Original research article

Functional genetic analysis of *Arabidopsis thaliana SYNC1* in *Lotus corniculatus* super-growing roots using the FOX gene-hunting system

Tsubasa Yano[1, 4], Hidenori Tanaka[2, 4], Taiki Kurino[3], Akihiro Yamamoto[2], Hisato Kunitake[2], Yuichi Saeki[2] and Ryo Akashi[2]

[1] Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuen Kibana-dai-nishi, Miyazaki 889-2192, Japan
[2] Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[3] Graduate School of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[4] These authors contributed equally to this work.
Corresponding author: R. Akashi, E-mail: rakashi@cc.miyazaki-u.ac.jp, Phone: +81-985-58-7257, Fax: +81-985-58-7257

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Abstract: To analyze the function of *SYNC1*, an *Arabidopsis* asparaginyl-tRNA synthetase gene, the FOX-hunting system using super-growing roots (SR) from the legume species *Lotus corniculatus* was employed. One transformed line, FSL#121, was compared to parental SR and to an SR line harboring the empty vector (Control), all of which were grown *in vivo* using vermiculite pots. The level of several free amino acids was higher in FSL#121 than SR. Concomitantly, FSL#121 had a distinct phenotype of greater shoot length, stem diameter and shoot fresh weight compared with SR. Also, the root length, root diameter and fresh root weight were greater in FSL#121 than SR. Furthermore, the greater number of nodules in FSL#121 increased the nitrogen fixation activity per whole plant. Therefore, *SYNC1* overexpression caused distinct changes in plant growth, increased the number of root nodules, and may be involved in increasing the amount of free amino acids, especially asparagine.

Keywords: *Arabidopsis thaliana*, FOX-SR line, *Lotus corniculatus*, *SYNC1*

Introduction

Legumes, such as peas, beans, and peanuts, are important crops and account for more than 25% of the global primary crop production (Graham and Vance 2003) due to their high protein content. Leguminous crops can grow on poor land because their nitrogen nutrition is obtained not only from soil via water flux but also from root nodules. The search for genes that can enhance growth in legume plants, including genes affecting nodulation, is a first step in producing higher yielding legumes.

In the post-sequencing era, emphasis on functional genomics approach, such as loss-of-function knockout mutants and activation-tagged gain-of-function approach had brought about the elucidation of the functions of individual genes in model plants (Kondou et al. 2009, Bolle et al. 2011). The Full-length cDNA Over-eXpressor gene-hunting system, known as the FOX-hunting system, was developed as an alternative gain-of-function approach to analyze gene functions in plants, and was initially used as a systematic assessment for the role of individual genes in *Arabidopsis thaliana* (Ichikawa et al. 2006). By ectopically over-expressing one to two full-length c-DNAs (fl-cDNAs), large numbers of dominant mutations can be generated in model plants, as reported in *A. thaliana* and rice (Ichikawa et al. 2006; Nakamura et al. 2007). Thus, this system is an indispensable tool for the comprehensive characterization of novel and important traits and gene functions that is beneficial for basic research and the applied sciences, especially the improvement of crop production.

Lotus corniculatus is one of the most important and widely distributed leguminous plants (Díaz et al. 2005). Another advantage of using *L. corniculatus* as

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a model plant is that super-growing roots (superroots; SR) were developed more than a decade ago. SR have the capacity for a higher rate of root growth than *L. corniculatus* plant roots and can grow continuously in hormone-free liquid media as root monocultures (Akashi et al. 1998, 2003). Furthermore, transgenic SR can be obtained from leaves and hairy roots through *Agrobacterium tumefaciens*-mediated (Tanaka et al. 2008) and *Agrobacterium rhizogenes*-mediated transformation (Jian et al. 2009), respectively. Hence, SR is a unique and promising experimental system for developmental studies, such as root formation, regeneration or nodulation *in vitro* (Tanaka et al. 2008; Jian et al. 2009).

