CLE-HAR1 Systemic Signaling and NIN-Mediated Local Signaling Suppress the Increased Rhizobial Infection in the *daphne* Mutant of *Lotus japonicus*

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Legumes survive in nitrogen-limited soil by forming a symbiosis with rhizobial bacteria. During root nodule symbiosis, legumes strictly control the development of their symbiotic organs, the nodules, in a process known as autoregulation of nodulation (AON). The study of hypernodulation mutants has elucidated the molecular basis of AON. Some hypernodulation mutants show an increase in rhizobial infection in addition to developmental alteration. However, the relationship between the AON and the regulation of rhizobial infection has not been clarified. We previously isolated *daphne*, a *nodule inception* (*nin*) allelic mutant, in *Lotus japonicus*. This mutant displayed dramatically increased rhizobial infection, suggesting the existence of NIN-mediated negative regulation of rhizobial infection. Here, we investigated whether the previously isolated components of AON, especially CLAVATA3/ESR (CLE)-RELATED-ROOT SIGNAL1 (CLE-RS1), CLE-RS2, and their putative receptor HYPERNODULATION AND ABERRANT ROOT FORMATION1 (HAR1), were able to suppress increased infection in the *daphne* mutant. The constitutive expression of *LjCLE-RS1/2* strongly reduced the infection in the *daphne* mutant in a HAR1-dependent manner. Moreover, reciprocal grafting analysis showed that strong reduction of infection in *daphne* rootstock constitutively expressing *LjCLE-RS1* was canceled by a scion of the *har1* or *klavier* mutant, the genes responsible for encoding putative LjCLE-RS1 receptors. These data indicate that rhizobial infection is also systemically regulated by CLE-HAR1 signaling, a component of AON. In addition, the constitutive expression of *NIN* in *daphne har1* double-mutant roots only partially reduced the rhizobial infection. Our findings indicate

that the previously identified NIN-mediated negative regulation of infection involves unknown local signaling, as well as CLE-HAR1 long-distance signaling.

Keywords: autoregulation of nodulation, CLE, infection thread, legume, NIN, nodulation, symbiosis

Legumes form symbiotic relationships with nitrogen-fixing bacteria, rhizobia, enabling the host plants to grow in nitrogen-limited soil. During the formation of this symbiosis, rhizobia invade the plant tissues through the formation of an infection thread (IT), while nodule organogenesis occurs in the host plant root cells. Upon rhizobial infection, the host legumes negatively regulate further symbiotic developments. This phenomenon, in which the first nodules prevent further nodule development in the same region of the root, was reported as the autoregulation of nodulation (AON) (Caetano-Anollés and Gresshoff 1990, 1991; Kosslak and Bohloul 1984).

The molecular basis of AON is mediated by systemic signals transduced between the roots and shoots (Nishida and Suzaki 2018a; Reid et al. 2011b; Suzaki et al. 2015). The systemic signals derived from the root were identified in various model legumes: CLAVATA3/ESR (CLE)-RELATED-ROOT SIGNAL1 (CLE-RS1) and CLE-RS2 peptides were identified in *Lotus japonicus* (Okamoto et al. 2009, 2013a), CLE12/13 was found in *Medicago truncatula* (Mortier et al. 2012), and RHIZOBIA-INDUCED CLE1 (RIC1) and RIC2 were discovered in *Glycine max* (Lim et al. 2011; Reid et al. 2011a). These systemically functioning small peptides are predicted to be posttranslationally arabinosylated by the enzymes PLENTY in *L. japonicus* and ROOT DETERMINED NODULATION 1 (RDN1) in *M. truncatula* (Kassaw et al. 2017; Schnabel et al. 2011; Yoro et al. 2019), after which they are secreted into the xylem and transported to the shoot. In the shoot, these peptides are thought to bind to phloem-expressed leucine-rich repeat receptor-like protein kinases (LRR-RLKs), specifically HYPERNODULATION AND ABERRANT ROOT FORMATION1 (HAR1) in *L. japonicus* (Okamoto et al. 2013a), which is thought to form a heterodimer with another LRR-RLK, KLAVER (KLV) (Miyazawa et al. 2010). HAR1 orthologs have been identified as SUPER NUMERIC NODULES (SUNN) in *M. truncatula* and NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in *G. max*. These LRR-RLKs were originally all identified as the proteins mutated in hypernodulation mutants (Krusell et al. 2002; Nishimura et al. 2002; Sagan and Duc 1996; Schnabel et al. 2005; Searle et al. 2003).

