against pathogens through inducing systemic resistance, secretions of antagonistic antimicrobial molecules, and competition for nutrient utilization [73–75].

2.1.3. Symbiotic nitrogen fixation

In legumes, symbiotic nitrogen fixation (SNF) is the biological process in which a group of bacteria, collectively known as Rhizobia, enzymatically transform atmospheric nitrogen during symbiosis in the host plant's roots into an organic form that can be easily utilized and absorbed by plant tissues [76, 77]. These bacteria infect plant roots to form specialized organs called nodules [78]. The most common bacterial symbionts are found within the alpha-proteobacteria *Rhizobiales* order [76]. This phylogenetic group contains important nitrogen-fixing bacterial genera, notably *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium* [76, 77, 79].

2.2. Initiating the rhizobia-legume interaction

The establishment of legume-Rhizobia symbiosis first occurs in nitrogen-limited soils through complex molecular mechanisms and signal exchange processes, where plants secrete flavonoids that get perceived and recognized by compatible, specific Rhizobia strains [80, 81]. The recognition of flavonoid metabolites by rhizobia triggers the induction of *nod* genes/proteins, their synthesis, and the eventual secretion of nodulation (Nod) factors [81, 82]. Plants perceive the Nod factors signals as a sign for a potential symbiont; this transduction triggers a cascade of reactions within plant roots to suppress the plant's immune system, to welcome symbiotes, and to initiate nodule formation.

2.2.1. Recognition of rhizobia and the formation of their host organ, nodules.

The perception of signaling Nod factors by the host root cells leads to changes in ion concentrations and the eventual depolarization of the root cell membrane [82]. Such fluctuations include variations in calcium concentration, named "calcium spiking" [83], inside epidermal root hair cells. Calcium spiking triggers changes in gene expression on these cells, resulting in nodule development [82, 83]. The epidermal root hairs are then deformed, coupled with early nodulin gene expression and the eventual development of nodule primordia [81, 82].

Rhizobia are then trapped in the deformed root hair cells to form curled, colonized infection pockets containing microcolonies of dividing rhizobia [81, 82]. These newly formed infection threads penetrate down towards nodule primordial cells, where the microcolonies of multiplying rhizobia get endocytosed inside a symbiotic membrane to form a unique organelle called a symbiosome, in which rhizobia grow, divide, and differentiate into bacteroids [80, 82, 84]. The differentiation stages are disclosed in Section 2.3. Eventually, it transforms into a nodule containing a functional organelle capable of synthesizing the nitrogenase enzyme, which catalyzes the reduction of atmospheric nitrogen N2 to ammonia NH_3^+, an easily accessible source of nitrogen [81], according to the following reaction [84]:

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

Chapter 3. Immunity during symbiosis

3.1. Plants can remember past stimuli

3.1.1. Defense priming

Plants must constantly cope with fluctuating environmental conditions, where sensing and responding to warning signals, both biotic and abiotic, is crucial to ensuring their survival. Interestingly, plants have evolved a plethora of mechanisms that allow them to remember certain past stimuli, which often prepare or prime them against potential future stresses [85]. By doing so, plants can react faster and stronger to future unpredictable stress occurrences [5, 85]. This type of mechanism in plants is termed "defense priming," providing them with a fitness advantage to better cope with the variability of repeated environmental stress [23, 86]. Induced systemic resistance (ISR) is considered one of the types of induced signaling resistance cascades that mediate defense priming [5, 87].

3.1.1.1. Induced systemic resistance

Beneficial arbuscular mycorrhizal fungi (AMF) and PGPR often simulate plant growth and provide them with protection against harmful pathogens. The suppression of pests and phytopathogens by the induction of defense signaling pathways is known as induced systemic resistance (ISR) [5, 87, 88]. Beneficial microbes sensitize and provide plants with MAMPs that activate ISR, where this mechanism not only primes plants against biotic stress but also increases their tolerance to abiotic stress [5].

3.2. Modulation of immunity during symbiosis

Aside from beneficial rhizobia, legumes often interact with other mutualistic and pathogenic microbes in the rhizosphere, where it is crucial for such plants to distinguish between positive and negative interactions and to modulate their immune response accordingly. As a result, legumes have developed highly specific recognition mechanisms to select ideal symbiotic microbes. It has been demonstrated that the innate immune system of plants regulates such a selection by identifying and inhibiting the invasion of certain rhizobia species [89, 90]. Symbiotic rhizobia have, in return, evolved ways to evade and modulate their host defenses through the secretion of special signaling molecules that suppress the host's immune system to initiate the symbiotic interaction for successful infection and establishment within the plant's nodules [89].

Current research [89–92] highlights that the establishment of such interactions can be achieved through a complex signal exchange between plants and compatible symbiotic rhizobia (Chapter 2.). After the initial infection and interaction process, the survival of compatible beneficial rhizobia inside nodules is tightly linked to the expression of nodule-specific genes, collectively known as NODULINS [91]. In the R108 ecotype of *Medicago truncatula* (Medtr), the control and suppression of immunity during symbiosis is governed by the expression of NODULINS, which include the genes *DEFECTIVE IN NITROGEN FIXATION 2* (*DNF2*), encoding a phospholipase-C phosphatidyl-dependent protein-like [90, 91, 93]; *Symbiotic CYSTEINE-RICH KINASE-LIKE* (*SymCRK*) responsible for the synthesis of a cysteine-rich receptor kinase [91, 94]; *REGULATOR OF SYMBIOSOME DIFFERENTIATION* (*RSD*) that encodes a C2H2 transcription factor (TF) [91, 95]; *NODULES WITH ACTIVATED DEFENSE 1* (*NAD1*) that gives a transmembrane protein [91, 96]; and a *NODULE INCEPTION* (*NIN*) gene that encodes a symbiotic TF [91, 97].

A study on the control of immunity during earlier infection steps [98] highlights that *DNF2*, *SymCRK*, and *RSD* can act consecutively during the bacterial internalization into the symbiotic cells step to repress defense-like reactions and prevent the death of rhizobia inside nodules. Additionally, other studies reveal that the lack of the genes *NAD1* [96, 99], *DNF2* [93], *SymCRK* [94], and *RSD* [95] causes an elevated induction of defense and stress-related genes as well as the secretion of phenolic compounds, compromising, as a result, bacterial viability and causing the early symbiotic arrest of rhizobia during the terminal differentiation of bacteroids (TDB) process [91]. Alternatively, identification of defense-activating signals in the mutants *dnf2*, *symCRK*, and *rsd* revealed that in response to environmental elicitors such as ulvan, *DNF2* prevents defenses [100], while in response to bacterial infection and/or bacteroid differentiation, *SymCRK* and *RSD* repress immunity [98].

Introduction

Throughout the course of evolution, plants have developed sophisticated defensive strategies to cope with biotic and abiotic stress. These strategies include the expression of various defense-related genes encoding PRRs, phytohormones, phytoalexins, phenolic compounds, signaling compounds [24, 101], and defense molecules, such as the pathogenesis-related (PR) genes, which encode for proteins that play numerous roles in the growth, development, as well as biotic and abiotic stress resistance of plants [24].

Among PRs, the class 10 of the pathogenesis-related (PR10) genes has been characterized in many plant species [51–53, 56]. Accordingly, PR10s have been assigned to play a pivotal role in the development and stress tolerance of plants. Despite the extensive evidence on the involvement of PR10s in plant defense mechanisms, our present study aims to explore the role of PR10s during nodule defense responses and the overall legume-rhizobia symbiosis. The methodology used in this study is as follows:

1. Preparation of *Medicago truncatula* plant material for expression analyses.
2. Construction of the phylogenetic tree of PR10 proteins.
3. Determination of *PR10* gene duplication through collinearity and synteny analysis.
4. Detection of TFs that bind cis-regulatory elements in the 5'-UTR upstream region of *PR10* genes.
5. Assessment of the ligand-binding activities of PR10 proteins using molecular docking.
6. Determination of cell death in nodules using the aniline blue stain.
7. Comparison of protein sequences of PR10s and evaluation of the conservation degree of their functional domains.
8. Subcellular localization of PR10 proteins.

The next sections provide the methods and materials used to obtain and construct the findings of this study.

Part II. Materials and methods

1. Plant genotype, plant inoculation and growth conditions

The *Medicago truncatula* ecotypes R108 [102], the derived Tnt1 transposon tagged-lines nf737 (*symCRK*) (provided by the Noble Research Institute) [100], and a MERE1 insertion mutant line ms240 (*dnf2-4*) (which corresponds to a somaclonal variant obtained by the regeneration of a T-DNA-tagged Medicago line) were used in this study [93].

To begin the experimental setup, Medicago seeds were surface sterilized [98] and vernalized for at least 48 h at 4 °C in the dark on a solid medium (Bacto-agar 1% w/v). Seeds were then germinated for 24 h in the dark at 24 °C before transferring them to the buffered nodulation medium (BNM) [103] solidified with 1.5% (w/v) Bacto Agar for *in vitro* cultivation. Plants were then cultivated under chamber growth conditions at 24 °C and 60% humidity under a photoperiod of 16 h light/8 h dark with a 150 µE light intensity.

Overnight cultures of bacterial strains were pelleted and washed twice with sterile water. $OD_{600\ nm}$ was adjusted to 0.1 in water by re-suspension. The roots of eight seedlings per plate were co-inoculated with 1 mL of bacterial cell suspensions.

2. RNA-seq data analysis

RNA-seq data was provided by Ratet Lab as well as other data extracted from the genome database MtExpress V3 [104]. These data were then analyzed using the built-in data manipulation and visualization tools (Fasta Tools, Blast GUI Wrapper, Heatmap Illustrator, and Table Manipulator.) on the Toolbox for Biologists (TBtools) software [105] following the web resources and manuals page of the software (TBtools Cookbook).

3. RNA extraction, cDNA synthesis, and expression analysis

A standardized protocol [106] for RNA extraction, cDNA synthesis, and RT-qPCR was followed and performed. Nodules collected from 16 plants (grown in vitro conditions) and 5 plants (grown in sand/perlite) per experiment were frozen in liquid nitrogen and then manually crushed with beads in a 2 mL microcentrifuge tube. The total RNA was then extracted using the TRI Reagent® RT procedure recommended by the Molecular Research Center manufacturer. DNA was removed from samples using the DNase I kit following the Invitrogen manufacturer's protocol. The concentration and RNA quality were checked using the Thermo Scientific NanoDrop 1000 spectrophotometer. Reverse transcription (RT) was then performed following the supplier's protocol (Life Technologies) on 0.5 µg or 1 µg of total RNA (DNA-

free) using oligo (dT) and SuperScript II Reverse Transcriptase in a final volume of 20 mL. For each tested gene, the primers amplified 200–300 nucleotides of the cDNA sequence, and the quantification was made using qPCR on the LightCycler 480 (Roche Life Science) with the LightCycler FastStart DNA Master SYBR green I kit following the Roche Life Science manufacturer's instructions. Temperatures of 94 °C, 58–62 °C, and 72 °C were used, respectively, for denaturation, annealing, and extension steps. In all analyzed samples, expression levels were normalized using the housekeeping gene MtACT (Actin 11) (Supplemental Table S6 [10]; [107]).

4. Phylogenetic analysis

To infer potential relationships between different *PR10* gene members, phylogenetic tree construction was performed using PR10 protein sequences downloaded from the NCBI database (NCBI Protein). The obtained sequences were used to construct the phylogenetic tree using the Molecular Evolutionary Genetics Analysis (MEGA) 11 software [108] with the Maximum Likelihood method, JTT matrix-based model [109, 110], and default settings (Bootstrap 1000). The file obtained from phylogenic analysis by MEGA was then saved in Newick (NWK) tree format and used on the Interactive Tree of Life (iTOL) [111] website to enhance the appearance of the phylogenetic tree for the final layout.

5. Circular genome viewing and gene collinearity analysis

The circular genome viewing for synteny analysis was conducted using the TBtools built-in Advanced Circos visualization tool [112]. The circular genome graph was constructed following the TBtools documentation and tutorials on their website (TBtools advanced circos). Chromosome length and the relative positions of *PR10* genes in Medtr were determined using the assembly or whole genome fasta file and the general feature format (gff) file of Medtr, which were downloaded from the Joint Genome Institute (JGI) data portal and the Medicago A17 r5.0 genome portal. The files were then analyzed using the Multiple Collinearity Scan Toolkit X (MCScanX) algorithm [113, 114] to detect intra-species collinearity and synteny. Collinearity and the gene-linked information files generated by the MCScanX package were then visualized using the advanced Circos package of TBtools to scan for potential syntenic relationships between different members of the *PR10* gene family in *M. truncatula*.

