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Full Length Research Paper

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

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Recently, we found that transgenic *Arabidopsis* plants with the forced expression of rice *45S rRNA* gene enhanced 2-fold growth of above ground tissues. Thus, we made transgenic tobacco plants harboring rice *45S rRNA* driven by maize *ubiquitin* promoter (UbiP::*Os45SrRNA*) or cauliflower mosaic virus 35S promoter (35SP::*Os45SrRNA*). Only whole plant size and dry weight of 35SP::*Os45SrRNA* and UbiP::*Os45SrRNA* transgenic tobacco plants were increased compared to those of the control plants. On the other hand, relative DNA content of leaf cell, size of stomata cell, and density of stomata per unit leaf area were similar between the transgenic and control plants. Transgenic tobacco plant harboring UbiP::*Os45SrRNA* and 35SP::*Os45SrRNA* transgene were increased dry weight to 2.1 and 1.4 fold compared to the control plant at 45 days after sowing (45 DAS), respectively. Transgenic plant harboring 35SP::*Os45SrRNA* transgene bloomed flowers at 107 DAS compared to 152 DAS of the control plant. Therefore, transgenic plants could produce the number of their fruits to 3 times than the control plant. Although concentrations of nicotine, a secondary metabolite of tobacco plant, in leaves were similar between transgenic and control plants, total amount of nicotine in the transgenic plant was produced more than twice than that in the control plant. Although the mechanism underlying the increased growth remains to be understood, forced expression of foreign *45S rRNA* gene will contribute to the production of secondary metabolites of plants as well as the growth increase of cereals, vegetables, and bio-energy plants.

Keywords: 45S ribosomal RNA, forced expression, growth increase, nicotine, transgenic plants.

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

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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 endoreplication (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 *HindIII* site between kanamycin (NPTII) and hygromycin (HPT) resistance cassettes (Figure. 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). *HindIII*-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°C 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 (mm)

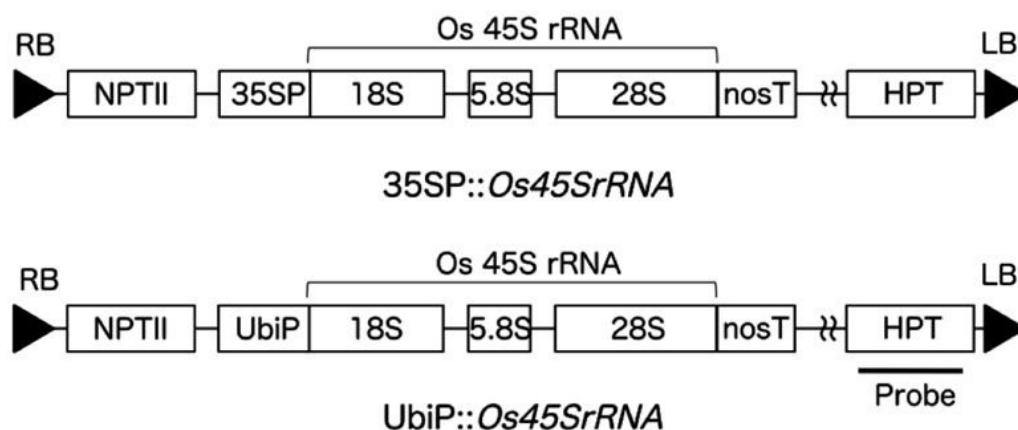


Figure 1: Schematic representation of T-DNA regions in the binary vectors used in this study. Full-length 45S rRNA gene (*Os45SrRNA*, 5.8 kb) of *Oryza sativa* was linked to the maize *ubiquitin* promoter (UbiP) or the CaMV 35S promoter (35SP). The chimeric gene was inserted into *Hind*III (H) site between 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.

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 (Figure. 1), respectively. Transgenic tobacco lines harboring a single-copy of UbiP::*Os45SrRNA* (U9) or 35SP::*Os45SrRNA* (S1) transgene were selected using Southern blot analysis (Figure. 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 (Figure. 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 (Figure. 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 Figure. 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 (Figure. 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) (Figure. 5C).

