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Exploration of Biomass-related Genes in the FOX-superroot Lines of *Lotus corniculatus* and its Impact on Agronomic Traits

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ABSTRACT

Background: Identifying biomass-related genes to meet the increasing demand for food supply is important approach for agricultural and scientific efforts. In this study, we employed the Full-length cDNA Over-eXpressing gene hunting system (FOX hunting system) to identify biomass-related genes through ectopic expression of *Arabidopsis thaliana* full-length cDNA in bird's-foot trefoil (*Lotus corniculatus*).

Methods: A total of 66 FOX superroot lines (FSLs) from *Lotus corniculatus* cv. Viking were transferred in vermiculite after two weeks in half-strength Murashige and Skoog (MS) medium. These FSLs were then grown in a growth chamber for four weeks, during which their plant length and root length were measured. High biomass lines were selected from this group. FSL #83 and #121 were then grown for an additional four months in a greenhouse until they reached maturity, at which point they were evaluated for their agronomic traits.

Result: Out of the 66 analyzed FSLs, 15 displayed superior growth characteristics in plant length and total root length. *Arabidopsis* cDNA, categorized into genes related to various functions such as membrane transport, photosystems, phytohormone synthesis, amino acid synthesis and unknown genes, was introduced into these lines. Two lines were grown to maturity in the greenhouse and their agronomic traits were analyzed in detail. FSL #83 expressed brassinosteroid biosynthetic pathway gene (*CPD*) led to significant differences in internode length, number of stems, number of flowers and above-ground dry matter weight compared to non-transgenic Super-growing roots (SR). On the other hand, FSL #121 expressed Asparaginyl-tRNA synthetase (*SYNC1*) gene exhibited increases in plant length and the number of stem nodes.

Key words: Biomass-related genes, *CPD,* FOX gene-hunting system, *Lotus corniculatus*, *SYNC1.*

INTRODUCTION

Birdsfoot trefoil (*Lotus corniculatus* L.) native to Europe is a widely used perennial forage legume crop globally (Steiner and de los Santos, 2001). It is known for its cross-pollination characteristics and tetraploid nature (2n = 4x = 24) (Grant and Small, 1996). It belongs to the same genus as the model plant *L. japonicus*, a diploid species for which a complete genome sequence is readily accessible (Li *et al*. 2020). Super-growing roots (SR) is a first-growing root culture found in *L. corniculatus* that allows continuous root cloning, direct somatic embryogenesis and mass regeneration of plants under entirely hormone-free culture conditions (Akashi *et al*., 1998, 2003). This plant material has sustained its characteristics for 25 years after isolation and also allows for protoplast culture (Akashi *et al*., 2000) and genetic transformation (Tanaka *et al*., 2008), which makes it a useful research material for the analysis of root physiology and gene function.

As the world population grows, it becomes essential to improve crop production and efficiency. To meet this demand, agriculture must increase production through conventional or genetic modification breeding technique. Genetic transformation as a powerful tool that can answer this problem offers the opportunity to improve and modify plants for useful traits. In parallel, the sequencing of entire genomes for many plant species (Kersey, 2019; Sugumar *et al*., 2024), has provided a wealth of genetic information, yet the functions of many genes remain unclear. This underscores the need to identify and understand agronomically useful genes to fully harness the potential of genetic transformation.

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Full-length cDNA Over-eXpressor gene-hunting system (FOX gene-hunting system) enables comprehensive analysis of gene function in plants by ectopically overexpressing full-length cDNA. This system has been developed in *Arabidopsis thaliana* (Ichikawa *et al*., 2006) and rice (Nakamura *et al*., 2007), which were limited to model plants. SR unique culture system was applied to develop a FOX line with *Arabidopsis* cDNA introduced into *L. corniculatus* (Himuro *et al*., 2011). Therefore, this FOX line has made it possible to explore agronomic useful genes in legume crops.

In this study, we explored biomass-related genes by evaluating 66 FSLs integrated with *Arabidopsis* cDNA. Specifically, two FSLs with distinctive characteristics were grown in the greenhouse until the flowering stage and their agronomic traits were investigated in detail. This study represents the first attempt to utilize the FOX hunting system to search for agriculturally useful genes in legume crops.

