Running head: NODULE INCEPTION gene inhibits rhizobial infection.

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Title: A positive regulator of nodule organogenesis, NODULE INCEPTION, acts as a negative regulator of rhizobial infection in *Lotus japonicus*.

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Summary:

A transcription factor, known as a positive regulator of nodule organogenesis occurred in root cortex, act as a negative regulator of epidermal infection event by rhizobia.
Footnotes:

Financial source: This research was also supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [23012038 (T.S.); 22128006 (M.K.)], Yoshida Scholarship Foundation (E.Y.).

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Legume–rhizobium symbiosis occurs in specialized root organs called nodules. To establish the symbiosis, two major genetically controlled events, rhizobial infection and organogenesis, must occur. For a successful symbiosis, it is essential that the two phenomena simultaneously proceed in different root tissues. Although several symbiotic genes have been identified during genetic screenings of non-symbiotic mutants, most of the mutants harbor defects in both infection and organogenesis pathways, leading to experimental difficulty in investigating the molecular genetic relationships between the pathways. In this study we isolated a novel non-nodulation mutant, *daphne*, in *Lotus japonicus*, that shows complete loss of nodulation but a dramatically increased number of infection threads. Characterization of the locus responsible for these phenotypes revealed a chromosomal translocation upstream of *NODULE INCEPTION (NIN)* in *daphne*. Genetic analysis using a known nin mutant revealed that *daphne* is a novel nin mutant allele. Although the *daphne* mutant showed reduced induction of *NIN* after rhizobial infection, the spatial expression pattern of *NIN* in epidermal cells was broader than that in the wild type. Over-expression of *NIN* strongly suppressed hyper-infection in *daphne*, and *daphne* phenotypes were partially rescued by cortical expression of *NIN*. These observations suggested that *daphne* mutation enhanced the role of NIN in the infection pathway due to a specific loss of the role of NIN in nodule organogenesis. Based on the results, we provide evidence that a bifunctional transcription factor NIN negatively regulates infection but positively regulates nodule organogenesis during the course of the symbiosis.
Introduction

Legumes develop a specialized symbiotic organ on their roots, the root nodule, in response to rhizobial infection. Benefiting from symbiotic nitrogen fixation by rhizobia in the nodule, plants can grow under nitrogen-limited conditions. The signaling pathways in nodule development are divided into two major events, rhizobial infection and organogenesis. For a successful symbiotic association, it is essential that the two phenomena simultaneously proceed in different root tissues (Crespi and Frugier, 2008; Madsen et al., 2010; Oldroyd, 2013). Rhizobial infection occurs in the epidermal cells of the root. Rhizobia penetrate the root tissues from curled root hair cells and progress toward root cortex through an intracellular channel called infection thread, IT (Vasse and Truchet, 1984; Gage, 2004; Jones et al., 2007; Fournier et al., 2008; Murray, 2011). In contrast, organogenesis begins with the re-initiation of cell division in the root cortex. Several non-nodulation or low-nodulation mutants have been identified by genetic mutant screening in the model legumes *Lotus japonicus* and *Medicago truncatula*. Those mutants are impaired in rhizobial infection processes at different steps, from earlier (root hair deformation and bacterial colonization; e.g., *Ljnfr5*/Mtnfp, *LjsymRK*/Mtdmi2, and *Ljccamk*/Mtdmi3), to later steps (IT initiation, IT progression, and bacterial release; e.g., *Ljcyclops*/Mtipd3 and *Ljcerberus*/Mtlin) (Kouchi et al., 2010; Popp and Ott, 2011). The respective mutated genes are involved in each event. These genes show diverse spatial expression patterns in the epidermis, cortex, and nodule (Popp and Ott, 2011). This complexity has made it difficult to
elucidate the molecular mechanism of the interrelationship between the two major signaling pathways of nodule development, occurring in both the epidermis and cortex at different developmental stages.

A few reports have recently focused on the crosstalk or independence of these two pathways, using different approaches. By characterization of phenotypes of various double mutant/transgenic plants harboring 14 individual infection-defective mutations and three spontaneous-nodule-formation mutations/transgenes, symbiotic genes in *L. japonicus* were categorized into four groups (Madsen et al., 2010): (1) genes for only infection, such as *NAP1, PIR1*, and *CERBERUS*; (2) genes for organogenesis and indirectly for infection, such as *SymRK, NUP85*, and *POLLUX*; (3) genes for both infection and organogenesis, such as *NIN, NSP1*, and *NSP2*; (4) genes for crosstalk between infection and organogenesis, such as *CCaMK* and *CYCLOPS*. Another study of expression systems under the control of a tissue-specific promoter investigated the special contribution of *MtNFP/LjNFR5, MtDMI3/LjCCaMK* to infection thread formation and nodule organogenesis in a tissue-autonomous manner (Rival et al., 2012). A third study of *LjsymRK* mutant alleles proposed different contributions of *LjSYMRK* to each pathway depending on different domains (Kosuta et al., 2011). These studies have shown that the SYMRK-CCaMK-CYCLOPS signaling cascade has two roles: rhizobial infection in the epidermis and nodule organogenesis in root cortex. However, the tissue-specific role or cellular interactions between the epidermal event
and the inner-tissue event of transcription factors functioning in later symbiotic signaling such as NIN, NSP1, and NSP2 remain still unclear.

