

MINIREVIEW

Type III effector provides a novel symbiotic pathway in legume–rhizobia symbiosis

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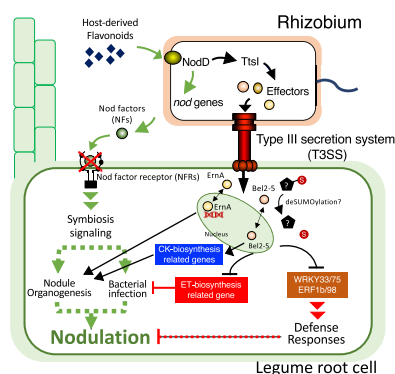
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ABSTRACT

Rhizobia form nodules on the roots of legumes and fix atmospheric nitrogen into ammonia, thus supplying it to host legumes. In return, plants supply photosynthetic products to maintain rhizobial activities. In most cases, rhizobial Nod factors (NFs) and their leguminous receptors (NFRs) are essential for the establishment of symbiosis. However, recent studies have discovered a novel symbiotic pathway in which rhizobia utilize the type III effectors (T3Es) similar to the pathogenic bacteria to induce nodulation. The T3Es of rhizobia are thought to be evolved from the pathogen, but they have a unique structure distinct from the pathogen, suggesting that it might be customized for symbiotic purposes. This review will focus on the recent findings from the study of rhizobial T3Es, discussing their features on a symbiont and pathogen, and the future perspectives on the role of rhizobial T3Es in symbiosis control technology.

Graphical Abstract



Legume-rhizobium symbiosis involves molecular interaction between the two partners. Besides the Nod factor, rhizobial type III effectors can activate the symbiotic signaling.

Keywords: symbiosis, leguminous, rhizobia, type III secretion system (T3SS), effectors

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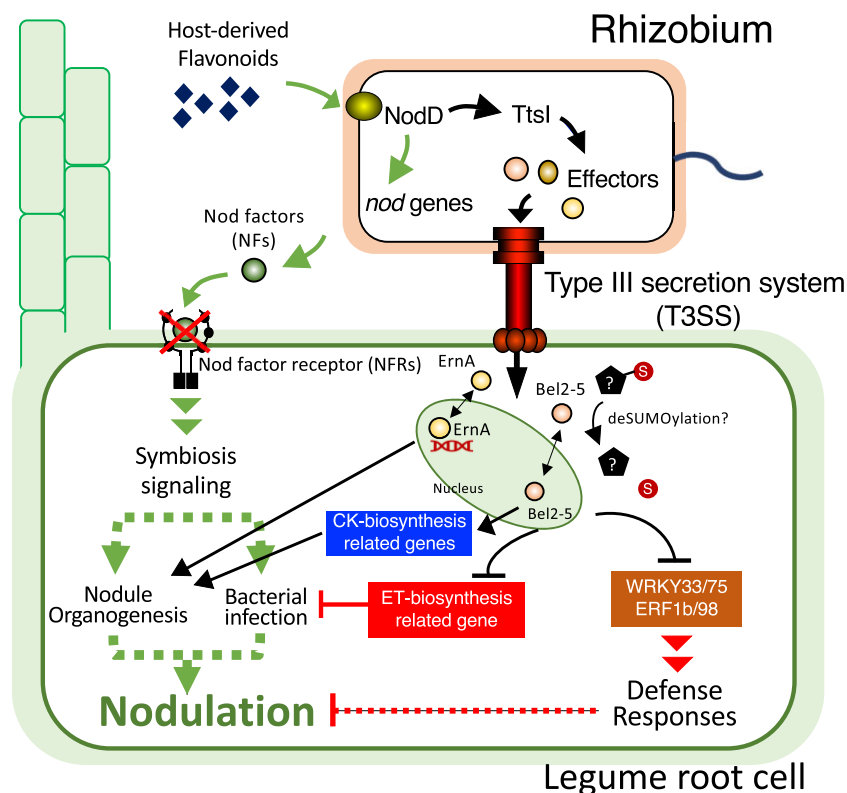


Figure 1. Model of NF signaling and the function of rhizobial T3 effectors triggering NF-independent nodulation (modified from Ratu et al. 2021a). Leguminous root-derived flavonoids induce the production of Nod factors (NFs) in rhizobia. Recognition of NFs by NF receptors triggers a signaling cascade leading to nodulation. The flavonoids also induce rhizobia to produce and inject effector proteins into legume root cells. Rhizobial effectors (ErnA and Bel2-5) can directly activate the nodulation signaling in an NF-independent manner. Both effectors localize in the plant nucleus. ErnA is likely to be associated with nucleic acids, while Bel2-5 may function as SUMO protease to regulate symbiosis with their respective hosts. Information shown is based on studies by Teulet et al. 2019; Ratu et al. 2021a.

