Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection

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Contents

Abstract

Leguminous plants form root nodules, within which soil bacteria called rhizobia symbiotically fix atmospheric nitrogen gas into ammonia, and provide the legumes with organic nitrogen compounds, such as ureides or asparagine, in exchange for the energy derived from photosynthates. This symbiotic relationship enables the legumes to grow in nitrogen-deficient soils. However, excessive nodulation damages the host growth by over-consuming nutrients in the plant. It can be assumed that legumes have evolved diverse mechanisms to regulate root nodule number according to the host's own energy demands.

Autoregulation of nodulation (AON) is a mechanism that regulates root nodule number, which is systemically controlled by roots and shoots via long-distance signaling factors. In AON, it is thought that a root-derived signal, generated upon infection by rhizobia, is transported to the shoot, eliciting a shoot-derived signal, which is translocated down to the roots to inhibit root nodulation. In addition to systemic regulation via long-distance signals derived from roots and shoots (AON), local regulation via short-distance signaling that occurs only in the root has been postulated as another mechanism for controlling root nodule formation. Plants also defend themselves against bacteria and other pathogens by the induction of localized acquired resistance (LAR) surrounding local lesions formed by hypersensitive response (HR) in the infected areas, and of systemic acquired resistance (SAR) or/and induced systemic resistance (ISR) to survive. Herein, I show that the number of root nodules is suppressed by programmed cell death (PCD), and is simultaneously controlled by SAR and ISR in soybean (*Glycine max* [L.] Merr.).

I observed that both the numbers of root nodule primordia (Stage1 + Stage2) and the ratios of Stage2 to (Stage1 + Stage2) in wild-type soybean Williams 82 were noticeably fewer than in hypernodulation mutant NOD1-3 on d4, d5, and d6, indicating that root nodule formation and development are suppressed in wild type during d4-d6, which also suggests that these suppression phenomena are genetically and biochemically regulated during d0-d4 after inoculation of the wild-type soybean roots with rhizobium. I also discovered characteristics of PCD accompanied by accelerated DNA degradation at 3.5 (d0), 26.5 (d1), 37 (d1), 66 (d2), 78 (d3), 97.5 (d4), and 122 h (d5), enhanced generation of reactive oxygen species (visualized by 3, 3′-diaminobenzidine staining) on d2, and markedly more cell death (detected on staining with trypan blue) and fewer

root nodule primordia on d3 after rhizobia inoculation in wild-type soybean Williams 82 than hypernodulation mutant NOD1-3. These findings suggest that the number of root nodules in wild-type soybean is suppressed by PCD. In addition, I conducted microarray, gene ontology and pathway analyses to detect the transcriptional response controlling the number of root nodules at rhizobia infection sites. Numbers of up-regulated genes associated with defense responses in wild type were several times larger than those in hyper-nodulation mutant (incidentally, it is shown that many genes were upregulated on d0 and/or d5, and were downregulated on d2 and/or d4 in Williams 82 compared with NOD1-3, suggesting that many genes are probably involved in the suppression of formation and development of root nodule primordia on d0 and/or d5, whereas these genes possibly participate in delaying the formation and development of root nodule primordia on d2 and/or d4 in wild type compared with the hypernodulation mutant.); essential factors for HR or disease resistance such as resistance genes, proteins generating H_2O_2, mitogen-activated protein kinase cascade, SAR, salicylic acid, jasmonic acid, ethylene etc., were activated in wild-type plants; the total numbers of sequences and enzymes participated in the pathways involved in defense responses including primary and secondary metabolisms in wild type were obviously larger than those in hyper-nodulation mutant. The data obtained from these analyses also corroborate the above finding that soybean controls its root nodule number by PCD suggesting simultaneously through SAR and ISR. I further show that most nodulins do not move in tandem with the regulatory mechanisms of defense response except for 2 nodulins. These findings provide new insight into the control of nodulation to balance nutritional requirements and energy status in legumes.

Like SAR and ISR, AON is induced by bacterial infection, and thus, exhibits systemic resistance against bacteria. In the present study, I elucidated the mechanisms underlying the suppression of root nodule formation in soybean using wild-type cv. Williams 82 and hypernodulation mutant NOD1-3. In the present study, I show that the containment of root nodule number in soybean is associated with PCD and HR, and is simultaneously controlled by SAR and ISR, suggesting that AON or local regulation is a part of SAR and ISR. It seems that LAR is induced around the local lesions in the infected areas by HR from pathogen infection, and shows a high degree of resistance in wild-type soybean Williams 82; and that the reason why it exhibits strong resistance in LAR is that the LAR around the HR site includes genes expressed both in the SAR and ISR.

Abbreviations

ABA: abscisic acid

AON: autoregulation of nodulation

bp: base pairs

CC: coiled-coil

DAB: 3, 3′-diaminobenzidine

DEGs: differentially expressed genes

ERF1b: ethylene-responsive transcription factor1b

ET: ethylene

ETI: effector-triggered immunity

GO: gene ontology

HAR1: HYPERNODULATION ABERRANT ROOT FORMATION1

HR: hypersensitive response

ISR, induced systemic resistance

JA: jasmonic acid

KEGG: the Kyoto Encyclopedia of Genes and Genomes

LAR: localized acquired resistance

LRR: leucine-rich repeats

MAPK: mitogen-activated protein kinase

MAPKK: MAPK kinase

miRNAs: micro RNAs

NBS: nucleotide-binding site

Nod factors: nodulation factors

NPR1: non-expressor of PR1

PAMP: pathogen-associated molecular pattern

PCD: programmed cell death

PCR: Polymerase chain reaction

PR genes: pathogenesis-related genes

PTGS: posttranscriptional gene silencing

PTI: PAMP-triggered immunity

RdRP: RNA-dependent RNA polymerase

R genes: resistance genes

RLKs: receptor-like kinases

ROS: reactive oxygen species

RT: reverse transcription

SA: salicylic acid

SAR: systemic acquired resistance

siRNAs: interfering RNAs

smRNAs: small RNAs

St1: Stage1

St2: Stage2

TE: tracheary element

TFs: transcription factors

TIR: Toll interleukin-1 receptor

TMV: tobacco mosaic virus

USDA: United States Department of Agriculture

UV: ultraviolet

WSR: wound-induced systemic resistance

Chapter 1 Introduction

Leguminous plants form root nodules, within which soil bacteria called rhizobia symbiotically fix atmospheric nitrogen gas into ammonia (Udvardi and Day 1990), and provide the legumes with organic nitrogen compounds, such as ureides or asparagine, in exchange for the energy derived from photosynthates (Udvardi et al. 1988). This symbiotic relationship enables the legumes to grow in nitrogen-deficient soils. However, excessive root nodulation is harmful to plants because of the overconsumption of energy from photosynthates (Nishimura et al. 2002). It can be assumed that legumes have evolved diverse mechanisms to regulate root nodule number according to the host's own energy demands.

Autoregulation of nodulation (AON) is a mechanism that regulates root nodule number, which is systemically controlled by roots and shoots via long-distance signaling factors (Kosslak and Bohlool 1984, Delves et al. 1986, Caetano-Anolles and Gresshoff 1991). In AON, it is thought that a root-derived signal, generated upon infection by rhizobia, is transported to the shoot, eliciting a shoot-derived signal, which is translocated down to the roots to inhibit root nodulation (Oka-Kira and Kawaguchi 2006). AON was discovered by researchers investigating the suppression of new root nodule formation by earlier developed nodules (Nutman 1952). Many studies have been conducted to identify both the root- and the shoot-derived signaling factors, including their receptors in the signaling pathway. For example, several leucine-rich repeat receptor-like kinases and other proteins, including HYPERNODULATION ABERRANT ROOT FORMATION1 (HAR1), NITRATE TOLERANT SYMBIOSIS1 (NTS1; NODULE AUTOREGULATION RECEPTOR KINASE [NARK]), SUPER NUMERIC NODULES (SUNN), and SYMBIOSIS29 (SYM29), which function in the shoots of *Lotus japonicus*, *Glycine max*, *Medicago truncatula*, and *Pisum sativum*, respectively, have been identified as key components of AON (Krusell et al. 2002,

Nishimura et al. 2002, Searle et al. 2003, Schnabel et al. 2005). All these proteins are orthologs of CLAVATA1 (CLV1) in *Arabidopsis*, which controls shoot and floral meristem size (Clark et al. 1997). In *L. japonicus*, two peptides, CLE-ROOT SIGNAL1 (CLE-RS1) and CLE-RS2, are candidates for root-derived mobile signaling factors that are specifically induced in infected roots. CLE-RS2 glycopeptides are transported through the xylem to the shoot where they directly bind to HAR1 (Okamoto et al. 2009, Okamoto et al. 2013). TOO MUCH LOVE was identified as a root-acting AON factor acting downstream of HAR1 to suppress nodulation (Magori et al. 2009, Takahara et al. 2013). As shoot-derived signaling factors, cytokinins systemically regulate root nodulation downstream of the CLE-RS1/2-HAR1 signaling pathway in AON (Sasaki et al. 2014). Mutants defective in AON showed supernodulation and increased number of lateral roots (Searle et al. 2003). During root nodule development, legumes respond to nodulation (Nod) factors produced by rhizobia. Perception of these factors by receptor kinases triggers a signaling cascade in the root epidermis (Suzaki et al. 2012). This induces dedifferentiation of some of the cortical cells, which subsequently divide to form the root nodule primordia (Szczyglowski et al. 1998, Oldroyd and Downie 2008, Oldroyd et al. 2011). During the course of root nodule development, rhizobia invade the dividing cortical cells via a tubular structure called the infection thread (Murray 2011), indicating that AON regulates the number of root nodules by controlling the redifferentiation of root cortex cells or cortical cell division, which eventually leads to the formation of root nodule primordia via shoot- or/and root-derived substances. Soybean hypernodulation mutant NOD1-3 was isolated from the cv. Williams (Gremaud and Harper 1989). Although the mutated site(s) have not been reported, evidence (Akao and Kouchi 1992, Vuong et al. 1996, Vuong and Harper 2000, Nishimura et al. 2002, Searle et al. 2003) suggests that the mutated locus is *GmNARK* (*Glycine max* nodule autoregulation receptor kinase) which controls AON in soybean (Searle et al. 2003, Ito et al. 2008).

In addition to systemic regulation via long-distance signals derived from roots and shoots

(AON), local regulation via short-distance signaling that occurs only in the root has been postulated as another mechanism for controlling root nodule formation (Yoshida et al. 2010). Susceptibility to infection by rhizobia is limited to the zone of root elongation and emerging root hairs (Bhuvaneswari et al. 1980), implying that the control of root system development indirectly leads to the regulation of root nodule formation. A few studies have reported that phytohormones affect local regulation. Root nodule formation is negatively affected by phytohormones, such as ethylene, abscisic acid (ABA), and jasmonic acid (JA) (Cho and Harper 1993, Penmetsa and Cook 1997, Suzuki et al. 2004, Nakagawa and Kawaguchi 2006, Sun et al. 2006). The position of root nodule primordia formation (usually opposite the xylem poles) is controlled by ethylene (Heidstra et al. 1997). Ethylene also exerts an inhibitory effect on infection thread initiation, infection thread growth after initiation, root hair deformation, early gene expression, and calcium spiking (Penmetsa and Cook 1997, Oldroyd et al. 2001). The *sickle*, an ethylene-insensitive mutant of *M. truncatula*, is characterized by hyperinfection of rhizobia (Penmetsa and Cook 1997). Generally, at the infection sites, coordination at various levels of controls represses production of excess nodules and failure of this coordination allows thousands of rhizobia to invade the root cells (Yoshida et al. 2010).

As mentioned above, it appears that leguminous plants manipulate systemic (AON) and local regulation harmoniously in order to maintain the appropriate level of root nodulation. Alternatively, plants defend themselves against countless bacteria and other microbial pathogens by the induction of both localized and systemic responses (Vijayan et al. 1998).

A nearly ubiquitous feature of plant-pathogen interactions is host cell death, which entails a rapid collapse of tissue, termed the hypersensitive response (HR; Dangl et al. 1996). The HR is triggered locally upon invasion by a pathogen, thus, inducing a defense response leading to the death of infected cells that appear as necrotic lesions (Hammond-Kosack and Jones 1996). The HR is genetically programmed in the plant, and is a consequence of transcription and translation of a unique set of genes that are induced upon infection in the host (Dixon et al. 1994, Godiard et al.

1994). Typically, HR is controlled by classic disease resistance (R) genes in the host plant (Dangl 1995, Staskawicz et al. 1995, Bent 1996). The local HR is often associated with the onset of systemic acquired resistance (SAR; Chester 1933, Ross 1961a, Enyedi et al. 1992, Ryals et al. 1994, 1996) that immunizes the entire plant against further infection (Ryals et al. 1996). Preceding the initiation of SAR, a high degree of resistance is induced in the local tissues surrounding the infected areas, which is termed as localized acquired resistance (LAR) (Ross 1961b). Invariably, sites of HR are focal points for transcriptional induction of plant defense genes in the neighboring cells (Somssich et al. 1988, Schmelzer et al. 1989). Subsequent biosynthesis of protective secondary metabolites and cell wall fortification around the HR site are also thought to contribute to overall pathogen containment (Dangl et al. 1996).