Among the non-nodulation mutants, the nin (nodule inception) mutant is defective in both IT initiation and nodule formation. It is believed that NIN, a transcription factor containing a RWP-RK domain, functions in both the infection and organogenesis pathways (Schauer et al., 1999; Borisov et al., 2003; Marsh et al., 2007). The expression pattern of the GUS reporter gene driven by the NIN promoter (ProNIN) indicates that epidermal expression a short time after inoculation is correlated with rhizobial infection, whereas expression in the root cortex at a later stage contributes to cell division (Heckmann et al., 2011; Kosuta et al., 2011; Popp and Ott, 2011). NIN transcription is highly induced only after rhizobial inoculation, and constitutive expression of NIN can lead to the ectopic division of cortical cells in the absence of rhizobia. These results indicate that NIN plays a central role in nodule organogenesis (Schauer et al., 1999; Tirichine et al., 2007; Suzaki et al., 2012; Soyano et al., 2013).

Cytokinin also plays an important role in nodule organogenesis, given that a loss-of-function mutation in the putative cytokinin receptors LOTUS HISTIDINE KINASE 1 (LHK1) in L. japonicus and the knockdown of CYTOKININ RESPONSE 1 (CRE1) in M. truncatula cause the low-nodulation phenotypes (Gonzalez-Rizzo et al., 2006; Murray et al., 2007). In contrast, the spontaneous nodule formation 2 (snf2) mutant which has a gain-of-function mutation in LHK1
yields a spontaneous-nodulation phenotype in *L. japonicus* (Tirichine et al., 2007). Likewise, ectopic cortical cell division and *NIN* expression are induced by exogenous cytokinin application without rhizobial infection (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Heckmann et al., 2011).

Cytokinin activates the cortical expression of *NIN* but does not induce the epidermal expression of *NIN*, suggesting that cytokinin activates only the organogenesis pathway and not the infection pathway mediated by *NIN* (Heckmann et al., 2011).

In this study, we identified a novel *nin* mutant allele, named *daphne*, which showed the interesting phenotypes of non-nodulation and hyper-IT formation in *L. japonicus*. The mutant showed an altered expression pattern of *NIN*. In view of the relationship between the spatio-temporal expression pattern of *NIN* and the symbiotic phenotype of *daphne*, we proposed a new cellular communication model controlled by *NIN* involving in crosstalk between infection and organogenesis, for regulating rhizobial infection processes.

**Results**

**Isolation of the *daphne* mutant, which showed non-nodulation and dramatically increased infection of rhizobia in *L. japonicus***

To date several host genes necessary for nodule development have been identified (Madsen et al., 2010; Oldroyd, 2013). However, most of non-nodulation mutants have defect in both the rhizobial
infection and organogenesis pathways, so that the molecular relationship between these two pathways and the mechanisms for controlling each pathway has remained obscure. To find new components involved in the infection or organogenesis pathways, we first screened the ion-beam-mutagenized *L. japonicus* Miyakojima MG-20 seeds (3400 M1 lines) for the-non-nodulation mutants. We next evaluated their ability of infection. As a final step, we focused on a mutant, named *daphne*, displaying the novel phenotype of non-nodulation and hyper-infection. The *daphne* mutant was completely defective in nodule formation, being different from *hit1-1*, which was previously isolated as a hyper-infection mutant able to form a few nodules (Supplemental Fig. S1; Murray et al., 2007). In the *daphne* mutant, no nodules were observed even 28 days after inoculation (DAI) (Fig. 1A–C, I). *daphne* showed a typical non-nodulation phenotype, with pale-yellow leaves and growth delay under low-nitrogen conditions (Fig. 1A). However, in *daphne* the number of infection threads (ITs) per root was dramatically increased, to 15-fold greater than that on the MG-20 wild type (Fig. 1D–G, J). In the wild type, ITs tend to be formed in small restricted regions called susceptible zone (Vasse et al., 1993; Pennmetsa and Cook, 1997; Krusell et al., 2002; Gage, 2004). On the other hand, ITs were observed on almost all regions of *daphne* roots. This extended rhizobial susceptibility has been previously observed in *nin* mutant (Schauser et al., 1999). Additionally, IT elongation in *daphne* was visible in root hair but aborted and burst in the epidermal cell layer, and no cortical infection thread was observed (Fig. 1I; Supplemental Fig. S2).
To identify the step of the organogenesis pathway that is blocked in daphne, we created daphne snf2 double mutant and evaluated its ability of spontaneous nodule formation. snf2 has a gain-of-function mutation in the LHK1 gene, which encodes a cytokinin receptor protein. In this mutant, only genes downstream of cytokinin signaling are constitutively active. snf2 can accordingly form a nodule-like structure without rhizobial infection (Tirichine et al., 2007). The non-spontaneous nodule formation phenotype of the daphne snf2 double mutant indicates that the non-nodulation phenotype of daphne is caused by defects downstream of cytokinin signaling (Fig. 1H, K).