Recently, the application of the FOX-hunting system was expanded to SR of *L. corniculatus*. Himuro et al. (2011) developed SR transformation lines that used FOX hunting system (Ichikawa et al. 2006) to systematically assess the role of individual *A. thaliana* genes in *L. corniculatus*. This method introduces individual *A. thaliana* cDNAs that are under the control of the CaMV35 promoter into SR for overexpression analysis. A total of 130 *Arabidopsis* FOX-superroot lines (FOX-SR) were generated for the systematic functional analysis of genes expressed in roots and for the selection of induced mutants with interesting root growth characteristics (Himuro et al. 2011). One of the FOX-SR lines (FSLs) that harbored the aquaporin gene *AtPIP1*;*2*, had specific phenotypic changes, including decreased stem number, shoot dry weight, root mass and nodule number, and an increased ratio of shoot to root dry weight (Himuro et al. 2010). These phenotypes corresponded to those of other plants investigated for aquaporin expression (Kaldenhoff et al. 1998, Aharon et al. 2003). The root tissue of another FSL line expressing the *rolB* gene, FSL#35, had longer roots and a greater number of lateral roots than parental SR due to alterations in their cell size profiles and respiration activity (Yano et al. 2014). These phenotypic differences might be caused by the expression of the *rolB* gene that functions in ROS metabolism and programmed cell death (Bulgakov et al. 2012, Gorpenchenko et al. 2012). Furthermore, FSL#6, #55, #78, and #125, each expressing a different *A. thaliana* cDNA, had longer roots than the control (Himuro et al. 2011). Thus, FSLs are useful for investigating the phenotypes caused by exogenous gene expression.

In this study, we have utilized similar FOX-SR FSL#121 plants, derived from the previous study of Himuro et al. (2011), which is currently part of the NBRP core collection (Hashiguchi et al. 2012). FSL#121 expresses *SYNC1*, an asparaginyl-tRNA synthetase that catalyzes the synthesis of asparaginyl-tRNA during translation (Peeters et al. 2000). Our aim was to analyze the expression of *A. thaliana SYNC1* in *L. corniculatus* SR that were grown *in vivo* using vermiculite with the goal of characterizing a gene that would promote high yield in legumes. We investigated the phenotype of FSL#121, one of the lines that had longer roots than untransformed SR, (Himuro et al. 2011). In fact, regenerated plants of FSL#121 expressing the *SYNC1* did not show difference in root character in *in vitro* assay (Himuro et al. 2011). However, we observed unique differences *in vivo* using vermiculite. To acquire more information of *SYNC1* expression, we analyzed the plant phenotypes of shoot and root elongation and nodulation pattern. Furthermore, physiological parameters such as amino acid content and nitrogen fixation activity were analyzed to investigate *SYNC1* function.

Materials and Methods

Plant materials and growth conditions

Parental SR, the empty-vector control (Control) and FSL#121 harboring *SYNC1* were used in this study. SR was distributed world-wide through legumebase, the National BioResource Project (NBRP), University of Miyazaki, Japan (http://www.legumebase.brc.miyazaki-u.ac.jp). The Control and FSL#121 were constructed in our previous study (Himuro et al. 2011). The empty-vector control contains the sequence of the empty vector of the FOX-hunting system. Plant materials were maintained in plant boxes (AGC Techno Glass, Shizuoka, Japan) containing hormone-free 1/2-MS medium (Murashige and Skoog 1962) solidified with 0.7% agar in a growth chamber at 27ºC and a photoperiod of 17h of 140 μmol m^{-2} s^{-1} light.

To investigate plant growth, the top 3 cm of shoot tips from SR, the Control and FSL#121 maintained on 1/2 MS media were cut and placed on new 1/2 MS solid media to re-differentiate root tissue. After 2 weeks, regenerated plants were transferred to plastic pots containing vermiculite and were cultured 4 weeks under the same conditions. Root tips were inoculated with *Mesorhizobium loti* NZP2037 (1.0 x 10^7 cells per plant); nodule number and nitrogen fixation activity of nodules were measured after 4 weeks and compared with the non-inoculated controls.

