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

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SUMMARY
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 prompted detailed studies of the underlying mechanisms. We performed 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 may be separated from the shoot phenotype. In amsh1 mutants, rhizobia initially became entrapped in infection threads with thickened cells walls. Some rhizobia were released into plant cells much later than observed for the wild-type; 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 involving the AMSH1 deubiquitinating enzyme are thus involved in both infection and organogenesis in Lotus japonicus.

Keywords: Lotus japonicus, nodulation mutant, AMSH1, deubiquitination, symbiosis, LORE1.

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-\(\beta\) glucosamine oligomers linked by \(\beta\) 1,4 bonds, in which the N acetyl group on the non-reducing 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 NFR1 and NFR5 bind Nod factors from compatible bacteria \textit{in vitro} 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 (Kouchi et al., 2010; Madsen et al., 2010). Promotion of epidermal infection relies on the activities of the pectate lyase Nodulation pectate lyase (NPL), the ubiquitin ligase CERBERUS, the Nck-associated protein 1 (NAP1), 12IF-specific p53 inducible RNA (PIR1) and Actin-related protein component 1 (ARPC1) proteins required for actin rearrangement, and the transcription factors Nodule inception protein (NIN), Nodulation signaling pathway 1 (NSP1) and Nodulation signaling pathway 2 (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; Yano et al., 2008, 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 leucine-rich repeat kinase Symbiosis receptor-like kinase (SymRK), and three nucleoporins (NUP133, NUP85 and NENA), together with CASTOR and POLLUX cation channel proteins (Schauser et al., 1998; Szczylowska et al., 1998; Endre et al., 2002; Kawaguchi et al., 2002; Stracke et al., 2002; Imaiizumi-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 Ca²⁺ and calmodulin-dependent receptor kinase (CCaMK) (Levy et al., 2005; Kanamori 2007) relay the organogenesis signal (Madsen, 2008; Ye and Rape, 2009).

Subsequently, CCaMK activates organogenesis via cytokinin signaling through the LHK1 receptor (Tirichine et al., 2006a). Several of the key nodulation proteins 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., 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).

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Ubiquitination is a reversible process that regulates protein degradation, trafficking, DNA repair, apoptosis and signal transduction, making it an important post-translational regulatory mechanism. It involves linking the C 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 the target protein is determined by the identity of the lysine residue involved in poly-ubiquitin 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 deubiquitinating enzymes (DUBs) play an important role in cellular homeostasis (Kimura and Tanaka, 2010). DUBs are isopeptidases that detach ubiquitin from target proteins. They are divided into five groups: ubiquitin-specific proteases, ubiquitin C terminal hydrolases, Otubain proteases, Machado Joseph disease proteases and JAB1/MPN/Mov34 metalloenzymes (JAMMs) (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 EX₁₇HX₁₁₀D, which is believed to be the active site involved in metal binding (Maytal-Kivity et al., 2002; Tran et al., 2003).

In Arabidopsis, three homologs of ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM (AMSH), which belong to the JAMM zinc metalloprotease class, have been identified: AMSH1, AMSH2 and AMSH3. Their catalytic MPN⁺ domain is homologous to the MPN⁺ domain 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 (Isono et al., 2010; Katsiarimpa et al., 2011, 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 LORE1 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 specificity for K63-linked ubiquitin, 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 performed a forward-genetic screen for nodulation-deficient mutants. We identified a LORE1 family segregating plants without mature nodules, although 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, but root growth was less affected (Figure 1a e). Additionally, the mutants did not survive until seed set.

