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Roles of the Plant Immune Response in Root Nodule Symbiosis

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Authors' contributions

This work was carried out in collaboration between both authors. Author MMC prepared the figures. Authors MMC and KPM wrote the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

The symbiotic relationship between legume plants and soil bacteria has been an important focus of research for several decades because of its agricultural and environmental potential to create a variety of nitrogen-fixating plants capable of fertilizing the soil and producing crops with high protein content. The symbiosis is largely plant-controlled, with the plant's innate immune responses playing an important role in initiating and regulating the symbiosis. This review elucidates the role of plant immune responses in forming symbiosis with microorganisms, including how molecular cues between host and symbiont suppress the plant defense pathways, as well as how the plant can dominate the symbiosis through defense-like reactions at key signaling steps in the symbiosis. Understanding the mechanisms of regulated defense responses during nitrogen-fixing symbiosis may help us transfer this ability into non-legume plants, creating crops with higher nutrient yields and promoting sustainable and environmentally-friendly agricultural practices.

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1. INTRODUCTION

Nitrogen fixation involves the reduction of atmospheric nitrogen (N_2) into biologically available forms such as ammonia and nitrate. Only a few microorganisms are able to fix nitrogen, among them the soil bacteria in the genus *Rhizobium*. Leguminous plants have evolved mutually symbiotic relationships with *Rhizobium* bacteria and are responsible for the majority of nitrogen released into the biosphere. In exchange for the reduced nitrogen, rhizobia gain a constant supply of photosynthates and a carefully controlled microaerobic environment for the oxygen-sensitive nitrogenase [1]. The rhizobia are contained in root structures called nodules, which are formed after the recognition of molecular cues between specific host and bacterial species. Flavonoids released by the plant roots interact with transcriptional factors that initiate the expression of a set of nodulation genes in the rhizobia. Nodule formation is induced by the secretion of bacterial Nod factors, a class of lipochitooligosaccharides with various chemical ornaments characteristic to each strain of bacteria [1]. The Nod factors stimulate the elongation of root hairs, which then curl around the bacteria and develop into infection threads that allow the bacteria to penetrate the root cortex tissue. Once embedded into the root's cortex, the pericycle tissue fuses to the growing nodule and vascular tissue develops around the nodule to facilitate the exchange of materials. The bacteria are released from the infection thread via exocytosis and sequestered into specialized vesicles called symbiosomes where they differentiate into their nitrogen-fixating forms called bacteroids [1]. The symbiosome is an organelle-like structure derived from plant membrane that is carefully regulated throughout the symbiotic relationship due to the high amount of traffic and crosstalk between plant and rhizobia [1]. Within the symbiosome, the plant contributes iron and sugars to drive the nitrogen fixation in the bacteria, which then export nitrogen products such as ammonium for protein synthesis in the plant. Oxygen has a high binding affinity to the ligand binding sites in nitrogenase and thus has to be regulated to prevent inactivating the nitrogen fixing process. The specialized anaerobic environment of the symbiosomes is maintained by membranes that prevent the diffusion of gas and the presence of leghemoglobin. The high binding affinity of oxygen to leghemoglobin also helps to buffer oxygen in the symbiosome, but more importantly it provides oxygen to the bacteria for respiration without interfering with nitrogen fixation. It was demonstrated that the leghemoglobin holoprotein is formed by contributions from both the plant (enzyme) and the bacteria (heme) [2].

After the onset of nitrogen fixation in the bacteroids, the nodules age in two distinct patterns according to the species involved. In *Lotus japonicus* and several species of tropical legumes, the nodules are determinate, characterized by a spherical shape and loss of meristematic activity [3]. These nodules are homogenous in terms of infected and uninfected cells, and aging occurs radially, beginning with the cells in the center of the nodule. In indeterminate nodules found in *Medicago truncatula* and most other legumes, the nodules are divided into four distinct zones: zone I, the persistent meristematic tissue that initiates new nodule growth; zone II, the infection zone full of undifferentiated rhizobia; zone III, the metabolically active area where mature bacteroids fix nitrogen; and finally zone IV, the senescent zone [4]. Aging progresses from the tip of the nodule towards the root tissue where nutrients and cellular materials are recycled by the plant upon senescence.