Expression analyses

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed to confirm the expression of *SYNC1* in FSL#121. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen,

Hilden, Germany) from SR, the Control and FSL#121 leaves, stems and roots as templates for cDNA synthesis following the manufacturer's instructions. Extracted total RNA was reverse transcribed using PrimeScript RT Master Mix (Perfect Real Time) (Takara, Shiga, Japan). The synthesized cDNA and primers for *SYNC1*, AtFSL#121Fw (5′-CGTAGATAACGAACAATGGCTGATG-3′)/AtFSL#121Rv (5′- GATCTCTTCCGGATTTTACCCAAC-3′) and for *ACTIN1* as a reference gene, Lc-actin-Fw (5′-ACAATGAGTTGCGTGTTGCT-3′)/Lc-actin-Rv (5′-ACTCACACCATCACCGGAAT-3′), were used to perform RT-PCR. These primers for detecting FSL#121 expression were designed by sequence analysis of genomic DNA extracted from FSL#121, and the sequences for the reference gene primers were selected based on the results of our previous study (Himuro et al. 2011). The PCR conditions for the investigation of *SYNC1* and *ACTIN1* gene expression were as follows: 5 min at 94°C for denaturation, followed by 30 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 1 min at 72°C for elongation. PCR products were then separated by 3% agarose gel electrophoresis, and band sizes were checked by ethidium bromide staining and image analysis using a FUJI LAS-4000 CCD camera system (Fujifilm, Tokyo, Japan).

Measurement of free amino acid content

Amino acids were extracted using the method reported by Yamamoto et al. (2003). Plants of SR, the Control and FSL#121 cultured 4 weeks in a growth chamber were sampled for amino acid measurements. Fresh leaf, stem and root tissues (100 mg) were powdered in liquid nitrogen using a mortar and pestle. Then, 1 mL of 5 mM HCl was added to the powdered samples, and they were homogenized. The samples were centrifuged at 2,500 x *g* for 15 min at 4ºC to remove the supernatant liquid. Proteins were removed by a 20 min incubation on ice with 100 g/L sulfosalicylic acid, followed by centrifugation at 2,500 x *g* for 15 min at 4ºC. After pH adjustment with 2N NaOH to pH 6-8, sample solutions were used to measure the amino acid contents. The filtered sample (50 µL), 100 µL acetonitrile and 50 µL LP/LC grade water were mixed in a 1.4 mL round-bottom vial. Sealed vials were used to measure the amino acid content with a UF-Amino Station (Shimadzu, Kyoto, Japan), an LC/MS that can simultaneously measure 38 amino acids.

Morphological traits of FSL#121

We investigated several morphological traits including shoot length, stem diameter, branch number, total root length and fresh weights of shoots and roots, in 4-week-old plants. Total root length was measured by image analysis using WinRHIZO Arabidopsis software (Regent Instruments Inc., Quebec, Canada).

Measurement of nitrogen fixation activity

The root nodule number and nitrogen fixation activity were measured using SR, the Control and FSL#121 inoculated with *Mesorhizobium loti* NZP2037 for 4 weeks. Prior to counting the number of nodules, vermiculite was removed from the plants. Nitrogen fixation activity was measured by acetylene reduction activity (ARA) (Hardy et al. 1968). Approximately 3 cm of root tissues having several nodules were sampled in 7 mL vials. Immediately, 0.7 mL of air was withdrawn from the vials by syringe and replaced with acetylene gas. The vials were incubated at 26ºC for 3 hours in the dark. Ethylene production was assayed using a GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan) with a 100 cm stainless steel column packed with Porapak N (Waters Associates Inc., Massachusetts, USA).

Statistical analysis

Tukey's test was used to compare SR, the empty vector control and FSL#121. A difference of $P < 0.05$ was considered significant.

Results

Expression of SYNC1 in FSL#121

Sequence analysis verified that *SYNC1* was incorporated in the FSL#121 genome, and expression analysis of *SYNC1* was carried out. Total RNAs of SR, the Control and FSL#121 were extracted from the leaves, stems and roots of each regenerated plant. After reverse transcription of total RNA, we performed RT-PCR to investigate the expression of *SYNC1*. Amplification of the *SYNC1* fragment was detected in FSL#121 cDNA (Fig. 1); however, no such expression was detected in SR or Control cDNA. These results show that *SYNC1* was overexpressed in the leaves, stems and roots of FSL#121.