Initially, we were unable to identify the causative insertion based on our LORE1 sequencing data, as none of the
annotated insertions co-segregated with the phenotype. After performing sequence-specific amplification polymorphism analysis optimized for LORE1 insertion detection (Urbanski et al., 2013), followed by Sanger sequencing of candidate PCR products, we detected a co-segregating insertion (amsh1 2) in exon 8 of the Amsh1 gene. Because Amsh1 was not included in the Lotus version 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 with a set of Lotus contigs assembled from short Illumina reads (data not shown). After re-running the analysis of the LORE1 sequencing data, including the Amsh1 genomic sequence, we discovered an additional insertion in exon 1 of the Amsh1 gene in an independent LORE1 mutant line (amsh1 1) (Figure 1f). In addition to Amsh1, the Lotus version 2.5 genome contains two additional Amsh homologs that appear 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. As 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 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 expansion of the LORE1 population. These additional alleles (amsh1 3 to amsh1 8; Figure 1f) all displayed phenotypes very similar to the amsh1 1 and amsh1 2 alleles, re-confirming correct identification of the causal mutations (Figure S3).

**The amsh1 mutants accumulate 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), suggesting that the Lotus AMSH1 protein may be a metalloprotease enzyme with DUB activity (Figure S4). In contrast to ubiquitin ligases, DUBs cleave ubiquitin chains from target proteins, and loss of DUB function may lead to accumulation of ubiquitin conjugates (Wilkinson et al., 1995; Amerik et al., 1997). To test whether 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 (CDK2 kinase) (Riabowol et al., 1989; Madrid et al., 2007) was used as a loading control. The amsh1 1 mutant clearly had the highest level of ubiquitin conjugates (Figure 2a), and there were no apparent differences between protein samples from the remaining genotypes.
conjugates in protein extracts from AMSH1 minimal glycine of a linked ubiquitin (Amerik and (K) residues to form poly-ubiquitin chains, and signaling activity.

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 ’Z line’ wt L1, wild type plant derived from the amsh1 ’Z 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. The numbers 1, 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.

Lotus AMSH1 specifically cleaves K63-linked ubiquitin chains
Ubiquitin molecules may be linked through various lysine (K) residues to form poly-ubiquitin chains, and signaling responses may 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 the C terminal glycine of a linked ubiquitin (Amerik and Hochstrasser, 2004). To examine whether AMSH1 is an active DUB, the fusion protein GST LJAMSH1 was expressed in the Escherichia 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 that specifically cleaves K63-linked poly-ubiquitin 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
As the amsh1 mutants displayed a severely stunted shoot phenotype, and as Amsh1 was expressed strongly across all Lotus tissues (Figure S5), the amsh1 mutation may 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 and Figure S6). Although nitrogen starvation inhibited shoot growth in uninoculated wild-type plants, the 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 mutants and the wild-type with respect to the number of lateral roots produced per cm of main root, but the total number of lateral roots per plant was reduced (Figure 3e,f). Despite the relatively severe amsh1 shoot phenotype, root growth and development were 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 vapaerin mutant (Murray et al., 2011).

Although root growth was not severely affected, Nod factor signaling and/or nodule development may 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 of 26 amsh1 mutants, although their shoots remained stunted compared to the wild-type controls (Figure 4, Table S1 and 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 3b).

Wild-type plants produced nodule primordia within days of inoculation with Mesorhizobium loti, indicating that the pink nodules on the complemented plants had contributed fixed nitrogen to the amsh1 seedling shoots. Despite the premature 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 in both the wild-type and amsh1 mutants (Figure S8a-c). We then used DoRed-labeled Mesorhizobium 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 (complete traversal of root hair cells) (Figure 5a-c).

At 8 days post-inoculation (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 approximately one IT per 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 S8d-g).

At 21 dpi, approximately 30 and 10 long ITs per cm were observed in the wild-type and in the amsh1 1 and amsh1 2 mutants, respectively. The number of incipient and elongating ITs was similar in amsh1 1, 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 (Figures 5e and 6). To investigate whether the infection status of the amsh1 primordia explained 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 \textit{hemA::lacZ} reporter gene. Dark blue 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 \textit{amsh1 1} and \textit{amsh1 2}, suggesting limited colonization (Figure 6a).

Next, we examined the \textit{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 \textit{amsh1 1} and \textit{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 \textit{amsh1} mutants.

At 6 wpi, the infected cells of wild-type nodules showed symbiosomes containing up to two or three bacteroids, while in the occasional larger \textit{amsh1 1} and \textit{amsh1 2} nodule primordia, a higher number of branching ITs with aberrant morphology were present (Figures 4c and 6d,e). Release of bacteria was observed for a few of the older \textit{amsh1} mutant primordia (Figure 6d,e). These released bacteria did not differentiate, and symbiosomes were not observed (Figure 6d,e).

