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The deubiquitinating enzyme AMSH1 is required for rhizobial infection and nodule organogenesis in

*Lotus japonicus*

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**Running title**

AMSH1 promotes infection and organogenesis

**Abstract**

The legume-rhizobium symbiosis contributes large quantities of fixed nitrogen to both agricultural and natural ecosystems. This global impact and the selective interaction between rhizobia and legumes culminating in development of functional root nodules have motivated detailed studies of the underlying mechanisms. We conducted a screen for aberrant nodulation phenotypes using the *Lotus japonicus* LORE1 insertion mutant collection. Here, we describe the identification of *amsh1* mutants that only develop small nodule primordia and display stunted shoot growth, and show that the aberrant nodulation phenotype caused by LORE1 insertions in the Amsh1 gene can be separated from the shoot phenotype. In *amsh1* mutants, rhizobia initially became entrapped in infection threads with thickened cells walls. Much delayed, some rhizobia were released into plant cells, however no typical symbiosome structures were formed. Furthermore, cytokinin treatment only very weakly induced nodule organogenesis in *amsh1* mutants, suggesting that AMSH1 function is required downstream of cytokinin signaling. Biochemical analysis showed that AMSH1 is an active deubiquitinating enzyme and that AMSH1 specifically cleaves K63-linked ubiquitin chains. Post-translational ubiquitination and deubiquitination processes comprising the AMSH1 deubiquitinating enzyme are thus involved in both infection and organogenesis in *Lotus japonicus*. 

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Introduction

Many legumes are rich sources of protein due to their symbiosis with nitrogen fixing rhizobia accommodated in root nodules. The symbiotic signaling process is initiated when rhizobia secrete nodulation (Nod) factors upon sensing (iso)flavonoids produced by compatible legumes. The Nod factors consist of N-acetyl-D-glucosamine oligomers linked by β-1, 4 bonds, where the N-acetyl group on the nonreducing end is replaced by a fatty acid (Lerouge et al. 1990; Spaink et al. 1991). Genetic studies of loss-of-function and gain-of-function mutants in *Lotus japonicus* (*Lotus*) and *Medicago truncatula* (*Medicago*) show that the *Lotus* Nod factor receptors NFR1 and NFR5 and the corresponding proteins LYK3 and NFP in *Medicago* (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006; Mulder et al. 2006; Smit et al. 2007) are pivotal for perception of rhizobial Nod factors, and *in vitro* NFR1 and NFR5 bind Nod factors from compatible bacteria with high affinity (Broghammer et al. 2012). In the susceptible root zone, Nod factor perception then leads to initiation of a nodulation signaling cascade, which bifurcates into branches promoting epidermal root hair infection and cortical nodule organogenesis, respectively (Kouchi et al. 2010; Madsen et al. 2010). Promotion of epidermal infection relies on the activities of the pectate lyase NPL, the ubiquitin ligase CERBERUS, the NAP1, PIR1 and ARPC1 proteins required for actin rearrangement, and the transcription factors NIN, NSP1 and NSP2 (Schäuser et al. 1999; Kalo et al. 2005; Smit et al. 2005; Heckmann et al. 2006; Murakami et al. 2006; Marsh et al. 2007; Yano et al. 2008; Yano et al. 2009; Yokota et al. 2009; Hossain et al. 2012; Xie et al. 2012).

An overlapping set of proteins act downstream of NFR1 and NFR5 to trigger the organogenesis process in the root cortex. The LRR receptor kinase SymRK, three nucleoporins, NUP133, NUP85 and NENA, together with CASTOR and POLLUX cation channel proteins (Schäuser et al. 1998; Szczyglowski et al. 1998; Endre et al. 2002;
Kawaguchi et al. 2002; Stracke et al. 2002; Imaizumi-Anraku et al. 2005; Kanamori et al. 2006; Saito et al. 2007) are essential for the nuclear calcium spiking signal that is in turn interpreted by CCaMK (Levy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006a). Subsequently, CCaMK activates organogenesis via cytokinin signaling through the LHK1 receptor (Tirichine et al. 2006b; Murray et al. 2007; Tirichine et al. 2007), but also cross-signals to the infection pathway by phosphorylating and activating the transcription factor CYCLOPS (Yano et al. 2008; Liao et al. 2012; Singh et al. 2014). Further downstream, the transcription factors NIN, NSP1 and NSP2 (Schauser et al. 1999; Kalo et al. 2005; Smit et al. 2005; Heckmann et al. 2006; Murakami et al. 2006; Marsh et al. 2007) relay the organogenesis signal (Madsen et al. 2010).

Several of the key nodulation genes mentioned above are associated with ubiquitination, either because they are putative targets for ubiquitination, or because they share sequence similarity with ubiquitin ligases. CERBERUS belongs to the latter category, whereas the Medicago Nod factor receptor LYK3 phosphorylates the E3 ubiquitin ligase Plant U-box Protein 1 (PUB1) (Mbengue et al. 2010), and SymRK was suggested to interact with the E3 ubiquitin ligases SymRK INTERACTING E3 UBIQUITIN LIGASE (SIE3) in Lotus, and SEVEN IN ABSENTIA (SINA) in Medicago (Den Herder et al. 2008; Hervé et al. 2011; Den Herder et al. 2012; Yuan et al. 2012).

