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Original Paper

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

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Abstract An increase in plant biomass production is desired to reduce emission of carbon dioxide emissions and arrest global climate change because it will provide a more source of energy production than fossil fuels. Recently, we found that forced expression of the rice *45S rRNA* gene increased aboveground growth by *ca*. 2-fold in the transgenic *Arabidopsis* plants. Here, we created transgenic tobacco plants harboring the rice *45S rRNA* driven by the maize *ubiquitin* promoter (UbiP::*Os45SrRNA*) or cauliflower mosaic virus 35S promoter (35SP::*Os45SrRNA*). In 35SP::*Os45SrRNA* and UbiP::*Os45SrRNA* transgenic tobacco plants, the leaf length and size were increased compared with control plants, leading to an increase of aboveground growth (dry weight) up to 2-fold at the early stage of seedling development. Conversely, leaf physiological traits, such as photosynthetic capacity, stomatal characteristics, and chlorophylls and RuBisCO protein contents, were similar between the transgenic and control plants. Flow cytometry analysis indicated that the transgenic plants had enhanced cell-proliferation especially in seedling root and leaf primordia. Microarray analysis revealed that genes encoding transcription factors, such as GIGANTEA-like, were more than 2-fold up-regulated in the transgenic plants. Although the mechanism underlying the increased growth has yet to be elucidated, this strategy could be used to increase biomass production in cereals, vegetables, and bio-energy plants.

Key words: 45S ribosomal RNA, cell proliferation, growth increase, photosynthetic properties, transgenic plants.

Introduction

We recently reported that the growth of transgenic *Arabidopsis* plant 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 and ploidy level of the 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; Hochholdinger and Hoecker 2007; Lippman and Zamir 2007; Meyer et al. 2004). The hybrid vigor appears only at the early stage of plant development through the enhancement of cell-proliferation, which is probably mediated by circadian rhythms (Chen 2010; Fujimoto et al. 2012; Ni et al. 2009). Polyploidization can also increases plant biomass through the enlargement of cell size (Kondrosi et al. 2000; Sugimoto-Shirasu and Robert 2003). Generally, hybrid vigor and polyploidization cause 1.2–1.5-fold increases in the 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 the 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

Abbreviations: 35SP, cauliflower mosaic virus 35S promoter; ARP, auxin repressed protein; DAS, days after sowing; ETR, electron transport rate; φPSII, quantum yield of photosystem II; FEE45, forced expression of exogenous *45S rRNA*; GI, GIGANTEA; hpt, hygromycin phosphotransferase; ITS, internal transcribed spacers; MS, Murashige and Skoog; NPQ, non-photosynthetic quenching; nosT, nopaline synthase gene terminator; PAM, pulse amplitude modulation; rRNA, ribosomal RNA; UbiP, maize *ubiquitin* promoter.

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modulation (PAM) (Woo et al. 2008). The 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 gas exchange of carbon dioxide, water vapor, and oxygen through stomata significantly affects on the photosynthetic capacity, plants optimize their gas exchange efficiency by regulating stomatal aperture size, stomatal density, stomatal pore openness, and stomatal 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 (Byrne 2009; Fujikura et al. 2009; Horiguchi et al. 2011; Horiguchi et al. 2012; Ito et al. 2000; Rosado et al. 2012; Van Lijsebettens et al. 1994; Zsogon et al. 2014). Thus, some ribosome-related process might be involved in the co-ordination of cell-proliferation and cell-expansion in the leaf development.

Makabe et al (2016) found that the 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 and Dvorak 1982). As the three *rRNA* sequences within the *45S rRNA* transcripts 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 the 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 (Figure 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°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.

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′-CGC GAT ACC ACG AGC T AAA TCC AC-3′-ITS3P2: 5′-GTC CGA GGC GTT CGC TCT CGG TGC-3′ and actin5P: 5′-GAA A ATG GTG AAG GCT GGT TTT G-3′-actin3P: 5′-AGG ATT GAT CCT CCG ATC CAG A-3′ were designed to amplify ITS (ITS1-5.8S-ITS2) region of rice *45S rRNA* and *actin* mRNA (positive control), respectively.