6. Molecular docking

First, we downloaded seven varying phenolic compound structures from the PubChem DB [115], and the SDF files were converted to pdbqt files using the Open Babel toolbox version 3.1.1 [116] for format conversion. The PDB files containing the 3D structures of PR10 proteins were obtained from the AlphaFold database [117], using their respective accessions from the Phytozome platform [118]. All the ligand and protein files were then geometrically optimized and used for the determination of the binding affinity of some PR10 proteins to the seven phenolic compounds using the molecular docking software Autodock Vina [119].

7. Aniline blue Staining

14-dpi nodules were embedded in 6% (w/v) agarose and sliced into 70- µm sections using the vibratome VT1200S. Aneline blue staining was carried out by the incubation of nodule slices in a 50 mM Tris-HCl buffer (pH 7.2) containing 0.1% (w/vol) of aniline blue for about 15 min. The stained slices were then washed 4–6 times with Tris-HCl. The samples were then observed using the AZ100 Nikon microscope.

8. Protein sequence comparison

Sequences of PR10 proteins were downloaded from the NCBI database (NCBI Protein), with the keyword "pathogenesis" being used to search for PR10 protein sequences. The genes corresponding to PRs were then isolated and analyzed using the BioEdit software [120] along with the ClustalW algorithm that was used for sequence alignments [121].

9. Protein subcellular localization in tobacco leaves

PR10 full length cDNA was amplified by Q5 high fidelity Taq Polymerase using the recommended condition by the manufacture. The PCR products were then separated by gel electrophoresis using TBE gelled with 2% (W/V) of agarose. The amplified products were then purified using the GeneJET PCR Purification Kit and the purified PCR fragments were then cloned using the vector Gateway™ pDONR™221 plasmid. The recombinant plasmids were then transferred into *Escherichia coli*. After 24 hours of incubation at 37°C on LB growth medium, the amplified plasmids from the transformed *E. coli* were extracted and the cloned PR10s were sequenced to ensure the absence of mutations in the amplified sequence.

The insert were then transferred from p221 to pR81 plasmid containing GFP gene at the N terminal cloning site. Finally the obtained pR81 PR10::GFP plasmids were transferred into *Agrobacterium tumefaciens* strains.

Leaves of tobacco plants cultivated for 3 weeks in growth chambers were infiltrated with suspension of p81 PR10 GFP-transformed *A. tumefaciens* strains that were grown in YEB liquid medium for 72 h at 30°C. The GFP fluorescence was then observed using confocal microscopy (Zeiss LSM 880) for 24 hours after transformation inside tobacco leaves.

Part III. Results

1. PR10s show two distinct phylogenetic branches in *Medicago truncatula*

A study on the control of nodule immunity and senescence during *M. truncatula* symbiosis [10] has already highlighted and described some *PR10* genes. Here, 34 gene members of PR10 family were identified in *M. truncatula*'s genome. To understand the evolution of *PR10* genes in *M. truncatula*, phylogenetic analysis was performed on corresponding PR10 proteins and revealed the presence of two distinct phylogenetic branches (Figure 1). The first phylogenetic branch, named phylogenetic branch I (PBI; in green), includes genes located on chromosomes 2, 3, 4, and 6. Conversely, the second branch, named phylogenetic branch II (PBII; in blue) displays genes located on chromosomes 1 and 8. Interestingly, no genes encoding PR10s were found on chromosomes 5 and 7. This result indicates the presence of two distinct phylogenetic branches of PR10s in *M. truncatula*'s genome.

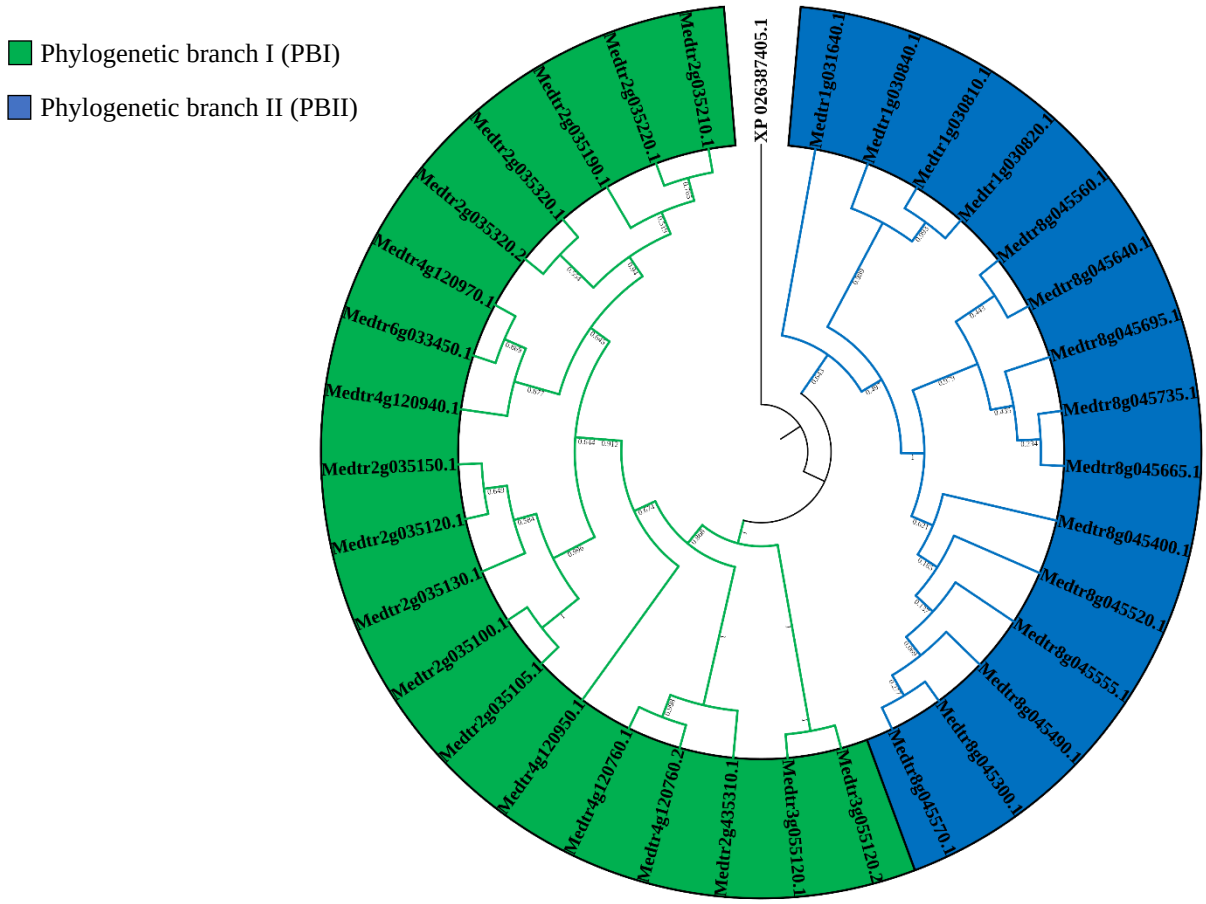


Figure 1. Phylogenetic tree analysis of PR10 reveals two distinct branches. The evolutionary history of the 34 identified PR10 proteins was obtained using the maximum likelihood method and the JTT matrix-based model [109]. The tree with the highest log likelihood (-5641.69) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 35 amino acid sequences. There was a total of 189 positions in the final dataset. Evolutionary analysis was conducted using the MEGA 11 software [108] and the Interactive Tree of Life (iTOL) [111] website to enhance the appearance of the phylogenetic tree and give its final layout.

2. PR10s belonging to PBI are stimulated during nodule defenses response

To study which of the identified PR10s are the most induced during nodule defense responses, the RNA-seq analysis, previously generated by the Ratet Lab (unpublished data), was performed. The RNA-seq analysis was carried out on nodules collected from the wild type (WT) of *M. truncatula* plants and the mutant *symCRK* that develops nodules showing defense responses [94, 98]. RNA-seq data reveals that genes belonging to the PBI are mostly up

regulated in nodules of *symCRK* compared to the WT (Figure 2A), whereas genes of the PBII are mainly not stimulated or even repressed in *symCRK* compared to the WT. These observations suggest that PBI genes are preferentially stimulated during nodule defenses compared to PBII genes.

To confirm this hypothesis, Reverse Transcription (RT)-qPCR analysis was performed on nodules collected from WT and two mutants producing defense response in the nodules, *symCRK* and *dnf2*. The analysis revealed that genes *Medtr2g03210*, *Medtr2g035190*, *Medtr2g035320*, *Medtr2g035520* and *Medtr4g120940* belonging to PBI genes are significantly induced (Figure 2B) in *symCRK* and *dnf2* nodules compared to the WT, whereas the genes Medtr8g045590, Medtr8g045560, Medtr8g045570 and Medtr8g045640 from the PBII show no significant variations in the studied mutants compared to the WT (Figure 2C). Together, these data indicate that during the defense responses within nodules, PR10s from PBI are more stimulated compared to those belonging to PBII.

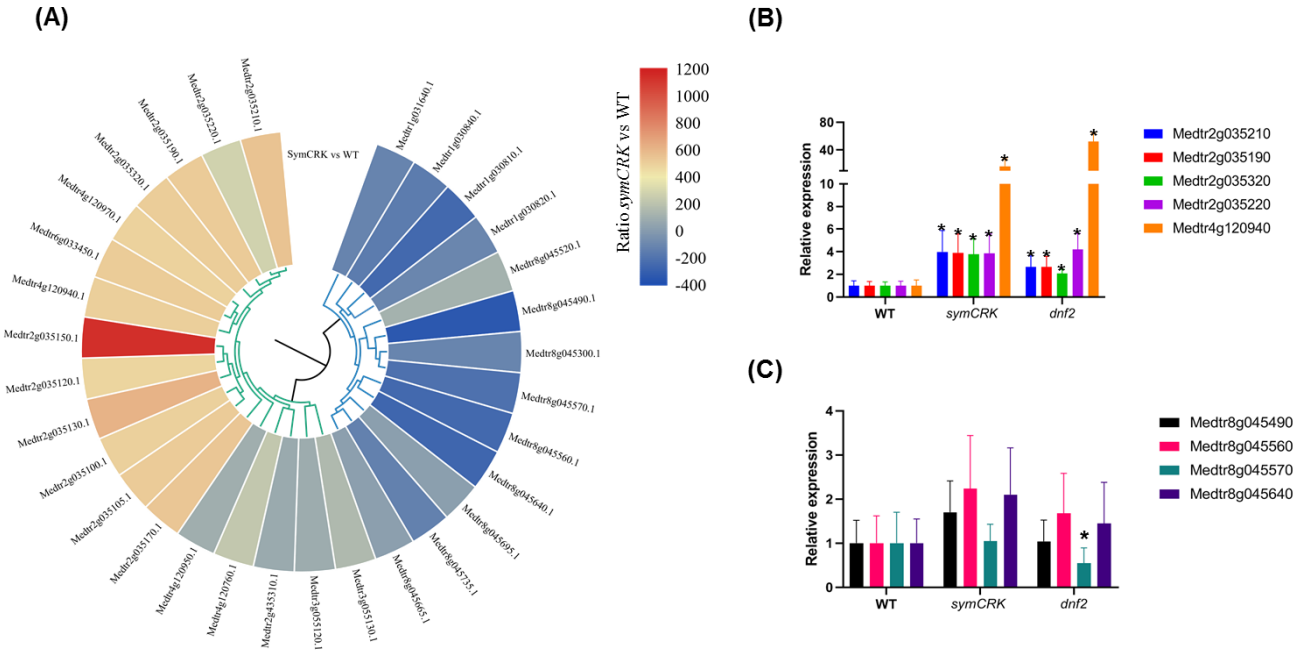


Figure 2. PR10s of PBI are stimulated during nodule defense responses. (A) RNA-seq analysis reveals the upregulation of PR10s from PBI in symCRK VS. WT nodules. Data were generated from *M. truncatula* nodules at 21 days post inoculation (dpi) with *Sinorhizobium medicae 419*. Eight plants were analyzed from one biological repetition. (B) The expression analysis confirm the upregulation of PR10 of PBI in the nodules showing defense responses. (C) PR10s from PBII don't show a significant expression variation in *symCRK* and *dnf2* nodules compared to the WT. The expression analysis was performed on WT, *symCRK* and *dnf2* nodules at 21-dpi with *S. medicae 419*. Three biological repetitions of nodules collected from eight plants for each repetition were analyzed. The error bar is represented by the standard error (SE). The asterisk shows the significant variation (p-value <5%) using the Mann-Whitney Test [122].

