Genetic Suppressors of the *Lotus japonicus har1-1* Hypernodulation Phenotype

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Submitted 23 March 2006. Accepted 12 June 2006.

Lotus japonicus har1 mutants respond to inoculation with *Mesorhizobium loti* by forming an excessive number of nodules due to genetic lesions in the *HAR1* autoregulatory receptor kinase gene. In order to expand the repertoire of mutants available for the genetic dissection of the root nodule symbiosis (RNS), a screen for suppressors of the *L. japonicus har1-1* hypernodulation phenotype was performed. Of 150,000 M2 plants analyzed, 61 stable *L. japonicus* double-mutant lines were isolated. In the context of the *har1-1* mutation, 26 mutant lines were unable to form RNS, whereas the remaining 35 mutant lines carried more subtle symbiotic phenotypes, either forming white ineffective nodules or showing reduced nodulation capacity. When challenged with *Glomus intraradices*, 18 of the 61 suppressor lines were unable to establish a symbiosis with this arbuscular mycorrhiza fungus. Using a combined approach of genetic mapping, targeting induced local lesions in genomics, and sequencing, all non-nodulating mutant lines were characterized and shown to represent new alleles of at least nine independent symbiotic loci. The class of mutants with reduced nodulation capacity was of particular interest because some of them may specify novel plant functions that regulate nodule development in *L. japonicus*. To facilitate mapping of the latter class of mutants, an introgression line, in which the *har1-1* allele was introduced into a polymorphic background of *L. japonicus* ecotype MG20, was constructed.

Additional keywords: root architecture.

The ability to form beneficial root symbioses with nitrogen-fixing rhizobia and arbuscular mycorrhiza (AM) fungi provides plants with a significant advantage in terms of the efficacy with which they obtain growth limiting nutrients, such as nitrogen and phosphate. A reciprocal molecular dialogue precedes

the establishment of these symbioses that ultimately leads to the accommodation of a microsymbiont within the plant roots and the development of symbiosis-specific structures, nitrogen-fixing root nodules or fungal arbuscules, which serve to facilitate nutrient exchange (Oldroyd et al. 2005).

The evolution of the nitrogen-fixing symbiosis (NFS) of legumes and rhizobia has been postulated to involve, at least in part, the recruitment of pre-existing plant functions (Rodríguez-Llorente et al. 2004; Szczygłowski and Amyot 2003). Following the seminal discovery of genetic loci in pea that are required for, and shared by, NFS and the more promiscuous AM symbiosis (Duc et al. 1989), a detailed genetic dissection of similar functions in *Lotus japonicus* led to the identification of seven independent loci that specify the so-called “common symbiosis” pathway (Kistner et al. 2005). Thus far, parallel efforts in *L. japonicus*, *Medicago sativa*, *M. truncatula* and *Pisum sativum* have revealed the molecular identity of five of these genes, *SYMRK/NORK/DMI2/SYM19*, *CASTOR*, *POLLUX/DMI1*, *NUP133*, and *LjCCaMK/DMI3* (Ané et al. 2004; Endre et al. 2002; Imaizumi-Anraku et al. 2005; Kanamori et al. 2006; Lévy et al. 2004; Stracke et al. 2002; Tirichine et al. 2006a). The existence of common symbiotic genes supports the notion of recruitment and points to the ancient origin (approximately 400 million years ago based upon the predicted origin of the mycorrhizal symbiosis) (Remy et al. 1994) of at least a portion of the signaling mechanism used by extant legumes to sustain NFS.

Key perception or transduction regulatory elements that are specific to the NFS also have been characterized. The presumed Nod factor (NF) receptors, NFR1 and NFR5 from *L. japonicus* (Madsen et al. 2003; Radutoiu et al. 2003), and *M. truncatula* entry receptors LYK3 and LYK4 (Limpens et al. 2003) have been identified as elements of the root perception apparatus. While NFR1 and NFR5 are required for the earliest responses of roots to NF signaling, the function of LYK3 and LYK4 has been linked to the mechanism that regulates infection thread (IT) formation in an NF-dependent manner.

Acting downstream from the receptors and the common symbiosis genes, a putative transcription factor NIN from *L. japonicus* and its pea ortholog, SYM35 (Borisov et al. 2003; Schauser et al. 1999), as well as two *M. truncatula* GRAS-family transcriptional regulators, NSP1 and NSP2 (Kaló et al. 2005; Smit et al. 2005), have been shown to be required for IT

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formation, nodule primordium initiation, and NF-induced changes in gene expression. Rapid progress in sequencing the *L. japonicus* and *M. truncatula* genomes constitutes a major driving force behind these increasingly successful cloning experiments, based on which the molecular skeleton of the signal transduction pathway or pathways that underlie the mechanisms of microsymbiont accommodation and the organogenesis of symbiont-specific structures begins to emerge.

Superimposed on the root-localized events is the developmental root-shoot-root regulatory feedback mechanism, called autoregulation. Autoregulation controls the extent of nodulation events in the root and, thus, serves to preserve the homeostasis of the NFS. In *L. japonicus*, the Har1 receptor kinase (Krusell et al. 2002) and the *KLAVIER* locus (Oka-Kira et al. 2005) were defined as essential elements of autoregulation. Mutations in these loci impair the ability of the plant to regulate nodule development systemically, such that a significantly increased number of nodules are formed.

Collectively, approximately 400 mutant lines, representing at least 35 independent symbiotic loci, have been identified in *L. japonicus* (Sandal et al. 2006). Genes have been identified for 12 of these loci, including three genes involved in the regulation of nodule number (Krusell et al. 2002; Nishimura et al. 2002; Oka-Kira et al. 2005) and eight genes, including five loci from the “common symbiosis pathway,” that regulate early signaling events (Imaizumi-Anraku et al. 2005; Kanamori et al. 2006; Madsen et al. 2003; Radutoiu et al. 2003; Schauser et al. 1999; Stracke et al. 2002). A single *L. japonicus* locus, *Sst1*, that is crucial for proper nodule functioning also has been identified and was shown to encode a nodule-specific sulfate transporter (Krusell et al. 2005).

