Nicotianamine synthase specifically expressed in root nodules of *Lotus japonicus*

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Abstract In dicotyledonous plants, nicotianamine synthase (NAS) is thought to play a role in the intercellular transport of iron (Fe). Fe is an essential metal for nitrogen-fixing root nodules of legumes, prompting us to characterize the role of the NAS gene in detail. We previously compared gene-expression profiles in ineffective nodules formed on a *Lotus japonicus* Fix$^-$ mutant, *sen1*, with those in wild-type effective nodules, and showed that expression of an expressed sequence tag (EST) clone encoding an NAS (EC 2.5.1.43) homologue was repressed in the ineffective nodules. In the present study, two EST clones encoding NAS homologues were found in the EST database. We named them *LjNAS1* and *LjNAS2*. Both were detected as single-copy genes in the *L. japonicus* genome, and conferred NAS activities in transformed *Saccharomyces cerevisiae*. *LjNAS2* was expressed only in nodules, but *LjNAS1* was expressed mainly in leaves, stems, and cotyledons. The level of *LjNAS2* transcripts was highest in the nodules 24 days after inoculation with *Mesorhizobium loti*, and was localized in vascular bundles within the nodules. Expression of *LjNAS2* was suppressed in ineffective nodules formed on Fix$^-$ mutants other than *sen1*. By contrast, nitrogenase activities of nodules were not influenced in *LjNAS2*-suppressed plants. We discuss the role of LjNAS2 from the aspect of Fe translocation in nodules.

Keywords Fix$^-$ mutant · Iron · *Lotus* · Nicotianamine synthase · Nitrogen fixation

Abbreviations

Cp Crossing-point

EST Expression sequence tag

NA Nicotianamine

NAS Nicotianamine synthase

RNAi RNA interference

SAM S-adenosylmethionine

Introduction

Rhizobia fix atmospheric nitrogen in a symbiotic association with legumes: the nitrogen fixed by rhizobia supports host plant growth, while the nitrogen fixation is dependent on the host plants. Rhizobia receive flavonoid compounds secreted from compatible host plants, and in turn produce lipo-chitooligosaccharide signals, known as Nod factors, which trigger the initiation of organogenesis of a symbiosis-specific organ, known as the root nodule (Geurts and Franssen 1996; Long 2001). During nodule formation, rhizobia invade cortical cells, differentiate into bacteroids, and start nitrogen fixation. Host plants establish a microenvironment in the nodule cells in which oxygen-sensitive nitrogenase is able to work, and control the expression and synthesis of nitrogen-fixing apparatus in the rhizobia (David et al. 1988). They also provide a source of energy and reductants for rhizobial nitrogenase (Udvardi and Day 1997). Thus, a number of host plant genes are predicted to be involved in various aspects of symbiotic nitrogen fixation. However, the molecular mechanisms that regulate symbiotic nitrogen fixation are not fully understood.

To identify host plant genes that contribute to the establishment of symbiotic nitrogen fixation, we compared gene expression in the nodules of a *Lotus japonicus* Fix^- mutant, *sen1*, with that in wild-type 'Gifu' nodules using cDNA macroarray (Suganuma et al. 2004). The *sen1* mutant forms nodules endocytosed by rhizobia, but the nodules completely lack nitrogen-fixing activity (Kawaguchi et al. 2002; Suganuma et al. 2003). Genes with repressed expression in ineffective *sen1* nodules are likely to be involved in the establishment of symbiotic nitrogen fixation. Here, we focus on one such gene that encodes nicotianamine synthase (NAS).

In graminaceous plants, iron (Fe) chelators called phytosiderophores are secreted from the roots, and play an essential role in acquiring sparingly soluble Fe in the rhizosphere (Herbik et al. 1999; Higuchi et al. 1999). This mechanism of Fe acquisition is called Strategy

II (Marschner et al. 1986). NAS is a key enzyme in the synthesis of nicotianamine (NA) from S-adenosylmethionine (SAM), which is a precursor of phytosiderophores (Supplementary Fig. 1). NA is ubiquitously present not only in graminaceous plants, but also in non-graminaceous plants, which do not use Strategy II to acquire Fe or produce phytosiderophores. In such non-graminaceous plants that produce no phytosiderophores, however, NA is not secreted but rather chelates metal cations including Fe in plants, suggesting a role in the internal transport of Fe and other metals (Ling et al. 1999; Pich et al. 2001; Takahashi et al. 2003).

As Fe is an essential component of nitrogenase and leghemoglobin, mobilization of Fe in legume nodules has been studied extensively. For example, Ragland and Theil (1993) showed that the Fe storage protein ferritin accumulates in the early stages of soybean nodule development, and that the Fe in ferritin can be utilized for nitrogenase and leghemoglobin. Furthermore, the nodule-enhanced divalent metal transporter GmDMT1, which transports ferrous Fe across the peribacteroid membrane, was identified in soybean nodules (Kaiser et al. 2003). However, little is known about NA and NAS genes in legume–*Rhizobium* symbiosis. Here, we describe cDNA cloning, expression profiles, and functional analyses of *L. japonicus* NAS genes, and discuss possible functions of a nodule-specific isoform of NAS.

Materials and methods

Plant materials

Seeds of *L. japonicus* ‘Gifu B-129’ were obtained from The National BioResource Project (*L. japonicus* and *G. max*) Office, Department of Agriculture, Miyazaki University (Miyazaki, Japan). They were surface-sterilized and inoculated with *Mesorhizobium loti* MAFF 303099. The plants were grown in vermiculite watered with a nitrogen-free nutrient solution in a

controlled chamber with a 16-h day/8-h night cycle at 26 °C, as described by Imaizumi-Anraku et al. (1997). The Fix$^-$ mutants *sen1* (Kawaguchi et al. 2002; Suganuma et al. 2003), *sst1* (Kawaguchi et al. 2002; Krusell et al. 2005), and *fen1* (Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002), derived from 'Gifu', were grown similarly.

Sequence analysis

Expressed sequence tag (EST) clones MWM057e03 and GNf070f09 were obtained from the Kazusa DNA Research Institute (Chiba, Japan) (Asamizu et al. 2004; Kouchi et al. 2004). Plasmids containing each clone were isolated, and the nucleotide sequence of each clone was determined by means of an automatic ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, USA). Deduced amino-acid sequences were predicted, and similarities were analyzed by using the GENETYX program (Software Development, Tokyo, Japan).