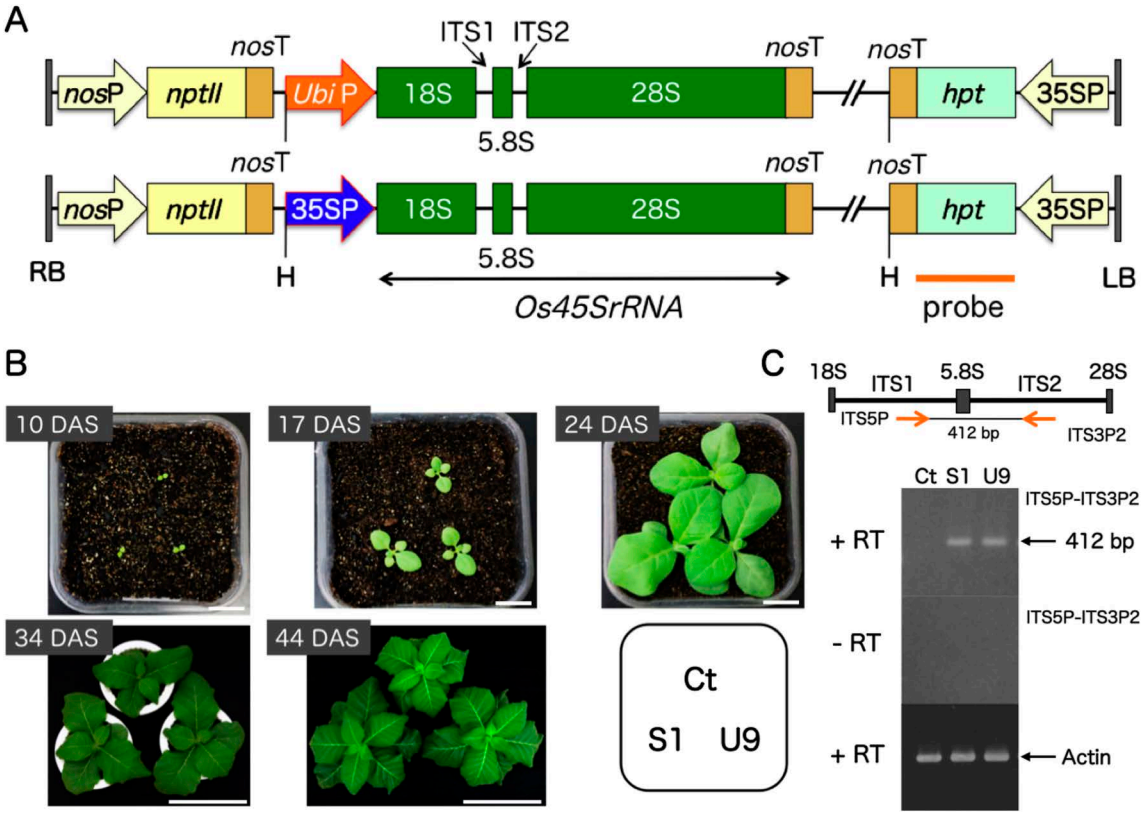

Figure 1. Production of transgenic tobacco plants with the forced expression of the rice *45S rRNA* gene using the maize *ubiquitin* or CaMV 35S promoter. (A) Schematic representation of transgenes; Full-length *45S rRNA* gene (*Os45SrRNA*, 5.8 kb) of *Oryza sativa* ecotype Indica cultivar N16 line was 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 (Ct) 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 Figure 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 Ct plant and without adding reverse transcriptase (−RT). *Actin* mRNA was also amplified as an internal standard.

Plant growth analysis in a growth chamber

Transgenic (S1, U9) and control (Ct) 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 μmol mol^{-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 (Ct) 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°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×absI×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×(Fo′/F′)] were calculated using the software ImagingWin. Data represent means±SE

Analysis of photosynthetic activity

Seeds of transgenic (S1, U9) and control (Ct) 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 −80°C 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

Microarray analysis

Total RNA was isolated from aerial parts of transgenic (S1, U9) and control (Ct) 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 (Figure 1A), respectively. Transgenic lines harboring a single-copy transgene were selected by Southern blot analysis (Supplementary Figure S1) and the 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 (Ct) 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 control (Ct) plants at 10 DAS (Figure 1B). In contrast, the aboveground growth of the two transgenic S1 and U9 plants was greatly increased compared with the 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* transcripts at similar and low levels when compared with the *actin* gene used as an amplification standard (Figure 1C).

Detailed measurements at 44 DAS showed that leaf-length and leaf-area of the 1st to 3rd leaves from the top were similar between transgenic (S1, U9) and control (Ct) plants, but started to increase from the 4th leaf in the transgenic plants compared with those of the control plants (Figure 2A, B). The total leaf-area and dry-weight of transgenic S1 and U9 plants were maximized up to 2-fold compared with the control plant at 34 DAS (Figure 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 Figure 3A, transgenic (S1, U9) and control (Ct) plants were grown in growth chamber condition 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 the 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 Figure S2). In the greenhouse conditions, the transgenic S1 and U9 plants showed 1.4 and 2.1-fold increase in the dry weight of aerial tissues, respectively, compared with the Ct plant at 45 DAS (Makabe et al. submitted). The S1 plants grew bigger than the Ct plants at 75 DAS in a greenhouse (Figure 3B). The S1 plants produced their first flowers at 107 DAS (Figure 3C–E). This was much earlier than the flowering of the Ct plants at 152 DAS. Therefore, the fruit number of the S1 plants was *ca*. 2.8-fold higher than that of the Ct plants. In addition, the 500-seed weights of the S1 and U9 plants were 1.1–1.2-fold greater than that of the Ct 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 transgenic S1 and U9 leaves, respectively, compared with the Ct leaves.