The mutant collections used to identify the symbiosis-related functions in *L. japonicus* have been generated via a variety of mutagenesis approaches in the wild-type ecotype Gifu (Buzas et al. 2005; Kawaguchi et al. 2002; Márquez et al. 2005; Perry et al. 2003; Schauser et al. 1998; Szczyglowski et al. 1998; Webb et al. 2000). Similarly, in other legume species, wild-type plants were used for mutagenesis (e.g.; Catoira et al. 2000; Sagan et al. 1995). We report here on the outcome of an alternative approach that has been designed to identify genetic suppressors of the mutant *L. japonicus har1-1* hypernodulation phenotype. Two main goals for this screen initially were put forward. First, because the *har1-1* mutation impairs the systemic mechanism of nodule autoregulation, a screen for suppressor mutations potentially could identify additional components of this interesting regulatory pathway. Second, given the hypermorph nature (hypernodulation) of the *har1-1* mutation, a variety of symbiotic phenotypes, including those difficult to select for in the wild-type genetic background, were predicted to be identified.

RESULTS

Screening for suppressors of the *har1-1* hypernodulation phenotype.

In the absence of rhizobia, the *L. japonicus har1-1* mutant exhibits altered root architecture, a nonsymbiotic root phenotype, which is characterized by the inhibition of root elongation and a concomitant increase in lateral root formation (bushy root phenotype) (Wopereis et al. 2000). When challenged by *Mesorhizobium loti*, *har1-1* mutant roots develop the hypernodulation phenotype, where supernumerary nitrogen-fixing nodules are formed. This exaggerated symbiotic development of *har1-1* roots constituted the basis for a screen for genetic suppressors of *har1-1* hypernodulation. Any mutation that counteracted *har1-1*-dependent hypernodulation, including those resulting in non-nodulating (Nod⁻) phenotypes, has been defined for the purpose of this work as a suppressor mutation.

Of 20,000 *har1-1/har1-1* seed treated with ethyl methane-sulfonate (EMS), approximately 10,000 fertile M1 plants were obtained. Three-week-old M1 plants, grown in the absence of rhizobia, were uprooted and the morphology of their root systems was evaluated visually to identify any modification of root architecture resulting from a dominant mutation. All M1 plants displayed the bushy root morphology of the uninoculated *har1-1* plant; therefore, they were replanted and grown to maturity to produce M2 progeny. In all, approximately 150,000 M2 plants derived from 100 independent pools (discussed below) were subjected to further phenotypic analysis by scoring their nodulation phenotypes at 21 days after inoculation (dai). The stable mutant lines at the M4/M5 generation level were analyzed further at 10, 21, and 48 dai using *Mesorhizobium loti* strain NZP2235 that carried a constitutive reporter *hemA::lacZ* cassette, which permitted histochemical detection to observe the progression of infection events. Overall, 26 suppressor lines were found to be characterized by easily identifiable mutant phenotypes, showing strong defects in the development of root nodule symbiosis (RNS), whereas the additional 35 mutant lines carried more subtle symbiotic aberrations. The molecular analysis, using a *har1-1* allele-specific cleaved amplified polymorphic sequence (CAPS) marker, confirmed that all selected suppressor lines were homozygous for the *har1-1* mutant allele (data not shown). Consequently, they were assumed to represent double mutants that carry secondary mutations that are responsible for the observed departure of their nodulation phenotype from the hypernodulation of the *har1-1* parental line. This assumption was tested and confirmed through classical genetic and molecular analyses (discussed below).

Nodulation-defective suppressor lines.

When evaluated at 21 dai, 26 *L. japonicus* suppressor lines did not form root nodules (Table 1). The *har1-1* mutant was hypernodulated at this stage, developing, on average, 41 ± 0.5 ($n = 10$) pink nodules. Further phenotypic evaluation, at 48 dai, revealed that 6 of the 26 lines that showed a Nod⁻ phenotype at 21 dai developed a few nodules on some plants (Nod^{+/+}) (Table 1), whereas the rest remained Nod⁻. These nodulation patterns clearly contrasted with the hypernodulation phenotype of the *har1-1* parental line which, at 48 dai, developed an average of 56 ± 4.1 nodules. When grown in the absence of rhizobia, all 26 mutant lines displayed a short and bushy root phenotype, characteristic of the *har1-1* parental line.

In order to further classify the above-described suppressor lines, two experimental avenues, the assessment of their capacity to form AM symbiosis and the genetic mapping of the underlying mutant loci, were followed. Of the 26 Nod⁻ mutants tested, 16 failed to establish a structurally intact symbiosis with the AM fungus, *Glomus intraradices*, as evidenced by the inability of the fungus to effectively enter or to form arbuscules and vesicles within the host roots (Table 1). In addition, line LjB13-B, and LjB74-A, which were capable of developing nodules (Table 2), were impaired in their ability to form AM symbiosis, bringing the total number of mycorrhiza-defective plants identified by the suppressor screen to 18 (Tables 1 and 2).

Mapping suppressor lines with strong defects in root symbiosis.

Given a rapidly growing number of characterized symbiotic loci in *L. japonicus* (Sandal et al. 2006), genetic mapping provides a convenient way of categorizing the newly discovered mutants as representing alleles of a previously characterized locus or as new loci. The 26 double-mutant lines (Table 1) with strong nodulation or AM phenotypes (homozygous for

both the *har1-1* allele and the presumed mutations limiting nodulation) were crossed to *L. japonicus* ecotype Miyakojima (MG20) in order to establish mapping populations and to test segregation against the predicted 9:3:3:1 ratio (wild-type/*har1-1*/single mutant/double mutant). Most symbiotic phenotypes segregated as predicted, whereas ratios for line LjB90-A deviated from the expected segregation for two independent recessive loci (Table 1).