Assay of NAS activity

The coding regions for *LjNAS1* and *LjNAS2* were amplified from the EST clones MWM057e03 and GNf070f09 by PCR using forward and reverse primers containing the *Kpn* I and *Not* I sites, respectively. The sequences of the forward and reverse primers were 5′-GGGGTACCATGGTTTGCCAAGAAGAGC-3′ and 5′-GTTGAGGAGCAACTCTCGTGAGCGGCCGC-3′ for *LjNAS1*, and 5′-GGGGTACCATGGAGAACCAGAAGGAGG-3′ and 5′-ACCATTGATGAGCATGCTTAAGCGGCCGC-3′ for *LjNAS2*, respectively. The amplified PCR fragments were ligated into the *Kpn* I and *Not* I cloning sites of the pYES2 yeast expression vector containing the *GAL1* promoter (Invitrogen, Carlsbad, CA, USA). The

resultant constructs were introduced into *Saccharomyces cerevisiae* INVSc1 (Invitrogen). Transformants were selected by uracil prototrophy. The production of recombinant proteins was induced by the addition of galactose as a carbon source according to the manufacturer's instructions (Invitrogen).

NAS activity was measured essentially as described by Suzuki et al. (1999). Transformed yeast cells were broken with glass beads in reaction buffer containing 50 mM Tris HCl (pH 8.7), 1 mM EDTA, 3 mM DTT, 10 μM ρ-amidinophenyl methanesulfonyl fluoride (APMSF), and 10 μM E-64, and the supernatant was obtained by centrifugation. Supernatant containing 50 μg protein was mixed with [^{14}C]SAM to give a final concentration of 20 μM [^{14}C]SAM. After incubation at 25 °C for 30 min, thin-layer chromatography (TLC) analysis was performed after applying the reaction mixture to a silica gel TLC plate. The plates were developed with phenol:1-butanol:formate:water (12:3:2:3, by vol.). The [^{14}C]NA synthesized by the enzymatic reaction was detected by autoradiography. The identity of this [^{14}C]NA was confirmed by comparison with chemically synthesized NA (Toronto Research Chemicals, Toronto, ON, Canada), which can be detected with ninhydrin spray.

Expression analyses

Southern-blot and northern-blot analyses were performed as described previously (Suganuma et al. 2004). Genomic DNA was isolated from leaves of 3-month-old *L. japonicus* plants, and digested to completion with *Bam* HI, *Eco* RI, and *Hind* III. Total RNA was isolated from tissues and from the roots and nodules of *L. japonicus* plants at the following stages of development: nodules, roots, and cotyledons were harvested from 3-week-old plants; stems, leaves, flowers, and pods were obtained from 3-month-old plants; and 7-day-old uninoculated roots, 10-day-old inoculated roots, and 14-day-old, 17-day-old, 21-day-old, and 24-day-old

effective nodules were also used. Digested DNA and total RNA were electrophoresed and then transferred to nylon membranes. The DNA inserts for *LjNAS1* and *LjNAS2* were isolated from their plasmids, and were labeled with [^{32}P]dCTP. After hybridization and stringent washings, the membranes were exposed to X-ray film. In-situ hybridization was carried out using the method described by Kouchi and Hata (1993). RNA probes were labeled with digoxigenin-11-UTR (Roche Diagnostics, Basel, Switzerland), and the hybridization signals were detected using anti-digoxigenin-alkaline phosphate conjugate with nitro-blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (Roche Diagnostics).

Generation of transgenic plants

An RNA interference (RNAi) plasmid was constructed as described by Shimomura et al. (2006). A cDNA fragment of *LjNAS2* was amplified by PCR from the EST clone GNf070f09 with the primers 5′-TTCCATCCTCGAGCAAGGAAGCATTCGGT-3′ (forward) and 5′-TTATCGATGGTACCAAGCCTGCTCATAGCA-3′ (reverse). The amplification products were digested with *Xho* I/*Kpn* I and *Bam* HI/*Cla* I, and ligated into the pHANNIBAL plasmid vector (Wesley et al. 2001), in which the sense and antisense *LjNAS2* RNA sequences were placed in tandem, with a pyruvate dehydrogenase kinase intron between them; this intron-spliced hairpin RNAi construct was placed downstream of the Cauliflower mosaic virus (CaMV) 35S promoter. The entire RNAi construct was excised and subcloned into the binary vector pCAMBIA1300 (CAMBIA, Canberra, ACT, Australia) by *Sac* I and *Pst* I. The construct was introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw procedure.

L. japonicus was transformed according to the method described by Stiller et al. (1997) with some minor modifications. In brief, hypocotyls excised from *L. japonicus* ‘Gifu

B-129’ seedlings were infected with *A. tumefaciens* harboring the above-mentioned binary vector construct. Generated calluses were screened for hygromycin resistance, and the regenerated plants were grown to maturity in vermiculite pots for harvesting of T_1 seeds. More than 20 independent T_1 transgenic lines were generated, and plants with sufficient fertility were selected and propagated to the T_2 generation. Among them, two lines in which the highest suppression in *LjNAS2* expression was detected were used for phenotypic analysis.

Quantitative RT-PCR

Nodules of T_2 transgenic plants were harvested 40 days after inoculation with *M. loti* MAFF 303099, and the total RNA of these nodules was isolated using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. RNA was treated with DNase (RQ1; Promega, Madison, WI, USA) and reverse-transcribed by Superscript II (Invitrogen) with oligo-(dT_{18}) as a primer. The resultant cDNA (equivalent to about 50 ng RNA) was used as the template for real-time PCR with a Lightcycler model 350S (Roche Diagnostics) according to the standard procedures described in the manual, with the *LjNAS2* primers 5′-CAGAAGGAGGTGATTGTGGG-3′ (forward) and 5′-AGGAACTTGAGTGCAGTGTG-3′ (reverse). Ubiquitin was used as an internal control with the primers 5′- TTCACCTTGTGCTCCGTCTTC-3′ (forward) and 5′-AACAACAGCACACACAGCCAATCC-3′ (reverse), as described by Flemetakis et al. (2000). The expression data were recorded as crossing-point (Cp) values. The Cp value defines the cycle number at which the fluorescence signal of the sample exceeds the background fluorescence. The Cp value was normalized to ubiquitin, and relative amounts of the *LjNAS2* transcripts were calculated using the following formulae:

$$nCp = Cp_{LjNAS2} - Cp_{LjUbi,}$$

Relative amount = $2^{-nCpLjNAS2}$.

