Growth increase of transgenic plants by the forced expression of rice *45S rRNA* gene

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Growth increase of transgenic plants by the forced expression of rice *45S rRNA* gene

（イネ *45S rRNA* 遺伝子の強制発現による形質転換植物の生長量の増大）

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Chapter 1

General introduction

Because population is growing in the world, especially African countries, demands against foods and energies are also increasing continuously. In addition, as most energy sources are fossil fuels, such as oil, coal, and natural gas, their burning to take energy, such as electricity and motive power, is responsible to increase of carbon dioxide in the atmosphere. Carbon dioxide and methane gases, so call greenhouse gas, can absorb infrared, which accumulate heats like a greenhouse (Lashof and Ahuja 1990). Now, these accumulated heats are causing climate changes, such as elevation of sea level and global warming. The global warming causes drastic changes of amount of rainfall to producing either drought or water-lodging areas and has a great influence on agriculture (e.g., Cline 2007, Farell et al. 2006, Ragauskas et al. 2006).

Plants can produce organic materials by the photosynthetic pathway, where atmospheric carbon dioxide and water from ground are bound using chemical energy of sunlight. Therefore, burning of organic materials from living plants to take energy is not increase amount of atmospheric carbon dioxide because the same amount of carbon dioxide is absorbed by the photosynthesis to produce organic materials (carbon neutral). Thus, organic materials, so call biomass, produced by living plants are recognized as renewable and sustainable energy sources.

The plant-based biomass, however, is not widely used for the energy source because of high cost compared with the fossil fuels. To use biomass is required costs for several processes, such as gathering and drying plants, and exchanges of starches and cellulose to alcohol and of lipids to biodiesel (Naik et al. 2010). One of ways to reduce the cost is probably increase biomass production of plants. If one could increase biomass of a plant up to twice, initial cost of its production were reduced to half. Therefore, we are interested to develop a way to increase biomass, mass of overall aerial tissues of plant.

Hybrid vigor is known as a genetic phenomenon, which brings vigor growth of a first filial (F_1) individual more than that of maternal and paternal parents (Darwin 1876). As the hybrid vigor occurs only in the F_1 generation, F_1 variety has been bred though laborious and time-consuming processes of cross hybridizations. The hybrid vigor can improve 20-80% increase of production capacity (Meyer et al. 2004). Although the mechanism underlying the hybrid vigor remains unclear, the F_1 plant shows accelerated initial growth, which might be promoted through cell proliferation under the control of circadian rhythm (Ni et al. 2009, Fujimoto et al. 2012).

Tetraploid plant can be induced from the diploid plant and generally produces its biomass more than the original diploid plant (Sugimoto-Shirasu and Roberts 2003). The tetraploidization invokes the growth increase of plants through cell enlargement, which

is observed as swelling of stomata in the leaves and measured as doubling of relative DNA content of cells using flow cytometric analysis. An increase in cell volume induced by the tetraploidization is due to an increase in turgor pressure of water in cell, so dry matter weight does not increase corresponding to the fresh weight (Bagheri and Mansouri 2015). Therefore, the tetraploid plants usually decrease concentrations of secondary metabolites compared with the original diploid plant.

Development of plants is determined by processes of cell proliferation and cell enlargement (Tsuge et al. 1996, Sugimoto-Shirasu and Roberts 2003). The proliferation and enlargement of cells are controlled by the interactions among many regulatory genes of plants (Palatnik et al. 2003). Also the growth of cells is limited by the available organic components, which are derived from storage materials in the seeds and photosynthesis in the leaves. If area of leaves were increased by the modification of a regulatory gene in the transgenic plant, thickness of the leaves must tend to be decreased. Therefore, it will be difficult to increase overall biomass of plants without the additional supply of organic components.

Green revolution (Peng et al. 1999) brought a remarkable increase of seed yields in cereal crops, such as wheat and rice, which had shorter stature. Semi-dwarf genes, such as *Rht* genes in wheat and *Sd-1* gene in rice, were used to reduce the plant height to 70-

80% of the original cultivar (Hedden 2003). In this case, organic components to make cells of leaves, stems, and roots are saved and the saved materials are transported to increase the number of seeds because amounts of available organic materials are similar between semi-dwarf and original cultivar. Thus, the increase of yield by the usage of semi-dwarf gene is considered not to be the results of the biomass increase of plant.

Organic materials of plants are produced through the functions of enzymes, which are synthesized as proteins on the ribosome in the cells. The ribosome also synthesizes structural proteins to control size and shape of cells. Of course, production of these proteins is strictly regulated by the amount of photosynthetic products. As the ribosome occupies 80% and more of components in the cells, it is likely to play important roles in regulating amount of the biomass production.

Three ribosomal RNAs, 18S, 5.8S, and 28S rRNA, are transcribed by RNA polymerase I (PolI) complex as a single transcriptional unit, *45S rRNA*, which is processed to the three rRNA molecules to form ribosome (Hershey et al. 2000). Other *5S rRNA* gene is transcribed by PolIII. The arrays of *45S* and *5S rRNA* genes contains hundreds copies of the tandem repeats in the nuclear genome. Although the sequences of the three *rRNA* within the *45S rRNA* gene are highly homologous among species of plants, two internal transcribed spacer (ITS) sequences, ITS1 between *18S* and *5.8S rRNA* and

ITS2 between *5.8S* and *28S rRNA*, show species-specificity. The *45S rRNA* gene is known to be transcribed by PolI of eukaryotes in the species-specific manner. However, the interactions between ITS sequences and PolI complex have been not resolved.

In yeast, a mutant strain lacking the function of PolI has been isolated because its second largest subunit is defective (Nogi et al. 1991). Thus, this strain cannot transcribe genomic *35S rRNA* gene, which is essential to be alive. However, if the PolI-deficient strain were transformed using a multicopy plasmid harboring *35S rRNA* gene driven by GAL7 promoter, which is recognized by PolII complex that transcribes various mRNAs, the strain could survive. This result suggests that *rRNA* molecules transcribed by the PolII are functional at least in yeast.

Hybrid vigor appears in F1 hybrid between indica and japonica rice (*Oryza sativa* L). In the F1 hybrid, *45S rRNA* genes from indica and japonica co-exist in the same nucleus. And the expressions of both *45S rRNA* genes are probably interfered each other because the hybrid vigor disappear even in a heterozygous plant of F2 generation (Charlesworth and Willis 2009). Therefore, we would like to try to express indica *45S rRNA* gene in japonica cultivar using a promoter, recognized by the RNA polymerase II.

In this study, full-length of *45S rRNA* gene (*ca*. 5.8 kb) was amplified by PCR as a template DNA extracted from indica cultivar N16. The isolated *45S rRNA* (*Os45SrRNA*)

gene was linked to between maize *ubiquitin* promoter (UbiP) and *nopaline synthase* terminator (nosT) or between califlower mosaic virus 35S promoter (35SP) and nosT. The obtained chiameric genes, UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA*, were inserted into between kanamycin and hygromycin resistance cassettes of a binary vector pEKH. The constructed binary vectors were mobilized into *Agrobacterium tumefaciens* strain EHA101. Transgenic *Arabidopsis* and tobacco plants were produced by floral-dip and leaf-disc inoculation methods, respectively. Growth profiles and physiological characteristics of the transgenic plants were compared with those of the control plants in this study.

Chapter 2

Growth increase of *Arabidopsis* by forced expression of rice *45S rRNA* gene

Introduction

The increase in carbon dioxide emissions from using of fossil fuels, such as petroleum, coal, shale and natural gas could result in significant climate change (Rahmstorf et al. 2007). These fossil fuels were produced by photosynthesis in plants and other microorganisms over millions of years. Plant photosynthesis is carbon neutral and can supply energy without increasing the net amount of carbon dioxide in the atmosphere (Odum et al. 1969). The production of plants with increased biomass (for review Rojas et al. 2010) through the enhancement of photosynthesis (Kebeish et al. 2007), cell division (Matsuda et al. 2008), and cell enlargement by endoreduplication (Breuer et al. 2012), has been the focus of many researchers. Modulation of the hormone synthesis pathway of plants, such as gibberellic acid (Eriksson et al. 2000, Nelissen et al. 2012) and brassinosteroid (Sakamoto et al. 2006), has been shown to increase biomass and grain yield. In the case of rice, improvement of yield factors, such as grain weight (Ishimaru et al. 2013) and number (Yoshida et al. 2013), resulted in a 15% and 50% increase in yield, respectively.

Biomass production of plants is regulated by the sizes of the source (photosynthesis amount) and sink (organ mass) (Herzog 1982). If either the sink or source is enlarged more than the other, biomass production would not be increased. Therefore, to increase biomass production, it is necessary to increase the sizes of both the source and the sink. In plants, as many genes are involved in photosynthesis and cell proliferation, it is difficult to simultaneously increase both the capacity of photosynthesis and the number of cells.

Hybrid vigor (Darwin 1876) is a method used to increase the biomass production of various plants. Most cultivars of vegetables and cereals are bred by practical application of hybrid vigor, which promotes a 1.2 – 1.5-fold increase in vegetable biomass and yield of seeds to the original cultivar. Hybrid vigor appears only in the F_1 hybrid between distantly-related parental cultivars and disappears in the next generation. The mechanism underlying hybrid vigor remains elusive (Meyer et al. 2004, Lippman et al. 2007, Hochholdinger and Hoecker 2007). Seed companies must strive to produce seeds of F_1 cultivars. Polyploidization (Wood et al. 2009), particularly tetraploidization, is also able to increase biomass through the expansion of cell volume. Therefore, the size of stomatal guard cells is considered a good marker of the ploidy level (Masterson et al. 1994). Tetraploidization has been used in plant breeding to increase the sizes of flowers, seeds, and fruits.

In *Arabidopsis*, mutants of ribosomal protein genes result in a range of specific developmental changes in leaf shape and defects of embryo morphogenesis, inflorescence development, transition to the flowering stage, and plant stature (Van Lijsebettens et al. 1994, Rosado et al. 2012, Ito et al. 2000, Horiguchi et al. 2012, Zsogon et al. 2014). For example, Fujikura et al. (2009) identified three *oligocellula* (*oli*) mutants, *oli2*, *oli5*, and *oli7*, the double mutants of which showed reduced number of expanded cells. As these three genes encode ribosome-related proteins, ribosome-related processes might be involved in the co-ordination between cell proliferation and cell expansion. Although the mechanism underlying these processes is unclear, it is plausible that ribosomal proteins may have the extraribosomal function required for the specific development of plants.

Ribosome RNA (rRNA) is a component of ribosome, which is central machinery of protein production in cells (Hershey and Merrick 2000). The endogenous *45S rRNA*

gene is transcribed by RNA polymerase I (PolI), and then transcribed to *45S rRNA* precursors, which are split into *18S*, *5.8S*, and *28S rRNAs*, and integrated into small or large subunits of ribosome. Two short internal transcribed spacers, ITS1 and ITS2, are present between *18S* and *5.8S* and between *5.8S* and *28S rRNA* within *45S rRNA* transcripts, respectively. Although these ITS sequences are species-specific (Appels and Dvořák 1982), there is no information available showing that ITS regions play a role in any physiological function in cells.

This study was initially aimed to express Indica rice specific *45S rRNA* gene in Japonica rice because a hybrid between Japonica and Indica showed hybrid vigor (Jones 1926). Japonica rice has a single *45S rRNA* array on chromosome 9 whereas Indica rice contains an additional *45S rRNA* array on chromosome 10 (Fukui et al. 1994). Thus, we isolated the Indica-specific *45S rRNA* gene from Indica line N16. In order to express the Indica-specific *45S rRNA* gene in Japonica rice, the isolated *45S rRNA* gene was linked to the promoter sequence of the maize *ubiquitin* gene. Unfortunately, we could not produce transgenic Japonica rice harboring the chimeric construct, therefore, *Arabidopsis thaliana* Col-0 line was transformed by the same construct using the floral-dip method (Clough and Bent 1998).

In this study, we found that the forced expression of the rice *45S rRNA* gene caused up to a 2-fold growth increase in the transgenic *Arabidopsis* plants.

Materials and methods

Plant material

Seeds of *Arabidopsis thaliana* Columbia 0 (Col-0) ecotype were obtained from the Arabidopsis Biological Resources Center, Ohio State University, OH, USA. For phenotypic analysis, surface-sterilized seeds were simultaneously sown on MS medium (pH 5.8) (Murashige and Skoog 1962) containing 0.8% agar (w/v) and 1% sucrose (w/v) (Breuer et al (2009). Seedlings (14 day after sowing: DAS) were transplanted to soil and plants were grown under the same long day conditions (22℃, 16 h light/8 h dark).

Production of transgenic Arabidopsis

Full-length *45S rRNA* gene (5.8 kb) of *Oryza sativa* ssp. Indica N16 line (a cultivar collected in Nepal) was amplified by PCR using a pair of primers, Os45SrRNA-5P and Os45SrRNA-3P (Fig. 2-1a). PCR product was cloned into pCR2.1-TOPO vector (Life Technologies Japan, Ltd., Tokyo, Japan) and the sequence was determined (deposited to DDBJ databank as accession No. LC086814). The *Os45SrRNA* fragment was ligated into Sub221 plasmid (Takesawa et al. 2002) between maize *ubiquitin* promoter (1.0 kb without the first intron) and *nopaline synthase* gene terminator (*nos*T) by In-Fusion HD Cloning Kit (TaKaRa Co. Kyoto, Japan) using a pair of primers, Os:nosTIF-5P and Os:UbiPIF-3P, to construct UbiP::*Os45SrRNA* chimeric gene. Cauliflower mosaic virus (CaMV) 35S promoter was also used to express rice *45S rRNA* gene (35SP::*Os45SrRNA*). The chimeric genes were inserted into the binary vector $pEKH_2$ (Takesawa et al. 2002) between kanamycin and hygromycin resistance cassettes (Fig. 2-1b). The obtained binary vectors,

pEKH$_2$ UbiP::*Os45SrRNA* and pEKH$_2$ 35SP::*Os45SrRNA* were mobilized to *Agrobacterium tumefaciens* EHA101 and transformation of *A. thaliana* Col-0 was done by floral-dip method (Breuer et al. 2009). Transgenic plants were selected on kanamycin-containing media. The primers used for chimeric gene constructions were listed in Table S1.

Southern Blot analysis

Leaf samples were frozen using liquid nitrogen and crushed into fine powder using a Multi-beads Shocker (Yasui Kikai, Kyoto, Japan). Genomic DNA was extracted from 100 mg of leaf tissues using the modified CTAB method (Doyle and Doyle 1987). For Southern blot analysis, *Hin*dIII-digested genomic DNA (5 μg) was separated through 0.9% agarose gel, blotted to nylon membrane (Immobilon-Ny$^+$; Millipore Corporation, USA), and hybridized with a digoxigenin-labeled probe of hygromycin phosphotransferase (*hpt*) gene according to the supplier's instructions (Roche Diagnostics, Mannheim, Germany). Hybridization with the DIG-labeled *hpt* probe was carried out at 39℃ for 16 h. The membrane was treated with anti-DIG alkaline phosphatase and substrate CPD-star (Roche Diagnostics, Mannheim, Germany). Then, the membrane was exposed to Hyperfilm TM-MP X-ray film for 30 min at room temperature.

Segregation analysis

Surface-sterilized seeds of T_3-generation from individual T_2 transgenic plants were sown on MS medium containing 1% sucrose, 0.8% agar, and 50 mg L^{-1} kanamycin. The numbers of resistant and non-resistant seedlings were scored at 14 DAS. T_2 transgenic plants showing 3:1 segregation ratio were selected for further analysis.

RT-PCR and semi-quantitative RT-PCR

Total RNAs were extracted from leaves (100mg) of transgenic and control plants (12 DAS) using Plant RNA Reagent (Life Technologies, USA). First strand cDNA were synthesized from 1 µg of total RNA in a 20 µl reaction volume using Superscript Transcriptase III (Life Technologies, USA) with oligo-dT20 primer. Primers were designed to amplify cDNA of ITS (ITS1 – 5.8S – ITS2) region of rice *45S rRNA*, five up-regulated genes, and *actin* gene (positive control). The primers used for RT-PCRs were listed in Table S1.

Phenotypic analysis

Dry weights of aerial parts were measured using 10 plants of transgenic U10 and Col-0 lines at 24 DAS. The primary root length and number of secondary roots were measured using 10 plants at 14 DAS. Leaf area of forth leaf was measured in U10 and Col-0 plant at 16 DAS. Preliminary data of the 1000 seed weight and total seed yield were obtained using 3 plants of U10 and Col-0 lines at 48 DAS.

Microscopy observation and Flow cytometry analysis

Leaf tissue was washed in 70% ethanol for 4 h and cleared in chloral hydrate:H_2O:glycerol (8:2:1) overnight at room temperature. Stomatal and pavement cells were observed at 400 × magnification using BX60 microscope (Olympus Co., Tokyo, Japan) equipped with a digital DP72 camera.

Relative DNA content per nuclei (ploidy level) in the forth and fifth leaf-cells was measured using laser flow cytometer PAS CA-IV (Partec GmbH, Germany) according to the method of Niimi et al (2015). A piece (0.5 cm × 0.5 cm) of fresh leaf was excised and

put on a plastic Petri dish, and chopped with a razor blade in 1.0 ml of solution composed of 10 mM Tris-Cl, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-100 and 2 mg L^{-1} 4,6-diamidino-2-phenylindole (DAPI), pH 7.5, and filtered through a 30 μm nylon mesh. The obtained nuclei suspension was subjected to flow cytometer for determining the relative nuclear DNA contents on a linear scale histogram.