MATERIALS AND METHODS

Plant materials and culture conditions

The plant material was 66 FOX superroot lines (FSLs), which were previously generated by introducing the *Arabidopsis*-FOX *Agrobacterium* library (Ichikawa *et al*., 2006) into bird's-foot trefoil (*Lotus corniculatus*) cv. Viking (Himuro *et al*., 2011). These FSLs were maintained in half-strength Murashige and Skoog (1/2 MS) medium without hormones as described by Murashige and Skoog (1962), solidified with 0.7% (w/v) agar and grown in a growth chamber with a 16/8 photoperiod. The experiment was conducted from 2014 to 2016 at the Frontier Science Research Center, University of Miyazaki, Japan.

Measurement of root length and plant length in FOX superroot lines

Sixty-six FSLs maintained in 1/2 MS solid medium were cut at the internodes of the third to fourth expanded leaves and transferred to fresh 1/2 MS solid medium. Two weeks later, they were transplanted into 6.5 cm^2 pot filled with vermiculite. Following four weeks of cultivation in a growth chamber with a 16/8 photoperiod, the plant length and root length was measured with three replications on each line. Total root length was measured by image analysis using WinRHIZO™ *Arabidopsis* software (Regent Instruments Inc., Canada).

Confirmation of *CPD* (FSL#83) and *SYNC1* (FSL#121) genes expression by reverse Transcriptase-polymerase chain reaction (RT-PCR)

To further examine the agronomic traits of distinctive morphology FSL#83 and FSL#121, the expression of their transgenes, *CPD* and *SYNC1*, respectively was confirmed by RT-PCR. The extraction of total RNA from leaf, stem and root tissues followed the TRIzol Reagent (Invitrogen) protocol. Complementary cDNAs were synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). RT-PCR was conducted with gene-specific primers, including *CPD* primers: 5′-TCCTCCTCCTCTCTTCCATCGCCG-3′ and 5′-ACGGCGCTTCACGAAGATCGGGTA-3′ and *SYNC1* primers: 5′-CGTAGATAACGAACAATGGCTGATG-3′ and 5'- GATCTCTTCCGGATTTTACCCAAC-3′. Internal control for normalization utilized a primer pair amplifying cDNAs for *ACTIN*1: 5′-ACAATGAGTTGCGTGTTGCT-3′ and 5′-ACTCA CACCATCACCGGAAT-3′. The RT-PCR was performed by preheating 94°C for 5 min and followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min. PCR products were separated by 2.0% agarose gel electrophoresis.

Measurement of morphological traits in FSL #83 and #121

The FSL#83 and #121 were transferred to a 1/5000a pot filled with sterile soil (Miyazaki Shodo) and grown until the flowering stage in the greenhouse of University of Miyazaki, Japan for another four months. Morphological traits such as stem diameter, internode length, leaf length and width and stem, branches, node and flower number were measured in the fourth month of cultivation. Additionally, dry matter weight was measured after harvest as summarized in Table 1. The data were analyzed using one-

Table 1: Measurement of *Lotus corniculatus* agronomic traits in FOX superroot lines

Trait	Measurement unit	Note
Plant length	cm	Plant length from ground to top of plant
Stem diameter	mm	The maximum diameter of stem
Internode length	cm	The length between nodes
Leaf length	mm	The length of middle leaflet of the biggest leaf
Leaf width	mm	The width of middle leaflet of the biggest leaf
Number of stems	count	Number of stems per plant
Number of branches	count	Number of branches from a single stem
Number of stem nodes	count	Number of nodes per stem
Number of branch nodes	count	Number of nodes per branch
Number of flowers	count	Number of flowers per plant
Under-ground dry weight	g	The mass of underground dry matter
Above-ground dry weight	g	The mass of above-ground dry matter
Plant regrowth	cm	Plant length after 1 month of cutting