3. PR10s show tandem gene duplication.

To further characterize the *PR10* gene family in the model legume *M. truncatula*'s genome, analysis and visualization of gene collinearity, location, and organization were assessed using the MCScanX algorithm and TBtools' built-in advanced Circos plugin. The obtained results revealed that PR10s were unevenly distributed across six *M. truncatula* chromosomes, along with various collinear gene pairs connected by gray curves, and spread across all 8 chromosomes (1–8). A total of three collinear *PR10* gene pairs are connected by red curves and were found to be distributed across chromosomes 2 and 4 (Figure 3). Interestingly, these three *PR10* gene pairs which correspond to Medtr2g035100.1-Medtr4g120970.1, Medtr2g035130.1-Medtr4g120940.1, and Medtr2g435310.1-Medtr4g120760.2 present potential collinear blocks. In addition to that, these genes mostly show an upregulation compared to the other PR10s, indicating that these PR10s were potentially subjected to tandem gene duplication and transfer, possibly, due to their conservation of important PR10 protein functions.

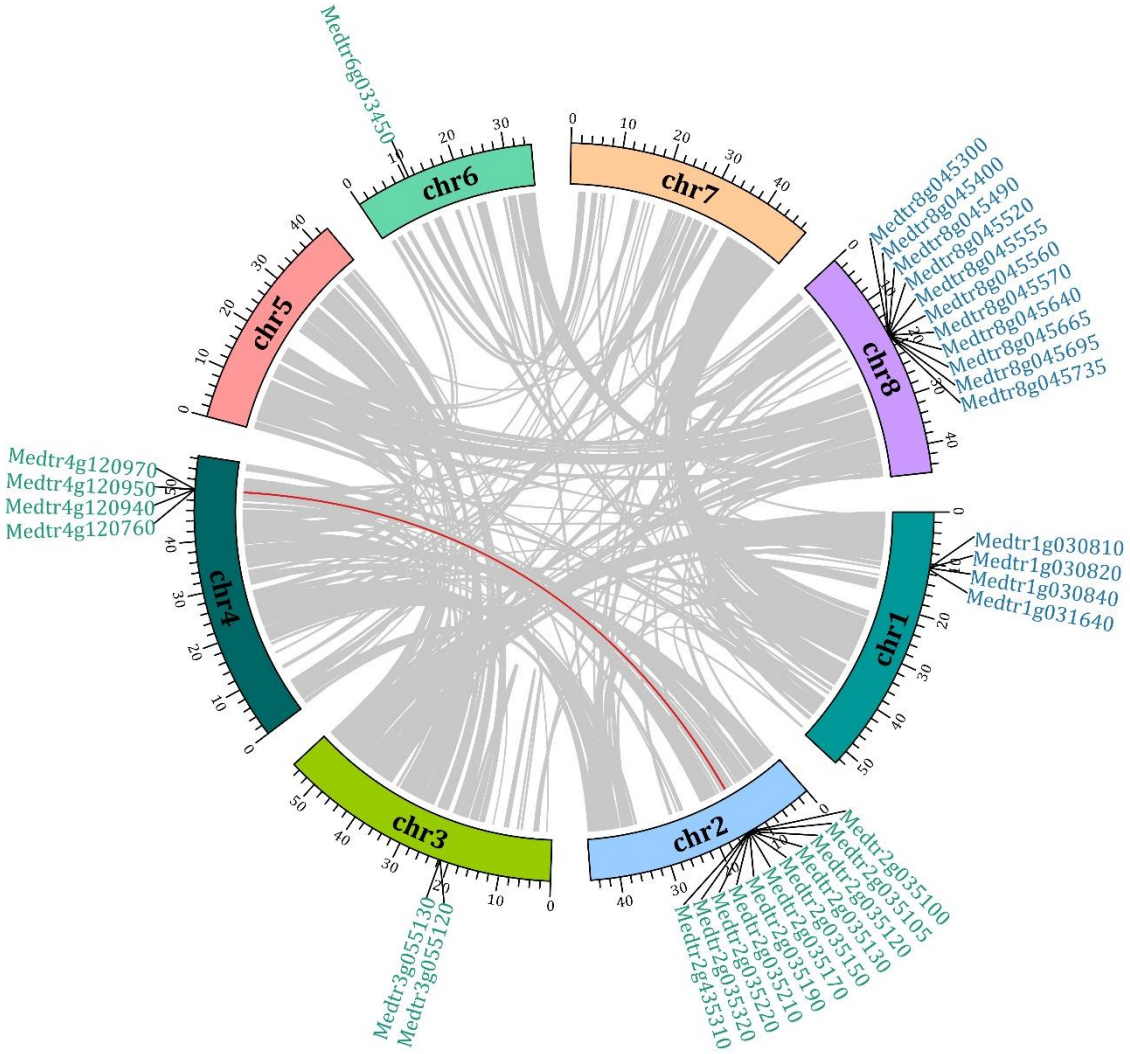


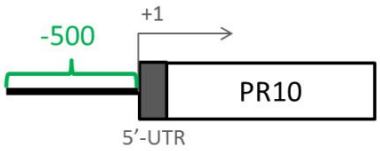
Figure 3. Circular chromosomal distribution and collinearity of *PR10* genes in *M. truncatula* show *PR10* gene location and duplication on chromosomes. Collinearity analysis was conducted using the MCScanX algorithm, where the e-value was set to 1e-5 to determine the significance of sequence matches [113, 114]. The obtained gene-linked information file was then modified by changing the RGB color of the linked genes of interest (PR10s) to a red color, which are shown in the graph to be connected by red curves. A common gray color was picked for the rest of the linked genes that are connected by the gray curves. The files used for the final circular chromosomal distribution and collinearity diagram were the modified gene linked information file for visualizing collinearity, the chromosome length file for the visualization of chromosomes and their lengths, and the genome feature list, which had the names of *PR10* genes alongside their positions on chromosomes. The advanced Circos plugin in TBtools was then used to visualize the results, revealing an organization of eight chromosomes of different sizes and various collinear gene pairs connected by gray curves and spread all across these chromosomes. Additionally, our findings also show a wide distribution of PR10s across six chromosomes (Chr1-4, 6, and 8), which appear to be subjected to tandem duplication. Among this distribution, a total of three *PR10* gene pairs in chromosomes 2 and 4 seem to be connected by red curves (Medtr2g035100-Medtr4g120970; Medtr2g035130-Medtr4g120940; and Medtr2g435310-Medtr4g120760), meaning that these PR10s present potential collinear blocks.

4. PR10s of PBI show binding sites for the transcription factors bZIP, NAC and Myb on their up-stream 5'-UTR region.

The upregulation of PR10s belonging to PBI during nodule defense responses can be explained by a conservation of important cis-regulatory elements in the corresponding promoter between PBI members, which leads to the recruitment of the same transcription factors during the immune response. Accordingly, *PR10* genes show tandem gene duplication in the genome of *M. truncatula*, which could affect the coding and the promoter sequence. To study the hypothesis of the conservation cis-elements, multiple sequence alignments using the ClustalW algorithm was performed on the 500 nucleotides up-stream the 5'-UTR region (Figure 4A) on multiple PBI *PR10* genes (Medtr2g035100, Medtr2g035105, Medtr2g035130, and Medtr2g035150). The analysis revealed a high degree of conservation of the nucleotide sequence between the -1 and -238 nucleotides in the studied PR10s (Figure 4B). Interestingly, analysis of the conserved sequences of *PR10* genes from PBI and the corresponding conserved sequences of PBII *PR10* genes using the *Arabidopsis thaliana* PlantPAN 4.0 database [123] revealed the presence of 252 binding sites of transcription factors for PBI, whereas only 133 sites were found on similar regions for PBII (Figure 4C). The prediction of transcription factors regulating PBI revealed the bZIP, NAC, and Myb transcription factors, which show numerous binding regions with respectively 95, 36, and 16 different sites. Conversely, PBII members displayed only two binding sites for each of the three transcription factors.

Together, these observations suggest a conservation of cis-elements between different *PR10* genes belonging to PBI, with the transcription factors bZIP, NAC, and Myb as potential activators of these genes during nodule defense responses.

(A)



(C)

	PPBI	PPBII
Total sites	252	133
bZIP	95	2
NAC	36	2
Myb	16	2
AP2-B3	2	5
bHLH	5	2

(B)

Figure 4. Sequence analysis of the 500 nucleotide upstream region of PR10s revealed a conservation of putative Cis-elements. A sequence alignment of 500 nucleotides up-stream the 5'-UTR region (A) revealed a high conservation of -238 nucleotides (B). Moreover, prediction analysis of the cis-elements using PlantPAN 4.0 revealed numerous binding sites for the bZIP, NAC, and Myc transcription factors of PR10s belonging to PBI, whereas few sites were identified for PR10s of PBII for the same region (C).

5. PR10s bind to phenolic compounds

It was previously reported that PR10 proteins have a role in the synthesis of phenolic compounds during plant defenses [26, 27, 54, 124]. The mutants showing impaired defense responses in nodules, such as *symCRK*, display an accumulation of phenols inside the symbiotic organ, nodules. We thus investigated the potential role of *M. truncatula*'s PR10s in the biosynthesis of phenolic compounds during nodule defense responses.

As a first approach, we examined the binding ability of the two phylogenetic branches of PR10 proteins, PBI and PBII, to various phenolic compounds. The interaction site for each PR10 protein was geometrically determined by XYZ coordinates within a grid box measuring 40*40*40 Å. Results in Table 2 summarize the binding affinity of PR10 proteins belonging to

PBI (Medtr4g120970, Medtr2g035150) and PBII (Medtr1g030810, Medtr1g030840, Medtr8g045570, and Medtr8g045560).

All tested PR10 proteins show an important binding affinity (binding value > 5), with the two PBI proteins, Medtr2g035150 and Medtr4g120970, showing the highest binding values (binding value > 8.5). These results suggest that PBI PR10s have the ability to bind phenolic compounds with the highest affinity for PBI compared to PBII.

Table 2. Binding affinity analysis of PR10s to phenolic compounds.

Proteins / Molecules	Medtr1g030810	Medtr1g030840	Medtr8g045560	Medtr8g045570	Medtr2g035150	Medtr4g120970
Apigenin	6	6.1	6.9	6.6	9.6	9.2
Daidzein	5.7	5.9	6.6	6.9	9.2	9.6
Flavone	5.8	7.1	7.1	6.6	9.6	9.6
Genistein	5.5	5.9	6.9	6.9	9.1	9.6
Naringenin	6.1	6.1	6.9	6.7	9.8	9.4
Quercetin	5.8	6.3	7	7.3	9.7	9.2
Resveratrol	5.4	5.9	5.6	6.1	8.8	8.5

To further investigate the phenol binding ability of PR10 proteins of interest, the amino acid sequences involved in the interaction between PR10 and the phenolic compound quercetin were identified (Figure 5). The results show a high conservation of three key amino acids, Tyr120, Lys 139, and Ile 85, which are involved in the binding of PR10s from PBI to quercetin, whereas PR10s from PBII require different amino acids when interacting with the studied phenol.