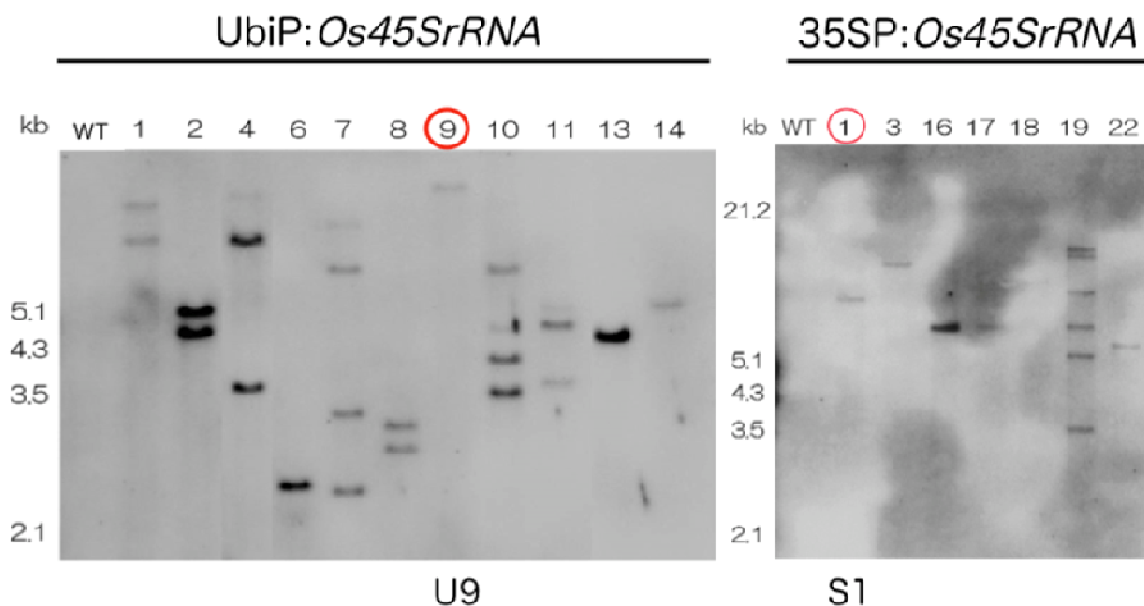


Figure 2: Southern blot analysis of transgenic tobacco plants harboring UbiP::*Os45SrRNA* or 35SP::*Os45SrRNA* transgene. *Hind*III-digested genomic DNAs extracted from leaves were subjected to agarose gel electrophoresis. Transgenic U9 and S1 lines showed a single band indicating the integration of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

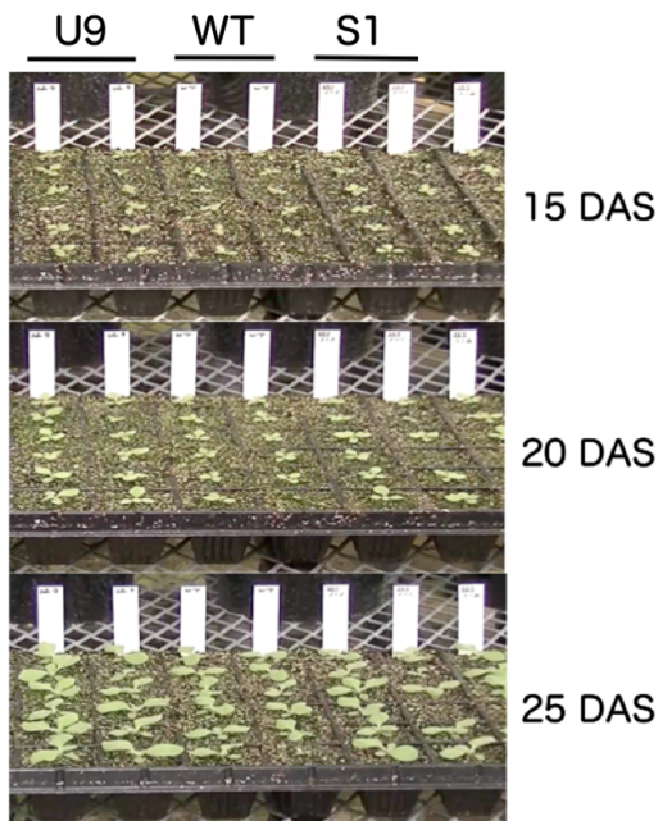


Figure 3: Comparison of seedling growth between transgenic and control plants. Transgenic (S1, U9) and control (WT) seedlings were grown side by side in the same tray and photographs were taken at 15, 20, and 25 DAS.