\textbf{AMSH1 is required downstream of cytokinin signaling}

The severe infection defects in the \textit{amsh1} mutants may explain the arrested progression of organogenesis observed in response to rhizobia. However, \textit{amsh1} mutants may 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 \textit{et al.}, 2011). After 6 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 five white nodules per wild-type plant, while only approximately two small primordia per plant were seen in \textit{amsh1 1} plants, and very few primordia were induced in \textit{amsh1 2} (Figure 7a). The BAP-induced primordia in the \textit{amsh1} mutants were much smaller than the white nodule-like structures observed on the wild-type roots (Figure 7b,c).

Additionally, we tested whether application of BAP rescued the \textit{amsh1 1} and \textit{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 for AMSH1 downstream of cytokinin signaling.

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

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, 2013). Here, we describe the efficient use of the LORE1 resource in a forward genetics approach aimed at identification of novel genetic regulators of nodulation. We performed a LORE1 family screen, whereby multiple mutant individuals displaying the amsh1
phenotype were detected within distinct segregating families, facilitating reliable detection of the aberrant phenotype. In the case of *amsh1*, the family screen was also critical in allowing ready access to heterozygous *amsh1* individuals, as the homozygous *amsh1* mutants did not produce seeds. The LORE1 collection also provided a large number of additional *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 *amsh1* alleles characterized in detail here, *amsh1* 1 and *amsh1* 2, showed very similar phenotypes, although *amsh1* 2 had a tendency to show slightly more severe defects. The large number of *amsh1* LORE1 lines with consistent phenotypes suggests that, at least in this case, background mutations and/or variation in LORE1 insertion site position within the target gene did not strongly influence the observed phenotypes.

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

Three *AMSH1* homologues have been identified in Arabidopsis, among which At*AMSH3* appears to be the major DUB that cleaves both K48- and K63-linked ubiquitin chains (Isono et al., 2010). Whereas the homozygous Arabidopsis *amsh3* mutant is seedling-lethal (Isono et al., 2010), *amsh1* and *amsh3* mutants show synergistic interaction, suggesting partially redundant functions (Katsiarimpa et al., 2013).

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

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

At first glance, the *amsh1* mutant phenotype appears 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 the biochemical function of *AMSH1* as a DUB that presumably acts on a number of target proteins. *AMSH1* therefore clearly does not act exclusively in nodulation pathways. Nevertheless, it still has a strong nodulation phenotype that may be decoupled from the shoot phenotype, as 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, as *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 impaired IT progression and bacterial release from the ITs, and amsh1 nodule development is arrested at the primordium stage.

AMSH1 may target nodulation proteins

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

The simplest hypothesis is that AMSH1 promotes infection and organogenesis by acting on a single nodule component, leaving the transcriptional regulators NIN, NSP1 and NSP2 as candidate AMSH1 targets, 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 by formation of larger nodule-like structures. The infection mutants nap, pir, arpc1, cyclops and cerberus thus display abnormal IT development, but nodule organogenesis proceeds further than in amsh1. Conversely, loss of organogenesis-specific proteins, such as the cytokinin receptor LHK1 (Tirichine et al., 2006b, 2007) and the DNA topoisomerase VI components VAG1 and SUNER1 (Suzuki et al., 2014; Yoon et al., 2014), does not hinder epidermal infection, but results in reduced or absent organogenesis. 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 target- ing of one of the corresponding proteins explains the requirement for AMSH1 downstream of cytokinin signaling.

An alternative hypothesis to targeting a single nodule- component required for both infection and organogenesis is that AMSH1 targets multiple components within 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 may now be tested using biochemical approaches to compare the ubiquitination status and protein abundance of known nodule signaling compo- nents 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 the effect of ubiquitination on nodule- signaling in legumes.