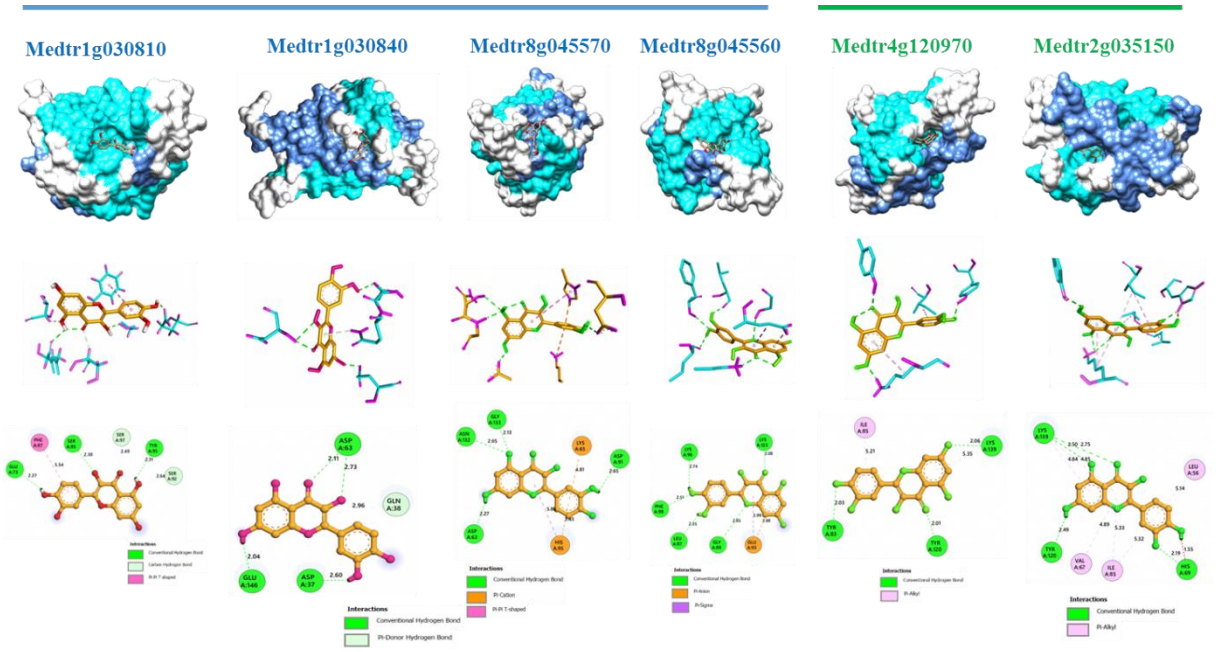


Figure 5. The molecular interaction between PR10 proteins and quercetin. The 3-dimensional view of the interaction between PR10 proteins belonging to PBI (Medtr4g12970 and Medtr2g035150) and PBII (Medtr1g030810, Medtr1g030840, Medtr8g045570, and Medtr8g045560) with quercetin. The lower part corresponds to the key amino acids that display putative interactions with quercetin.

6. The nodules of *symCRK* display cell death

PR10s are known for their role in the activation of programmed cell death [27, 101, 125]. In order to evaluate the potential impact of the over-expression of *PR10s* during the activation of the host's PCD in nodules, aniline blue staining was performed on nodule slices obtained from WT or *symCRK* plants cultivated *in vitro* after 14 dpi with *S. medicae WSM419*. *symCRK* shows a high accumulation of aniline blue compared to the WT (Figure 6A). The analysis of cell types (infected and non-infected) that show the highest accumulation rate of the stain revealed that the infected cell types accumulate the aniline blue stain more than the non-infected ones. These data indicate that the mutant *symCRK*, which displays an upregulation of PR10s from PBI, also displays an increased death of infected cells.

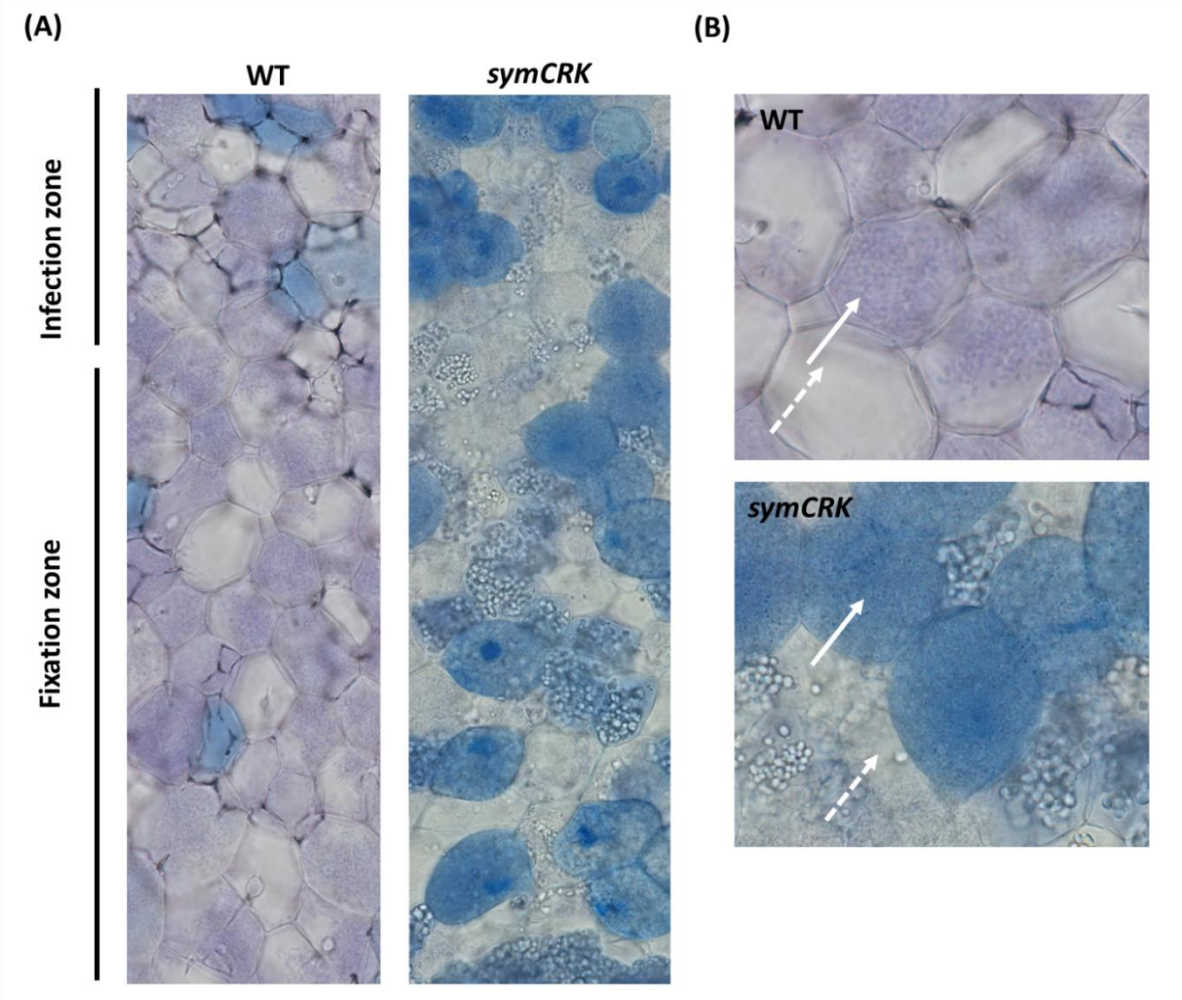


Figure 6. *symCRK* mutants show the death of infected cells compared to the WT. The analysis of aniline blue accumulation (which stains dead cells in blue) between the WT and the mutant *symCRK* nodule slices reveals a higher accumulation of the stain in *symCRK*'s infected cells compared to the WT.

7. PR10s of PBI display a conservation of RNase domains

Multiple studies have shown that the ability of PR10 proteins to induce cell death can be linked to their RNase activity [24, 27, 124, 126]. To evaluate the potential conservation of RNase activity among the studied PR10s, the identification of NTPase and the ribonuclease motifs was assessed on PR10 proteins belonging to PBI (Figure 7A) and PBII (Figure 7B). The results indicate that PR10s from PBI show a conservation of canonical motifs of both the NTPase and the ribonuclease domains, whereas PR10s from the second branch, PBII, show a loss of this conservation. These results suggest that PR10s from PBI display a conservation of the RNase activity and could potentially be responsible for the activation of cell death during nodule defense responses.

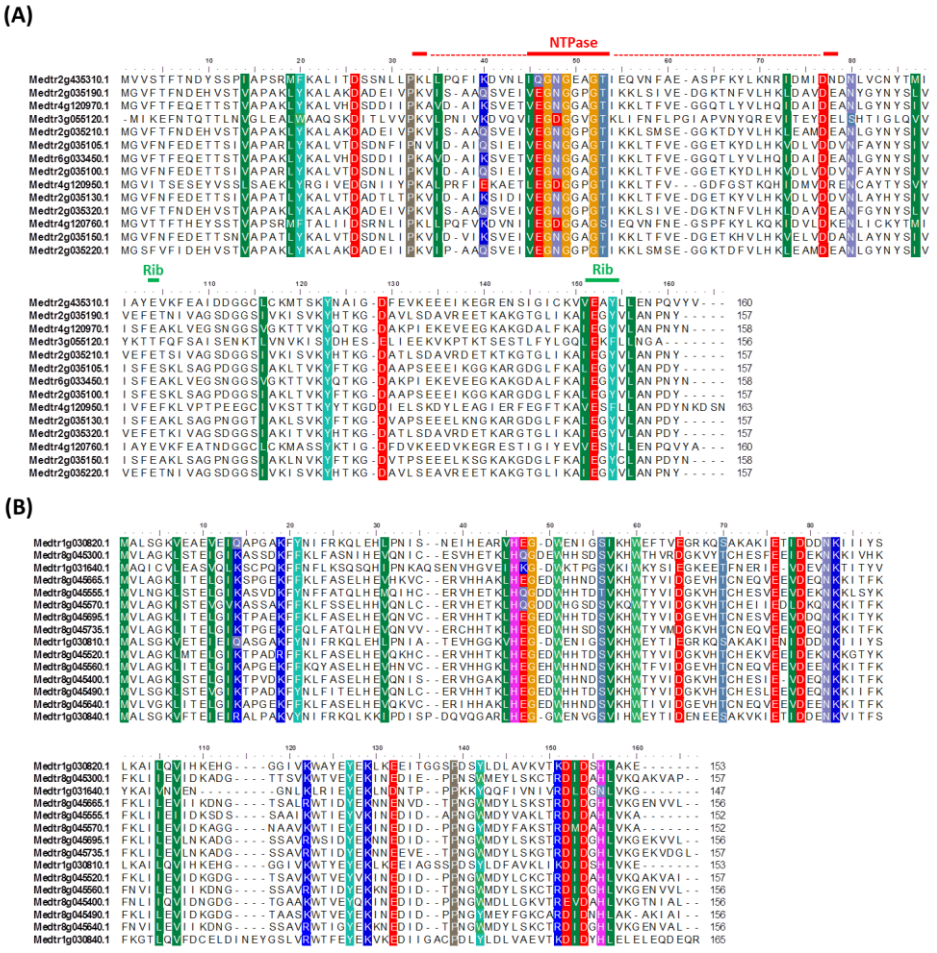


Figure 7. PR10s of PBI show a conservation of NTPase and ribonuclease domains. Multiple sequence alignments using the ClustalW algorithm reveal a conservation of the NTPase and ribonuclease motifs of PR10s belonging to PBI (A) and a loss of these motifs in PR10s from PBII (B).

8. PR10s are found in various places inside the cell

To identify the site of action of PR10s inside the symbiotic cells of nodules, an assessment of the location of PR10s was performed (Figure 8). As an initial step, protein enrichment data [127] was used for the detection of PR10 proteins from PBI (Figure 8A) and PBII (Figure 8B) inside fractionated WT and *symCRK* nodules. PR10 proteins were detected in three cell fractions: the symbiosome, the microsome, and the remaining proteins of the plant's cytosol fractions. These results reveal a higher accumulation of PBI PR10 proteins in *symCRK* nodules compared to the WT, while PBII proteins were less accumulated in both, *symCRK* and the WT nodules. The microsomal and the cytosol fractions were the most enriched fractions in the WT and *symCRK*. Interestingly, some PR10s were detected in the symbiosome fraction of *symCRK*, in contrast to the symbiosome fraction of the WT where no PR10 proteins were detected.

Furthermore, two PR10s (Medtr4g120970 and Medtr8g045665) were cloned and fused with the green fluorescent protein (GFP) at their N-terminal region, as demonstrated in Figure 8C. These constructs were then introduced and expressed inside tobacco cells. The subcellular localization of the GFP-tagged proteins was then analyzed, as shown in Figure 8D. Based on the fluorescence intensity, the PBI PR10, Medtr4g120970, showed a predominant presence in the cytoplasmic membrane, cytosol, and nuclear compartments compared to the PBII PR10.

Together, these data indicate that PR10s can be located in three main cell compartments: the cytoplasmic membrane, the cytosol, and the nucleus. Moreover, during nodule defense responses, these proteins can additionally be accumulated inside the symbiosome.