Rhizobia are nitrogen-fixing soil bacteria that have a symbiotic relationship with legumes via the formation of root nodules on host roots. In nodules, rhizobia live as bacterioides (pleomorphic forms) and fix the atmospheric nitrogen (N_2) to ammonia (NH_4^+), thus rendering it suitable for host nutrition. In exchange, plants supply carbon sources and other nutrients to support bacterial activities. This interaction substantially contributes to the agronomic importance and global nitrogen cycle (Soumare et al. 2020). For the establishment of symbiosis, rhizobia release specific nodulation signals called Nod factors (NFs) that are recognized by legume receptors (NFRs), leading to nodule organogenesis and bacterial infection (Figure 1). The nodulation program has been considered to be (almost) exclusively controlled by NFs signaling (Oldroyd et al. 2011). However, rhizobia have gained alternative tools to promote infection or survival in legume nodules. For example, surface polysaccharides include lipopolysaccharide (LPS), exopolysaccharide (EPS), or K-antigen polysaccharide (KPS) that serve as a protective barrier or effector proteins via type III (T3SS), type IV (T4SS), or type VI (T6SS) secretion systems that modulate host cellular functions, have been shown to affect symbiosis (Frayse et al. 2003; Nelson and Sadowsky 2015). Of these, T3SS has been recognized as a central player in the interaction of bacteria with diverse host legumes.

Many but not all rhizobia possess T3SS, which was previously thought to be an attribute of pathogenic bacteria to deliver “effector proteins (T3Es)” for suppressing host immunity and promoting pathogen infections. In most legume-rhizobium

symbioses, T3SS in rhizobia is dispensable, however, it favors or impedes nodule formation on legumes depending on host genotypes (Deakin and Broughton 2009; Miwa and Okazaki 2017).

T3SS is a complex secretory machinery that forms a needle-like structure and serves as a transport channel for delivering bacterial effectors into the cytoplasm of eukaryotic cells. Bacterial pathogens heavily rely on a functional T3SS for their virulence, whereas in rhizobia, the system can promote symbiosis and expand its role beyond virulence (Tampakaki 2014; Staehelin and Krishnan 2015). Comparative genomics studies between animal/phytopathogens and rhizobia provide many similarities and variations of the T3SSs.

Rhizobial T3SS gene clusters are designated *tts* (type three secretion) and are generally located either in symbiotic plasmids or in chromosomal symbiotic islands on which nodulation and nitrogen fixation genes are encoded. Rhizobial T3SS is divided into four sub-groups rhizobia conserved (*rhc*) based on T3E core components, annotated as α -*rhcI* to α -*rhcIII*, and β -*rhc*. This *rhc*-T3SS is similar to phytopathogenic hypersensitive reaction and pathogenicity (*hrp*), which is classified as *hrp/hrc1*, and *hrp/hrc2*. Expression of rhizobial T3SS and T3Es is under the control of host-derived flavonoids and a transcriptional regulator TtsI (Figure 1). Upon delivery, rhizobial T3Es directly alter plant cell functions and facilitate host specificity (Tampakaki 2014; Staehelin and Krishnan 2015; Miwa and Okazaki 2017). In this review, we provide a current overview of T3Es in legume-rhizobium symbioses and compare their features as symbiont and pathogen factors (Table 1).