Measurement of the free amino acid content

The free amino acid content was measured in leaf, stem and root tissues to investigate the effects of *SYNC1* on amino acid production *in planta*. The asparagine contents of FSL#121 leaves and roots were higher than SR by 50% and 15%, respectively. In contrast, the amount of asparagine in stems was lower by 42% (Table 1). Also, the aspartate content of

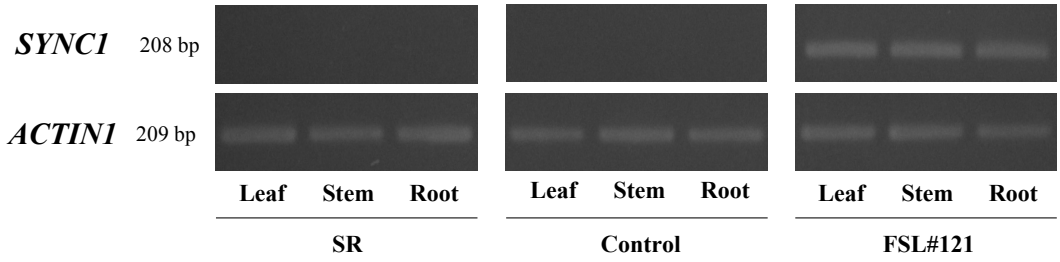

Fig. 1. Expression analysis of the *SYNC1* gene by RT-PCR in SR, the Control and FSL#121. The upper panels show the expression of *SYNC1*; the lower panels show the expression of the *ACTIN1* gene used as an internal standard.

Table 1. Amino acid content of FSL#121, SR and Control tissues

	SR	Control	FSL#121
Asn (μmol mg^{-1} FW)			
Leaf	56.1 ± 1.5a	57.3 ± 1.1a	84.3 ± 0.9b
Stem	143.5 ± 6.8a	131.4 ± 7.1a	83.0 ± 8.4b
Root	136.1 ± 0.8a	132.9 ± 5.4a	156.4 ± 1.5b
Asp (μmol mg^{-1} FW)			
Leaf	6.3 ± 0.4a	5.6 ± 0.7a	9.2 ± 0.3b
Stem	13.8 ± 1.9a	12.5 ± 1.7a	8.8 ± 1.6b
Root	14.2 ± 0.2a	14.1 ± 0.6a	16.7 ± 0.9b
Val (nmol mg^{-1} FW)			
Leaf	141.7 ± 29.6a	155.5 ± 14.9a	225.6 ± 9.9b
Stem	306.2 ± 12.9a	238.3 ± 13.8a	286.9 ± 47.1a
Root	354.4 ± 22.2a	349.5 ± 13.4a	498.6 ± 16.7b
Lys (nmol mg^{-1} FW)			
Leaf	10.3 ± 2.7a	10.5 ± 3.2a	33.6 ± 9.3b
Stem	83.3 ± 11.3a	100.7 ± 9.7a	94.6 ± 9.0a
Root	82.1 ± 27.5a	84.1 ± 29.3a	114.8 ± 17.0a
Leu (nmol mg^{-1} FW)			
Leaf	15.9 ± 11.1a	29.5 ± 12.6a	121.5 ± 37.8b
Stem	33.0 ± 8.6a	32.4 ± 7.6a	41.0 ± 10.0a
Root	55.3 ± 7.7a	63.6 ± 5.9a	98.8 ± 11.3b

Results are expressed as means ± SD from 5 replications. Different letters indicate significant differences by multiple comparison of Tukey's test ($P < 0.05$).

FSL#121 leaves and roots was greater than those of SR by 48% and 17%, respectively; although the amount of aspartate in stems was 36% lower than that in SR. In addition, the valine, lysine and leucine contents of FSL#121 leaves were significantly higher than those of SR as were the root valine and leucine contents. The abundance of other amino acids was not significantly different between SR and FSL#121. Also, we detect significant differences in the amino acid content of the empty-vector control and FSL#121 like between SR and FSL#121.

Growth of regenerated plants

To investigate the effect of *SYNC1* on plant phenotypes, we regenerated plants from SR, the Control and FSL#121. FSL#121 had distinctly different phenotypes from SR. Shoot length and stem diameter of FSL#121 plant were significantly higher than SR plants (Fig. 2AC, Fig. 3AB). Fresh shoot weight of FSL#121 was 24% higher compared with SR (Fig. 3D). In contrast, the number of FSL#121 branches was low in comparison with SR (Fig. 2AC, Fig. 3C). The total root length and root diameter of FSL#121

were higher than those of SR (Fig. 4AB). Fresh root weight of FSL#121 was 27% higher than that of SR (Fig. 4C). Additionally, there was no significant difference between SR and the Control (Figs. 2, 3 and 4). This result suggests that FSL#121 had a specific growth pattern resulting from the overexpression of *SYNC1*.