Comparison of various photosynthetic parameters

Chlorophyll fluorescence images were analyzed using seedlings at 10-, 17-, and 24-DAS under the growth

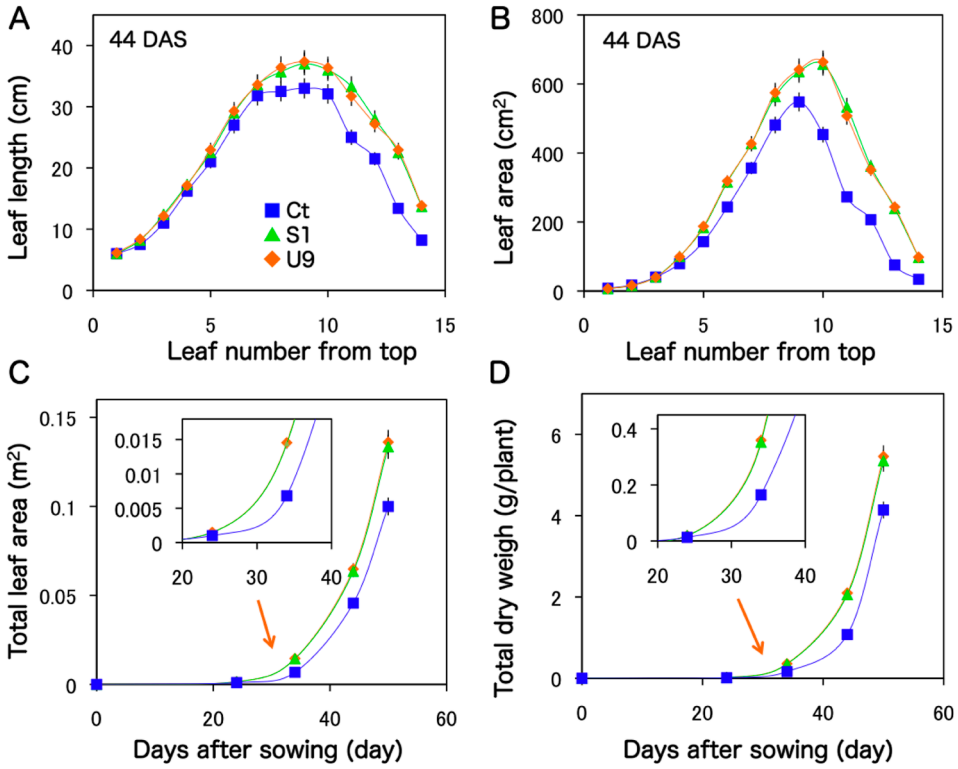

Figure 2. Comparison of aboveground growth between transgenic and control plants. Transgenic (S1, U9) and control (Ct) plants were grown in small pots within a growth chamber and the leaf characters were measured at 44 DAS. (A) Length (cm) of each leaf. (B) Area (cm^2) of each leaf. (C) Time-course of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

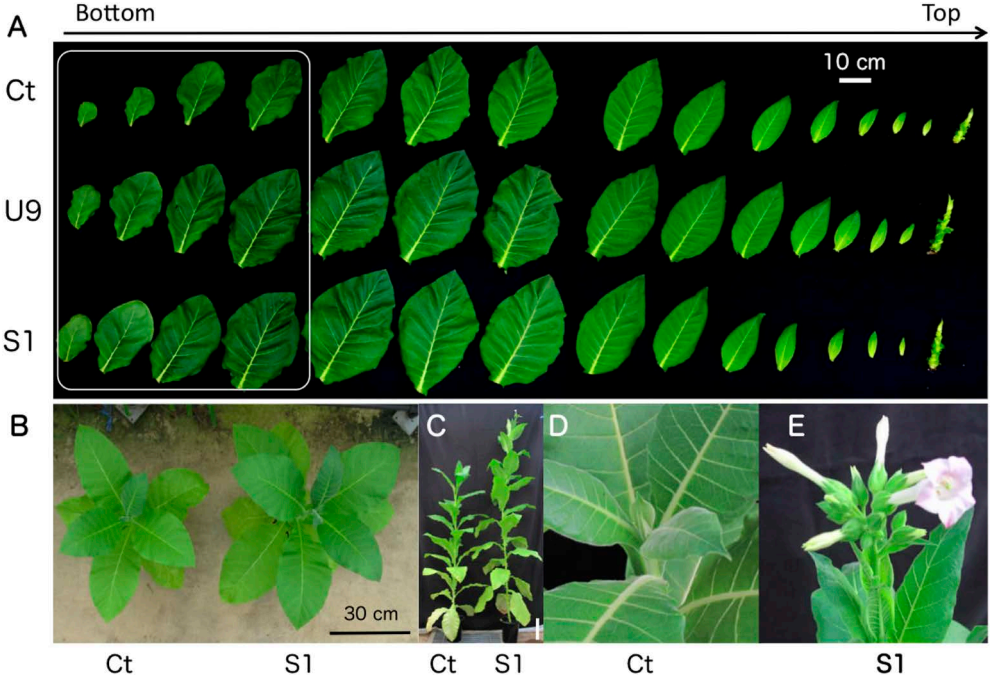

Figure 3. Comparison of growth between transgenic and control plants. (A) Leaves of transgenic (S1, U9) and control Ct plants were aligned from the bottom to the top. The first four leaves of transgenic plants were clearly bigger than those of the control plant (box). (B) Photograph of transgenic S1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