Identification of daphne mutation by map-based cloning and inverse PCR

We roughly identified two loci on chromosome II and III linked to the non-nodulation phenotype of daphne using a small F2 mapping population by map-based cloning (http://www.kazusa.or.jp/lotus/; Sandal et al., 2006) (Supplemental Fig. S3). This result was apparently inconsistent with the observation that the F2 population segregated in approximate 3:1 ratio, indicating that daphne is a recessive mutant. (Supplemental Table S1). We further explored the locus in using a large F2 population with markers on linkage group (LG) III, and the translocation fusion point was identified by reverse transcription-PCR (RT-PCR) (Fig. 2; Supplemental Fig. S4). We finally detected the fused sequences originated from chromosome II and III in daphne genome by inverse PCR. This finding suggested that the ion beam irradiation had induced a reciprocal
chromosomal translocation. The translocation points lie in the second intron of the TIM gene (chr3.CM0423.360.r2.d) on chromosome III and in an intergenic region (IGR) on chromosome II. The IGR sequence on IGR lies approximately 7kb upstream of NIN, which is known to be an essential gene for nodule development (Schauser et al., 1999). No mutation in NIN coding region was detected in the daphne genome.

**daphne is a novel nin mutant allele, different from the nin null mutant**

As described above, we determined two candidate loci responsible for the non-nodulation phenotype in daphne: TIM, on chromosome III, and NIN, on chromosome II. Although we introduced TIM cDNA under the ubiquitin promoter (ProLjUBQ; Mackawa et al., 2008) by hairy-root transformation, the non-nodulation phenotype was not complemented (Supplemental Fig. S4). We next hypothesized that a translocation near the NIN locus causes the daphne phenotype, a notion supported by previous characterization of a nin mutant which displays non-nodulation and no IT formation (Schauser et al., 1999). By crossing daphne (Miyakojima MG-20) and nin-2(Gifu B-129), we tested whether daphne is a nin mutant allele. The success of crossing experiments using pollen of nin-2 was judged by the accumulation of anthocyanin, the dominant characteristic phenotype of Gifu B-129 (nin-2), in stems. All daphne x nin-2 F1 plants originating from three independent seed pods exhibited the non-nodulation (Fig. 3A). These results suggested that daphne
and nin-2 are allelic for the non-nodulation phenotype. Although normal infection phenotype of daphne x wild type F1 plants and F2 segregation ratio indicates that daphne phenotypes of both non-nodulation and hyper-infection are recessive (Supplemental Table S1), daphne x nin-2 F1 plants showed hyper infection. We also observed that the excessive root hair deformation phenotype in daphne was similar to the phenotype of the nin mutant (Supplemental Fig. S5). For the subsequent analysis described below, we used nin-9 (Suzaki et al., 2012) as a canonical nin mutant because it has same genetic background as daphne.

**daphne has completely lost the NIN expression induced by cytokinin application**

NIN is a putative key transcription factor that plays a role in the infection and nodule organogenesis pathways. The expression level of NIN is strongly elevated in an inoculation-dependent manner (Schauser et al., 1999). Because the allelism test showed that daphne was a nin mutant allele, we next investigated the NIN expression pattern in daphne. Expression in whole roots was slightly induced by inoculation with *Mesorhizobium loti*. The transcript levels of NIN at earlier stages were almost identical in the wild type and daphne, whereas at 7 DAI the level of NIN expression in daphne is almost 1/3 of that in the wild type (Fig. 3B). The induction level of NF-YA, known as a downstream target of NIN (Soyano et al., 2013), also indicates that daphne
partially retains the function of NIN, compared to almost no induction of \textit{NF-YA} in a typical \textit{nin} mutant, \textit{nin-9} (Fig. 3C).

We next evaluated the cytokinin-induced expression level of \textit{NIN}, finding it to be completely absent in \textit{daphne} (Fig. 3D). This loss is in good agreement with the finding that the \textit{snf2} mutation spontaneously activating cytokinin signal does not rescue the non-nodulation phenotype of \textit{daphne} (Fig. 1H, K), because cytokinin is believed to induce only the organogenesis and not the infection pathway (Heckmann et al., 2011). The cytokinin-induced expression of \textit{LjRR6} (den Camp et al., 2011) was detected even in \textit{daphne}, suggesting that \textit{daphne} retains the cytokinin responsiveness of gene other than \textit{NIN} (Fig. 3E).

\textit{daphne} shows broad epidermal expression patterns of \textit{NIN}

How is the lower expression of \textit{NIN} in the \textit{daphne} mutant implicated in the increased number of IT? To identify the underlying mechanism of increased infection events, we investigated the spatial expression pattern of \textit{NIN}. We cloned ~approximately 4kb of \textit{ProNIN} and ~approximately 2kb of the \textit{NIN} terminator (\textit{TerNIN}) for promoter-\textit{GUS} analysis. 10 out of 14 \textit{nin-9} plants were rescued their infection threads formation by introducing the \textit{ProNIN::NIN::TerNIN} construct. This indicated the \textit{NIN} promoter was sufficient for the function of \textit{NIN} at least involved in rhizobial infection (Table 1; Supplemental Fig. S5). In the wild type, blue staining was restricted into several small epidermal
regions of the root (Fig. 4A, C, E), as previously reported (Radutoiu et al., 2003; Kosuta et al., 2011). The inner cells of nodule primordia in the wild type was also stained, as previously observed (Fig. 4I; Heckmann et al., 2011). In contrast, we observed a broad range, almost whole root area, of the NIN promoter activity in the daphne root (Fig. 4B, D, F). The broader activity of ProNIN::GUS::TerNIN in daphne coincided with the region where excessive root hair curling and IT formation occur. (Fig. 4A–H).