Measurement of nodule number and nitrogen fixation activity

The number of nodules per 1 cm root length of FSL#121 is approximately 2-fold higher compared with SR (Fig. 5A). To investigate nodulation in more detail, we analyzed the nitrogen fixation activity of the nodules, and calculated these values per plants. Total nodules FSL#121, that is to say nodules per FSL#121 plant had a high capacity for ethylene conversion as determined by ARA analysis comparison with SR and Control (Fig. 5B). However, ARA of FSL#121 nodules per weight was not different with SR and the Control nodules (Data not shown). These results suggested that the FSL#121 has a property of nodulation, not nodule activity.

Fig. 2. Comparison of phenotypes in SR, Control and FSL#121 cultured for 2 weeks. (A) SR, (B) Control, and (C) FSL#121. White arrows indicate branch stems. The white bar indicates 5 cm.

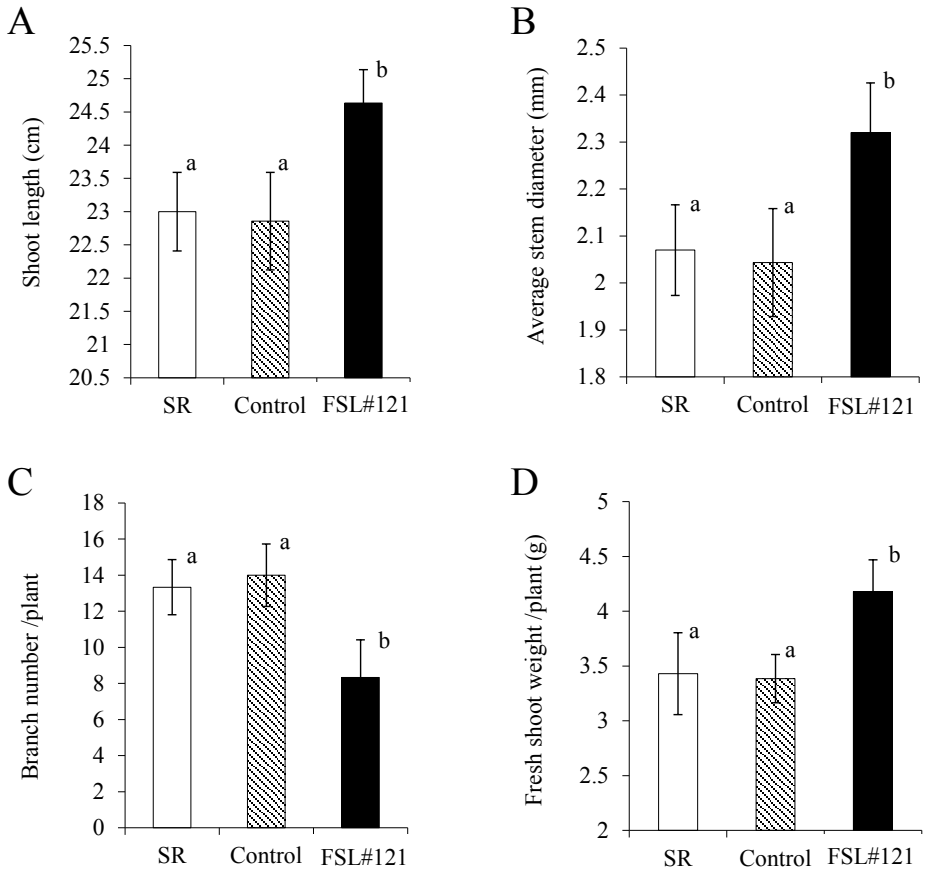

Fig. 3. Evaluation of shoot phenotypes in SR, the Control and FSL#121. (A) shoot length, (B) stem diameter, (C) branch number, and (D) shoot fresh weight. Results are expressed as the means ± SD from 5 independent experiments. The data were analyzed by multiple comparison of Tukey's test. Different letters indicate significant differences ($P < 0.05$).