SAR is a mechanism that confers systemic resistance against a broad spectrum of plant pathogens (Durrant and Dong 2004). Long-distance signals initiated at the HR site lead to the induction of specific pathogenesis-related (PR) genes at the infection site as well as in uninfected parts of the plant, which is thought to contribute to resistance (Ryals et al. 1996). Signaling molecules, such as salicylic acid (SA), methyl jasmonate, ethylene, hydrogen peroxide, and superoxide radicals, have been proposed to be involved in induction and coordination of these plant responses (Hammond-Kosack and Jones 1996, Ryals et al. 1996). However, SA has been ascribed a central role in localized response and SAR (Delaney et al. 1994, Ryals et al. 1995, 1996, Dangl et al. 1996, Baker et al. 1997, Vijayan et al. 1998). In response to SA, the positive regulator protein NPR1 (non-expressor of PR1) moves to the nucleus, where it interacts with TGA transcription factors (binding specifically to variants of the palindrome TGACGTCA) to induce defense gene expression, thus, activating SAR (Durrant and Dong 2004).

Another type of systemic resistance is known as induced systemic resistance (ISR), which is induced by nonpathogenic rhizobacteria, and is phenotypically similar to pathogen-induced SAR (van Loon et al. 1998). The rhizobacteria-mediated ISR has been demonstrated against fungi,

bacteria, and viruses in Arabidopsis, bean, carnation, cucumber, radish, tobacco, and tomato. However, the inducing bacteria and the challenging pathogen remain spatially separated (van Loon et al. 1998). Some rhizobacteria induce resistance through the SA-dependent SAR pathway, whereas others do not and require perception of JA and ethylene by the plant for ISR to develop (van Loon et al. 1998). The key regulatory protein NPR1 functions downstream of the JA and ethylene response in the ISR pathway (Pieterse et al. 1998). Van Wees et al. (2000) demonstrated the enhancement of induced disease resistance by simultaneous activation of the SAR and ISR pathways in Arabidopsis.

Like SAR and ISR, AON is induced by bacterial infection, and thus, exhibits systemic resistance against bacteria (Nakagawa and Kawaguchi 2006). In the present study, I elucidated the mechanisms underlying the suppression of root nodule formation in soybean using wild-type cv. Williams 82 and hypernodulation mutant NOD1-3. In the present study, I show that the containment of root nodule number in soybean is associated with programmed cell death (PCD) and HR, and is simultaneously controlled by SAR and ISR, suggesting that AON or local regulation is a part of SAR and ISR.

These findings provide new insight into the control of nodulation to balance nutritional requirements and energy status in legumes, and reveal how legumes could preserve the mechanism of restraint of rhizobium infection for tens of millions of years. Thus, our findings will be able to fill various gaps in the agricultural field with regard to problems associated with the inoculation of root nodule bacteria into soybean, and I believe that these findings will provide a baseline for the development of a microbial inoculation technology useful in crop production.

Chapter 2 Materials and methods

2.1. Bacterial materials

The root nodule bacterium *Bradyrhizobium diazoefficiens* USDA110 (10 µl liquid taken from 15% glycerol stock) was inoculated into approximately 100 mL yeast-mannitol broth (Somasegaran and Hoben 1994) liquid culture medium in a 300-mL Erlenmeyer flask. This flask was wrapped in aluminum foil and shaken in an incubator under dark conditions at 118 rpm at 25°C for 5 days. The incubated USDA110 was washed thrice with 1X phosphate buffered saline using a centrifuge (at 10,000 rpm at 4°C for 3 minutes per time), and the absorbance was adjusted to 0.073 (λ = 600 nm) using sterilized nitrogen-free plant nutrient solution prior to inoculation of plants (Somasegaran and Hoben 1994).

2.2. Plant materials

Wild-type soybean (*Glycine max* [L.] Merr. cv Williams 82) and hypernodulation mutant NOD1-3 (a mutant of soybean *Glycine max* [L.] Merr. cv Williams, Gremaud and Harper 1989) seeds were surface-sterilized and sown in troughs formed using paper wicks of autoclaved seedling growth pouches (177 mm by 163 mm) that were filled with autoclaved 17 mL sterilized nitrogen-free plant nutrient solution (Somasegaran and Hoben 1994) in clean benches. The root area of the pouch was wrapped in aluminum foil to block out light. The sown soybean seeds were grown hydroponically in a growth chamber under the following conditions: temperature 25°C, relative humidity 60%, 14 h light/10 h dark photoperiod, illuminance 13,000 lx (adjusted using ultraviolet [UV]-fluorescent lamps meant for plants), and UV intensity 0.026 mW/cm^2. Two days after sowing, when root hairs emerged, a mark was made on the pointed end of the vascular bundle (red color mark) near the root tip. Four days after sowing, when lateral roots emerged, the zone (approximately 2 cm) from the

boundary between the shoot and root up to the mark was designated as the position (approximately 2 cm) of inoculation, and was inoculated with 1 mL suspension of *B. diazoefficiens* USDA110 adjusted to absorbance 0.073 (λ = 600 nm), which contained approximately 1.0×10^8 bacterial cells. In this experiment, I designated the onset of the inoculation as day 0 (d0), and cuttings (as mentioned above, approximately 2-cm-long portion of main roots, including lateral roots) adjacent to the inoculation site were taken on d0 (immediately following rhizobium inoculation), d2 (at the time point of 48 h after inoculation), d4 (at 96 h), d5 (120 h), and d6 (144 h) to be used as the experimental material. The cuttings taken on d6 were utilized only for histological observations and counting of root nodule primordia under a stereomicroscope (SZX16, Olympus, Tokyo, Japan).

As a side note, susceptibility to infection by rhizobia is limited to the zone of root elongation and emerging root hairs (Bhuvaneswari *et al.* 1980). On d0, I applied rhizobia to the zone where root hairs had just emerged, thus infection of the rhizobia is considered to have occurred mostly on d0. Then, on other days, I sampled roots from the same zone inoculated on d0. Therefore, under our experimental condition, the infection timing of rhizobium and the developmental status of the soybean root-nodules is thought to be almost synchronized.

2.3. Histological observations and counts of root nodule primordia

The plant materials collected were fixed in formaldehyde:acetic acid:70% (v/v) ethyl alcohol (5:5:90, v/v/v), and stained with 0.03% (w/v) toluidine blue solution, and then observed under a stereomicroscope to identify the differences in the number of root nodule primordia between wild-type plant Williams 82 and hypernodulation mutant NOD1-3, and to count the number of root nodule primordia.

2.4. RNA isolation and confirmation of total RNA quality

The marked positions where (approximately 2-cm-long portion of main roots, including lateral

roots) fresh roots of wild-type soybean Williams 82 and hypernodulation mutant NOD1-3 seedlings had been inoculated, were cut and collected on d0, d2, d4, and d5. The roots were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. The root infection sites from more than three independent seedlings were pooled as a single biological replicate, and three biological replicates were obtained from more than nine independent seedlings at each time point. Total RNA was isolated using RNAiso Plus reagent (Takara Bio, Shiga, Japan). Quality characterization of RNA samples was determined and confirmed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA samples were analyzed for the absence of genomic DNA contamination as follows: as positive controls, total RNAs were subjected to reverse transcription (RT) using Super Script III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions; as negative controls, RNase-free water was added instead of the RT enzymes in the RT reaction; after positive RT reaction, cDNAs were amplified by Polymerase chain reaction (PCR) using primers amplifying the endogenous control ubiquitin (ubiquitin [SUBI-1] F [5′-GAAGTCGAAAGCTCCGACAC-3′] and ubiquitin [SUBI-1] R [5′-TTTTGGGAACACATCCAACA-3′]), which hybridize to exon sequences of the *SUBI-1* (soybean polyubiquitin) genes; negative controls, which did not contain the RT enzymes in the PCR mix, were also subjected to PCR using the same primer sets. As shown in Supplementary Fig. S2, the bands in the positive controls were amplified from cDNAs (lanes 10-17), whereas no bands were observed in negative controls without RT enzymes (lanes 2-9), indicating that there was no genomic DNA contamination in our samples. In this experiment, 1.5% (w/v) agarose gel was used and stained with ethidium bromide. The gel pattern was photographed using an electronic UV transilluminator system (Funakoshi, Tokyo, Japan) and a bioprinter (Bio Craft, Tokyo, Japan). I also conducted a total RNA quality confirmation for all the 24 samples composed of the three biological replicates using an electrophoresis apparatus MultiNA (Shimadzu, Kyoto, Japan).

2.5. Microarray analysis, data processing, and detection of transcriptional response

After quality confirmation as described above, total RNAs were subjected to RT reaction using Super Script III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's instructions. The synthesized cDNA samples were subjected to microarray analysis using Affymetrix Soybean Gene 1.1 ST Array Strip, which contains 66,473 probe sets of *G. max* and 8,250 probe sets of *B. diazoefficiens* (http://www.affymetrix.com). For data processing, Affymetrix Power Tool (a cDNA microarray analysis system) was used to generate CEL files (raw data sets) to detect transcriptional response of soybean controlling the number of root nodules at rhizobia infection sites. CHP files (normalized expression data sets) were generated from the CEL files using the Expression Console software (Version 1.1; Affrymetrix). The CHP files were loaded into DNASTAR ArrayStar software (Version 5.0, http://www.dnastar.com/) using which differentially expressed genes (DEGs) whose expressions were upregulated or downregulated more than 2.9-fold in wild-type Williams 82 compared with hypernodulation mutant NOD1-3, in at least one time point on d0, d2, d4, or d5, were extracted.

2.6. Hierarchical cluster analysis

Hierarchical cluster analysis of the DEGs was carried out by DNASTAR ArrayStar software (Version 5.0, http://www.dnastar.com/).

2.7. Gene ontology (GO) analysis

To compare the functions of the DEGs in wild-type Williams 82 and hypernodulation mutant NOD1-3 in response to rhizobia, GO analysis was conducted using the Blast2GO (Version 2.5; Conesa *et al.* 2005; Götz *et al.* 2008) online service (https://www.blast2go.com) through the following steps: (1) loading sequences, in which the FASTA format sequence data file "SoyGene-1_0-st-v1.glyma1.transcript_cluster.fa" (Affymetrix) was loaded into Blast2GO; (2)

BLAST, in which Blast2GO used the Basic Local Alignment Search Tool (BLAST; Altschul *et al*. 1990) to find sequences similar to our query set; (3) mapping, the process of retrieving GO terms associated to the hits obtained after a BLAST search; (4) GO annotation, the process of selecting GO terms from the GO pool obtained from the mapping step and assigning them to the query sequences. Then, the sequence distribution was filtered by #Seqs: cutoff = 15.0 (selecting scores containing more than 15 sequences).

2.8. Pathway analysis

The GO-annotated upregulated sequences were used to carry out the main pathway analysis through the processes "Enzyme Code and KEGG" and "Load KEGG maps" in the Blast2GO online service (KEGG, the Kyoto Encyclopedia of Genes and Genomes; https://www.blast2go.com).

2.9. DNA degradation analysis

DNA degradation analysis was performed according to Yamada *et al*. (2003) with modifications. Genomic DNA was isolated from roots using Plant DNA Isolation Reagent (Takara Bio) according to the manufacturer's instructions. DNA was isolated from infected areas of roots at different time points, i.e., 3.5, 26.5, 37, 66, 78, 97.5, and 122 h, after rhizobia inoculation. The root infection sites from more than three independent seedlings were pooled as a single biological replicate, and three biological replicates were obtained from more than nine independent seedlings at every time point from wild type (Williams 82) and hypernodulation mutant (NOD1-3). Extracted DNA was electrophoresed in a 3% (w/v) agarose gel and stained with GelRed™ (Biotium, https://biotium.com/). The gel patterns obtained from three biological replicates were photographed with GelDoc^{TM} XR Plus system (Bio-Rad, http://www.bio-rad.com/). DNA degradation analysis using the photographs was conducted as follows. First, the intensities of Band2 (approximately 180 base pairs [bp] DNA) and the intensities of Band1 (undegraded DNA) in each lane were quantified

by GelDoc^{TM} XR Plus system (Bio-Rad) and the ratios of intensities (Band2)/(Band1) in each lane were calculated. Then, the second ratios, calculated as ([Band2]/[Band1] in Williams 82)/([Band2]/[Band1] in NOD1-3), obtained at the time points mentioned above were compared between Williams 82 and NOD1-3.

2.10. H_2O_2 detection by 3, 3'-diaminobenzidine (DAB) DAB Staining

To visualize H_2O_2 at the sites of infection by rhizobia, DAB staining was performed as described by Fester and Hause (2005), with slight modification. Intact soybean roots were collected on d2 after inoculation and incubated in DAB buffer (1 mg/mL DAB buffered in 100 mM sodium citrate, pH 3.7) for 2-4 h at room temperature. Stained samples were transferred into 10% (v/v) lactic acid for rinsing. Then, the samples were transferred into freshly prepared 10% (v/v) lactic acid and photographs were taken using a digital camera (DMC-TZ20, Panasonic, Osaka, Japan).

2.11. Cell death detection by trypan blue staining

Trypan blue staining was conducted as described by Koch and Slusarenko (1990), with slight modification (Kobayashi *et al.* 2011). Soybean roots were sampled on d3 after inoculation and boiled for 10 min in trypan blue solution (10 mL lactic acid, 10 mL glycerol, 10 g phenol, 10 mL distilled water, 40 mL ethanol, and 10 mg trypan blue). Then, the stained roots were decolorized in trichloroacetaldehyde monohydrate (2.5 g of trichloroacetaldehyde monohydrate dissolved in 1 mL of distilled water) for 24-48 h and observed under a stereomicroscope (SZX16, Olympus) to detect cell death.