Loci for the 26 mutant lines were positioned on the *L. japonicus* genetic map within approximately 10-centimorgan (cM) intervals bounded by microsatellite markers (Table 1). All 26 suppressor loci mapped to locations in the *L. japonicus* genome that contained previously characterized symbiotic loci. Prediction of a candidate gene or locus was made based on the best fit between the map position and the available phenotypic data (Table 1). For example, AM-defective lines were likely to represent new alleles for one of the seven *L. japonicus* common symbiosis genes, *SYM7*, *SYM8*, *SYM9*, *SYM10*, *SYM11*, *SYM12*, *SYM13*, *SYM14*, *SYM15*, *SYM16*, *SYM17*, *SYM18*, *SYM19*, *SYM20*, *SYM21*, *SYM22*, *SYM23*, *SYM24*, *SYM25*, *SYM26*, *SYM27*, *SYM28*, *SYM29*, *SYM30*, *SYM31*, *SYM32*, *SYM33*, *SYM34*, *SYM35*, *SYM36*, *SYM37*, *SYM38*, *SYM39*, *SYM40*, *SYM41*, *SYM42*, *SYM43*, *SYM44*, *SYM45*, *SYM46*, *SYM47*, *SYM48*, *SYM49*, *SYM50*, *SYM51*, *SYM52*, *SYM53*, *SYM54*, *SYM55*, *SYM56*, *SYM57*, *SYM58*, *SYM59*, *SYM60*, *SYM61*, *SYM62*, *SYM63*, *SYM64*, *SYM65*, *SYM66*, *SYM67*, *SYM68*, *SYM69*, *SYM70*, *SYM71*, *SYM72*, *SYM73*, *SYM74*, *SYM75*, *SYM76*, *SYM77*, *SYM78*, *SYM79*, *SYM80*, *SYM81*, *SYM82*, *SYM83*, *SYM84*, *SYM85*, *SYM86*, *SYM87*, *SYM88*, *SYM89*, *SYM90*, *SYM91*, *SYM92*, *SYM93*, *SYM94*, *SYM95*, *SYM96*, *SYM97*, *SYM98*, *SYM99*, *SYM100*, *SYM101*, *SYM102*, 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*SYM903*, *SYM904*, *SYM905*, *SYM906*, *SYM907*, *SYM908*, *SYM909*, *SYM910*, *SYM911*, *SYM912*, *SYM913*, *SYM914*, *SYM915*, *SYM916*, *SYM917*, *SYM918*, *SYM919*, *SYM920*, *SYM921*, *SYM922*, *SYM923*, *SYM924*, *SYM925*, *SYM926*, *SYM927*, *SYM928*, *SYM929*, *SYM930*, *SYM931*, *SYM932*, *SYM933*, *SYM934*, *SYM935*, *SYM936*, *SYM937*, *SYM938*, *SYM939*, *SYM940*, *SYM941*, *SYM942*, *SYM943*, *SYM944*, *SYM945*, *SYM946*, *SYM947*, *SYM948*, *SYM949*, *SYM950*, *SYM951*, *SYM952*, *SYM953*, *SYM954*, *SYM955*, *SYM956*, *SYM957*, *SYM958*, *SYM959*, *SYM960*, *SYM961*, *SYM962*, *SYM963*, *SYM964*, *SYM965*, *SYM966*, *SYM967*, *SYM968*, *SYM969*, *SYM970*, *SYM971*, *SYM972*, *SYM973*, *SYM974*, *SYM975*, *SYM976*, *SYM977*, *SYM978*, *SYM979*, *SYM980*, *SYM981*, *SYM982*, *SYM983*, *SYM984*, *SYM985*, *SYM986*, *SYM987*, *SYM988*, *SYM989*, *SYM990*, *SYM991*, *SYM992*, *SYM993*, *SYM994*, *SYM995*, *SYM996*, *SYM997*, *SYM998*, *SYM999*, *SYM1000*, *SYM1001*, *SYM1002*, *SYM1003*, *SYM1004*, *SYM1005*, *SYM1006*, *SYM1007*, *SYM1008*, *SYM1009*, *SYM1010*, *SYM1011*, *SYM1012*, *SYM1013*, *SYM1014*, *SYM1015*, *SYM1016*, *SYM1017*, *SYM1018*, *SYM1019*, *SYM1020*, *SYM1021*, *SYM1022*, *SYM1023*, *SYM1024*, *SYM1025*, *SYM1026*, *SYM1027*, *SYM1028*, *SYM1029*, *SYM1030*, *SYM1031*, *SYM1032*, *SYM1033*, *SYM1034*, *SYM1035*, *SYM1036*, *SYM1037*, *SYM1038*, *SYM1039*, *SYM1040*, *SYM1041*, *SYM1042*, *SYM1043*, *SYM1044*, *SYM1045*, *SYM1046*, *SYM1047*, *SYM1048*, *SYM1049*, *SYM1050*, *SYM1051*, *SYM1052*, *SYM1053*, *SYM1054*, *SYM1055*, *SYM1056*, *SYM1057*, *SYM1058*, *SYM1059*, *SYM1060*, *SYM1061*, *SYM1062*, *SYM1063*, *SYM1064*, *SYM1065*, *SYM1066*, *SYM1067*, *SYM1068*, *SYM1069*, *SYM1070*, *SYM1071*, *SYM1072*, *SYM1073*, *SYM1074*, *SYM1075*, *SYM1076*, *SYM1077*, *SYM1078*, *SYM1079*, *SYM1080*, *SYM1081*, *SYM1082*, *SYM1083*, *SYM1084*, *SYM1085*, *SYM1086*, *SYM1087*, *SYM1088*, *SYM1089*, *SYM1090*, *SYM1091*, *SYM1092*, *SYM1093*, *SYM1094*, *SYM1095*, *SYM1096*, *SYM1097*, *SYM1098*, *SYM1099*, *SYM1100*, *SYM1101*, *SYM1102*, *SYM1103*, *SYM1104*, *SYM1105*, *SYM1106*, *SYM1107*, *SYM1108*, *SYM1109*, *SYM1110*, *SYM1111*, *SYM1112*, *SYM1113*, *SYM1114*, *SYM1115*, *SYM1116*, *SYM1117*, *SYM1118*, *SYM1119*, *SYM1120*, *SYM1121*, *SYM1122*, *SYM1123*, *SYM1124*, *SYM1125*, *SYM1126*, *SYM1127*, *SYM1128*, *SYM1129*, *SYM1130*, *SYM1131*, *SYM1132*, *SYM1133*, *SYM1134*, *SYM1135*, *SYM1136*, *SYM1137*, *SYM1138*, *SYM1139*, *SYM1140*, *SYM1141*, *SYM1142*, *SYM1143*, *SYM1144*, *SYM1145*, *SYM1146*, *SYM1147*, *SYM1148*, *SYM1149*, *SYM1150*, *SYM1151*, *SYM1152*, *SYM1153*, *SYM1154*, *SYM1155*, *SYM1156*, *SYM1157*, *SYM1158*, *SYM1159*, *SYM1160*, *SYM1161*, *SYM1162*, *SYM1163*, *SYM1164*, *SYM1165*, *SYM1166*, *SYM1167*, *SYM1168*, *SYM1169*, *SYM1170*, *SYM1171*, *SYM1172*, *SYM1173*, *SYM1174*, *SYM1175*, *SYM1176*, *SYM1177*, *SYM1178*, *SYM1179*, *SYM1180*, *SYM1181*, *SYM1182*, *SYM1183*, *SYM1184*, *SYM1185*, *SYM1186*, *SYM1187*, *SYM1188*, *SYM1189*, *SYM1190*, *SYM1191*, *SYM1192*, *SYM1193*, *SYM1194*, *SYM1195*, *SYM1196*, *SYM1197*, *SYM1198*, *SYM1199*, *SYM1200*, *SYM1201*,