Here, nCp is the normalized Cp value.

Phenotypic analysis

Transgenic plants were grown as described above. At 40 days after inoculation with *M. loti* MAFF 303099, the fresh weights of the plants and nodules, the numbers of nodules, and the nitrogenase activity were determined. The nitrogenase activity was assayed by the acetylene reduction assay (Suganuma et al. 2004).

Results

Primary structures of NASs in *L. japonicus*

Previously, we showed by cDNA macroarray analysis that the EST clone GNf070f09, with repressed expression in ineffective nodules induced on the Fix^- mutant *sen1*, is homologous to NASs identified from various plant species in comparison with wild-type nodules (Suganuma et al. 2004). We searched for more homologous clones to the NAS gene in the *L. japonicus* EST database (Asamizu et al. 2004; Kouchi et al. 2004), and identified MWM057e03. Predicted proteins for MWM057e03 and GNf070f09 contained 318 and 312 amino acids, respectively. The homology of their amino-acid sequences was 62.8%. We named MWM057e03 and GNf070f09 as *LjNAS1* (accession number AB480829) and *LjNAS2* (accession number AB480830), respectively. Multiple alignments of the deduced amino-acid sequences allowed us to divide the known NASs into monocotyledonous and dicotyledonous types (Fig. 1 and Supplementary Fig. 2). Both LjNAS1 and LjNAS2 were categorized as

dicotyledonous types, but LjNAS2 was found to belong to the same clade as NAS from the legume species *Medicago truncatula* (MtNAS).

Southern-blot analysis using entire cDNA sequences as probes gave a single band for *LjNAS1* and two bands for *LjNAS2* (Fig. 2). As *Bam* HI, *Eco* RI, and *Hin*d III restriction sites were present in the coding region of *LjNAS2*, these results indicate that both genes are present as a single copy in the *L. japonicus* genome.

To confirm the NAS activities of these gene products, we introduced each cDNA into *S. cerevisiae* under the control of the *GAL1* promoter. The *S. cerevisiae* NAS gene has not been identified. NA synthesis from [^{14}C]SAM was clearly detected in the cell-free extract of *S. cerevisiae* transformed with either *LjNAS1* or *LjNAS2* only when cultured with added galactose (Fig. 3). These results demonstrate that *LjNAS1* and *LjNAS2* both encode NAS.

Expression analysis of NAS genes

Northern-blot analysis revealed that *LjNAS2* was expressed only in nodules (Fig. 4). By contrast, *LjNAS1* was expressed mainly in leaves, stems, and cotyledons, and only slightly in nodules. Levels of *LjNAS2* transcripts in nodules increased strongly 24 days after inoculation with *M. loti*, but expression of *LjNAS1* remained low throughout nodule development (Fig. 4). In-situ hybridization revealed that *LjNAS2* transcripts were localized at vascular bundles in nodules (Fig. 5). In ineffective *sen1* nodules, expression of *LjNAS2* is repressed (Suganuma et al. 2004). To ascertain whether this effect is specific to the *sen1* mutant, we examined the expression of *LjNAS2* in other types of ineffective nodule, and found that it was also repressed in those formed on two other Fix$^-$ mutants, *sst1* (Kawaguchi et al. 2002; Krusell et al. 2005) and *fen1* (Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002), as well as in *sen1* (Fig. 6). By contrast, slightly more *LjNAS1* transcripts were observed in *sen1*, *sst1*, and *fen1* nodules

than in effective nodules of wild-type ‘Gifu’ plants.

Phenotypic analysis of *LjNAS2*-suppressed transgenic plants

The repression of *LjNAS2* in three types of ineffective nodule suggests that LjNAS2 plays a role in symbiotic nitrogen fixation in nodules. To elucidate this role, we generated transgenic plants in which the expression of *LjNAS2* was suppressed by RNA silencing, and analyzed their phenotypes at 40 days after *M. loti* inoculation. We expected a longer period to be required to detect phenotypic differences of *LjNAS2*-suppressed transgenic plants, as *LjNAS2* is expressed in the later stages of nodule development. Suppression of *LjNAS2* expression in two transgenic lines was confirmed by quantitative RT-PCR (Fig. 7). Expression of *LjNAS1* was not affected (data not shown). No clear differences were observed between transgenic line 1 and wild-type plants in the fresh weights of plants and nodules and in the numbers of nodules, but the values were reduced significantly in the transgenic line 2. However, the nitrogenase (acetylene reduction) activities of both lines were similar to that of the control (Fig. 7).

Discussion

Two genes encoding NASs were identified in a model legume, *L. japonicus*, and the NAS activities of both gene products were confirmed by heterologous expression in *S. cerevisiae*. *LjNAS1* was expressed in leaves, stems, and cotyledons, but the expression of *LjNAS2* was specific to nodules. These results suggest that *LjNAS1* is a housekeeping gene, and that *LjNAS2* probably arose from *LjNAS1* and functions exclusively in nodules. Phylogenic analysis placed LjNAS2 and MtNAS at a distinct distance from other NASs in dicotyledonous plants. MtNAS is likely to be an orthologue of LjNAS2, and both LjNAS2 and MtNAS are

likely to be functionally different from other NASs.

NA has been proposed to play a role in the intercellular transport of Fe and other metal ions in dicotyledonous plants (Ling et al. 1999; Pich et al. 2001; Takahashi et al. 2003), which is supported by our detection of *LjNAS2* transcripts in vascular bundles in nodules. NA produced in vascular bundles is likely to bind Fe, and the Fe–NA complex is probably then transported to nodule cells. However, the expression of *LjNAS2* was induced strongly 24 days after inoculation with *M. loti*. Under the same culture conditions, the expression of leghemoglobin genes in nodules was detected 10 days after inoculation, reaching a maximum at 14 days (Suganuma et al. 2004). The nitrogenase activity of nodules was concomitantly detected at 14 days (Suganuma et al. 2003). Therefore, in the early stages of nodule development, LjNAS2 is not involved in the acquisition of Fe for functional nitrogenase and leghemoglobin.