Dissection analysis of UbiP::*Os45SrRNA* transgene

The *Os45SrRNA* gene cloned into pCR2.1 was dissected using four different restriction enzymes, *Xba*I (D1), *Bam*HI (D2), *Nhe*I (D3), and *Not*I (D4), as illustrated in Fig. 2-6a. The obtained dissected constructs were ligated into Sub221 plasmid (Takesawa et al. 2002) between maize *ubiquitin* promoter and *nos* terminator by the In-Fusion HD Cloning kit using a pair of primers, Os:nosTIF-5P and Os:UbiPIF-3P. Simultaneously, *Arabidopsis thaliana 45S rRNA* gene was cloned into pCR2.1 vector through PCR using At45SrRNA-5P and At45SrRNA-3P primers. *Os45SrRNA* and *At45SrRNA* genes were ligated into S221s plasmid (Takesawa et al. 2002) between cauliflower mosaic virus (CaMV) 35S promoter and *nos* terminator by the In-Fusion HD Cloning kit using pairs of primers, Os:nosTIF-5P / Os:35SPIF-3P and At:nosTIF-5P / Os:35SPIF-3P, to construct 35SP::*Os45SrRNA* and 35SP::*At45SrRNA*, respectively. These constructs were independently integrated into EKH_2 binary vector (Takesawa et al. 2002) and transformed into Col-0 plants using the floral-dip method. Obtained kanamycin-resistant transgenic plants harboring each dissected construct were grown to maturity and homologous lines for the transgene were selected at the T_3 generation. Growth of four seedlings from the homologous line was observed for each dissected construct.

Microarray analysis

Total RNAs were isolated from aerial tissues of transgenic U10 and Col-0 seedlings at 12 DAS (2 samples) and 14 DAS (1 sample) using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and cDNAs were synthesized with 700 ng of total RNA using M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA). Cy3- or Cy5-labelled cRNAs were synthesized from the cDNA as a template and hybridized for 17 h at 60 °C with Arabidopsis oligo-DNA microarray ver. 4 (Agilent Technologies, Palo Alto, CA). After hybridization, washed slides were scanned with Agilent Microarray Scanner and obtained images were processed by Agilent Features Extraction Software (ver. 7.1). We attempted dye swap experiments to reduce errors. Data mining of microarray results was performed according to Satou et al. (2014). Pathway analysis was also done using Mapman tool (http://MapMan.gabipd.org) to overview functional categories of up- or down-regulated genes in the transgenic U10 plant.

Glucosinolate content

Glucosinolates of leaves in lines U10 and Col-0 (24 DAS, 6 plants) were extracted according to Takahata et al. (2006). Total content of glucosinolates was measured four times by Triple Quadrupole Liquid Chromatograph Mass Spectrometer LC-MS/MS 8030 (Shimadzu Scientific Instrument, Japan) using silica-based reverse phase column TSKgel ODS-100V 3 μm (2.0 mm×150 mm) (Tosoh Co. Japan). Aliquot (2 μl) was injected to the column and mobilized (1 ml/1 min) using water-acetonitrile mixtures at 35℃. Most glucosinolates were detected at 190 – 340 nm wavelength. One of glucosinolates, glucoraphanin (Cayman Chemical Co., USA) was used as a standard.

Results

Production of transgenic Arabidopsis harboring UbiP::*Os45S rRNA* gene

Isolated Indica-specific *45S rRNA* gene (5.8 kb in Fig. 2-1a) shared 99% nucleotide identity (5752 bp identity/5782 bp) to that of Japonica rice. The structure of T-DNA region between right and left border of the constructed binary vector, $pEKH_2$ UbiP::*Os45rRNA*, was shown in Fig. 2-1b. By using floral-dip transformation of *A. thaliana* Col-0 line with *A. tumefaciens* harboring $pEKH_2$ UbiP::*45SrRNA*, eleven transgenic T_1 seedlings were selected on MS media containing 50 mg L^{-1} kanamycin. In the obtained transgenic plants, the endogenous *45S rRNA* gene was transcribed by RNA polymerase I (PolI) whereas the UbiP::*Os45SrRNA* transgene was probably transcribed by PolII as illustrated in Fig. 2-1c.

Analysis of copy number of UbiP::*Os45S rRNA* transgene

Southern blot analysis using a probe of the hygromycin resistance (*hpt*) gene revealed three T_1 lines (lines U3, U7 and U10) out of the 11 transformants with a single locus (Fig. S1). T_2 seeds of six selected lines (lines U1, U3, U4, U5, U7, U10) were sown on kanamycin plates and Mendelian inheritance of a single kanamycin resistance (*nptII*) gene was confirmed as a ratio of 3 (resistant): 1 (sensitive) in four lines (U3, U5, U7, U10), but two lines U1 and U7 did not fit into this ratio (Table S2). These results indicated that the UbiP::*Os45SrRNA* transgene, along with kanamycin and hygromycin resistance genes, was inserted as a single-copy into the genome of lines U3 and U10. Thus, we used lines U3 and U10 for further experiments.

Growth of transgenic plants harboring UbiP::*Os45SrRNA* transgenic plant

T_3 seeds of U3 and U10 homozygous lines were sown on MS media containing 1% sucrose (Breuer et al. 2009) and these seedlings were transplanted to pods 14 days after sowing (14 DAS). Maximum rosette diameters of U3 and U10 plants were bigger than those of control plant at 15 DAS and these differences were kept until 24 DAS (Fig. S2). As shown in Fig. 2-2a and 2-2b, the plants of lines U10 and U3 had significantly more and larger leaves compared to those of the control plants at 24 DAS. Both fresh and dry weights of the aerial parts also increased in lines U10 and U3 (Fig. 2-2c and 2-2d). Also, the dry weights of lines U10 and U3 were 2.3- and 1.9-fold increases, respectively, higher than those of the control plants.

Next, we checked the root system of the transgenic and control plants at 14 DAS. Interestingly, the plants of the transgenic lines U3 and U10 had well developed roots compared to their control counterparts (Fig. 2-3a). The length of the primary root and the number of secondary roots significantly increased in the transgenic lines (Fig. 2-3b and 2-3c). Together, these results suggest that the expression of rice *45S rRNA* gene in *Arabidopsis* significantly increased plant architecture in transgenic lines compared to the control plants.

Number of stomatal and pavement cells and ploidy level of leaf-cells in transgenic plants

The U10 plant showed bigger leaf area (59.6 mm^2) of the forth rosette leaf compared to the Col-0 plant (51.1 mm^2) at 16 DAS (Fig. 2-4a). Although longitudinal diameter of stomatal guard cells within the adaxial epidermis of the forth leaf in U10 plant

were little shorter compared to Col-0 plant, U10 plant showed total numbers of stomata (14.7×10^3) and pavement cell (20.6×10^3), which were 1.7 and 1.3 time higher than Col-0 plant (8.6×10^3 and 15.1×10^3), respectively.

Flow cytometric patterns of the fifth leaf-cells were similar between U10 and Col-0 plants at 16 DAS (Fig. 2-4b). Frequencies of 2C, 4C, and 8C cells in the forth and fifth leaves of U10 plant were similar to those of Col-0 plant (Table S3). These results indicated that polyploidization of somatic cells was not enhanced in the U10 plant.

Expression of introduced UbiP::*Os45S rRNA* gene in transgenic Arabidopsis

The expression of the introduced *Os45SrRNA* gene at 15 DAS was analyzed by RT-PCR using primers, ITS5P – ITS3P1 or ITS5P – ITS3P2, designed against the ITS1 and ITS2 regions of *Os45SrRNA* (Fig. 2-5a). A weak PCR product (326 bp), corresponding to ITS5P – ITS3P1, was amplified in the U10 plant but not in the Col-0 plant and – RT control (Fig. 2-5b). Another weak PCR product (412 bp) was also amplified using a pair of ITS5P and ITS3P2 in the U10 plant. An equal amount of PCR product (983 bp) was detected using primers for the *actin* gene both in U10 and Col-0 plants. These data indicate that the introduced *Os45SrRNA* gene was expressed in the U10 transgenic plants at a very low level.

Dissection analysis of UbiP::*Os45S rRNA* transgene

To confirm whether the forced expression of the full-length *Os45SrRNA* gene was required for the enhanced growth in the transgenic plants, four dissected constructs (D1

– D4) were created using four different restriction enzymes, respectively (Fig. 2-6a). At 14 DAS, root developments of transgenic plants harboring each dissected construct were similar to those of the control Col-0 plants and transgenic plants harboring the CaMV 35S promoter (35SP) regulating the expression of *Arabidopsis thaliana 45S rRNA* gene (Fig. 2-6b), whereas the transgenic plants harboring 35SP::*Os45SrRNA* showed similar enhanced root growth to the U10 plant. Primary root length of UbiP::*Os45SrRNA* (U10) and 35SP::*Os45SrRNA* plants were 1.4 – 1.8 times longer than that of the control (Col-0), D1 – D4, and 35SP::*At45SrRNA* plants (Fig. 2-6c). This result indicates that forced expression of the rice full-length *Os45SrRNA* gene, not the endogenous one, was necessary to produce the enhanced growth observed in the transgenic plants.

Phenotype of transgenic *Arabidopsis* harboring 35SP::*Os45rRNA*

As shown in Fig. 2-7, transgenic plant harboring 35SP::*Os45SrRNA* had bigger leaves and thicker petiole compared to the control plant. Petiole and leaf color of 35SP::*Os45SrRNA* plant (T_2) were thicker and whiter than those of UbiP::*Os45SrRNA* plant (T_3), respectively. Although detail measurement should be done later, growth rate of 35SP::*Os45SrRNA* plant was smaller than that of UbiP::*Os45SrRNA* plant.

Microarray analysis of UbiP::*Os45S rRNA* transgenic Arabidopsis

We employed microarray analysis to characterize up- or down-regulated genes in the UbiP::*Os45SrRNA* transgenic U10 plants. Although expression profiles of all Arabidopsis genes were quite differentiated between U10 and Col-0 plants and between 12 and 14 DAS samples (Fig. S3), 80 and 45 genes were commonly up- or down-regulated

by more than 1.7 fold in the U10 plant at both 12 and 14 DAS, respectively (Tables S4, S5). We performed pathway analysis using Mapman software to overview functional categories, metabolism (Fig. S4), ribosomal proteins (Fig. S5), cellular response (Fig. S6). In the metabolism overview, several genes related to starch degradation and secondary metabolism were up-regulated in U10 plant. Genes encoding for ribosomal proteins showed similar expression level between U10 and Col-0 plants. In cellular response overview, several genes, involved in ethylene and jasmonate responses, were up-regulated in U10 plants.

Although transgenic plants showed *ca* 2-fold increase of aboveground growth, genes involved in the regulation of cell cycle and cell division did not significantly up-regulated in the transgenic U10 plants (Fig. S7). Most of up- or down-regulated genes were found in the development and biotic/abiotic stresses categories. In biotic stress response overview (Fig. S8), out of five transcription factor families, such as ERF, bZIP, WRKY, MYB, and DOF, only ethylene-responsive transcription factor (ERF) was up-regulated in U10 plant. Signaling pathway genes for auxin and ethylene responses were up-regulated whereas salicylic acid (SA) and jasmonic acid (JA) responsive genes were weakly down-regulated. Gene for redox state was down-regulated whereas genes for heat shock protein and secondary metabolite production were up-regulated.

Out of 233 genes, which were commonly expressed in U10 seedling in the concerted manner, 14 up- and down-regulated genes were listed in Table 2-1. Development related genes, encoding senescence–associated protein and nodulin family protein, were up-regulated. On the other hand, expression of cruciferin A storage protein gene was quite low level. Thioredoxin peroxidase gene involved in redox state was down-

regulated whereas indole 3-acetonitrile nitrilase gene involved in IAA signal transduction and secondary metabolite (glucosinolate) production was up-regulated. One ORA47 ethylene-responsive transcription factor (ERF18) was highly expressed (2.15-fold) and other three ERF factors, ERF1, ERF2, ERF104, were also weakly to moderately up-regulated in the transgenic U10 plant.

Preliminary data of yields in the mature transgenic plant

Forty-eight days after sowing (48 DAS), we collected seeds from each three plants of transgenic and control plants. Average 1000 seed weight of the U10 plant was 18.6 ± 1.2 mg, which was 1.2-fold heavier than that of the Col-0 plant (14.9 ± 0.1 mg). U10 plant, however, produced total seed yield of 367.3 ± 50.0 mg, 2-fold more than that of the control Col-0 plant (161.8 ± 2.6 mg). The glucosinolate concentration of leaves determined using LC-MS was similar level in the transgenic U10 (3.3 ± 0.1 mg g^{-1}) and Col-0 (2.9 ± 0.1 mg g^{-1}) plants. These results in this study indicated that the physiological and morphological properties of cells in the U10 plant were similar to those in the Col-0 plant but its growth increased *ca*. 2-fold compared to the control Col-0 plant.

Discussion

Two independent transgenic *Arabidopsis* U3 and U10 plants grew bigger than the Col-0 plant (Fig. 2-2a). In comparison with the Col-0 plant, two transgenic plants showed a *ca*. 2-fold increased growth of aerial parts (24 DAS), such as enlarged leaves and thick leaflets (Fig. 2-2b), as well as well-developed root system (14 DAS), such as a longer primary root and increased number of secondary roots (Fig. 2-3). These data indicate that the entire, not partial, growth of the transgenic plants was enhanced. Development of the root system was particularly character specific to the transgenic plants.

Transgenic U10 plants had bigger forth leaf than the Col-0 plants (Fig. 2-4a). Although the average longitudinal diameters of guard cell were similar between U10 and Col-0 plants, numbers of stomata and pavement cell of forth leaf were much higher in the U10 plant compared to the Col-0 plant. Relative DNA content per cell were similar in the forth and fifth leaves between U10 and Col-0 plants (Fig. 2-4b, Table S3). These results indicate that the cell size and frequency of the ploidy level were similar between U10 and Col-0 plants. The higher numbers of stomata and pavement cells in the U10 plants corresponded to differences in the final leaf size. These data suggest that the increase in cell number, not cell size, was probably responsible for the growth increase in the transgenic plant.

In this study, the intron-less *ubiquitin* promoter (Bourdon et al. 2001) was used for the forced expression of the rice *45S rRNA* gene. The transgenic *Arabidopsis* plant (T_2) harboring CaMV 35SP::*Os45SrRNA* did not grow bigger than the UbiP::*Os45SrRNA* (U10) plant (Fig. 2-7). Although the 35S promoter was much stronger than the intron-less

ubiquitin promoter, *ubiquitin* acts in every type of cells whereas 35S promoter preferentially acts in vascular tissue cells. The rice *45S rRNA* transcripts could not directly contribute to the growth increase because the expression of *Os45SrRNA* messages was at a low level in the U10 plants (Fig. 2-5). The expressed rice *45S rRNA* transcripts might trigger off unknown machinery to enhance the growth increase.

Transgenic plants harboring four dissected (D1 – D4) constructs of UbiP::*Os45SrRNA* showed similar growth of root system with the control Col-0 plant (Fig. 2-6). Although the 35SP::*At45SrRNA* transgenic plant and Col-0 plants showed similar root development, 35SP::*Os45SrRNA* and UbiP:*Os45SrRNA* (U10) plants showed well-developed root systems. These results indicate that the forced expression of the exogenous full-length *45S rRNA* gene was required to increase growth in the transgenic plants.

Within *45S rRNA* transcripts, nucleotide sequences are highly conservative in *18S* (95%), *5.8S* (96%), *28S* (92%) *rRNAs* between *Arabidopsis* and rice, whereas two short internal transcribed sequences (ITS) were differentiated in ITS1 (51%) and ITS2 (56%) between the two species. A large number of studies have reported species-specificity of the ITS sequences, the function of which remains elusive. Therefore, it is most likely that an unknown cellular mechanism to monitor a subtle amount of exogenous ITS transcripts might be involved in the enhanced growth. This monitoring mechanism is probably involved in the regulation of the cell cycle and recognition of species identity.

Microarray analysis was carried out to look for genes responsible for growth increase found in the U10 plant. Although expression profiles of all transcripts were drastically changed between 12 and 14 DAS samples, we tried to search up- and down-

regulated genes in the concerted manner between 12 and 14 DAS samples using Mapman software. Pathway analysis showed that genes regulating cell division and cell cycles (Fig. S7), and ribosomal protein genes (Fig. S5) were not significantly up-regulated in the U10 plant. As the result of the pathway analysis, 14 genes were extracted in Table 2-1. Interestingly, several ethylene responsive transcription factors were up-regulated whereas expression of other transcription factors, such as bZIP, WRKY, MYB, DOF, were similar level between U10 and Col-0 plants (Fig. S8). We could not understand the reasons for up-regulation of nodulin gene and down-regulation of thioredoxin peroxidase gene in the U10 plant.

Although ORA47 ethylene-responsive transcription factor ERF18 was particularly up-regulated in the U10 plant, ethylene responsive factors are usually involved in the stress responses (Lorenzo et al. 2003) and the developmental process (Licausi et al. 2013). Because seedlings used for microarray experiments were grown on the culture media under less biotic/abiotic stress condition, forced expression of rice *45S rRNA* gene possibly caused 'genomic incompatibility' (Brennan et al. 2014), which induced ethylene production (Yamada et al. 2001). Niimi et al. (2015) found that intergenic hybrids between *Brassica oleracea* and *Raphanus sativus* showed curing leaf phenotype (epinasty), which could be suppressed by the addition of the ethylene inhibitor aminoethoxyvinylglycine (AVG). The ethylene production of the transgenic plants might be a result of growth increase and genomic incompatibility because ethylene generally inhibits growth and flowering of plants.

Our findings demonstrate that the forced expression of the rice *45S rRNA* gene increased growth by increasing number of cells in transgenic *Arabidopsis* plants. To the

best of our knowledge, a more than 2-fold increase in the entire above-ground growth using genetic engineering technology has not been reported (Miyagawa et al. 2001, Gonzalez et al. 2010), although the leaf size of transgenic plants was enlarged by over-expression of various genes, such as *AVP1* (Li et al. 2005), *GFR5* (Horiguchi et al. 2005), *JAW* (Palatnik et al. 2003), *BRI1* (Wang et al. 2001), and *GA20OX* (Huang et al. 1998).