contents into the epidermal cell. We named this locus *Epidermal Entry Defective (EED)*, to reflect on the presumed function of the underlying gene in mediating the entry of *Mesorhizobium loti* into the *L. japonicus* root system.

Class 2: Root hair development mutants.

This class was represented by 10 independent suppressor mutant lines, reflecting four distinct *L. japonicus* loci, where defects in root hair development led to an altered nodulation pattern of the mutant roots (Table 2). A detailed phenotypic and genetic characterization of these lines was reported earlier (Karas et al. 2005).

Class 3: Nod⁺ hyperinfected mutants.

The group of five mutants belonging to this phenotypic class formed normal ITs and several pink nodules. A subset of these plants was hyperinfected, and three of these lines (S30-AA, S32-AA, and S56-HA) had a low number of often misshapen and pink nodules that were flattened in appearance (Fig. 2); no nodule primordia were evident, but the roots had a swollen ap-

pearance. The latter three mutants were shown to represent an allelic series by complementation crosses. The remaining class 3 mutant suppressor lines, S47-A and S95, had many infection threads that managed to descend and partially ramify within underdeveloped nodule structures. Interestingly, when analyzed 10 dai, line LjS95 showed only a very limited number of nodule formation events in spite of the ongoing abundant infection events at the root epidermis (data not shown). This line, however, became supernodulated at 21 dai.

Class 4: Nod⁺ mutants.

Eleven mutants of class 4 formed intact ITs and pink nodules that were fewer in number compared with the *har1-1* parental line. Mutants of this class generally were larger and healthier than Nod⁻ plants when grown in the presence of 0.5 mM KNO₃, indicating that at least some of their nodules were able to fix nitrogen. Interestingly, two of these lines, LjS51-3 and LjS12-5, in addition to the low-nodulation phenotype, showed altered root architecture. While LjS51-3 had a pleiotropic phenotype, including prolific development of very short lateral

Table 2. Nodulation phenotypes of *Lotus japonicus* suppressor lines with more subtle defects in root nodule symbiosis^a

Line name	Nodule count at 21 dai	Nodule primordia/white bumps	Comments
Gifu	9 ± 1.6	3 ± 1.5	Wild type
<i>har1-1</i>	41 ± 0.5	6 ± 1.4	Hypernodulated, parental line
Class 1 (white bumps)			
LjS28-2B ^b	3 ± 1.3	41 ± 5.2	Many microcolonies, few ITs, many disintegrated ITs
LjS57	1 ± 0.7	73 ± 11.7	Many microcolonies, few ITs, many disintegrated ITs
LjS29-1	5 ± 2.0	41 ± 6.8	Many microcolonies, few ITs, some disintegrated ITs
LjS90-D	3 ± 1.1	58 ± 12.9	Many microcolonies, few ITs, many disintegrated ITs
LjS51-1	1 ± 0.8	45 ± 8.1	Few microcolonies, few ITs, many disintegrated ITs
LjS3-BA	1 ± 0.7	51 ± 16.4	Many ITs, failure to ramify
LjB31-C	2 ± 1.3	63 ± 7.3	Many ITs, many disintegrated ITs, failure to ramify
LjB32-BB	4 ± 2.2	70 ± 11.4	ITs present, many disintegrated ITs, failure to ramify
LjS14	4 ± 1.2	37 ± 7.9	ITs present, many disintegrated ITs, failure to ramify
Class 2 (root hair development)			
LjS16-2 ^c	1 ± 0.7	37 ± 4.5	Root hair mutant, <i>Ljrh1-1</i>
LjB13-C ^c	nd	nd	Root hair mutant, <i>Ljrh1-2</i>
LjS3-1 ^c	nd	nd	Root hair mutant, <i>Ljrh1-3</i>
LjS24-B ^c	7.2 ± 1.7	19 ± 3.7	Root hair mutant, <i>Ljprh1-1</i>
LjS67-B ^c	nd	nd	Root hair mutant, <i>Ljprh1-2</i>
LjS88-5A ^c	21 ± 5.0	31 ± 5.5	Root hair mutant, <i>Ljsrh1</i>
LjB69-A ^c	14 ± 1.9	1 ± 0.5	Root hair mutant, <i>Ljvrh1-1</i>
LjB12-1B ^c	nd	nd	Root hair mutant, <i>Ljvrh1-2</i>
LjS49-AA ^c	nd	nd	Root hair mutant, <i>Ljvrh1-3</i>
LjS36-1	nd	nd	Root hair mutant, <i>Ljvrh1-4</i>
Class 3 (Nod ⁺ , hyperinfected)			
LjS32-AA ^d	5 ± 2.8	0	Hyperinfected, deformed nodules, delayed ccd
LjS30-AA ^d	nd	nd	Hyperinfected, deformed nodules, delayed ccd
LjS56-HA ^d	nd	nd	Hyperinfected, deformed nodules, delayed ccd
LjS47-A	9 ± 2.8	27 ± 5.6	Hyperinfected, delayed ccd
LjS95-E	63 ± 12.8	44 ± 10.3	Many ITs, delayed ccd
Class 4 (Nod ⁺)			
LjSup11	2 ± 1.3	11 ± 6.7	Many ITs, exaggerated root hair response
LjB74-A	3 ± 1.4	2 ± 0.7	Few ITs, AM ⁻
LjB13-B ^e	6 ± 0.7	16 ± 2.8	Exaggerated root hair response, AM ⁻ , <i>symRK-14</i>
LjS97-CA	10 ± 2.6	32 ± 4.7	
LjS7-3 ^f	8, 28	1, 3	Only two plants examined at 21 dai
LjS13-AB	22 ± 3.3	4 ± 1.9	Few ITs
LjB88-A	25 ± 5.1	6 ± 2.5	Many ITs, exaggerated root hair response
LjS8-1A	30 ± 6.1	25 ± 5.5	Many ITs
LjS24-1B ^g	nd	nd	Candidate <i>ALB1</i> allele
LjS12-5	11 ± 1.9	7 ± 2.4	Less bushy root
LjS51-3	nd	nd	Supernumerary short lateral roots, fertility problem