The role of the plant's immune system in determining and regulating the interactions between plant cells and bacterial cells is an important feature of these symbioses and understanding the complex network of signals may facilitate the establishment of the nitrogen fixating relationship into non-leguminous crops. Additionally, understanding the mechanisms involved in the plant-controlled regulation of the developmental zones in determinate nodules may help extend the nitrogen-fixating phase of aging, increasing the nitrogen content in the plant and the protein content in the seeds. Suppression of the plant's immune system during the initiation of symbiosis is a key characteristic that makes the relationship between plant and rhizobia possible. However, the immune response is not simply turned off to allow the rhizobial infection, but is carefully controlled via a dynamic reciprocal signaling network between bacteria and plant cells throughout the symbiosis. Although the defense pathways are

functioning in different respects, the similarities between symbiosis and nodule-specific genes and proteins and the molecular components involved in pathogen response is a remarkable feature of the symbiosis and will be the main focus of this review.

2. NODULE ORGANOGENESIS

To initiate the root nodule symbiosis, the rhizobia must be able to evade the plant's defense systems to establish infection threads. The high specificity of the symbiosis is a determining factor in whether or not the plant will accept the rhizobia. Before nodulation begins, the plant releases flavonoids that attract specific species of rhizobia to the roots. Flavonoids can trigger two possible pathways in rhizobia: (1) the traditional Nod factor (NF) and NF receptor (NFR) pathway, or (2) a type III secretion system pathway. Both pathways induce the expression of regulatory transcription factors NODULE INCEPTION gene (*NIN*) and ETHYLENE RESPONSE FACTOR REQUIRED FOR NODULATION 1 (*ERN1*) [5].

In the first pathway, the plant-secreted flavonoids stimulate the production of bacterial nodulation factors (Nod factors) that then interact with a variety of receptors on the host plant plasma membrane (Fig. 1). Reverse genetic analyses identified lysine motif receptor-like kinases (LysM-RKs) that are necessary for Nod factor recognition. These kinases bind oligosaccharides found in peptidoglycan bacterial cell walls. Important LysM-RKs are NOD FACTOR RECEPTOR 1 (NFR1) and NOD FACTOR RECEPTOR 5 (NFR5) in *L. japonicus* as well as LysM RECEPTOR KINASE 3 (LYK3) and NOD FACTOR PERCEPTION (NFP) in *Medicago truncatula*. These receptors are activated by nanomolar concentrations of Nod factors and act as heterodimers or in heterocomplexes [6]. NFR1 contains an essential kinase domain that promotes the auto- or trans-phosphorylation of NFR5's cytoplasmic domain that then initiates a cascade of events required for nodulation [7]. LYK3 and NFP function in a similar mechanism.

The host-symbiont specificity is determined by the LysM-RKs, particularly the LysM2 domain of NFR5 and NFP. Nodulation is achieved after the NFR5/NFP recognizes the Nod factors released by the rhizobia and NFR1/LYK3 activates regions of NFR5/NFP that initiate downstream signaling [8]. The LysM2 domains of NFR5 and NFP are able to recognize specific oligosaccharides on the cellular surfaces of bacteria in a similar mechanism to the plant's immune response to pathogens. Pathogen-associated molecular patterns (PAMPs), often associated with lipooligosaccharides and peptidoglycan in bacteria and chitin in fungi, trigger a broad immune response in plants that results in ion fluxes, callose deposition, salicylic acid production, and reactive oxygen species activation [9]. Calcium oscillations and hormone production play an important role in the symbiotic relationships with rhizobia by contributing to massive changes in gene expression [10]. PAMPs are recognized by LysM-containing receptor-like proteins similar to LysM-RKs that recognize Nod factors [9]. The extracellular LysM-containing proteins LYM1 and LYM3 perceive peptidoglycan components in *Arabidopsis thaliana* and act as heterodimers along with CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) [8]. CERK1 is able to recognize chitinous elements in both bacteria (lipochitooligosaccharides, or LPOs) and fungi (chitin) and is important in the defense pathways against both types of pathogens. CERK1 shares structural similarity to NFR1 and LYK3 and, like NFR1 and LYK3, contains kinase domains responsible for downstream signaling [8]. The similarity between these kinases suggests a similar recognition and gene expression mechanism that is supported by the activation of a symbiotic response in *L. japonicus* when treated with chitin. The subtle differences in the above described kinase domains are key factors responsible for the activation of an immune versus symbiotic response to bacterial (and fungal) molecular patterns but the mechanistic underpinnings of this process still remain to be elucidated.