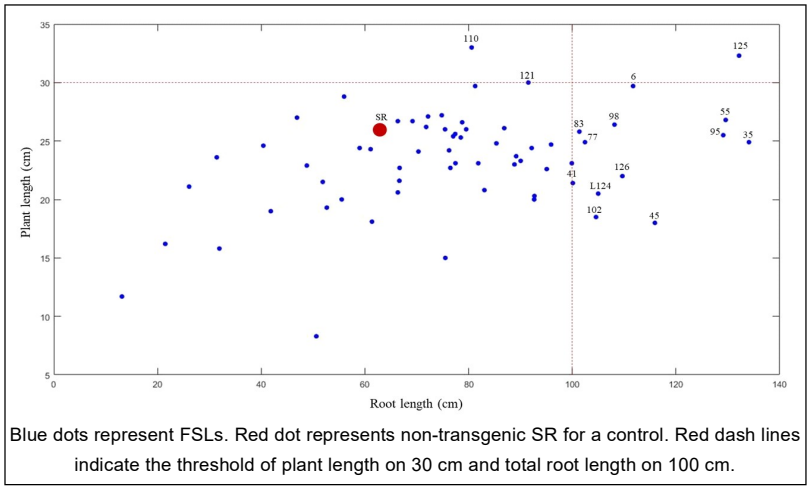
way analysis of variance (ANOVA) to determine significant differences among the three plant lines for each of the 13 measured traits with five replications. Following significant ANOVA results, Tukey's Honestly Significant Difference (HSD) post-hoc test was conducted to identify specific group differences.

RESULTS AND DISCUSSION

Phenotypic variations and selection of high biomass lines in FSLs

In this study, we analyze the functionality of *Arabidopsis* cDNA in SR using the FOX hunting system, with a focus on biomass and demonstrated its usefulness in legume crops. Sixty-six lines were used to measure plant length and total root length as biomass traits in early plant growth stage. The trait values were widely distributed around the SR and their morphology was affected by the introduced *Arabidopsis* cDNA (Fig 1). The non-transgenic SR used as a control had a plant length of 25.8 cm and a total root length of 63.1 cm. Among the tested lines, there were 18 lines displayed greater plant length and 48 lines exhibited greater total root length than SR. To identify lines with superior biomass, a threshold of 30 cm in plant length and 100 cm in total root length was set. Fifteen lines surpassed these criteria were selected and their respective transgenes were listed in Table 2.

The initial stages of plant growth are influenced by both above-ground and under-ground development, making it a crucial factor in biomass production (Berhongaray *et al*., 2013; Singh *et al*., 2016). Identifying the genetic factors that control plant growth is essential for developing crops with improved yields. Three FSLs (#110, #121 and #125) surpassed the threshold of 30 cm in plant length. Their transgenes were *Arabidopsis* Selenoprotein.Rdx type: At5g58640 (FSL#110), *SYNC1* gene: At5g56680 (FSL#121) and Serine/arginine repetitive matrix-like protein: At4g22190



Blue dots represent FSLs. Red dot represents non-transgenic SR for a control. Red dash lines indicate the threshold of plant length on 30 cm and total root length on 100 cm.