^a dai = Days after inoculation, IT = infection thread, nd = not determined, ccd = cortical cell divisions, and AM = arbuscular mycorrhiza.

^b Locus positioned between markers TM0095 and TM0909, chromosome 5 (*SYM7* candidate).

^c Karas and associates (2005).

^d These lines form a complementation group.

^e *SYMRK* allele.

^f Actual numbers of nodules and nodule primordia are given for *n* = 2.

^g Locus positioned between markers TM0002 and TM0036 on chromosome 1,2.

roots, the root system of LjS12-5A was more elongated and less bushy compared with the *har1-1* parental line (Fig. 3).

Notably, this class included two lines, LjB13-B and LjB74-A, that were AM⁻ (Table 2). The LjB13-B line was mapped and confirmed as an unusual Nod⁺ allele of *SYMRK* (Table 3).

Mapping suppressor lines with subtle phenotypes.

An unambiguously scoreable phenotype is essential to identify homozygous mutant individuals in a mapping population, an essential step in a map-based cloning project. All low-nodulating suppressor lines were clearly identifiable in the context of the *har1-1* mutation. However, with the exception of very few lines, such as LjS28-2B (discussed above), these phenotypes turned out to be difficult to select for in either of the wild-type backgrounds (Gifu or MG20). For example, when backcrossed to wild-type Gifu, the low-nodulation phenotype of LjS32-AA segregated, although it was difficult to distinguish between the single mutant plants and occasional less-

well-nodulated wild-type plants. As a result, it was necessary to confirm all presumed homozygous single-mutant plants by progeny testing in order to verify the initial selection. Only those plants for which the corresponding progeny showed a fully elongated root system and the low-nodulation phenotype (typically one to four nodules per plant), with the concomitant presence of enlarged nodules, were scored as single mutants (Fig. 2). Based on this rather lengthy selection scheme, the predicted 9:3:3:1 ratio (86 wild-type:27 *har1-1* phenotype:24 suppressed *har1-1* phenotype:10 double-mutant LjS32-AA, $\chi^2 = 0.68$) (Fig. 2) was recovered, supporting a recessive mode of inheritance for this mutation.

The difficulty in the unambiguous selection of single mutants was enhanced in the background of the relatively low-nodulating *L. japonicus* ecotype MG20. This feature extended the overlap between distributions of the nodule numbers for wild-type and mutant populations, further confounding the selection of the homozygous single mutants. When the LjS32-

Table 3. Suppressor lines carrying new alleles, as confirmed by sequencing, are listed together with the identified mutations^a

Line name	Mutated gene	Nucleotide change ^b	Amino-acid change	Allele designation
LjB21-1	<i>NIN</i>	C ₁₇₈₅ to T	Q ₅₁₉ to stop	<i>nin-8</i>
LjB46-C	<i>NUP133</i>	G ₂₃₅₃ to A	W ₆₅₆ to stop	<i>nup133-5</i>
LjB62-D	<i>NUP133</i>	G ₁₃₄₂ to A	W ₃₅₂ to stop	<i>nup133-6</i>
LjB80-A	<i>NUP133</i>	G ₄₄₉₆ to A	E ₁₀₂₉ to K	<i>nup133-7</i>
LjB88-B2	<i>NUP133</i>	C ₂₃₂₁ to T	Q ₆₇₉ to stop	<i>nup133-8</i>
LjSup12	<i>NUP133</i>	G ₃₅₈₇ to A	W ₈₅₈ to stop	<i>nup133-9</i>
LjB85-E	<i>SYM70/LjNSP2</i>	C ₁₃₅₇ to T	Q ₄₅₃ to stop	<i>ljnsp2-6</i>
LjB90-A	<i>SYM70/LjNSP2</i>	C ₈₆₉ to T	A ₂₉₀ to V	<i>ljnsp2-7</i>
LjB32-BA	<i>CASTOR</i>	G ₁₅₉₄ to A	E ₃₆₃ to K	<i>castor-21</i>
LjS41-1	<i>CASTOR</i>	G ₃₀₅₃ to A	W ₄₈₃ to stop	<i>castor-22</i>
LjB5-2	<i>CASTOR</i>	G ₁₆₅₅ to A	G ₃₈₃ to E	<i>castor-23</i>
LjB68-B	<i>CASTOR</i>	G ₁₀₃₅₃ to A	W ₈₄₀ to stop	<i>castor-24</i>
LjS46-1	<i>CASTOR</i>	G ₁₂₅₁ to A	W ₃₂₈ to stop	<i>castor-25</i>
LjS50-1	<i>SYMRK</i>	G ₃₇₇₇ to A	A ₆₂₀ to T	<i>symRK-13</i>
LjB13-B ^c	<i>SYMRK</i>	C ₁₉₆₅ to T	P ₃₈₆ to T	<i>symRK-14</i>
LjS4-1	<i>NFR5</i>	G ₁₄₁₅ to A	G ₄₇₂ to E	<i>nfr5-4^d</i>
LjS58-1	<i>NFR5</i>	G ₁₂₁₂ to A	W ₄₀₄ to stop	<i>nfr5-5^d</i>
LjB31-E	<i>NFR5</i>	C ₂₃₇ deleted	Frameshift	<i>nfr5-6^d</i>
LjB91-B	<i>LjCCaMK</i>	G ₃₇₁ to A	G ₁₂₄ to D	<i>ccamk-5</i>
LjS95-B	<i>LjCCaMK</i>	G ₆₁₀ to A	G ₂₀₄ to R	<i>ccamk-6</i>
LjB12-1A	<i>POLLUX</i>	G ₁₂₀₅ to A	W ₃₂₀ to stop	<i>pollux-11</i>
LjB50-C	<i>POLLUX</i>	G ₁₆₄₄ to A	Splice site	<i>pollux-12</i>
LjS49-D	<i>POLLUX</i>	G ₅₆₈₂ to A	Splice site	<i>pollux-13</i>
LjSup3	<i>POLLUX</i>	C ₄₈₀₄ to A	P ₇₁₉ to L	<i>pollux-14</i>