Another kinase important in crosstalk between nodulation and the immune responses is the leucine-rich repeat receptor kinase (LRR-RK). The LRR-RK FLAGELLIN SENSITIVE 2 (FLS2) recognizes the flg22 peptide of flagellin, a conserved protein component of flagella in bacteria, and initiates defenses through a cascade of mitogen activated protein kinases (MAPKs). FLS2 has an analogous gene called symbiosis receptor-like kinase (*SYMRK*) in *L. japonicus* that is required for Nod factor signaling. Although little is known about *SYMRK*'s functionality, the FLS2 model may provide useful insights not only into the mechanisms for Nod factor perception but the key differences between signaling that allows a symbiotic relationship in one organism and a

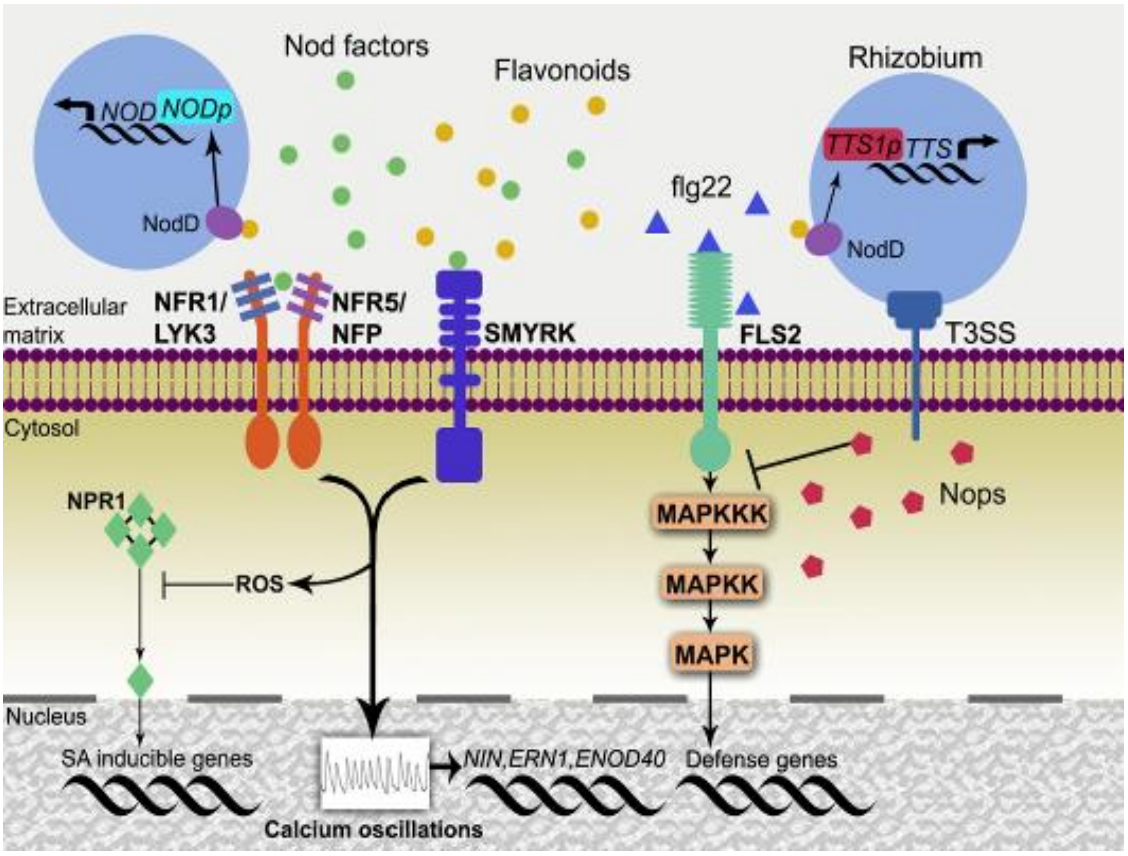

Fig. 1. Model describing the molecular signaling pathways between plant roots and rhizobia