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Downstream of the CLE peptide-receptor signaling pathway, shoot-derived inhibitors (SDIs) of nodulation are produced and transported from the shoot to the roots via the phloem. Several SDI candidates have been reported (Kinkema and Gresshoff 2008; Reid et al. 2012; Sasaki et al. 2014). Unlike the role of SDIs proposed so far, a recently identified microRNA, miR2111, acts as a shoot-derived activator of nodulation in *L. japonicus* and downregulates expression of *TOO MUCH LOVE (TML)* (Tsikou et al. 2018), a negative regulator of nodulation. TML is a root-acting kelch-repeat F-box protein involved in the proteasome-mediated degradation of an unknown positive regulator of nodulation (Magori et al. 2009; Takahara et al. 2013). It is possible that the miR2111-TML module could contribute to controlling the inhibitory effect of AON on nodulation.

In addition to their hypernodulation phenotypes, *har1*, *tml*, and *plenty* showed an increased number of ITs, although the degree of increase in ITs was milder than that in nodules (Magori et al. 2009; Wopereis et al. 2000; Yoshida et al. 2010), suggesting that these molecular components of AON control not only nodulation, but also rhizobial infection processes. On the other hand, some of the no- or low-nodulation mutants, including *daphne*, *lotus histidine kinase1 (lhl1)* (formerly known as *hyper infected1*), *sunergos1*, and *vagrant infection thread1* in *L. japonicus* (Miri et al. 2016, 2019; Murray et al. 2007; Suzuki et al. 2014; Yoon et al. 2014; Yoro et al. 2014), also produced extranumerary ITs, as reviewed by Suzuki et al. (2015). These mutant phenotypes suggest that nodule organogenesis negatively regulates further infections. In particular, our previous work with *daphne*, a *nodule inception (nin)* allelic mutant, in *L. japonicus*, proposed a model in which NIN has an antagonistic role in rhizobia infection in different root tissues: that is, the positive regulation of IT formation by NIN expressed in the epidermis and the negative regulation by NIN in the inner cortical cells. Hereafter, they are referred to as epidermal NIN and cortical NIN, respectively.

Nodule organogenesis is driven by the cortical NIN and causes the subsequent inhibition of epidermal rhizobial infection (Yoro et al. 2014). Recently, mutants showing excessive infection of the *nin* allele have also been reported as a *daphne-like* mutant in *M. truncatula* (Liu et al. 2019b; Roy 2019). The study of the *daphne-like* mutant identified the remote *cis* element essential for specific expression of NIN in the inner pericycle and strongly supported our model of IT control coordinated by each NIN expressed in two different tissues. The NIN transcription factor promotes nodule organogenesis (Marsh et al. 2007; Schauser et al. 1999; Soyano et al. 2013; Suzuki et al. 2012), while also negatively regulating nodulation by directly inducing the expression of *CLE-RS1/2* (Soyano et al. 2014). These findings suggest that NIN negatively regulates rhizobial infection by promoting the CLE-HAR1 signaling pathway.

In this study, we investigate whether hyper-IT formation in the *daphne* mutant is caused by the loss of CLE-RS1/2-HAR1 signaling. The constitutive expression of *CLE-RS1* suppresses hyperinfection of the *daphne* mutant depending on HAR1 and KLV in the shoot. However, the constitutive expression of *NIN* still suppresses ITs on the *daphne har1* double mutant, indicating the presence of a NIN-mediated unknown IT control mechanism that is different from CLE-HAR1 signaling.

RESULTS

The constitutive expression of *CLE-RS1/2* strongly suppresses the hyperinfection of the *daphne* mutant in a HAR1-dependent manner.

To test the hypothesis that the lower expression of *NIN* in the *nin*-allelic mutant *daphne* reduces the subsequent induction of *CLE-RS1/2* expression, we quantified *CLE-RS1/2* expression

following rhizobial inoculation. The expression levels of both *CLE-RS1* and *CLE-RS2* were lower in the *daphne* mutant than in the wild type, MG-20 (Fig. 1), indicating that the lower expression of *CLE-RS1/2* might cause the hyper-IT phenotype in the *daphne* mutant.