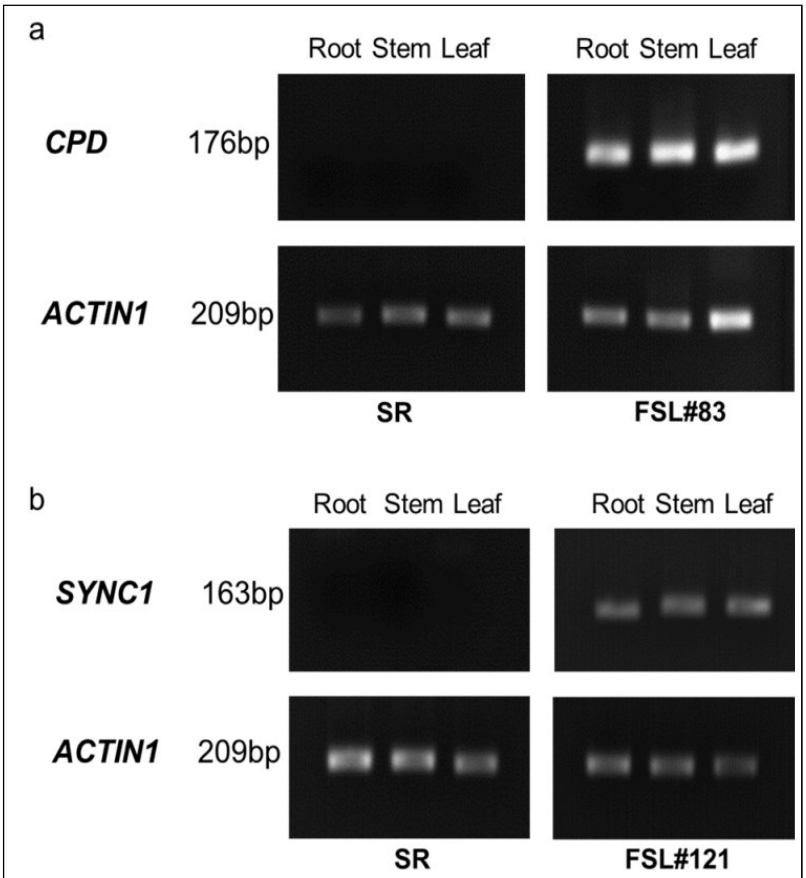
Fig 1: Phenotypic variations in plant length and root length of FOX superroot lines after 4 weeks of cultivation.

Table 2: The selected FOX superroot lines of *Lotus corniculatus* with exceeding 30 cm in plant length and 100 cm in total root length and their introduced proteins/genes (Himuro *et al*., 2011).

FOX-SR line	AGI code	Annotation in database
6	At2g33180	Unknown
35	At5g42960	Outer envelope pore 24B-like protein
41	At3g11410	Protein phosphatase 2CA
45	At2g44870	Replicase polyprotein 1ab protein
55	At5g50915	CKG, Cytokinin-Responsive growth regulator
77	At5g49440	Unknown protein
83	At5g05690	CPD, Brassinosteroid biosynthetic pathway gene
95	At5g42980	TRX3, Thioredoxin 3
98	At1g49820	MTK, S-methyl-5-thioribose kinase
102	At4g10340	LHCB5, Light harvesting complex of photosystem II 5
110	At5g58640	Selenoprotein.Rdx type
121	At5g56680	SYNC1, Asparaginyl-tRNA synthetase gene
124	At1g18210	Calcium-binding EF-hand family protein
125	At4g22190	Serine/arginine repetitive matrix-like protein
126	At1g32360	Hakai-Interacting zinc finger protein 1

(FSL#125). Selenoprotein Rdx belongs to the thioredoxin-like family and plays a critical role in various aspects of plant biology, such as photosynthesis, metabolism, growth, development and responses to environmental stresses (Dikiy *et al*., 2007; Chauhan *et al*., 2017). The introduction of serine/arginine proteins in FSL#125 not only contribute to increased plant length but also to longer root development. The *SYNC1* gene is responsible for encoding an asparaginyl-tRNA synthetase, which catalyzes the synthesis of key amino acids in seeds, influencing plant morphology and resulting in a significant increase in plant length, number of branches and number of branch nodes, all of which are yield components of soybean (Arifin *et al*., 2019). Serine/arginine proteins are vital splicing factors that play crucial roles in spliceosome assembly and splicing regulation. Loss of function of these proteins can lead to various changes in plant morphology and development, including serrated leaves, late flowering, shorter roots and abnormal silique phyllotaxy (Yan *et al*., 2017).