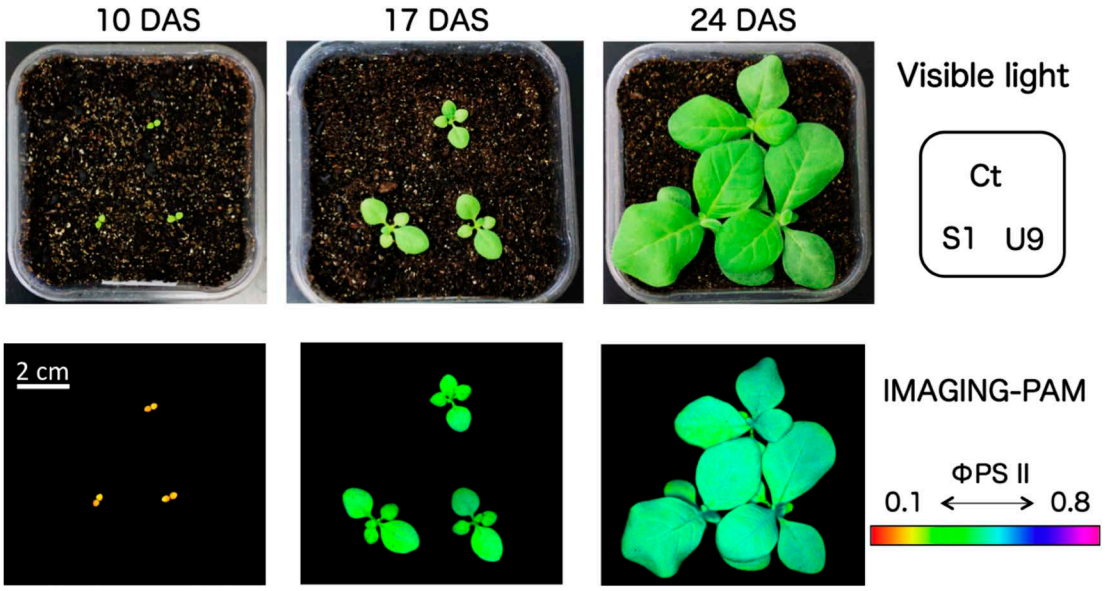

Figure 4. Comparison of photosynthetic capacity between transgenic and control plants using IMAGING-PAM analysis. Seedlings of transgenic (S1, U9) and control (Ct) plants were grown together in the same pot and images were taken at 10, 17, and 24 DAS. Top: images under visible light. Bottom: Two-dimensional 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.