To investigate whether the CLE-RS1/2 peptides actually function in IT suppression, we constitutively expressed *CLE-RS1/2* (*Pro35S::CLE-RS1/2*) in hairy roots of the *daphne* mutant. The roots transformed with a negative control vector (*Pro35S::GUS*) exhibited extranumerary ITs typical of the *daphne* mutant; however, those transformed with *Pro35S::CLE-RS1* or *Pro35S::CLE-RS2* showed strong suppression of the hyper-IT phenotype (Fig. 2A). The strong suppression effects were observed not only in the green fluorescent protein (GFP)-positive transformed roots, but also in the GFP-negative untransformed roots in the same plant. This indicates that the CLE-RS1/2 peptides expressed in transformed roots move to the shoot and produce a systemic signal to suppress ITs, not only in *CLE-RS1/2*-expressing roots but also in nontransformed roots.

To further explore the involvement of HAR1 in the suppression of IT formation by *Pro35S::CLE-RS1/2*, the same hairy root transformation was performed using the *daphne har1-8* double mutant. In this background, the suppression of IT formation by *Pro35S::CLE-RS1/2* was completely abolished (Fig. 2B), suggesting that the CLE-RS1/2 peptides strongly suppress increased formation of ITs in the *daphne* mutant via the HAR1 signaling pathway, as has been observed for nodule suppression (Okamoto et al. 2009).

The strong suppression of IT formation by CLE-RS1/2 is mediated by HAR1 and KLV in the shoot.

To clarify whether hyper-IT formation in the *daphne* mutant is determined by the shoot or the root, we performed grafting experiments with the following combinations: MG-20 (scion)/*daphne* (rootstock) and *daphne*/MG-20, with MG-20/MG-20 and *daphne*/*daphne* included as controls (Fig. 3A). Hyper-IT formation was determined by the root genotype, and only when the *daphne* mutant was used as the rootstock did the grafted plants display this phenotype.

In CLE-HAR1 signaling, the CLE-RS1/2 peptides are produced in the root, and their putative receptors, HAR1 and KLV, are expressed in the shoot (Miyazawa et al. 2010; Nishimura et al. 2002). Therefore, we crossed the *daphne* mutant with a stable transformant constitutively expressing *CLE-RS1*

Fig. 1. Postinoculation *CLE-RS1/2* expression is reduced in the *daphne* mutant. **A**, *CLE-RS1* and **B**, *CLE-RS2* expression in the wild type (Miyazawa et al. 2010; Nishimura et al. 2002) MG-20; black bars) and the *daphne* mutant (gray bars) before inoculation (0 days) and at 1 and 3 days after inoculation (DAI) was analyzed using quantitative reverse-transcription PCR. Fold changes in expression are shown relative to the expression level at 0 DAI. Error bars indicate the standard deviation for three biological replicates. Asterisks indicate significant differences between the genotypes (Student's *t* test; *P* < 0.05).

(*Pro35S::CLE-RS1*) (Sasaki et al. 2014) and performed grafting experiments using the transformants as the rootstock and MG-20, *har1*, or *klv* as the scion. The combination of the *daphne* mutant expressing *CLE-RS1* with an MG-20 wild-type scion resulted in

strong suppression of IT formation; however, the combinations using a scion from *har1* or *klv* lost this IT suppression (Fig. 3B and C). These findings suggest that, in addition to the inhibition of nodule formation, the suppression of IT by the constitutive

Fig. 2. HAR1-dependent CLE-RS1/2-mediated suppression of hyper-infection thread (IT) production in the *daphne* mutant. *Agrobacterium rhizogenes*-mediated transformation of hairy roots of **A**, the *daphne* mutant and **B**, the *daphne har1-8* double mutant at 21 days after inoculation. The constructs *Pro35S::GUS* (negative control vector), *Pro35S::CLE-RS1*, and *Pro35S::CLE-RS2* were used for each transformation. Red fluorescence (upper panel) indicates ITs derived from *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRED*. Green fluorescent protein fluorescence (middle panel) indicates transformed hairy roots. Bright-field images (lower panel) provide an overview of the hairy roots. Yellow dashed lines indicate the border between transformed and nontransformed roots. Scale bars = 5 mm.

expression of *CLE-RS1* is mediated by *HAR1* and *KLV* in the shoot. The expression of *CLE-RS1* was natively induced only in the root (Okamoto et al. 2009); however, when we grafted the scion of *Pro35S::CLE-RS1* to the untransformed *daphne* rootstock, we observed that IT formation had been suppressed (Fig. 3B and C). This indicates that shoot-expressed *CLE-RS1* is also able to suppress IT formation, as was previously observed for nodule suppression (Sasaki et al. 2014).