defense response in another. Interestingly, the defense response to flg22 is significantly suppressed after the addition of Nod factors in both legume and non-legume plants. The production of reactive oxygen species (ROS) was reduced by 25% when Nod factors were present in soybean [11]. In the non-legume *Arabidopsis thaliana*, a similar effect was observed, although the suppression is weaker and slower compared to soybean. In legume mutants with defective NFR1 or NFR5 kinases, the PAMP-triggered immunity (PTI) is suppressed after addition of Nod factors even though symbiosis is lost. In *A. thaliana*, *lyk3* mutants are unable to suppress ROS production. Conversely, overexpression of LYK3 results in an enhanced suppression of ROS production with the addition of Nod factors. These results indicate that the perception of Nod factors is conserved among non-legumes, offering potentially valuable pathways for introducing symbiotic relationships into these plants. Additionally, plants respond to Nod factors by suppressing the immune response, which may elucidate how these symbiotic relationships with rhizobia have evolved and how the bacterial load is maintained within the plant cells during the symbiosis.

In symbionts that either lack NFs or NFRs or have incompatible NFs/NFRs, the type III secretion system (T3SS) pathway may bypass the NF/NFR system to initiate nodulation. The flavonoids interact with the NodD transcription factor in rhizobia to trigger expression of a gene cluster that encodes several proteins required for nodulation. NodD activates expression of Ttsl protein that in turn binds to the *tts* box on the *tts* gene cluster promoter to drive their expression, leading to the production of the components of the T3SS apparatus and the nodulation proteins that are secreted by it. The *tts* cluster is located in a physical proximity to genes required for nodulation and nitrogen fixation.

The nodulation of *Glycine max* by its symbiont *Bradyrhizobium elkanii* is a T3SS-dependent pathway [12]. In this system, NFs and T3SS proteins, called nodulation proteins (Nops) act synergistically to promote optimal numbers of

nodules formed. BErhcJ is a mutated strain of *B. elkanii* that is defective in the *rhcJ* gene that encodes a key component of the T3SS apparatus; however, it is still capable of infecting the wild-type strain of *G. max*. In En1282, a mutant *G. max* that is defective in *nfr1*, *B. elkanii* still infects the cells, although nodulation is reduced, while BErhcJ is unable to infect any cells [12]. This data suggests that the NF/NFR pathway and the T3SS pathway can act independently to induce nodulation or synergistically for more effective nodulation. Microarray analysis of gene expression of common and required nodulation genes, *NIN* and *ENOD40* (a root nodule extension gene), revealed that both pathways contribute to the expression of these genes [12]. Although the mechanism for T3SS activation of *NIN* and *ENOD40* is undetermined, it is possible that the Nops may interact with several kinases involved in the expression of nodulation genes.

The T3SS is a mechanism shared by pathogenic and symbiotic bacteria for introducing proteins required for suppressing or evading the plant defense systems, allowing for bacterial infection (Fig. 1). Previously, T3SSs were thought to be unique to pathogenic bacteria, but are now known to be present in some symbiotic species. Although many genetic and structural similarities exist, there are some key differences between the secretion system, the proteins secreted, and its function in phytopathogenic and symbiotic species that utilize this mechanism. In pathogenic species of bacteria, the T3SS is required for infection and the proteins secreted by the apparatus offer various methods of increased pathogenicity for the bacterium, by modifying the cell cycle of the plant or hijacking genetic or structural elements in the plant cells to aid infection [13-15]. The effector proteins released by pathogenic T3SSs are an important trigger for the plant immune response and constitute a cornerstone of the co-evolutionary relationship between pathogen and host [16]. If the effectors are detected by the plant, a strong immune response called effector-triggered immunity (ETI) is activated, often resulting in hypersensitive response, a form of programmed cell death. Effectors are detected by resistance, or R, proteins in the plant that belong to a class of Toll-interleukin receptor/nucleotide-binding leucine-rich repeat (TIR-NBS-LRR) proteins or coiled-coil NBS-LRR (CC-NBS-LRR) proteins conserved among several plant, mammal, and invertebrate species. With few exceptions, these NBS-LRR proteins do not interact with the effectors directly, but merely detect effector-mediated disruption of key native proteins. RPM1 INTERACTING PROTEIN 4 (RIN4) is a well-studied plant protein that is guarded by the NBS-LRR R proteins [17,18]. RIN4 interacts with R proteins as well as membrane-bound ATPases that play a role in regulating the aperture of stomata, a common entryway for pathogens [19]. RIN4 is targeted by several *Pseudomonas syringae* effectors, and once modified or damaged by pathogenic interactions, can activate the R proteins and initiate a signaling cascade that results in ion fluxes and ROS production, key components of the hypersensitive response. By monitoring internal targets of effector proteins, plants are able to defend themselves against a wide range of pathogens with only a few classes of proteins. In legumes, LRR proteins may play a role in regulating the size and number of bacteria in the nodules in addition to controlling host-symbiont specificity.