Polyploidization is a method used to increase the biomass and stress resistance of plants (Chen et al. 2010). Productivity, however, of secondary metabolites was decreased in several cases because cell size was expanded but plant size was reduced in polyploid (Lavania et al. 2012). In this study, although total growth increased two-fold, U10 and Col-0 plants contained a similar content of glucosinolates, functional ingredients in the Brassicaceae family. This result suggests that the forced expression of the exogenous *45S rRNA* gene is one means of producing secondary metabolites of plants.

The preliminary time course-experiment showed that the maximum diameters of rosettes in U3 and U10 plants were 1.6 – 1.7-fold longer than those of the Col-0 plants at 15 DAS and the differences were maintained until 24 DAS (Fig. S2). This result suggests that the increased growth of the transgenic plants was initiated at the early seedling stage after germination. Although 1000 seed weight of the U10 plant was slightly heavier (1.2-fold) compared to the Col-0 plant, this could not explain the difference in growth between transgenic and Col-0 plants.

Hybrid vigor or heterosis is another way to increase the biomass of plants and it occurs at the early stages of seedling development of F_1 hybrid (Fujimoto et al. 2012). Ni et al. (2009) suggested that altered expression of regulatory genes for circadian rhythm

might be involved in the hybrid vigor, which appears in early seedling stage and enhances the cell cycle to increase the number of cells. The cell numbers of transgenic *Arabidopsis* harboring UbiP::*Os45SrRNA* also showed increased cell number to achieve growth increase, which might start at the early seedling stage. This resemblance suggests that there might be a similar underlying mechanism between hybrid vigor and forced expression of the exogenous *45S rRNA* gene.

Our experiments demonstrate that the forced expression of the rice *45S rRNA* gene caused growth increase in transgenic *Arabidopsis*. Unlike hybrid vigor, this trait of growth increase is heritable and can be genetically fixed as the homozygous line. This detail could be useful when considering the practical application of this technology to agriculture and industry. Furthermore, if the mechanism underlying the growth increase can be resolved, it will be possible to increase biomass production more than 2-fold. Due to bio-safety concerns, as the final product of the *45S rRNA* gene is RNAs, it is scarcely to produce allergenic substances. The procedure reported in this paper is very simple, but it is a potential breakthrough in the production of plants with increased biomass, which will provide sustainable resources for foods, materials, and bio-energy.

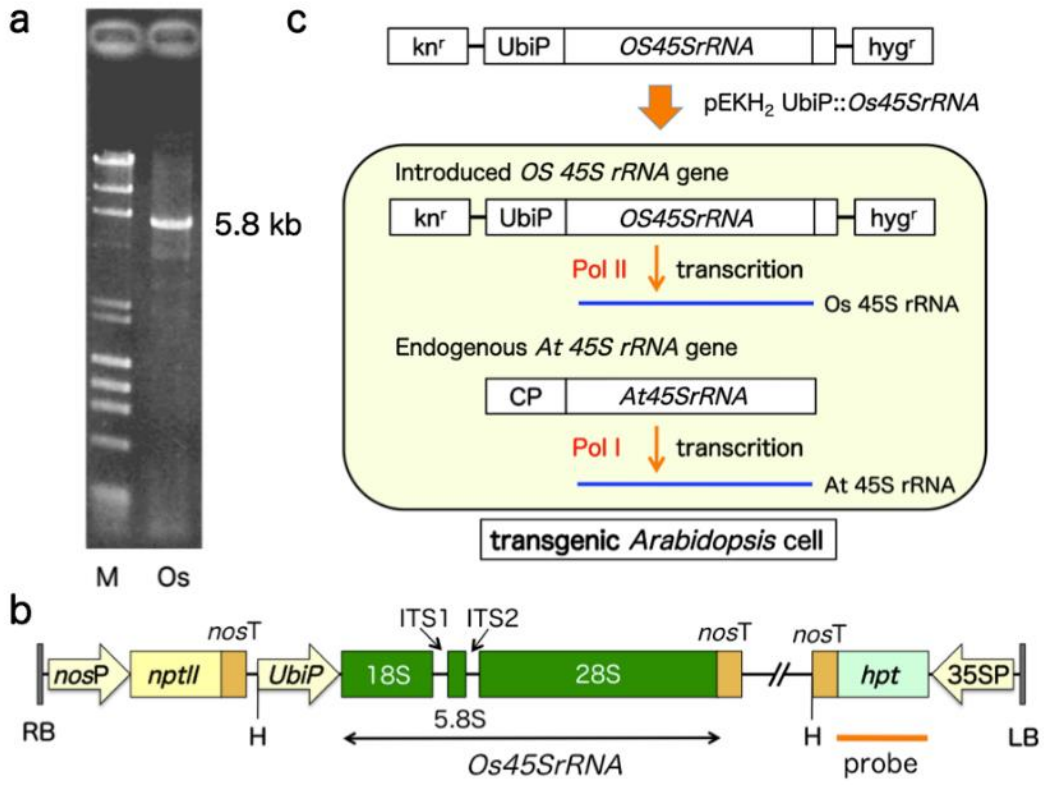


Figure 2-1 Production of transgenic *Arabidopsis* plants with forced expression of rice *45S rRNA* gene using maize ubiquitin promoter.

a Full-length *45S rRNA* gene (5.8 kb) was amplified from Indica cultivar N16 using PCR. **b** Isolated rice *45S rRNA* (*Os45SrRNA*) gene, consisting of *18S*, *5.8S*, *28S rRNAs* and two internal transcribed spacers (ITS1, ITS2), was linked to maize *ubiquitin* promoter (*Ubi*P) and nopaline synthase terminator (*nos*T). The chimeric gene was inserted into *Hin*dIII (H) site between kanamycin (*nos*P-*nptII*-*nos*T: kn^r) and hygromycin (35SP-*hpt*-*nos*T: hyg^r) resistance cassettes of binary vector $pEKH_2$. T-DNA region between right (RB) and left (LB) border repeats of pKH_2 UbiP;;*Os45SrRNA* was illustrated. PCR product of *hpt* gene was used as probe for Southern blot analysis. **c** Schematic illustration of the gene expression in transgenic *Arabidopsis* cell carrying UbiP::*Os45SrRNA*. In the transgenic *Arabidopsis* cells, endogenous *45S rRNA* gene was transcribed from cognate promoter (CP) by RNA polymerase I (PolI) whereas introduced exogenous rice *45S rRNA* gene was transcribed from *ubiquitin* promoter by PolII.

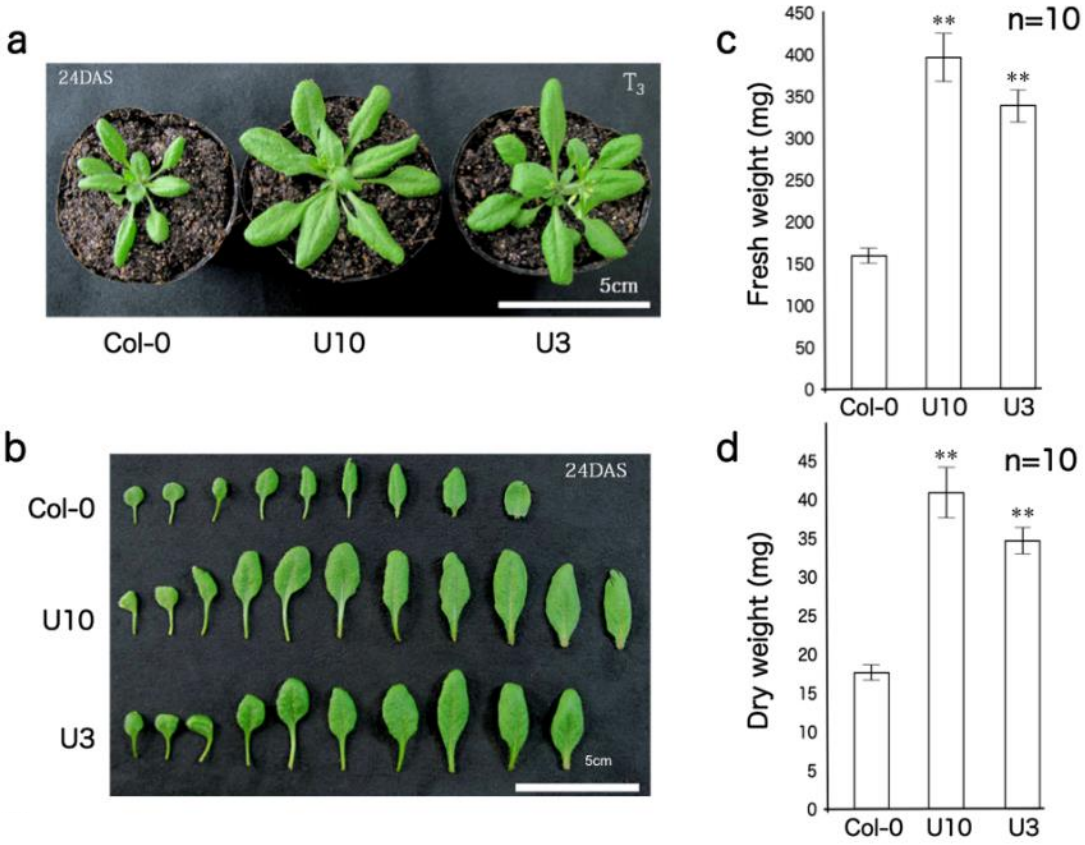


Figure 2-2 Comparison of aboveground growth between the transgenic and control plants.

a Phenotype of transgenic (U3, U10) and control (Col-0) plants under long-day condition at 24 DAS (bar: 5cm). Transgenic plants started bolting earlier than control plant. **b** Alignment of leaves from left (old) to right (young). Two cotyledons were not shown (bar: 5 cm). **c** and **d** Fresh and dry weight (mg) of aboveground tissues. Data represent mean plus standard errors (n=10). ** $P < 0.01$ compared with Col-0.

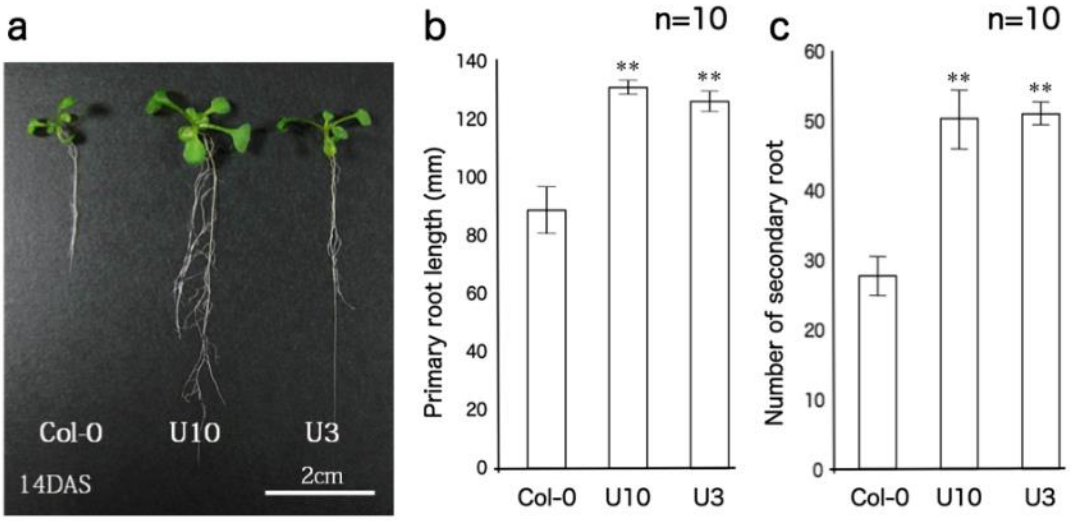
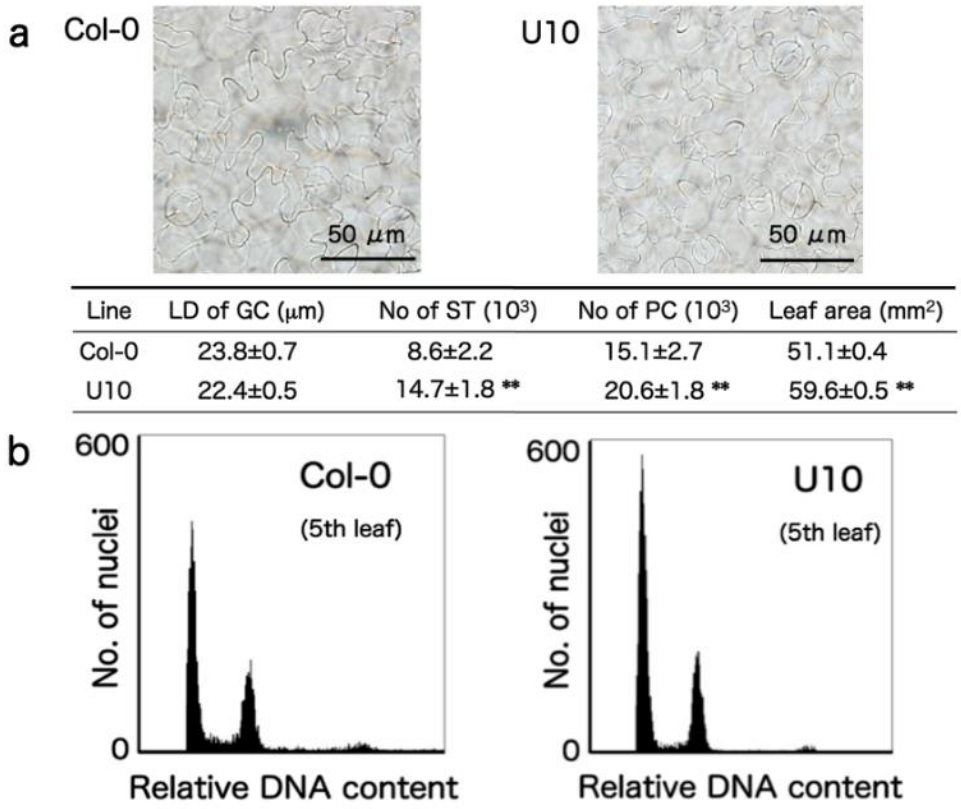


Figure 2-3 Comparison of root system development between the transgenic and control plants.

a Side view of root system in the transgenic (U3, U10) and control (Col-0) plants at 14 DAS (bar: 2 cm). **b** Primary root length (mm), C: Number of secondary roots. Data represent mean plus standard errors (n=10). ** $P < 0.01$.



Line	LD of GC (μm)	No of ST (10^3)	No of PC (10^3)	Leaf area (mm^2)
Col-0	23.8±0.7	8.6±2.2	15.1±2.7	51.1±0.4
U10	22.4±0.5	14.7±1.8 **	20.6±1.8 **	59.6±0.5 **

Figure 2-4 Number of stomata and pavement cells and ploidy level of leaf-cells in the U10 and Col-0 plants.

a Photograph of adaxial epidermal surface of forth leaf on the same magnification in the transgenic (U10) and control (Col-0) leaves at 16 DAS. Longitudinal diameter (LD) of guard cell (GC), total number of stomata (ST), total number of pavement cell (PC), and leaf are of forth leaf were measured in U10 and Col-0 plants. Data represent mean plus standard errors (n=4). ** $P < 0.01$. **b** Relative DNA content per nuclei of leaf-cells measured by using flow cytometer in fifth leaf of U10 and Col-0 plants.

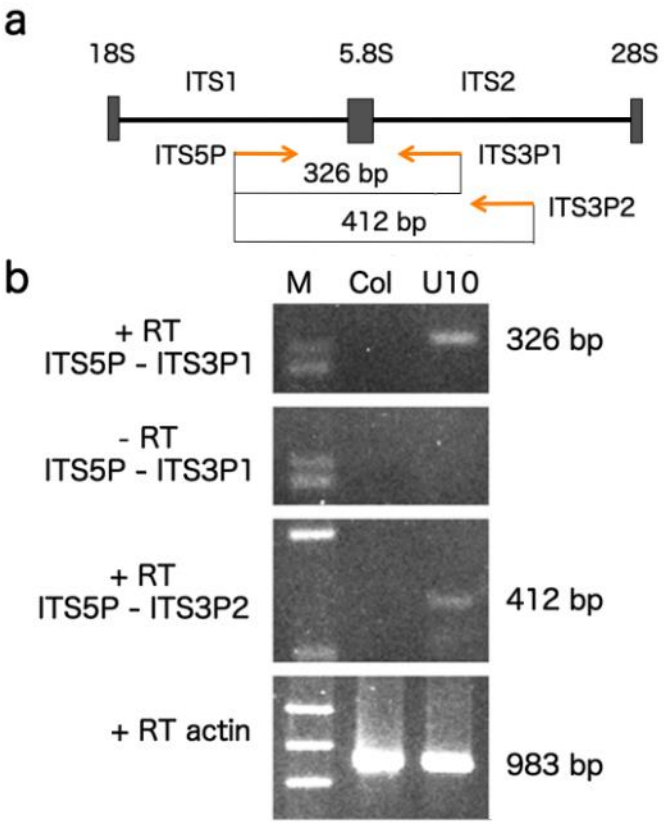


Figure 2-5 RT-PCR of ITS region within rice *45S rRNA* transcripts in the U10 and Col-0 leaves.

a Schematic illustration of ITS region of rice *45S rRNA* gene. Two RT-PCR products (326 bp and 412 bp) were amplified using two pair of primers (ITS5P - ITS3P1 and ITS5P - ITS3P2), designed based on ITS1 and ITS2 sequences, respectively. **b** Agarose gel pattern of RT-PCR products with or without reverse transcriptase (RT) between the transgenic (U10) and control (Col) leaves at 12 DAS. The same amount of RT-PCR product (983 bp) was amplified from *actin* mRNA in the transgenic and control plants.