Table 1. Rhizobial T3Es and their functions in nitrogen-fixing nodulation

No	Secreted Nops	Rhizobial strains—host species	Putative function	Homologs in pathogens	Localization compartment	Putative Associated-Host genes/Host target	Effect on nodulation	References
(a) Rhizobial TEs that conserved in both rhizobia and pathogenic bacteria								
1	Bel2-5	<i>Bradyrhizobium elkanii</i> USDA61– <i>nif1</i> mutant, R/4 soybeans	Cysteine protease (C48)	<i>XopD</i> (<i>Xanthomonas campestris</i>)	Nucleus		Triggers NF-independent nodulation on <i>nif1</i> mutant, but restricts nodule formation on R/4 soybeans Suppresses nodules formation on <i>T. vogelii</i>	Faruque et al. 2015; Ratu et al. 2021a, 2021b
2	NopD	<i>Bradyrhizobium</i> sp. XS1105– <i>Tephrosia vogelii</i> <i>Sinorhizobium fredii</i> HH103– <i>Glycine max</i>	Cysteine protease (C48)	<i>XopD</i> (<i>Xanthomonas campestris</i>)	Nucleus	Soybean F-Box/LRR-repeat (FBD/LRR) (<i>Glyma.19g068600</i>) and protein phosphatase 2C (PP2C) (<i>Glyma.19g069200</i>)*	Regulates nodulation either positively or negatively on 10 soybean germplasms tested in QTL analysis Triggers the inhibition of USDA61 infection in <i>Lotus</i> spp.	Xiang et al. 2020 Wang et al. 2020
3	NopF	<i>Bradyrhizobium elkanii</i> USDA61– <i>Lotus</i> spp.	Acyl-CoA N-acyltransferase	HopBG1 (<i>Pseudomonas syringae</i> pv. <i>maculicola</i> ES4326)				
4	NopJ	<i>Rhizobium</i> sp. NGR234– <i>Crotalaria juncea</i>	Cysteine protease (C55)	<i>YopJ</i> (<i>Yersinia pestis</i>) <i>AvrRxxv</i> (<i>Xanthomonas</i> pv. <i>vesicatoria</i>)			Minor negative effect on the nodulation of <i>C. juncea</i>	Kusakabe et al. 2020
5	NopM	<i>Rhizobium</i> sp. NGR234– <i>Lablab purpureus</i> or <i>Lotus japonicus</i> <i>Bradyrhizobium</i> sp. ORS3257– <i>Aeschynomene indica</i> <i>Bradyrhizobium elkanii</i> USDA61– <i>Lotus</i> spp.	E3 ubiquitin ligase	<i>IpaH9.8</i> , <i>IpaH1.4</i> (<i>Shigella flexneri</i>), <i>SspH1</i> , <i>SspH2</i> , <i>SlrP</i> (<i>Salomonella enterica</i>), <i>HpX29</i> (<i>Ralstonia solanacearum</i>)	Plasma membrane	MAPK signaling pathway	Promotes nodulation on <i>L. purpureus</i> , whereas reduce nodule formation of <i>Lotus japonicus</i> Positively promotes nodulation on <i>A. indica</i>	Kambara et al. 2009 Xin et al. 2012; Xu et al. 2018
6	NopT	<i>Rhizobium</i> sp. NGR234– <i>Phaseolus vulgaris</i> , <i>Tephrosia vogelii</i> , <i>Crotalaria juncea</i> , and <i>C. pallida</i> <i>Sinorhizobium fredii</i> USDA257 (expressing NopT of NGR234)– <i>Glycine max</i> <i>Sinorhizobium</i> (<i>Ensifer</i>) <i>fredii</i> HH103– <i>Glycine max</i>	Cysteine protease (C58)	<i>AvrPphB</i> (<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>) <i>YopT</i> (<i>Yersinia pestis</i>)			Induces ETI-like response, thereby nodule early senescence in <i>Lotus</i> spp. Affects nodulation positively on <i>P. vulgaris</i> , <i>T. vogelii</i> , and suppresses nodulation of <i>C. juncea</i> and <i>C. pallida</i> Impairs nodulation in <i>G. max</i> cv. Nenfeng 15	Dai et al. 2008; Kambara et al. 2009 Khan et al. 2022
					Plasma membrane	Associated with soybean protein kinases, <i>GmpPBS1-1</i> (<i>Glycine max</i> <i>AvrPphB</i> Susceptible 1) Associated with peroxidase superfamily protein (<i>Glyma.02G135100</i>) and aspartyl protease family protein 2 (<i>Glyma.02G100800</i>)*	Regulates nodulation either positively, negatively or with no effects on 30 soybean germplasms tested in QTL analysis	Liu Y et al. 2021

Table 1. Continued.