The T3SSs in symbiotic bacteria are often dispensable for nodulation and have variable degrees of activity depending on bacteria and host species. Deletion of the *tts* genes in *Mesorhizobium loti* results in a reduction of nodules in *Lotus corniculatus* but an increase in nodule formation in *Lotus halophilus* [12,13]. These results indicate that T3SS proteins may be involved in host-symbiont specificity. The T3SS may confer advantages to rhizobia that lack the appropriate Nod factors to infect a host by offering alternative methods of infection. In the soybean with a mutated NFR1, *B. elkanii* infected the roots without the traditional root hair curling or infection thread formation [12]. Instead, crack entry infection or intercellular infection are other, albeit less efficient, methods of gaining entry into the root cells. Crack entry infection involves the invasion of fissures and wounds in epidermal cells to gain direct access to cortical cells. Intercellular infection is a process by which bacteria can invade the gaps between cells to navigate to the cortical tissue and infect the cells through cell wall invagination [20]. However, akin to pathogenic T3SSs and effector proteins, if the plant R proteins detect the presence of Nops, a defense response is activated and the rhizobial infection is terminated [21]. This phenomenon reiterates the importance of plant-rhizobia specificity in the symbiosis and the role of the plant immune system in initiating and regulating the symbiosis.

3. NODULE DIFFERENTIATION

After the rhizobia infect the root hairs, the infection thread elongates into the cortical tissue of the host plant's roots where the bacteria are endocytotically released into membranous sacs called symbiosomes. The bacteria are permanently and irreversibly sequestered in these symbiosomes where they will fix nitrogen until the plant initiates senescence and recycles the cellular contents. Once deposited into the symbiosomes, the bacteria differentiate into non-growing bacteroids that are capable of fixing nitrogen. The symbiosome is a unique, organelle-like structure that serves as an interface for the interactions, material exchanges, and signals between plant cells and bacteroids [1]. The environment within the symbiosome is carefully controlled to allow nitrogenase activity. An endodermal layer surrounds infected nodule zones and acts as a gas barrier that reduces oxygen pressure within the infected region to allow functionality of oxygen-labile nitrogenase [1]. Although up to a third of the rhizobial genome is dedicated to the maintenance of the symbiosome, the entire structure is largely plant-controlled. Most of the proteins associated with the symbiosome membrane are of plant origin and non-metabolic. The proteins in the symbiosome space, however, are bacterially originated metabolic enzymes that may operate as a functional citric acid-like cycle to provide bacteroids with a dedicated carbon source [1].

An interesting feature of the bacteroids are the differentiation pathways that are dependent upon the host species. In legumes such as *Medicago* that belong to the inverted repeat-lacking clade (IRLC), bacteroids undergo a terminal differentiation that results in loss of reproductive activity, cellular elongation, and polyploidy [22]. However, in non-IRLC legumes such as *L. japonicus*, bacteroids retain normal cell and genome sizes as well as reproductive ability. The structure of the infected nodules is morphologically different in IRLC and non-IRLC legumes [22]. Because *Rhizobium* strains that are capable of infecting both types of legumes experience different bacteroid fates, differentiation is determined to be a plant-controlled process [23]. Nodule-specific cysteine-rich peptides (NCRs) are restricted to infected plant cells and have activity similar to antimicrobial peptides (AMPs) that can control membrane permeability and cell division [22]. The *NCR* genes are not expressed in *L. japonicus*, but *NCR* ectopic overexpression forces the bacteroids into a terminal state, with cell elongation, loss of reproductive ability and abnormal membrane permeability [24]. NCR peptides are absorbed by the bacteroids, where they are able to inhibit cytokinesis and force DNA reduplication events resulting in enlarged, polyploid cells. Remarkably, NCR peptides-treated free-living bacterial cells are rapidly killed, but the bacteroids in nodules are kept alive [22]. There may be more complex interactions with different peptides or genes in the bacteroids that abolish the proliferative ability of the bacteroids but keep them alive for nitrogen fixation. The NCR peptides are a mechanism by which the plants can dominate the symbiosis by controlling the cell cycle of the bacteroids and alter the genetic content for higher metabolic efficiency.