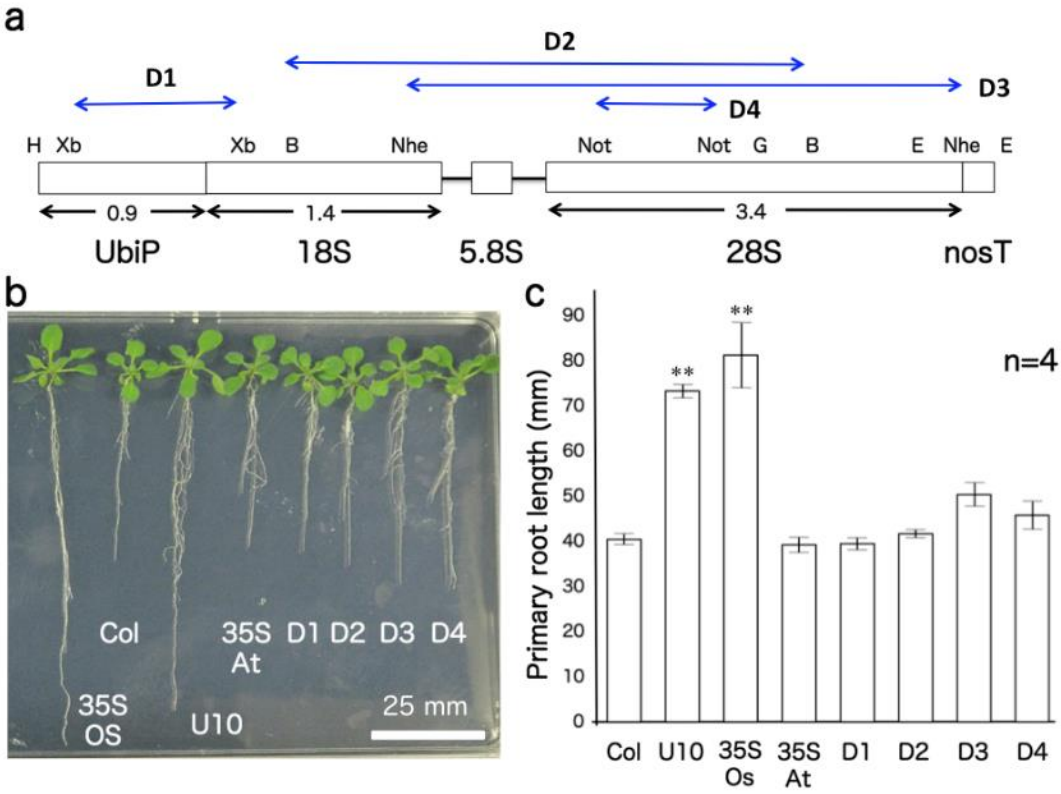


Figure 2-6 Dissection analysis of UbiP::*Os45SrRNA* gene.

a four dissected constructs were created using restriction enzyme, *Xba*I (D1), *Bam*HI (D2), *Nhe*I (D3), and *Not*I (D4). **b** Root system development of transgenic plants harboring UbiP::*Os45SrRNA* (U10), dissected constructs (D1 – D4), 35SP::*Os45SrRNA* (35SOs), 35SP::*At45SrRNA* (35SAt), and control (Col) plants at 14 DAS (bar: 25 mm). **c** Primary root length (mm) of the transgenic and control plants (14 DAS). Data represent mean plus standard errors (n=4). ** $P < 0.01$.

Figure 2-7 Aboveground phenotypes of transgenic and control plants.

Transgenic *Arabidopsis* harboring 35SP::*Os45SrRNA* (center, T_2 generation) or UbiP::*Os45SrRNA* (right, T_3). control Col-0 plant (left). Photo was taken at 28 DAS.

Table 2-1 List of up- and down-regulated genes by pathway analysis in the U10 plant

Category	AGI No.	Name	Fold change
Biotic stress	AT2G43620	chitinase	-1.49
Development	AT5G44120	Cruciferin A storage protein	-2.91
	AT1G53903	Senescence-associated protein-related	1.74
	AT1G70260	Nodulin MtN21 family protein	2.42
Heat	AT3G12580	Heat shock protein 70	1.62
Peroxiredoxin	AT1G48130	Thioredoxin peroxidase	-1.73
Ethylene	AT1G74930	ORA47 ethylene responsive transcription factor ERF18	2.15
	AT4G17500	Ethylene responsive responsive factor ERF1	1.19
	AT5G47220	Ethylene responsive responsive factor ERF2	1.46
	AT5G61600	Ethylene responsive responsive factor ERF104	1.13
Transcription factor, other	AT1G25550	Myb family transcription factor	1.24
	AT3G04070	Arabidopsis NAC domain containing protein 47	1.49
IAA & glucosinolate	AT3G44300	Indole-3-acetonitrile nitrilase	2.01
Anthocyanins	AT2G38240	Oxidoreductase, oxygenase family protein	2.42

Chapter 3

Growth profiles of transgenic tobacco plants expressing rice *45S rRNA* gene

Introduction

As the biomass is created by photosynthesis from atmospheric carbon dioxide and water using energy from sunlight, the usage of bio-energy from biomass, instead of fossil energy, is desired to stop the increase in carbon dioxide emission, which is considered to be responsible for global climate change (Edward et al. 2008). The photosynthesis of plants, algae, and cyanobacteria produces oxygen and organic compounds, which are required by most of organisms on Earth. Organic compounds, i.e. biomass, are expected to be converted to renewable bio-energy (Chisti 2008).

We recently reported that the transgenic *Arabidopsis* plant achieved a growth increase by forced expression of the rice *45S rRNA* gene (Makabe et al. 2016). The transgenic *Arabidopsis* plants increased *ca*. 2-fold growth compared to the control plants, with showing no difference in cell size and ploidy level of the leaves. Although the mechanism underlying the growth increase remains to be resolved, these results showed that forced expression of the rice full-length *45S rRNA* transgene was necessary for growth increase of the transgenic *Arabidopsis*. To probe that similar growth increase could be reproducible in other plants, we tried to produce transgenic tobacco plants harboring the rice *45S rRNA* gene under the regulation of the maize *ubiquitin* promoter (UbiP::*Os45SrRNA*) and cauliflower mosaic virus (CaMV) 35S promoter (35SP::*Os45SrRNA*).

There are many reports about production of plants with increased biomass (Rojas et al. 2010) using enhancement of photosynthetic capacity (Kebeish et al. 2007), cell division activity (Masuda et al. 2008), and cell expansion by endoreduplication (Breuer

et al. 2012). Over-expression of genes responsible for plant hormone synthetic pathway, such as increased content of gibberellic acids in poplar tree (Eriksson et al. 2000) and in maize (Nelissen et al. 2012) showed increased biomass production and leaf growth, respectively. Brassinosteroid deficiency in rice leaf has been shown to increase grain yield (Sakamoto et al. 2006). Improvement of yield factors, such as grain weight (Ishimaru et al. 2013) and spikelet number (Yoshida et al. 2013), in rice resulted in a 15% and 50% increase in yield, respectively.

Hybrid vigor is a genetic phenomenon where the F_1 hybrid can increase growth more than parental lines (Darwin 1876, Meyer et al. 2004, Lippman and Zamir 2007, Hochholdinger and Hoecker 2007). Hybrid vigor cannot be genetically fixed because it appears only at the early growing stage of the F_1 hybrid and disappears in the next generation. Therefore, in order to apply hybrid vigor to biomass production, F_1 hybrid seeds have to be prepared by laborious and time-consuming hybridization between the parental lines. Polyploidization is another way to increase plant biomass through the enlargement of cell size (Kondrosi et al. 2000, Sugimoto-Shirasu et al. 2003). Thus, the sizes of stomata and guard cells are considered as good indicators of the ploidy level in somatic cells (Wood et al. 2009). There is usually a 1.2 – 1.5 fold increase in the biomass of cultivars by using hybrid vigor and polyploidization compared to those of the original cultivar (Duvick 1999).

The eukaryotic *45S rRNA* gene, consisting of *18S*, *5.8S*, and *28S rRNA*, is transcribed as a single transcription unit and post-transcriptionally processed to the three rRNA molecules (Appels et al. 1982). As the sequences of three *rRNA* genes within *45S rRNA* transcripts are highly conservative between rice and tobacco, the expression of two internal transcribed spacers (ITS) might be involved in the above ground biomass increase.

Although variations of ITS sequences are known as species-specific, there is no report that the expression of ITS sequences has any physiological function in the growth of plants.

In this study, we produced transgenic tobacco plants with forced expression of the rice *45S rRNA* gene and analyzed characteristics of their growth.

Materials and methods

Plant material

Plantlets of *Nicotiana tabacum* 'Petit Havana' SR-1 line were maintained in culture bottles under sterile condition and used as starting materials for the production of transgenic plants.

Production of transgenic tobacco plants

Full-length of *45S rRNA* gene (DDBJ Accession No. LC086814) in *Oryza sativa* ssp. Indica N16 line was amplified (Makabe et al. 2016). The *Os45SrRNA* fragment (5.8 kb) was linked between maize *ubiquitin* promoter (UbiP: 1.0 kb without the first intron) (Christensen and Quail 1996) and *nopaline synthase* terminator (nosT) or between CaMV 35S promoter and nosT to construct UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA* chimeric gene, respectively (Makabe et al. 2016). These chimeric genes were inserted into a binary vector pEKH (Takesawa et al. 2002) at *Hin*dIII site between kanamycin (NPTII) and hygromycin (HPT) resistance cassettes (Fig. 3-1). The binary vector was mobilized to *Agrobacterium tumefaciens* EHA101 by freeze-thaw method and transformation of tobacco plant was done by leaf-disc method (Horsch et al. 1985). Transgenic plants were selected on 50 mg L^{-1} kanamycin-containing media.

Southern blot analysis

Leaf samples were frozen using liquid nitrogen and crushed into fine powder using a Multi-beads Shocker (Yasui Kikai, Kyoto, Japan). Genomic DNA was extracted from 100

mg of leaf tissues using the modified CTAB method (Doyle and Doyle 1987). *Hin*dIII-digested genomic DNA (5 μg) was separated through 0.9% agarose gel, blotted to Immobilon-Ny+ membrane (Millipore Corporation, USA), and hybridized with a digoxigenin (DIG)-labeled probe of hygromycin phosphotransferase (*hpt*) gene according to the supplier's instructions (Roche Diagnostics, Mannheim, Germany). Hybridization with the DIG-labeled *hpt* probe was carried out at 39℃ for 16 h. The membrane was treated with anti-DIG alkaline phosphatase and substrate CPD-star (Roche Diagnostics, Mannheim, Germany). Then, the membrane was exposed to Hyperfilm TM-MP X-ray film for 30 min at room temperature.

Plant growth analysis

Seeds of the transgenic (S1. U9) and control (WT) plants were sown side by side in a tray in a greenhouse. Serial photographs of seedlings were taken at 15, 20, and 25 DAS. Aerial tissues of plants were harvested at 45 DAS and used to measure dry weight. Leaf samples at 75 DAS were used to measure stomatal characteristics, length and width (μm) of stomata, and density of stomata per unit leaf area (mm^2). Number of fruits and 500 seed weight were measured after plants were grown to maturity.

Nicotine content

Crude extracts of nicotine were prepared from above ground tissues (10 mg) at 45 DAS according to the CORESTA Recommended Method (CRM No. 72) (CORESTA

2013). The crude extract was subjected to liquid chromatography mass spectrophotometer LC-MS/MS 8030 (Shimadzu Scientific Instrument, Japan).

Flow cytometric analyses

Relative DNA content per nuclei of leaf cells at 20 DAS was measured in triplicate using the laser flow cytometer PAS CA-IV (Partec GmbH, Germany) (Mishiba and Mii 2000). The suspension of nuclei stained with 4, 6-diamidino-2-phenylindole (DAPI) was subjected to flow cytometer to determine the relative DNA contents on a linear scale histogram. Ploidy levels were determined by comparing the position of dominant 2C peaks between transgenic (S1, U9) and control WT plants.

Results

Production of transgenic tobacco plants expressing rice *45S rRNA* gene

We produced fourteen and three transgenic tobacco plants harboring UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* transgene (Fig. 3-1), respectively. Transgenic tobacco lines harboring a single-copy of UbiP::*Os45SrRNA* (U9) or 35SP::*Os45SrRNA* (S1) transgene were selected using Southern blot analysis (Fig. 3-2). These two homozygous lines for the transgenes were used to measure various growth profiles.

Comparison of growth between transgenic and control lines

Seedling growth was compared between the transgenic tobacco (S1, U9) and control (WT) plants, which were grown side by side in the same tray (Fig. 3-3). Although sizes of transgenic (S1, U9) and control (WT) seedlings seemed to be similar at 15 DAS, both transgenic seedlings grew bigger than the control seedlings at 25 DAS. Transgenic (S1) and control (WT) plants were grown in pots until maturity in a greenhouse condition (Fig. 3-4). Size and number of leaves and plant height of transgenic S1 plant were increased more than those of WT plant at 44 and 75 DAS.

Comparison of dry weight and seed production

As shown in Fig. 2-5A, dry weight of the transgenic S1 and U9 plants was 3.7 g and 5.6 g, which were 1.4 and 2.1 fold heavier than that of the control WT plant (2.7 g/plants) at 45 DAS. As S1 plant bloomed a first flower at 107 DAS, it was much earlier than the

flowering of the WT plant (152 DAS). Therefore, the fruit number of the S1 plant was *ca*. 3-fold higher than that of the WT plant (Fig. 3-5B). In addition, 500-seed weights of S1 (41.2 ± 0.6 mg) and U9 (45.7 ± 0.4) were 17% heavier than that of the WT plant (39.0 ± 0.7 mg) (Fig. 3-5C).

Comparison of nicotine content in leaves

Although nicotine contents of leaves per dry weight (mg/g) were similar among two transgenic and control plants (Fig. 3-6A), total nicotine production per plant was much increased in transgenic U9 (2.4 fold) and S1 (1.5 fold) plants compared to that in the control plants because of increased dry weight in the transgenic plants (Fig. 3-6B).

Comparison of stomatal characteristics

Stomatal characteristics of leaves in the transgenic (S1, U9) and control (WT) plants were measured at 75 DAS. There were no significant differences on length and width of stomata between the transgenic and control plants (Table 3-1). The densities of stomata per unit leaf area (mm^2) were also similar between the transgenic (S1, U9) and control WT plants.

Flow cytometric analysis

Relative DNA contents of leaf cells were compared between transgenic (S1, U9) and control (WT) plant. The positions of major 2C peaks were identical between transgenic

and control plants (Fig. 3-7). There was no other peak except a minor 4C peak in the flow chart.

Discussion

Dry weights of transgenic S1 and U9 plants were 1.4 and 2.1 fold compared to the control WT plant (Fig. 3-5A). However, we could not detect any evidence of cell enlargement because size and density of stomata and relative DNA content of leaf cells were similar between the transgenic and control plants (Table 3-1, Fig. 3-7). This result suggested that cell proliferation was mainly contributed to the growth increase in the transgenic plants.

Growth increases in the transgenic S1 and U9 plants were found in the early stage of seedlings compared to the control WT plants at 20 - 25 DAS (Fig. 3-3). As shown in Fig. 3-4, the growth increase of transgenic S1 plant was observed at 44 and 75 DAS. Later, transgenic S1 plant bloomed flowers at 107 DAS, which was much earlier than the control WT plants (152 DAS). This earliness of flowering time in the transgenic S1 plant was responsible for producing 3-fold seed yield compared the control WT plant (Fig. 3-5B).

The 500-seed weight of the transgenic S1 plants was 17% heavier than that of the control WT plant (Fig. 3-5C). However, this difference of seed weight was difficult to account for the 2-fold growth increase of the transgenic seedlings. Although mechanism underlying growth increase in the transgenic plant is unclear, forced expression of rice *45S rRNA* gene probably triggers to enhance the growth increase.

Sequence homology of *18S*, *5.8S*, and *28S rRNAs* within *45S rRNA* transcripts is more than 90% between rice and tobacco. Whereas homology of internal transcribed spacer (ITS) sequence between two species is about 60%. As the ITS sequences are excised from *45S rRNA* transcripts of eukaryotes to create mature *18S*, *5.8S*, and *28S*

rRNAs, which are components to form ribosome (Udem 1972), the expression of species-specific ITS sequences of full-length *45S rRNA* transcripts might be involved in the growth increase found in the transgenic tobacco and *Arabidopsis* (Makabe et al. 2016).

In this study, transgenic U9 and S1 plants increased dry weight of total aerial tissues to 2.1 and 1.4-fold compared to the control WT plants (Fig. 3-5A), respectively. As hybrid vigor of F_1 line generally increases growth of whole body up to 1.2 – 1.5 fold compared to parental line, the forced expression of rice *45S rRNA* gene using maize ubiquitin promoter can enhance growth more than he hybrid vigor. The UbiP::*Os45SrRNA* transgene could enhance the growth more than 35SP::*Os45SrRNA* because ubiquitin promoter acts in all cells of tobacco plant whereas 35S promoter mainly induces gene expression in vascular tissues (Battraw and Hall 1990).

The forced expression of rice *45S rRNA* is simple technology, which will contribute to enhance the growth increase of transgenic plants. Unlike hybrid vigor, the growth increase by this technology can be fixed as a homozygous allele in cultivars of practical plant species. Because nicotine content was similar between the transgenic and control plant, this technology will be can increase biomass without reducing content of secondary metabolites in medicinal plants. And this technology will contribute to breeding of high yielding cultivars in cereals, vegetables, trees, and especially biomass plants for bio-energy production.

Table 3-1 Measurements of stomatal characteristics in leaves.

Transgenic (S1, U9) and control (WT) leaves were subjected to measure stomatal characteristics at 75 DAS.

	Stomatal density (mm^{-2})	Stomatal length (μm)	Stomatal width (μm)
WT	110.4±2.2	33.9±0.6	29.1±0.5
S1	106.3±6.5	34.7±0.5	29.3±0.4
U9	107.7±4.1	33.8±0.8	28.8±0.5

There were no significant differences. Data represent mean plus standard errors. n = 5.