Over half of the lines displayed total root lengths higher than SR, with 13 FSLs surpassing the 100 cm threshold (Fig 1). These 13 FSLs with superior root growth (#6, #35, #41, #45, #55, #77, #83, #95, #98, #102, #124, #125 and #126) were each introduced with specific *Arabidopsis* cDNA with various plant functions, such as membrane transport (#35), growth and development (#41, #55), viral replication (#45), hormone biosynthesis (#83), redox regulation (#95), metabolic pathways (#98), photosynthesis (#102), calcium signaling (#124) and Zinc Finger Protein associated with transcription factors (#126). For instance, FSL#35 exhibited the highest total root length of 134 cm and expressed the outer envelope pore 24B-like protein: At5g42960, essential for transporting nutrients like sugars, ATP and amino acids crucial for plant growth (Zybailov *et al*., 2008). Similarly, the expression of Cytokinin-Responsive Growth Regulator influenced root development in FSL#55, as cytokinins regulate processes such as cell division and root regeneration (Higuchi *et al*., 2004; Marhavı *et al*., 2011). Moreover, the extensive root length in FSL#95 may be



(A) The expression of *CPD* gene in SR and FSL#83. The upper panels show the expression of *CPD*; the lower panels show the expression of *ACTIN1* for an internal control. (B) The expression of *SYNC1* gene in SR and FSL#121. The upper panels show the expression of *SYNC1*; the lower panels show the expression of *ACTIN1*, for an internal control.

Fig 2: Transgene expression of *SYNC1* and *CPD* gene by RT-PCR.

attributed to redox regulation induced by Thioredoxin 3: At5g42980, which participating in thiol-disulfide exchange reactions and contributing to the maintenance of the redox homeostasis necessary for various cellular processes in plants (Gelhaye *et al*., 2005). Understanding the role of these genes related to biomas production in *L. corniculatus* can be useful for improving pasture productivity. Furthermore, we expect that this basic research will lead to applications in breeding other legume crops.

Transgene expression in FSLs #83 and #121

Among the 15 superior FOX superroot lines (FSLs), #83 integrated with the Constitutive Photomorphogenic Dwarf (*CPD*) gene: At5g05690 demonstrated longer total root length and plant length similar to SR, while also displaying an increased number of side branches. Similarly, #121 carrying the Asparaginyl-tRNA synthetase (*SYNC1*) gene: At5g56680, exhibited a higher plant length and thicker stems. To further examine the agronomic traits of distinctive morphology FSL#83 and FSL#121, the expression of their transgenes in the leaf, stem and root was initially confirmed by RT-PCR. Individual gene-specific primers were used to amplify each transgene, with *ACTIN1* serving as an internal control. The RT-PCR products were then separated in a 2.0% agarose gel, using the SR line as a control in each experiment. The results revealed that under the regulation of the CaMV35S promoter, *CPD* and *SYNC1* were overexpressed constantly in all tissues of FSL #83 (Fig 2a) and #121 (Fig 2b), respectively.

The effect of *CPD* and *SYNC1* gene overexpression on morphological traits in FSLs #83 and #121

SR, FSL#83 and FSL#121 were cultivated for 4 months until they reached maturity to evaluate the influence of overexpressing the *CPD* and *SYNC1* genes on agronomic traits. The results were summarized in Table 3 and Fig 3, where it was observed that FSL#83 showed higher internode length, number of stems, number of flowers and above-ground dry matter weight compared to SR. The *CPD* gene encodes the cytochrome P450 enzyme CYP90A1, which roles an important process in the brassinosteroid biosynthesis pathway. Brassinosteroids play crucial roles in various physiological and developmental processes in plants, including cell expansion, cell division, vascular development and stress responses (Sui *et al*., 2012; Godara *et al*., 2017). Our results showed that the increase

Table 3: Comparison of morphological traits between SR, FSL#83 and FSL#121.