Discussion

Expression of SYNC1 increased asparagine levels in FSL#121

SYNC1 encodes an asparaginyl-tRNA synthetase that catalyzes the synthesis of asparaginyl-tRNA used in translation (Peeters et al. 2000). In plants, amino-acyl-tRNA synthetase (aaRSs) genes such as *SYNC1* are encoded in the nuclear, mitochondrial and chloroplast genomes. Mitochondrial genomes lack several aaRSs. Some aaRSs that are encoded in the nucleus and translated in the cytoplasm are translo-cated to mitochondria by an N-terminal transit peptide sequence (Duchêne et al. 2005). *A. thaliana* has three *SYNC* genes called *SYNC1*, *SYNC2* and *SYNC3*. *SYNC1* operates in the cytoplasm because this gene does not have a transit sequence in the N-terminal region (Peeters et al. 2000).

To investigate the function of *SYNC1* on amino acid production, the free amino acid content was measured in plants regenerated from SR, the Control and FSL#121. Our results suggested that *SYNC1* expression induced not only increases in the asparagine and aspartate content, but also increases in the levels of other amino acid whose catalytic reactions are located downstream of asparagine and aspartate in the biosynthetic pathway. These data are similar to a previous report about the overexpression of *A. thaliana* lysyl-tRNA synthetase in carrot cells and *Zea mays* (Wu et al. 2003, 2007). Our results showed that levels of asparagine and a few other amino acids increased as a result of *SYNC1* expression, supporting the hypothesis that *SYNC1* regulates amino acid content.

Expression of SYNC1 influenced the growth of FSL#121

Expression of *SYNC1* in FSL#121 was coincident with increases in shoot and root biomass (Figs. 2 and 3). aaRSs are essential components for protein synthesis