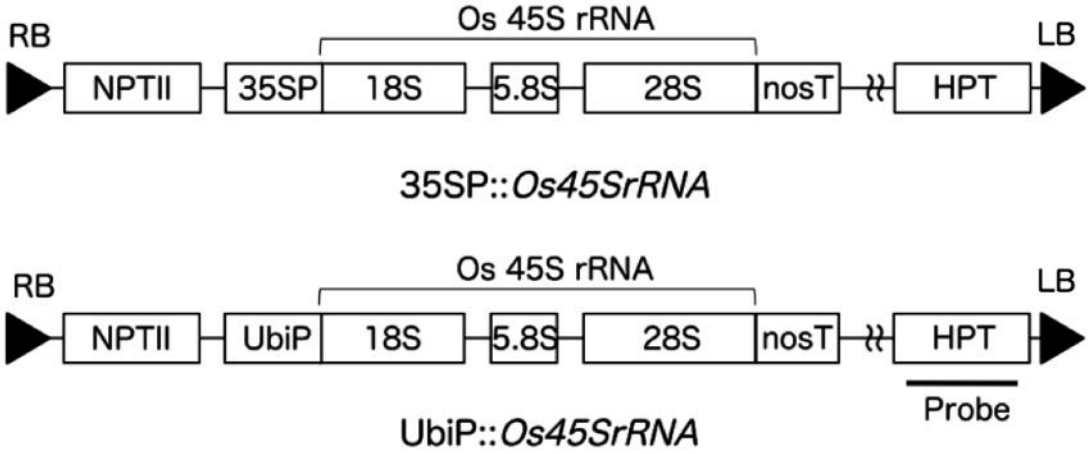


Figure 3-1 Schematic representation of T-DNA regions in the binary vectors used in this study. Full-length *45S rRNA* gene (*Os45SrRNA*, 5.8 kb) of *Oryza sativa* was linked to the maize *ubiquitin* promoter (UbiP) or the CaMV 35S promoter (35SP). The chimeric gene was inserted into *Hin*dIII (H) site between kanamycin (NPTII) and hygromycin (HPT) resistance cassettes of binary vector pEKH to construct pEKH UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA*. (Makabe et al. 2016). PCR product of *hpt* gene was used as probe for Southern blot analysis. RB: right border, LB: left border.

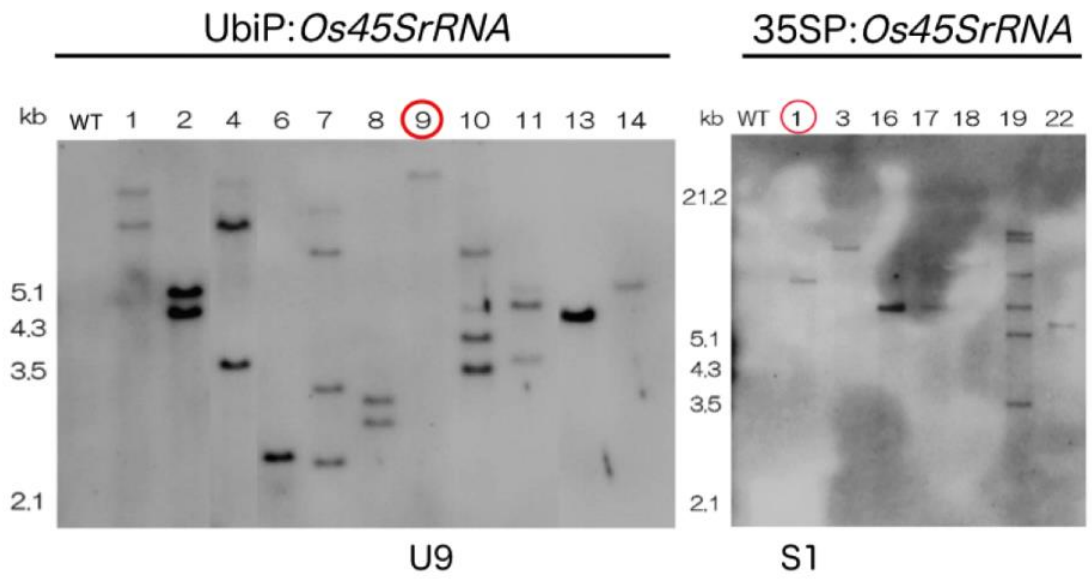


Figure 3-2 Southern blot analysis of transgenic tobacco plants harboring UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA* transgene. *Hin*dIII-digested genomic DNAs extracted from leaves were subjected to agarose gel electrophoresis. Transgenic U9 and S1 lines showed a single band indicating the integration of single-copy of UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* into the nuclear genome, respectively.

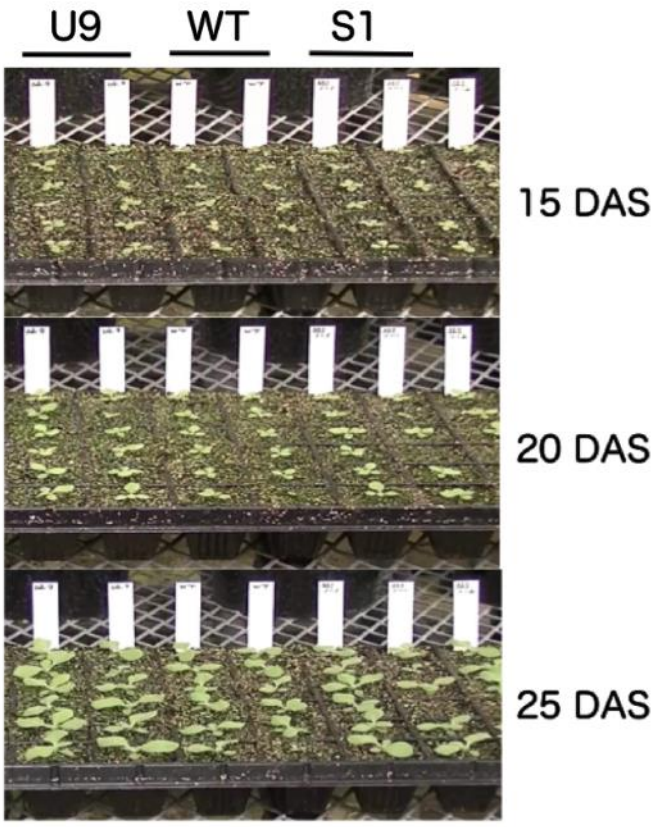


Figure 3-3 Comparison of seedling growth between transgenic and control plants. Transgenic (S1, U9) and control (WT) seedlings were grown side by side in the same tray and photographs were taken at 15, 20, and 25 DAS.

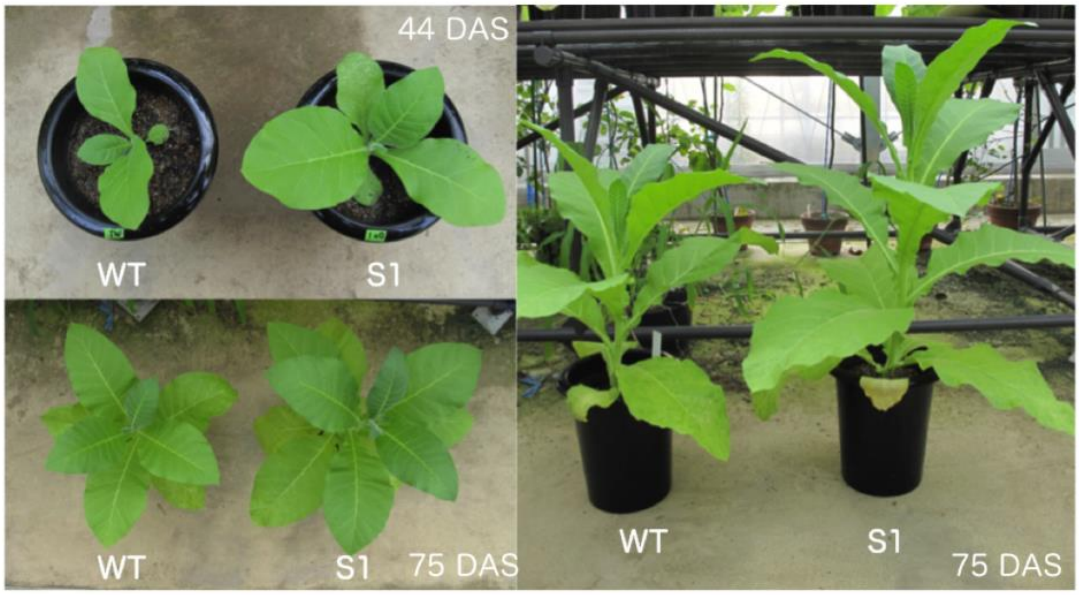


Figure 3-4 Comparison of growth between transgenic and control plants. Transgenic S1 and control WT plants were grown with pots to the maturity in a greenhouse and photographs were taken at 44 and 75 DAS.

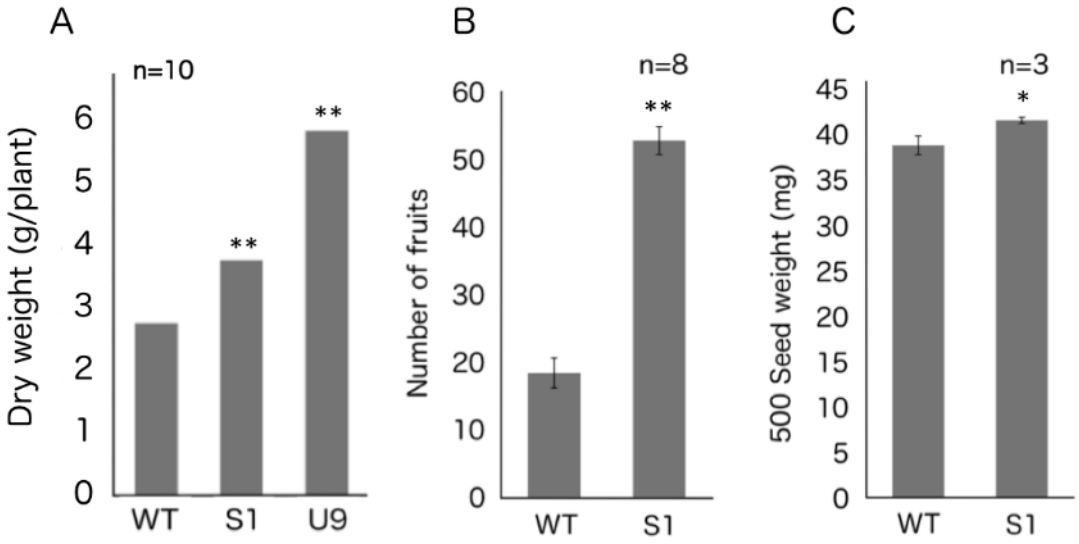


Figure 3-5 Comparison of dry weight between transgenic and control plants. Dry weight (g/plant) of above ground tissues of transgenic (S1, U9) and control (WT) plants were measured at 45 DAS (A). Fruit number (B) and 500 seed weight (C) of transgenic S1 and control WT plants were also measured at the end of the cultivation.

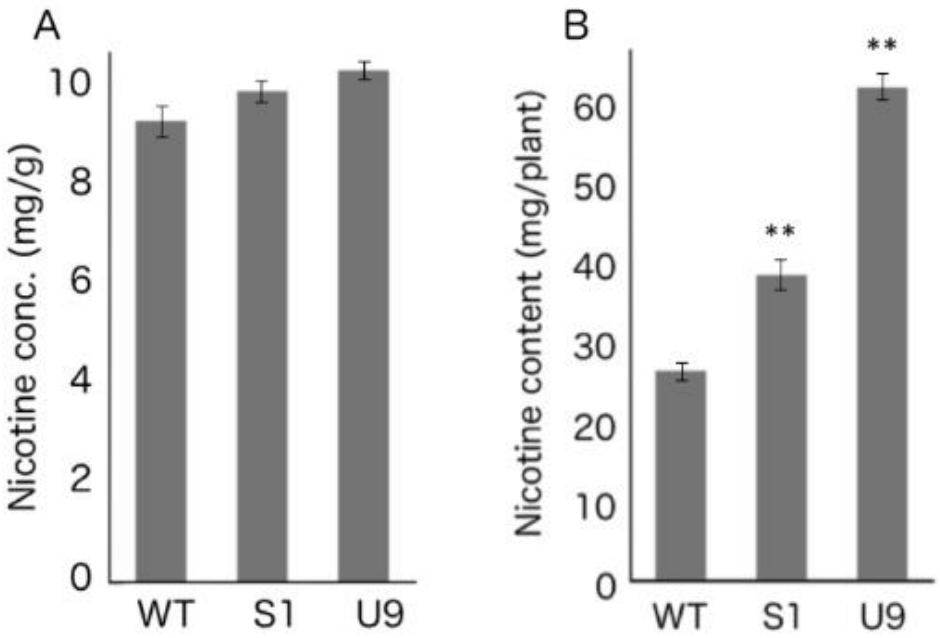


Figure 3-6 Comparison of nicotine content in leaves between transgenic and control plants. Nicotine content per dry weight (mg/g) of transgenic (S1, U9) and control WT leaves were measured at 45 DAS (A). And total nicotine content per plant (mg/plant) was calculated based on the dry weight (B).

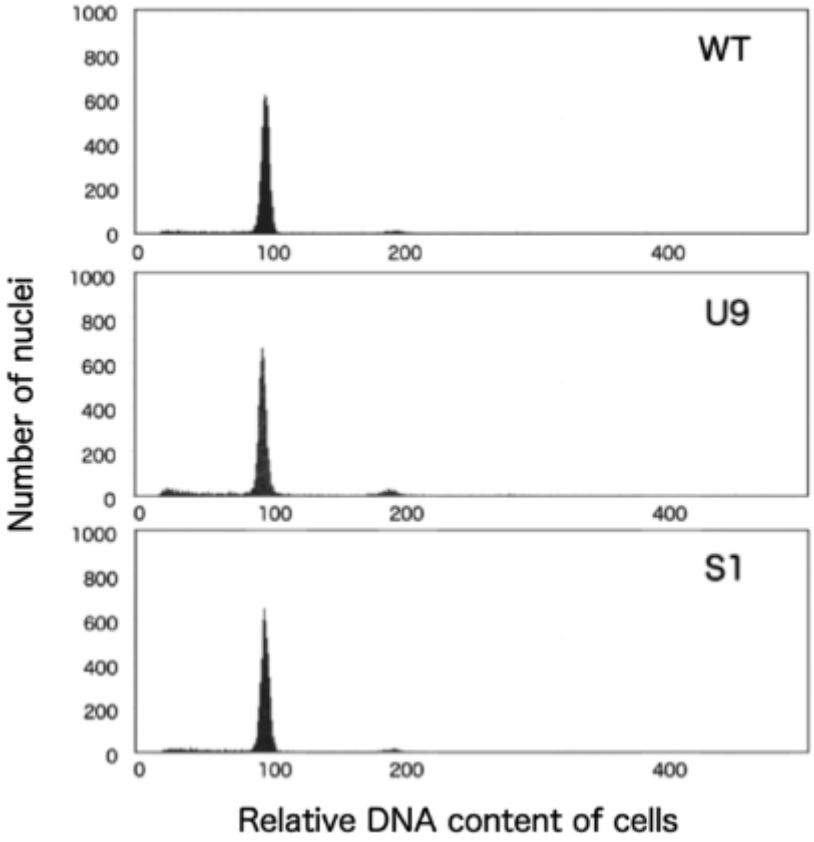


Figure 3-7 Comparison of ploidy level between transgenic and control plants. Relative DNA content leaf cells of the transgenic (S1, U9) and control WT plants were measured at 20 DAS using the flow cytometer.

Chapter 4

Expression of rice 45S rRNA promotes cell proliferation, leading to enhancement of growth in transgenic tobacco

Introduction

We recently reported that the growth of transgenic *Arabidopsis* plants was increased by forced expression of the rice *45S rRNA* gene (Makabe et al. 2016). The transgenic *Arabidopsis* plants showed *ca*. 2-fold increased growth compared with control plants without showing any difference in the size or ploidy level of leaf cells. Although the mechanism underlying this growth increase is unclear, the results showed that forced expression of the rice, not *Arabidopsis*, full-length *45S rRNA* gene was required to increase the growth of transgenic *Arabidopsis*. To confirm that this phenomenon was reproducible in another plant, we produced transgenic tobacco plants harboring the rice *45S rRNA* gene under the control of the maize *ubiquitin* promoter (UbiP::*Os45SrRNA*) and cauliflower mosaic virus (CaMV) 35S promoter (35SP::*Os45SrRNA*).

Growth increases in organisms can be caused by hybrid vigor (Darwin 1876, Meyer et al. 2004, Lippman and Zamir 2007, Hochholdinger and Hoecker 2007). Hybrid vigor appears only at the early stage of plant development through the enhancement of cell proliferation, which is probably mediated by circadian rhythms (Ni et al. 2009, Chen 2010, Fujimoto et al. 2012). Polyploidization can also increase plant biomass through the enlargement of cell size (Kondrosi et al. 2000, Sugimoto-Shirasu et al. 2003). Generally, hybrid vigor and polyploidization cause 1.2–1.5-fold increases in biomass compared with the original plants (Duvick 1999).

Plant growth is regarded as the product of cell number and cell size if sufficient organic materials are supplied by photosynthesis. Therefore, photosynthetic capacity is one of the determinants of plant growth. The photosynthetic capacity is mediated by various factors, such as the integrity of the photosynthetic machinery, leaf morphology,

and environmental stresses (Saibo et al. 2009). Recently, the physiological state of photosynthesis in intact leaves has been analyzed using pulse amplitude modulation (PAM) (Woo et al. 2008). PAM analysis can measure chlorophyll fluorescence to estimate a wide range of photosynthetic parameters, such as the photosynthesis rate, non-photochemical quenching (NPQ), stomatal conductance, and the electron transport rate. This method can also generate a two-dimensional image of the photosynthetic rate by scanning chlorophyll fluorescence from photosystem II (φPSII). Simultaneously, because the exchange of carbon dioxide, water vapor, and oxygen through stomata significantly affects the photosynthetic capacity, plants optimize their gas exchange efficiency by regulating stomatal aperture size, density, pore openness, and distribution patterns, all of which affect stomatal conductance (Scheidegger et al. 2000).