^a DNA sequences deposited to the GenBank under the following accession numbers were used as *Lotus japonicus* Gifu reference: AJ238956 for *NIN*, AJ890252 for *NUP133*, AB162016 for *CASTOR*, AP004579 for *SYMRK*; AJ575254 for *NFR5*, AM230792 for *LjCCaMK*, AB162017 for *POLLUX*, and AB241456/DQ665943 for *SYM70/LjNSP2*, a putative orthologue of *Medicago truncatula* *NSP2* (Allan Downie and Shinji Kawasaki, *personal communication*).

^b Adenine of the ATG initiator codon is designated as position 1.

^c This line is Nod⁺.

^d Allele previously reported by Sandal and associates (2006).

Fig. 1. Symbiotic phenotype of the LjS28-2B suppressor line. **A**, Confocal image of a curled root hair encapsulating an enlarged colony of the *lacZ:GFP*-tagged *Mesorhizobium loti* strain NZP2235 (green). **B**, Negative of light microscopy image of a curled root hair containing *lacZ:GUS*-tagged *M. loti* (blue GUS staining appears red on the image). **C**, White bumps/nodule primordia on LjS28-2B root 21 days after inoculation.

AA single mutant, which was selected from the cross to the Gifu background, was hybridized with *L. japonicus* MG20, only 1 of 10, instead of the expected 1 of 4, of the M2 plants could be selected unambiguously as homozygous LjS32-AA mutants. Using 19 homozygous individuals, the underlying locus has been positioned preliminarily to the lower half of the *L. japonicus* chromosome IV (discussed below).

Construction of the MG20_ *har1-1* introgression line.

Similar difficulties in the unambiguous selection of homozygous mutants, as described above for line LjS32-AA, have been encountered with numerous suppressor lines with more subtle symbiotic phenotypes, thus hampering the mapping effort. To overcome this obstacle and to make mapping and subsequent cloning procedures more efficient, two approaches were considered. First, mapping based on the selection of double mutants only, which could be identified clearly among segregating phenotypes after the cross with MG20, was envisaged. Following this scenario, only 1 of 16 of plants from the segregating F2 population were predicted to be informative. Alternatively, the construction of an introgression line, where the *har1-1* allele ("Gifu" background) has been moved into the polymorphic background of the ecotype MG20 (a mapping partner) via marker-assisted backcrossing, was considered. The latter scenario would allow for the efficient scoring and mapping of new mutant phenotypes in the uniform background of the *har1-1* mutation (i.e., as double mutants). Given the relatively large number of suppressor lines which remain to be analyzed, the latter has been chosen, because it offers a larger proportion of informative individuals (predicted one fourth of the segregating population), which should facilitate a robust mapping approach.

The homozygous *L. japonicus* Gifu *har1-1* line was hybridized with the ecotype MG20. After performing four consecutive backcrosses aided by marker-assisted selection, an individual introgression line (called MG20_ *har1-1*) carrying MG20 markers throughout the entire genome, except for an approximately 6-cM region on the bottom of chromosome 3 which contained Gifu DNA encompassing the *har1-1* locus, was selected (Fig. 4). The efficacy of the MG20_ *har1-1* line for mapping suppressor lines was tested by crossing it with the LjS32-AA double mutant. In the F2, a perfect 3:1 segregation ratio of *har1-1* and LjS32-AA (192:64) was obtained. The selected LjS32-AA double-mutant segregants subsequently were used to unambiguously position the LjS32-AA locus to a 0.4-cM region on the bottom of *L. japonicus* chromosome IV (Fig. 5). We named this locus *hyperinfected 1* (*HIT1*) to reflect on

the most distinctive early phenotypic feature of the inoculated LjB32-AA mutant root; namely, the formation of a large number of ITs (Table 2).

DISCUSSION

The analysis of genetic suppressors is a powerful tool for the identification of genes that may directly or indirectly interact with the original mutant locus (Kwon et al. 2004; Xiao et al. 2004; Yu et al. 2000). We mutagenized the *L. japonicus har1-1* mutant and screened for genetic suppressors of its hypernodulation and root developmental phenotype. The large number of mutants and variety of phenotypes recovered from this screening procedure indicates that many independent symbiotic loci were successfully targeted.

Two classes of mutants were expected. One class reduces nodulation by interfering directly with the ability of the plant to form nodules. The vast majority of the mutants recovered in this screen belong to this category, including mutants in the common symbiosis pathway and mutants that are impaired in root nodule development as a pleiotropic secondary effect of their defective root hair development. Another predicted class was expected to restore the wild-type nodulation behavior by directly interfering with the autoregulatory mechanism of nodulation.

Although, at the present stage of the phenotypic analysis, it is difficult to categorize all of the obtained mutants unambiguously to either of the two categories, several lines with signifi-

Fig. 3. Root nodule phenotypes of LjB51-3 and LjS12-5A double-mutant lines compared with the *har1-1* parental line, 48 days after inoculation. Note proliferating short lateral roots in line LjB51-3. Line LjS12-5A showed a less bushy root phenotype than *har1-1*, with one to eight nodules per plant. Bar scale = 2 mm.