Ubiquitination is a reversible process, which regulates protein degradation, trafficking, DNA repair, apoptosis and signal transduction, making it an important post-translational regulatory mechanism. It involves linking the carboxy-terminal glycine residue of ubiquitin to lysine residues of specific protein targets, in many cases followed by ubiquitin polymerization (Kimura and Tanaka 2010). The fate of target protein can be determined by the identity of the lysine residue involved in polyubiquitin chain formation. If the poly-Ubiquitin chain is generated by K48 linkage, the substrate is targeted to the proteasome for degradation. On the
other hand, formation of K63-linked chains promotes DNA repair, endocytosis and vesicular trafficking or ribosomal protein synthesis (Mukhopadhyay and Riezman 2007; Sato et al. 2008; Ye and Rape 2009).

Accurate control of protein ubiquitination status is crucial for the cell, which is why the deubiquitinating enzymes (DUBs) play an important role in cellular homeostasis (Kimura and Tanaka 2010). DUBs are isopeptidases, which detach ubiquitin from target proteins. They are divided into five groups: ubiquitin-specific proteases (USP), ubiquitin carboxy-terminal hydrolases (UCH), Otubain proteases (OTU), Machado–Joseph disease proteases and JAB1/MPN/Mov34 metalloenzymes (JAMM) (Isono and Nagel 2014). All DUBs belong to the cysteine protease group, except for the JAMMs, which are zinc metalloproteases (Amerik and Hochstrasser 2004; Nijman et al. 2005; Sato et al. 2008; Isono and Nagel 2014). The metalloproteases contain an MPN$^+$ domain with a conserved JAMM motif (EX$_3$HXH$_{10}$D), which is believed to be the active site involved in metal binding (Maytal-Kivity et al. 2002; Tran et al. 2003). In Arabidopsis three ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM (AMSH) homologs, which belong to the JAMM zinc metalloproteases class were identified: AMSH1, AMSH2 and AMSH3. Their catalytic MPN$^+$ domain is homologous to MPN$^+$ in human AMSH. AMSH1 and AMSH3 interact with Endosomal Sorting Complex Required for Transport III (ESCRT III), which is involved in sorting of ubiquitinated membrane proteins to multivesicular bodies (MVB) (Isono et al. 2010; Katsiarimpa et al. 2011; Katsiarimpa et al. 2013). Recently it was shown that AMSH3 is involved in the degradation of AvrPtoB-activated CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Katsiarimpa et al. 2014).

Despite the aforementioned associations between proteins from the nodulation pathways and the ubiquitination machinery, the role of ubiquitination in nodulation signaling is not well understood. Using an unbiased forward genetics approach, we screened the LOREI collection
(Fukai et al. 2012; Urbanski et al. 2012) for mutants with aberrant nodulation phenotypes, and found that loss of a gene encoding the deubiquitinating enzyme AMSH1 prevented nodulation. Our analysis showed that Lotus AMSH1 is an active DUB with ubiquitin K63-link specificity and highlights the importance of post-translational protein modification and ubiquitin signaling in the developmental events leading to root infection and nodule organogenesis.

**Results**

**Identification of two independent LORE1 mutants with similar aberrant nodulation phenotypes**

Following the successful establishment and annotation of a small-scale LORE1 insertion mutant population (Fukai et al. 2012; Urbanski et al. 2012), we initiated a forward genetic screen for nodulation deficient mutants. We identified a LORE1 family segregating plants without mature nodules, where no insertions in known nodulation genes were registered in the LORE1 database. In addition to displaying arrest of nodule development at the primordium stage, the mutant plants showed severely stunted shoot growth, while root growth was less affected (Figure 1a-e). Additionally, the mutants failed to survive till seed set.

Initially, we were unable to identify the causative insertion based on our LORE1 sequencing data, since none of the annotated insertions co-segregated with the phenotype. After performing Sequence Specific Amplification Polymorphism (SSAP) analysis optimized for LORE1 insertion detection (Urbanski et al. 2013) followed by Sanger sequencing of candidate PCR products, we detected a co-segregating insertion in exon 8 (amshl-2) of the Amsh1 gene, which was not part of the Lotus v. 2.5 reference genome sequence. We then
cloned and sequenced the full length *Amsh1* cDNA sequence and identified the *Amsh1* genomic DNA sequence by aligning the cDNA sequence to a set of Lotus contigs assembled from short Illumina reads (not shown). After re-running the analysis of the *LORE1* sequencing data including the *Amsh1* genomic sequence, we discovered an additional insertion in the *Amsh1* gene in exon 1 in an independent *LORE1* mutant line (*amsh1-1*) (Figure 1f). In addition to *Amsh1*, the Lotus v. 2.5 genome comprised two additional *Amsh* homologs, which appeared to be putative orthologs of the Arabidopsis *AMSH2* and *AMSH3* genes (Figure S1).

**LORE1 insertions in the Amsh1 gene cause the aberrant nodulation phenotype**

The phenotypes of the two independent *amsh1* alleles were compared. Since the homozygous *amsh1* mutants did not produce seeds, offspring from heterozygous plants were used in all analyses. We found that all plants with stunted shoots and small nodule primordia carried homozygous *LORE1* insertions in the *Amsh1* gene. To confirm that the two *LORE1* insertions in the *Amsh1* gene were the causal mutations, heterozygous *amsh1-1* and *amsh1-2* plants were crossed to test for non-complementation in the F1 generation. *amsh1-1/amsh1-2* heteroallelic individuals displayed phenotypes indistinguishable from the homozygous *amsh1-1* and *amsh1-2* mutants (Figure S2). *amsh1-1* and *amsh1-2* are therefore allelic and we conclude that the *LORE1* insertions in the *Amsh1* gene are the causal mutations.