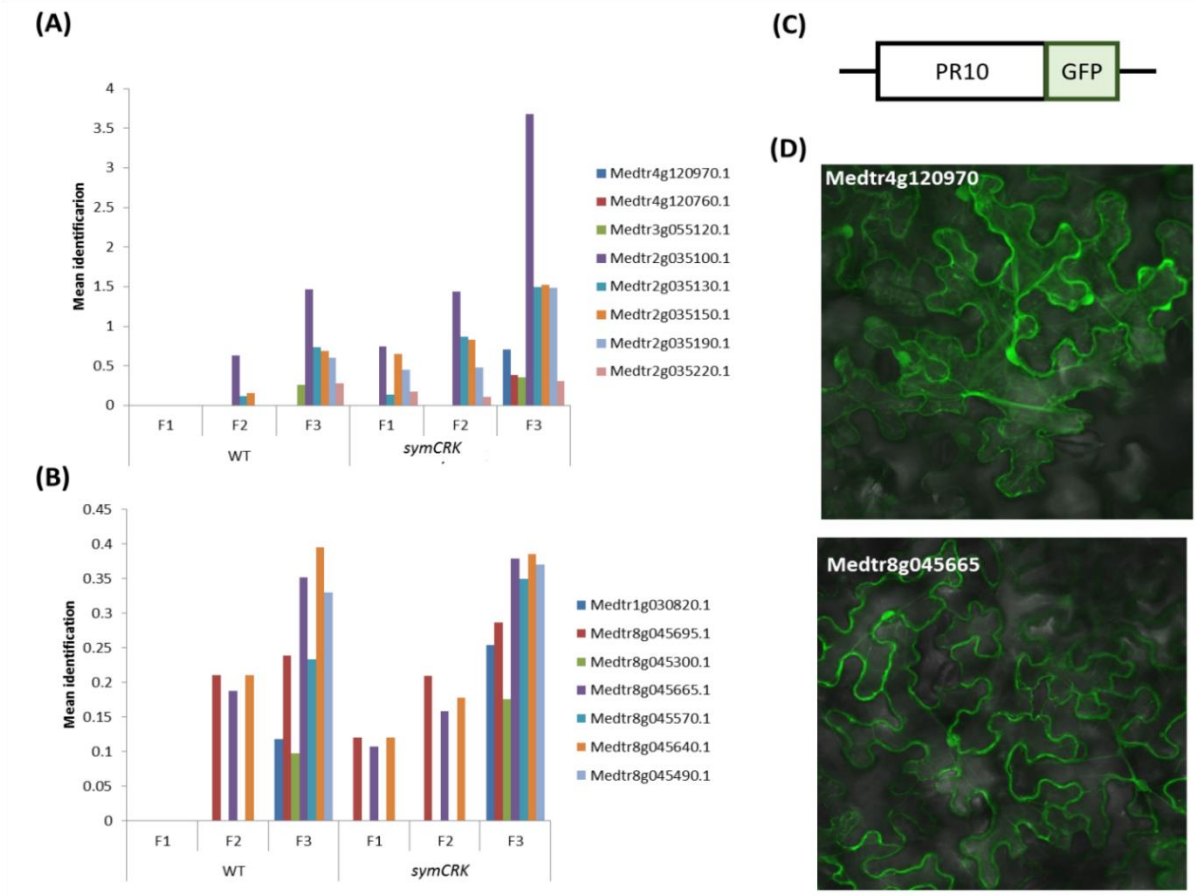


Figure 8. PR10s can be found in the cytoplasmic membrane, the cytosol, and the nucleus. Protein enrichment data of PR10s reveals the presence of PBI (A) and PBII (B) PR10 proteins in three nodule fractions: F1, which is the symbiosome fraction; F2, which consists of proteins extracted from the microsome; and F3, which includes the remaining soluble cytoplasmic proteins of the plant. The selected PR10s were fused with GFP at their N-terminal position (C), followed by cloning and expression in tobacco leaves. Subsequent fluorescence microscopy (D) reveals GFP signals within the cytoplasmic membrane, cytosol, and nuclear compartments of the two PR10 GFP-tagged proteins, with a prevalent abondance of the PBI PR10 protein.

Part IV. Discussion

The family of PR10 proteins is considered an important, unique, and highly conserved group of PR proteins distributed across a wide range of plants, primarily due to their various roles during the defense and development of plants [24, 48, 128]. These roles, along with the ribonuclease and ligand-binding activities of PR10s, have been assessed in numerous studies of various plant species [9, 26, 27, 48, 49, 57]. However, little is known about their role in the establishment and maintenance of legume nodules, as well as their diversity within this group of plants.

In this study, we characterized PR10s and investigated their role during legume symbiosis. We first conducted a phylogenetic analysis on different *PR10* genes found in *M. truncatula*, where the analysis revealed the presence of two phylogenetic branches (PB) of PR10s, PBI and PBII. PBI had 19 PR10s found on chromosomes 2, 3, 4, and 6, while PBII had 15 PR10s found on chromosomes 1 and 8. Similar results were found on a study of PR10s from the legume *Medicago sativa* [129], where a total of 29 PR10s were divided into two subclasses based on the size of their hydrophobic internal cavity and the number of β-bulges. Here, our findings suggested that PR10s of *M. truncatula* could have been a subject to potential gene duplication events, which could have possibly led to a functional divergence between the two groups. To further explore these possibilities, we analyzed the expression rate of the two PR10 groups during nodule defense responses in the WT and two mutants, *symCRK* and *dnf2*. Interestingly, the obtained results showed that *PR10* genes of PBI were predominantly upregulated in the nodules of the two mutants compared to the WT, whereas PR10s of PBII had no significant expression rate between the mutants and the WT. The two mutants, *symCRK* and *dnf2*, are known to have impaired immune defense responses due to mutations on key nodule immunity suppression genes (*NODULINS*) [91], and as a result, show an upregulation of defense markers inside nodules [10, 91, 100]. These findings indicate that PR10s of PBI are more stimulated compared to the ones of PBII during defense responses within nodules. This can be linked to the conservation of key PR10 functions and regulatory elements among PBI members, and that the wide distribution of *PR10* genes across the chromosomes could have also been the result of tandem gene duplication events. To test such a hypothesis, synteny and collinearity analyses were conducted on all *PR10* genes, and the findings demonstrated a distribution of PR10s across chromosomes 1-4, 6, and 8, while no PR10s were found on chromosomes 5 and 7. In addition to that, three *PR10* gene pairs belonging to PBI were found to show potential collinear blocks, meaning that these pairs have remained intact and in the same order and were potentially

subjected to tandem gene duplication and transfer events, a characteristic that has already been seen in many *PR10* genes from different plant species [52]. This could explain their upregulated expression compared to other PR10s, which could possibly be due to the conservation of key PR10 functional domains, which render them invaluable during nodule defense responses.

Moreover, multiple sequence alignments of the 500 nucleotides upstream region of PR10s were conducted to confirm the hypothesis that the upregulation of PBI PR10s could be related to the conservation of key regulatory elements (Cis-elements). The results revealed a high conservation of 238 nucleotides that were used on a prediction server to investigate for any transcription factors binding the putative cis-regulatory elements. The latter revealed 252 binding sites for bZIP, NAC, Myb, and other transcription factors (TFs) of PBI PR10s compared to 133 binding sites of TFs of PBII. These results clearly indicate a conservation and enrichment of binding sites for transcription factors of PBI PR10s, with the bZIP, NAC, and Myb transcription factors acting as putative regulators of PBI PR10s. bZIP TFs are a wildly known family of transcription factors that play numerous roles during the defense responses to biotic and abiotic stress [130]. Similar results were found in a study of PR10s in western white pine species, where cis-regulatory elements of PR10s were found to be recognized by the TF bZIP910 [131].

Multiple studies have shown that PR10 proteins can bind to a wide variety of ligands [26, 27, 49, 53]. Mutants, like *symCRK*, show an accumulation of phenolic compounds in nodules [93, 94], suggesting that PR10 proteins could potentially have a role in the biosynthesis of phenolic compounds during plant defense responses. Here, we investigated the binding activity and affinity of the two PR10 groups, PBI and PBII, and found that PBI members can bind more effectively to phenolic compounds compared to the second PR10 group (PBII). Furthermore, the analysis of the amino acid sequences involved in the interaction between PR10s of interest and quercetin revealed a conservation of the amino acids Tyr120, Lys 139, and Ile 85, which are essential for their binding, while PR10s from PBII require different amino acids when interacting with the studied phenol. Together, these results indicate that PR10s of PBI are more functional and more employed during the synthesis of phenolic compounds and the overall nodule defense responses compared to the second group of PR10s (PBII).

The ability of PR10s to activate PCD has been demonstrated in many studies [24, 25, 27]. To evaluate this ability during the activation of PCD in the nodules of *symCRK*, a staining procedure using aniline blue stain was conducted on the nodules of the WT and the mutant *symCRK*. The results showed a high accumulation of the stain in the nodules of the mutant

compared to the WT. This demonstrates that the *symCRK* nodules display a higher cell death rate, highlighting the role of the symCRK gene in inducing, through signaling cascades, the synthesis of PR10 proteins [10, 94], that sequentially activate PCD through the synthesis and accumulation of phenolic compounds in nodules. Additionally, PR10 proteins have been shown to induce PCD through their ribonuclease activity [24, 27, 124, 126]. To investigate the conservation of the RNase activity between PR10s of PBI and PBII, multiple sequence alignments and the identification of the NTPase and the ribonuclease motifs were done on the two PR10 groups. Interestingly, the findings illustrated a conservation of the canonical motifs of both the NTPase and the ribonuclease domains of PR10s belonging to PBI, in contrast to PBII PR10s, which show a loss of this conservation. Together, these findings prove that the induction of PCD during nodule defense responses is most likely caused by the RNase activity of PR10 proteins belonging to PBI and that these PR10s are primarily recruited by cells compared to PBII members, notably due to the high conservation of key motifs that are important for various PR10 protein functions.

Protein enrichment data of PR10 proteins inside the symbiotic cells of fractionated nodules have revealed that PR10s can be found in all the three nodule fractions of *symCRK*, the symbiosome, the microsome, and the remaining plant cytosol protein fractions, with PBI members as the prevalent detected PR10 protein group compared to PBII, conversely to the WT, where no PR10 proteins were detected in the symbiosome fraction, suggesting that during nodule defense responses, PR10 proteins can accumulate in the symbiosome, and could directly compromise the survival of bacterial symbiotes inside it. Additionally, the subcellular localization of two PR10 proteins, one from PBI and the other from PBII, highlight the abondance of the two in the cytoplasmic membrane, cytosol, and nuclear compartments, which concords with multiple research findings [24, 56, 132], with the PBI PR10 showing more abundance compared to the other PBII PR10. Altogether, all the obtained results show that PR10s of PBI are more conserved and highly functional compared to the second PR10 group of PBII.

Conclusion

In conclusion, this work highlights the role of PR10 proteins during legume symbiosis. We first identified a total of 34 *PR10* genes in the model legume, *Medicago truncatula*, which were divided into two distinct groups (PBI and PBII) based on phylogenetic analysis. To gain a better understanding of the functional conservation between the two groups, we investigated their chromosomal distribution, expression rate, collinearity, phenolic compound binding activities, and conservation of their cis-regulatory elements and functional motifs, which revealed that the first branch of *PR10* genes was the most expressed, functional, and conserved compared to the second branch. Moreover, the protein detection data and the subcellular localization of PR10s between the WT and mutants with impaired nodule immunity genes showed that PR10 proteins belonging to PBI can be found in the cytoplasmic membrane, cytosol, nuclear compartments, and additionally, in the symbiosome, where they directly comprise the survival of symbiotes inside nodules during nodule defense responses. Further studies are needed to determine the cells expressing *PR10* genes, their binding to different phenols inside cells, the subcellular localization of PR10 proteins inside nodule cells, and the functional diversity of the second PR10 group.