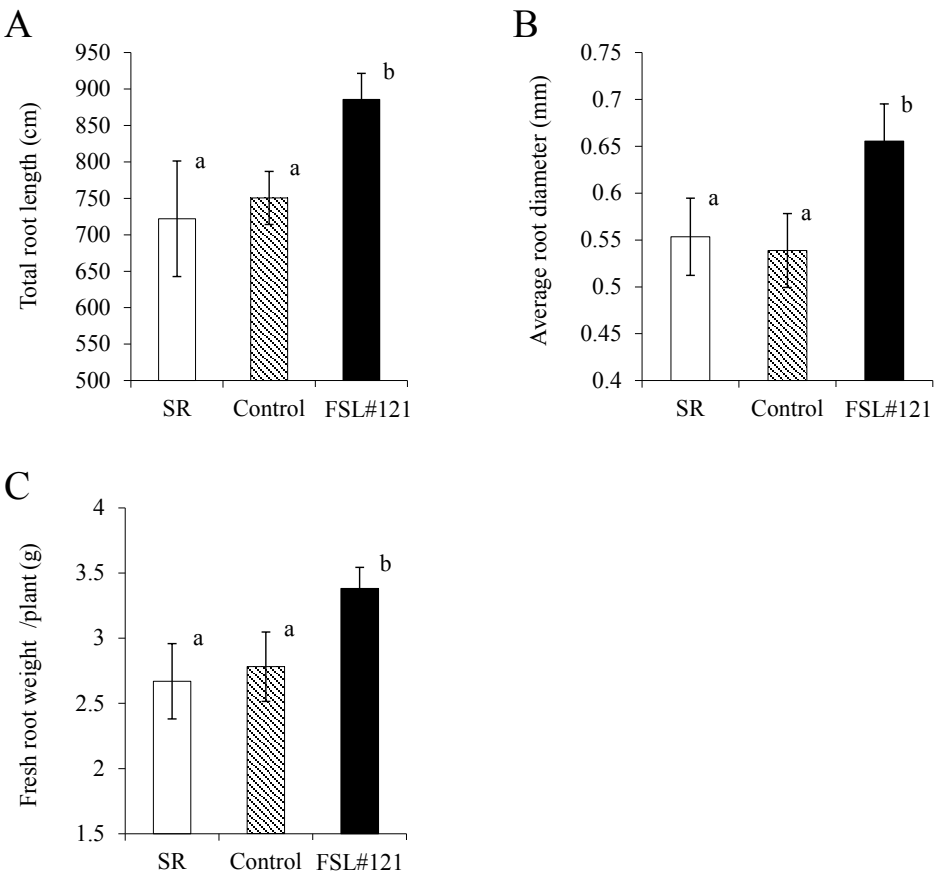

Fig. 4. Evaluation of root phenotypes in SR, the Control and FSL#121. (A) root length, (B) root diameter, and (C) fresh root weight. Results are expressed as the means ± SD from 5 independent experiments. The data were analyzed by multiple comparison of Tukey's test. Different letters indicate significant differences ($P < 0.05$).

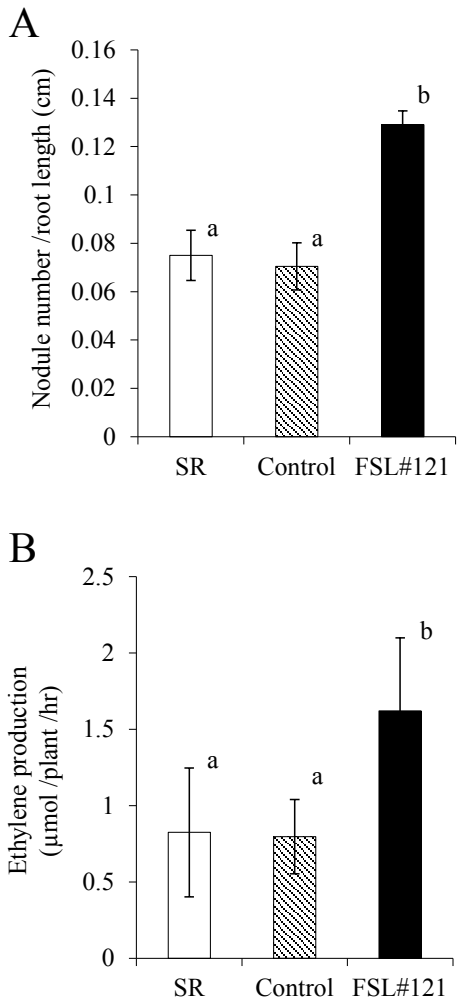

Fig. 5. Measurement of nodule number and ethylene production in SR, the Control and FSL#121 nodules. (A) nodule number per root length and (B) ethylene production per plant of SR, the Control and FSL#121 nodules. Results are expressed as the means ± SD from 5 independent experiments. The data were analyzed by multiple comparison of Tukey's test. Different letters indicate significant differences ($P < 0.05$).

in three different plant cell compartments; elimination of this basal function is lethal in early development. The disruption of aaRSs gene function in either the chloroplast or the mitochondria, consequently induced seed abortion at the transition stage of embryogenesis and caused ovule abortion before and immediately after fertilization, respectively (Berg et al. 2005). Similarly, the absence of *SYNC1* gene function can also induce defects in the embryo (Berg et al. 2005), whereas inactivation of a glycyl-tRNA synthetase in *A. thaliana* suppresses plant embryo development (Uwer et al. 1999). These studies have shown that an increased abundance of aminoacyl-tRNA and enhanced expression of aaRSs may influence plant growth and development; however, little is known about the overexpression of asparaginyl-tRNA synthetases, such as *SYNC1*, in plants.

Asparagine is a biosynthetic product derived from asparaginyl-tRNA, which is made from aspartate in the tRNA-dependent transamidation pathway (Becker and Kern 1998). In leguminous plants, such as *Lotus japonicus* and *Lupinus albus*, much of the nitrogen is transported in the xylem away from the nodule in the form of asparagine (Lea and Morot-Gaudry 2001). Asparagine in *L. albus* serves as a predominant nitrogen nutrient during seed development (Atkins *et al*. 1975). Additionally, asparagine enhances starch accumulation in the development and germination of lupin seeds (Borek *et al*. 2013). These reports suggest that increasing the level of asparagine results in higher biomass and yield in legumes. In this study, increasing the asparagine content by overexpressing the *SYNC1* gene increased the shoot and root biomass in FSL#121. This is the first report of increasing the asparagine content to improve root and shoot biomass in Lotus species. Our results may contribute to improving the yield of feed plants. Furthermore, this type of analysis might identify the function of *SYNC1* in legumes in relation to nitrogen-fixed transport by amides because *SYNC1* mig ht affect amino acid metabolism.