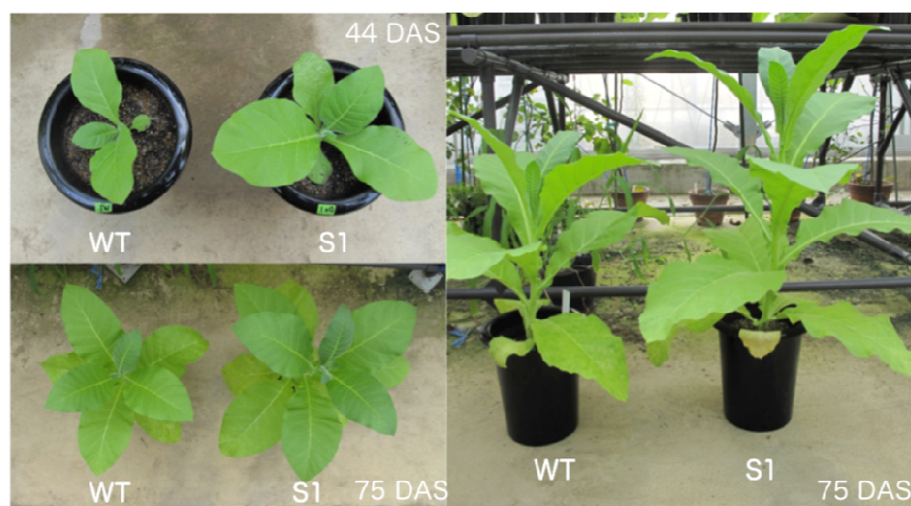


Figure 4: Comparison of growth between transgenic and control plants. Transgenic S1 and control WT plants were grown with pots to the maturity in a greenhouse and photographs were taken at 44 and 75 DAS.

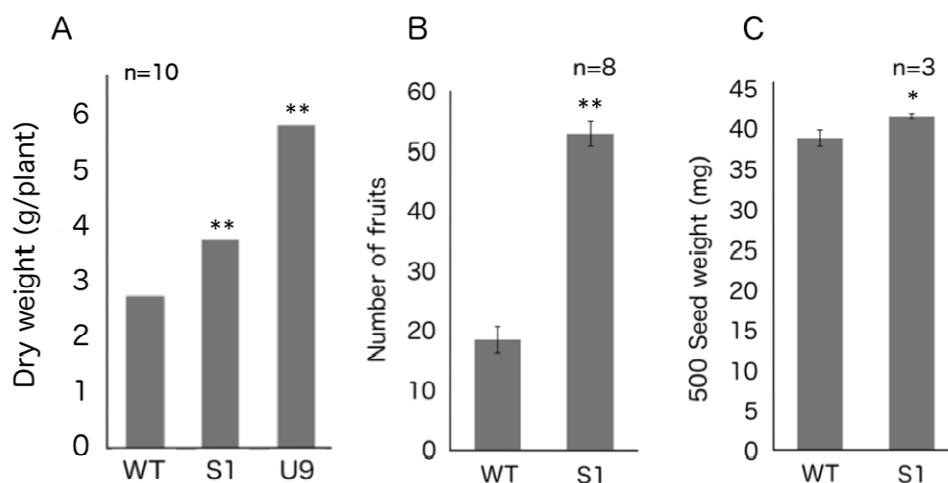


Figure 5: Comparison of dry weight between transgenic and control plants. Dry weight (g/plant) of above ground tissues of transgenic (S1, U9) and control (WT) plants were measured at 45 DAS (A). Fruit number (B) and 500 seed weight (C) of transgenic S1 and control WT plants were also measured at the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

Comparison of nicotine content in leaves

Although nicotine contents of leaves per dry weight (mg/g) were similar among two transgenic and control plants (Figure. 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 (Figure. 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. 1). The densities of stomata per unit leaf area (mm^2) were also similar between the transgenic (S1, U9) and control WT plants.