Leaf development in plants is an important factor that affects the photosynthetic capacity (Tsukaya 2006). To increase the photosynthetic capacity, plants have to enlarge their leaf area to the widest extent possible to capture the sun's energy. In leaf development, cell division first occurs in the primordium and then cell expansion follows to achieve the final leaf size. To date, much knowledge has been accumulated on the genetic regulation of cell proliferation and cell expansion in leaves. However, the detailed mechanism underlying the determination of final leaf size has yet to be elucidated. Several *Arabidopsis* mutants for ribosomal protein genes have shown developmental changes in leaf size (Van Lijsebettens et al. 1994, Ito et al. 2000, Byrne 2009, Fujikura et al. 2009, Horiguchi et al. 2011, Horiguchi et al. 2012, Rosado et al. 2012, Zsogon et al. 2014). Thus, some ribosome-related process might be involved in the co-ordination of cell proliferation and cell expansion in leaf development.

Makabe et al. (2016) found that forced expression of the rice *45S rRNA* gene caused a growth increase in transgenic *Arabidopsis.* The eukaryotic *45S rRNA* gene, consisting of the *18S*, *5.8S,* and *28S rRNAs*, is transcribed as a single transcription unit and post-transcriptionally processed into three rRNA molecules (Appels et al. 1982). As the three *rRNA* sequences within the *45S rRNA* transcript are highly conserved between rice and *Arabidopsis*, the expression of the two internal transcribed spacers (ITSs), ITS1 between the *18S* and *5.8S rRNAs* and ITS2 between the *5.8S* and *28S rRNAs*, might be involved in the growth increase in transgenic *Arabidopsis*.

In this study, we produced transgenic tobacco plants with forced expression of the rice *45S rRNA* gene and analyzed their leaf photosynthetic and morphological traits in detail.

Materials and methods

Plant material

Plantlets of *Nicotiana tabaccum* 'Petit Havana' SR-1 line were maintained in culture bottles under sterile condition and used for the production of transgenic plants.

Production of transgenic tobacco plants

Full-length of *45S rRNA* gene (DDBJ Accession No. LC086814) in *Oryza sativa* ssp. Indica N16 line was amplified (Makabe et al. (2016). The *Os45SrRNA* fragment (5.8 kb) was ligated between maize *ubiquitin* promoter (1.0 kb without the first intron) (Christensen and Quail 1996) and nopaline synthase gene terminator (*nos*T) or between CaMV 35S promoter and *nos*T to construct UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA* chimeric gene, respectively (Makabe et al. 2016). These chimeric genes were inserted into a binary vector pEKH (Takesawa et al. 2002) at *Hin*dIII site between kanamycin and hygromycin resistance cassettes (Fig. 4-1B). The binary vector was mobilized to *Agrobacterium tumefaciens* EHA101 by freeze-thaw method and transformation of tobacco plant was done by leaf-disc method (Horsch et al. 1985). Transgenic plants were selected on 50 mg L^{-1} kanamycin-containing MS media (Murashige and Skoog 1962).

Southern blot analysis

Leaf samples were frozen using liquid nitrogen and crushed into fine powder using a Multi-beads Shocker (Yasui Kikai, Kyoto, Japan). Genomic DNA was extracted from 100 mg of leaf tissues using the modified CTAB method (Doyle and Doyle 1987). *Hin*dIII-

digested genomic DNA (5 µg) was separated through 0.9% agarose gel, blotted to Immobilon-Ny+ membrane (Millipore Corporation, USA), and hybridized with a digoxigenin-labeled probe of hygromycin phosphotransferase (*hpt*) gene according to the supplier's instructions (Roche Diagnostics, Mannheim, Germany). Hybridization with the DIG-labeled *hpt* probe was carried out at 39℃ for 16 h. The membrane was treated with anti-DIG alkaline phosphatase and substrate CPD-star (Roche Diagnostics, Mannheim, Germany). Then, the membrane was exposed to Hyperfilm TM-MP X-ray film for 30 min at room temperature.

RT-PCR analysis

Total RNAs were extracted from leaves (100 mg) of transgenic and non-transformation plants using Plant RNA Reagent (Life Technologies, USA). First strand cDNA were synthesized from 1 µg of total RNA in a 20 µl reaction volume using Superscript Transcriptase III (Life Technologies, USA) with oligo dT (20) primer. Two pairs of RT-PCR primers, ITS5P: 5'-CGCGATACCACGAGCT AAATCCAC-3' – ITS3P2: 5'-GTCCGAGGCGTTCGCTCTCGGTGC-3' and actin5P: 5'-GAAA ATGGTGAAGGCTGGTTTTG-3' – actin3P: 5'-AGGATTGATCCTCCGATCCAGA-3' were designed to amplify ITS (ITS1 – 5.8S – ITS2) region of rice *45S rRNA* and *actin* mRNA (positive control), respectively.

Plant growth analysis in a growth chamber

Transgenic (S1. U9) and control (WT) plants were grown in an environmentally controlled growth chamber (Yamori et al. 2011). The chamber for all the plants was

operated with a day/night temperature of 25/20˚C, a PPFD of 500 µmolm^{-2} s^{-1}, a 12h photoperiod and a CO_2 concentration of 400 µmolmol^{-1}. Plants were grown in garden mix containing approximately 2 gL^{-1} of a slow-release fertilizer (Osmocote, Scotts Australia, Castle Hill, Australia). Plant growth analysis was performed at 24, 34, 44, and 50 DAS.

Gas-exchange and chlorophyll fluorescence measurements

CO_2 gas exchange and chlorophyll a fluorescence was measured in fully expanded leaves of the transgenic (S1, U9) and control (WT) plants with a portable gas exchange system (LI-6400, LI-6400-40 leaf chamber fluorometer, LI-COR) (Yamori et al. 2011). The light response of CO_2 assimilation rate was measured at a CO_2 concentration of 400 µmol mol^{-1} and 25^{0}C and 65% relative humidity. Non-photochemical quenching (NPQ) was calculated as NPQ = (Fm – Fm')/Fm'. The quantum yield of photosystem II (φPSII) was calculated as φPSII = (Fm' – F')/Fm', and the electron transport rate (ETR) was calculated as ETR = 0.5 x absI x φPSII, where 0.5 is the fraction of absorbed light reaching PSII and absI is absorbed irradiance taken as 0.84 of incident irradiance. Data represent means ± SE.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence images were taken in plants of 10-, 17- and 24-DAS with a chlorophyll fluorescence imaging system (IMAGING-PAM, Walz, Effeltrich, Germany) (Yamori et al. 2011). Leaves were dark adapted for 20 min prior to determination of chlorophyll fluorescence. Then, plants were placed at 500 μmol photons $m^{-2}s^{-1}$, which is similar to the growth light condition, for 20 min. The quantum yield of photosystem II [φPSII = (Fm' – F')/Fm'], photochemical quenching [qP = (Fm' – F')/(Fm' – Fo')], non-photochemical quenching [NPQ = (Fm – Fm')/Fm'], and the fraction of PSII centers in the open state (with QA oxidized) [qL = qP x (Fo'/F')] were calculated using the software ImagingWin. Data represent means ± SE.

Analysis of photosynthetic activity

Seeds of transgenic (S1, U9) and control (WT) lines were sown in small pots within an environmental controlled growth chamber as described (Yamori et al. 2011). Leaves were exposed to strong light at 2,000 μmol photons $m^{-2}s^{-1}$ at the corresponding temperature for 90 min. The fraction of active PSII (Fv/Fm) was measured after dark incubation for 30 min. Data represent means ± SE, n = 5.

Quantifications of photosynthetic components

Immediately after the measurements of gas exchange, leaf samples were taken, immersed in liquid nitrogen and stored at −80ºC until determinations of chlorophyll and RuBisCO. Contents of leaf chlorophyll and RuBisCO were quantified according to Yamori et al. (2011). Data represent means ± SE, n = 5.

Flow cytometric analyses

Relative DNA content per nuclei of somatic cells in cotyledon, root and hypocotyl of seedling at 7, 8, 10, 14, 20 days after sowing (DAS) were measured in triplicate using the laser flow cytometer PAS CA-IV (Partec GmbH, Germany) (Mishiba and Mii 2000). Cell division activity was indicated as the 4C/2C ratio. Mature leaf cells were also analyzed to check polysomaty at 30 DAS. Data represent means ± SE.

Nicotine content

Crude extracts of nicotine were prepared from aboveground tissues (10 mg) at 30 DAS according to the CORESTA Recommended Method (CRM No. 72) (CORESTA 2013). The crude extract was subjected to liquid chromatography mass spectrophotometer LC-MS/MS 8030 (Shimadzu Scientific Instrument, Japan).

Microarray analysis

Total RNA was isolated from aerial parts of transgenic (S1, U9) and control (WT) seedlings (12 DAS) using RNeasy Plant Mini Kit (Qiagen, Valencia, CA). We entrusted

microarray analysis to DNA Chip Research Institute (Yokohama, Japan) using Agilent tobacco oligo-DNA microarray (Agilent Technologies, Palo Alto, CA).

Results

Production of transgenic tobacco plants expressing the rice *45S rRNA* gene

We produced 14 and seven transgenic tobacco plants harboring the UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* transgenes (Fig. 4-1A), respectively. Transgenic lines harboring a single-copy transgene were selected by Southern blot analysis (Supplementary Fig. S1) and homozygous lines for the transgene were obtained by genetic analysis for kanamycin resistance (data not shown).

Comparison of the initial growth of transgenic tobacco in a growth chamber

Seedlings of T_2 transgenic lines, which were homozygous for the UbiP::*Os45SrRNA* (U9) and 35SP::*Os45SrRNA* (S1) transgenes, and control (WT) plants were grown together in small pots within a growth chamber. Under these conditions, the transgenic S1 and U9 plants showed similar growth to the WT plants at 10 DAS (Fig. 4-1B). In contrast, the aboveground growth of the transgenic S1 and U9 plants was greatly increased compared with control plants at 17 DAS and later. Semi-quantitative RT-PCR showed that the two transgenic (S1, U9) plants expressed the ITS region of the rice *45S rRNA* transcript at similar low levels compared with the *actin* gene used as an amplification standard (Fig. 4-1C).

Detailed measurements at 44 DAS showed that the leaf length and leaf area of the 1st to 3rd leaves from the top were similar between transgenic (S1, U9) and control plants, but started to increase from the 4th leaf in the transgenic plants compared with the control

plants (Fig. 4-2A, B). The total leaf area and dry weight of the S1 and U9 plants were increased up to 2-fold compared with control plants at 34 DAS (Fig. 4-2C, D). The values of U9 plants were a bit higher than those of S1 plants. Later, these growth differences were reduced to *ca*. 1.4-fold at 50 DAS in this experiment.

Comparison of plant growth between transgenic and control plants

As shown in Fig. 4-3A, transgenic (S1, U9) and control (WT) plants were grown in growth chamber conditions and their leaves were aligned from the bottom to the top at 44 DAS. Although clear growth differences were observed in the first four leaves, these differences became smaller in later leaves, probably because of the limited fertilizer in the small pots. U9 plants increased in size compared with S1 plants when they were planted in a large tray within a growth chamber (Supplementary Fig. S2). In the greenhouse conditions, the S1 and U9 plants showed 1.4- and 2.1-fold increases in the dry weight of aerial tissues, respectively, compared with the WT plants at 45 DAS (Makabe et al. 2017). The S1 plants grew bigger than the WT plants at 75 DAS in a greenhouse (Fig. 4-3B). The S1 plants produced their first flowers at 107 DAS (Fig. 4-3C–E). This was much earlier than the flowering of the WT plants at 152 DAS. Therefore, the fruit number of the S1 plants was *ca*. 2.8-fold higher than that of the WT plants. In addition, the 500-seed weights of the S1 and U9 plants were 1.1–1.2-fold greater than that of the WT plants. In addition, although the nicotine concentration in the leaves, a secondary metabolite of tobacco, was similar between the transgenic and control plants, total nicotine production was increased by 1.4- and 2.1-fold in the S1 and U9 leaves, respectively, compared with the WT leaves.

Comparison of photosynthetic parameters

Chlorophyll fluorescence images were analyzed using seedlings at 10, 17, and 24 DAS under the growth light intensity. In the IMAGING-PAM analysis, the fluorescence changed from orange to pale-blue, indicating low to high photosynthetic capacities. There were no differences between transgenic (S1, U9) and control plants in the two-dimensional fluorescence images (Fig. 4-4) or photosynthetic parameters, such as φPSII, the reduction state of PSII (1-qL), and NPQ at any DAS (Supplementary Table S1).

The light-intensity responses of several photosynthetic parameters in leaves were measured at 44 DAS (Fig. 4-5). The photosynthetic rate, stomatal conductance, electron transport rate, and NPQ at a CO_2 concentration of 400 μm mol^{-1} were all similar between the transgenic and control plants. These data indicate that the S1, U9 and WT plants had the same photosynthetic capacity per unit leaf area.

Comparison of photosynthetic components

The contents of RuBisCO and chlorophyll per unit leaf area were similar between the transgenic (S1, U9) and control plants at 44 DAS (Table 4-1). All stomatal characteristics, including stomatal size, density and index, were also similar between the transgenic and control plants.

Cell division activities of tobacco seedlings

Transgenic U9 seedlings developed true leaves and root hairs earlier than WT seedlings at 10 DAS (Fig. 4-6A) and their secondary roots started to grow at 14 DAS

(data not shown). Flow cytometry analysis showed that cell division activity, i.e. relative DNA content per nucleus of 4C (G2/M phase) *vs*. 2C (G1 phase), increased in roots (10 DAS) and hypocotyls (14 DAS) in U9 and WT seedlings (Fig. 4-6B, raw data was shown in Supplementary Table S2). Because it was difficult to excise hypocotyls from roots at 7 DAS, these tissues were analyzed together as roots. In contrast, cotyledon cells had very low 4C/2C ratios in both seedlings. U9 seedlings showed higher 4C/2C ratios in their roots (7 DAS and 14–20 DAS) and hypocotyls (8 DAS) than WT seedlings.

Microarray analysis

Microarray analysis of the aerial parts of 12 DAS seedlings revealed that 37 and 45 genes were more than 2-fold up- or down-regulated, respectively, in both S1 and U9 plants compared with WT plants (Supplementary Tables S3, S4). Of the 37 up-regulated genes, most genes encoded functional and structural proteins, such as 5-epi-aristolochene synthase, P-rich protein NtEIG-C29, and glutathione S-transferase. Genes involved in transcription, translation, and signal transduction are listed in Table 4-2. These 23 genes encoded a transformer SR ribonucleoprotein, 60S ribosomal protein L30-like, blue light photoreceptor PHR2, translation initiation factor 5A1, MOB kinase activator like 1, two receptor kinases, four transcription factors (2 GIGANTEA-like, homeobox leucine-zipper HAT7-like, Lateral Organ Boundaries (LOB) domain-containing protein 41-like), and 12 auxin repressed protein (ARP)-like proteins. Of the 45 down-regulated genes, eight genes encoding two mitochondrial 39S ribosomal protein L41A-like proteins, a splicing specificity factor, three signal transduction related proteins, and two transcription factors (TGA10 and LEUNIG-like corepressor) are listed in Table 4-2.

Discussion

Transgenic tobacco seedlings harboring a single copy of the 35SP::*Os45SrRNA* (S1) or UbiP::*Os45SrRNA* (U9) transgene (Fig. 4-1A) showed increased growth at 17 DAS compared with the control (WT) plants in growth chamber conditions (Fig. 4-1B). As shown in Fig. 4-2A and 4-2B, the enlargement of transgenic (S1, U9) leaves started at the 4–5th leaf from the top. At this leaf stage, because cell expansion becomes more prominent than cell proliferation, the proliferation of leaf cells might have occurred in the transgenic seedlings at an earlier stage. Although the total leaf area and dry weight in S1 and U9 plants were increased by up to 2-fold compared with WT plants at 34 DAS, the difference in growth was reduced to *ca*. 1.4-fold at 50 DAS (Fig. 4-2C, D). This growth retardation during the late stage was considered to be due to fertilizer deficiency and/or limited growth of the root system because the plants were grown in small pots within a growth chamber. In fact, U9 plants seemed to grow bigger than S1 plants when they were grown in a larger tray (Supplementary Fig. S2).

In the greenhouse, transgenic tobacco plants reached the flowering stage much earlier than control plants (Fig. 3C–E). Therefore, it is possible to produce a 2–3-fold seed yield increase in transgenic tobacco plants because the fruit number in S1 plants was increased by 2.8-fold compared with WT plants. Although the 500-seed weight of the U9 plants was 17% greater than that of the WT plants, this difference cannot account for the 2-fold growth increase of the transgenic seedlings. In addition, transgenic (S1, U9) leaves had a 14% higher nicotine content than WT leaves at 30 DAS. As older leaves have a higher nicotine content than younger leaves in tobacco plants (Igaki 1929), this probably

reflects a difference in leaf age between the transgenic and control plants (Fig. 4-3C).

In the IMAGING-PAM analysis, transgenic (S1, U9) and WT plants showed the same photosynthetic capacity (Fig. 4-4). Four different parameters affecting the photosynthetic capacity, including the photosynthesis rate, stomatal conductance, NPQ, and the electron transport rate under various light intensities, showed the same values in transgenic and control plants (Fig. 4-5A–D). In addition, the RuBisCO and chlorophyll contents and all stomatal characteristics were similar between the transgenic and control plants (Table 4-1). These data indicate that forced expression of the rice *45S rRNA* gene promotes up to 2-fold increased aboveground growth without changing the photosynthetic and stomatal characteristics of transgenic plants.

Because tobacco plants do not show much polysomaty, like *Arabidopsis thaliana*, the cell division activity was inferred from the 4C/2C ratio using a flow cytometer. Interestingly, the maximum peaks of cell division activity differed between (secondary) roots (10 DAS) and hypocotyls (14 DAS) in both U9 and WT seedlings (Fig. 4-6B). These data suggest that root system development might occur faster than aerial tissue development in tobacco plants. When compared with WT seedlings, U9 seedlings showed higher cell division activity in 7 DAS roots and 8 DAS hypocotyls. Similarly, transgenic *Arabidopsis* seedlings showed a well-developed root system compared with control seedlings (Makabe et al. 2016). Therefore, higher cell division activity in the root and leaf primordia at the early seedling stage is probably important for the enhanced growth in the transgenic plants.