**Overexpression of NIN strongly represses the hyper-infection in daphne**

The above result showed that daphne exerts broad ProNIN-activity in the epidermis, indicating a broader susceptible zone for rhizobial infection than in the wild type. Based on the results, we hypothesized that NIN itself negatively regulates rhizobial infection. To address the negative function, we accordingly over-expressed NIN under ProLjUBQ in daphne roots and observed the IT formation phenotype with *M. loti* expressing DsRED. Surprisingly, the hyper-infection phenotype of daphne was strongly suppressed in NIN-over-expressing transgenic roots, whereas GUS-over-expressing roots and non-transformed (green fluorescent protein (GFP)-negative) roots retained the excessive IT formation phenotype (Fig. 5A–C, I–K; Table 1). The hyper-infection phenotype of non-transformed roots indicates that the negative feedback regulation of IT formation is not long-distance signaling mediated by the shoot, in contrast to the regulation of number of
nODULES (Okamoto et al., 2009). We also observed no normal nodules, but some lateral roots with
enlarged tips and bumps in ProLjUBQ::NIN transgenic roots in daphne (Table 1; Supplemental Fig.
S6; Suzaki et al., 2012; Soyano et al., 2013).

Next, we confirmed the positive function of NIN in rhizobial infection in daphne mutant
background. We expressed a chimeric protein of NIN and the SRDX domain, a transcriptional
repressor domain in the Arabidopsis SUPERMAN repressor (Oshima et al., 2011).

ProLjUBQ:NIN::SRDX dominantly repressed the target gene expression of NIN in MG-20 wild type,
causing a reduction in the number of nodules (Supplemental Fig. S7, Table 1). In daphne, the ITs
almost disappeared only in ProLjUBQ:NIN::SRDX expressing transgenic roots (Fig. 5D, H, J; Table
1), phenocopying the previously observed phenotype of a typical nin mutant (Schauser et al., 1999;
Marsh et al., 2007). This suggests that daphne maintains the positive function of NIN in rhizobial
infection, unlike a typical nin mutant.

These results indicate that NIN plays not only positive but also negative roles in IT formation, and
daphne maintains the positive role, but loses the negative role. In contrast to daphne, MG-20 wild
type plants formed a few number of ITs (less than 20 per root) (Fig. 1J), which may account for the
observation that ProLjUBQ:NIN had apparently no strong suppressive effects on IT number in wild
type (Table 1).
Cortical but not epidermal expression of NIN was specifically lost in *daphne*

Both positive and negative roles of NIN in rhizobial infection had now been demonstrated. To further investigate the underlying mechanism, we hypothesized that the positive and negative actions of NIN are generated by epidermis and cortex, respectively, given that the lack of cytokinin-induced NIN in *daphne* results in an increase in number of IT and a typical *nin* mutant does not form IT. We speculated that a less negative role of NIN in IT formation (cytokinin-induced NIN) resulted in excessive IT formation.

To address the tissue specific activity of NIN, we attempted to express *NIN* using a cortex- and endodermis-specific enhancer isolated from *Arabidopsis thaliana* J0571 (Miyashima et al., 2011). The J0571 enhancer element was identified from the Arabidopsis GAL4-GFP enhancer-trap lines (http://www.plantsci.cam.ac.uk/haseloff; http://www.arabidopsis.org/abrc/haseloff.jsp). First, we tested the fluorescent marker (*mCherry–NLS*) expressed by *J0571* in hairy roots of *L. japonicus*. Although no marker expression was detected in the epidermis, signal was detected in inner layers of the root including cortex and endodermis (Supplemental Fig. S8), suggesting that the cortex- and endodermis-specific expression of *J0571* is conserved in *L. japonicus*. Both the non-nodulation phenotype and excessive IT formation in *daphne* were partially rescued by *J0571 >> NIN*, confirming that the *daphne* phenotype was caused by loss of *NIN* expression specifically in the cortex. (Fig. 6A–C, F–H, K–M; Table 1).
Discussion

*NIN* was first identified as a gene responsible for the non-nodulation phenotype in legumes (Schauser et al., 1999). Since then, it has been believed that NIN, a putative transcription factor, plays a positive role in nodule organogenesis and IT formation. Meanwhile, it has been discussed the possibility that NIN has also negative role for rhizobial infection processes based on the excessive root-hair response or the expanded *ENOD11* expression pattern in *nin* mutants and the lower expression of *NIN* in another hyper IT mutant, *hit1-1* (Schauser et al., 1999; Marsh et al., 2007; Murray et al., 2007). In this study, we identified the *daphne* mutant, a novel *nin* mutant allele displaying excessive IT formation as well as non-nodulation. The spatio-temporal expression patterns of *NIN* gene in *daphne* indicated a new evidence of negative feedback regulation of infection process mediated by NIN. In *daphne*, the level of *NIN* transcription from whole roots was less than that in wild type. In contrast, the epidermal expression of *NIN* was broader than that in the wild type, indicating that the susceptible zone for rhizobial infection was enlarged in *daphne*. This increased susceptibility for infection could account for the excessive IT formation in *daphne*. Furthermore, although over-expression of *NIN* suppressed excessive infection, inner-cell-layer-specific expression of *NIN* rescued nodule formation in *daphne*. Based on these observations, we propose a negative feedback regulation of rhizobial infection mediated by NIN (Fig. [www.plantphysiol.org](http://www.plantphysiol.org)).
7). In this model, NIN plays two important roles, one in infection and the other in organogenesis. NIN functioning in infection is located in the epidermis in an earlier stage (in the susceptible zone) for proceeding with IT formation, whereas NIN functioning in organogenesis act in a later stage. NIN functioning in organogenesis has not only a positive role in promoting cell division in the cortex but also a negative role in inhibiting rhizobial infection. In *daphne*, owing to the loss of expression of such NIN functioning in organogenesis, the root area for rhizobial infection becomes broader. Several reports have already suggested that genes downstream of cytokinin signaling or NIN itself are involved in preserving the balance of the nodule symbiosis (Murray et al., 2007; Mortier et al., 2012; Saur et al., 2011). Our study has experimentally confirmed one of those mechanisms, a negative role of NIN in rhizobial infection. Excessive root hair curling of a typical *nin* mutant also may be explained by our working model of NIN.