Chapter 3 Results

3.1. Histological observation of soybean roots at rhizobia infection sites

Sampling for histological observation was performed on d3 (3 days), d4, d5, and d6 after rhizobium inoculation by fixing and staining with toluidine blue (Fig. 1A). Histological observation was conducted under a stereomicroscope to identify differences in the number of root nodule primordia between wild-type soybean Williams 82 and hypernodulation mutant NOD1-3 (Fig. 1B). Root nodule primordia were classified into Stage1 (St1) and Stage2 (St2; Fig. 2A, a and b). St1 signifies division in deep cortical tissue without an upheaval of cortical cells and St2 is represented by bulging out of the cortical layer without any constriction (Fig. 2A, a and b; Yamaya and Arima 2010). Lateral root primordia develop from the vascular bundles (Fig. 2A, b and c). Different developmental stages of root nodule primordia were counted on d4, d5, and d6 by observation under a stereomicroscope (Fig. 2B). On d3, observation of root nodule primordia under the stereomicroscope was extremely difficult because their structures could not be clearly discriminated. Starting from d4, a significant difference was observed in the number of root nodule primordia between wild type and hypernodulation mutant (Fig. 2B). The number of St1 root nodule primordia reached a peak on d5 in wild type and on d4 in hypernodulation mutant (Fig. 2B; Supplementary Fig. S1). The ratios of St2 to total primordia (St1 + St2) were 46% on d4, 83% on d5, and 100% on d6 in hypernodulation mutant NOD1-3, whereas they were 26% on d4, 46% on d5, and 78% on d6 in wild-type Williams 82 (Supplementary Fig. S1; Fig. 2B). The higher ratios of St2 in NOD1-3 throughout d4-d6 indicate that development of root nodules is faster in the mutant than in wild type. In addition, the significantly fewer number of root nodule primordia (St1 + St2) on d4, d5, and d6 in wild type compared with hypernodulation mutant indicate that formation of root nodules is suppressed in wild type (Fig. 2B). Since the total number of primordia and the St2 ratio, both were

lesser in the wild type than in the mutant on d4, I suggest that suppressive regulation of root nodule formation and development starts during d0-d4 in wild-type soybean roots.

3.2. Detection of transcriptional response through microarray analysis

Total RNA was extracted from 24 samples composed of three biological replicates of wild type and hypernodulation mutant, collected on d0, d2, d4, and d5 after rhizobium inoculation, and was confirmed for the absence of genomic DNA contamination (Supplementary Fig. S2) and the quality (Supplementary Fig. S3). The 24 RNA samples, whose A260/A280 ratio was 1.7-2.1 (Affymetrix recommended), were subjected to microarray analysis and a total of 591 DEGs, whose expressions were either upregulated or downregulated by more than 2.9-fold at least at one time point in wild-type Williams 82 compared with hypernodulation mutant NOD1-3, were extracted (Table 1).

The data showing percentages of these DEGs expressed on d0, d2, d4, and d5 out of the total 591 DEGs have been presented in Table 1. The number of genes upregulated in wild type decreased from d0 (244) to d2 (33) and d4 (11), and again increased on d5 (254; Table 1). At the beginning (d0 and d2) and end (d5) of the rhizobium infection period, a higher number of genes were upregulated (244 on d0, 33 on d2, and 254 on d5) compared with downregulated genes (30 on d0, 0 on d2, and 32 on d5; Table 1). These data indicate that these genes induced in wild type at different time points are possibly involved in the suppression of formation and development of root nodules. In contrast, in the middle stage of rhizobium infection (d4), there were more genes downregulated (62 on d4) than upregulated (11 on d4) in wild type (Table 1). This result indicates that expression of many genes is repressed on d4 in wild type compared with hypernodulation mutant to delay the formation and development of root nodule primordia.

To analyze the similarities and differences in expression patterns among the 591 DEGs, hierarchical cluster analysis was carried out (Supplementary Fig. S4). In wild-type plants, the expression patterns on d0 and d5 were similar, suggesting that there are many common early-term

(immediately after infection on d0) and late-term (on d5) responses. However, I consider that short-term (d2) and middle-term (d4) responses are different from the early- (d0) and late- (d5) term responses.

3.3. GO analysis of DEGs

To determine the functions of the 591 DEGs in wild-type Williams 82 and hypernodulation mutant NOD1-3 in response to rhizobia, I conducted GO analysis. The results of GO analysis are shown in Supplementary Table S1. In the GO analysis, 429 out of these 591 DEGs were annotated. GO terms with more than 15 sequences were filtered among GO terms for the annotated genes and are shown in Supplementary Table S2. I further selected the top 10 categories based on highest scores, as shown in Supplementary Table S2. Fig. 3 shows the percentage of score obtained out of the total score (778) for the top 10 categories, which are oxidation-reduction process (7%), DNA-dependent regulation of transcription (6%), protein phosphorylation (4%), organic substance catabolic process (4%), signal transduction (3%), and defense response (3%), as shown in Fig. 3. I focused on 5 out of these 10 categories (Fig. 4) because of many previous reports demonstrating that the processes of these 5 categories are associated with defense responses in plants (Dangl *et al.* 2000; Dangl and Jones 2001; Dong 2001; Asai *et al.* 2002; Gershenzon 2002; Hoeberichts and Woltering 2003; see 'Discussion' section). I showed that the number of genes upregulated from these five categories in wild-type Williams 82 was several times higher than the genes upregulated in hypernodulation mutant NOD1-3 (Fig. 4). Based on previous studies on defense responses in plants, from these five categories, I selected genes recognized to be associated with HR or disease resistance (Supplementary Table S3; Dangl *et al.* 2000; Dangl and Jones 2001; Dong 2001; Asai *et al.* 2002; Gershenzon 2002; Hoeberichts and Woltering 2003; see 'Discussion' section), i.e., I selected 23 out of 52 genes functional in oxidation-reduction process, 16 out of 44 genes in DNA-dependent regulation of transcription, 10 out of 33 genes in protein phosphorylation, 13 out of 26 genes in

signal transduction, and 23 out of 25 genes in defense response (Supplementary Table S3). Table 2 was constructed using the data presented in Supplementary Table S3 to highlight the genes functional in defense response out of all the other loci shown in Supplementary Table S3 that have similar functions. Many genes (51 in Table 2; 85 in Supplementary Table S3) included in the five categories are associated with HR or plant disease resistance in wild type (see 'Discussion' section for details).

3.4. Analysis of pathways where DEGs are functional based on the two types of soybeans' response to rhizobia

The pathways where 359 out of the total 59l DEGs annotated and upregulated in wild type (Williams 82) and 74 out of 591 DEGs annotated and upregulated in hypernodulation mutant (NOD1-3) are functional were analyzed (Supplementary Table S4 and Supplementary Image S1; Supplementary Table S5 and Supplementary Image S2). Table 3 was constructed from Supplementary Tables S4 and S5, focusing on primary and secondary metabolism in terms of disease resistance. The total number of sequences (#Seqs) and enzymes (#Enzs) participating in the pathways involved in defense responses, including primary and secondary metabolism pathways induced by rhizobia, was higher in wild type (#Seqs = 40, #Enzs = 29) than hypernodulation mutant (#Seqs = 11, #Enzs = 7; Table 3). The six pathways induced, i.e., starch and sucrose metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway, and phenylalanine metabolism in primary metabolism, and phenylpropanoid biosynthesis and isoflavonoid biosynthesis in secondary metabolism were common to both the plants (Table 3). However, the same #Seqs and #Enzs were shared by the two soybeans only in phenylalanine metabolism pathway (#Seqs = 3, #Enzs = 1) in primary metabolism and isoflavonoid biosynthesis pathway (#Seqs = 1, #Enzs = 1) in secondary metabolism. In the other four pathways, there were more #Seqs and #Enzs in wild type than hypernodulation mutant (Table 3). Seven pathways, including oxidative phosphorylation,

phenylalanine, tyrosine, and tryptophan biosynthesis, and tryptophan metabolism in primary metabolism, and flavonoid, flavones, and flavonol biosynthesis, cyanoamino acid metabolism, and nicotinate and nicotinamide metabolism in secondary metabolism, were induced only in wild type, but not in hypernodulation mutant (Table 3).

3.5. PCD in the infected areas of wild-type plant Williams 82

Total DNA was extracted from infected areas at 3.5, 26.5, 37, 66, 78, 97.5, and 122 h after inoculation of wild-type soybean Williams 82 and hypernodulation mutant NOD1-3 with rhizobia. Extracted DNA was electrophoresed in an agarose gel and stained. A photograph of DNA degradation with faint laddering of multiple bands of size approximately 180 bp was taken (Fig. 5A). DNA degradation appears to be a hallmark of animal apoptosis, a type of PCD (Wyllie 1980; Jacobson *et al.* 1997). Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 bp and multiples thereof (McCabe *et al.* 1997; Danon and Gallois 1998; Enari *et al.* 1998). This effect can be used to detect apoptosis via the DNA laddering assay (Wyllie 1980). The DNA ladder is characteristic of apoptosis and can arise during PCD in plants (Ryerson and Heath 1996). DNA degradation analysis was conducted in Williams 82 and NOD1-3 using three gel patterns obtained from three biological replicates (Fig. 5). The averages of second ratios calculated as ([Band2]/[Band1] in Williams 82)/([Band2]/[Band1] in NOD1-3) were derived from three biological replicates and three technical replicates (quantification of intensity of bands in the three biological replicates was performed thrice). The average ratios of ([Band2]/[Band1] in Williams 82)/([Band2]/[Band1] in NOD1-3) at 3.5, 26.5, 37, 66, 78, 97.5, and 122 h were 3.0, 2.3, 20.0, 5.5, 1.8, 4.9, and 6.4, respectively (Fig. 5B). Similar DNA degradation result was obtained in a repeat experiment using four to five biological replicates (data not shown). These data indicate that DNA degradation with faint laddering of multiples of approximately 180 bp occurs in the infected areas

of both wild-type soybean Williams 82 and hypernodulation mutant NOD1-3. Additionally, Fig. 5B shows that the DNA degradation rate in wild-type Williams 82 is higher than in hypernodulation mutant NOD1-3, suggesting that progression of PCD possibly involves the control of root nodule number in wild-type soybeans.

The production of hydrogen peroxide (H_2O_2) has been recognized as an important feature of plant cells that undergo PCD during host-pathogen interaction (Liu and Friesen 2012). I performed DAB staining which helps in visualization of H_2O_2 accumulation in the form of a brown precipitate (Thordal-Christensen *et al.* 1997). Initially, stronger polymerization of DAB was observed within approximately 2 cm of the inoculated areas in roots of wild type (Williams 82) compared with hypernodulation mutant (NOD1-3) on d2 (Fig. 6). This result indicates that the infected areas of wild-type soybeans generate more reactive oxygen species (ROS), including H_2O_2, than those of hypernodulation mutant.

As clear and countable root nodule primordia (Fig. 2 and Supplementary Fig. S1) and generation of ROS (Fig. 6) were detected on d4 and d2, respectively, observation of dead cells was performed on d3. To detect and compare dead cells in infected areas of wild-type Williams 82 and hypernodulation mutant NOD1-3, roots were stained with trypan blue and toluidine blue, which stain dead cells and root nodule primordia, respectively, after rhizobia inoculation on d3 (Fig. 7). The results indicated a higher amount of cell death in wild type than hypernodulation mutant, whereas the number of nodule primordia was lesser in wild type than hypernodulation mutant (Fig. 7). This suggests that the number of root nodule primordia in wild-type Williams 82 is restrained by cell death.

3.6. Relationship between nodulins and mechanism of regulation of defense response in wild-type soybean Williams 82

Additionally, I investigated if there was an association between nodulins and mechanism of

regulation of defense response in wild-type soybean Williams 82 based on the results obtained from microarray analysis and the data shown in Supplementary Table S1 (Table 4). Table 4 shows patterns of changes in the amount of sequences annotated as nodulin genes. Pattern 6 was similar to the one presented in Table 2, which showed a higher fold change on d0 and/or d5, as compared with d2 and d4, for most of the genes associated with HR or disease resistance, whereas patterns 1-5 were different from those shown in Table 2. This suggests that most nodulins are involved in nodule organogenesis, but do not function in tandem with the regulatory mechanisms of defense response; however, the two nodulins showing pattern 6 are probably associated with these mechanisms.

Chapter 4 Discussion

I observed that both the numbers of root nodule primordia (St1 + St2) and the ratios of St2 to (St1 + St2) in wild-type soybean Williams 82 were noticeably fewer than in hypernodulation mutant NOD1-3 on d4, d5, and d6 (Fig. 1B and 2B, and Supplementary Fig. S1), indicating that root nodule formation and development are suppressed in wild type during d4-d6, which also suggests that these suppression phenomena are genetically and biochemically regulated during d0-d4 after inoculation of the wild-type soybean roots with rhizobium. I also discovered characteristics of PCD accompanied by accelerated DNA degradation at 3.5 (d0), 26.5 (d1), 37 (d1), 66 (d2), 78 (d3), 97.5 (d4), and 122 h (d5), higher amount of ROS generation on d2, and markedly more cell death and fewer root nodule primordia on d3 after rhizobia inoculation in wild-type soybean Williams 82 than hypernodulation mutant NOD1-3 (Fig. 5-7). These findings suggest that the number of root nodules in wild-type soybean is probably suppressed by PCD. In addition, I conducted microarray, GO, and pathway analyses to detect the transcriptional response controlling the number of root nodules at rhizobia infection sites (Table 2 and Table 3). The data obtained from these analyses also corroborate the above finding that soybean controls its root nodule number by PCD. Incidentally, it is shown that many genes were upregulated on d0 and/or d5, and were downregulated on d2 and/or d4 in Williams 82 compared with NOD1-3 (Table 2 and Supplementary Fig. S4), suggesting that many genes are probably involved in the suppression of formation and development of root nodule primordia on d0 and/or d5, whereas these genes possibly participate in delaying the formation and development of root nodule primordia on d2 and/or d4 in wild type compared with the hypernodulation mutant.