However, the expression of *LjNAS2* was repressed in ineffective nodules formed on three types of Fix^- mutant, *sen1*, *sst1*, and *fen1*, suggesting that LiNAS2 is involved in symbiotic nitrogen fixation. To understand the role of LjNAS2 in the establishment of symbiotic nitrogen fixation, we generated transgenic plants in which the expression of *LjNAS2*, but not of *LjNAS1*, was suppressed. In the NAS-deficient tomato mutant *chloronerva*, growth is retarded, intercostal chlorosis in young leaves is induced, and flowers are sterile (Ling et al. 1999). These abnormal phenotypes are also observed in transgenic tobacco plants that consume NA as a result of the overexpression of NA aminotransferase (Takahashi et al. 2003), and in which NAS activity is suppressed by the introduction of an antisense *Arabidopsis* NAS gene (Herbik et al. 1999). Here, we did not observe such remarkable abnormalities in plant growth in *LjNAS2*-suppressed plants. These results were expected, as LjNAS2 acts exclusively in nodules. Nevertheless, 40 days after *M. loti* inoculation, when we expected LjNAS2 to be abundantly expressed in normal nodules, nitrogenase activities were

not affected by suppression of *LjNAS2*. This implies that the Fe required for symbiotic nitrogen fixation is supplied to nodules without LjNAS2, not only in the early stages but also in the later stages of nodule development.

When Fe was artificially supplied to soybean plants, it was mostly deposited in leaves and nodules, and the levels in each organ increased during plant development (Burton et al. 1998). During the reproductive stage of soybean, approximately 50% of the Fe in leaves and nodules is translocated to seeds. Here, the expression of *LjNAS1* was strongest in leaves, and that of *LjNAS2* was strongest in nodules. These results suggest that LjNAS1 and LjNAS2 play roles in the export of Fe to other plant parts, such as seeds, from leaves and from nodules, respectively. Legumes are able to acquire Fe for development when they do not form a symbiotic association with rhizobia. In symbiotic conditions, however, they utilize a large amount of Fe on the development and functioning of nodules. When nodules start to senesce, their Fe is exported to shoots, and so LjNAS2 might play such a role. This hypothesis is supported by the observed late expression of LjNAS2. The reduced expression of *LjNAS2* in ineffective nodules might be attributable to insufficient nodule development due to the lack of nitrogen fixation. We did not observe consistent defects in the growth of plants and nodules 40 days after rhizobial inoculation in two transgenic lines in which the expression of *LjNAS2* was suppressed: longer periods might be required to observe clear defects. In addition, the distribution of Fe in each organ, especially in the seeds and nodules, remains to be determined in future experiments.

NA is also involved in the transport of other metals, such as copper (Cu), manganese (Mn), and zinc (Zn; Benes et al. 1983; Stephan and Scholz 1993; von Wirén et al. 1999). In transgenic tobacco plants in which overproduced NA aminotransferase consumes NA, concentrations of Cu, Mn, and Zn, as well as Fe, were decreased significantly in leaves and flowers (Takahashi et al. 2003). LjNAS2 might also be involved in the translocation of other

metals from nodules to shoots.

Our results show that *L. japonicus* nodules have an NAS that is expressed exclusively in the vascular bundles of nodules during the late stage of nodule development. This provides a new insight into the translocation of Fe in legumes. Further analysis of *LjNAS2*-suppressed plants will unravel the exact role of LjNAS2 in legume–*Rhizobium* symbiosis.

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Figure legends

Fig. 1 Phylogenetic relationship of deduced amino-acid sequences of LjNAS1 and LjNAS2 with other nicotianamine synthases from plants. The phylogram was created by the unweighted pair-group maximum average method using GENETYX-MAC software. Branch lengths reflect sequence diversity counted as the number of substitutions per site. At: *Arabidopsis thaliana*; Le: *Lycopersicon esculentum*; Mt: *Medicago truncatula*, Hv: *Hordeum vulgare*; Zm: *Zea mays*; Os: *Oryza sativa*. Sequence accession numbers were as follows: AtNAS1 (AB021934); AtNAS2 (AB021935); AtNAS3 (AB021936); AtNAS4 (AB181237); LeNAS (AJ242045); MtNAS (A2Q2X7); HvNAS1 (AB010086); HvNAS2 (AB011265); HvNAS3 (AB011264); HvNAS4 (AB011266); HvNAS6 (AB011269); HvNAS7 (AB019525); HvNAS8 (AF136941); HvNAS9 (AF136942); ZmNAS1 (AB061270); ZmNAS3 (AB042551); OsNAS1 (AB021746); OsNAS2 (AB023818); OsNAS3 (AB023819).

Fig. 2 Genomic Southern-blot analysis of *LjNAS1* and *LjNAS2* genes. Genomic DNA was isolated from leaves of 3-month-old *Lotus japonicus* plants. Genomic DNA (10 μg) was digested with *Bam* HI (B), *Eco* RI (E), and *Hin*d III (H), fractionated in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes.

Fig. 3 Thin-layer chromatography analysis of LjNAS1 and LjNAS2 activities. Crude extracts isolated from transformed *Saccharomyces cerevisiae* cultured without (–) or with (+) galactose were mixed with radiolabeled S-adenosylmethionine. After incubation, assay mixtures were applied to thin-layer chromatography for separation of produced nicotianamine. Radiolabeled S-adenosylmethionine and nicotianamine were detected by autoradiography.