No	Secreted Nops	Rhizobial strains—host species	Putative function	Homologs in pathogens	Localization compartment	Putative Associated-Host genes/Host target	Effect on nodulation	References
		<i>Mesorhizobium amphore</i> CCNWGS0123— <i>Robinia pseudoacacia</i>			Plasma membrane and nucleus	ATP-citrate synthase alpha chain protein 2 (ATP-CSACP2) and hypersensitive-induced response protein (HIRP)	Minor positive effect on the nodulation of <i>R. pseudoacacia</i>	Luo et al. 2020
		<i>Bradyrhizobium sp.</i> ORS3257— <i>Aeschynomene indica</i> or <i>Vigna</i> spp.					Promotes nodule formation <i>V. mungo</i> and <i>V. unguiculata</i> and plays lesser role on <i>A. indica</i>	Teulet et al. 2019; Songwattana et al. 2021
(b) Rhizobial-specific T3Es								
7	ErnA	<i>Bradyrhizobium sp.</i> ORS3257— <i>Aeschynomene indica</i>		Rhizobium-specific	Nucleus		Triggers NF-independent nodulation and cell-division on <i>A. indica</i>	Teulet et al. 2019
8	InnB	<i>Bradyrhizobium elkanii</i> USDA61- <i>nif1</i> mutant soybean <i>Bradyrhizobium elkanii</i> USDA61- <i>Vigna</i> spp.	Unknown	Rhizobium-specific			Positively affects nodulation on <i>nif1</i> mutant soybean Restricts nodulation on KPS1, but promotes nodulation on <i>V. mungo</i>	Ratu et al. 2021a Nguyen et al. 2018
9	NopAA	<i>Sinorhizobium fredii</i> HH103— <i>Glycine max</i> , <i>Vigna unguiculata</i>	Glycoside hydrolase 12 (GH12)	Rhizobium-specific		GmARP (<i>Glyma.19g074200</i>)*;	Promotes rhizobial infection on soybean, but has detrimental effect on <i>V. unguiculata</i> cv. Red Caloona	Jiménez-Guerrero et al. 2019; Dorival et al. 2020; Wang et al. 2022
10	NopAB	<i>Bradyrhizobium sp.</i> ORS3257— <i>Aeschynomene indica</i> or <i>Vigna</i> spp.	Unknown	Rhizobium-specific			Promotes nodules formation on <i>V. mungo</i> and <i>V. unguiculata</i> and plays lesser role on <i>A. indica</i>	Teulet et al. 2019; Songwattana et al. 2021
11	NopC	<i>Sinorhizobium fredii</i> HH103— <i>Glycine max</i> , <i>Vigna unguiculata</i> , <i>Lotus japonicus</i> GIFU	Unknown	Rhizobium-specific			Positively affects nodulation on <i>G. max</i> cv. William 82 and <i>Vigna unguiculata</i> , but blocks nodulation on <i>Lotus japonicus</i> GIFU	Jiménez-Guerrero et al. 2015, 2020
12	NopE	<i>Bradyrhizobium diazoefficiens</i> USDA110— <i>Vigna</i> spp. <i>Glycine max</i> . <i>Macroptilium atropurpureum</i>	Calcium binding protein	Rhizobium-specific			Promotes nodulation on <i>G. max</i> , <i>M. atropurpureum</i> , and <i>V. radiata</i> cv. KPS1, but has negative effect on <i>V. radiata</i> cv. KPS2 via SA-mediated ETI-responses	Wenzel et al. 2010; Piromyou et al. 2021

Table 1. Continued.

No	Secreted Nops	Rhizobial strains—host species	Putative function	Homologs in pathogens	Localization compartment	Putative Associated-Host genes/Host target	Effect on nodulation	References
13	NopL	<i>Sinorhizobium fredii</i> NGR234—Phaseolus vulgaris, <i>Flemingia congesta</i>	Protein phosphorylation	Rhizobium-specific	Nucleus	MAP Kinase	Suppresses the expression of pathogenesis-related (PR) proteins on <i>L. japonicus</i> , inhibits <i>P. vulgaris</i> nodule-senescence, promotes nodulation of <i>F. congesta</i> Positively affects nodulation on <i>nfr1</i> mutant soybean	Marie et al. 2003; Bartssev et al. 2004; Zhang et al. 2011;
14	NopP	<i>Bradyrhizobium elkanii</i> USDA61— <i>nfr1</i> mutant soybean <i>Rhizobium</i> sp. NGR234— <i>Vigna unguiculata</i> , <i>Flemingia congesta</i> , <i>Tephrosia vogelii</i> <i>Bradyrhizobium</i> USDA110— <i>Glycine max</i> <i>Bradyrhizobium diazoefficiens</i> USDA122— <i>Glycine max</i> <i>Sinorhizobium fredii</i> HH103— <i>Glycine max</i>	Protein phosphorylation	Rhizobium-specific	Endoplasmic reticulum (ER)	GmNNL1	Promotes nodulation on <i>F. congesta</i> and <i>T. vogelii</i> but inhibits nodulation on <i>V. unguiculata</i> Inhibits root hair infection on soybean carrying GmNNL1 Severely restricts nodulation with R/2-soybean via ETL-like responses Regulates nodulation either positively or negatively on 10 soybean germplasm tested in QTL analysis	Ratu et al. 2021a Ausmees et al. 2004; Skorpil et al. 2005 Zhang et al. 2021 Sugawara et al. 2018 Wang et al. 2018
		<i>Mesorhizobium amorphae</i> — <i>Robinia pseudoacacia</i>			Plasma membrane	Associated with pathogenesis-related 5 (PR5) family (<i>Glyma.12g031200</i>) and mitogen-activated protein kinase 3 (MAPK3) (<i>Glyma.12g073000</i>)* Trafficking protein particle complex subunit 13-like protein (TRAPPC13)	Plays roles at the early stage of <i>M. amorphae</i> infection. Minor positive effect on nodule biomass, but significantly promotes N-fixing activity of <i>M. amorphae</i> on <i>R. pseudoacacia</i>	Liu D et al. 2021

* means putative associated-host gene/host target of effector was identified by the Quantitative trait locus (QTL) analysis on the respective host.