To further validate correct identification of the causal mutations, we identified six independent *LORE1* lines following the expansion of the *LORE1* population. These additional *amsh1-3* to *amsh1-8* alleles (Fig. 1f) all displayed phenotypes very similar to the *amsh1-1* and *amsh1-2* alleles, re-confirming correct identification of the causal mutations (Figure S3).
amsh1 accumulates ubiquitin conjugates in vivo

Alignment of the conceptual Lotus AMSH1 amino acid sequence with AMSH1 protein sequences from human and Arabidopsis AMSH metalloproteases showed that they share 42% and 64% overall amino acid sequence identity, respectively (McCullough et al. 2004; Isono et al. 2010), which suggested that the Lotus AMSH1 protein might be a metalloprotease enzyme with DUB activity (Figure S4). In contrast to ubiquitin ligases, DUBs cleave ubiquitin chains off target proteins, and loss of DUB function can lead to accumulation of ubiquitin conjugates (Wilkinson et al. 1995; Amerik et al. 1997). To test if amsh1 mutants accumulated ubiquitinated proteins, we used an anti-ubiquitin antibody to detect ubiquitin conjugates in total extracts from wild type, amsh1-2, heterozygous Amsh1/amsh1, nfr1-1, and nfr5-2 seedlings. Cyclin-dependent kinase (CDC2 kinase) (Riabowol et al. 1989; Madrid et al. 2007) was used as loading control. amsh1 clearly stood out as the genotype with the highest level of ubiquitin conjugates (Figure 2a), while there were no apparent differences between the protein samples from the remaining genotypes.

Lotus AMSH1 specifically cleaves K63-linked ubiquitin chains

Ubiquitin molecules can be linked through different lysine (K) residues to form poly-ubiquitin chains, and signaling responses can be modulated by variations in the patterns of lysine links (Komander and Rape 2012). DUBs act by cleaving the amide bond between the lysine and carboxy-terminal glycine of a linked ubiquitin (Amerik and Hochstrasser 2004). To examine if AMSH1 is an active DUB, the fusion protein GST-LjAMSH1 was expressed in the E. coli Rosetta strain, purified and tested for DUB activity. K48-linked and linear ubiquitin chains remained intact upon LjAMSH1 treatment, whereas K63-linked chains were cleaved to ubiquitin monomers (Figure 2b). We conclude that Lotus AMSH1 is an active DUB, which specifically cleaves K63-linked polyubiquitin chains in vitro. These results are
consistent with the previously reported K63 chain specificities of human AMSH and Arabidopsis AMSH1 (McCullough et al. 2004; Katsiarimpa et al. 2013).

**AMSH1 acts in Lotus roots to promote nodulation**

Since the amsh1 mutants displayed a severely stunted shoot phenotype, and since Amsh1 was expressed strongly across all Lotus tissues (Figure S5), the amsh1 mutation could indirectly affect root growth and progression of nodulation through its effect on shoot function and/or delivery of photosynthates to the root. To determine whether this was the case, we first examined root and shoot growth rates in the wild type and in the amsh1-1 and amsh1-2 mutants. The growth rate was measured at 1, 2 and 3 weeks for plate grown plants with or without rhizobial inoculation (Figure 3, Figure S6). Although nitrogen starvation inhibited shoot growth in uninoculated wild type plants, amsh1 shoot growth rate was significantly lower than that of the wild type both with and without inoculation (Figure 3a, c). In contrast, root growth rates were similar across all genotypes independent of inoculation, despite the short initial lengths of the amsh1 roots (Figure 3b, d). With respect to lateral root formation, there were no significant differences between amsh1 and the wild type in the number of lateral roots produced per cm of main root, whereas the total number of lateral roots per plant was reduced (Figure 3e-f). Despite the relatively severe amsh1 shoot phenotype, root growth and development was thus not strongly affected, suggesting that the indirect shoot effect on the root phenotype is minimal in plate-grown amsh1 mutants. As further testimony to the relatively normal general functionality of amsh1 roots, they supported mycorrhizal colonization (Figure S7), which distinguishes amsh1 from the Medicago vapyrin mutant (Murray et al. 2011).

Although root growth was not severely affected, Nod factor signaling and/or nodule development could potentially still be affected by limited shoot growth and functionality. To
examine this possibility, we used Agrobacterium rhizogenes-mediated transformation to generate transgenic roots expressing the wild type Amsh1 cDNA sequence. Using this system, the transgene is exclusively expressed in the transformed roots, allowing us to separate shoot and root effects. Expressing Amsh1 from the CaMV 35S promoter restored development of large pink nodules in 17 out of 26 amsh1 mutants, while their shoots remained stunted compared to the wild type controls (Figure 4, Table S1, Figure S8). Vibratome sections (Figure 4) showed that these large pink nodules were fully infected and appeared indistinguishable from wild type nodules at the same developmental stage. However, closer inspection of the complemented nodules using electron microscopy showed that, although symbiosomes were detected in the complemented amsh1 nodules, they were less frequent than in wild type nodules and appeared to be partly degraded (Figure S8).

To determine if the degraded symbiosomes observed at 6 wpi in the complemented plants had been functional at earlier stages, we quantified the shoot lengths of amsh1 plants in the complementation experiment. Complemented amsh1 plants with pink nodules had significantly longer shoots than the amsh1 mutants without pink nodules (p=0.0048, Student’s t-test) (Figure 4e), indicating that the pink nodules on the complemented plants had contributed fixed nitrogen to the amsh1 seedling shoots. Despite the premature symbiosome senescence observed, transformation of roots with the 35S:Amsh1 construct thus restored both infection and, at least partly, nitrogen fixation in the amsh1 mutants, and we conclude that AMSH1 acts in the root to promote nodulation.