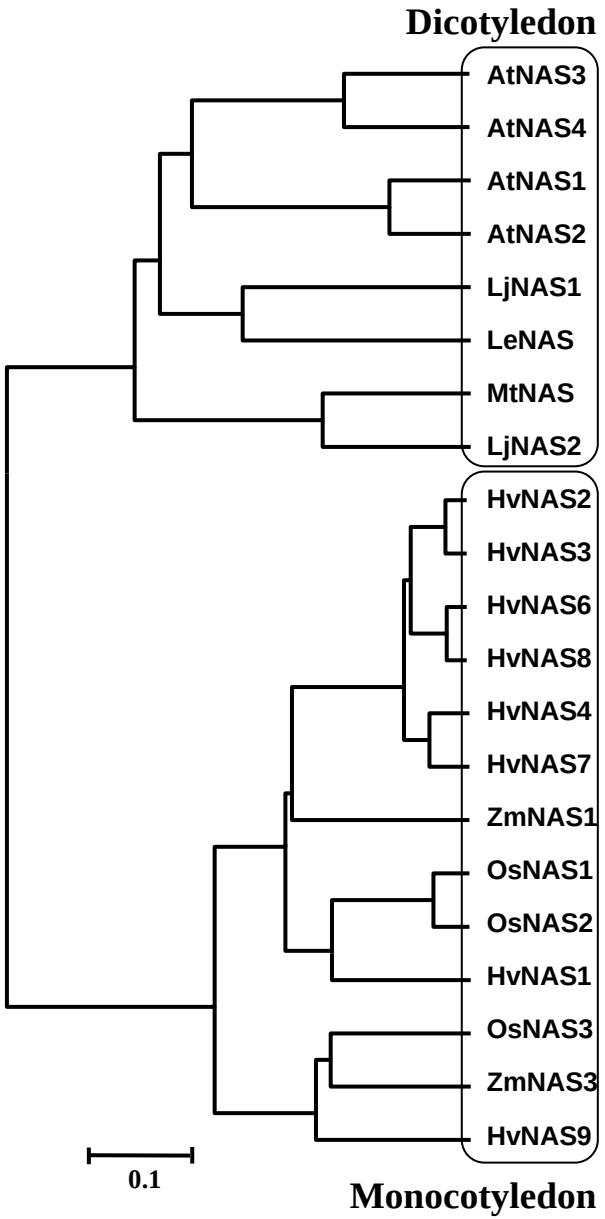
Fig. 4 Northern-blot analysis of *LjNAS1* and *LjNAS2* messenger RNAs. Total RNA was isolated from tissues (left), and from roots and nodules (right), of *Lotus japonicus* plants at the following stages of development: nodules (N), roots (R), and cotyledons (C) of 3-week-old plants; stems (S), leaves (L), flowers (F), and pods (P) of 3-month-old plants; and 7-day-old uninoculated roots (7), 10-day-old inoculated roots (10), and effective nodules (14, 17, 21, and 24 days after sowing). Each sample of total RNA (5 μg) was electrophoresed in a 1.25% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with radiolabeled probes. Ribosomal RNA (rRNA) was stained with ethidium bromide.

Fig. 5 In-situ localization of *LjNAS2* mRNA in effective nodules harvested from 4-week-old plants. Longitudinal (**a**, **c**) and traverse (**b**, **d**) sections (10 μm) through nodules were hybridized with digoxigenin-labeled antisense (**a**, **b**) or sense (**c**, **d**) probes. Hybridization signals are visible as purple or blue areas (arrow heads). Scale bar represents 100 μm; all micrographs were taken at the same magnification.

Fig. 6 Northern-blot analysis of *LjNAS1* and *LjNAS2* mRNAs from effective nodules (Gifu) and ineffective nodules induced on *sen1*, *sst1*, and *fen1* mutants harvested from 4-week-old plants. Each sample of total RNA (5 μg) was electrophoresed in a 1.25% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with radiolabeled probes. Ribosomal RNA (rRNA) was stained with ethidium bromide.

Fig. 7 Phenotypic analysis of transgenic plants in which expression of *LjNAS2* was suppressed by RNA silencing. Control plants containing empty vector (V) and two lines of *LjNAS2*-suppressed plants (1 and 2) were analyzed. Suppression of *LjNAS2* expression was confirmed by quantitative RT-PCR (**a**). At 40 days after inoculation, fresh weights of plants

(**b**), numbers of nodules (**c**), fresh weights of nodules formed per plant (**d**), and acetylene reduction activities (ARAs) (**e**) were determined. All values are the means of nine plants, and the vertical bars represent standard errors. An asterisk indicates a significant difference from the vector control according to the Student's *t*-test ($p < 0.05$).


Dicotyledon
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AtNAS4
AtNAS1
AtNAS2
LjNAS1
LeNAS
MtNAS
LjNAS2
HvNAS2
HvNAS3
HvNAS6
HvNAS8
HvNAS4
HvNAS7
ZmNAS1
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ZmNAS3
HvNAS9
0.1
Monocotyledon