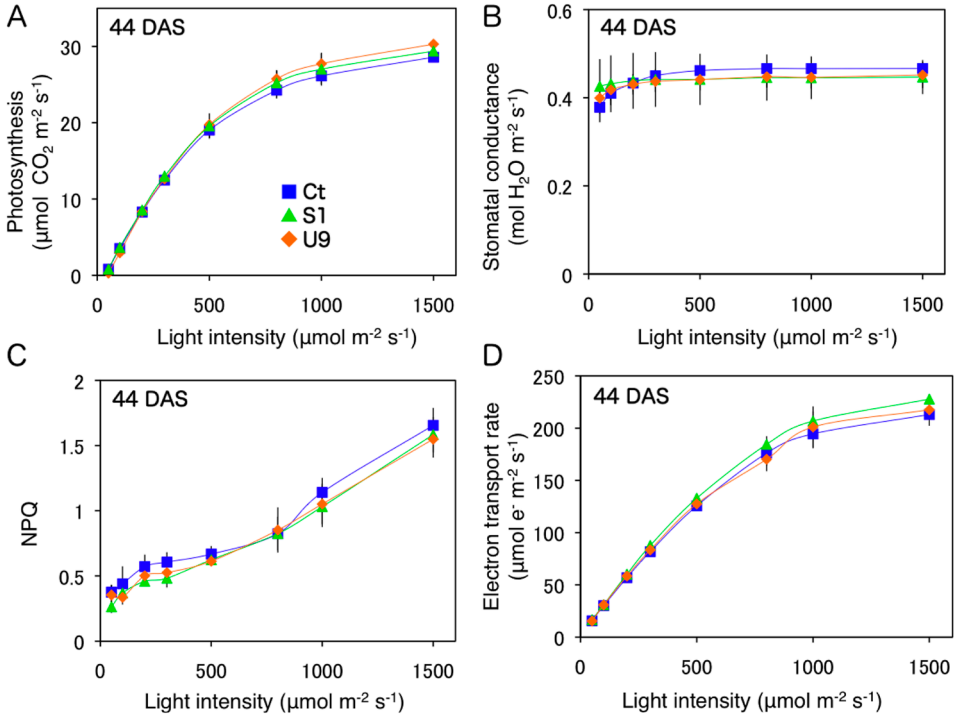

Figure 5. Comparison of photosynthetic parameters between transgenic and control plants. Four different photosynthetic parameters of transgenic (S1, U9) and control (Ct) leaves were measured under various intensities of light at 44 DAS. (A) Photosynthesis rate (μmol CO_2 $m^{-2}s^{-1}$), (B) Stomatal conductance (μmol H_2O $m^{-2}s^{-1}$), (C) Non photosynthetic quenching (NPQ), (D) Electron transport rate (μmol $e^{-}m^{-2}s^{-1}$).

light intensity. The IMAGING-PAM analysis showed that the fluorescence changed from orange to pale-blue, indicating low to high level of photosynthetic capacities. There were no differences between transgenic (S1, U9) and control (Ct) plants in the two-dimensional fluorescence images (Figure 4) and in the photosynthetic parameters, such as the quantum yield of photosystem II PSII (φPSII), the reduction state of PSII (1-qL), and the non photosynthetic quenching (NPQ) at each DAS (Supplementary Table S1).

The light-intensity responses of several photosynthetic parameters in leaves were measured at 44 DAS (Figure 5).

Table 1. Measurements of RuBisCO and chlorophyll contents, and stomatal characteristics in leaves. Transgenic (S1, U9) and control (Ct) 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)
Ct	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 transgenic (S1, U9) and control (Ct) plants. Data represent mean plus standard errors. $n=5$.

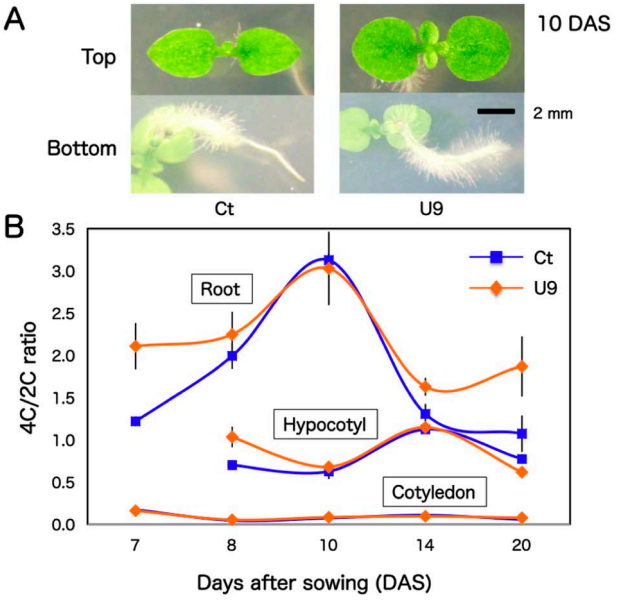

Figure 6. Comparison of phenotype and relative DNA content per nuclei of somatic cells between transgenic and control plants. (A) Photos of transgenic U9 and control Ct seedlings were taken from top and bottom view at 10 DAS, (B) relative DNA content per nuclei of somatic cells in detached cotyledons, 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.

The photosynthetic rate, stomatal conductance, electron transport rate, and non-photosynthetic quenching (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 transgenic (S1, U9) and control (Ct) 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 (Ct) plants at 44 DAS (Table 1). All stomatal characteristics, including stomatal size, density and index, were also substantially similar between the transgenic and control plants.

Cell division activities of tobacco seedling

Transgenic U9 seedlings developed true leaves and root hairs earlier than control Ct seedling at 10 DAS (Figure 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 nuclei of 4C (G2/M phase) *vs.* 2C (G1 phase), maximized in roots (10 DAS) and hypocotyls (14 DAS) in transgenic U9 and control Ct seedlings (Figure 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. Transgenic U9 seedlings showed higher 4C/2C ratios in their roots (7 DAS and 14–20 DAS) and hypocotyls (8 DAS) than Ct seedlings.

Microarray analysis

Microarray analysis of aerial parts of 12 DAS seedlings revealed that 37 and 45 genes were more than 2-fold up- or down-regulated, respectively, in both transgenic S1 and U9 plants compared with Ct 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 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 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).

Discussion

Transgenic tobacco seedlings harboring a single-copy of the 35SP::*Os45SrRNA* (S1) or UbiP::*Os45SrRNA* (U9) transgene (Figure 1A) showed increased growth at 17 DAS compared with the control (Ct) plants in growth chamber conditions (Figure 1B). As shown in Figures 2A and 2B, the enlargement of transgenic (S1, U9) leaves was 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

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

Probe Name	Description	Fold change	
		S1 vs Ct	U9 vs Ct
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

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 those of the Ct plants at 34 DAS, the differences in the growth was reduced to *ca*. 1.4-fold at 50 DAS (Figure 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 Figure S2).