Infection thread progression is delayed in amsh1 mutants

To determine the exact nature of the amsh1 nodulation defect, we characterized the formation of infection threads (ITs) in root hairs. These ITs represent the early stages of rhizobial infection that allow the bacteria to traverse plant epidermal cells. The earliest morphological
response to rhizobia is root hair curling, which we observed both in the wild type and amsh1 mutants (Figure S9a-c). We then used DsRed-labelled M. loti to track IT progression, and categorized ITs into three groups: 1) Incipient (microcolonies/short ITs not progressing), 2) Elongating (incomplete traversal of root hair cells) and 3) Long (completely traversing the root hair cell) (Figure 5a-c).

At 8 dpi, the wild type had approximately 30 long ITs per cm (Figure 5d) and also showed a number of incipient and elongating ITs. In contrast, the number of long ITs was significantly reduced to ~one IT/cm in amsh1-1 and amsh1-2 (Figure 5d). Instead, we found mainly incipient or elongating ITs in amsh1-1 and amsh1-2, some with aberrant morphology (Figure S9d-g).

Subsequently, at 21 dpi, ~30 and ~10 long ITs per cm were observed in the wild type and in amsh1-1 and amsh1-2, respectively. The number of incipient and elongating ITs was similar in amsh1-1 and amsh1-2 and wild type plants, suggesting that the infection process was progressing at a reduced speed in the amsh1 mutants (Figure 5d). It thus appears that amsh1-1 and amsh1-2 are able to perceive rhizobial signals and initiate the infection process, but that IT progression is aberrant and delayed, resulting in decreased infection.

**amsh1 infection threads display aberrant morphology and bacterial release**

The slower infection process in amsh1 mutants was also reflected at the level of nodule organogenesis (Figure 5e). Wild type plants produced nodule primordia within days of infection and functional pink nitrogen fixing nodules were present at 11 dpi. In contrast, none of the amsh1 mutants had fully developed mature nodules at 6 wpi (Figure 5e and Figure 6).

To investigate if the infection status of the amsh1 primordia could explain the lack of mature nodules, we visualized the infection of wild type nodules and amsh1 nodule primordia 6 weeks after inoculation with M. loti expressing a hemA::lacZ reporter gene. Dark blue

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staining was observed at the center of the wild type nodules, indicating full colonization. In contrast, only weak staining near primordia surfaces was detected in amsh1-1 and amsh1-2 primordia, suggesting limited colonization (Figure 6a).

Next, we examined the amsh1 primordia and wild type nodules at 3 and 6 wpi using light and transmission electron microscopy. At 3 wpi, wild type nodules were fully colonized with ITs present within the nodules (Figure 6b, c). In some amsh1-1 and amsh1-2 primordia, ITs had entered cortical cells and these ITs were irregular, bulbous, and enclosed in unusually thick cell wall-like structures (Figure 6b-c). Release of bacteria into the plant cells was not observed in the amsh1 mutants.

At 6 wpi, the infected cells of wild type nodules showed symbiosomes containing up to 2-3 bacteroids, while in the occasional larger amsh1-1 and amsh1-2 nodule primordia a higher number of branching ITs with aberrant morphology were present (Figure 4c and Figure 6d-e). For a few of the older amsh1 mutant primordia, release of bacteria could be observed (Figure 6d-e). These released bacteria did not differentiate and symbiosomes were not observed (Figure 6d-e).

**AMSH1 is required downstream of cytokinin signaling**

The severe infection defects in the amsh1 mutants could explain the arrested progression of organogenesis observed in response to rhizobia. However, amsh1 mutants could suffer defects in nodule organogenesis independent of their infection deficiencies. To investigate this possibility, we determined the extent of organogenesis induced by exogenous application of cytokinin in the absence of rhizobia and infection (Heckmann et al. 2011). Following six weeks of treatment with $10^{-8}$ M of the cytokinin analog 6-benzylaminopurine (BAP), no apparent growth rate differences were observed between plants growing on medium with and without BAP (Figure S10). BAP treatment induced formation of more than 5 white nodules.
per wild type plant, while only ~2 small primordia per plant were seen in amsh1-1 and very few primordia were induced in amsh1-2 (Figure 7a). The amsh1 BAP-induced primordia were much smaller than the white nodule-like structures observed on the wild type roots (Figure 7b-c).

Additionally, we tested if application of BAP could rescue the amsh1-1 and amsh1-2 nodulation phenotype. We did not observe a difference in primordium number compared to the plants grown on medium without BAP (Figure S10). The wild type plants formed approximately three pink nodules while amsh1 mutants produced only two nodule primordia. Likewise, we found no effect of BAP treatment on the amsh1 infection phenotype, and the cytokinin-treated amsh1-1 and amsh1-2 primordia were not properly colonized, but had a high abundance of branching and bulbous infection threads enclosed in thick cell wall-like structures as previously observed without BAP treatment (Figure 7d-g). In conclusion, amsh1 mutants appear to have organogenesis defects that are independent of infection, suggesting a requirement of AMSH1 downstream of cytokinin signaling.