Fig. 1

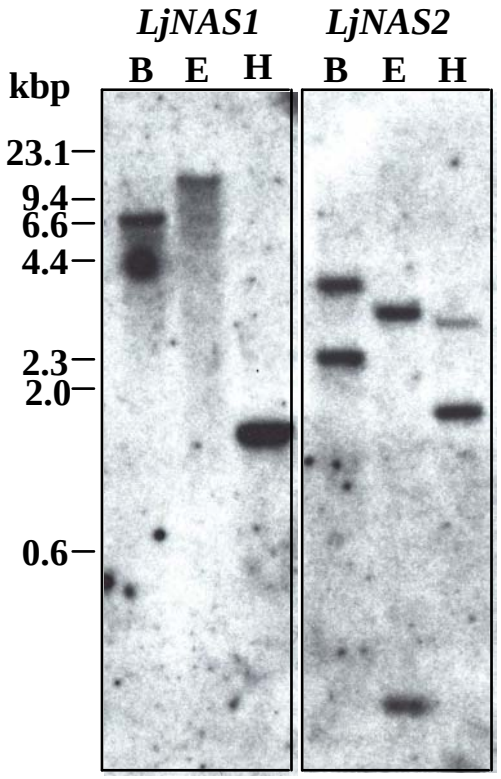


Fig. 2

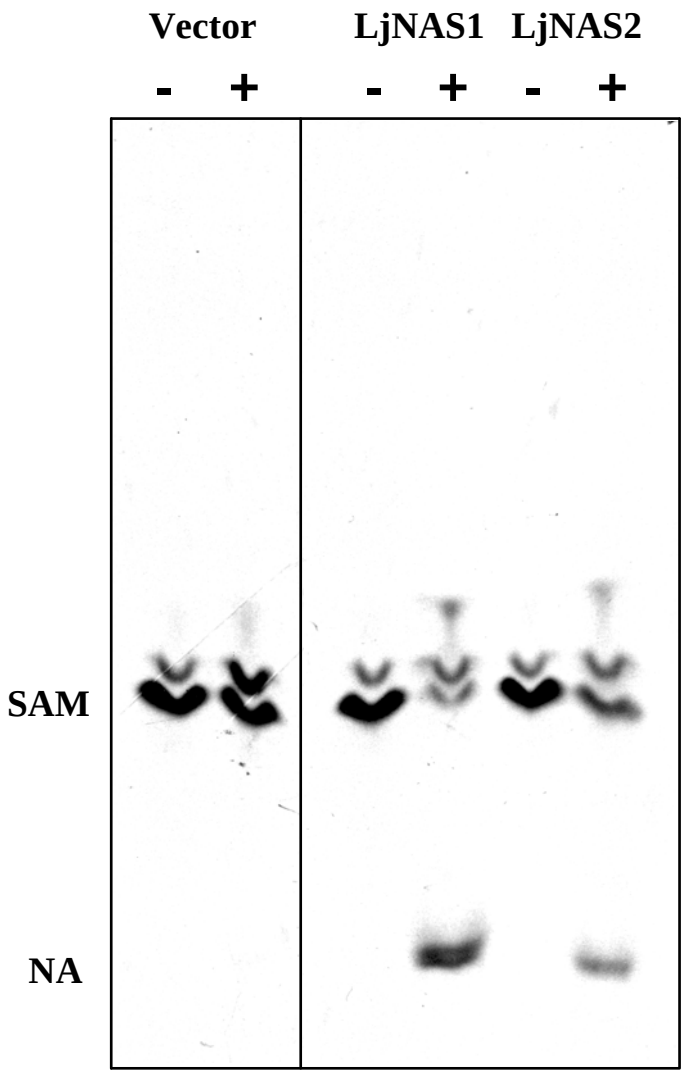


Fig. 3

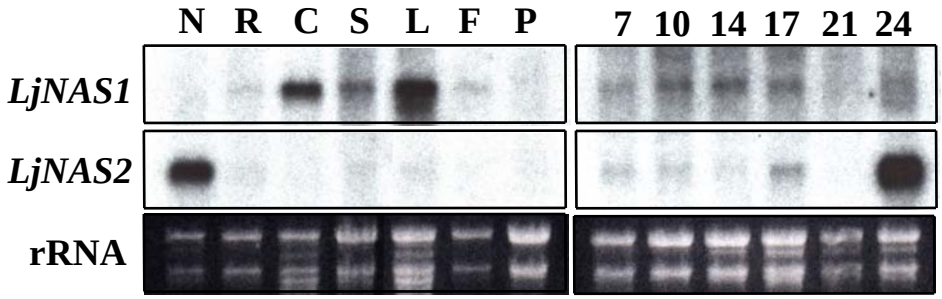


Fig. 4

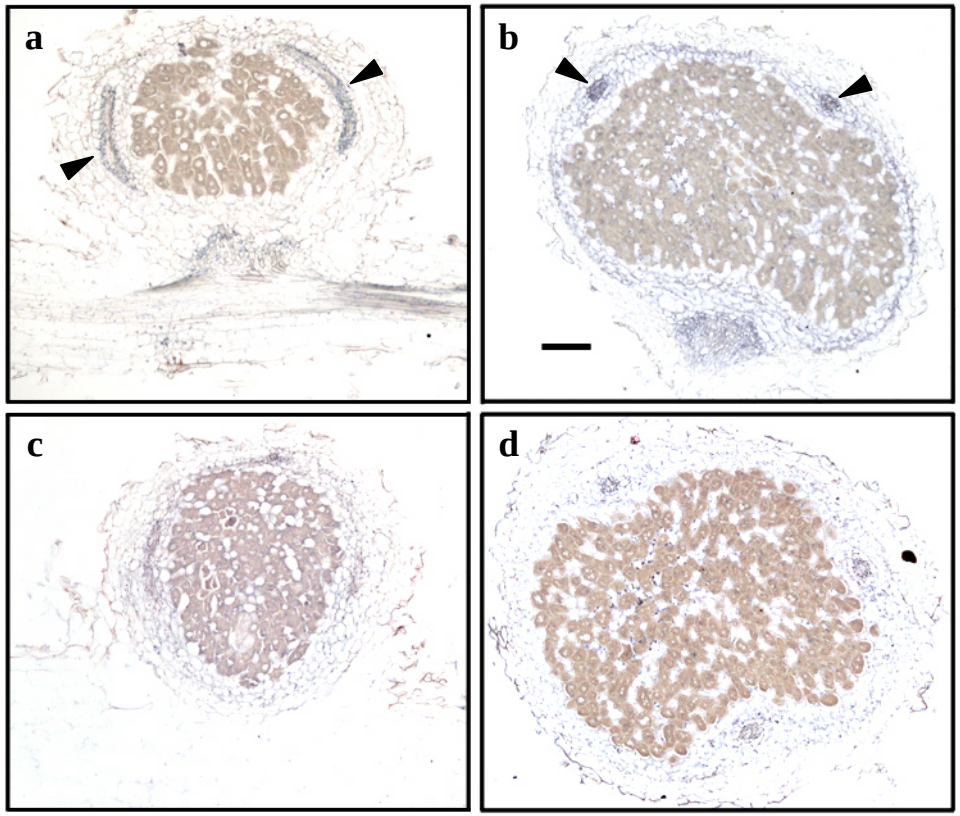


Fig. 5

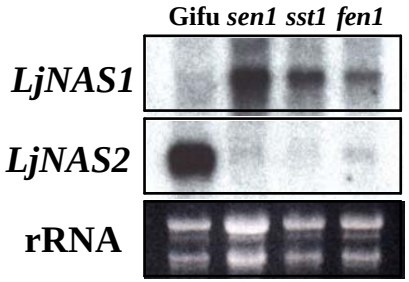
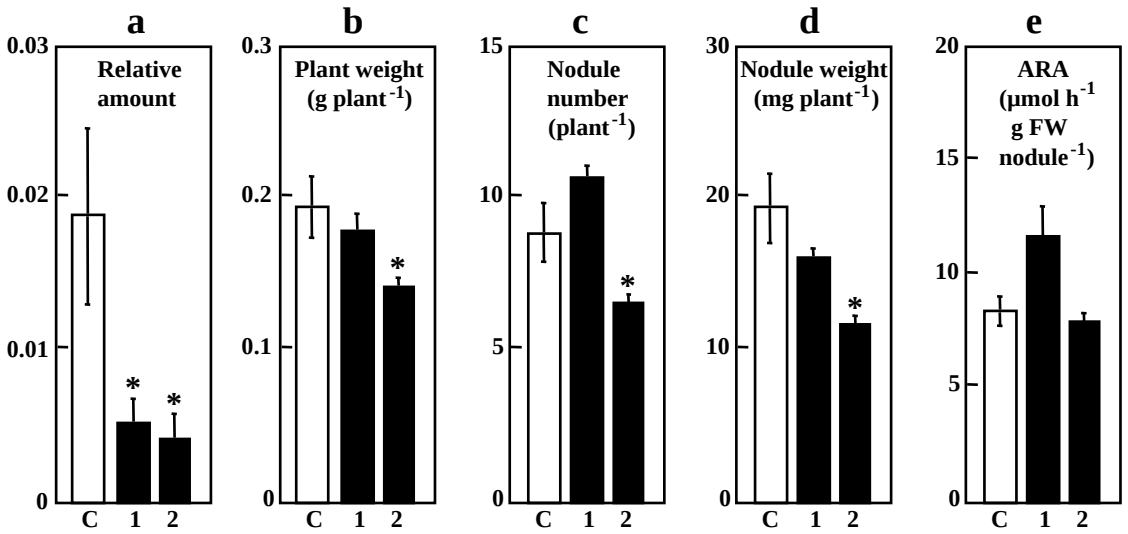