How can two different biological events, infection and organogenesis, be controlled by the same transcription factor, NIN? How can NIN act both positively and negatively in a stage- or tissue-dependent manner during nodule organogenesis? Recent reports in *A. thaliana* indicate that a transcription factor may act as a bifunctional transcription factor and mediate a wide variety of biological events. WUSCHEL acts as both a repressor and an activator in a domain-dependent manner (Ikeda et al., 2009). In our study, however, an NIN chimeric repressor can repress IT formation in the epidermis (Fig. 5; Table 1), so that NIN functions only as an activator in both
infection and organogenesis. If NIN functions as a repressor of IT formation, the NIN chimeric
repressor should not repress IT formation. Another possibility is that different actions of NIN are
dependent on tissue or stage specific downstream targets including in co-transcriptional regulators
and. The putative cortex- or late-nodule-specific downstream factors might suppress the function of
NIN for IT formation transcriptionally or post-transcriptionally. This potential mechanism is
supported by several studies. Studies of LFY and AP1 transcription factors indicate that a single
transcription factor can bind to different groups of targets by interacting with individual cis-regions
or co-factors following the developmental stages (Gregis et al., 2008; Liu and Mara, 2010).
Moreover, several reports of crosstalk in defense signaling propose a potential mechanism by which
single transcription factors, such as WRKYs and TGAs, play both positive roles in the SA-dependent
pathway and negative roles in the JA-dependent pathway (Li et al., 2004; Gao et al., 2011; Van der
Does et al., 2013).

We next discuss the candidates for co-factors or downstream target genes of NIN. In terms of cell
proliferation activity of NIN, a positive role for organogenesis, the contribution of NSP2 and
NF-YA/YB has been demonstrated (Soyano et al., 2013). For the negative regulation of infection,
ethylene responsive factors are strong candidates, given that both of the hyper-infection mutants

_Mtsickle_ and _Mtefd_ harbor mutations in ethylene-signaling molecules. Although a few
hyper-infection mutants, _Ljhit1-1, Mtsickle_ and _Mtefd_ (Penmetsa and Cook, 1997; Murray et al.,
suggest the existence of regulatory mechanisms for controlling rhizobial infection processes, such regulation is far less well known than that of the number of nodules (van Brussel et al., 2002; Oka-Kira and Kawaguchi, 2006; Kouchi et al., 2010). Furthermore, although *Ljhit1-1* exhibits low-nodulation, *Mtsickle* and *Mtefd* exhibit increased the number of nodules, leaving mysterious the putative crosstalk between the infection and organogenesis pathways.

According to our model, the phenotype of *Ljhit1-1* is caused by lower expression of NIN functioning in organogenesis and inhibiting infection, similar to *daphne*. In contrast, in *Mtsickle* and *Mtefd* NIN may be more highly expressed, but inhibitory mechanisms of infection mediated by NIN may be lost.

Our study indicates that NIN may switch between positive and negative influence on rhizobial infection in different tissues or nodule developmental stages. NIN could be controlling the balance between infection and organogenesis. Future study of *daphne* will shed new light on co-factors or downstream target genes of NIN that differ between those two pathways.

Although NIN is a key transcriptional factor in nodule development, the functional NIN promoter region necessary for nodule organogenesis has not yet been elucidated. Only IT formation, and not nodule formation was rescued in *nin-9* by the introduction of ProNIN(~4kb)::NIN::TerNIN (Table 1; Supplemental Fig. S5). In this study, we identified a novel mutant allele of *nin, daphne* whose genome was changed approximately 7kb upstream of *NIN* by chromosomal translocation (Fig. 2). These results raise at least two possibilities. One is that a cis-regulatory element necessary for the
organogenesis pathway including the cytokinin response element has been lost from the upstream region of NIN in the daphne genome. In other words, a 7kb segment of the NIN promoter region is sufficient for the function of NIN in the infection pathway. The other possibility is that epigenetic alteration leads to a different NIN expression pattern in daphne. Furthermore, ProUBQ::NIN induced aberrant roots including bumps in wild type, daphne, and nin-9 (Supplemental Fig. S6), but we failed to rescue IT formation in nin-9 by the introduction of ProUBQ::NIN (Supplemental Fig. S5;Table1). This implies that the induction mechanism of NIN transcript is more complex than so far anticipated; spatial and temporal expression of NIN may need to be strictly controlled in order to achieve its function in both infection and organogenesis pathways. The elucidation of the mechanism remains an important challenge.