4. NODULE SENESCENCE

Senescence is characterized by a reversal of the suppression of the plant immune response required for the onset of the symbiosis. Although little is known about the signaling pathways that initiate senescence, it is believed to be a plant-controlled process that is divided into two developmental phases: (1) the degradation of the symbiosome, and (2) the degradation of nodule (plant) cells. Senescence is a slow form of cell death that is carefully controlled to maximize on recycled nutrients and cellular components [25]. Early nodule senescence can be triggered by many stress factors or by a mismatch in recognition factors and signaling between host and symbiont [25]. This form of senescence occurs much more rapidly and resembles a strong defense response rather than natural senescence. Some common features of senescence include a shift in the electron content of the cytoplasm, a range of proteolytic enzymatic activity, the appearance of ghost membranes and vesicles as materials are reabsorbed by the plant, as well as the synthesis of a number of transporter proteins such as ATP-binding cassette (ABC) transporters that move the degraded materials to other parts of the plant for use [25].

A few key players in nodule senescence include the important regulatory hormones abscisic acid (ABA), jasmonic acid (JA), and ethylene (ET). ABA is involved with inhibiting cell growth, and JA and ET are both involved in senescence. Many of these hormones have roles in plant defense against pathogens [26]. It is believed that an ABA-mediated pathway is involved in

inducing proteolytic activities within the nodule cells [27]. ET biosynthetic enzyme S-adenosyl-Met synthetase is up-regulated during nodule senescence [28]. Likewise, JA accumulation is indicated in nodule senescence by the expression of lipoxygenase genes that catalyze an early step in the JA biosynthetic pathway. Although not much is known about the transduction of these pathways, the implication of these hormones in senescence points to a carefully controlled, plant-regulated induction of nodule degradation.

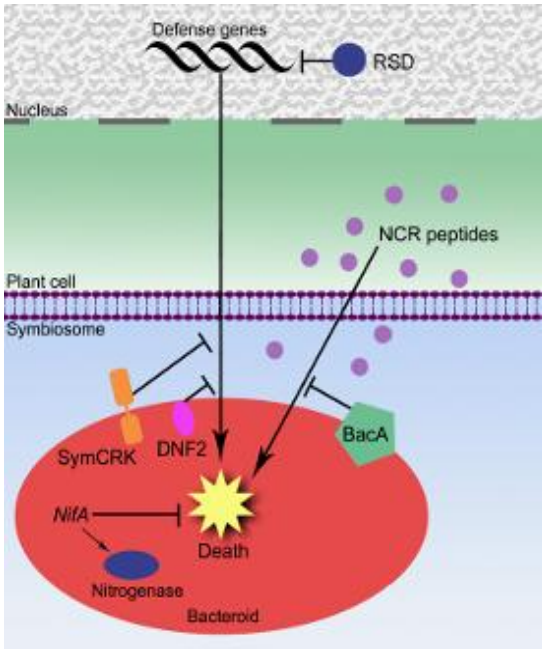

Fig. 2. Maintenance of symbiosis requires controlled suppression of defense responses