In the greenhouse, transgenic tobacco plants reached the flowering stage much earlier than control plants (Figure 3C–E). Therefore, it is possible to produce a 2–3-fold seed yield increase in the transgenic tobacco plants because the fruit number in S1 plants was increased by 2.8-fold compared with Ct plants. Although the 500-seed weight of the U9 plants was 17% heavier than the Ct 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 Ct 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 substantial leaf-age between the transgenic and control plants (Figure 3C).

In the IMAGING-PAM analysis, transgenic (S1, U9) and control Ct plants showed the same photosynthetic capacity (Figure 4). Four different parameters affecting the photosynthetic capacity, including the photosynthesis rate, stomatal conductance, non-photochemical quenching (NPQ), and the electron transporter rate under various light intensities, showed the same values in transgenic and control plants (Figure 5A–D). In addition, the RuBisCO and chlorophyll contents and all stomatal characteristics were similar level between the transgenic and control plants (Table 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 the 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 Ct seedlings (Figure 6B). These data suggest that root system development might occur faster than aerial tissues development in tobacco plants. When compared with Ct 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 (Ct) seedlings were found at 10 DAS (Figure 6A). Among the more than 2-fold up- and down-regulated genes, 23 (of 37) and 8 (of 45) genes are listed in Table 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). Because Ni et al. (2009) and Chen (2010) suggested that altered circadian rhythms promote growth in the hybrid vigor, circadian genes are probably responsible for the cell proliferation in the hybrid vigor and growth increase found in this study. Thus, the analysis of the circadian genes controlling the expression of tobacco *GI* genes will be necessary to reveal the mechanism of the growth increase.

The up-regulated ribosomal L30 and down-regulated mitochondrial S41A-like genes were 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 plant because over-expression of the *ARP1* gene represses plant growth (Zhao et al. 2014). There is no down-regulated gene that is responsible for the control of cell cycle.

In case of 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 transgenic plants expressed the rice *45S rRNA* at a similar level (Figure 1C). However, the expressed rice *45S rRNA* transcripts might not play roles as rRNA molecules because they were expressed at a quite low level. Because the sequences of the *18S*, *5.8S*, and *28S rRNAs* within the *45S rRNA* transcripts are highly homologous between rice and tobacco, the expression of species-specific ITS sequences might be responsible for the growth increase found in the transgenic tobacco and *Arabidopsis* (Makabe et al. 2016).

Plant leaf development is governed through the 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 1) and the ploidy level of leaf cells (Supplementary Figure S3) were similar between the transgenic and control leaves. Stomatal size is considered as good indicators 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 those of 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 to each other 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.

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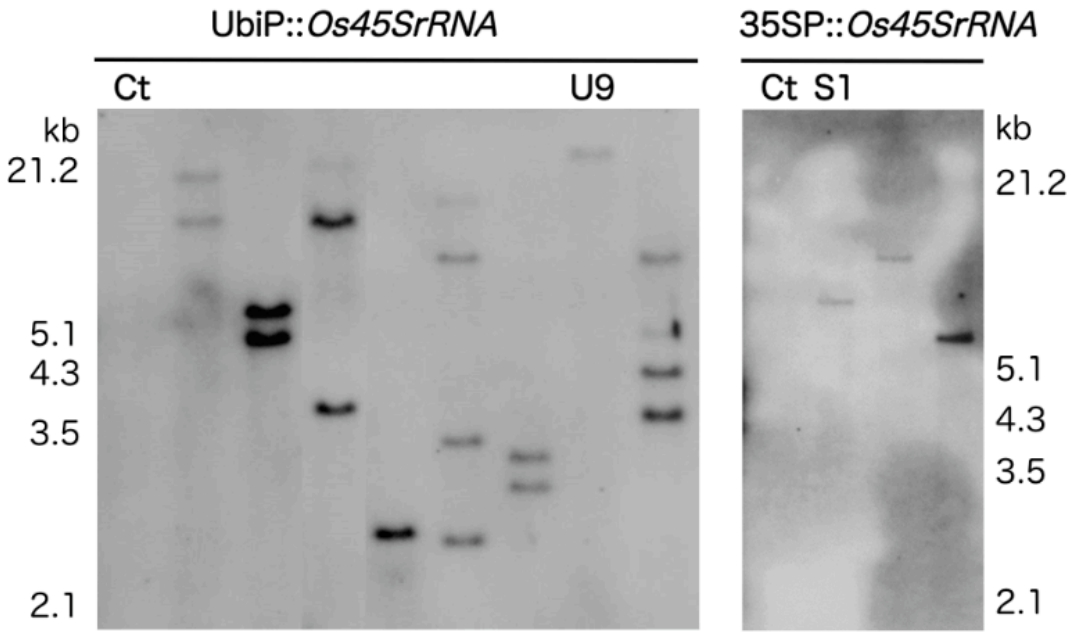

Figure S1 Southern blot analysis for hygromycin gene in transgenic plants. A: Transgenic tobacco plants containing single-copy (U9 and S1) of hygromycin resistance (*hpt*) gene were selected by Southern blot analysis. Total genomic DNAs were digested with *Hin*dIII and hybridization was done using labeled PCR product of *hpt* gene as a probe.