**Discussion**

*The LORE1 resource facilitated efficient identification and validation of the amsh1 mutants*

The LORE1 mutant collection represents a substantial reverse genetics resource, providing access to loss-of-function alleles of more than 20,000 unique *Lotus* genes (Fukai et al. 2012; Urbanski et al. 2012; Urbanski et al. 2013). Here, we exemplify the efficient use of the LORE1 resource in a forward genetics approach aimed at identification of novel genetic regulators of nodulation. We carried out a family-wise screen, where multiple mutant individuals displaying the amsh1 phenotype were detected within distinct segregating...
families, facilitating reliable detection of the aberrant phenotype. In the case of \textit{amsh1}, the family-wise screen was also critical in allowing ready access to heterozygous \textit{amsh1} individuals, since the homozygous \textit{amsh1} mutants did not produce seeds. Following identification of \textit{LORE1} insertions that co-segregated with the aberrant phenotype, the \textit{LORE1} collection provided a large number of additional \textit{amsh1} alleles, which ensured rapid validation of the causal mutation and the corresponding gene, allowing us to proceed quickly with the biochemical and phenotypic characterization. In general, the two \textit{amsh1} alleles, \textit{amsh1-1} and \textit{amsh1-2} characterized in detail here, showed very similar phenotypes, although \textit{amsh1-2} had a tendency to show slightly more severe defects. The large number of \textit{amsh1} \textit{LORE1} lines with consistent phenotypes suggests that, at least in this case, background mutations and/or variation in \textit{LORE1} insertion site position within the target gene did not strongly influence the observed phenotypes.

**AMSH1 acts as a K63-specific deubiquitinating enzyme**

In \textit{Arabidopsis} three AMSH1 homologues were identified, among which \textit{AtAMSH3} appears to be the major DUB that cleaves both K48- and K63-linked ubiquitin chains (Isono et al. 2010). Whereas the homozygous Arabidopsis \textit{amsh3} mutant is seedling lethal (Isono et al. 2010), \textit{amsh1} and \textit{amsh3} mutants show synergistic interaction, suggesting partially redundant functions (Katsiarimpa et al. 2013).

Our biochemical analysis revealed that \textit{Lotus} AMSH1 is an active DUB that specifically cleaves K63- but not K48-linked ubiquitin chains. A specificity it shares with human AMSH and Arabidopsis AMSH1 (McCullough et al. 2004; Katsiarimpa et al. 2013). Previously, it was suggested that K63 ubiquitin chains could act as a signals for endocytosis (Mukhopadhyay and Riezman 2007; Woelk et al. 2007). Supporting this suggestion, \textit{Arabidopsis} AMSH1 and AMSH3 interact with ESCRT-III components involved in
endocytosis (Katsiarimpa et al. 2013; Katsiarimpa et al. 2014). It is thus possible that *Lotus* AMSH1 function could also be related to endocytosis.

**amsh1 mutants display a pleiotropic phenotype including a severe nodulation defect**

At first glance, the *amsh1* mutant phenotype seems pleiotropic because of the strong effects on shoot growth, plant viability, and nodulation. Such global phenotypic deviations are consistent with the ubiquitous expression of *Amsh1* and with the biochemical function of AMSH1 as a DUB, which presumably acts on a number of target proteins. AMSH1 therefore clearly does not act exclusively in the nodulation pathways. Nevertheless, it still has a strong nodulation phenotype, which can be decoupled from the shoot phenotype, as we have demonstrated here using *A. rhizogenes*-mediated transgenic root complementation (Figure 4). Within the root, the nodulation defect appears to be the most prominent phenotypic aberration, since *amsh1* roots are able to support mycorrhization (Figure S7), grow at rates comparable to the wild type, and produce lateral roots (Figure 3). Despite this overall normal appearance of *amsh1* roots, they fail to produce functional nodules. Instead, *amsh1* displays the impaired IT progression as well as bacterial release from the ITs and *amsh1* nodule development is arrested at the primordium stage.

**AMSH1 could target nodulation proteins**

The most likely explanation for these observations is that one or more critical components of the nodulation signaling machinery are highly sensitive to K63-linked ubiquitination, and require AMSH1 DUB activity to retain functionality.

The simplest hypothesis is that AMSH1 promotes infection and organogenesis by acting on a
single nodulation component, leaving the transcriptional regulators NIN, NSP1, and NSP2 as candidates, because they are required for both processes (Madsen et al. 2010; Heckmann et al. 2011). The remaining nodulation proteins are less likely candidates under this hypothesis, because they either have more specific infection- or organogenesis-related functions, or because their corresponding loss-of-function mutants, unlike amsh1, respond to cytokinin treatment with formation of larger nodule-like structures. The infection mutants nap, pir, arpC1, cyclops, and cerberus thus display abnormal IT development, but nodule organogenesis that proceeds further than in amsh1. Conversely, loss of organogenesis-specific proteins, such as the cytokinin receptor LHK1 (Tirichine et al. 2006b; Tirichine et al. 2007) and the DNA topoisomerase VI components VAG1 and SUNER1 (Suzaki et al. 2014; Yoon et al. 2014), does not hinder epidermal infection, but result in absence of, or reduced, organogenesis. Finally, the nfr1, nfr5, symrk, nup133, nup85, castor, pollux, ccamk, pir and cyclops mutants all respond to cytokinin treatment with formation of nodule-like structures (Heckmann et al. 2011), making it unlikely that AMSH1 targeting one of the corresponding proteins could explain the requirement of AMSH1 downstream of cytokinin signaling.