The biological meaning of controlling the susceptibility of rhizobia has not been established. At least under our experimental conditions, daphne exhibited no difference in plant growth between the non-inoculated and inoculated conditions (Supplemental Fig. S9). IT formation may be a less energy-consuming process than nodule formation or nitrogen fixation (van Brussel et al., 2002; Oka-Kira and Kawaguchi, 2006; Kouchi et al., 2010). Alternatively, it is possible that plant may need to avoid excessive bacterial infection even during an interaction with symbiotic bacteria. It may be related to a common mechanism for establishment of plant-symbiont and plant-pathogen interactions (Vasse et al., 1993; Bozso et al., 2009; Soto et al., 2009; Nakagawa et al., 2011).
Our study identified a novel nin mutant allele, *daphne*. We demonstrated that NIN, known to date as a positive factor for IT formation and nodule organogenesis, has a negative role in rhizobial infection processes. The multiple functions of the transcription factor NIN will afford an opportunity to investigate potential crosstalk between infection in the epidermis and cell division in the cortex during the course of establishment of the nodule symbiosis.

**Materials and Methods**

**Plant materials and growth conditions**

The *daphne* mutant was isolated by screening of M2 progeny derived from *L. japonicus* Miyakojima MG-20 wild type seeds mutagenized by irradiation with a carbon ion beam (C\(^5^+\)). The details of the ion beam irradiation have been previously reported (Oka-Kira et al., 2005; Magori et al., 2009; Yoshida et al., 2010). Seeds were sown in sterilized vermiculite soaked in autoclaved vermiculite supplied with Broughton and Dilworth (B&D) solution (Broughton and Dilworth, 1971) containing 0.5mM KNO\(_3\) with or without *M. loti* MAFF 303099, respectively, under 16-h light/8-h dark cycles. Cytokinin treatment was applied by incubation of seedlings in vermiculite supplied with B&D solution containing 10\(^{-7}\)M benzylaminopurine (BAP) for 16 h. ITs were observed or counted after inoculation of rhizobia *M. loti* constitutively expressing *LacZ* (Yoshida et al., 2010) or *DsRED*. *nin-2* was kindly provided by Jens Stougaard (Schauer et al., 1999) and was used for the allelism
test. *nin-9* (Suzaki et al., 2012) was used for gene expression analysis and complementation testing.

**Microscopic observation**

Bright-field and fluorescence images were viewed with an SZX12/16 stereomicroscope, BX50 microscope (Olympus). Images were acquired with a DP Controller (Olympus). Confocal images were viewed with an A1 confocal laser-scanning microscope (Nikon) and NIS Elements (Nikon).

**The quantification of ITs**

At 5 days after germination, plants were inoculated with *M. loti* constitutively expressing *LacZ*. At 7 days after inoculation, roots were stained for beta-galactosidase activity. ITs on all parts of the root were counted under the microscope (BX50, Olympus).

**Map-based cloning and inverse PCR**

The *daphne* locus was mapped using F$_2$ progeny of *daphne* and Gifu B-129. Two loci located on LG II and LG III were identified using 52 F$_2$ plants. Fine mapping was performed in by 2048 F$_2$ plants. The newly developed genetic markers in this study are shown in Supplemental Table S2. The deleted region located on CM0423 (chromosome III) was identified. *daphne* genomic DNA was extracted with a DNeasy Plant Mini Kit (Qiagen) and digested with EcoO109I, ApoI, and EcoRI. The digested
DNA fragments were self-ligated with T4 DNA ligase (TaKaRa, Japan). Then, using inverse PCR analysis with two sets of primers designed on sequences in CM0423, the fused sequences originated from CM0423 (chromosome III) and CM0102 (chromosome II) (Fig. 2) was detected. The primers used in inverse PCR analysis are shown in Supplemental Table S3.

Expression analysis

Total RNA was isolated from each plant tissue using the RNeasy Plant Mini Kit (Qiagen).

First-strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed using an ABI Prism 7000 (Applied Biosystems) with a THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) or with a QuanTitect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer’s protocol. The expression of ubiquitin or EF-1a was used as the reference. The primers used in expression analysis are shown in Supplemental Table S3. Data are the mean ± SD of three biological and three technical replicates.

Plant transformation

The recombinant plasmids were introduced into Agrobacterium rhizogenes strain AR1193 and were transformed into roots of L. japonicus by a hairy-root transformation method previously described (http://www.bio-protocol.org/wenzhang.aspx?id=795; Okamoto et al., 2009).
Cloning of NIN promoter constructs and promoter–GUS assay

A 1.7 kb gateway-cassette (GW) fragment was excised from DR5::GFP-NLS construct (Suzaki et al., 2012), and inserted into the BamHI site of pCAMBIA1300-GFP, named pCAMBIA1300-GW-GFP.

Next, the GFP in the vector was removed using XhoI, and PCR-amplified GFP-LjLTI6b (Suzaki et al., 2012) was inserted into the XhoI site to create a new binary vector pCAMBIA1300-GW-GFP-LjLTI6b. Then, using two sets of primers for amplification of ~approximately 4 kb of ProNIN and ~approximately 2 kb of TerNIN, two fragments were cloned into pCAMBIA1300-GW-GFP-LjLTI6b. In the final step, GUS in pDONR221 (Invitrogen) which was provided by Detlef Weigel, and the NIN cDNA in pENTR/D-TOPO (Suzaki et al., 2012) were inserted between the ProNIN and TerNIN by an LR recombination reaction (Invitrogen). A T-DNA construct expressing ProNIN::GUS::TerNIN was transformed into MG-20 and daphne. GFP fluorescence was checked as a marker for transformation. Transformed roots were inoculated with M. loti MAFF303099. At 7 DAI, a GUS staining procedure was performed as previously described (Jefferson et al., 1987).