Constitutive *NIN* expression suppresses rhizobial infection through both *HAR1*-dependent and -independent pathways.

Using the *daphne* root, which showed dramatically increased IT, we provided evidence that CLE-HAR1 signaling inhibited

rhizobial infection. We next explored whether IT suppression by CLE-HAR1 signaling could be completely overlapped with the *NIN*-mediated IT suppression proposed in our previous study (Yoro et al. 2014). In the *daphne har1-8* double-mutant background, we constitutively expressed *NIN* (*proLjUBQ::NIN*) and *GUS* (*proLjUBQ::GUS*; negative control). Some of the transformed roots constitutively expressing *NIN* (8/18) still showed the hyper-IT phenotype (Fig. 4A; photos in central column), as did the negative control (Fig. 4A; photos in left column), while the others (10/18) showed a strong reduction in IT numbers (Fig. 4A; photos in right column) despite the presence of the *har1-8* mutation. This indicates that *NIN* can act as a negative regulator for IT formation even in the absence of *HAR1*. In other words, the *NIN*-mediated IT control and

Fig. 3. Grafting experiments using the *daphne* mutant expressing *CLE-RS1*, MG-20 (wild type), and *har1* and *klv* mutants. **A**, Box plots of the number of infection threads (ITs) on grafted plants formed with wild type and *daphne* materials at 7 days after infection (DAI). **B**, Box plots of the number of ITs on grafted plants formed from combinations of MG-20 (wild type), MG-20 constitutively expressing *CLE-RS1* (*Pro35S::CLE-RS1*), *daphne*, and *daphne* constitutively expressing *CLE-RS1* (*daphne CLE-RS1*), *har1-8*, and *klv* at 21 DAI. Data from each plant are represented as a dot. Boxes represent the interquartile range, and the horizontal lines within each box indicate the median. Different lowercase letters represent statistically significant differences (Tukey's honestly significant difference; $P < 0.05$). The number of grafted plants used for the analysis is indicated at the top of each graph. **C**, Images of grafted plants using *daphne* or *daphne CLE-RS1*. Grafting combinations are indicated at the top of each panel. Red fluorescence (upper panel) indicates ITs derived from *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRED*. Green fluorescent protein fluorescence (middle panel) shows roots successfully crossed with a stable transformant constitutively expressing *CLE-RS1*. Bright-field images (lower panel) provide an overview of roots from grafted plants. Scale bars = 5 mm.

CLE-HAR1-mediated pathway are only partially overlapped. In addition, the phenotypic differences between persistence of hyper-ITs (Fig. 4A; photos in central column) and strong reduction (Fig. 4A; photos in right column) might be associated with the ratio of NIN in the epidermis and cortex introduced by *proLjUBQ*, because epidermal and cortical NIN act positively and negatively, respectively. Furthermore, the strong reduction of ITs by *proLjUBQ::NIN* was restricted to the transgenic (GFP-positive) roots (Fig. 4A; photos in right column), which was different from the *daphne* roots constitutively expressing *LjCLE-RS1/2* (Fig. 2A). This local inhibition can be explained well by a lack of systemic regulation due to a *har1* mutation.

DISCUSSION

In this study, we demonstrated that the constitutive expression of *CLE-RS1/2* suppressed the hyper-IT phenotype in both transformed and nontransformed roots of the *daphne* mutant. In particular, the suppression in nontransformed roots is in good agreement with the systemic effects of CLE-RS1/2 peptides. The involvement of HAR1 or KLV expressed in the shoot for the suppression of ITs was further proven by a series of grafting experiments. The systemic control of rhizobial infection is compatible with the recent report that the miR2111-TML module, a downstream signaling component of the CLE-HAR1

Fig. 4. Systemic and local suppression by NIN of hyper-infection thread (IT) formation in the *daphne har1-8* double mutant. **A**, *Agrobacterium rhizogenes*-mediated transformed *daphne har1-8* hairy roots at 21 days after infection. The construct *ProLjUBQ::GUS* (negative control vector) or *ProLjUBQ::NIN* was used for each transformation. Red fluorescence (upper panel) indicates ITs derived from *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRED*. Green fluorescent protein fluorescence (middle panel) shows transformed hairy roots. Bright-field images (lower panel) provide an overview of the hairy roots. Yellow dashed lines indicate the border between transformed and nontransformed roots. Scale bars = 5 mm. **B**, A model of the two IT suppression pathways mediated by cortical NIN (black bold lines). The first pathway is the HAR1-dependent systemic suppression of ITs. The suppression starts with induction of *CLE-RS1/2* genes by cortical NIN, corresponding with canonical autoregulation of nodulation. CLE-HAR1 signaling functions not only in nodule suppression, but also in IT suppression. The second pathway is the HAR1-independent local suppression of IT, based on the observation of local suppression of ITs by constitutively expressed NIN in the absence of HAR1. The molecules comprising the second pathway are completely unknown.