The alternative hypothesis to AMSH1 targeting a single nodulation component required for both infection and organogenesis is that AMSH1 targets multiple components distributed on both signaling pathways, for instance LHK1 and one or more of the proteins required for infection.

With the availability of multiple amsh1 mutant alleles, these hypotheses can now be tested using biochemical approaches to compare the ubiquitination status and protein abundance of known nodulation signaling components in amsh1 and wild type backgrounds. The AMSH1 characterization presented here thus opens new avenues of investigation with the potential for greatly advancing the understanding of ubiquitin impact on nodulation signaling in legumes.
Experimental procedures

Bacterial strains

*Mesorhizobium loti* strain NZP2235 was used for forward screening under greenhouse conditions, while for most of the studies on plates and in Magenta containers, *M. loti* strain MAFF303099 expressing DsRed was used (Maekawa et al. 2009). The *M. loti* strain NZP2235 expressing hemA::lacZ reporter gene was used for lacZ staining (Schauser et al. 1998; Wopereis et al. 2000). An OD$_{600}$ = 0.06 was used for all the studies involving rhizobia. *Agrobacterium rhizogenes* strain AR1193 was used for hairy root transformation (Stougaard et al. 1987).

Plant materials

*amsh1* mutants were obtained from the LOREI collection (Fukai et al. 2012; Urbanski et al. 2012). The isolation of *nfr1-1* and *nfr5-2* was described by Schauer et al. (Schauer et al. 1998). *Lotus japonicus* B-129 Gifu is the wild type for all these mutants (Handberg and Stougaard 1992).

Seeds were scarified by treatment with 98% sulfuric acid for 15 min, or using sand paper, and then sterilized with 0.5% sodium hypochlorite for 20 min. They were rinsed 5 times with sterile water and incubated on a shaker for 3 h at room temperature. Seeds were germinated on moist, sterile paper on vertical plates in growth chambers under the following conditions: 16/8h light/darkness and 21°C/18°C temperature for 3 days. Subsequently, for nodulation tests they were moved to plates containing ¼-strength B&D medium (Broughton and Dilworth 1971) and 1.4% agar noble slants without nitrate, or to Magenta containers (Sigma) filled with Leca (Optiroc) and Vermiculite (3:1) and 80 ml of ¼-strength B&D medium without nitrate. On plates, roots were shielded from light using a metal spacer. For cytokinin experiments, ¼-strength B&D medium was supplemented with BAP to a final concentration

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of $10^8$ M. For complementation, 3 days old seedlings were moved to $\frac{1}{2}$-strength Gamborg’s B5 basal salt mixture (Duchefa Biochemie) supplemented with Gamborg’s vitamin solution (Sigma).

For each experiment, a segregating population of *amsh1* plants was used. Preparation of 200 seeds usually yielded approximately 20 *amsh1* plants. For root growth assays, 20 Gifu plants, 17 *amsh1-1* and 15 *amsh1-2* were counted. For nodule counts, 20 plants from each genotype were used. For infection thread counts 10 plants from each genotype was used at 8 and 21 dpi. For western blot the protein extract from one seedling was used and it was repeated three times. For hairy root complementation 1000 seeds from segregating population were used. 500 seeds were transformed with empty vector control and 500 with 35S::*Amsh1* cDNA. After 4 weeks the small plants were moved to Magenta containers and inoculated. 6 weeks later plants were scored, pictures were taken and plants were genotyped.

**Arbuscular Mycorrhiza**

One week old plants from segregating population of *amsh1* and wild type Gifu seedlings were transferred to *Rhizophagus irregularis*-colonized chive nurse pots as previously described (Chabot et al. 1992; Kosuta et al. 2005; Kruger et al. 2012). Plants were co-cultivated for 5-6 weeks at 25°C. Each week they were fertilized with chive nutrient solution supplemented with potassium nitrate to a final concentration of 5.5 mM nitrate. Plants roots were scored for AM colonization by ink staining as previously described (Vierheilig et al. 1998).

**Genotyping**

DNA was extracted according to the standard CTAB method (Rogers and Bendich 1985). The genotyping PCR reactions were performed using the primers designed by the FSTpoolit
software as previously described (Urbanski et al. 2012), except that the touch-down step was skipped and a three-step, 30 cycle reaction was performed with a 62°C annealing temperature.

Cloning procedure

The *Lotus Amsh1* coding sequence was cloned from a *Lotus* cDNA library obtained from roots using the F1 and R1 primers (Table S2). The promoter region was amplified together with the 5’UTR from genomic DNA of Gifu using the F2 and R2 primers. The promoter region and cDNA were combined by using the F3 and R3 primers. The Phusion polymerase (Thermo Fisher Scientific) was used to amplify all of the fragments. They were cloned into pENTR/d-TOPO vector between attL1 and attL2 sites (Invitrogen). An LR reaction was performed according to the manufacturer’s instructions (Invitrogen) in order to transfer the constructs from pENTR/d-TOPO to a pIV10 expression vector containing a Gateway cassette (Stougaard et al. 1987).

Ligation Independent Cloning (LIC cloning) was performed according to the Novagen protocol to clone the *Amsh1* cDNA into pET41 for recombinant expression in *E. coli*. The F4 and R4 primers were used to amplify the *Amsh1* cDNA (Table S2).