Microarray analysis was performed using mRNAs extracted from the aerial parts of 12 DAS seedlings because phenotypic growth differences between the transgenic (S1,

U9) and control seedlings were found at 10 DAS (Fig. 4-6A). Among the more than 2-fold up- and down-regulated genes, 23 (of 37) and 8 (of 45) genes are listed in Table 4-2, respectively. The up-regulation of two GIGANTEA (GI)-like transcription factor genes is interesting because *Arabidopsis GI* genes are controlled by the circadian rhythm and regulate flowering time genes (Fowler et al. 1999). The up-regulated ribosomal L30 and down-regulated mitochondrial S41A-like genes are also interesting because mutations of several ribosomal protein genes affect the regulation of cell proliferation and expansion in *Arabidopsis* leaves (Tsukaya 2006). Although 12 auxin repressed protein ARP1-like genes were up-regulated, they were probably induced to suppress overgrowth of organs in the transgenic plants because overexpression of the *ARP1* gene represses plant growth (Zhao et al. 2014).

In the transgenic *Arabidopsis*, several ethylene-responsive transcription factor genes were up-regulated in 12–14 DAS seedlings (Makabe et al. 2016). However, such genes, up- or down-regulated in the transgenic *Arabidopsis*, were not detected in the microarray analysis of transgenic tobacco seedlings (12 DAS). Although the reasons for the differences in gene expression between *Arabidopsis* and tobacco are unclear, microarray analysis of seedlings at 12–14 DAS was too late to resolve the genes that were responsible for the growth increase in the transgenic *Arabidopsis*. The flow cytometry analysis in this study suggests that the transcriptomes in the root and leaf primordia of tobacco seedlings need to be analyzed before 7 DAS.

Semi-quantitative PCR showed that the S1 and U9 plants expressed the rice *45S rRNA* at a similar level (Fig. 4-1C). However, the expressed rice *45S rRNA* transcripts might not play roles as rRNA molecules because they were expressed at a low level.

Because the sequences of the *18S*, *5.8S*, and *28S rRNAs* within the *45S rRNA* transcript are highly homologous between rice and tobacco, the expression of species-specific ITS sequences might be responsible for the growth increase in the transgenic tobacco and *Arabidopsis* (Makabe et al. 2016).

Plant leaf development is governed through mechanisms that regulate the number and size of leaf cells (Palatnik et al. 2003). Therefore, co-ordination between cell proliferation and post-mitotic cell expansion mediates the final leaf size (Gonzalez et al. 2010). Several *Arabidopsis* mutants with defective genes for cell proliferation show increased cell expansion in their leaves (Horiguchi et al. 2005). Polyploidization can also cause a growth increase in plants through cell expansion (Miller et al. 2012). The aboveground growth increase in the transgenic tobacco plants was not caused by cell expansion because the size and number of stomatal guard cells (Table 4-1) and the ploidy level of leaf cells (Supplementary Fig. S3) were similar between the transgenic and control leaves. Stomatal size is considered a good indicator of the ploidy level in plant cells (Wood et al. 2009), and only the total leaf area and dry weight of transgenic tobacco plants were increased by *ca*. 2-fold compared with control plants. Taking previous findings together with the results of this study, forced expression of the rice *45S rRNA* accelerates cell proliferation without changing the morphological and physiological traits of somatic cells in the transgenic plants.

The forced expression of exogenous *45S rRNA* (FEE45) is a simple technology that will contribute to increasing the growth of transgenic plants. Unlike hybrid vigor, the growth increase by FEE45 can be fixed as a homozygous allele in practical cultivars. The mechanism of the growth increase through the enhancement of cell proliferation at the early seedling stage might be related between hybrid vigor and FEE45. The FEE45

technology could be applied to increase the production of secondary metabolites in medicinal plants and to breed high-yielding cultivars of cereals, vegetables, trees, and especially biomass plants for bio-energy production.

Table 4-1 Measurements of RuBisCO and chlorophyll contents, and stomatal characteristics in leaves.

Transgenic S1/U9 and control WT leaves at 44 DAS were subjected to measure stomatal characteristics and content of RuBisCO and chlorophyll according to Yamori *et al*. (2011).

	RuBisCO ($g\ m^{-2}$)	Chlorophyll ($g\ m^{-2}$)	Stomatal density (mm^{-2})	Stomatal index	Stomatal length (μm)	Stomatal width (μm)
WT	1.17±0.07	0.366±0.015	270±12.8	0.278±0.009	24.7±0.4	15.5±0.3
S1	1.20±0.06	0.370±0.018	274±12.5	0.254±0.028	24.7±0.5	15.0±0.4
U9	1.24±0.04	0.373±0.013	278±10.5	0.263±0.007	24.4±0.6	15.1±0.3

There were no significant differences between S1/U9 transgenic and WT control plants. Data represent mean plus standard errors. n = 5.

Table 4-2 More than 2-fold up- or down-regulated genes in both S1 and U9 transgenic plants

Probe Name	Description	Fold change	
		S1 vs WT	U9 vs WT
A_95_P005211	Transformer-SR ribonucleoprotein	5.67	5.82
A_95_P225937	Blue-light photoreceptor PHR2 (LOC104104471	4.54	6.29
A_95_P093968	60S ribosomal protein L30-like (LOC104229770)	4.38	4.29
A_95_P091298	Eukaryotic translation initiation factor 5A-1 (LOC104242538)	4.64	2.77
A_95_P258451	LOB domain-containing protein 41-like (LOC104239409)	4.22	2.72
A_95_P297428	GIGANTEA-like (LOC104104191)	2.45	3.97
A_95_P108877	Auxin-repressed protein (ARP1)-like	3.17	3.20
A_95_P114717	Auxin-repressed protein (ARP1)-like	2.92	3.08
A_95_P105487	Auxin-repressed protein (ARP1)-like	2.58	3.38
A_95_P163447	Membrane located receptor kinase-like protein (NtC7)	2.04	3.88
A_95_P105232	Auxin-repressed protein (ARP1)-like	2.67	3.24
A_95_P176997	Auxin-repressed protein (ARP1)-like	2.48	3.37
A_95_P110457	Auxin-repressed protein (ARP1)-like	2.71	2.87
A_95_P106487	Auxin-repressed protein (ARP1)-like	2.69	2.87
A_95_P114372	Auxin-repressed protein (ARP1)-like	2.71	2.74
A_95_P177002	Auxin-repressed protein (ARP1)-like	2.59	2.81
A_95_P310088	G-type lectin S-receptor-like serine/threonine-protein kinase	2.63	2.71
A_95_P106782	Auxin-repressed protein (ARP1)-like	2.42	2.84
A_95_P107032	Auxin-repressed protein (ARP1)-like	2.16	2.67
A_95_P092983	Homeobox-leucine zipper protein HAT7-like (LOC104232387)	2.46	2.32
A_95_P003171	Auxin-repressed protein (ARP1)-like	2.06	2.63
A_95_P094463	MOB kinase activator-like 1 (LOC104233287)	2.18	2.29
A_95_P025081	GIGANTEA-like (LOC104222517)	2.04	2.07
A_95_P125607	Splicing specificity factor subunit 3-I-like (LOC104216883)	-25.23	-2.05
A_95_P233824	Mitochondrial 39S ribosomal protein L41A-like (LOC104224887)	-4.26	-4.39
A_95_P014791	Mitochondrial 39S ribosomal protein L41A-like (LOC104224887)	-4.19	-4.34
A_95_P131377	T-complex protein 1 subunit eta (LOC104236456)	-5.97	-2.54
A_95_P239499	Putative GEM-like protein 8 (LOC104228486)	-3.35	-2.44
A_95_P299943	Putative virus-specific-signaling-pathway regulated protein	-2.10	-3.12
A_95_P034838	TGA10 transcription factor	-2.39	-2.62
A_95_P065840	Transcriptional corepressor LEUNIG-like (LOC104246300)	-2.07	-2.13

Probe name: based on Agilent tobacco oligo-DNA microarray

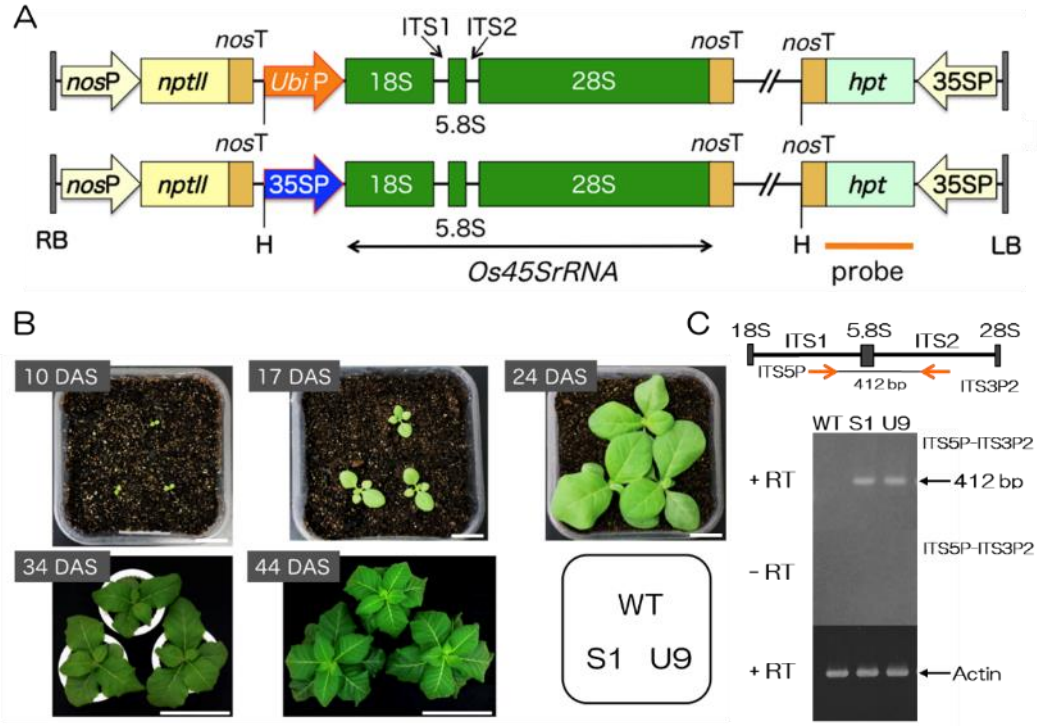


Figure 4-1 Production of transgenic tobacco plants with the forced expression of the rice *45S rRNA* gene using the maize *ubiquitin* or CaMV 35S promoter. (A**)** Schematic representation of transgenes; Full-length *45S rRNA* gene (*Os45SrRNA*, 5.8 kb) of *Oryza sativa* ecotype Indica cultivar N16 line was linked to the maize *ubiquitin* promoter (*Ubi*P) or the CaMV 35S promoter (35SP). The chimeric gene was inserted into *Hin*dIII (H) site between kanamycin (*nos*P-*nptII*-*nos*T) and hygromycin (35SP-*hpt*-*nos*T) resistance cassettes of binary vector pEKH to construct pEKH UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA* (Makabe et al. 2016). PCR product of *hpt* gene was used as probe for Southern blot analysis. (B**)** Comparison of growth between transgenic (S1, U9) and control (WT) plants in a growth chamber. Transgenic S1 and U9 plants having a single-copy of transgene were selected by Southern blot analysis for hygromycin resistance gene and segregation analysis for kanamycin resistance gene (Supplementary Fig. S1). Photographs were taken at 10, 17, 24, 34, and 44 DAS. 10, 17 and 24 DAS (bar = 2 cm), 34 DAS (bar = 20 cm), 44 DAS (bar = 40 cm). (C) Semi-quantitative RT-PCR was performed to detect the expression of ITS regions within rice 45S rRNA transcripts in transgenic S1 and U9 plants. A pair of primers, ITS5P and ITS3P2, was designed based on the rice ITS sequences. S1 and U9 plants showed similar amount of PCR product, which was absent in WT plant and without adding reverse transcriptase (−RT). *Actin* mRNA was also amplified as an internal standard.

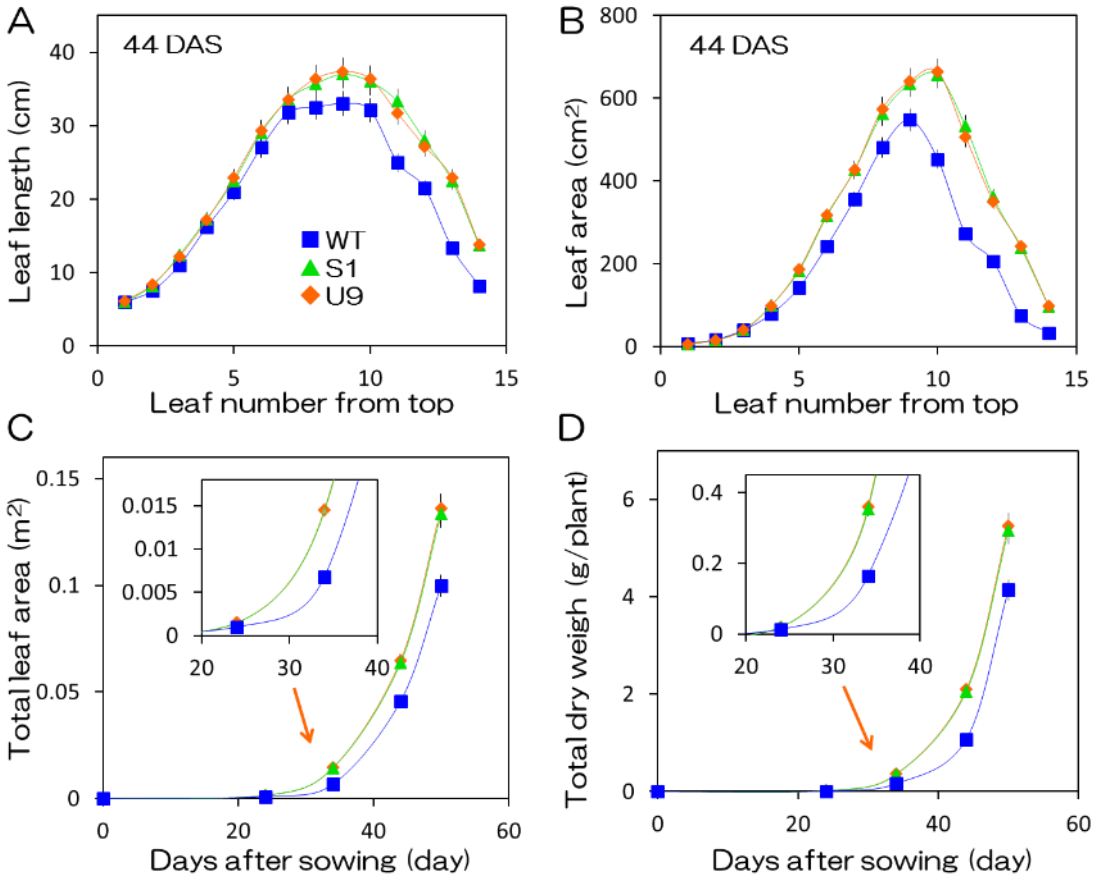


Figure 4-2 Comparison of aboveground growth between transgenic and control plants. Transgenic (S1, U9) and control (WT) plants were grown in small pots within a growth chamber and the leaf characters were measured at 44 DAS. (A) Length (cm) of each leaf. (B) Area (cm2) of each leaf. (C) Time-course of total leaf area (cm2). (D) Time-course of total dry weight (g/plant). Transgenic S1 and U9 plants showed similar growth patterns in this condition.

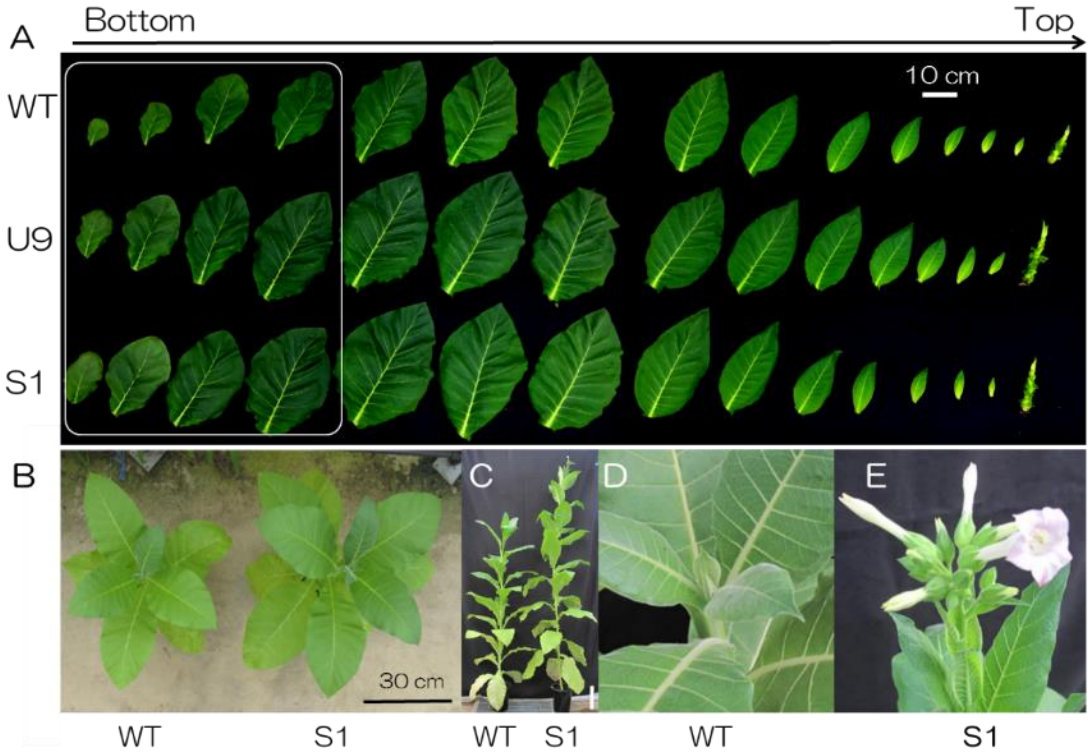


Figure 4-3 Comparison of growth between transgenic and control plants. (A) Leaves of transgenic (S1, U9) and control WT plants were aligned from the bottom to the top. The first four leaves of transgenic plants were clearly bigger than those of the control plant (box). (B) Photograph of transgenic S1 and control WT plants at 75 DAS. (C) Photograph of transgenic S1 and control WT whole plants at 107 DAS, bar = 20 cm. At that time, S1 plant started flowering (D) while WT plant did not have any flower bud (E).