pathway, also controls susceptibility for infection (Tsikou et al. 2018), with the constitutive expression of *miR2111* resulting in the hyper-IT phenotype. It was also shown that the increase in the number of ITs of *lhk1* or *lhk1 har1* mutants was explained well by lower expression levels of *TML* (Miri et al. 2019). These findings indicate that the components of AON via CLE-HAR1 signaling negatively regulate not only nodulation, but also rhizobial infection processes.

It has been proposed that the loss of *NIN* expression causes an increase in rhizobial infection, indicating a negative role for *NIN* in IT formation (Murray et al. 2007; Schauser et al. 1999). Isolation of *nin* alleles that specifically lost *NIN* expression in the inner cortex or pericycle led to a model in which epidermal *NIN* expressed at early stages positively regulates IT formation, whereas cortical *NIN* expressed at late stages negatively regulates IT formation (Liu et al. 2019b; Yoro et al. 2014). A study of *LHK1* that functions upstream of *NIN* also showed that the negative regulation of epidermal infection originates from the root cortex (Miri et al. 2019). Given that *CLE-RS1* and *CLE-RS2* are both direct targets of *NIN* (Soyano et al. 2014), the IT suppression mediated by CLE-HAR signaling may be a part of the IT control mediated by inner cortical *NIN* that we proposed previously (Yoro et al. 2014) (Fig. 4B; upper pathway). Of note, even in the absence of *HAR1* the constitutive expression of *NIN* suppressed IT formation in the *daphne* mutant, and the suppressive effect was restricted to the transformed roots. This local suppression proposes a novel *NIN*-mediated and *HAR1*-independent local IT suppression pathway (Fig. 4B; lower pathway). Namely, the loss of both systemic and local inhibition originating from cortical *NIN* may result in the hyper-IT phenotypes of *daphne* or *daphne-like* mutants.

The molecular targets functioning downstream of *NIN* in the local pathway are completely unknown but are presumed to involve ethylene- or nitrate-mediated local signaling. Ethylene signaling is known to be a negative regulator of rhizobial infection (Lohar et al. 2009; Oldroyd et al. 2001), and a study of the *sickle* mutant in *M. truncatula* showed that nodule inhibition by ethylene was independent of *SUNN*, the *HAR1* ortholog (Penmetsa et al. 2003). In addition, a role for cytokinin–ethylene crosstalk in the local regulation of infection has also been discussed in the analysis of *lhk1* showing hyper-ITs (Miri et al. 2019). However, *NIN* is not likely to suppress infection by altering ethylene production, because the ethylene production induced by rhizobial infection is not required for *NIN* activity (Reid et al. 2018). Nitrate has also been known to negatively regulate rhizobial infection processes (Barbulova et al. 2007; Malik et al. 1987). Recently, *NIN*-like proteins (NLPs), NITRATE UNRESPONSIVE SYMBIOSIS1 (*NRSYM1*) in *L. japonicus* and *NLP1* in *M. truncatula*, were identified as key regulators of the nitrate-induced control of nodulation (Lin et al. 2018; Nishida et al. 2018; Nishida and Suzaki 2018b). In particular, the regulation of infection by *NRSYM1* is independent of *HAR1*, which differs from nodule suppression. In *G. max*, on the other hand, the NITRATE-INDUCED CLE PEPTIDE1 (*NIC1*)-NARK receptor signaling was found to function in the nitrate-mediated local inhibition of nodulation (Ferguson et al. 2019; Reid et al. 2011a); that is, the same NARK receptor was shared between both systemic and local pathways, unlike our proposed *HAR1*-independent local pathway in *L. japonicus*.

A recent root hair transcriptomic study of *M. truncatula* revealed other signaling modules acting downstream of *NIN* that influence a variety of biological processes, including infection, cell wall modification, gibberellin biosynthesis, and nutrient uptake (Liu et al. 2019a). Future research may identify new targets among these candidates that function in the *NIN*-mediated local suppression of rhizobial infection.