References

1. Vishwakarma, K., Kumar, N., Shandilya, C., Mohapatra, S., Bhayana, S., Varma, A.: Revisiting Plant–Microbe Interactions and Microbial Consortia Application for Enhancing Sustainable Agriculture: A Review. Front Microbiol. 11, (2020). https://doi.org/10.3389/fmicb.2020.560406

2. Li, J., Wang, C., Liang, W., Liu, S.: Rhizosphere Microbiome: The Emerging Barrier in Plant-Pathogen Interactions. Front Microbiol. 12, (2021). https://doi.org/10.3389/fmicb.2021.772420

3. Berg, G.: The plant microbiome and its importance for plant and human health. Front Microbiol. 5, (2014). https://doi.org/10.3389/fmicb.2014.00491

4. Newman, M.-A., Sundelin, T., Nielsen, J.T., Erbs, G.: MAMP (microbe-associated molecular pattern) triggered immunity in plants. Front Plant Sci. 4, (2013). https://doi.org/10.3389/fpls.2013.00139

5. Jamil, F., Mukhtar, H., Fouillaud, M., Dufossé, L.: Rhizosphere Signaling: Insights into Plant–Rhizomicrobiome Interactions for Sustainable Agronomy. Microorganisms. 10, 899 (2022). https://doi.org/10.3390/microorganisms10050899

6. Wan, J., He, M., Hou, Q., Zou, L., Yang, Y., Wei, Y., Chen, X.: Cell wall associated immunity in plants. Stress Biology. 1, 3 (2021). https://doi.org/10.1007/s44154-021-00003-4

7. Newman, M.-A., Sundelin, T., Nielsen, J.T., Erbs, G.: MAMP (microbe-associated molecular pattern) triggered immunity in plants. Front Plant Sci. 4, (2013). https://doi.org/10.3389/fpls.2013.00139

8. Nishad, R., Ahmed, T., Rahman, V.J., Kareem, A.: Modulation of Plant Defense System in Response to Microbial Interactions. Front Microbiol. 11, (2020). https://doi.org/10.3389/fmicb.2020.01298

9. Fang, Y., Gu, Y.: Regulation of Plant Immunity by Nuclear Membrane-Associated Mechanisms. Front Immunol. 12, (2021). https://doi.org/10.3389/fimmu.2021.771065

10. Berrabah, F., Bernal, G., Elhosseyn, A.-S., El Kassis, C., L'Horset, R., Benaceur, F., Wen, J., Mysore, K.S., Garmier, M., Gourion, B., Ratet, P., Gruber, V.: Insight into the control of nodule immunity and senescence during *Medicago truncatula* symbiosis. Plant Physiol. 191, 729–746 (2023). https://doi.org/10.1093/plphys/kiac505

11. Wang, S., Boevink, P.C., Welsh, L., Zhang, R., Whisson, S.C., Birch, P.R.J.: Delivery of cytoplasmic and apoplastic effectors from *Phytophthora infestans* haustoria by distinct secretion pathways. New Phytologist. 216, 205–215 (2017). https://doi.org/10.1111/nph.14696

12. Uhse, S., Djamei, A.: Effectors of plant-colonizing fungi and beyond. PLoS Pathog. 14, e1006992 (2018). https://doi.org/10.1371/journal.ppat.1006992

13. Büttner, D., He, S.Y.: Type III Protein Secretion in Plant Pathogenic Bacteria. Plant Physiol. 150, 1656–1664 (2009). https://doi.org/10.1104/pp.109.139089

14. Zboralski, A., Biessy, A., Filion, M.: Bridging the Gap: Type III Secretion Systems in Plant-Beneficial Bacteria. Microorganisms. 10, 187 (2022). https://doi.org/10.3390/microorganisms10010187

15. Polonio, Á., Pérez-García, A., Martínez-Cruz, J., Fernández-Ortuño, D., de Vicente, A.: The Haustorium of Phytopathogenic Fungi: A Short Overview of a Specialized Cell of Obligate Biotrophic Plant Parasites. Presented at the (2020)

16. Oliveira-Garcia, E., Tamang, T.M., Park, J., Dalby, M., Martin-Urdiroz, M., Rodriguez Herrero, C., Vu, A.H., Park, S., Talbot, N.J., Valent, B.: Clathrin-mediated endocytosis facilitates the internalization of *Magnaporthe oryzae* effectors into rice cells. Plant Cell. 35, 2527–2551 (2023). https://doi.org/10.1093/plcell/koad094

17. Van Ghelder, C., Parent, G.J., Rigault, P., Prunier, J., Giguère, I., Caron, S., Stival Sena, J., Deslauriers, A., Bousquet, J., Esmenjaud, D., MacKay, J.: The large repertoire of conifer NLR resistance genes includes drought responsive and highly diversified RNLs. Sci Rep. 9, 11614 (2019). https://doi.org/10.1038/s41598-019-47950-7

18. Malinovsky, F.G., Fangel, J.U., Willats, W.G.T.: The role of the cell wall in plant immunity. Front Plant Sci. 5, (2014). https://doi.org/10.3389/fpls.2014.00178

19. Naveed, Z.A., Wei, X., Chen, J., Mubeen, H., Ali, G.S.: The PTI to ETI Continuum in Phytophthora-Plant Interactions. Front Plant Sci. 11, (2020). https://doi.org/10.3389/fpls.2020.593905

20. Li, N., Lin, Z., Yu, P., Zeng, Y., Du, S., Huang, L.-J.: The multifarious role of callose and callose synthase in plant development and environment interactions. Front Plant Sci. 14, (2023). https://doi.org/10.3389/fpls.2023.1183402

21. Ušák, D., Haluška, S., Pleskot, R.: Callose synthesis at the center point of plant development—An evolutionary insight. Plant Physiol. 193, 54–69 (2023). https://doi.org/10.1093/plphys/kiad274

22. Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M., Bakker, P.A.H.M.: Induced Systemic Resistance by Beneficial Microbes. Annu Rev Phytopathol. 52, 347–375 (2014). https://doi.org/10.1146/annurev-phyto-082712-102340

23. Hönig, M., Roeber, V.M., Schmülling, T., Cortleven, A.: Chemical priming of plant defense responses to pathogen attacks. Front Plant Sci. 14, (2023). https://doi.org/10.3389/fpls.2023.1146577

24. Lopes, N. dos S., Santos, A.S., de Novais, D.P.S., Pirovani, C.P., Micheli, F.: Pathogenesis-related protein 10 in resistance to biotic stress: progress in elucidating functions, regulation and modes of action. Front Plant Sci. 14, (2023). https://doi.org/10.3389/fpls.2023.1193873

25. Islam, Md.M., El-Sappah, A.H., Ali, H.M., Zandi, P., Huang, Q., Soaud, S.A., Alazizi, E.M.Y., Wafa, H.A., Hossain, Md.A., Liang, Y.: Pathogenesis-related proteins (PRs) countering environmental stress in plants: A review. South African Journal of Botany. 160, 414–427 (2023). https://doi.org/10.1016/j.sajb.2023.07.003

26. Kumar Sinha, R., Shankar Verma, S., Rastogi, A.: Role of Pathogen-Related Protein 10 (PR 10) under Abiotic and Biotic Stresses in Plants. Phyton (B Aires). 89, 167–182 (2020). https://doi.org/10.32604/phyton.2020.09359

27. dos Santos, C., Franco, O.L.: Pathogenesis-Related Proteins (PRs) with Enzyme Activity Activating Plant Defense Responses. Plants. 12, 2226 (2023). https://doi.org/10.3390/plants12112226

28. Anisimova, O.K., Shchennikova, A. V., Kochieva, E.Z., Filyushin, M.A.: Pathogenesis-Related Genes of PR1, PR2, PR4, and PR5 Families Are Involved in the Response to Fusarium Infection in Garlic (Allium sativum L.). Int J Mol Sci. 22, 6688 (2021). https://doi.org/10.3390/ijms22136688

29. Ali, S., Ganai, B.A., Kamili, A.N., Bhat, A.A., Mir, Z.A., Bhat, J.A., Tyagi, A., Islam, S.T., Mushtaq, M., Yadav, P., Rawat, S., Grover, A.: Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiol Res. 212–213, 29–37 (2018). https://doi.org/10.1016/j.micres.2018.04.008

30. Kaur, A., Kaur, S., Kaur, A., Kaur Sarao, N., Sharma, D.: Pathogenesis-Related Proteins and Their Transgenic Expression for Developing Disease-Resistant Crops: Strategies Progress and Challenges. In: Case Studies of Breeding Strategies in Major Plant Species. IntechOpen (2023)

31. Antoniw, J.F., Ritter, C.E., Pierpoint, W.S., Van Loon, L.C.: Comparison of Three Pathogenesis-related Proteins from Plants of Two Cultivars of Tobacco Infected with TMV. Journal of General Virology. 47, 79–87 (1980). https://doi.org/10.1099/0022-1317-47-1-79

32. Ali, S., Ganai, B.A., Kamili, A.N., Bhat, A.A., Mir, Z.A., Bhat, J.A., Tyagi, A., Islam, S.T., Mushtaq, M., Yadav, P., Rawat, S., Grover, A.: Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiol Res. 212–213, 29–37 (2018). https://doi.org/10.1016/j.micres.2018.04.008

33. Van Loon, L.C.: Regulation of Changes in Proteins and Enzymes Associated with Active Defence against Virus Infection. In: Active Defense Mechanisms in Plants. pp. 247–273. Springer US, Boston, MA (1982)

34. Melchers, L.S., Groot, M.A., Knaap, J.A., Ponstein, A.S., Sela-Buurlage, M.B., Bol, J.F., Cornelissen, B.J.C., Elzen, P.J.M., Linthorst, H.J.M.: A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. The Plant Journal. 5, 469–480 (1994). https://doi.org/10.1046/j.1365-313X.1994.05040469.x

35. Métraux, J.P., Streit, L., Staub, Th.: A pathogenesis-related protein in cucumber is a chitinase. Physiol Mol Plant Pathol. 33, 1–9 (1988). https://doi.org/10.1016/0885-5765(88)90038-0

36. Green, T.R., Ryan, C.A.: Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects. Science (1979). 175, 776–777 (1972). https://doi.org/10.1126/science.175.4023.776

37. Vera, P., Conejero, V.: Pathogenesis-Related Proteins of Tomato. Plant Physiol. 87, 58–63 (1988). https://doi.org/10.1104/pp.87.1.58

38. Lagrimini, L.M., Burkhart, W., Moyer, M., Rothstein, S.: Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: Molecular analysis and tissue-specific expression. Proceedings of the National Academy of Sciences. 84, 7542–7546 (1987). https://doi.org/10.1073/pnas.84.21.7542

39. Somssich, I.E., Schmelzer, E., Bollmann, J., Hahlbrock, K.: Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. Proceedings of the National Academy of Sciences. 83, 2427–2430 (1986). https://doi.org/10.1073/pnas.83.8.2427

40. Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N. V, Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J.: Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell. 7, 573–588 (1995). https://doi.org/10.1105/tpc.7.5.573

41. Epple, P., Apel, K., Bohlmann, H.: An Arabidopsis thaliana Thionin Gene Is Inducible via a Signal Transduction Pathway Different from That for Pathogenesis-Related Proteins. Plant Physiol. 109, 813–820 (1995). https://doi.org/10.1104/pp.109.3.813

42. García-Olmedo, F., Molina, A., Segura, A., Moreno, M.: The defensive role of nonspecific lipid-transfer proteins in plants. Trends Microbiol. 3, 72–74 (1995). https://doi.org/10.1016/S0966-842X(00)88879-4

43. Wei, Y., Zhang, Z., Andersen, C.H., Schmelzer, E., Gregersen, P.L., Collinge, D.B., Smedegaard-Petersen, V., Thordal-Christensen, H.: An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. Plant Mol Biol. 36, 101–112 (1998). https://doi.org/10.1023/A:1005955119326

44. Zhang, Z., Collinge, D.B., Thordal-Christensen, H.: Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. The Plant Journal. 8, 139–145 (1995). https://doi.org/10.1046/j.1365-313X.1995.08010139.x

45. Okushima, Y., Koizumi, N., Kusano, T., Sano, H.: Secreted proteins of tobacco cultured BY2 cells: Identification of a new member of pathogenesis-related proteins. Plant Mol Biol. 42, 479–488 (2000). https://doi.org/10.1023/A:1006393326985

46. Custers, J.H.H.V., Harrison, S.J., Sela-Buurlage, M.B., Van Deventer, E., Lageweg, W., Howe, P.W., Van Der Meijs, P.J., Ponstein, A.S., Simons, B.H., Melchers, L.S., Stuiver, M.H.: Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence. The Plant Journal. 39, 147–160 (2004). https://doi.org/10.1111/j.1365-313X.2004.02117.x