Quantitative RT-PCR analysis

Total RNA was isolated from different tissues at 3 wpi inoculated with *M. loti MAFF303099* or mock using modified Lithium Chloride - TRIzol LS method as described by Holt and associates (Holt et al. 2015). cDNA was synthesized using oligodT primer and MuLV RT from Thermo Fisher Scientific as described in Holt et al. (Holt et al. 2015). The F5 and R5 primers were used to amplify *Amsh1* transcripts. *Ubiquitin-conjugating enzyme (LjUBC)* was used as a reference gene. Primers for *LjUBC* were F6 and R6 (Table S2). Real-Time PCR
was performed using the LightCycler 480 II (Roche Molecular Biochemicals) using LightCycler 480 SYBR Green I Master (Roche Molecular Biochemicals).

**Protein extraction, DUB assays, and Western blots**
The GST-AMSH1 fusion protein was expressed from a pET41 vector in the E. coli Rosetta strains (DE3) (Merck Chemicals) at 18°C and purified using Glutathione Sepharose 4B (GE Healthcare) beads. Protein was eluted from the beads by incubation for 30 min at 4°C with 100 mM Glutathione. DUB assays were performed according to Isono (2010). 8 pmol of LjAMSH1 enzyme in DUB buffer were incubated with 125 ng µl⁻¹ of K48-, K63-linked or linear ubiquitin chains (2-7 ubiquitins, Enzo Life Sciences) for 120 min at 30°C. The reaction was then stopped by adding 2.5 µl SDS of sample buffer. The samples were run on the NuPAGE Novex Bis-Tris 4-12% gel (Invitrogen) in MES buffer according to the instructions provided by Invitrogen. The antibodies used were: anti-GST (1:2000, raised in goat, GE Healthcare), anti-CDC2 kinase (1:5000, raised in rabbit, Santa Cruz Technology), anti-Ub P4D1 (1:2500, raised in mouse, Cell Signaling Technology), anti-mouse HRP conjugated antibody (1:2000, Pierce), anti-goat AP conjugated antibody (1:1000, Sigma), anti-rabbit AP (1:4000, Sigma). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was used for HPR reaction. The BCIP and NBT for Alkaline Phosphatase (AP) detection were purchased from AppliChem.

**Microscopic observation, staining and image processing**
Characterization of nodulation phenotypes was carried out using M. loti MAFF303099 expressing DsRed. The confocal microscope Zeiss 780LSM was used for counting of ITs. ITs were counted at 8 and 21 dpi. To visualize nodule colonization, plants were inoculated with M. loti NZP2235 expressing hemA::lacZ, and lacZ staining was performed as previously
described (Wopereis et al. 2000). The root lengths were measured using ImageJ. 6 wpi nodules/primordia were sectioned using a Leica vibratome. Transmission electron microscopy was performed on ultrathin sections (80 nm thick) of fixed and resin-embedded nodules and nodule primordia according to Madsen et al. (2010), and were viewed and photographed using a JEOL JEM1400 TEM.

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Accession numbers
The Amsh1 sequences have Genbank accession numbers KR270441.

Author contributions:
A.M., D.F.U., N.S. performed the forward screening. D.F.U. and A.M. carried out the cloning and the SSAP analysis. A.M. characterized the mutant. E.K.J. carried out the light and transmission electron microscopy. E.I. supervised the biochemical assays. S.U.A and J.S. supervised the work. A.M. and S.U.A wrote the manuscript with input from JS.

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Supplemental Tables and Figures

Supplemental Figure 1. Alignment of Lotus and Arabidopsis AMSH homologs

Supplemental Figure 2. *amsh1-1* and *amsh1-2* are allelic

Supplemental Figure 3. Phenotypes of the *amsh1-3* to *amsh1-8* alleles

Supplemental Figure 4. Alignment of human AMSH (HsAMSH), *Arabidopsis* AMSH1 (AtAMSH1) and *Lotus* AMSH1 (LjAMSH) protein sequences

Supplemental Figure 5. Relative expression of *Amsh1* in roots, nodules and leaves of inoculated and uninoculated wild type plants

Supplemental Figure 6. Comparison of shoot and root growth in the wild type and *amsh1*

Supplemental Figure 7. *amsh1* mutants are able to establish arbuscular mycorrhiza with *Rhizophagus irregularis*

Supplemental Figure 8. Root specific complementation restores normal infection in *amsh1-1.1*

Supplemental Figure 9. *amsh1-1* and *amsh1-2* mutants respond to bacteria but fail to form long infection threads

Supplemental Figure 10. The application of exogenous cytokinin does not revert the *amsh1* shoot and nodulation phenotype

Supplemental Table 1. Complementation of the *amsh1-1* mutant

Supplemental Table 2. Primers used in this study
References


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Figure legends

Figure 1. amsh1 nodulation phenotype. (a)-(b) Plants grown on ¼-strength B&D plates, 6 wpi with M. loti rhizobia. (c)-(e) Plant root systems at 6 wpi. Fully developed nodules are indicated by arrows and nodule primordia by arrowheads. Scale bar indicates 0.5 mm. Plant genotypes are indicated at the bottom of the panels. (f) Structure of the L. japonicus Amshl gene (GenBank accession KR270441). The exon-intron structure was obtained by alignment of the sequenced full length Amshl cDNA to Lotus contigs assembled from short Illumina reads (not shown). The Amshl gene has 14 exons, covers ~6.2 Kb of genomic sequence and encodes a protein of 58 kD. The LOREl insertion sites are shown for the amshl mutant alleles.
Figure 2. AMSH1 deubiquitination activity. (a) Western blot using an anti-ubiquitin antibody (P4D1) to detect ubiquitin conjugates in protein extracts from plant seedlings. An anti-CDC2 kinase antibody was used to detect the CDC2 kinase loading control. Wt – Gifu, het L1 – heterozygous Amsh1/amsh1 plant derived from the amsh1-2 line, wt L1 – wild type plant derived from the amsh1-2 line. (b) Deubiquitination assay using linear, K48- and K63-linked ubiquitin chains as substrates. The ubiquitin chains were incubated with or without Lotus AMSH1 for 0 or 120 min. Numbers from 1 to 5 indicate the number of ubiquitin units in the chains. An anti-ubiquitin antibody (P4D1) was used to detect ubiquitin chains and an anti-GST antibody was used to detect the presence of the GST-AMSH1 protein.