The roles of T3Es that are conserved in both rhizobia and pathogenic bacteria

Certain rhizobial T3Es have homologs in animal/phytopathogens and function similarly in dampening host immunity to promote bacterial infection (Table 1a). However, these pathogenic-like effectors can also severely restrict nodulation on certain legumes. The restriction of nodulation is most likely caused by direct or indirect recognition by host resistance (R) genes (eg Rj2, Rj4, and Rfg1), followed by the induction of effector-triggered immunity (ETI)-like plant defense responses (Yang et al. 2010; Yasuda et al. 2016). This ETI often results in a hypersensitive response (HR) to halt pathogen invasion and disease development in the infected host (Jones and Dangl 2006). We discuss the features and functions of several T3Es below:

The NopD is a rhizobial T3E with a putative small ubiquitin related-modifier (SUMO) protease domain of the C48 peptidase [ubiquitin-like protease (Ulp)] similar to the *Xanthomonas* spp. XopD effector. The NopD of *Bradyrhizobium* sp. XS1150 induces ETI-like responses, thus negatively affecting *Tephrosia vogelii* nodulation (Xiang et al. 2020). *Sinorhizobium fredii* HH103 NopD interferes with soybean F-Box/LRR-repeat (FBD/LRR) and protein phosphatase 2C (PP2C) to promote rhizobial infection (Wang et al. 2020). The NopD and XopD have been shown to have a similar substrate preference for plant SUMOs, indicating that they may interact with proteins that are sumoylated in a similar manner (Xiang et al. 2020).

NopF carries a putative Acyl-CoA N-acyltransferase that is identical to the HopBG1 effector of the phytopathogen *P. syringae* pv. *maculicola* ES4326. NopF inhibits *Bradyrhizobium elkanii* USDA61 crack entry infection in *Lotus japonicus* GIFU via ETI-like responses at an early stage of symbiosis (Kusakabe et al. 2020).

NopJ is a homolog of YopJ/AvrRxv C55 peptidases found in many T3SS-possessing pathogens. YopJ was shown to target small SUMO-conjugated proteins to alter mitogen-activated protein kinase (MAPK) and nuclear factor kappaB (NF-κB) signaling pathways via acetyltransferase activity (Sweet et al. 2007). NopJ of *Rhizobium* sp. NGR234 negatively affects the nodulation of *Crotalaria juncea* (Kambara et al. 2009).

NopM belongs to IpaH (invasion-plasmid antigen H) family effector that is present in various human pathogens. NopM exploits the host ubiquitination pathway via its C-terminus E3 ubiquitin ligase (NEL) domains and its variable N-terminal containing leucine-rich repeat (LRR) domain to interfere with the MAPK signaling pathway (Xin et al. 2012; Xu et al. 2018). In *Lotus* spp., USDA61 NopM induces ETI-like responses, thus mediating early nodule senescence and/or restricting nodule formation (Kusakabe et al. 2020). The NopM of ORS3257 and NGR234 promote nodulation in *Aeschynomene indica* and *Lablab purpureus*, respectively (Xin et al. 2012; Xu et al. 2018; Teulet et al. 2019).

NopT belongs to the C58 cysteine family found in *Pseudomonas syringae* AvrPphB and *Yersinia* YopT effectors. The N-terminus of NopT is predicted to be myristoylated and palmitoylated in plant cells (Dai et al. 2008). Rhizobial NopT localizes to the plasma membrane and/or nucleus of the host cell, thus targeting various host genes depending on the legume plants. Host genes-associated NopT appears to be involved in plant-related resistance signaling that may facilitate rhizobial infection (Luo et al. 2020; Liu Y et al. 2021; Khan et al. 2022). NopT of some rhizobia positively regulates nodulation on *A. indica*, *Vigna* spp., *Phaseolus vulgaris*, and *T. vogelii*, or negatively on *C. juncea* and *C. pallida* (Dai et al. 2008; Kambara et al. 2009; Teulet et al. 2019; Songwattana et al. 2021; Khan et al. 2022).