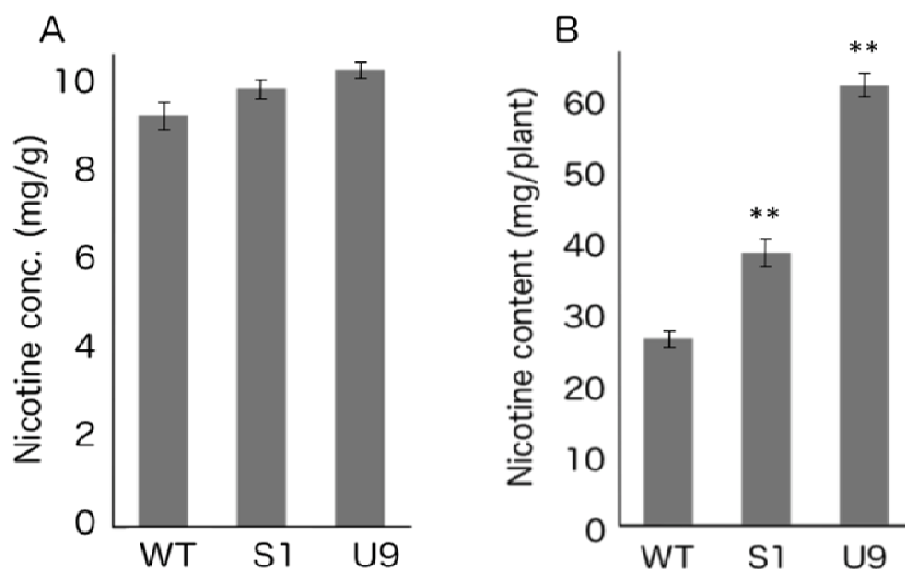


Figure 6: Comparison of nicotine content in leaves between transgenic and control plants. Nicotine content per dry weight (mg/g) of transgenic (S1, U9) and control WT leaves were measured at 45 DAS (A). And total nicotine content per plant (mg/plant) was calculated based on the dry weight (B).

Table 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.

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 (Figure. 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 (Figure. 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. 1, Figure. 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 (Figure. 3). As shown in Figure. 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 (Figure. 5B).

The 500-seed weight of the transgenic S1 plants was 17% heavier than that of the control WT plant (Figure. 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.

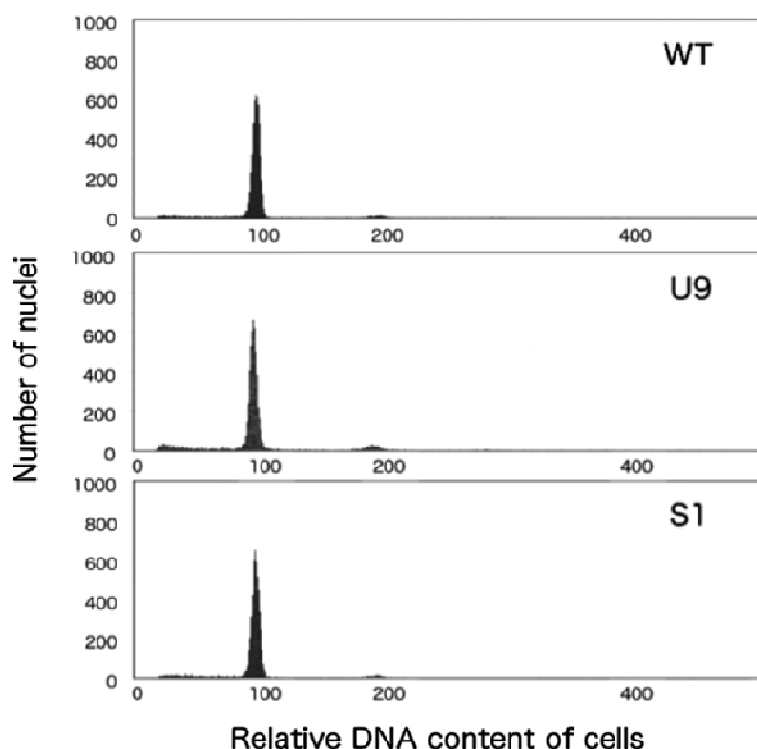


Figure 7: Comparison of ploidy level between transgenic and control plants. Relative DNA content leaf cells of the transgenic (S1, U9) and control WT plants were measured at 20 DAS using the flow cytometer.

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 (Figure. 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 the 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 able to 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.

REFERENCES

- Appels R, Dvorak J (1982). Relative rates of divergence of spacer and gene sequences within the rDNA region of species in the Triticeae: Implications for the maintenance of homogeneity of a repeated gene family. *Theor Appl Genet* 63: 361 – 365
- Battraw MJ, Hall TC (1990). Histochemical analysis of CaMV 35S promoter-beta-glucuronidase gene expression in transgenic rice plants. *Plant Mol Biol* 15:527-528
- Breuer C, Kawamura A, Ichikawa T, Tominaga-Wada R, Wada T, Kondou Y, Muto S, Matsui M, Sugimoto K (2009). The trihelix transcription factor GTL1 regulates ploidy-dependent cell growth in the *Arabidopsis* trichome. *Plant Cell* 21: 2307-2322
- Chisti Y (2008). Response to Reijnders: do biofuels from microalgae beat biofuels from terrestrial plants? *Trends Biotechnol* 26: 351–352
- Christensen AH, Quail PH (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgen Res* 5: 213 – 218
- CORESTA. (2013). Determination of tobacco-specific nitrosamines in smokeless tobacco products by LC-MS/MS. CRM No. 72