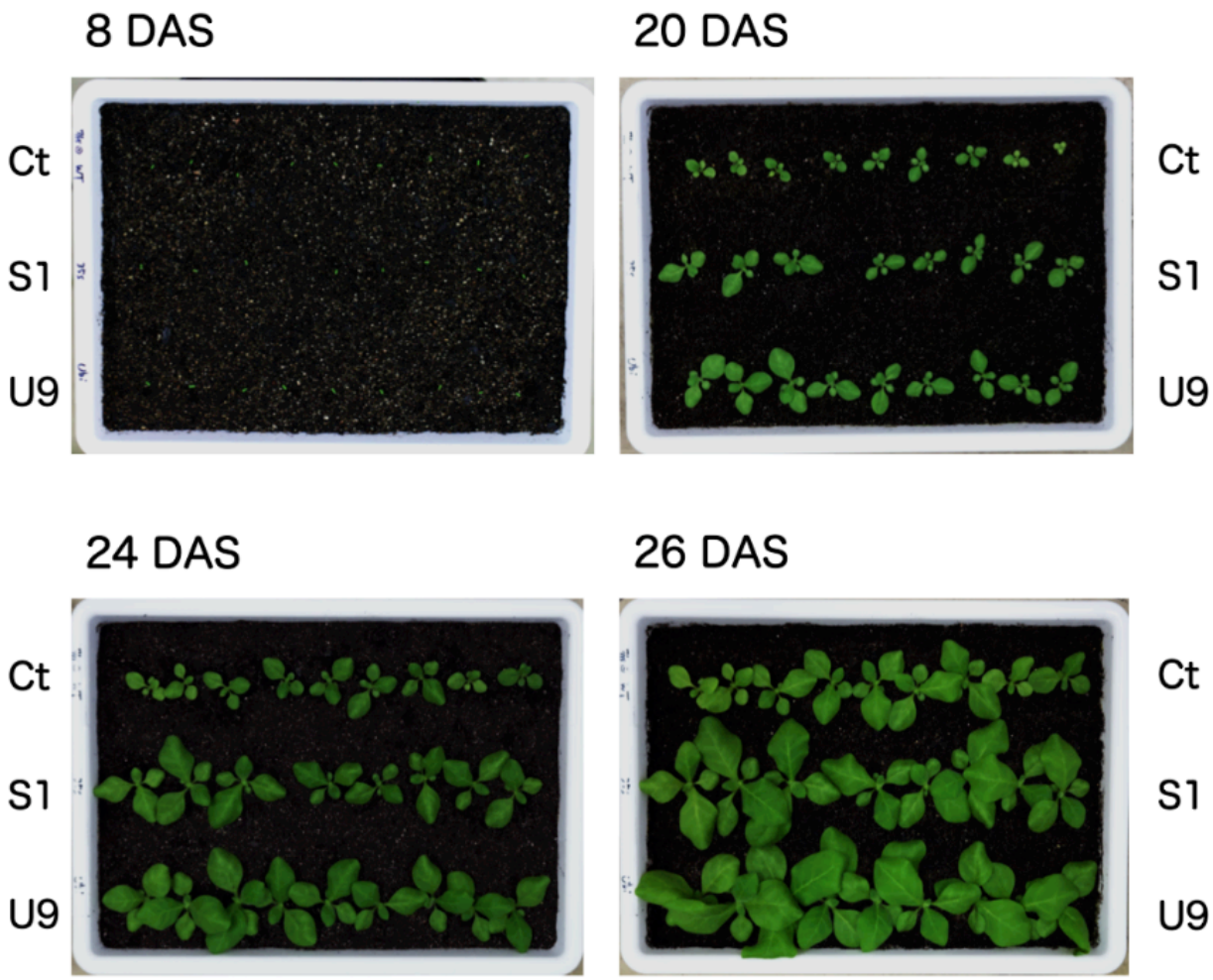

Figure S2 Comparison of growth between transgenic and control plants. Transgenic S1/U9 and control Ct plants were grown in large tray within a growth chamber. In this condition, U9 plants grew bigger than S1 plants during 20 to 26 DAS.