The roles of rhizobium-specific T3Es in nodulation

Certain rhizobia specifically produce T3Es that are distinct from pathogen effectors, possibly for symbiotic purposes (Table 1b). For instance, NopAA (GuaA) of *Sinorhizobium fredii* belongs to glycoside hydrolase family 12 (GH12) and exhibits cellulose-xylloglucan activity necessary for the breakdown of soybean cell walls to promote rhizobial infections, however, it is detrimental for symbiosis with cowpea (Jiménez-Guerrero et al. 2019; Dorival et al. 2020). In soybean, NopAA is associated with GmARP, which positively regulates nodulation (Wang et al. 2022). The phosphorylated effectors of NGR234 NopL, and NopP positively affect nodulation of *T. vogelii*, *Flemingia congesta*, *P. vulgaris*, and *V. unguiculata*, respectively (Marie et al. 2003; Ausmees et al. 2004; Bartsev et al. 2004; Skorpil et al. 2005; Zhang et al. 2011). NopL favors nodulation by mimicking a substrate of MAP kinases, thereby downregulating the expression of host defense genes (Zhang et al. 2011; Ge et al. 2016). Ectopic expression of *nopL* blocks the expression of chitinases in *L. japonicus* (Bartsev et al. 2004). Recently, NopL of USDA61 has been shown to play a minor role in the nodulation of the *nfr1* mutant soybean (Ratu et al. 2021a). In the case of NopP, the transgenic expression of an effector promotes the hairy root transformation of *L. japonicus*, presumably by suppressing stress-related plant reactions (Wang et al. 2021). The HH103 NopP putatively targets a pathogenesis-related protein and a MAPK of soybean, while NopP of *Mesorhizobium amorphae* interacts with the trafficking protein TRAPPC13 of *Robinia pseudoacacia* (Wang et al. 2018; Liu D et al. 2021). Intriguingly, specific NopP variants cause nodulation blockage depending on the soybean genotypes, including GmNNL1 and Rj2 (Rfg1) via early-induced ETI-like responses (Sugawara et al. 2018; Zhang et al. 2021).

NopAB, NopC, NopE, and InnB are also rhizobium-specific effectors. NopAB of ORS3257 is a major effector that determines nodule efficiency in *V. mungo* and *unguiculata*, while playing a lesser role in symbiosis with *A. indica* (Teulet et al. 2019; Songwattana et al. 2021). NopC of HH103 strain exerts a positive effect on symbiosis with soybean and *V. unguiculata*, but is responsible for the nodulation-blocking phenotype in *L. japonicus* GIFU at an early stage of interaction (Jiménez-Guerrero et al. 2015, 2020). *B. diazoefficiens* USDA110 secretes a NopE effector that positively and negatively affects nodulation of various legumes. In *V. radiata*, NopE reduces the salicylic acid (SA)-mediated ETI-response for an efficient nodule organogenesis (Piromyou et al. 2021). NopE1 carries two EF hand-like motifs and might be transported as a preprotein into the plant cytosol, thus exhibiting self-cleavage in the presence of calcium at its two autoproteolytic sites (Wenzel et al. 2010). A novel effector of USDA61 strain, InnB is an incompatible factor in symbiosis with KPS1, but it promotes nodulation in *V. mungo* cultivar. USDA61 carrying InnB failed to form root hair curling and weakly infected via ITs on KPS1 roots; however, a small number of active nodules occasionally formed at the base of lateral roots, implying that USDA61 infection may occur via the crack entry mode at lateral roots emergence sites (Nguyen et al. 2018).

Rhizobium T3Es provide an alternative pathway in nodulation

The perception of rhizobial NFs by legume receptors has been believed to be a key factor to initiate nitrogen-fixing nodulation. Intriguingly, in the absence of NF signaling, certain bradyrhizobia can activate the nodulation process in some legume plants