EXPERIMENTAL PROCEDURES

Bacterial strains

Mesorhizobium loti strain NZP2235 was used for forward screening under greenhouse conditions, but for most other studies, M. loti strain MAFF303099 expressing DaRed was used (Maekawa et al., 2009). The M. loti strain NZP2235 expressing hema::lacZ reporter gene was used for lacZ staining (Schauer et al., 1998; Wopereis et al., 2000). Cultures with an OD600 of 0.06 were used for all 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 LORE1 collection (Fukui et al., 2012; Urbanski et al., 2012). The isolation of nfr1 1 and nfr5 2 has been described by 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 five 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: 16/8 h light/darkness at 21°C/18°C for 3 days. For nodulation tests, they were moved to plates containing quarter strength B&D medium (Broughton and Dilworth, 1971) and 1.4% Noble agar (Difco, http://www.difco.com) without nitrate, or to Magenta containers (Sigma, http://www.sigmaalrich.com) filled with lightweight expanded clay aggregate (LECA, 2 4 mm diameter, Saint Gobain Weber A/S, http://www.weber.dk) and vermiculite size M (Damolin A/S http://www.damolin.dk) in a 4:1 mixture, and 90 ml of quarter strength B&D medium without nitrate. On plates, roots were shielded from light using a metal spacer. For cytokinin experiments, quarter strength B&D medium was supplemented with BAP to a final con- centration of 10−8 M. For complementation, 3 day old seedlings were moved to half strength Gamborg’s B5 basal salt mixture (Duchefa Biochemie, https://www.duchefa.biochemie.com) supple- mented 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 plants and 15 amsh1 2 plants were assayed. For nodule counts, 20 plants from each genotype were used. For infection thread counts, ten plants from each genotype were used at 8 and 21 dpi. For Western blots, the protein extract from one seedling was used, and blotting was repeated three times. For hairy root complementation, 1000 seeds from a segregating population were used; 500 seeds were transformed with empty vector con- trol and 500 with J5S::Amsh1 CDNA. After 4 weeks, the small plants were moved to Magenta containers and inoculated. Six weeks later, plants were scored, photographs were taken and plants were genotyped.

Arbuscular mycorrhiza

One week old plants from a segregating population of amsh1 and wild type Gifu seedlings were transferred to Rhizophagus irregu- laris 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 fertil- ized with chive nutrient solution supplemented with potassium nitrate to a final concentration of 5.5 mM nitrate. Plants roots were
DNA was extracted according to the standard CTAB method (Rogers and Bendich, 1985). The genotyping PCR reactions were performed using primers designed using FSTpoolit software (http://carb.au.dk/resources/) as previously described (Urbanski et al., 2012), except that the touch down step was omitted 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 F1 and R1 primers (Table S2). The promoter region was amplified together with the 5’UTR from genomic DNA of Gifu using F2 and R2 primers. The promoter region and cDNA were combined using F3 and R3 primers. Phusion polymerase (Thermo Scientific, https://www.lifeotechnologies.com/dk/en/home/brands/thermo-scientific.html) was used to amplify all of the fragments. They were cloned into pENTR/d TOPO vector (Invitrogen, http://www.lifetechnologies.com/dk/en/home/brands/invitrogen.html) between the attL1 and attL2 sites. An LR reaction was performed according to the manufacturer’s instructions in order to transfer the constructs from pENTR/d TOPO to a pIV10 expression vector containing a Gateway cassette (Stougard et al., 1987).

Ligation independent cloning was performed according to the Novagen, http://www.novagen.com/ cloning protocol. The cDNA used for cloning was from the Amsh1 coding sequence amplified from a Lotus cDNA library (Table S2). F4 and R4 primers were used to amplify the Amsh1 cDNA (Table S2).

**Quantitative real-time PCR analysis**

Total RNA was isolated from various tissues 3 weeks after inoculation with M. loti MAFF303099 or mock inoculated using the modified lithium chloride/Trizol LS method as described by Holt et al. (2015). cDNA was synthesized using an oligo(dT) primer and the MuLV reverse transcriptase (Thermo Scientific) as previously described (Urbanski et al., 2012), except that the touch down step was omitted and a three step 30 cycle reaction was performed with a 62°C annealing temperature.