47. Sooriyaarachchi, S., Jaber, E., Covarrubias, A.S., Ubhayasekera, W., Asiegbu, F.O., Mowbray, S.L.: Expression and β-glucan binding properties of Scots pine (Pinus sylvestris L.) antimicrobial protein (Sp-AMP). Plant Mol Biol. 77, 33–45 (2011). https://doi.org/10.1007/s11103-011-9791-z

48. Feng, Y., Ren, Y., Zhang, H., Heng, Y., Wang, Z., Wang, Y.: Halostachys caspica pathogenesis-related protein 10 acts as a cytokinin reservoir to regulate plant growth and development. Front Plant Sci. 14, (2023). https://doi.org/10.3389/fpls.2023.1116985

49. Aglas, L., Soh, W.T., Kraiem, A., Wenger, M., Brandstetter, H., Ferreira, F.: Ligand Binding of PR-10 Proteins with a Particular Focus on the Bet v 1 Allergen Family. Curr Allergy Asthma Rep. 20, 25 (2020). https://doi.org/10.1007/s11882-020-00918-4

50. Wu, J., Kim, S.G., Kang, K.Y., Kim, J.-G., Park, S.-R., Gupta, R., Kim, Y.H., Wang, Y., Kim, S.T.: Overexpression of a Pathogenesis-Related Protein 10 Enhances Biotic and Abiotic Stress Tolerance in Rice. Plant Pathol J. 32, 552–562 (2016). https://doi.org/10.5423/PPJ.OA.06.2016.0141

51. Agarwal, P., Agarwal, P.K.: Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. Mol Biol Rep. 41, 599–611 (2014). https://doi.org/10.1007/s11033-013-2897-4

52. Liu, J.-J., Ekramoddoullah, A.K.M.: The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiol Mol Plant Pathol. 68, 3–13 (2006). https://doi.org/10.1016/j.pmpp.2006.06.004

53. Morris, J.S., Caldo, K.M.P., Liang, S., Facchini, P.J.: PR10/Bet v1-like Proteins as Novel Contributors to Plant Biochemical Diversity. ChemBioChem. 22, 264–287 (2021). https://doi.org/10.1002/cbic.202000354

54. Peng, Q., Su, Y., Ling, H., Ahmad, W., Gao, S., Guo, J., Que, Y., Xu, L.: A sugarcane pathogenesis-related protein, ScPR10, plays a positive role in defense responses under Sporisorium scitamineum, SrMV, SA, and MeJA stresses. Plant Cell Rep. 36, 1427–1440 (2017). https://doi.org/10.1007/s00299-017-2166-4

55. Breiteneder, H., Kraft, D.: The History and Science of the Major Birch Pollen Allergen Bet v 1. Biomolecules. 13, 1151 (2023). https://doi.org/10.3390/biom13071151

56. Feki, K., Tounsi, S., Jemli, S., Boubakri, H., Saidi, M.N., Mrabet, M., Brini, F., Mhadhbi, H.: Genome-wide identification of PR10 family members and expression profile analysis of PvPR10 in common bean (Phaseolus vulgaris L.) in response to hormones and several abiotic stress conditions. Plant Growth Regul. (2023). https://doi.org/10.1007/s10725-023-00997-z

57. Lebel, S., Schellenbaum, P., Walter, B., Maillot, P.: Characterisation of the Vitis viniferaPR10 multigene family. BMC Plant Biol. 10, 184 (2010). https://doi.org/10.1186/1471-2229-10-184

58. Andrade, L.B. da S., Oliveira, A.S., Ribeiro, J.K.C., Kiyota, S., Vasconcelos, I.M., de Oliveira, J.T.A., de Sales, M.P.: Effects of a Novel Pathogenesis-Related Class 10 (PR-10) Protein from Crotalaria pallida Roots with Papain Inhibitory Activity against Root-Knot Nematode Meloidogyne incognita. J Agric Food Chem. 58, 4145–4152 (2010). https://doi.org/10.1021/jf9044556

59. Wang, W., Nie, J., Lv, L., Gong, W., Wang, S., Yang, M., Xu, L., Li, M., Du, H., Huang, L.: A Valsa mali Effector Protein 1 Targets Apple (Malus domestica) Pathogenesis-Related 10 Protein to Promote Virulence. Front Plant Sci. 12, (2021). https://doi.org/10.3389/fpls.2021.741342

60. Xie, Y.-R., Chen, Z.-Y., Brown, R.L., Bhatnagar, D.: Expression and functional characterization of two pathogenesis-related protein 10 genes from Zea mays. J Plant Physiol. 167, 121–130 (2010). https://doi.org/10.1016/j.jplph.2009.07.004

61. Gómez-Gómez, L., Rubio-Moraga, A., Ahrazem, O.: Molecular cloning and characterisation of a pathogenesis-related protein CsPR10 from *Crocus sativus*. Plant Biol. 13, 297–303 (2011). https://doi.org/10.1111/j.1438-8677.2010.00359.x

62. Park, C., Kim, K., Shin, R., Park, J.M., Shin, Y., Paek, K.: Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. The Plant Journal. 37, 186–198 (2004). https://doi.org/10.1046/j.1365-313X.2003.01951.x

63. Colditz, F., Niehaus, K., Krajinski, F.: Silencing of PR-10-like proteins in Medicago truncatula results in an antagonistic induction of other PR proteins and in an increased tolerance upon infection with the oomycete Aphanomyces euteiches. Planta. 226, 57–71 (2007). https://doi.org/10.1007/s00425-006-0466-y

64. Babalola, O.O., Fadiji, A.E., Enagbonma, B.J., Alori, E.T., Ayilara, M.S., Ayangbenro, A.S.: The Nexus Between Plant and Plant Microbiome: Revelation of the Networking Strategies. Front Microbiol. 11, (2020). https://doi.org/10.3389/fmicb.2020.548037

65. Mendes, R., Garbeva, P., Raaijmakers, J.M.: The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev. 37, 634–663 (2013). https://doi.org/10.1111/1574-6976.12028

66. Maitra, P., Hrynkiewicz, K., Szuba, A., Jagodziński, A.M., Al-Rashid, J., Mandal, D., Mucha, J.: Metabolic niches in the rhizosphere microbiome: dependence on soil horizons, root traits and climate variables in forest ecosystems. Front Plant Sci. 15, (2024). https://doi.org/10.3389/fpls.2024.1344205

67. Dlamini, S.P., Akanmu, A.O., Babalola, O.O.: Rhizospheric microorganisms: The gateway to a sustainable plant health. Front Sustain Food Syst. 6, (2022). https://doi.org/10.3389/fsufs.2022.925802

68. Huey, C.J., Gopinath, S.C.B., Uda, M.N.A., Zulhaimi, H.I., Jaafar, M.N., Kasim, F.H., Yaakub, A.R.W.: Mycorrhiza: a natural resource assists plant growth under varied soil conditions. 3 Biotech. 10, 204 (2020). https://doi.org/10.1007/s13205-020-02188-3

69. Siddiqui, Z.A., Pichtel, J.: Mycorrhizae: An Overview. In: Mycorrhizae: Sustainable Agriculture and Forestry. pp. 1–35. Springer Netherlands, Dordrecht (2008)

70. Ma, J., Wang, W., Yang, J., Qin, S., Yang, Y., Sun, C., Pei, G., Zeeshan, M., Liao, H., Liu, L., Huang, J.: Mycorrhizal symbiosis promotes the nutrient content accumulation and affects the root exudates in maize. BMC Plant Biol. 22, 64 (2022). https://doi.org/10.1186/s12870-021-03370-2

71. Pandey, D., Kehri, H.K., Zoomi, I., Akhtar, O., Singh, A.K.: Mycorrhizal Fungi: Biodiversity, Ecological Significance, and Industrial Applications. Presented at the (2019)

72. Genre, A., Lanfranco, L., Perotto, S., Bonfante, P.: Unique and common traits in mycorrhizal symbioses. Nat Rev Microbiol. 18, 649–660 (2020). https://doi.org/10.1038/s41579-020-0402-3

73. Lopes, M.J. dos S., Dias-Filho, M.B., Gurgel, E.S.C.: Successful Plant Growth-Promoting Microbes: Inoculation Methods and Abiotic Factors. Front Sustain Food Syst. 5, (2021). https://doi.org/10.3389/fsufs.2021.606454

74. Besset-Manzoni, Y., Rieusset, L., Joly, P., Comte, G., Prigent-Combaret, C.: Exploiting rhizosphere microbial cooperation for developing sustainable agriculture strategies. Environmental Science and Pollution Research. 25, 29953–29970 (2018). https://doi.org/10.1007/s11356-017-1152-2

75. Abhilash, P.C., Dubey, R.K., Tripathi, V., Gupta, V.K., Singh, H.B.: Plant Growth-Promoting Microorganisms for Environmental Sustainability. Trends Biotechnol. 34, 847–850 (2016). https://doi.org/10.1016/j.tibtech.2016.05.005

76. Bekele, M., Yilma, G.: Nitrogen Fixation Using Symbiotic and Non-Symbiotic Microbes: A Review Article. Biochemistry and Molecular Biology. 6, 92 (2021). https://doi.org/10.11648/j.bmb.20210604.12

77. Goyal, R.K., Mattoo, A.K., Schmidt, M.A.: Rhizobial–Host Interactions and Symbiotic Nitrogen Fixation in Legume Crops Toward Agriculture Sustainability. Front Microbiol. 12, (2021). https://doi.org/10.3389/fmicb.2021.669404

78. Vats, S., Srivastava, P., Saxena, S., Mudgil, B., Kumar, N.: Beneficial Effects of Nitrogen-Fixing Bacteria for Agriculture of the Future. Presented at the (2021)

79. Mousavi, S.A., Willems, A., Nesme, X., de Lajudie, P., Lindström, K.: Revised phylogeny of Rhizobiaceae: Proposal of the delineation of Pararhizobium gen. nov., and 13 new species combinations. Syst Appl Microbiol. 38, 84–90 (2015). https://doi.org/10.1016/j.syapm.2014.12.003

80. Oldroyd, G.E.D., Murray, J.D., Poole, P.S., Downie, J.A.: The Rules of Engagement in the Legume-Rhizobial Symbiosis. Annu Rev Genet. 45, 119–144 (2011). https://doi.org/10.1146/annurev-genet-110410-132549

81. Hawkins, J.P., Oresnik, I.J.: The Rhizobium-Legume Symbiosis: Co-opting Successful Stress Management. Front Plant Sci. 12, (2022). https://doi.org/10.3389/fpls.2021.796045

82. Roy, S., Liu, W., Nandety, R.S., Crook, A., Mysore, K.S., Pislariu, C.I., Frugoli, J., Dickstein, R., Udvardi, M.K.: Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. Plant Cell. 32, 15–41 (2020). https://doi.org/10.1105/tpc.19.00279

83. Charpentier, M., Oldroyd, G.E.D.: Nuclear Calcium Signaling in Plants. Plant Physiol. 163, 496–503 (2013). https://doi.org/10.1104/pp.113.220863

84. Mahmud, K., Makaju, S., Ibrahim, R., Missaoui, A.: Current Progress in Nitrogen Fixing Plants and Microbiome Research. Plants. 9, 97 (2020). https://doi.org/10.3390/plants9010097

85. Hilker, M., Schmülling, T.: Stress priming, memory, and signalling in plants. Plant Cell Environ. 42, 753–761 (2019). https://doi.org/10.1111/pce.13526

86. Mauch-Mani, B., Baccelli, I., Luna, E., Flors, V.: Defense Priming: An Adaptive Part of Induced Resistance. Annu Rev Plant Biol. 68, 485–512 (2017). https://doi.org/10.1146/annurev-arplant-042916-041132

87. Prusky, D., Romanazzi, G.: Induced Resistance in Fruit and Vegetables: A Host Physiological Response Limiting Postharvest Disease Development. Annu Rev Phytopathol. 61, 279–300 (2023). https://doi.org/10.1146/annurev-phyto-021722-035135

88. Martínez-Hidalgo, P., García, J.M., Pozo, M.J.: Induced systemic resistance against Botrytis cinerea by Micromonospora strains isolated from root nodules. Front Microbiol. 6, (2015). https://doi.org/10.3389/fmicb.2015.00922

89. Poole, P., Ramachandran, V., Terpolilli, J.: Rhizobia: from saprophytes to endosymbionts. Nat Rev Microbiol. 16, 291–303 (2018). https://doi.org/10.1038/nrmicro.2017.171