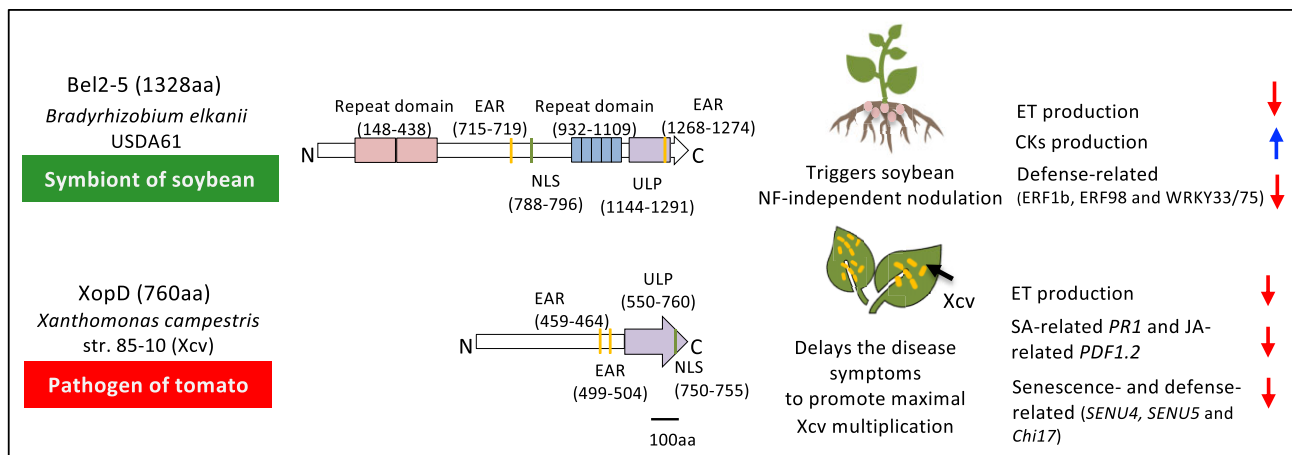


Figure 2. Structural and functional comparison between *Bradyrhizobium elkanii* USDA61 Bel2-5 and *Xanthomonas campestris* str. 85-10 (Xcv) XopD effectors on their respective hosts. Bel2-5 and XopD contain similar putative domains/motifs, including EAR motifs, a NLS motif, and ULP domain, except for repeat domains that are limited to rhizobia. Bel2-5 can trigger soybean nodulation in an NF-independent manner, while XopD delays tomato leaf chlorosis and necrosis to maximal Xcv multiplication. Both effectors can suppress ethylene (ET) production and host immune systems to promote bacterial invasion. However, Bel2-5 expands its role for symbiotic purpose by enhancing cytokinin (CKs) production on soybean roots. Red arrow with represents suppression while blue arrow represents enhancement. Information shown is based on studies by Hoston et al. 2003; Kim et al. 2008, 2013; Ratu et al. 2021a, 2021b.

that rely on a T3SS-dependent mechanism (Figure 1). The T3SS of ORS3257 and USDA61 strains can form nitrogen-fixing nodules on *A. indica* and soybean *nfr1* mutant, respectively, in an NF-independent manner (Okazaki et al. 2013, 2016).

ErnA (effector required for nodulation-A) is a novel ORS3257 effector that induces nodule organogenesis on *A. indica*, which is nodulated by NF-deficient rhizobia (Table 1b). Ectopic expression of *ernA* triggered root cell division, resulting in the formation of a nodule-like structure. Homology analysis showed that ErnA is a rhizobium-specific effector broadly dispersed throughout T3SS-carrying bradyrhizobium strains. Introduction of *ernA* into a strain unable to nodulate *A. indica*, conferred nodulation ability. ErnA targets the plant nucleus, thus it may associate with nucleic acids to manipulate the host gene expression (Teulet et al. 2019).

Bel2-5 is a USDA61 effector that shows a remarkable role in triggering NF-independent nodulation on *nfr1* mutant soybean (Table 1a). The C-terminus of Bel2-5 contains the ULP domain, hence it belongs to the C48 peptidase family along with NopD and XopD (Ratu et al. 2021a). Bel2-5 is located outside of the symbiotic island, suggesting that USDA61 may acquire the *bel2-5* gene by an independent horizontal acquisition event or as a result of fragmentation of the acquired symbiotic island. Amino acid sequences of Bel2-5 and ErnA show limited level similarity, thus they may have different symbiotic routes to activate NF-independent nodulation specifically on their respective hosts.

Multiple conserved domains/motifs are shared by rhizobium and pathogen T3Es

Bel2-5 is a pathogenic-like effector containing multiple putative conserved domains/motifs, including two internal repeat domains, two ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs, a nuclear localization sequence (NLS), and a ULP domain (Figure 2). Intriguingly, except for repeat domains, which are unique to rhizobia, other domains/motifs are present in Xcv XopD (Ratu et al. 2021a, 2021b). Following their translocation into plant cells, both Bel2-5 and XopD act as nuclear-localized effectors.

Phenotypic analyses showed that domains/motifs possessed by these effector homologs were confirmed to be essential for bacterial activities in controlling either a symbiotic or a pathogenic interaction with their respective hosts (Hotson et al. 2003; Kim et al. 2008, 2013; Ratu et al. 2021a, 2021b).

Both the symbiont and the pathogen can interfere with host immune systems via the suppression of defense-related phytohormones/genes to enhance their colonization of plants. In infected tissue, Xcv XopD delays the onset of tomato leaf chlorosis and necrosis in the late infection stage to sustain bacterial growth by targeting ethylene (ET) transcription factor (TF) SIERF4 and substantially suppressing ET production. Defense- and senescence-associated genes (*SENU4*, *SENU5*, and *Chi17*) are also suppressed by XopD (Kim et al. 2008, 2013). Another XopD variant delays gibberellin acid (GA)-induced DELLA degradation and promotes disease tolerance (Tan et al. 2014). Intriguingly, the presence of Bel2-5 can suppress the expression of ET-biosynthesis gene/responsive factors (eg *ACO1*, *ERF1b/98*) and plant defense response (eg *WRKY33/75*) in soybean roots, which are most likely to promote rhizobial infection. However, in rhizobia, the Bel2-5 effector expands its roles for symbiotic purposes by inducing the expression of cytokinin (CK)-related biosynthesis genes preferably for nodule organogenesis (Ratu et al. 2021a).