Trait	SR	FSL#83	FSL#121
Plant length (cm)	51.1±3.6^{c}	60.0±6.4^{b}	81.8±4.9^{a}
Stem diameter (mm)	1.5±0.3 ^{b}	1.6±0.2^{ab}	2.0±0.3^{a}
Internode length (cm)	39.1±4.3^{b}	53.4±3.3^{a}	34.1±2.1^{b}
Leaf length (mm)	15.6±1.7^{a}	16.5±1.1^{a}	13.3±1.2^{b}
Leaf width (mm)	5.9±0.7^{a}	5.5±0.9^{a}	5.7±0.7^{a}
Number of stems	4.2±0.8^{ab}	4.6±1.3^{a}	2.8±0.4^{b}
Number of branches	5.2±1.1^{a}	6.2±0.4^{a}	5.8±0.8^{a}
Number of stem nodes	15.0±1.0^{b}	14.4±0.5^{b}	20.2±1.9^{a}
Number of branch nodes	27.2±17.5^{b}	33.8±13.7^{ab}	53.6±12.9^{a}
Number of flowers	45.0±21.9^{b}	68.6±3.4^{a}	40.2±8.2^{b}
Dry matter of underground organs (g)	5.5±0.1^{ab}	5.7±0.2^{a}	5.2±0.2^{b}
Dry matter of above-ground organs (g)	6.8±1.0^{b}	8.2±0.6^{a}	6.6±0.7^{b}
Plant regrowth (cm)	35.7±7.5^{a}	36.3±9.1^{a}	32.4±7.8^{a}

Value represents the mean±standard deviation of five replicates. Letters indicate significant differences among the plant lines (Tukey's test, p<0.05).



Pot size was 17.5 cm in diameter.

Fig 3: Phenotypes of SR, FSL#83 and FSL#121 after 4 months of cultivation.

in number of flowers and above-ground dry matter weight in FSL #83 as a result of *CPD* expression, aligns with previous research on the overexpression of brassinosteroid synthesis genes in *Arabidopsis* (Choe *et al*., 2001), pear (Zheng *et al*., 2020), rice and maize (Sun *et al*., 2021). FSL#121 exhibited higher plant lengths and number of stem nodes compared to SR. *SYNC1*, the Asparaginyl-tRNA synthetase gene, is crucial for transferring amino acids to cognate tRNA molecules, essential for accurate protein translation (Friedman *et al*., 2019). Higher expression levels of the *SYNC1* gene enhance the activity of enzymes involved in the synthesis of amino acids such as asparagine, lysine, aspartic acid, alanine and histidine, leading to improved plant growth, including increased plant height, branching, stem thickness and fresh weight of shoots in transgenic soybean (Arifin *et al*., 2019). In this study, the exploration of biomass-related genes using the *L. corniculatus* FOX Line is expected to be applied to the same legume crops. Transformation of the *SYNC1* gene into soybean resulted in the plant growing to twice the height compared to the wild type, along with an increased number of branches, which aligns with the findings of this study (Arifin *et al*., 2019). Next, we would like to create transgenic soybean with overexpressed *CPD* gene and determin its effect of increased number of flowers on the yield.

Conclusion

Identifying genes associated with biomass is crucial for meeting the growing demand for food. In our study, we explored these genes by studying 66 FOX superroot lines (FSLs) that integrated with *Arabidopsis* cDNA. We used the FOX hunting system to analyze the functionality of *Arabidopsis* cDNA in FSLs, focusing on biomass traits and demonstrated its potential in legume crops. The 66 FSLs showed diverse plant heights and root lengths during the early growth stage, with some lines exhibiting superior growth. Fifteen FSLs were identified with superior growth characteristics, surpassing thresholds of 30 cm for plant length and 100 cm for root length. These superior FSLs were each integrated with *Arabidopsis* cDNA, categorized into genes related to various functions such as membrane transport, photosystems, phytohormone synthesis, amino acid synthesis and two unknown genes. Two FSLs with distinct characteristics were grown for 4 months in a greenhouse until the flowering stage and their agronomic traits were investigated in detail. FSL #83, expressing the brassinosteroid biosynthetic pathway gene (CPD), displayed significant higher in internode length, stem count, flower number and above-ground dry matter weight compared to non-transgenic SR. On the other hand, FSL #121 expressed Asparaginyl-tRNA synthetase (*SYNC1*) gene exhibited increased plant height and the number of stem nodes. The information on candidate genes related on biomass obtained in this study can be expected to improve of other legume crops in the future.

Conflict of interest

All authors declare that they have no conflict of interest.

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