Analysis of the IT phenotype with over-expressing NIN or chimeric repressor of NIN

NIN cDNA without a stop codon in pENTR/D-TOPO was generated from NIN cDNA in
pENTR/D-TOPO (Suzaki et al., 2012) by site-directed mutagenesis with primers (Supplemental Table S3). A \textit{GW::SRDX} fragment was amplified from the pDEST-BCKH plasmid (Oshima et al., 2011), and inserted between the KpnI and AscI sites of pUB-GFP (Maekawa et al., 2008), named pUB-GW-SRDX-GFP. \textit{NIN} cDNA in pENTR/D-TOPO (Suzaki et al., 2012) and \textit{NIN} cDNA without a stop codon were inserted into the GW site of pUB-GW-GFP (Maekawa et al., 2008) and pUB-GW-SRDX-GFP, respectively, with the LR recombination reaction (Invitrogen). As a control, \textit{GUS} in pDONR221 (Invitrogen) was inserted into the GW site of pUB-GW-GFP by the LR recombination reaction (Invitrogen). \textit{daphne} plants were treated with \textit{M. loti} MAFF303099 constitutively expressing \textit{DsRED}. At 14 DAI, ITs were observed.

\textbf{Analysis of tissue specificity using a cortex- and endodermis-specific expression system}

GAL4-VP16 and NOS terminator sequence with flanking genomic region were amplified by PCR from an enhancer trap line J0571 in \textit{A. thaliana} (http://www.plantsci.cam.ac.uk/Haseloff/; Miyashima et al., 2011) and cloned into the HindIII site of pGW501:5xUAS (Goh et al., 2012) using an In-fusion HD cloning kit (TaKaRa, Japan), named pGW501:5xUAS-J0571. Next, \textit{GW::SRDX::TerNOS} fragment was excised from pUB-GW-SRDX-GFP by KpnI and ScaI double digestion, and inserted into pCAMBIA1300-GFP, named pCAMBIA1300-GW-SRDX-GFP. A J0571-GAL4-VP16-TerNOS-5xUAS-35Sminimal promoter was amplified by PCR from the
template plasmid pGWB501:5xUAS-J0571 and inserted into the KpnI site of
pCAMBIA1300-GW-GFP or pCAMBIA1300-GW-SRDX-GFP, named
pCAMBIA1300-J0571-GW-GFP and pCAMBIA1300-J0571-GW-SRDX-GFP, respectively. In the
final step, NIN and mCherry-NLS coding sequence (Suzaki et al., 2012), or NIN and mKO2 coding
sequence without a stop codon was inserted into the GW site of pCAMBIA1300-J0571-GW-GFP or
pCAMBIA1300-J0571-GW-SRDX-GFP by the LR recombination reaction (Invitrogen). As a
control, mKO2 without a stop codon was amplified from the plasmid including mKO2 [Medical and
Biological Laboratories (Sakaue-Sawano et al., 2008)] by PCR, and cloned into pENTR/D-TOPO
vector using a TOPO cloning kit (Invitrogen). Primers used for these constructs are listed in
Supplemental Table S3. At 21 DAI, nodules and ITs were observed in transformed hairy roots.

Acknowledgements

We thank Jens Stougaard for the mutant seeds of nin-2, Krzyztof Szczyglowski for the mutant seeds
of hit1-1, Detlef Weigel for the GUS construct, Makoto Hyashi for M. loti MAFF303099 expressing
DsRED, Shusei Sato for the genomic data of L. japonicus,

Masaru Ohme-Takagi for the SRDX construct, Masanao Sato and Kiyoshi Tatematsu for valuable
comments, Functional Genomics Facility and Spectrography and Bioimaging Facility of NIBB Core
Research Facilities and Model Plant Facilities of NIBB Bioresource Center for technical support, and Enago (www.enago.jp) for the English language review.

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\textbf{Figure 1.} Isolation of a novel non-nodulation mutant, \textit{daphne}. A, Shoot and root phenotype of the \textit{daphne} mutant (left) and the Miyakojima MG-20 wild type (right) at 28 days after incubation (DAI).

B, Nodulation phenotype of Miyakojima MG-20. Arrowheads indicate nodules. C, The non-nodulation phenotype of \textit{daphne}. D-G, ITs formation of the Miyakojima MG-20 root (D,E) and of the \textit{daphne} root (F,G, I) following inoculation with \textit{M. loti} MAFF303099 constitutively expressing \textit{DsRED}. Red fluorescence images of roots (D,F). Linear red signals indicate infection threads (ITs). Red fluorescence images and transmitted light images are merged (E,G,I).