90. Grundy, E.B., Gresshoff, P.M., Su, H., Ferguson, B.J.: Legumes Regulate Symbiosis with Rhizobia via Their Innate Immune System. Int J Mol Sci. 24, 2800 (2023). https://doi.org/10.3390/ijms24032800

91. Berrabah, F., Benaceur, F., Yin, C., Xin, D., Magne, K., Garmier, M., Gruber, V., Ratet, P.: Defense and senescence interplay in legume nodules. Plant Commun. 5, 100888 (2024). https://doi.org/10.1016/j.xplc.2024.100888

92. Cao, Y., Halane, M.K., Gassmann, W., Stacey, G.: The Role of Plant Innate Immunity in the Legume-Rhizobium Symbiosis. Annu Rev Plant Biol. 68, 535–561 (2017). https://doi.org/10.1146/annurev-arplant-042916-041030

93. Bourcy, M., Brocard, L., Pislariu, C.I., Cosson, V., Mergaert, P., Tadege, M., Mysore, K.S., Udvardi, M.K., Gourion, B., Ratet, P.: *Medicago truncatula* <scp>DNF</scp> 2 is a <scp>PI</scp> - <scp>PLC</scp> - <scp>XD</scp> -containing protein required for bacteroid persistence and prevention of nodule early senescence and defense-like reactions. New Phytologist. 197, 1250–1261 (2013). https://doi.org/10.1111/nph.12091

94. Berrabah, F., Bourcy, M., Eschstruth, A., Cayrel, A., Guefrachi, I., Mergaert, P., Wen, J., Jean, V., Mysore, K.S., Gourion, B., Ratet, P.: A non <scp>RD</scp> receptor-like kinase prevents nodule early senescence and defense-like reactions during symbiosis. New Phytologist. 203, 1305–1314 (2014). https://doi.org/10.1111/nph.12881

95. Sinharoy, S., Torres-Jerez, I., Bandyopadhyay, K., Kereszt, A., Pislariu, C.I., Nakashima, J., Benedito, V.A., Kondorosi, E., Udvardi, M.K.: The C_2H_2 Transcription Factor REGULATOR OF SYMBIOSOME DIFFERENTIATION Represses Transcription of the Secretory Pathway Gene *VAMP721a* and Promotes

Symbiosome Development in *Medicago truncatula*. Plant Cell. 25, 3584–3601 (2013). https://doi.org/10.1105/tpc.113.114017

96. Wang, C., Yu, H., Luo, L., Duan, L., Cai, L., He, X., Wen, J., Mysore, K.S., Li, G., Xiao, A., Duanmu, D., Cao, Y., Hong, Z., Zhang, Z.: *NODULES WITH ACTIVATED DEFENSE 1* is required for maintenance of rhizobial endosymbiosis in *Medicago truncatula*. New Phytologist. 212, 176–191 (2016). https://doi.org/10.1111/nph.14017

97. Liu, J., Rasing, M., Zeng, T., Klein, J., Kulikova, O., Bisseling, T.: NIN is essential for development of symbiosomes, suppression of defence and premature senescence in *Medicago truncatula* nodules. New Phytologist. 230, 290–303 (2021). https://doi.org/10.1111/nph.17215

98. Berrabah, F., Ratet, P., Gourion, B.: Multiple steps control immunity during the intracellular accommodation of rhizobia. J Exp Bot. 66, 1977–1985 (2015). https://doi.org/10.1093/jxb/eru545

99. Domonkos, Á., Kovács, S., Gombár, A., Kiss, E., Horváth, B., Kováts, G., Farkas, A., Tóth, M., Ayaydin, F., Bóka, K., Fodor, L., Ratet, P., Kereszt, A., Endre, G., Kaló, P.: NAD1 Controls Defense-Like Responses in Medicago truncatula Symbiotic Nitrogen Fixing Nodules Following Rhizobial Colonization in a BacA-Independent Manner. Genes (Basel). 8, 387 (2017). https://doi.org/10.3390/genes8120387

100. Berrabah, F., Bourcy, M., Cayrel, A., Eschstruth, A., Mondy, S., Ratet, P., Gourion, B.: Growth Conditions Determine the DNF2 Requirement for Symbiosis. PLoS One. 9, e91866 (2014). https://doi.org/10.1371/journal.pone.0091866

101. Kattupalli, D., Srinivasan, A., Soniya, E.V.: A Genome-Wide Analysis of Pathogenesis-Related Protein-1 (PR-1) Genes from Piper nigrum Reveals Its Critical Role during Phytophthora capsici Infection. Genes (Basel). 12, 1007 (2021). https://doi.org/10.3390/genes12071007

102. Hoffmann, B., Trinh, T.H., Leung, J., Kondorosi, A., Kondorosi, E.: A New *Medicago truncatula* Line with Superior in Vitro Regeneration, Transformation, and Symbiotic Properties Isolated Through Cell Culture Selection. Molecular Plant-Microbe Interactions®. 10, 307–315 (1997). https://doi.org/10.1094/MPMI.1997.10.3.307

103. Ehrhardt, D.W., Atkinson, E.M., Long, S.R.: Depolarization of Alfalfa Root Hair Membrane Potential by *Rhizobium meliloti* Nod Factors. Science (1979). 256, 998–1000 (1992). https://doi.org/10.1126/science.10744524

104. Carrere, S., Verdier, J., Gamas, P.: MtExpress, a Comprehensive and Curated RNAseq-based Gene Expression Atlas for the Model Legume *Medicago truncatula*. Plant Cell Physiol. 62, 1494–1500 (2021). https://doi.org/10.1093/pcp/pcab110

105. Chen, C., Chen, H., Zhang, Y., Thomas, H.R., Frank, M.H., He, Y., Xia, R.: TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. Mol Plant. 13, 1194–1202 (2020). https://doi.org/10.1016/j.molp.2020.06.009

106. Berrabah, F., Salem, E.H.A., Garmier, M., Ratet, P.: The Multiple Faces of the Medicago-Sinorhizobium Symbiosis. Presented at the (2018)

107. Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., Crespi, M., Frugier, F.: MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. The Plant Journal. 65, 622–633 (2011). https://doi.org/10.1111/j.1365-313X.2010.04447.x

108. Tamura, K., Stecher, G., Kumar, S.: MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 38, 3022–3027 (2021). https://doi.org/10.1093/molbev/msab120

109. Jones, D.T., Taylor, W.R., Thornton, J.M.: The rapid generation of mutation data matrices from protein sequences. Bioinformatics. 8, 275–282 (1992). https://doi.org/10.1093/bioinformatics/8.3.275

110. Aldrich, J.: R. A. Fisher and the making of maximum likelihood 1912 - 1922. Statistical Science. 12, (1997). https://doi.org/10.1214/ss/1030037906

111. Letunic, I., Bork, P.: Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. Nucleic Acids Res. (2024). https://doi.org/10.1093/nar/gkae268

112. Chen, C., Wu, Y., Xia, R.: A painless way to customize Circos plot: From data preparation to visualization using TBtools. iMeta. 1, (2022). https://doi.org/10.1002/imt2.35

113. Wang, Y., Tang, H., DeBarry, J.D., Tan, X., Li, J., Wang, X., Lee, T. -h., Jin, H., Marler, B., Guo, H., Kissinger, J.C., Paterson, A.H.: MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res. 40, e49–e49 (2012). https://doi.org/10.1093/nar/gkr1293

114. Wang, Y., Tang, H., Wang, X., Sun, Y., Joseph, P. V., Paterson, A.H.: Detection of colinear blocks and synteny and evolutionary analyses based on utilization of MCScanX. Nat Protoc. (2024). https://doi.org/10.1038/s41596-024-00968-2

115. Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B.A., Thiessen, P.A., Yu, B., Zaslavsky, L., Zhang, J., Bolton, E.E.: PubChem 2023 update. Nucleic Acids Res. 51, D1373–D1380 (2023). https://doi.org/10.1093/nar/gkac956

116. O'Boyle, N.M., Banck, M., James, C.A., Morley, C., Vandermeersch, T., Hutchison, G.R.: Open Babel: An open chemical toolbox. J Cheminform. 3, 33 (2011). https://doi.org/10.1186/1758-2946-3-33

117. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D.: Highly accurate protein structure prediction with AlphaFold. Nature. 596, 583–589 (2021). https://doi.org/10.1038/s41586-021-03819-2

118. Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., Rokhsar, D.S.: Phytozome: a comparative

platform for green plant genomics. Nucleic Acids Res. 40, D1178–D1186 (2012). https://doi.org/10.1093/nar/gkr944

119. Trott, O., Olson, A.J.: AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 31, 455–461 (2010). https://doi.org/10.1002/jcc.21334

120. Hall, T.A.: BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. Nucleic Acids Symp Ser. 41, (1999)

121. Thompson, J.D., Higgins, D.G., Gibson, T.J.: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680 (1994). https://doi.org/10.1093/nar/22.22.4673

122. Mann, H.B., Whitney, D.R.: On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other. The Annals of Mathematical Statistics. 18, (1947). https://doi.org/10.1214/aoms/1177730491

123. Chow, C.-N., Yang, C.-W., Wu, N.-Y., Wang, H.-T., Tseng, K.-C., Chiu, Y.-H., Lee, T.-Y., Chang, W.-C.: PlantPAN 4.0: updated database for identifying conserved non-coding sequences and exploring dynamic transcriptional regulation in plant promoters. Nucleic Acids Res. 52, D1569–D1578 (2024). https://doi.org/10.1093/nar/gkad945

124. Jain, D., Khurana, J.P.: Role of Pathogenesis-Related (PR) Proteins in Plant Defense Mechanism. In: Molecular Aspects of Plant-Pathogen Interaction. pp. 265–281. Springer Singapore, Singapore (2018)

125. Choi, D.S., Hwang, I.S., Hwang, B.K.: Requirement of the Cytosolic Interaction between PATHOGENESIS-RELATED PROTEIN10 and LEUCINE-RICH REPEAT PROTEIN1 for Cell Death and Defense Signaling in Pepper. Plant Cell. 24, 1675–1690 (2012). https://doi.org/10.1105/tpc.112.095869

126. Huh, S.U., Paek, K.-H.: Plant RNA binding proteins for control of RNA virus infection. Front Physiol. 4, (2013). https://doi.org/10.3389/fphys.2013.00397

127. Berrabah, F., Balliau, T., Aït-Salem, E.H., George, J., Zivy, M., Ratet, P., Gourion, B.: Control of the ethylene signaling pathway prevents plant defenses during intracellular accommodation of the rhizobia. New Phytologist. 219, (2018). https://doi.org/10.1111/nph.15142

128. He, H., Liu, D., Zhang, N., Zheng, W., Han, Q., Ji, B., Ge, F., Chen, C.: The PR10 gene family is highly expressed in Lilium regale Wilson during Fusarium oxysporum f. sp. lilii infection. Genes Genomics. 36, 497–507 (2014). https://doi.org/10.1007/s13258-014-0185-x

129. Bahramnejad, B., Goodwin, P.H., Zhang, J., Atnaseo, C., Erickson, L.R.: A comparison of two class 10 pathogenesis-related genes from alfalfa and their activation by multiple stresses and stress-related signaling molecules. Plant Cell Rep. 29, 1235–1250 (2010). https://doi.org/10.1007/s00299-010-0909-6

130. Gai, W.-X., Ma, X., Qiao, Y.-M., Shi, B.-H., ul Haq, S., Li, Q.-H., Wei, A.-M., Liu, K.-K., Gong, Z.-H.: Characterization of the bZIP Transcription Factor Family in Pepper (Capsicum annuum L.): CabZIP25 Positively Modulates the Salt Tolerance. Front Plant Sci. 11, (2020). https://doi.org/10.3389/fpls.2020.00139

131. Liu, J.-J.: Root-specific expression of a western white pine PR10 gene is mediated by different promoter regions in transgenic tobacco. Plant Mol Biol. 52, 103–120 (2003). https://doi.org/10.1023/A:1023930326839

132. He, M., Xu, Y., Cao, J., Zhu, Z., Jiao, Y., Wang, Y., Guan, X., Yang, Y., Xu, W., Fu, Z.: Subcellular localization and functional analyses of a PR10 protein gene from Vitis pseudoreticulata in response to Plasmopara viticola infection. Protoplasma. 250, 129–140 (2013). https://doi.org/10.1007/s00709-012-0384-8