Fig. 2. Root nodule phenotypes of *har1-1*, LjS32AA double mutant, wild-type Gifu, and a representative LjS32-AA single mutant, 21 days after inoculation. The single mutant shown was selected from the F2 progeny derived from the cross between LjS32-AA double-mutant and wild-type Gifu and reconfirmed by progeny testing. Bar scale = 2 mm.

cantly decreased nodule number were discovered by the suppressor screening. It is possible that at least some of these mutant lines correspond to elements or targets of the HAR1 receptor-kinase autoregulatory pathway. On the other hand, their low-nodulation phenotypes may be reflective of lesions in genes that regulate nodule number or nodule organogenesis via developmental processes that are independent of HAR1. In this context, it is interesting to note that the *HIT1* locus described in this work was mapped within the genetic interval in the *L. japonicus* genome that encompasses the recently discovered *Spontaneous Nodule Formation 2* (*SNF2*) locus (Tirichine et al. 2006b). The relationship, if any, among *SNF2*, *HIT1*, and the HAR1-receptor kinase-dependent autoregulatory pathway constitutes an interesting subject of the ongoing investigation.

In theory, at least two types of downstream effectors for the HAR1-dependent pathway could be envisaged. First, if HAR1 kinase-dependent signaling targets a positive regulator of root susceptibility to rhizobial signaling, a lesion in such an element would be predicted to prevent nodulation. Consequently, the non-nodulating class of mutant could include lines carrying lesions in the elements or targets of the HAR1 kinase-signaling pathway, but also would incorporate defects in any gene required for root susceptibility that may not necessarily be a direct downstream target of HAR1-mediated signaling. Therefore, it remains uncertain whether any of the elements of the early root perception or signaling apparatus discovered so far, including two positive regulators of root susceptibility, *NFR1* and *NFR5*, constitute a direct downstream target for HAR1-kinase-dependent signaling.

An alternative scenario, in which the HAR1 kinase-dependent pathway activates a negative regulator of either root susceptibility, nodule organogenesis, or both also was envisaged. Under such a formulated hypothesis, one could expect to recover a gain of function or partial gain-of-function mutation that would result in restriction of nodulation in a HAR1-independent manner. No dominant gain-of-function mutation was identified in this screen, although a number of suppressor lines remain to be analyzed.

Despite screening more than 150,000 M2 individuals, not a single line could be recovered that fully restored the wild-type nodulation and root developmental phenotype of the *har1-1* mutant. Only line LjS12-5A, which carries a presumed secondary mutation that counteracts the effect of the primary *har1-1*

mutation with respect to both the root and nodulation phenotypes, came close to the predicted, complete suppressor phenotype. However, because LjS12-5A also showed a strong defect in shoot elongation (data not shown), further detailed analysis of its complex phenotype in both the single and double mutant background will be necessary to understand the underlying nature of its suppressor effect. Aside from this line and line LjS51-3, which represents a hypermorph with respect to lateral root development, all remaining mutant lines, although affected in nodulation, showed the typical bushy root phenotype of the parental *har1-1* mutant line.

Identification of new alleles for seven known symbiotic genes should contribute to the functional characterization of their products. For example, the phenotypic variation for nodulation ability that was observed among alleles for *NUP133*, *CASTOR*, *POLLUX*, and *SYMRK* might give valuable insights into the relevance of certain amino-acid residues for nodulation (Tables 1 to 3). In particular, the contrasting nodulation phenotypes of *symRK-13* (Nod⁻) and *symRK-14* (Nod⁺) may help to elucidate the role of different SYMRK receptor kinase domains in signaling events during RNS and AM. One line,

Fig. 5. Position of *LjS32-AA* locus on *Lotus japonicus* chromosome IV. Flanking microsatellite markers are shown. The positions of known symbiosis-related loci on chromosome IV are indicated.

Fig. 4. Genetic color map of MG20_*har1-1* introgression line. The microsatellite markers used for marker-assisted selection are shown. Numbers indicate a genetic position in centimorgans along six *Lotus japonicus* chromosomes.

LjB83-B, is presumed to carry a mutation in a novel common symbiosis locus in *L. japonicus*. This notion, however, needs to be confirmed through complementation analysis and by map-based cloning of the underlying locus.

The major obstacle encountered while attempting to analyze the suppressor lines characterized by more subtle symbiotic phenotypes was the inability to reliably select for the homozygous single-mutant individuals. The successful development of the MG20_ *har1-1* introgression line is now facilitating the rapid mapping of more difficult to select phenotypes. Indeed, the *har1-1/har1-1* background may prove to be essential for mapping and characterization of certain suppressor lines due to gene interactions. For example, mutant alleles of *POLTERGEIST* act as suppressors of *clv1* phenotypes but *pol* mutants are virtually indistinguishable from wild-type plants (Yu et al. 2000). The use of the MG20_ *har1-1* introgression line should allow cloning and characterization of genes underlying more subtle mutant symbiotic phenotypes identified by the suppressor screen. This likely will clarify their role, if any, in the autoregulatory pathway that limits nodulation in *L. japonicus*.

MATERIALS AND METHODS

har1-1 Suppressor screen.

Twenty thousand homozygous *har1-1/har1-1* *L. japonicus* Gifu mutant seeds were treated with 0.4% EMS for 3 h following the previously described protocol (Szczygłowski et al. 1998). Germinated seedlings (M1 plants) were transferred to pots (approximately 30 plants per pot) and were grown for 3 weeks, after which the surviving plants (approximately 10,000) were transferred to new pots (four plants per pot, 25 pots per flat). Each flat was considered a single pool. Approximately 1,500 M2 seed per single pool (approximately 150,000 in total) were analyzed by scoring their symbiotic phenotype 3 weeks after inoculation with *Mesorhizobium loti* NZP2235. All putative mutant lines, in which the nodulation characteristics differed from the *har1-1* hypernodulation phenotype, were transferred to new pots and grown to maturity. The symbiotic and root architecture phenotypes of the lines identified in the M2 generation were reevaluated in the M3 and M4 generations using histochemical staining of a *hemA:lacZ* tagged strain of *Mesorhizobium loti*. Selected stable mutant lines from the M4 generation were used in subsequent genetic or phenotypic analyses.

Plant growth conditions.