4.1. R genes are upregulated at the infection sites in wild-type soybean Williams 82

R genes of plants perceive attacks by various pathogens (Shirasu and Schulze-Lefert 2000), and are classified into two main classes: (1) Nucleotide-binding site (NBS)-leucine-rich repeats (LRR) containing NBS and C-terminal LRR, which can be further divided into Toll interleukin-1 receptor (TIR)-NBS-LRR and coiled-coil-NBS-LRR, and (2) extracellular LRR. Tobacco N genes that confer resistance to tobacco mosaic virus (TMV) are R genes (Whitham *et al.* 1994). In the present study, gene loci functional in signal transduction, defense response, and protein phosphorylation were upregulated in Williams 82, compared with NOD1-3, and these genes are considered to be R genes (Table 2). The defense-related genes shown in Table 2 are similar to At3g14460-like gene, and were upregulated in the root of wild-type soybean Williams 82 (Table 2). The Arabidopsis ortholog (At3g14460) of adenyl cyclases in *Zea mays* pollen is annotated as disease resistance protein belonging to the NBS-LRR family used for pathogen sensing with a role in defense responses and apoptosis-like cell death (PCD; DeYoung and Innes 2006; Gehring 2010). Moreover, the gene locus *Glyma18g36000.1*, functional in defense response (Table 2) was upregulated in Williams 82, but not in NOD1-3, particularly on d0 and d5. The function of Glyma18g36000.1 is predicted to be similar to RPM1-interacting protein 4, which is required for the induction of HR by RPM1 in Arabidopsis (Mackey *et al.* 2002; RPM1 is an NBS-LRR protein from *Arabidopsis thaliana* that confers resistance to *Pseudomonas syringae* expressing either avirulence [*avr*] *Rpm1* or *avrB* [Grant *et al.* 1995]).

4.2. Proteins participating in the generation of H_2O_2 are induced in wild-type soybean Williams 82

An oxidative burst leading to the generation of superoxide (O_2^-) and accumulation of H_2O_2 is a characteristic early feature of HR following the perception of an avirulent pathogen attack (Doke 1983; Jabs *et al.* 1996; Lamb and Dixon 1997). These ROS generated from this oxidative burst are directly protective and drive oxidative cross-linking of cell wall structural proteins that later become

lignified (Lamb and Dixon 1997). I detected higher ROS generation on d2, and observed more cell death and fewer root nodule primordia in wild-type soybean Williams 82 than hypernodulation mutant NOD1-3 on d3 after rhizobia inoculation (Fig. 6 and 7). Enzymes considered to be responsible for H_2O_2 generation include a peroxidase catalyzing oxidation of NADH (Gross *et al.* 1977), cell-wall-bound laccase (Bao *et al.* 1993), NADPH oxidase (Levine *et al.* 1994; Lamb and Dixon 1997; Sagi and Fluhr 2001; Yoshioka *et al.* 2003), and diamine oxidase (Federico and Angelini 1991; Møller and McPherson 1998). In the present study, as shown in Table 2, the gene loci functional in the category of oxidation-reduction processes, such as NADH-plastoquinone oxidoreductase subunit4, laccase diphenol oxidase family protein isoform1, laccase family protein, laccase-11-like, laccase17, peroxidase10-like, and peroxidase64 (for their detailed GO IDs, see Table 2), were markedly induced at the infection sites of wild-type roots Williams 82, particularly on d0 and d5, compared with NOD1-3. These data imply that these genes are induced in wild type for the generation of H_2O_2, inducing lignin that strengthens cell walls, inhibiting the extension of bacteria.

4.3. Kinases involved in mitogen-activated protein kinase (MAPK) cascade, which induces ROS and HR-like cell death, are activated in wild-type soybean Williams 82

Previous studies have indicated that MAPK cascade is involved in the control of ROS generation and HR-like cell death induction (Yang *et al.* 2001; Ren *et al.* 2002; Katou *et al.* 2003). MAPK kinase (MAPKK) and MAPK are identified as serine/threonine kinases (Matsuda *et al.* 1993; Katou *et al.* 2003). In the present study, as shown in Table 2, the nine gene loci categorized under protein phosphorylation pathway, such as serine/threonine-protein kinase nek2, probable serine/threonine-protein kinase At5g41260, serine/threonine-protein kinase ht1-like, probable LRR receptor-like serine/threonine-protein kinase At1g14390-like, and MAPKK kinase yoda-like, were upregulated at the infection sites in wild-type roots compared with hypernodulation mutant,

especially on d0 and d5. I suggest that these genes probably function in MAPK cascade and participate in the regulation of ROS generation and HR-like cell death.

4.4. Protein kinases related to calcium influx which triggers oxidative burst or cell death in wild-type soybean Williams82

Calcium influx into the cytoplasm (Chandra and Low 1997, Piedras et al. 1998) and changes in protein phosphorylation (Kauss and Jeblick 1995, Miura et al. 1995) are implicated in the activation process of the oxidative burst. In soybean cells, H_2O_2 stimulates Ca^{2+} influx to activate a physiological cell death program that is remarkably similar to apoptosis in animals (Levine et al. 1996). The gene loci described with calcium-dependent protein kinase 3-like and calmodulin-domain protein kinase 5 isoform 1 in the category of protein phosphorylation in Table 2 may be involved in the activation process of the oxidative burst or cell death.

4.5. Several primary and secondary metabolism pathways related to defense response are activated in wild-type soybean Williams 82

Nakane *et al.* (2003) revealed that high levels of proteins functional in glycolysis and shikimate pathway are necessary to condition downstream metabolism, such as isoprenoid and phenylpropanoid pathway, and that NADPH produced through the pentose phosphate pathway seems to participate in the oxidative burst. Their results demonstrated that signals from the pathogen activate not only the secondary metabolism but also the primary metabolism, leading to the rapid expression of defense response in potato tubers. This report appears to corroborate our finding that acceleration of primary metabolism and secondary metabolism (in the wild-type Williams 82 compared with NOD1-3; Table 3) results in hastening of defense response, including HR and so-called PCD, which substantially suppresses the number of root nodule primordia in Williams 82 as compared with NOD1-3 soybeans.

4.6. Suppression of the root nodule primordia in wild-type soybean is regulated by HR and is simultaneously controlled by SAR and ISR via phytohormones

In the present study, the gene locus *Glyma02g41700.1*, categorized under signal transduction in Table 2, is described in several GO IDs, such as JA-mediated signaling pathway, SA biosynthetic process, SAR, SA-mediated signaling pathway, regulation of plant-type HR, defense response to bacterial pathogens, MAPK cascade, and leaf senescence, and its expression is enhanced in the roots of wild-type soybean Williams 82, but not in NOD1-3, particularly on d0 and d5. This result suggests that the suppression of generation and development of root nodule primordia in wild-type soybean Williams 82 is regulated by HR, and that SAR, SA, JA, and MAPK cascade are involved in the suppressive regulation. Similarly, *Glyma04g30930.1* categorized under signal transduction in Table 2, is functional in SAR, ABA-mediated signaling pathway, response to ethylene stimulus, and SA biosynthetic process, as per the GO IDs, and its expression was elevated in the roots of Williams 82, but not in NOD1-3, particularly on d0 and d5. This result indicates that the suppression of the number of root nodule primordia in wild-type Williams 82 is also controlled by SAR, and that SA, ET (ethylene), and ABA are involved in the signaling pathway.

In the present study, the gene locus *Glyma11g34940.1*, categorized under defense response in Table 2, is described as coronatine-insensitive protein1-like, and is functional in JA-mediated signaling pathway, defense response to bacterial pathogens, and JA and ethylene-dependent systemic resistance, as per the GO IDs, and its expression was promoted in the roots of Williams 82, but not in NOD1-3, particularly on d5. In addition, the gene locus *Glyma13g18410.1*, categorized under DNA-dependent regulation of transcription was upregulated in wild type, but not in hypernodulation mutant on d0. *Glyma13g18410.1* is described as ethylene-responsive transcription factor1b (ERF1b)-like. ERF1b is known to act as a transcriptional activator, binding to GCC-box PR promoter element, and seems to be a key integrator of ET and JA signals in the regulation of ET/JA-dependent defenses (Solano *et al.* 1998; Lorenzo *et al.* 2003).

Similar to these reports, our results also suggest that the suppression of the number of root nodule primordia in wild-type soybean Williams 82 is regulated by JA and ET-dependent defense pathways.

It has become evident that plants utilize multiple pathways to transduce pathogenic signals to activate HR, SAR, and other resistance responses, and that SA-mediated SAR is not the only pathway that can lead to broad-spectrum disease resistance (Dong 1998). Emerging evidence strongly suggests the importance of JA and ET as alternative signals in the induction of resistance against microbial pathogens, along with their well-characterized roles in wound-induced systemic resistance (WSR) and ISR in plants (Doares *et al.* 1995; Dong 1998; Pieterse and van Loon 1999). ISR differs from pathogen-inducible SAR, and is not accompanied by the accumulation of SA or the systemic expression of PR genes, but requires JA and ET (Pieterse *et al.* 1996; Knoester *et al.* 1999; Van Wees *et al.* 2000). Van Wees *et al.* (2000) demonstrated the enhancement of induced disease resistance due to the simultaneous activation of SAR and ISR pathways in *Arabidopsis*.

Based on these results and the reports stated above, it appears that suppression of the number of root nodule primordia in wild-type soybean Williams 82 is regulated by HR and is simultaneously controlled by SAR and ISR (WSR).

4.7. Numeral suppression of nodule primordia in wild-type soybean is simultaneously regulated by LAR and SAR along with HR via WRKY network

Plants are capable of extensive reprogramming of their transcriptome in a highly dynamic and temporal manner (Pandey and Somssich 2009). This regulation in response, leading to adaptive plasticity of plants in highly variable environments, is mainly achieved by enforcement of a network of various transcription factors (TFs; Pandey and Somssich 2009). WRKY TFs are a large family of regulatory proteins forming such a network (Eulgem and Somssich 2007).

The WRKY TF superfamily consists of 74 and 109 members in Arabidopsis (*Arabidopsis*

thaliana) and rice (*Oryza sativa*), respectively (Eulgem and Somssich 2007, Ross et al. 2007, Pandey and Somssich 2009). The majority of the analyzed *WRKY* genes respond to pathogen attack and to the endogenous signal molecule SA (Eulgem and Somssich 2007, Pandey and Somssich 2009).

Plant innate immunity is composed of two inter-connected branches: (1) PTI, or pathogen-associated molecular pattern (PAMP)-triggered immunity, which is initiated by the recognition of molecular patterns (such as bacterial flagellin) of many pathogens by extracellular receptor-like kinases (RLKs) and often activates downstream MAPK cascades, defense genes, production of ROS, and deposition of callose to reinforce the cell wall at sites of infection; and (2) ETI, or effector-triggered immunity, driven by plant disease resistance proteins (major *R* gene products) that recognize directly or indirectly specific pathogen-derived effectors (Chisholm et al. 2006). PTI and ETI activate LAR as well as SAR, which are modulated by phytohormones, especially JA and SA (Durrant and Dong 2004, Bostock 2005). JA-dependent plant defenses are generally activated by necrotrophic pathogens and chewing insects, whereas SA-dependent defenses are often triggered by biotrophic pathogens. JA and SA signaling usually act antagonistically, but synergism between these two phytohormons has also been observed (Mur et al. 2006). These responses to pathogen attack require large-scale transcriptional reprogramming, including those of TF families such as *WRKY* genes (Eulgem 2005, Ryu et al. 2006, Naoumkina et al. 2008). In addition, WRKY TFs can modulate the expression of many *PR* genes via binding to a W-box ([T][T]TGAC[C/T]) in *PR*s promoters, and result in cell death (Yang et al. 2009, Rushton et al. 2010, Tang et al. 2013).

In the root of wild-type soybean Williams 82, the gene locus, *Glyma14g12260.1* belonging to the category of protein phosphorylation in Table 2 is described as lrr receptor-like kinase-family protein in Seq. Description, and its expression is promoted than in hypernodulation mutant NOD1-3, particularly on d0 and d4. The lrr receptor-like kinase-family protein may be

thought to belong to one of R genes, also be used to one of extracellular RLKs to recognize PAMP in PTI. Additionally, in the root of wild-type soybean Williams 82, the gene loci, *Glyma01g31920.1*, *Glyma14g38010.1*, *Glyma18g22730.1*, and *Glyma18g37630.1* belonging to the category of regulation of transcription, DNA-dependent in Table 2 are described as probable wrky transcription factor 33-like, probable wrky transcription factor 33, probable wrky transcription factor 21, and wrky transcription factor 21 family protein in Seq. Description, respectively, and their expressions are enhanced than in hypernodulation mutant NOD1-3, particularly on d0 and/or d5. Moreover, in the root of wild-type soybean Williams 82, the gene locus, *Glyma03g28850.1* belonging to the category of defense response in Table 2 is described as endo-beta- -glucanase in Seq. Description, and as carbohydrate metabolic process, and defense response in GO IDs, and its expression is promoted than in hypernodulation mutant NOD1-3, particularly on d5. Bata-glucanase represents a group of carbohydrate enzymes that break down beta-glucan, a polysaccharide made of several glucose sub-units. These glucans also create the cell wall of certain types of pathogens, such as fungi or bacteria. This hydrolase is known as a member of PR genes induced by the infection of microbes in plants. Furthermore, in the root of wild-type soybean Williams 82, the gene locus, *Glyma19g24520.1* belonging to the category of defense response in Table 2 is described as regulation of plant-type hypersensitive response and negative regulation of programmed cell death in GO IDs, and its expression is downregulated almost 3 times than in hypernodulation mutant NOD1-3 on d4, implying that the PCD triggered by HR is much more progressed in wild-type Williams 82 than in hypernodulation mutant NOD1-3, and which leads to the suppression of nodule primordia in wild-type soybean Williams 82.