Figure 3. amsh1 shoot and root growth and growth rates. (a) Shoot lengths of plate grown seedlings. (b) Root lengths of plate grown seedlings. (c) Average shoot growth rates from 1 to 3 weeks. (d) Average root growth rates from 1 to 3 weeks. (e) Average number of lateral roots per plant at 3 weeks. (f) Average number of lateral roots per cm of root at 3 weeks. The asterisks indicate statistically significant differences compared to the wild type (*: P < 0.05, **: P < 0.01, ***: P < 0.001, Student’s t-test). Error bars indicate SEM. 20 Gifu, 17 amsh1-1 and 15 amsh1-2 were assayed.

Figure 4. 35S::Amsh1 complementation of amsh1-1 in transgenic roots. (a) Plants with transgenic roots at 6 wpi. Arrowheads indicate nodule primordia, while arrows indicate pink well-developed nodules. Scale bars indicate 1 cm. (b) Close up views of root and nodules/nodule primordia at 6 wpi. The top panels are light micrographs. The red signal in the middle panels indicates DsRed-labeled M. loti rhizobia, and the lower panels are overlays of the two panels above. Scale bars indicate 0.5 mm. (c) Vibratome sections of wild type

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nodules and amsh1 nodule primordia at 6 wpi. The purple signal indicates DsRed-labeled rhizobia. The blue signal represents cell wall autofluorescence. A dashed white line indicates a region with branched infection threads typical of amsh1, while dashed yellow lines indicate groups of normally infected cells. Infected cells were rarely seen in amsh1-1. Scale bars indicate 50 µm. (d) Close up views of the regions marked by white boxes in (c). Scale bars indicate 100 µm. The construct used for transformation and the plant genotype are indicated at the top and bottom of the panels, respectively. (e) Shoot lengths of amsh1 plants. The mean shoot length of 35S::Amsh1 complemented amsh1 plants with pink nodules is significantly larger than the mean shoot length of 35S::Amsh1 transformed amsh1 plants without pink nodules and amsh1 plants transformed with an empty vector control (p=0.0048, Student’s t-test).

Figure 5. Root hair infection thread and nodule frequencies in wild type and amsh1 mutants. (a)-(c) Representative examples of IT categories, i.e. incipient, elongating and long ITs. Scale bars indicate 50 µm. (d) Number of infection threads of different categories at 8 and 21 days post infection (dpi). Error bars indicate SEM. 10 plants of each genotype were assayed. (e) Number of nodules and nodule primordia on wild type and amsh1-1 and amsh1-2 at 3 and 6 wpi. Error bars show SEM. 20 plants of each genotype were assayed.

Figure 6. Nodule infection thread phenotypes of amsh1 mutants. (a) Nodule colonization visualized by lacZ staining (blue) of M. loti expressing a hemA::lacZ reporter gene. Scale bars indicate 0.5 mm. (b) Micrographs of wild type nodules and amsh1-1 and amsh1-2 nodule primordia at 3 wpi. Scale bars indicate 100 µm. Red arrowheads indicate ITs. (c) Transmission electron micrographs of wild type nodules and amsh1-1 and amsh1-2 nodule
primordia at 3 wpi. Scale bars indicate 2 μm. The green arrow indicates symbiosomes. Infection thread structures are indicated by dashed red lines and thickened cell wall structures by dashed yellow lines. (d) Micrographs of wild type nodules and amsh1-1 and amsh1-2 nodule primordia at 6 wpi. Scale bars indicate 100 μm. Red arrowheads indicate ITs. (e) Transmission electron micrographs of wild type nodules and amsh1-1 and amsh1-2 nodule primordia at 6 wpi. Scale bars indicate 2 μm. Infection thread structures are indicated by dashed red lines. Green arrows indicate symbiosomes, while blue arrowheads indicate free bacteria. The genotypes are indicated at the bottom of the panels.

Figure 7. Cytokinin response in amsh1 mutants. (a) Number of BAP-induced nodules/nodule primordia in wt and amsh1-1 and amsh1-2. Error bars indicate SEM. (b)-(g) Plant tissue six weeks post BAP treatment. (b) Uninoculated wild type root with nodule-like structures (arrows) induced by BAP treatment. (c) Uninoculated amsh1 roots with a primordium (arrow) induced by BAP treatment. (d)-(g) Plant tissue six weeks post inoculation with rhizobia. (d)-(e) Sections of amsh1 nodule primordia. The red signal indicates DsRed-labeled rhizobia. The blue signal represents cell wall autofluorescence. Excessive IT branching (dashed white lines) was seen in amsh1. Scale bars indicate 50 μM. (f)-(g) Transmission electron micrographs of amsh1 primordia. Blue arrowheads indicate free bacteria. Infection threads are marked by dashed red lines. Scale bars indicate 2 μm. The genotypes are indicated at the bottom of the panels.
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