H, Spontaneous nodule formation in \textit{snf2} (left), the \textit{daphne snf2} double mutant (middle), and the \textit{daphne} mutant (right). Arrowheads indicate spontaneous nodules. I, Confocal microscopic image of \textit{daphne} root. \textit{z}-stack series are in Supplemental Fig. S2. J, Nodules were counted at 28 DAI with \textit{M. loti} MAFF303099. K, The number of ITs per root was counted at 7 DAI with \textit{M. loti} MAFF303099.
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Figure 2. Identification of the daphne mutation. A, Two genetic linkage maps of the regions of daphne loci in linkage group III (black, left) and linkage group II (gray, right). The newly developed marker (EY001-3) is shown in Supplemental Table S2. The number of recombination events (events / total chromosomes) is indicated. B, Physical maps of TAC clone LjT14H06 (black, left) and TAC clone LjT08G20b (gray, right). Arrows indicate the annotations from miyakogusa.jp release 2.5 (http://www.kazusa.or.jp/lotus/; Sandal et al., 2006). C, Outline of chromosomal translocation between chromosome (chr.) II and chr. III with sequences at the fusion point identified by inverse PCR amplified from the daphne genome. Black letters indicate the bases from CM0423 (chr.III) and gray letters indicate the bases from CM0102 (chr. II). Bases of unknown chromosomal origin are indicated by underlined letters. D, The location of translocation fusion point in the contigs and with gene annotations (exon shown as block and intron shown as thin line). Numbers on a ruler indicate the exact points (kb) in each contig. Asterisks (*) indicate the reciprocal chromosomal translocation points of each locus.
Figure 3. *daphne* is a novel *nin* mutant allele. A, Allelism tests by crossing *daphne* and *nin-2* mutants (Schauser et al., 1999). Nodules were counted at 14 DAI on each plant root. B-E Quantitative real-time reverse transcription-PCR analysis of *NIN* (B,D), *NF-YA* (C) and *RR6* (E) expression in Miyakojima MG-20 wild type (black bars), in *daphne* (gray bars) and in *nin-9* (white bars) at non-inoculated (0), 1, 3, and 7 DAI (B,C) or dependent on cytokinin treatment (10^{-7} M BAP) for 16 h (D,E). Each cDNA was prepared from total RNA derived from whole root. Fold changes in expression are shown relative to roots at 0 DAI (B,C) or before cytokinin treatment (D,E). Error bars indicate SD of three biological replicates. *P<0.05, **P<0.01,* Student’s t-test.

Figure 4. Spatial expression analysis of the *NIN* gene. GUS staining images of *Agrobacterium rhizogenes*-mediated transformed roots with Pro*NIN::GUS::TerNIN* at 7 DAI (with MAFF303099 wild type) on Miyakojima MG-20 wild type (A,C,E,G) and *daphne* (B,D,F,H). Blue stainings were observed with transformed (GFP positive) hairy roots in susceptible zones (A-F), including root hair cells (G,H) and immature nodule in wild type (I). Scale bars = 0.5mm in A-D; 0.1mm in E-I.

Figure 5. Ectopic expression of the *NIN* gene strongly suppresses excessive IT formation in *daphne*. Red fluorescence images (A-D), GFP fluorescence images (E-H), and transmitted light images (I-L) of *A. rhizogenes*-mediated transformed *daphne* roots at 21 DAI. Transformed with negative control

ITs were observed by inoculating M. loti MAFF303099 constitutively expressing DsRED (A-D).

Green fluorescent protein fluorescence showed transformed roots (E-H). Yellow dashed lines indicate the border between transformed and non-transformed roots. Scale bars = 2cm.

**Figure 6.** The non-nodulation phenotype in *daphne* is partially rescued by the cortical expression of *NIN*. Red fluorescence images (A-C), GFP fluorescence images (D-F), and transmitted light images (G-I) of *A. rhizogenes*-mediated transformed *daphne* roots at 21 DAI. Transformed with J0571 >> NIN (A-I). Excessive ITs (A,D,G), strongly suppressed ITs (B,E,H), and nodules (C,F,I) were observed by inoculating *M. loti* MAFF303099 constitutively expressing DsRED. GFP fluorescence showed transformed roots (F-J). Arrows and arrowheads indicate nodules and enlarged bumps, respectively. Scale bars = 2cm.

**Figure 7.** A model of inhibition of rhizobial infection processes mediated by NIN. In the wild type, NIN functions in both rhizobial infection (blue, in the epidermis) and organogenesis (red, in the cortex). In the earlier stage (#1), NIN (blue) is predominant but in the later stage (#3), the proportion of NIN (red) has increased with nodule development. It is assumed that a potential negative correlation between organogenesis and infection pathway (black bars) regulates the amount of
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Table 1. The phenotypic effect of the expression of the NIN or NIN chimeric repressor in MG-20 wild type, *daphne* and *nin*.

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<sup>a</sup> Roots with small size and number of nodules (see Supplemental Fig.S7); <sup>b</sup> Roots with aberrant lateral root such as enlarged tips and bumps (see Supplemental Fig.S6); <sup>c</sup> Roots with typical *daphne* phenotype, highly increased infection thread number; <sup>d</sup> Roots with wild type-like infection phenotype, normal infection thread number. *J0571>>NIN* rescued non-nodulation phenotype. *ProLjUBQ::NIN* and *J0571>>NIN* inhibits hyper infection in *daphne*. *ProLjUBQ::NIN::SRDX* showed the repression of both nodulation in MG-20 and infection in *daphne*. *ProNIN::NIN::TerNIN* rescued no infection phenotype of *nin*, yielding *NIN* promoter used in this study is sufficient for *NIN* expression for infection pathway. These important results are **highlighted in underlined boldface**.