These results and the reports mentioned above suggest that the suppression of the number of nodule primordia in wild-type soybean Williams 82 is simultaneously regulated by LAR and SAR, which require MAPK cascades, ROS, phytohormones, WRKY TFs and *PR* genes, along with HR during the nodule primordial appearance and the subsequent nodule formational and

developmental processes.

The importance of small RNAs (smRNAs), including micro RNAs (miRNAs) and small interfering RNAs (siRNAs), in plant processes related to adaptation to (a)biotic stresses is increasingly becoming evident, and the endogenous plant-derived smRNAs probably have broad implications in posttranscriptionally regulating plant response to pathogen attack (Navarro et al. 2006, Pandey and Baldwin 2007, Voinnet 2008, Pandey and Somssich 2009). The smRNAs regulate gene expression posttranscriptionally in a process often called RNA interference, RNA silencing, or posttranscriptional gene silencing (PTGS; Pandey and Somssich 2009). Previous data show the existence of a WRKY-smRNA interactome, where on the one hand, pathogen attacks trigger the expression of *WRKY* genes that regulate cellular smRNA populations, and on the other hand, several differentially regulated smRNAs modulate WRKY TF levels by targeting their transcripts (Zhang et al. 2008, Zhou et al. 2008, Pandey and Somssich 2009). RNA-dependent RNA polymerase (RdRP) synthesizes smRNAs, and is induced by SA and pathogen attacks activating plant defense response (Xie et al. 2001, Pandey and Somssich 2009). In the root of wild-type soybean Williams 82, the gene locus, *Glyma08g04490.1* belonging to the category of regulation of transcription, DNA-dependent in Table 2 is described as actin-related protein 4-like in Seq. Description, and as chromatin silencing by small RNA; production of miRNAs involved in gene silencing by miRNA in GO IDs, and its expression is upregulated than in hypernodulation mutant NOD1-3, particularly on d0 and d5.

Our results and the reports described above suggest that the suppression of the number of nodule primordia in wild-type soybean Williams 82 is also regulated by WRKY-smRNA interactome during the nodule primordia appearance and the subsequent nodule formational and developmental processes.

4.8. Activation of autophagy in wild-type soybean Williams 82

Research on plant PCD has focused mainly on two categories: PCD during normal development and PCD during HR triggering by pathogen infection (Lv et al. 2014). Previous studies have suggested that autophagy plays crucial roles in both these processes. Kwon et al. (2010) reported that autophagy occurred during tracheary element (TE) differentiation, and that RabG3b, being a component of autophagy, regulated the TE PCD *in vitro* in Arabidopsis cultures. Kwon et al. (2013) also showed that RabG3b plays a positive role in autophagy and promotes HR PCD in response to avirulent bacterial pathogens in *Arabidopsis*. Transgenic plants overexpressing *RabG3b* displayed accelerated, unrestricted HR PCD within 1 d of infection, in contrast to the autophagy-defective *atg5-1* mutant, which gradually developed chlorotic cell death of uninfected sites over several days (Kwon et al. 2013). Escamez et al. (2016) proposed that eukaryotic cells undergoing PCD tightly regulate the level of autophagy to avoid detrimental consequences for the surrounding cells. These reports suggest that autophagy has substantial implications for PCD. In the roots of wild-type soybean Williams 82, expression of the gene locus *Glyma09g41160.1*, categorized under protein phosphorylation and autophagy as per the GO ID, was upregulated in wild type, but not in hypernodulation mutant, particularly on d0 and d5 (Table 2). In corroboration with the reports stated above, our results suggest that activation of autophagy triggers PCD, or vice versa, to protect other cells from bacteria in wild-type soybean Williams 82. This result also implies that root nodule formation and development are suppressed by HR PCD in wild-type soybean Williams 82.

4.9. Detection of DNA degradation at infection sites in soybean roots

In most multicellular organisms, PCD is built into the routine processes of development, growth, and response to environmental stresses (Wang H *et al.* 1996; Ito and Fukuda 2002). Formation of DNA ladder on visualization by gel electrophoresis is characteristic of apoptosis, and can arise during PCD in plants (Ryerson and Heath 1996), although it does not occur in all forms of PCD (McCabe *et al.* 1997). In plants, the same apoptotic-like DNA ladder has been reported in PCD that

is associated with death of cereal aleurone or endosperm (Wang M *et al.* 1996; Young *et al.* 1997), senescence of the carpel, petal, and leaf (Orzáez and Granell 1997a; 1997b; Yen and Yang 1998), loss of cell viability in cell-suspension cultures (Callard *et al.* 1996), abiotic stress-induced cell death, such as by cold , salt stress, D-mannose, and UV-C radiation (Katsuhara 1997; Koukalova *et al.* 1997; Danon and Gallois 1998; Stein and Hanson 1999), and biotic stress-induced cell death, such as the HR to microbial pathogens (Ryerson and Heath 1996; Wang H *et al.* 1996).

Consistent with these studies, our results also show that DNA degradation with faint laddering of multiples of approximately 180 bp fragments occurs in the rhizobia-infected areas of soybean roots (Fig. 5), although Levin *et al.* (1996) reported that DNA in soybean cells treated with an avirulent strain of a pathogen seemed to be fragmented into large, ~50kb pieces. Moreover, the DNA degradation rate in wild-type Williams 82 is higher than in hypernodulation mutant NOD1-3 (Fig. 5), suggesting a possibility that PCD is accelerated in wild-type soybean, which leads to lesser nodulation in wild type compared with hypernodulation mutant.

Chapter 5 Conclusion

Our present study suggests that the formation of root nodules in wild-type soybean Williams 82 is regulated by PCD accompanied with HR, and is simultaneously controlled by SAR and ISR. It seems that LAR is induced due to HR around the local lesions caused by bacterial infection, and leads to a high degree of resistance in wild-type soybean Williams 82. The strong resistance exhibited by LAR is due to the expression of genes involved in both SAR and ISR in the regions surrounding the HR site.

To our knowledge, this is the first study indicating involvement of defense responses in AON. Further study is needed to understand the relationship between long-distance signaling factors already reported in AON and defense responses in regulation of nodule formation to balance nutritional requirements and energy status in soybean plants.

Figures

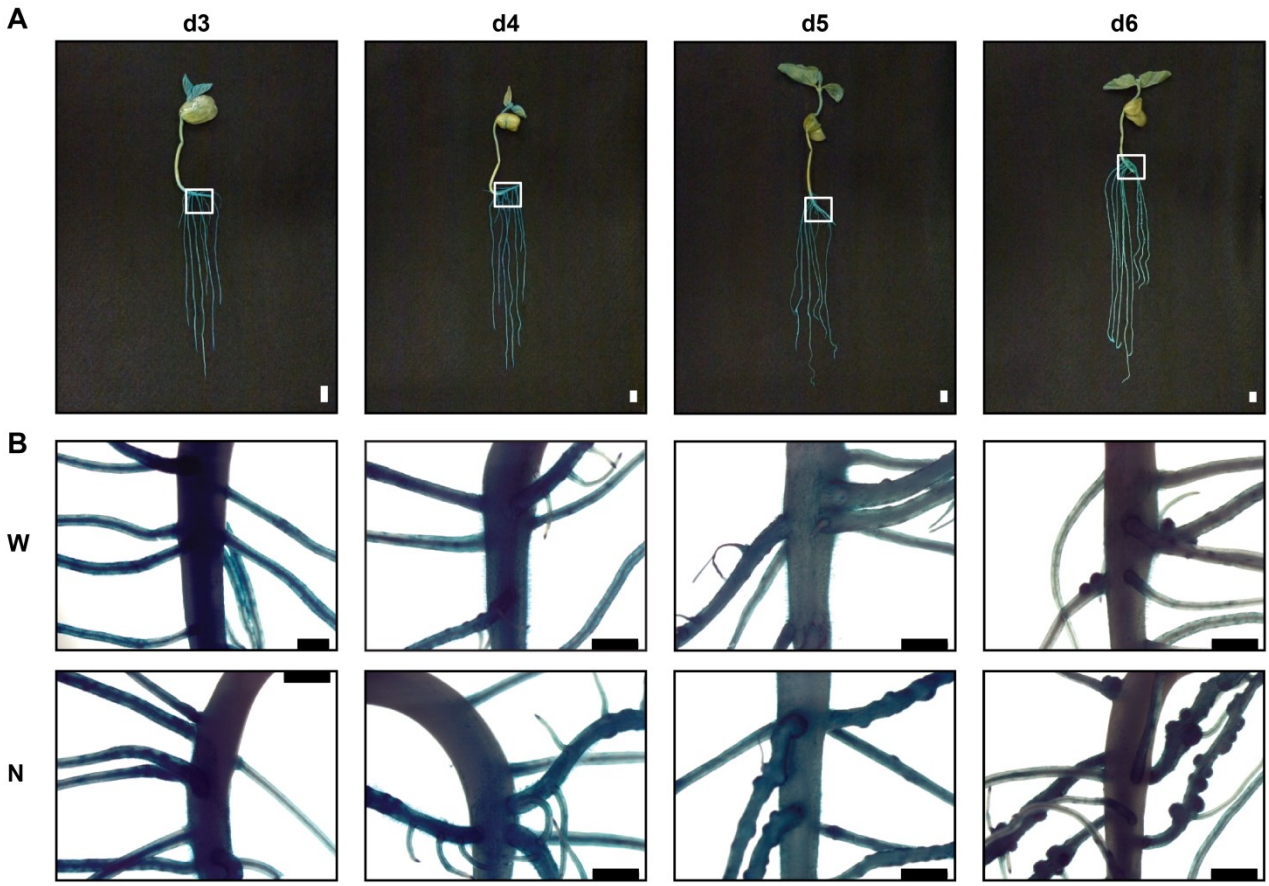


Figure 1. Histological observation under a stereomicroscope. A, Soybean plants stained with toluidine blue on d3 (3 days), d4, d5, and d6 after rhizobium inoculation. B, Histological observation under a stereomicroscope to identify the differences in number of root nodule primordia between wild-type plant Williams 82 (W) and hypernodulation mutant NOD1-3 (N). Square areas in A are imaged and magnified in B. Bars = 1 cm in A, 2 mm in B.

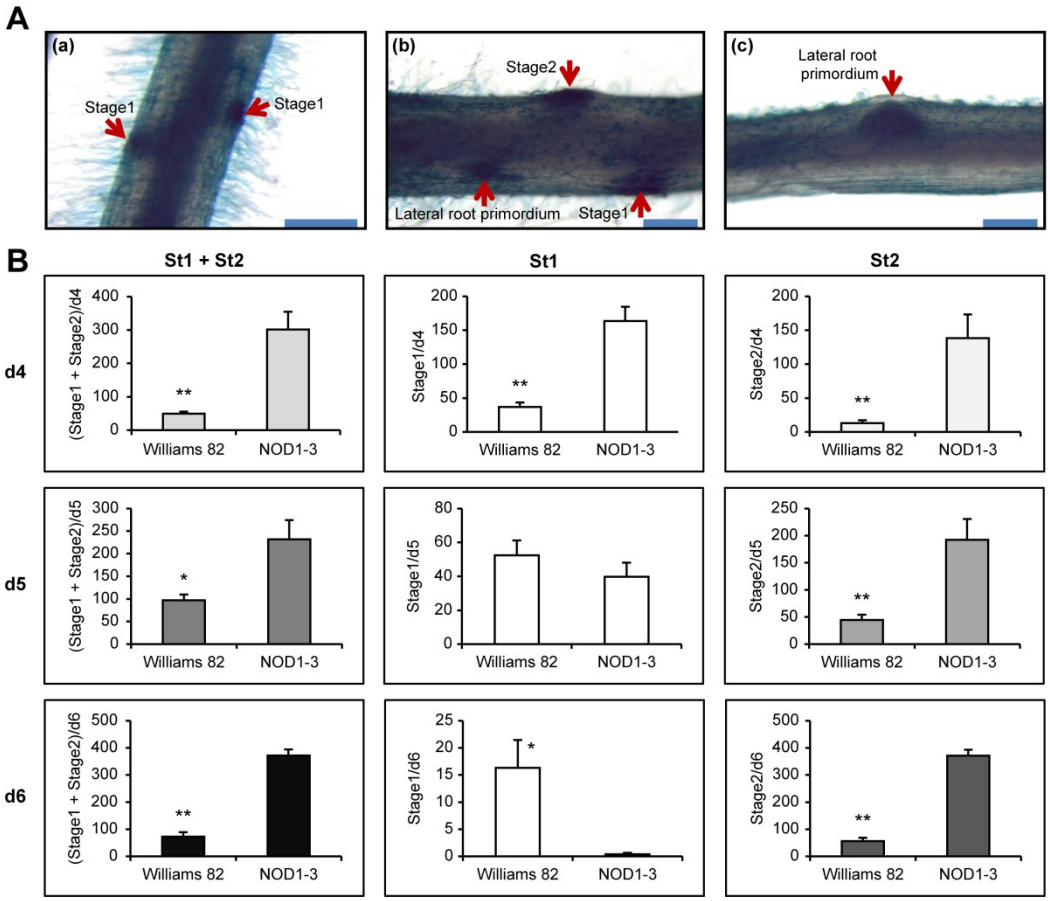


Figure 2. The number of nodule primordia in wild-type soybean Williams 82 and hypernodulation mutant NOD1-3. (A) Classification according to developmental stages of root nodule primordia. Stage1 represents dividing tissue in the deep cortical part without an upheaval of cortical cells (a and b). Stage2 is represented by a bulge in the cortical layer without any constriction (b). Lateral root primordia develop from the vascular bundles (b and c). Arrows point to Stage1, Stage2, or lateral root primordium. Bars = 2 mm in A. (B) The number of root nodule primordia (Stage1 = St1, Stage2 = St2, Stage1 + Stage2 = St1 + St2) on d4, d5, and d6 in wild type and hypernodulation mutant. Each bar represents the mean ± standard error obtained from five to eight different samples. Asterisks indicate significant differences at $P<0.01$ (**) and $P<0.05$ (*) between wild type and hypernodulation mutant (Welch's *t*-test, Mann-Whitney's U test, and Student's *t*-test).