**Protein extraction, DUB assays, and Western blots**

The GST AMSH1 fusion protein was expressed from a pET41 vector in E. coli Rosetta strain (DE3) (Merck Chemicals, http://www.merckmillipore.com) at 18°C, and purified using glutathione Sepharose 4B beads (GE Healthcare, http://www3.gehealthcare.com). Protein was eluted from the beads by incubation for 30 min at 4°C with 100 mM glutathione. DUB assays were performed as described by Isono (2010). LjAMSH1 enzyme (8 pmol) in DUB buffer were incubated with 125 ng μl−1 of K48, K63 linked or linear ubiquitin chains (two to seven ubiquitins, Enzo Life Sciences, http://www.enzolifesciences.com) for 120 min at 30°C. The reaction was then stopped by adding 2.5 μl of 4x LDS (Lithium dodecyl sulfate) sample buffer (Invitrogen). The samples were run on a NuPAGE Novex Bis/Tris 4-12% gel (Invitrogen) in MES buffer according to the manufacturer’s instructions. The antibodies used were anti GST (1:2000, raised in goat, GE Healthcare, http://www3.gehealthcare.com), anti CDC2 kinase (1:5000, raised in rabbit, Santa Cruz Technology, http://www.scbt.com), anti Ub P4D1 (1:2500), raised in mouse, Cell Signaling Technology, http://www.cellsignal.com), anti mouse horseradish peroxidase conjugated antibody (1:2000, Pierce, http://www.pierce antibodies.com), anti goat alkaline phosphatase conjugated antibody (1:1000, Sigma), and anti rabbit alkaline phosphatase conjugated antibody (1:4000, Sigma). SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific) was used for horseradish peroxidase reaction. The 5 bromo 4 chloro 3’ indolylphosphate and nitro blue tetrazolium chloride for alkaline phosphatase detection were purchased from AppliChem (http://www.applichem.com/).

**Microscopic observation, staining and image processing**

Characterization of noduleation phenotypes was performed using M. loti MAFF303099 expressing DsRed. A Zeiss (http://www.zeiss.com) 780LSM confocal microscope 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 (http://imagej.nih.gov/ij/). Nodules/primordia at 8 wpi were sectioned using a Leica (http://www.leica.com) vibratome. Trans mission electron microscopy was performed on ultra thin sections (80 nm thick) of fixed and resin embedded nodules and nodule primordia as described by Madsen et al. (2010), and samples viewed and photographed using a JEOL (http://www.jeol.co.jp/en/) JEM1400 TEM.

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**AUTHOR CONTRIBUTIONS**

A.M., D.F.U. and N.S. performed the forward screening, D.F.U. and A.M. performed the cloning and the sequence-specific amplification polymorphism analysis. A.M. characterized the mutant. E.K.J. performed the light and transcription polymerase chain reaction. A.M., D.F.U. and N.S. performed the forward screening.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Alignment of Lotus and Arabidopsis AMSH homologs.

**Figure S2.** amsh1 1 and amsh1 2 are allelic.

**Figure S3.** Phenotypes of the amsh1 3 to amsh1 8 alleles.
Figure S4. Alignment of human AMSH (HsAMSH), Arabidopsis AMSH1 (AtAMSH1) and Lotus AMSH1 (LjAMSH) protein sequences.

Figure S5. Relative expression of Amsh1 in roots, nodules and leaves of inoculated and uninoculated wild type plants.

Figure S6. Comparison of shoot and root growth in the wild type and amsh1 mutants.

Figure S7. amsh1 mutants are able to establish arbuscular mycorrhiza with Rhizopus irregularis.

Figure S8. Root specific complementation restores normal infection in amsh1 1.

Figure S9. amsh1 1 and amsh1 2 mutants respond to bacteria but fail to form long infection threads

Figure S10. Application of exogenous cytokinin does not reverse the amsh1 shoot and nodulation phenotype.

Table S1. Complementation of the amsh1 1 mutant.

Table S2. Primers used in this study.

REFERENCES