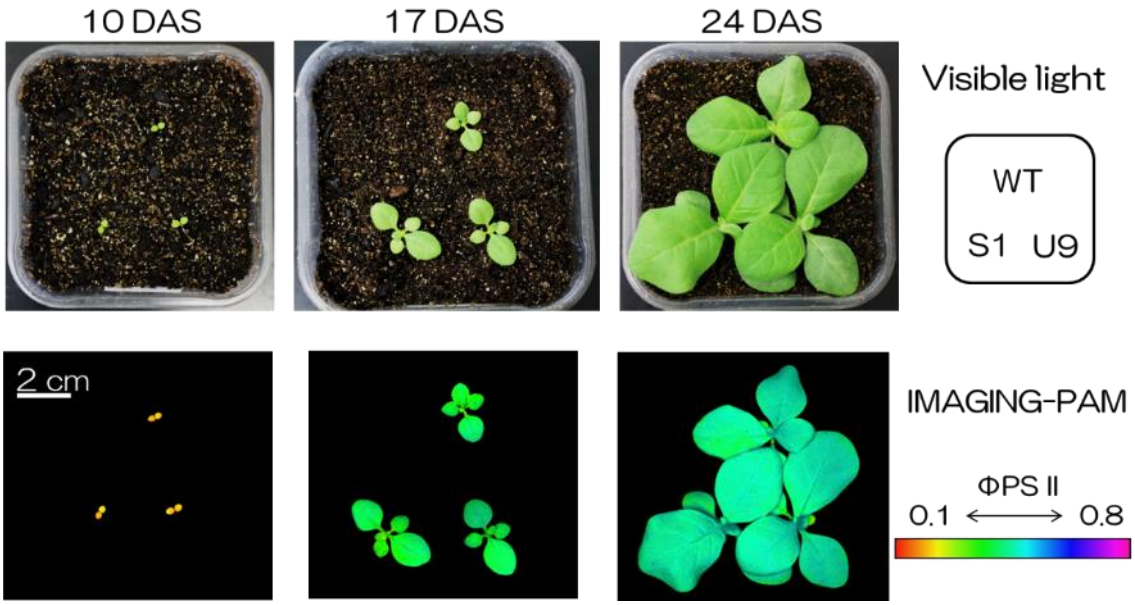


Figure 4-4 Comparison of photosynthetic capacity between transgenic and control plants using IMAGING-PAM analysis. Seedlings of transgenic (S1, U9) and control (WT) plants were grown together in the same pot and images were taken at 10, 17, and 24 DAS. Top: images under visible light. Bottom: Two-dimensional images by the IMAGING-PAM indicated low (orange at 10 DAS) to high (light blue at 24 DAS) photosynthetic capacity corresponding to φPSII indicator bar.

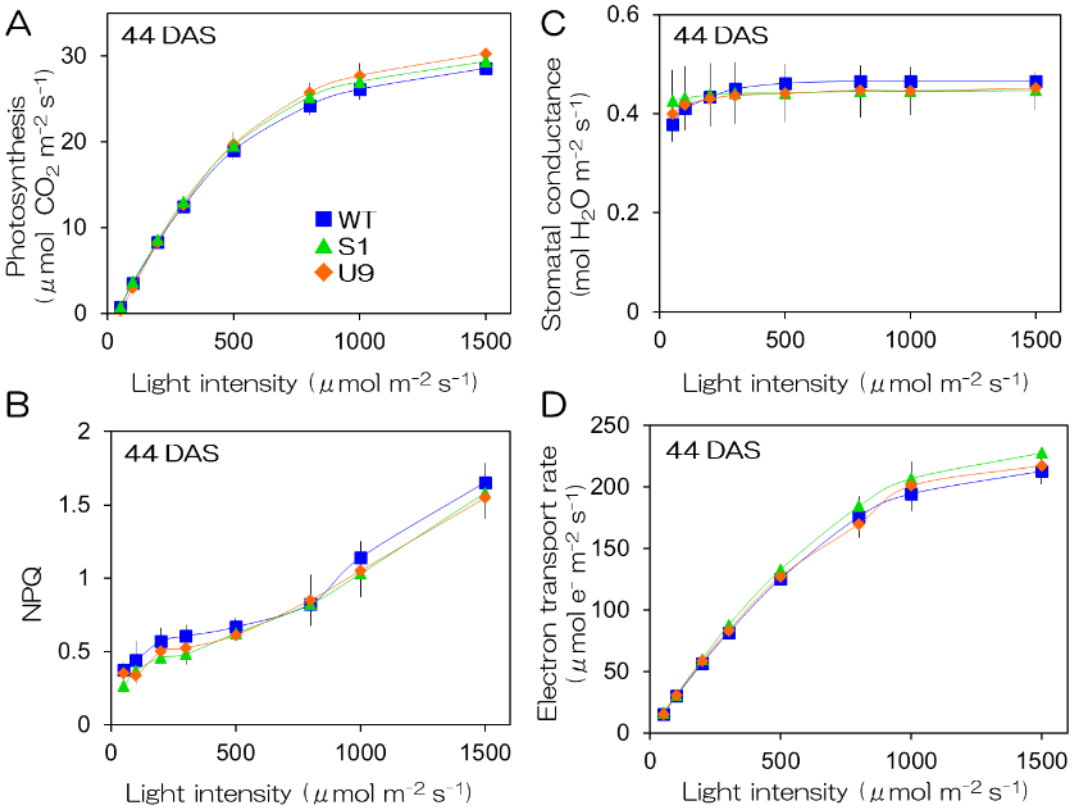


Figure 4-5 Comparison of photosynthetic parameters between transgenic and control plants. Four different photosynthetic parameters of transgenic (S1, U9) and control (WT) leaves were measured under various intensities of light at 44 DAS. (A) Photosynthesis rate (μmol CO2 m-2s-1), (B) Stomatal conductance (μmol H2O m-2s-1), (C) Non photosynthetic quenching (NPQ), (D) Electron transport rate (μmol e-m-2s-1).

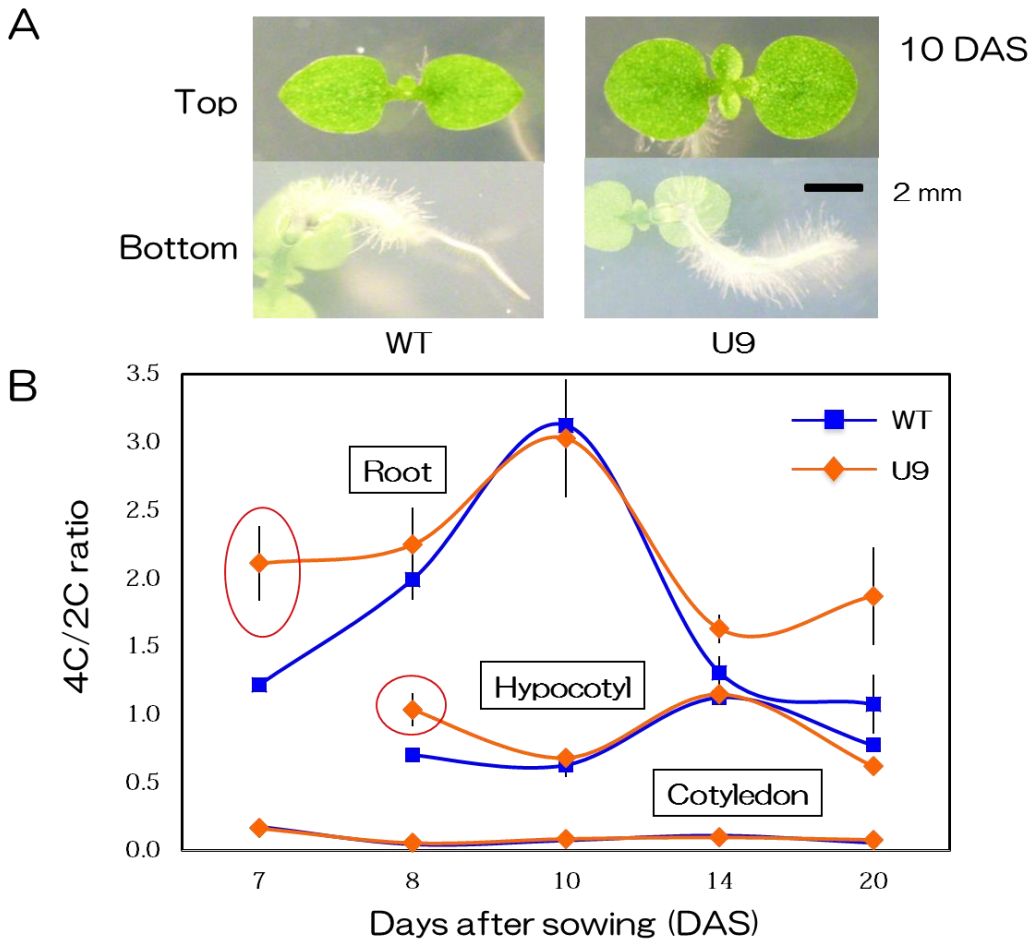


Figure 4-6 Comparison of phenotype and relative DNA content per nuclei of somatic cells between transgenic and control plants. (A) Photos of transgenic U9 and control WT seedlings were taken from top and bottom view at 10 DAS, (B) relative DNA content per nuclei of somatic cells in detached cotyledons, hypocotyls, and roots of U9 and Ct seedlings were measured during 7 – 20 DAS. The 4C (G2, M phase) / 2C (G1 phase) ratio probably corresponded to the activity of cell division.

Chapter 5

General discussion

Recently, increase on biomass production of plants and microalgae is an urgent task to reduce the concentration of atmospheric carbon dioxide in the world. The accumulation of carbon dioxide in the air causes global climate changes, which are responsible for localized heavy rain and drought. To reduce the amount of carbon dioxide emission, it will be required to stop to use fossil energy sources and necessary to use plant biomass as energy sources. Because plants can absorb aerial carbon dioxide and convert it to organic materials, i.e. biomass, by the photosynthesis, the use of biomass as energy sources does not increase the amount of the emission of carbon dioxide (carbon neutral).

Because world population is rapidly growing specially in south African countries, food supply is also important issues. To increase on the amount of food production, it will be necessary to improve yield capacity of cereal crops and staple plants per unit area of field because cultivation area suitable for agricultural production cannot increase in the world any more. One of possible ways to improve yield capacity of plants is the use of hybrid vigor which is genetic phenomena reported by Darwin (1876). A typical characteristic of this phenomenon is 1.2 – 1.5 fold growth increase of the hybrid compared with both parents. However, the detailed mechanism of hybrid vigor has not been fully elucidated.

Since we are interested in hybrid vigor, which is found in F_1 hybrids of indica and

japonica cultivars of rice, we would like to investigate the regulation for the expression of *45S rRNA* gene inherited from both parents in the hybrid plant. Therefore, we tried to isolate full-length of *45S rRNA* gene from indica rice and then perform forced expression of it in japonica rice by using the Pol II promoter. If growth of the transgenic rice were increased compared with the control plant, it would suggest that expression of distantly related *45S rRNA* gene was probably involved in the appearance of hybrid vigor. Unfortunately, we could not produce transgenic plants of japonica rice harboring such chimeric gene.

In this study, we isolated full-length of *45S rRNA* gene (*ca*. 5.8 kb) from indica rice cultivar N16. The isolated *45S rRNA* (*Os45SrRNA*) gene was linked to between maize *ubiquitin* promoter (UbiP) and *nopaline synthase* terminator (nosT) or between califlower mosaic virus 35S promoter (35SP) and nosT. The obtained chiameric genes, UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA*, were inserted into between kanamycin and hyromycin resistance cassettes of a binary vector pEKH. The constructed binary vectors were mobilized into *Agrobacterium tumefaciens* strain EHA101.

In the chapter 2, transgenic *Arabidopsis* plants were created by the floral dip method using *Agrobacterium* strain harboring pEKH UbiP::*Os45SrRNA*. Transgenic *Arabidopsis* plants showed 2-fold growth increase compared with the control plants. We

performed dissection analysis of *Os45SrRNA* transgene to determine which segment of the transgene was involved in the growth increase. Microarray analysis was also performed to reveal profiles of up- or down regulated gene.

In the chapter 3, transgenic tobacco plants were produced by the leaf-disc transformation using *Agrobacterium* strains harboring pEKH UbiP::*Os45SrRNA* and pEKH 35SP::*Os45SrRNA.* The transgenic tobacco plants harboring UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* also showed 2.1- and 1.4-fold increase of dry weight mass at 45 days after sowing (45 DAS), respectively. Ploidy level analyzed by flow cytometer and sizes of stomatal cells were similar between transgenic and control plants. Nicotine, typical secondary metabolite in tobacco, concentration in leaves was similar between transgenic and control plants. Thus, nicotine production per plant was increased 1.4- and 2.1-fold in 35SP::*Os45SrRNA* and UbiP::*Os45SrRNA* plants compared with the control plant, respectively.

In the chapter 4, transgenic tobacco plants harboring UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* were grown in a growth chamber and analyzed parameters of seedling growth, stomatal development, and photosynthetic capacities in detail. And microarray array analysis was performed to determine up- or down-regulated genes between

transgenic and control plants. Moreover, cell division activity of hypocotyl, root in young seedling was compared using flow cytometric analysis.

In the initial primodia of seedlings, if the number of cell division of transgenic plants were one cycle more than that of the control plant, their growth would be double. The forced expression of rice *45S rRNA* gene may trigger to alter control of cell cycle at the early germination stage. Because hybrid vigor is appears only at the early stage of plant development through the enhancement of cell-proliferation, which is probably mediated by circadian rhythm (Ni et al. 2009, Chen 2010, Fujimoto et al. 2012). Thus, the mechanism of the growth increase through the enhancement of cell proliferation might be related to each other between the hybrid vigor and the growth increase found in this study.

The forced expression of exogenous *45S rRNA* (FEE45) is simple technology, which will contribute to increase the growth of transgenic plants. Unlike hybrid vigor, the growth increase by FEE45 can be fixed as a homozygous allele in practical cultivars. The FEE45 technology will be applicable for the production of secondary metabolites in medicinal plants as well as the breeding of high yielding cultivars in cereals, vegetables, trees, and especially biomass plants for bio-energy production.

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Summary

Usage of plant biomass is being an attracted choice due to the depletion of fossil energy resources and deterioration of the global environment due to an increase in the emission of carbon dioxide. However, production cost of plant biomass has become higher than fossil energy, and it will be required to improve plants with further increased production per unit area.

In this study, the full-length *45S rRNA* gene isolated from the Indica cultivar N16 of rice was ligated to the promoter of the maize ubiquitin gene, the obtained chimeric gene was incorporated into a binary vector, and *Arabidopsis* was transformed using the floral dip method. In this case, the introduced rice *45S rRNA* gene is thought to be transcribed by RNA polymerase II (Pol II), rather than the Pol I. The transgenic lines produced showed a 2.3-fold increase at the maximum in dry weight of the aerial tissues as compared with the control lines. Since size of the guard cells and ploidy of the leaf cells of the transgenic lines were not different from the control line, it suggests that an increase in the number of cells than the cell enlargement was the cause of an increased growth of the transgenic plants. Likewise, when transgenic plants of tobacco SR1 line were produced, their growth increased about twice as compared with the control line. In addition, no difference was observed between the transgenic and the control lines in the physiological properties, such as photosynthetic capacity and nicotine producing ability. Flow cytometric analysis of hypocotyls and roots of the early seedlings revealed that the activity of cell division in the transgenic lines was significantly higher than that of the control line. The results of this study showed that biomass production can be increased without altering physiological properties by creating transgenic plants of which the 45S rRNA gene of rice is linked to a promoter recognized by Pol II.

概要 (Summary in Japanese)

化石エネルギー資源の枯渇および二酸化炭素の放出量の増加による地球環境の悪化を背景として植物バイオマスが注目を浴びている。しかし、植物バイオマスは、生産コストが高いことが大きなネックとなっており、単位面積当りの生産量が一層増大した植物の作出が求められている。

本研究では、インド型栽培イネ N16 系統から単離した全長の *45S rRNA* 遺伝子をトウモロコシのユビキチン遺伝子のプロモーターに連結して、得られたキメラ遺伝子をバイナリーベクターに組み込み、Floral dip 法によりアラビドプシスを形質転換した。この場合、導入したイネの *45S rRNA* 遺伝子は、本来の RNA ポリメラーゼ I (Pol I) ではなく、Pol II により転写されると考えられる。作出された形質転換系統は、原系統に比べて最大 2.3 倍の地上部乾物重の増大を示した。形質転換体の孔辺細胞のサイズや細胞の倍数性は、原系統と大きな差は見られないので、細胞の肥大より細胞数の増大が形質転換体の生長量が増大した原因であることを示唆している。同様に、タバコの SR1 系統の形質転換体を作出したところ、原系統に比べて生長量が約２倍に増大した。また、光合成能、ニコチン生産能などの生理的特性には、形質転換体と原系統との間で差異は認められなかった。発芽初期の実生の胚軸や根のフローサイト解析を行ったところ、形質転換体における細胞分裂の活性が原系統に較べて有意に高いことが認められた。本研究の結果は、イネの *45S rRNA* 遺伝子を Pol II が認識するプロモーターに繋いで導入した形質転換植物を作出することにより、生理的な特性を変更することなしにバイオマス生産量を増大できることを示している。

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