In this study, we demonstrated not only the involvement of the CLE-HAR1 signaling pathway in the control of rhizobial infection, but also the existence of a new *HAR1*-independent local control of infection mediated by *NIN*. It tends to be more difficult to evaluate the suppressive effect on IT formation than the suppression of nodules. Indeed, our previous study did not detect the effect of *CLE-RS1/2* expression on IT inhibition in *L. japonicus* MG-20 (Suzaki et al. 2012). The markedly increased infection and nonnodulation phenotypes of the *daphne* mutant enabled us to evaluate this effect for the first time. Therefore, mutants with markedly increased numbers of ITs, such as *lhk1*, *daphne* in *L. japonicus* (Miri et al. 2019; Murray et al. 2007; Yoro et al. 2014), and *daphne-like* in *M. truncatula* (Liu et al. 2019b), will help facilitate the identification and study of negative regulators of rhizobial infection that are distinct from those controlling nodulation.

MATERIALS AND METHODS

Plant materials and growth conditions.

Seeds were sown in sterilized vermiculite soaked in Broughton and Dilworth solution (Broughton and Dilworth 1971) containing 0.5 mM KNO₃ and *Mesorhizobium loti* MAFF 303099 constitutively expressing *DsRed* and then grown under 16 h of light and 8 h of dark cycles. The *daphne* (Yoro et al. 2014), *har1-8* (Suzaki et al. 2012), and *klv* (Miyazawa et al. 2010) mutants in *L. japonicus* were previously reported. The primers used to genotype *daphne* and *klv* were previously described (Miyazawa et al. 2010; Yoro et al. 2014). Genotyping of the *har1-7* mutation was performed using a dCAPS marker (forward primer: 5'-CCCGGAGAGCTTCTCAAACTG-3'; reverse primer: 5'-TCCCCGCTTTTGCATAGATCCTGC-3') and digestion with *Pst*I, which cuts only the *har1-7*-type fragment. The stable transformant (*P35S::CLE-RS1*) was made previously (Sasaki et al. 2014), and the construct for making the stable transformant was described previously (Okamoto et al. 2009). The F₂ population derived from crossing plants between the *daphne* mutant and the *CLE-RS1*-expressing transformant was screened for the presence of the *daphne* mutation and GFP fluorescence (indicating *CLE-RS1* constitutive expression).

Gene expression analysis.

Total RNA was isolated from whole roots at selected time points after rhizobial infection using an RNeasy Plant Mini Kit (Qiagen), and the first-strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative reverse-transcription PCR analysis was performed using an ABI Prism 7000 (Applied Biosystems) with THUNDERBIRD SYBR qPCR Mix (Toyobo) or with a QuantiTect SYBR Green RT-PCR Kit (Qiagen), according to the manufacturer protocols. *EF-1a* (GNf095a12) expression was used as the reference (Groth et al. 2010), and the relative expression levels were calculated using the 2^{-(Delta Delta C(T))} method (Livak and Schmittgen 2001). The primers used for analyzing the expression of the *CLE-RS1/2* genes were previously reported (Okamoto et al. 2009).

Grafting experiments.

The method used for the grafting experiments was previously described in detail by Yoshida et al. (2010). The grafted plants were kept on moistened filter paper for 7 days, after which the successfully grafted plants were transplanted into vermiculite inoculated with *Mesorhizobium loti* constitutively expressing *DsRed*. The number of ITs was determined at 7 days after inoculation (DAI) for combinations of the *daphne* mutant and wild-type MG-20 or at 21 DAI for the series of combinations using *daphne* rootstock constitutively expressing *CLE-RS1*.

ITs showing DsRed fluorescence were counted under the microscope.

Microscopy observation.

Bright-field and fluorescence images were observed using an SZX12 stereomicroscope (Olympus Corporation). Images were acquired with a DP controller (Olympus Corporation).

Plant transformation.

A series of constructs for expressing *GUS* or *CLE-RS1/2* genes under the control of the CaMV 35S promoter (*Pro35S::GUS*, *Pro35S::CLE-RS1*, and *Pro35S::CLE-RS2*), together with visible marker GFP under the control of the *roID* promoter, were provided by S. Okamoto (Okamoto et al. 2009). The generation of a construct for expressing *NIN* under the control of the *LjUBIQUITIN* promoter (*ProUBQ::NIN*) was previously (Yoro et al. 2014). The hairy root and stable transformations of *L. japonicus* were performed using the *Agrobacterium rhizogenes* AR1193 harboring the respective plasmids, as described previously (Okamoto et al. 2013b).

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