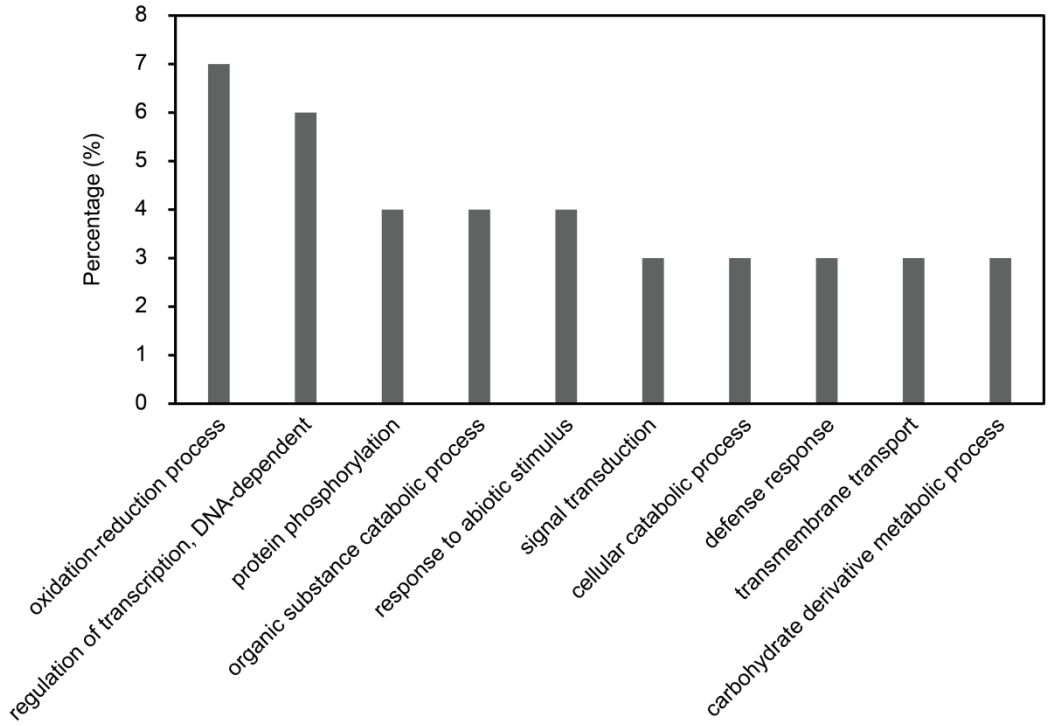


Figure 3. Functional category distribution of the top 10 biological categories obtained with highest scores. Percentages of the score of each category to the total score of that category, with more than 15 sequences filtered (as in Supplemental Table S2), are shown.

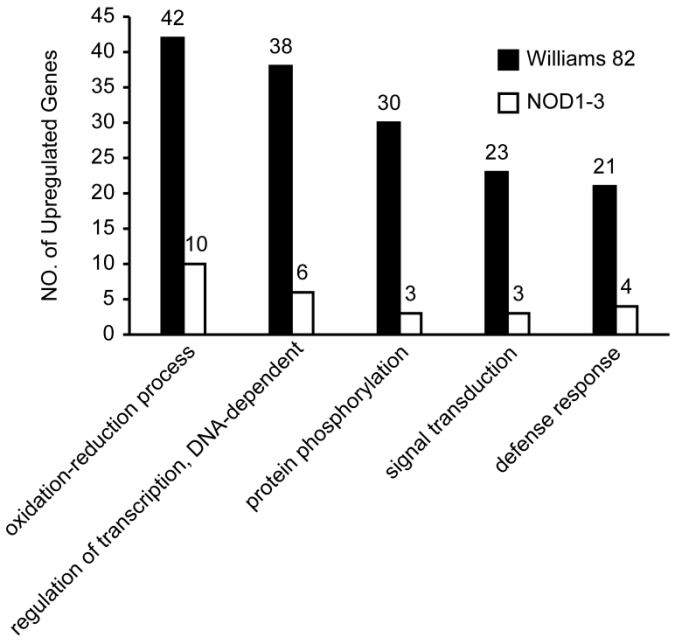
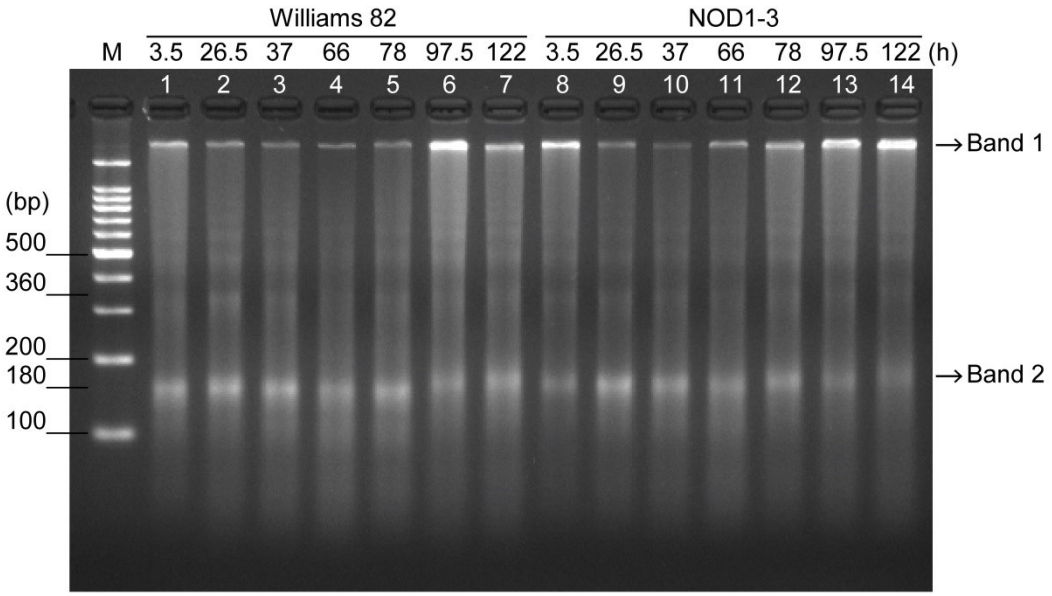


Figure 4. Number of genes upregulated in wild-type Williams 82 or hypernodulation mutant NOD1-3, under the five main categories, i.e., oxidation-reduction process, DNA-dependent regulation of transcription, protein phosphorylation, signal transduction, and defense response. Blast2GO web service was used for this analysis.

A



B

Time (h)	3.5	26.5	37	66	78	97.5	122
Williams 82 / NOD1-3	Lane1/Lane8	Lane2/Lane9	Lane3/Lane10	Lane4/Lane11	Lane5/Lane12	Lane6/Lane13	Lane7/Lane14
Averaged second ratio	3.0 ± 0.4	2.3 ± 1.3	20.0 ± 19.0	5.5 ± 4.8	1.8 ± 0.9	4.9 ± 1.4	6.4 ± 5.7

Figure 5. DNA degradation in the areas infected with rhizobia in wild type (Williams 82) and hypernodulation mutant (NOD1-3). Total DNA was extracted from infected areas at 3.5, 26.5, 37, 66, 78, 97.5, and 122 h after rhizobia inoculation. (A) The representative gel photograph out of the three replicates is shown. (B) Quantification and comparison of DNA degradation between Williams 82 and NOD1-3. First, the intensities of Band2 (approximately 180 bp DNA) and Band1 (undegraded DNA) from each lane shown in (A) were quantified and the ratio of intensities, i.e., (Band2)/(Band1), was calculated for each lane. Then, the second ratio calculated as ([Band2]/[Band1] in Williams 82)/([Band2]/[Band1] in NOD1-3), obtained at the time points 3.5, 26.5, 37, 66, 78, 97.5, and 122 h from Williams 82 and NOD1-3 were compared. Finally, the second ratios obtained at the same time points from three separate experiments (three biological replicates) were averaged and the mean ± standard errors are shown.

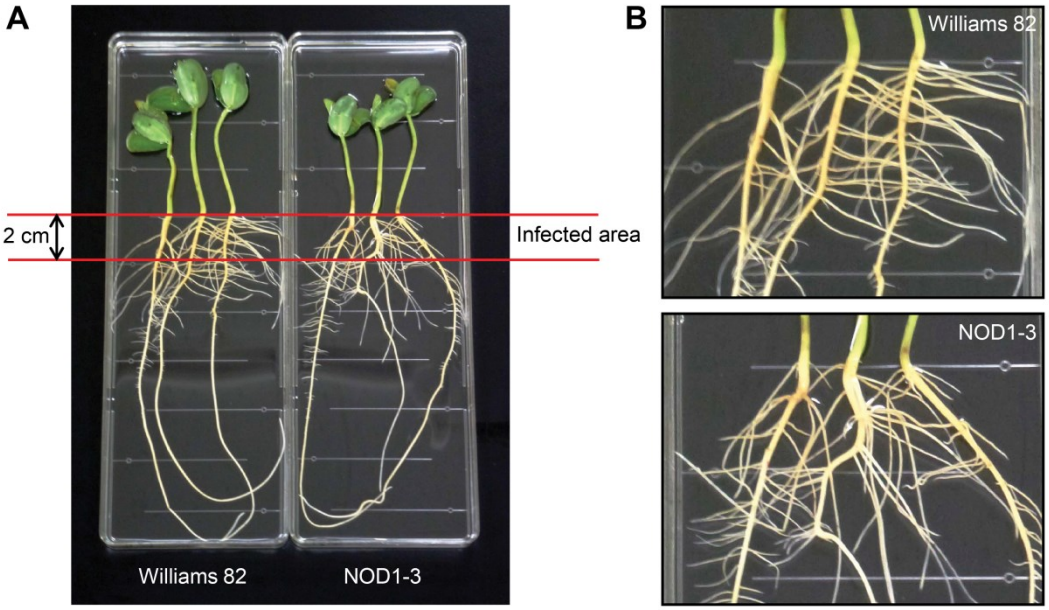


Figure 6. Detection and comparison of H_2O_2 generation in infected areas of wild type (Williams 82) and hypernodulation mutant (NOD1-3) on d2 after inoculation. A, Approximately 2 cm of the infected area stained brown with DAB in the two types of soybean. B, Magnification of A around the infected area.

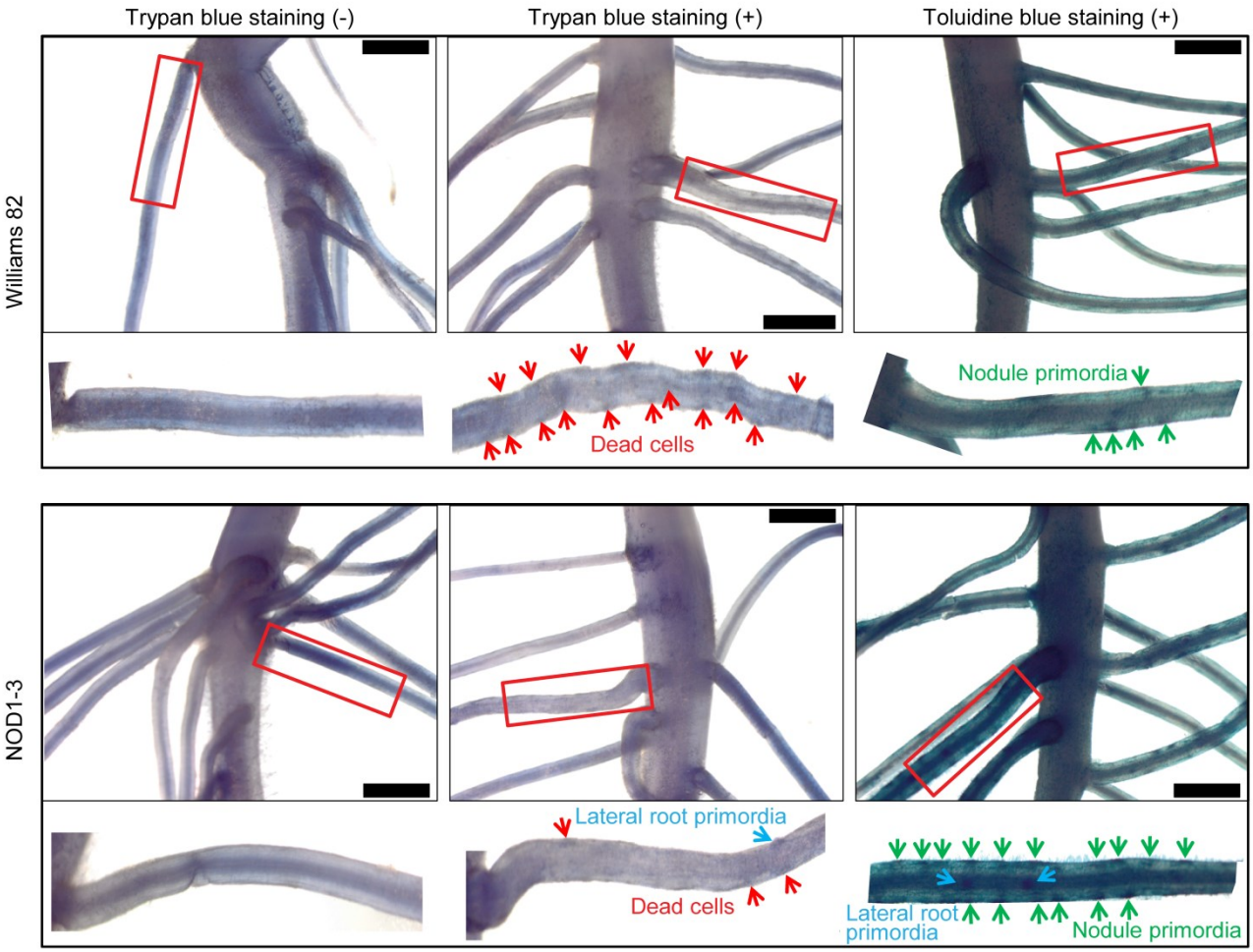


Figure 7. Comparison of dead cells in infected areas of wild type (Williams 82) and hypernodulation mutant (NOD1-3). To compare cell death in wild type and hypernodulation mutant, roots collected from infected (+) and noninfected plants (-) on d3 and stained with trypan blue and toluidine blue were observed under the stereomicroscope. Images of roots enclosed by red squares were magnified. Red, green, and blue arrowheads point to dead cells, nodule primordia, and lateral root primordia, respectively. Bars = 2 mm.