Fig. 6


a
Relative amount
0.03
0.02
0.01
0
C 1 2
b
Plant weight (g plant-1)
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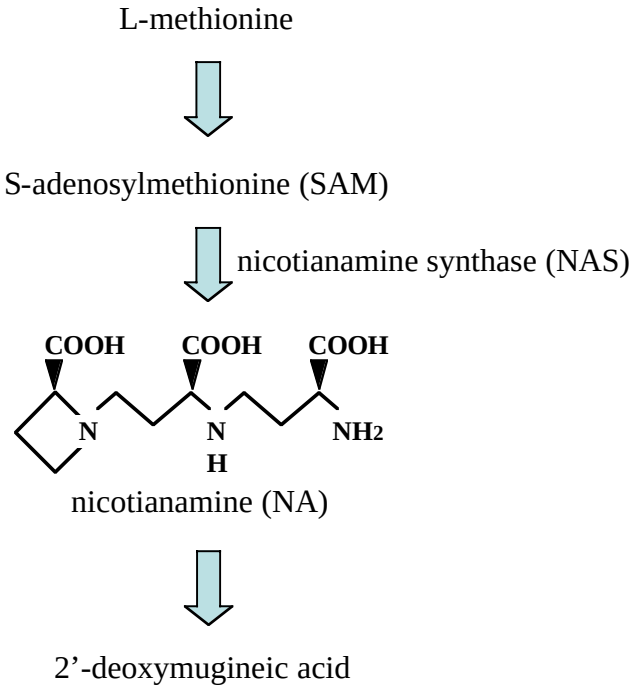
0.2
0.1
0
C 1 2
c
Nodule number (plant-1)
15
10
5
0
C 1 2
d
Nodule weight (mg plant-1)
30
20
10
0
C 1 2
e
ARA (µmol h-1 g FW nodule-1)
20
15
10
5
0
C 1 2

Fig. 7



Supplementary Fig. 1 Biosynthetic pathway of nicotianamine

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LjNAS1    MV----CQEE-LL--------IEKVCSLYSQIS-------------TLESLK----------PSK    29
LjNAS2    MEN---QKEV-IV-------GK-V-CEIYAKIS-------------KLENLN----------PSN    29
MtNAS     MDN---RQEV-II-------EK-V-CKIYDKLS-------------RLGSLN----------PPN    29
AtNAS1    MAC---QNNL-V--------VKQI-IDLYDQIS-------------KLKSLK----------PSK    29
AtNAS2    MAC---ENNL-V--------VKQI-MDLYNQIS-------------NLESLK----------PSK    29
AtNAS3    MG----CQDE-QL-------VQT-ICDLYEKIS-------------KLESLK----------PSE    29
AtNAS4    MGY---CQDD-QL-------VNK-ICDLYEKIS-------------KLETLK----------PCE    30
LeNAS     MV----CPNS-NP-------VVEKVCELYEQIS-------------RLENLS----------PSK    30
HvNAS1    -DAQN------K-E------VAA--L-IE-----------------KIAGIQAAIAELPSLSPSP    31
HvNAS2    MAAQN------N-Q------E-VDAL-VE-----------------KITGLHAAIAKLPSLSPSP    33
HvNAS3    MAAQN------N-N------KDVAAL-VE-----------------KITGLHAAIAKLPSLSPSP    34
HvNAS4    MDGQS------E-E------V--DAL-VQ-----------------KITGLHAAIAKLPSLSPSP    32
HvNAS6    MDAQN------K-E--------VDAL-VQ-----------------KITGLHAAIAKLPSLSPSP    32
HvNAS7    MDAQS------K-E------V--DAL-VQ-----------------KITGLHAAIAKLPSLSPSP    32
HvNAS8    MDAQN------K-E--------VDAL-VQ-----------------KITGLHAAIAKLPSLSPSP    32
HvNAS9    MGMEGCCSNKKVMEE-----------------E------A---LVKKITGLAAAIGELPSLSPSP    39
ZmNAS1    MEAQN------V-E--------VAAL-VQ-----------------KIAALHANITKLPSLNPSP    32
ZmNAS3    MAVMGKEEEEQQQQHKEE-EVVQGDVRVVVQQETAADEEAESALVRKISGLAAAIARLPSLSPSP    64
OsNAS1    MEAQN------Q-E------VAA--L-VE-----------------KIAGLHAAISKLPSLSPSA    32
OsNAS2    MEAQN------Q-E------VAA--L-VE-----------------KIAGLHAAISKLPSLSPSA    32
OsNAS3    MTVEVEAVTMAKEEQPEEEEVIE-----------------K-LVEKITGLAAAIGKLPSLSPSP    46

LjNAS2    NVDTLFTELVLTCMPPSP-IDVTNLTKNVQ-DIRSHLIRLCGEAEGHLESHYSTILGSHKN---P    89
LjNAS2    HVNELFTQLVTTCTTHC-ELDVTLLSQEVKETIAK-LIKLCGKAEGLLESHYSAIIGSHEN---P    89
MtNAS     QVNDLFTQLVTTCTTPCHEFDITQLSQEIKEKIAK-LITLCGKAEGLLESHYSTLIGSNEN---P    90
AtNAS1    NVDTLFGQLVSTCLPTDTNIDVTNMCEE-VKDMRANLIKLCGEAEGYLEQHFSTILGSLQEDQNP    93
AtNAS2    NVDTLFRQLVSTCLPTDTNIDVTEIHDEKVKDMRSHLIKLCGEAEGYLEQHFSAILGSFEDN--P    92
AtNAS3    DVNILFKQLVSTCIPPNPNIDVTKMCDRVQ-EIRLNLIKICGLAEGHLENHFSSILTSYQDN--P    91
AtNAS4    DVDTLFKQLVSTCIPPNPNIDVTKMSENIQ-EMRSNLIKICGEAEGYLEHHFSSILTSFEDN--P    92
LeNAS     DVNVLFTDLVHTCMPPNP-IDVSKLCQKIQ-EIRSHLIKLCGQAEGLLESHFSKILSSYEN---P    90
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HvNAS2    DVDALFTELVTACVPPSP-VDVTKLGPEAQEMREGL-IRLCSEAEGKLEAHYSDMLAAFDKP---    93
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OsNAS2    EVDALFTDLVTACVPASP-VDVAKLGPEAQAMREEL-IRLCSAAEGHLEAHYADMLAAFDNP---    92
OsNAS3    EVNALFTELVMTCIPPSS-VDVEQLGAEAQDMRGRL-IRLCADAEGHLEAHYSDVLAAHDNP---   106