Plants were surface sterilized and germinated as previously described (Szczygłowski et al. 1998). F2 plants were transferred to pots containing a 6:1 mixture of vermiculite/sand soaked with B&D nutrient solution (Broughton and Dilworth 1971), and containing a low concentration (0.5 mM) of KNO₃. The plants were maintained in a growth room under a regime of 16 h of day and 8 h of night, 200 to 250 $\mu\text{E s}^{-1} \text{m}^{-2}$ light intensity, at 22°C. After performing selection, the homozygous single-mutant plants were transplanted into a 4:4:1 mixture of Promix XP (Premier Horticulture, Quakertown, PA, U.S.A.), medium-size vermiculite (Therm-O-Rock East Inc. New Eagle, PA, U.S.A.), and perlite (Therm-O-Rock East Inc.) and watered with Hoagland's nutrient solution (Hoagland and Arnon 1950) or water as needed.

Mapping of mutant nodulation loci.

All lines that were confirmed as having no nodules or selected lines forming a few nodules were crossed to the wild-type Gifu and allowed to self-pollinate to generate F2 progeny. These plants were phenotyped to determine segregation ratios (Table 1). Homozygous single mutants were selected from the F2 segregating populations based on the nodulation or AM phenotype

and the absence of the *har1-1* allele. The latter was evaluated using a *har1-1*-specific CAPS marker, as described previously (Karas et al. 2005). A second cross involving *L. japonicus* ecotype Miyakojima MG20, a mapping partner, also was performed. The resulting F2 plants were evaluated 3 to 6 weeks after inoculation and scored as wild type, *har1-1*, or nodulation or AM defective (Table 1). Typically, 16 homozygous single-mutant F2 plants were selected and re-potted. One week later, leaves were harvested from each plant for DNA isolation (Karas et al. 2005). Simple sequence repeat markers were chosen from the Kasuza map based on the positions of known symbiotic loci (Sandal et al. 2006) and the AM phenotype of the line being tested. Once tentative linkage was detected, approximately 8 to 12 additional markers in the area were tested to confirm the linkage and establish the map interval. Candidate mutant loci were subjected to sequencing.

TILLING.

In parallel to the mapping approach, individual DNA preparations of each of the suppressor lines were included in an array that was subjected to TILLING of all sequence-identified genes required for nodulation (forward TILLING population) (Perry et al. 2003). In cases where putative mutations were identified through the mismatch-mediated cleavage system, the corresponding DNA fragment was amplified from the mutant to determine the precise mutation.

Sequencing.

The entire candidate locus was amplified from the corresponding suppressor line. The polymerase chain reaction products were sequenced directly on both strands. The DNA sequence of the entire locus was reconstituted using SeqMan 6.1 sequence analysis software (DNASTAR Inc., Madison, WI, U.S.A.) and compared with the corresponding wild-type sequences.

Evaluation of root-nodule phenotypes.

Plants were inoculated 2 weeks after germination (1 week after transfer to soil) with *Mesorhizobium loti* NZP2235 carrying either the *hemA:LacZ* reporter gene fusion or constitutively expressing green fluorescent protein (GFP). Roots were fixed, stained for β -galactosidase activity, and cleared as previously described (Wopereis et al. 2000). Plants were evaluated using brightfield light microscopy (Nikon SMZ1500) 10, 21, and 42 dai. Nodule counts were performed on at least 15 to 20 independent individuals for each line and were expressed as a mean nodule number per plant $\pm$ 95% confidence interval. For GFP detection, root samples were prepared and analyzed as described (Karas et al. 2005).

Culture of AM.

The AM fungus *G. intraradices* (Schenck and Smith) was cultured for at least 6 months on *Zea mays* L. planted in 1:1 sand/soil. Pot cultures were watered as needed with deionized water and given a low-phosphate nutrient solution approximately once per month. The final concentrations of the following nutrients were used: 0.4 mM KH₂PO₄, 2.5 mM Ca(NO₃)₂, 2 mM K₂SO₄, 1 mM MgSO₄, 0.2 mM FeCl₃, 50 μM KCl, 25 μM H₃BO₃, 2 μM ZnSO₄, 2 μM MnSO₄, 0.5 μM CuSO₄, and 0.5 μM Na₂MoO₄.

Evaluation of AM phenotypes.

Seven-day-old seedlings of *L. japonicus* were transplanted (one per pot) into small containers (Stuewe & Sons, Inc. Corvallis, OR, U.S.A.) filled with substrate. The substrate consisted of one part inoculum (substrate from *Z. mays* pot-culture containing desiccated colonized roots, spores, and hyphae) and

six parts autoclaved sand and Turface (mixed 1:1) watered to field capacity. Containers were covered with plastic wrap for the first 2 weeks of seedling growth, after which plants were watered sparingly with deionized water; those grown for 6 weeks were given low-phosphate nutrient solution at 3 weeks after transplanting. Containers were randomized into racks and plants were kept in a growth room at 24°C for 16-h days and 20°C for 8-h nights. During the day, seedlings received approximately 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux from both cool white fluorescent and incandescent bulbs. Eight replicates of each line for each harvest time point were prepared.

Microscopy of AM.

Upon harvest, the entire root system of each seedling was excised and placed in a separate vial containing 50% ethanol for fixation (1 to 2 h at room temperature). Roots then were transferred to 10% KOH and cleared for 40 min in a water bath at 75°C followed by a rinse in tap water and staining for 1 h at room temperature. The stain consisted of 5% Sheaffer black ink in 5% acetic acid (vol/vol) (Vierheilig et al. 1998). After staining, roots were placed in 2 ml of water with a drop or two of 5% acetic acid until mounting on slides in 50% aqueous glycerol and then examined under a cover-slip at $\times 100$ total magnification.

Between five and eight surviving replicates were evaluated per line.

ACKNOWLEDGMENTS

This work was funded by Agriculture and Agri-Food Canada Crop Genomics Initiative and National Sciences and Engineering Research Council (NSERC) of Canada grant award number 3277A01 to K. Szczyglowski, and an NSERC Discovery grant to L. Peterson. Initial stages of this work were supported by the National Science Foundation subgrant agreement number 61-2607, between Agriculture and Agri-Food Canada and Michigan State University, MI, U.S.A. The *L. japonicus* TILLING facility was supported by grants from the Biotechnology and Biological Sciences Research Council (BBSRC). We thank A. Molnar for his help in preparation of figures and L. Amyot and K. Boucher for their excellent technical help.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Kasuga DNA Research Institute *Lotus japonicus* genome sequencing project: www.kasuga.or.jp/lotus/index.html