Tables

Table 1. Number of differentially expressed genes (DEGs) in wild-type soybean Williams 82 upregulated (Up) and downregulated (Down) compared with NOD1-3 on d0 (immediately following rhizobium inoculation), d2 (2 days after inoculation), d4, and d5.

Gene Category	Number of Genes	Percentage in total DEGs
Up-d0	244	41.3
Up-d2	33	5.6
Up-d4	11	1.9
Up-d5	254	43
Down-d0	30	5.1
Down-d2	0	0
Down-d4	62	10.5
Down-d5	32	5.4

Table 2. Five categories of genes associated with hypersensitive response or disease resistance in Williams 82

Categories	Gene locus	Fold change				Seq. description	GO IDs
		d0	d2	d4	d5		
Oxidation-reduction process	Glyma01g35660.1	2.91	1.80	2.70	4.29	Abscisic acid 8 -hydroxylase 1-like	P:oxidation-reduction process
	Glyma02g43600.1	1.18	3.12	1.03	1.65	1-Aminocyclopropane-1-carboxylate oxidase	P:ethylene biosynthetic process; P:fruit ripening; P:oxidation-reduction process
	Glyma09g01110.1	1.51	1.42	1.27	3.56	1-Aminocyclopropane-1-carboxylate oxidase	P:ethylene biosynthetic process; P:oxidation-reduction process
	Glyma13g43850.1	5.87	1.85	2.21	1.88	Gibberellin 3-beta-dioxygenase 1-like	P:oxidation-reduction process
	Glyma15g01500.1	3.53	0.74	1.44	1.19	Gibberellin 3-beta-dioxygenase 1-like	P:oxidation-reduction process
	Glyma16g13830.1	3.06	0.97	0.46	2.79	NADH-plastoquinone oxidoreductase subunit 4	P:ATP synthesis coupled electron transport
	Glyma18g42520.1	3.16	1.02	0.48	2.77	Laccase diphenol oxidase family protein isoform 1	P:lignin catabolic process; P:oxidation-reduction process
	Glyma14g06760.1	3.51	0.78	0.59	2.41	Laccase family protein	P:secondary cell wall biogenesis; P:lignin catabolic process; P:oxidation-reduction process
	Glyma01g37930.1	3.68	1.33	0.87	2.56	Laccase-11-like	P:lignin catabolic process; P:oxidation-reduction process
	Glyma18g40070.1	2.21	1.43	0.63	4.33	Laccase 17	P:lignin catabolic process; P:oxidation-reduction process
	Glyma11g06180.1	1.07	1.25	0.88	5.28	Peroxidase 10-like	P:oxidation-reduction process; P:response to oxidative stress
	Glyma11g08520.1	1.57	1.05	0.74	3.13	Peroxidase 64	P:oxidation-reduction process; P:response to oxidative stress
	Other 11 gene loci						
Regulation of transcription, DNA-dependent	Glyma06g13040.1	3.62	1.11	0.79	1.12	Ethylene-responsive transcription factor crf3-like	P:regulation of transcription, DNA-dependent
	Glyma09g38370.1	3.09	1.05	0.44	1.41	AP2-like ethylene-responsive transcription factor bbm2-like	P:regulation of transcription, DNA-dependent
	Glyma13g18410.1	4.03	1.11	0.79	1.02	Ethylene-responsive transcription factor 1b-like	P:regulation of transcription, DNA-dependent
	Glyma20g33800.1	6.84	2.54	0.81	2.24	Ethylene-responsive transcription factor	P:regulation of transcription, DNA-dependent
	Glyma06g11700.1	2.40	1.07	1.28	3.08	Ethylene-responsive transcription factor ERF012-like	P:regulation of transcription, DNA-dependent
	Glyma11g01640.1	0.91	1.17	1.69	4.14	Ethylene-responsive transcription factor ERF021-like	P:regulation of transcription, DNA-dependent
	Glyma01g31920.1	3.72	1.43	1.07	2.94	Probable WRKY transcription factor 33-like	P:regulation of transcription, DNA-dependent
	Glyma14g38010.1	3.14	1.30	1.04	1.93	Probable WRKY transcription factor 33	P:regulation of transcription, DNA-dependent
	Glyma18g22730.1	2.43	0.88	0.63	4.99	Probable WRKY transcription factor 21	P:regulation of transcription, DNA-dependent
	Glyma18g37630.1	2.22	1.12	0.70	4.18	WRKY transcription factor 21 family protein	P:regulation of transcription, DNA-dependent
	Glyma08g04490.1	3.45	1.28	0.40	2.42	Actin-related protein 4-like	P:chromatin silencing by small RNA; P:production of miRNAs involved in gene silencing by miRNA
	Other 5 gene loci						
Protein phosphorylation	Glyma03g16340.1	3.41	1.66	0.51	2.61	Calcium-dependent protein kinase 3-like	P:response to salt stress; P:regulation of anion channel activity
	Glyma03g31330.1	2.46	1.12	0.65	3.05	Serine threonine-protein kinase nek2	P:protein phosphorylation
	Glyma03g15830.1	2.31	1.94	0.90	3.23	Probable serine threonine-protein kinase At5g41260	P:protein phosphorylation
	Glyma06g19440.1	3.25	1.29	0.70	2.46	Serine threonine-protein kinase ht1-like	P:protein phosphorylation
	Glyma09g41160.1	2.39	1.87	1.00	2.91	Probable receptor-like protein kinase At5g15080-like	P:protein phosphorylation; P:autophagy

(*Continued*)

Table 2. (*Continued*).

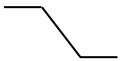
Categories	Gene locus	Fold change				Seq. description	GO IDs
		d0	d2	d4	d5		
	Glyma14g00320.1	4.35	1.37	1.10	2.18	Calmodulin-domain protein kinase 5 isoform 1	P:protein phosphorylation
	Glyma14g12260.1	3.09	0.74	2.95	1.15	LRR receptor-like kinase family protein	P:protein phosphorylation
	Glyma18g16880.1	1.77	1.53	1.25	3.02	Probable LRR receptor-like serine threonine-protein kinase At1g14390-like	P:protein phosphorylation
	Glyma18g24950.1	2.23	0.50	0.50	5.10	Mitogen-activated protein kinase kinase kinase yoda-like	P:protein phosphorylation
Signal transduction	Glyma02g41700.1	4.68	1.21	1.23	3.71	Ribulose bisphosphate carboxylase oxygenase activase chloroplastic-like	P:jasmonic acid-mediated signaling pathway; P:salicylic acid biosynthetic process; P:systemic acquired resistance, salicylic acid-mediated signaling pathway; P:regulation of plant-type hypersensitive response; P:defense response to bacterium; P:MAPK cascade; P:leaf senescence
	Glyma04g30930.1	2.77	1.30	1.00	4.02	ALA-interacting subunit 3-like	P:systemic acquired resistance; P:abscisic acid mediated signaling pathway; P:response to ethylene stimulus; P:salicylic acid biosynthetic process
	Glyma03g07000.1	2.18	3.11	1.62	4.13	Disease resistance protein (TIR-NBS-LRR class)	P:signal transduction
	Glyma03g07120.1	2.11	1.59	2.12	3.74	Disease resistance protein (TIR-NBS-LRR class)	P:signal transduction
	Glyma06g44520.1	2.12	0.99	0.73	3.56	Leucine-rich repeat family protein	P:signal transduction
	Other 7 gene loci						
Defense response	Glyma03g04530.1	2.07	1.30	1.81	2.97	Disease resistance protein At3g14460-like	P:defense response
	Glyma03g05350.1	3.18	1.77	1.22	3.75	Disease resistance protein At3g14460-like	P:defense response
	Glyma03g05420.1	1.36	1.60	1.31	2.97	Disease resistance protein At3g14460-like	P:defense response
	Glyma03g04030.1	2.25	0.84	0.74	2.92	Disease resistance protein At3g14460-like	P:defense response
	Glyma03g04140.1	4.18	1.20	1.40	2.27	Disease resistance protein At3g14460-like	P:defense response
	Glyma17g36420.1	4.72	1.17	0.77	2.40	Probable disease resistance protein At4g33300-like	P:defense response
	Glyma03g06920.1	2.32	3.04	1.25	3.83	TMV resistance protein N-like isoform X1	P:defense response; P:signal transduction
	Glyma06g40740.1	1.33	1.41	1.04	3.07	TMV resistance protein N-like	P:defense response; P:recognition of pollen; P:signal transduction
	Glyma04g40990.1	3.17	0.89	1.39	2.56	Probable 6-phosphogluconolactonase chloroplastic-like	P:cellular response to redox state; P:pentose-phosphate shunt; P:defense response to bacterium
	Glyma11g34940.1	1.87	1.22	0.66	3.29	Coronatine-insensitive protein 1-like	P:jasmonic acid mediated signaling pathway; P:defense response to bacterium; P:jasmonic acid and ethylene-dependent systemic resistance
	Glyma20g04280.1	3.51	0.99	0.48	1.91	NHL repeat-containing family protein	P:virus induced gene silencing; P:vegetative phase change; P:RNA splicing, via endonucleolytic cleavage and ligation
	Glyma18g36000.1	3.71	1.15	0.85	2.36	RPM1 interacting protein 4 transcript protein	P:cellular process; P:innate immune response; P:response to bacterium
	Glyma03g28850.1	1.23	1.08	1.40	3.08	Endo-beta- -glucanase	P:carbohydrate metabolic process; P:defense response
	Glyma19g24520.1	1.13	1.17	0.34	1.33	Lysine histidine transporter 1-like	P:regulation of plant-type hypersensitive response; P:negative regulation of programmed cell death
	Other 9 gene loci						

Table 3. Pathways related to defense response induced by pathogen signals in Williams 82 and NOD1-3

Cultivar	Metabolism	Pathway	#Seqs	#Enzs
Williams 82	Primary metabolism	Starch and sucrose metabolism	11	8
		Glycolysis / gluconeogenesis	3	2
		Pentose phosphate pathway	4	2
		Oxidative phosphorylation	2	1
		Phenylalanine, tyrosine and tryptophan biosynthesis	1	1
		Tryptophan metabolism	1	1
		Phenylalanine metabolism	3	1
	Secondary metabolism	Phenylpropanoid biosynthesis	4	2
		Flavonoid biosynthesis	3	3
		Flavone and flavonol biosynthesis	1	1
		Isoflavonoid biosynthesis	1	1
		Cyanoamino acid metabolism	3	3
		Nicotinate and nicotinamide metabolism	3	3
NOD1-3	Primary metabolism	Starch and sucrose metabolism	2	2
		Glycolysis / gluconeogenesis	1	1
		Pentose phosphate pathway	1	1
		Phenylalanine metabolism	3	1
	Secondary metabolism	Phenylpropanoid biosynthesis	3	1
		Isoflavonoid biosynthesis	1	1

Table 4. Patterns of fold changes of the sequences annotated as nodulin genes in wild-type soybean Williams 82 and hypernodulation mutant NOD1-3

Patterns 1-5 are based on microarray analysis and pattern 6 is from Supplementary Table S1. Fold change was calculated as the ratio of expression level of a gene obtained from Williams 82 compared with NOD1-3.