- Darwin CR (1876). The effects of cross- and self-fertilization in the vegetable kingdom, John Murry
- Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19: 11 – 15
- Duvick DN (1999). Heterosis: feeding people and natural resources. In: Coors JG, Pandey S (eds) *The Genetics and Exploitation of Heterosis in Crops*. Amer Soc of Agron, Crop Sci Soc of Amer, and Soil Sci Soc of Amer. pp. 19 – 29
- Edward A, Schuur G, Bockheim J, Canadell JG, Euskirchen E, Field CB, Goryachkin SV, Hagemann S, Kuhry P, Lafleur PM, Lee H, Mazhitova G, Nelson FE, Rinke A, Romanovsky VE, Shiklomanov N, Tarnocai C, Venevsky S, Vogel JG, Zimov SA (2008). Vulnerability of permafrost carbon to climate change: implications for the global carbon cycle. *BioScience* 58: 701-714
- Eriksson ME, Israelsson M, Olsson O, Moritz T (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat Biotechnol* 18: 784-788
- Hochholdinger F, Hoecker N (2007). Towards the molecular basis of heterosis. *Trends Plant Sci* 12: 427 – 432
- Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) Simple and general method for transferring genes into plants. *Science* 227: 1229 – 1231
- Ishimaru K, Hirotsu N, Madoka Y, Murakami N, Hara N, Onodera H, Kashiwagi T, Ujiie K, Shimizu B, Onishi A, Miyagawa H, Katoh A (2013). Loss of function of the IAA-glucose hydrolase gene *TGW6* enhances rice grain weight and increases yield. *Nat Genet* 45: 707-711
- Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsch H-J, Rosenkranz R, Stäbler N, Schönfeld B, Kreuzaler F, Peterhänsel C (2007). Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in *Arabidopsis thaliana*. *Nat Biotechnol* 25: 593 – 599
- Kondrosi E, Roundier F, Gendreau E (2000). Plant cell-size control growing by ploidy? *Curr Opin Plant Biol* 3: 488 – 492
- Lippman ZB, Zamir D (2007). Heterosis: Revisiting the magic. *Trends Genet* 23: 60 – 66
- Makabe S, Motohashi R, Nakamura I (2016). Growth increase of *Arabidopsis* by forced expression of rice *45S rRNA* gene. *Plant Cell Reports* doi:10.1007/s00299-016-2075-y
- Masuda HP, Cabral LM, De Veylder L, Tanurdzic M, de Almeida Engler J, Geelen D, Inzé D, Martienssen RA, Ferreira PC, Hemerly AS (2008) ABAP1 is a novel plant Armadillo BTB protein involved in DNA replication and transcription. *EMBO J* 27: 2746-2756
- Meyer RC, Törjék O, Becher M, Altmann T (2004) Heterosis of biomass production in *Arabidopsis*. Establishment during early development. *Plant Physiol* 134, 1813 – 1823
- Mishiba K, Mii M (2000). Polysomaty analysis in diploid and tetraploid *Portulaca grandiflora*. *Plant Sci* 156: 213 – 219
- Nelissen H, Rymen B, Jikumaru Y, Demuynck K, Van Lijsebettens M, Kamiya Y, Inzé D, Beemster GT (2012) A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. *Curr Biol* 22: 1183-1189
- Rojas CA, Hemerly AS, Ferreira PC (2010) Genetically modified crops for biomass increase. *Genes and strategies*. *GM Crops* 1: 137-142
- Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S, Tanaka H, Kitano H, Matsuoka M (2006). Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. *Nat Biotechnol* 24: 105-109
- Sugimoto-Shirasu K, Roberts K (2003). “Big it up”: endoreduplication and cell-size control in plants. *Curr Opin Plant Biol* 6: 544 – 553
- Takesawa T, Ito M, Kanzaki H, Kameya N, Nakamura I (2002). Over-expression of ζ glutathione S -transferase in transgenic rice enhances germination and growth at low temperature. *Mol Breed* 9: 93 – 101
- Udem SA, Warner JR (1972). Ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *J Mol Biol.* 65: 227–242
- Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH (2009). The frequency of polyploid speciation in vascular plants. *Proc Natl Acad Sci USA* 106: 13875 – 13879
- Yoshida A, Sasao M, Yasuno N, Takagi K, Daimon Y, Chen R, Yamazaki R, Tokunaga H, Kitaguchi Y, Sato Y, Nagamura Y, Ushijima T, Kumamaru T, Iida S, Maekawa M, Kyojuka J (2013). *TAWAWA1*, a regulator of rice inflorescence architecture, functions through the suppression of meristem phase transition. *Proc Natl Acad Sci USA* 110: 767-772