Expression of SYNC1 affected the nodulation pattern in FSL#121

FSL#121 showed higher nodule number not only per unit root length, but also per the whole plant than SR and Control (Fig. 3A, Fig. 4A). However, fresh nodule weight of FSL#121 was not different when compared with SR and the Control (Data not shown). Additionally, In FSL#121, nitrogen fixation activity of total nodules increased compared with SR and Control because FSL#121 could form nodules more than SR and Control (Fig. 4AB). These results suggested that *SYNC1* expression affected the nodulation pattern in FSL#121. High nitrogen fixation activity per whole plant in FSL#121 induced by increase in nodule number would relate increasing of biomass. By overexpressing an asparagine synthetase gene in alfalfa root nodules, the amount of asparagine increased (Shi et al. 1997). In *L. japonicus*, lowering the amount of glutamine synthetase resulted in higher asparagine content and higher nodule fresh and dry weights (Harrison et al. 2003). An artificial phloem feeding experiment demonstrated that asparagine has a role in N-feedback regulation, resulting in an increased concentration of asparagine in nodules and decreased nodule activity (Sulieman et al. 2010). Additionally, cycling of not only asparagine, but also several other amino acids, drives nitrogen fixation in

the legume-rhizobium symbiosis (Lodwig et al. 2003). Therefore, plant amino acids, including asparagine, affect nodulation and nodule activity. In this study, we observed an increase in the nodule number that may have been caused by the overexpression of *SYNC1* leading to changes in amino acid metabolism.

Conclusions

In this study, when an SR line (FSL#121) overexpressed *SYNC1*, an *A. thaliana* gene for asparaginyl-tRNA synthetase, the biomass of the transformant was greater than those of the Control or SR. Also, the expression of *SYNC1* affected nodulation patterns in FSL#121. That is, the nodulation activity, nodule number and the nitrogen fixation activity per plant in FSL#121 increased. Differences in the nodulation response could be induced also by altering the asparagine content. These results indicated that *SYNC1* might be a useful gene for agricultural use because overexpression of this gene promoted an increase in legume biomass. Additional investigation into the relationship between amino acid metabolism and the increase in biomass and nodulation may provide even more utility value.

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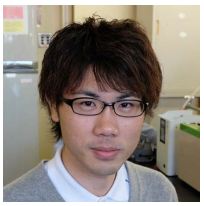
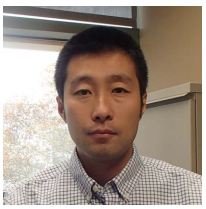


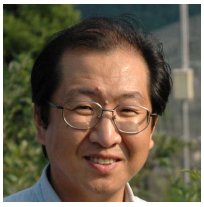
Mr. Tsubasa Yano has studied gene functions related phenotype and physiology in leguminous plant. He is interested in valuable genes enhanced growth and nodulation in leguminous plant.

Dr. Hidenori Tanaka has studied biotechnology and genetic analysis in microbes and plants. His recent research interest is genetic structure in leguminous plants.

Mr. Taiki Kurino studied biotechnology in Graduate School of Agriculture, University of Miyazaki. His current research is breeding of chestnuts in Miyazaki Agricultural Research Institute.



Dr. Akihiro Yamamoto has studied biochemical and physiological responses of higher plants to environmental stress. He is interested in bioactive nitrogen compounds, such as polyamine, under environmental stress conditions.



Dr. Hisato Kunitake has studied the genetic improvement of fruit crops by biotechnology. His recent research is focused on mechanism of self-incompatibility in Citrus species.

Dr. Yuichi Saeki is a soil microbiologist in the laboratory of soil science and plant nutrition in University of Miyazaki, Japan. His research theme is genomic ecology of soybean-nodulating rhizobia.

Dr. Ryo Akashi is a plant breeder and biotechnology in Faculty of Agriculture, University of Miyazaki, Japan. He has been studying the genetic analysis and root growth in leguminous plants.