Pattern of fold change	Column ID	Gene assignment	Gene symbol	Fold change			
				d0	d2	d4	d5
1	11882918	D13506 // LOC547773 // early nodulin // --- // 547773	LOC547773	1.08	1.25	0.83	1.22
	11889712	AF065435 // LOC547522 // nodulin // --- // 547522	LOC547522	1.08	1.10	1.02	1.09
	12154311	D13505 // LOC547772 // early nodulin // --- // 547772	LOC547772	1.02	0.97	0.96	0.85
	11828625	L12257 // LOC547967 // nodulin-26 // --- // 547967 /// AF047049 // SPCP1 // SPCP	LOC547967	1.00	1.34	1.31	1.23
	12056652	L12258 // LOC547794 // nodulin-26 // --- // 547794	LOC547794	0.98	1.08	1.35	1.27
	12104581	X05091 // LOC548101 // nodulin-27 (AA 1-213) // --- // 548101	LOC548101	0.94	0.98	1.09	0.86
	12047356	D38015 // LOC547778 // late nodulin // --- // 547778	LOC547778	0.92	0.94	0.94	0.83
	12047367	D38015 // LOC547778 // late nodulin // --- // 547778	LOC547778	0.91	0.95	1.12	1.05
	12071959	X03979 // LOC547903 // nodulin (E27) (aa 1-360) // --- // 547903	LOC547903	0.90	0.81	0.90	0.94
	11868439	X16488 // LOC547974 // nodulin-21 (AA 1-201) // --- // 547974	LOC547974	0.88	0.82	1.01	0.75
	12180188	X05092 // LOC547905 // nodulin-26b (AA 1-213) // --- // 547905	LOC547905	0.88	0.82	1.02	1.03
2	11765532	D13503 // LOC547771 // early nodulin // --- // 547771	LOC547771	1.18	1.29	0.37	0.66
	11990922	AF434718 // GmN6l // nodulin 6l // --- // 547629	GmN6l	0.99	1.06	0.42	0.66
	12134491	D13502 // LOC547770 // early nodulin // --- // 547770 /// X69157 // ENOD55-2 //	LOC547770	0.88	1.01	0.43	0.60
	11798675	D13504 // ENOD40-1 // early nodulin // --- // 547926 /// X69155 // ENOD40-1 // e	ENOD40-1	1.70	1.72	0.62	0.64
3	11909166	DQ418880 // SAN1A // senescence-associated nodulin 1A // --- // 100101864	SAN1A	1.91	2.54	0.91	2.23
	12201584	AB002809 // Uox // nodulin 35 // --- // 100037445	Uox	1.81	1.84	1.03	2.28
	11805771	X69156 // ENOD55-1 // nodulin // --- // 547927	ENOD55-1	0.81	1.43	0.55	1.05
4	11927963	X04782 // LOC547904 // nodulin // --- // 547904	LOC547904	0.88	1.04	1.53	0.87
5	11981141	AB002810 // LOC547453 // nodulin 35 // --- // 547453 /// M63743 // LOC547453 //	LOC547453	1.36	0.96	0.90	1.52
6	11878627	Nodulin 21-like transporter family protein	LOC100783910	2.49	1.65	1.80	3.75
	12087773	Early nodulin	LOC100818460	2.15	1.30	0.75	3.24

Supplementary figures

Fig. S1

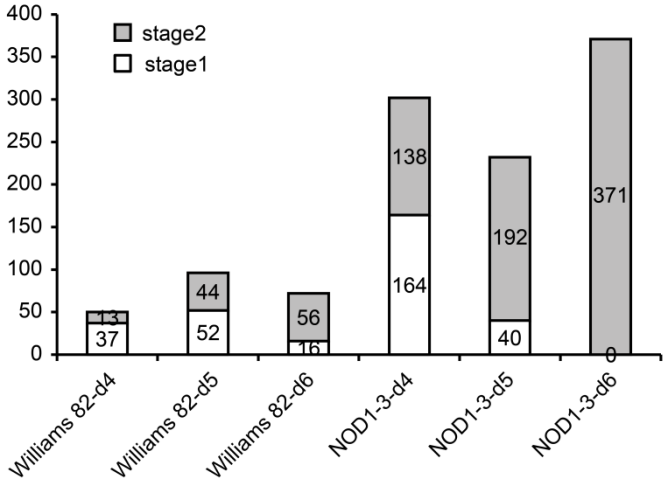


Figure S1. The number of root nodule primordia according to different developmental stages on d4, d5, and d6 in wild-type Williams 82 and hypernodulation mutant NOD1-3. The number of Stage1 reached a peak on d5 in wild type and on d4 in hypernodulation mutant. The root nodule development from Stage1 to Stage2 during d4-d6 in wild type is slower than in hypernodulation mutant.

Fig. S2

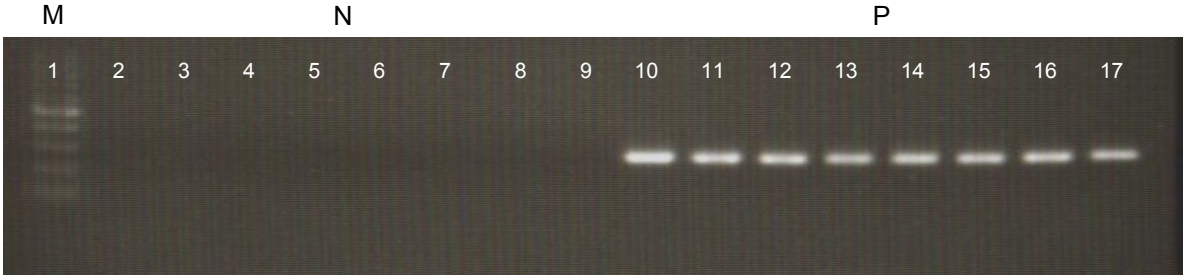


Figure S2. Confirmation of the absence of genomic DNA contamination in total RNA. A segment of the ubiquitin gene was amplified by PCR using ubiquitin (SUBI-1) F and ubiquitin (SUBI-1) R primers in positive control lanes from 10-17. Compared with positive control lanes, no amplification of the ubiquitin gene was obtained using the same primers from the negative controls (2-9). One out of the three biological replicates was selected at random for this experiment.

Lane 1: Marker
Lane 2: W (+) d0
Lane 3: N (+) d0
Lane 4: W (+) d2
Lane 5: N (+) d2
Lane 6: W (+) d4
Lane 7: N (+) d4
Lane 8: W (+) d5
Lane 9: N (+) d5
Lane 10: W (+) d0
Lane 11: N (+) d0
Lane 12: W (+) d2
Lane 13: N (+) d2
Lane 14: W (+) d4
Lane 15: N (+) d4
Lane 16: W (+) d5
Lane 17: N (+) d5

Lane 2-9: N (Negative controls): Water was added instead of a reverse transcriptase enzyme Super Script III RT.
Lane 10-17: P (Positive controls): The reverse transcriptase enzyme Super Script III RT was used.
W: Williams 82
N: NOD1-3
(+): rhizobium inoculation
d0: 0 day
d2: 2 days
d4: 4 days
d5: 5 days

Fig. S3

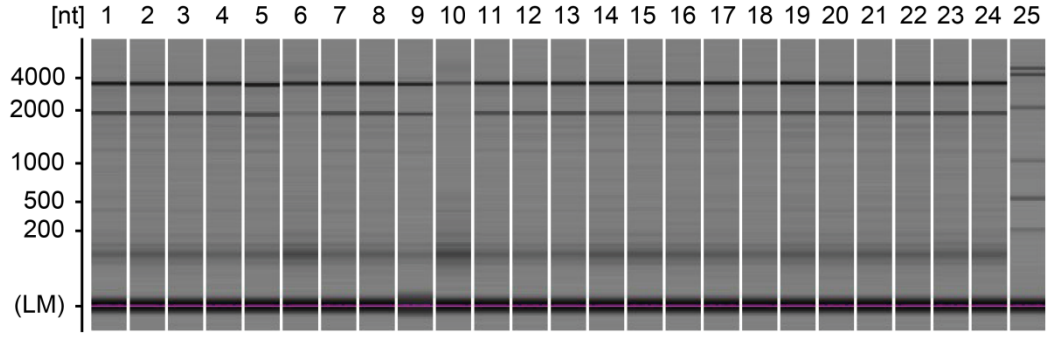


Figure S3. Total RNA quality confirmation of all the 24 samples using electrophoresis apparatus MultiNA. The far-right lane 25 is a size marker, and the rest of the lanes are samples. The sample in lane 10, in which two bands are not seen, was used only to confirm a peak in the apparatus.

Lane 1: W (+) d0-1
Lane 2: W (+) d0-2
Lane 3: W (+) d0-3
Lane 4: N (+) d0-1
Lane 5: N (+) d0-2
Lane 6: N (+) d0-3
Lane 7: W (+) d2-1
Lane 8: W (+) d2-2
Lane 9: W (+) d2-3
Lane 10: N (+) d2-1
Lane 11: N (+) d2-2
Lane 12: N (+) d2-3
Lane 13: W (+) d4-1
Lane 14: W (+) d4-2
Lane 15: W (+) d4-3
Lane 16: N (+) d4-1
Lane 17: N (+) d4-2
Lane 18: N (+) d4-3
Lane 19: W (+) d5-1
Lane 20: W (+) d5-2
Lane 21: W (+) d5-3
Lane 22: N (+) d5-1
Lane 23: N (+) d5-2
Lane 24: N (+) d5-3
Lane 25: Marker
W: Williams 82
N: NOD1-3
(+): rhizobium inoculation
d0: 0 day
d2: 2 days
d4: 4 days
d5: 5 days

Fig. S4

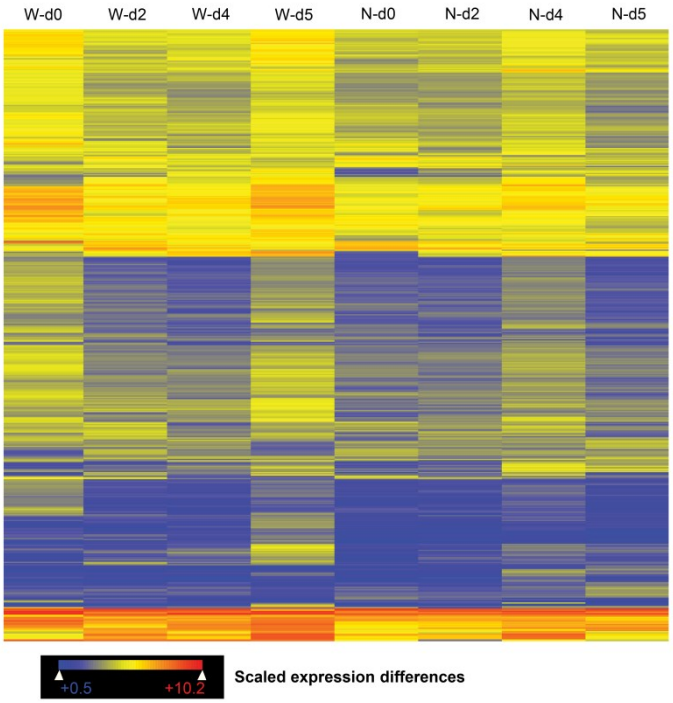


Figure S4. Hierarchical cluster analysis of 591 DEGs, based on the microarray data, at indicated times (d0, d2, d4, and d5) following rhizobium infection in Williams 82 (W) and NOD1-3 (N). Average of three biological replicates is shown in each column. The color key is indicated at the bottom.

Supplementary tables

Supplementary Table S1: (Table S1 is too large to be placed here. Please see the paper entitled "Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection" at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

Table S2. GO-terms filtered (by selecting more than 15 sequences) from the 429 annotated genes

GO-id	GO-term	Score	Percentage in total score
GO:0055114	oxidation-reduction process	52	6.7
GO:0006355	regulation of transcription, DNA-dependent	44	5.7
GO:1901575	organic substance catabolic process	33	4.2
GO:0006468	protein phosphorylation	33	4.2
GO:0009628	response to abiotic stimulus	28	3.6
GO:0007165	signal transduction	26	3.3
GO:0044248	cellular catabolic process	26	3.3
GO:0006952	defense response	25	3.2
GO:0055085	transmembrane transport	24	3.1
GO:1901135	carbohydrate derivative metabolic process	23	3.0
GO:0048513	organ development	23	3.0
GO:0009791	post-embryonic development	22	2.8
GO:0009117	nucleotide metabolic process	21	2.7
GO:0006996	organelle organization	21	2.7
GO:0048608	reproductive structure development	20	2.6
GO:0009888	tissue development	19	2.4
GO:0048367	shoot system development	19	2.4
GO:0046394	carboxylic acid biosynthetic process	19	2.4
GO:0006812	cation transport	19	2.4
GO:0006629	lipid metabolic process	18	2.3
GO:0032787	monocarboxylic acid metabolic process	18	2.3
GO:1901700	response to oxygen-containing compound	18	2.3
GO:0046907	intracellular transport	17	2.3
GO:0009653	anatomical structure morphogenesis	17	2.3
GO:1901566	organonitrogen compound biosynthetic process	17	2.3
GO:0016051	carbohydrate biosynthetic process	17	2.3
GO:0006520	cellular amino acid metabolic process	17	2.3
GO:0051707	response to other organism	17	2.3
GO:0009725	response to hormone stimulus	16	2.1
GO:0010035	response to inorganic substance	16	2.1
GO:1901657	glycosyl compound metabolic process	16	2.1
GO:0006073	cellular glucan metabolic process	16	2.1
GO:0071310	cellular response to organic substance	16	2.1
GO:0065008	regulation of biological quality	15	1.9
GO:0006820	anion transport	15	1.9
GO:0015031	protein transport	15	1.9
	Total score	**778**	

Supplementary Table S3: (Table S3 is too large to be placed here. Please see the paper entitled "Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection" at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

Supplementary Table S4: (Table S4 is too large to be placed here. Please see the paper entitled "Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection" at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

Supplementary Table S5: (Table S5 is too large to be placed here. Please see the paper entitled "Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection" at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

Supplementary images

Supplementary Image S1: (Image S1 [65 pathway maps] is too large to be placed here. Please see the paper entitled “Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection” at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

Supplementary Image S2: (Image S2 [15 pathway maps] is too large to be placed here. Please see the paper entitled "Involvement of programmed cell death in suppression of the number of root nodules formed in soybean induced by *Bradyrhizobium* infection" at SOIL SCIENCE AND PLANT NUTRITION, 2017 VOL. 63, NO. 6, 561–577. https://doi.org/10.1080/00380768.2017.1403842)

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