Another important feature of senescence is a class of genes involved in the suppression of the immune response during symbiosis (Fig. 2). The gene *DNF2* (*DOES NOT FIX NITROGEN 2*) encodes a phosphatidylinositol phospholipase C-like protein that is required for nitrogen fixation and preventing premature defense-like responses typical during senescence [29,30]. A coregulator of this gene is *SymCRK* (SYMBIOSIS CYSTEINE-RICH KINASE), which encodes a cysteine-rich kinase with a nonRD motif common to pattern recognition receptors (PRRs) involved in plant immune response [31,32]. *SymCRK* is expressed specifically in nodule tissue. In loss-of-function mutants of these genes, the early stages of symbiosis were unaffected, but later stages, including senescence, were negatively regulated. Additionally, NCR expression was reduced in these mutants, indicating a loss of control over bacteroid development. *DNF2* and *SymCRK* prevent early senescence by controlling and suppressing defense-like reactions during the nitrogen-fixing stages of the symbiosis [33]. The expression of these genes occurs in a chronological order, with *DNF2* acting before *SymCRK*, to regulate and control the stages of symbiosis [34]. Although more research is required, it is possible that senescence is triggered by a controlled silencing of these genes, allowing immune responses to degrade the bacteroids and plant cells for recycling.

5. NPR1 AND NODULATION

NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS 1 (NPR1) is a major regulator of plant defense responses against biotrophic and hemibiotrophic pathogens attack. NPR1 is believed to be a receptor for SA, a key hormone in establishing HR and SAR. Under normal, uninfected conditions, NPR1 exists as an oligomeric complex in the cytosol. After infection, short oxidative bursts degenerate the complex into NPR1 monomers that translocate to the nucleus to induce SA-related genes. NPR1 has an antagonistic role in rhizobial symbiosis. In the early stages of nodulation, SA levels are reduced, and addition of SA results in fewer curled root hairs. Similarly, overexpression of *NPR1* resulted in less curling. Legumes regulate the expression of NPR1 during symbiosis through ROS production. Although pathogenic and symbiotic reactions both involve ROS, the differential duration and localization of ROS determines which response (defense or symbiosis) is initiated. In defense reactions, the oxidative bursts peak at 4 hours post inoculation (p.i.) and are reduced at 24h p.i. [35]. However, during symbiosis, ROS peaks at 24h p.i. and remains high even 48h p.i. [35]. Additionally, while ROS during defense responses occurs throughout the cytoplasm, ROS during symbiosis is localized to the cytosol and nucleus, indicating that its primary function in symbiotic reactions is to prevent the activation and movement of NPR1 and thus suppress the defense response (Fig. 1). Understanding the role of NPR1 in nodulation may elucidate the key defense components that are downregulated to allow symbiosis, as NPR1 may be a primary target for suppression during nodulation.

6. CONCLUSION

The plant immune response plays an important role in various developmental stages of symbiosis between rhizobia and legume plants. The initiation of symbiosis requires many molecular recognition events between host and symbiont, which then induces a cascade of transcriptional responses in both participants to form the nodules that house the nitrogen-fixing bacteria. Nodule organogenesis is characterized by the evasion or suppression of the plant's immune response to microbial molecular patterns, and the continuation of the symbiosis is regulated through the suppression of the defense response to allow the plant to host large microbial loads of the symbiont. Finally, senescence is a reversal of the immune response suppression in a tightly regulated manner that allows the plant to recycle cellular and bacterial contents.

Enhancing our understanding of the function and regulation of the plant immune response in symbiotic relationships has many agricultural implications. Given that nitrogen fixation has obvious benefits like increased crop yields, higher protein content, and increased soil fertility, creating transgenic crops with the ability to establish symbiosis with rhizobia is an important area of research. Manipulating the plant's immune response to allow the rhizobial infection of root hairs is a promising avenue for integrating symbiosis into non-leguminous plants. However, there is still much to learn about the symbiotic relationship between rhizobia and legumes. For example, the similarities and differences between LysM-RKs, CRKs, and other kinases and proteins still need exploration to determine the various structural differences and functional pathways that result in defense response or symbiosis. Much of the signaling pathways that differentiate developmental stages, particularly senescence, are unknown. The regulation and maintenance of the symbiosome and periplasm that allows the cohabitation of both bacterial and plant molecules is a fascinating and little understood feature of the symbiosis. A particularly interesting area of research is the power play involved in the symbiosis and the unbalancing of the relationship due to plant-dominated mechanisms that control the bacteroid cell cycle without offering additional benefits to the rhizobia. Elucidating these and other areas of nodulation-centered research will facilitate our understanding of how the plant innate immunity can be manipulated to achieve contrasting functional outcomes and how this process can be used to introduce root nodule symbiosis into non-legumes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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