Nodulation test of ULP domain-swapped chimeras between Bel2-5 and XopD suggests that the ULP domain of effectors are partially exchangeable and may function similarly in their respective hosts. However, other features from the respective effector sequences may involve the differentiation of species-specific functions, either symbiotic or pathogenic, on the respective host plants. Yeast carrying only Bel2-5 ULP domain exhibited strong yeast growth inhibitory phenotypes that were comparable to the activity of Bel2-5 full-length, suggesting that the ULP domain of Bel2-5 can possess independent functional activities inside yeast cells (Ratu et al. 2021b). Recently, SUMO protease was found to be one of the major effector families among T3SS-carrying rhizobia, hence it may contribute significantly to nodulation (Teulet et al. 2020).

Two EAR motifs are found within Bel2-5 sequences that differ from Xcv XopD but are similar to that in PopP2, a T3E from phytopathogenic *Ralstonia solanacearum* (Ratu et al. 2021a, 2021b).

The EAR motif was previously described for plant transcriptional regulators (Kazan 2006). Mutation in PopP2 EAR motif caused loss of pathogen stabilization and activities in plant cells (Segonzac et al. 2017). In XopD, EAR motifs are required for the repression of SA-related PR1 and jasmonic acid (JA)-related PDF1.2, thus maximizing Xcv growth in tomato leaves (Kim et al. 2008). So far, the involvement of EAR in symbiotic activities remains unknown, however, disruption in EAR motifs of Bel2-5 affected the nodulation efficiency on legumes tested. Intriguingly, the EAR motifs were found in diverse rhizobial effectors, at least in Bel2-5 homologs (Ratu et al. 2021a, 2021b).

The Bel2-5 effector has additional repeat domains that are absent in Xcv XopD, which were later confirmed to be essential for nodulation (Ratu et al. 2021a, 2021b). The biological function of domains in legume–rhizobium symbiosis remains to be elucidated. However, the sequence composition or architecture of the repeat domains is predicted to be intimately linked to the effector's roles during bacterial infections (Mesarich et al. 2015). Although the Xcv XopD effector does not possess a repeat domain, it carries a DNA-binding domain (DBD) that is predicted to be involved in interaction with the host target (Kim, Taylor and Hotson 2013).

The T3SS-dependent NF-independent nodulation is predicted to constitute the ground state of rhizobium–legume interaction, considering the symbiosis lacks root-hair curling or infection–thread formation that is believed to be ancestral to infection pathway. The T3SS-dependent symbiosis likely occurs via cracks in the epidermis or via intercellular invasion between epidermal cells (Okazaki et al. 2013). This rhizobia infection process seems similar to that of phytopathogens, which multiply in the intercellular spaces of the plant tissue and/or the xylem, upon their invasion via natural openings, such as stomata/hydathodes or wounds (Kay and Bonas 2009).

In the initial stage, rhizobia may adopt Bel2-5 to modulate cytokinin signaling and induce nodulation. However, later, plants acquired the R protein to eliminate the infection of pathogenic bacteria carrying Bel2-5-/XopD-like effectors, which subsequently restricts the symbiosis, such as in Rj4 soybean (Faruque et al. 2015; Yasuda et al. 2016). In the second stage of nitrogen-fixing symbiosis, rhizobia gained the NF to overcome nodulation restriction by certain hosts. This secondarily invented NF might overlay the ancient effector-triggered nodulation by acquiring a more sophisticated process for developing fully differentiated root nodule symbiosis. Thus, the discovery of bradyrhizobium T3Es capable of triggering nodulation in a T3SS-dependent manner is a breakthrough toward nitrogen-fixing symbiosis.

Future perspectives

Rhizobia exploit the pathogenic secretion system and thus have developed specific effectors for symbiotic purposes. A comprehensive understanding of T3SS-related structure and function between pathogenic and symbiotic lifestyles is expected to illuminate various aspects related to the evolution of T3SS underlying plant–microbe interactions and beyond. Thanks to the increase in the number of genomes sequenced from T3SS-containing bacteria, an incredible diversity and complexity of T3SSs involved in host-specificity have been unveiled. Although many rhizobial effectors have been identified so far, their function inside host cells largely remains unknown. The challenge is to identify more host targets of rhizobial effectors to understand how nodulation regulatory networks that allow compatible or incompatible interactions depending on host geno-

types are affected. The discovery of effectors capable of initiating nodule organogenesis in an NF-independent manner may pave the way for transferring nitrogen-fixing nodulation to nonleguminous plants of agricultural importance. These efforts could be pursued via at least two major categories: (1) introducing effector-triggered nodulation into a broad range of free-living or associative N₂-fixing bacteria to improve their colonization on nonleguminous roots, and (2) identifying and engineering nonleguminous crops to carry receptors of effector-triggered nodulation to activate symbiosis signaling.

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