LjNAS1    LDHLHIFPYYNNYLKLGLLEYTILTQNSIHV--PEKI-AFIGSGPLPLTSIVLAS-NHLISTTFH   150
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MtNAS     LNHIKIFPYYKNYLKLTHLEFTMFTKHITQV-PSKL--AFIGSGPLPLTSIILATYYLTK-TCFH   151
AtNAS1    LDHLHIFPYYSNYLKLGKLEFDLLSQHSSHV--PTKI-AFVGSGPMPLTSIVLAK-FHLPNTTFH   154
AtNAS2    LNHLHIFPYYNNYLKLGKLEFDLLSQHTTHV--PTKV-AFIGSGPMPLTSIVLAK-FHLPNTTFH   153
AtNAS3    LHHLNIFPYYNNYLKLGKLEFDLLEQNLNGF-VPKSV-AFIGSGPLPLTSIVLAS-FHLKDTIFH   153
AtNAS4    LHHLNLFPYYNNYLKLSKLEFDLLEQNLNGF-VPRTV-AFIGSGPLPLTSVVLAS-SHLKDSIFH   154
LeNAS     LQHLHIFPYFDNYIKLSLLEYNILTKNTTNI--PKKI-AFIGSGPLPLTSLVLAT-KHLKTTCFH   151
HvNAS1    LDHLGLFPYYSNYVNLSRLEYELLARHVPGIAPAR--VAFVGSGPLPFSSLVLAAH-HLPETQFD   153
HvNAS2    LDHLGMFPYYNNYINLSKLEYELLARYVPGGY-RPARVAFIGSGPLPFSSFVLAAR-HLPDTMFD   156
HvNAS3    LDHLGIFPYYSNYINLSKLEYELLARYVRR-H-RPARVAFIGSGPLPFSSFVLAAR-HLPDTMFD   156
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HvNAS6    LDHLGMFPYYSNYINLSKLEYELLARYVPGGIARPA-VAFIGSGPLPFSSYVLAAR-HLPDAMFD   155
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LjNAS2    NYDMDPSANAKAYD-LVSSDPDLSKR--MFFHTTDIAHVSNALKEYNVVFLAALVGMDKKGKESV   212
MtNAS     NFDIDSLANSKAYD-LISKDNDLSKR--MLFHTSDIVDVKNELKEFNVVFLAALVGMDKKEKAKV   213
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AtNAS2    NFDIDSHANTLASN-LVSRDSDLSKR--MIFHTTDVLNAKEGLDQYDVVFLAALVGMDKESKVKA   215
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HvNAS1    NYDLCGAANE-RARKLF-GATADGVGARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKV   216
HvNAS2    NYDLCGAAN-DRASKLFRADRDVGA--RMSFHTADVADLAGELAKYDVVFLAALVGMAAEDKAKV   218
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MtNAS     INHLAKYMAPGAILVLRSAHGAKAFLYHVVDPSCDLK-G-FEVLSIFHPTDEVINSVIVARK-GL   275
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LjNAS2    N-PGI--LLS-----SK--CHELAA---------EGFNMLNHRNVID-ELSLTIDEHA---       312
MtNAS     --------VN-----Q----Q--------------GIT-----------------------       282
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AtNAS2    S-NGARGSQI-----G-R-CVVMPCN------CSKVHAILNNRGMEKNL----IEEYSAIE       320
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LeNAS     P--SVPLLD------GLG-AYVLPSK------CACAEIH--AFNPLNKMN-L-VEEFALEE       317
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HvNAS2    VHADGLGSGR-GAGGQYARGTV-PVVSPPCRFGE-MVADVTQNHKRDE-FANA--EVA-F-       335
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HvNAS4    VHEYGLGSGR-G-GR-YARGTVVPVVSPPCRFGE-MVADVTQ--KREE-FANA--EVA-F-       329
HvNAS6    VHANERPN---GRGGQY-RGAV-PVVSPPCRFGE-MVADVTH--KREE-FTNA--EVA-F-       328
HvNAS7    MFANGPRNGC-G-GR-YARGTV-PVVSPPCRFGE-MVADVTQ--KREE-FAKA--EVA-F-       329
HvNAS8    VHANERPN---GVVDST-RGAV-PVVSPPCRFGE-MVADVTH--KREE-FTNA--EVA-F-       329
HvNAS9    PPPVAADRDVPVNMPMPAQCAVA-VS-RPCLGCACELG-ARAHQKMKE-IA-ME-EME-A-       340
ZmNAS1    AHTKGLQNGH-VHAR----GTVPI-VSPPCKCCK-MEANALQK--REE-MATTT-ELS-I-       327
ZmNAS3    --PVVDEHAVAGVG-HA-HAHGA-VLSRPCLCC--EME-ARAHQKMEE-VA-ME-QLP-S-       359
OsNAS1    AAAAAAARRDE-LADS--RGVVLPVVGPPSTCC-KVEASAVE--KAEE-FAAN--KEL-SV       332
OsNAS2    -------RRGGGLAGA--RGAV-PVVSPPCKCC-KMEAAAGAFQKAEE-FAAK--R-L-SV       325
OsNAS3    --PVAAP-ALEGGDAHA-HGHGA-VVSRPCQRC--EME-ARAHQKMED-MSAME-KLP-SS       343
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Supplementary Fig. 2 Alignment of the deduced amino-acid sequences of the LjNAS1 and LjNAS2 with other nicotianamine synthase from plants. Identical amino-acid residues are highlighted. Dashed lines indicate gaps introduced into the sequences to maximized homology. Sequence accession numbers are described in Figure 1