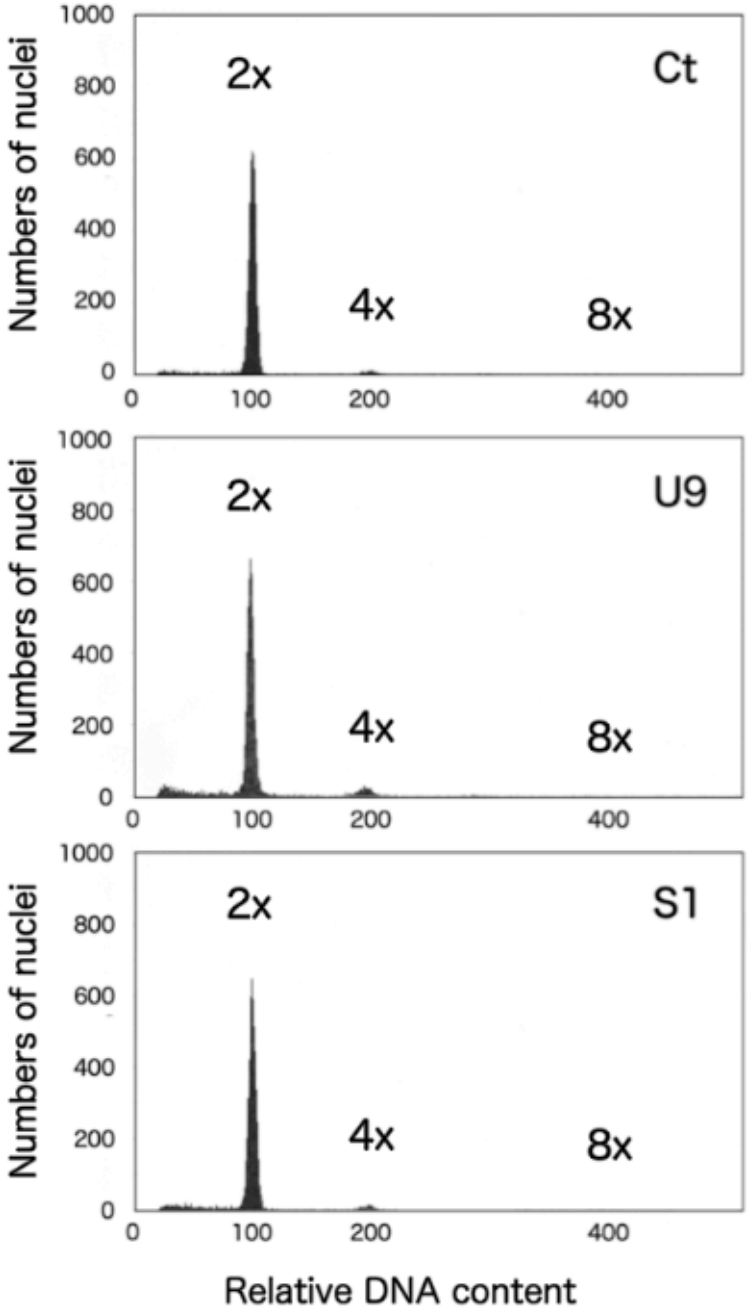

Figure S3 Relative DNA content per nuclei of leaf cells between transgenic and control plants. Fully expanded leaves of transgenic S1/U9 and control Ct plants were subjected to Partec flow cytometer at 30 DAS.

Table S1

Measurements of various photosynthetic parameters in transgenic and control leaves. Transgenic S1/U9 and control Ct plants were grown in a growth chamber and photosynthetic parameters of leaves were directly measured using IMAGING-PAM at 10, 17, and 24 DAS. φPSII: the quantum yield of photosystem II, 1-qL: the reduction state of PSII, NPQ: the non photosynthetic quenching.

	Line	φPS II	1-qL	NPQ
10 DAS	Ct	0.182±0.007	0.887±0.009	0.524±0.021
	S1	0.181±0.007	0.866±0.006	0.542±0.007
	U9	0.181±0.008	0.886±0.004	0.540±0.008
17 DAS	Ct	0.410±0.010	0.498±0.025	0.351±0.020
	S1	0.410±0.005	0.507±0.021	0.333±0.013
	U9	0.420±0.006	0.517±0.016	0.349±0.013
24 DAS	Ct	0.456±0.021	0.464±0.031	0.433±0.015
	S1	0.452±0.023	0.471±0.035	0.432±0.027
	U9	0.463±0.023	0.477±0.034	0.425±0.018

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

Table S2 Relative DNA content per nuclei of somatic cells in U9 transgenic and Ct control seedlings

Organ		7 DAS			8 DAS			10 DAS			14 DAS			20 DAS		
		2C	4C	4C/2C	2C	4C	4C/2C	2C	4C	4C/2C	2C	4C	4C/2C	2C	4C	4C/2C
Cotyledon	Ct	2062	419	0.20	2201	117	0.05	3101	198	0.06	1970	245	0.12	3575	180	0.05
		1239	166	0.13	2867	87	0.03	3183	220	0.07	3517	388	0.11	3275	175	0.05
		1163	201	0.17	418	47	0.11	3038	275	0.09	2439	240	0.10	2913	199	0.07
	Sum	4464	786	0.18	5486	251	0.05	9322	693	0.07	7926	873	0.11	9763	554	0.06
	U9	2773	467	0.17	3122	146	0.05	3278	281	0.09	3463	351	0.10	2862	245	0.09
		2642	286	0.11	2608	172	0.07	3166	280	0.09	2408	211	0.09	1519	71	0.05
		2145	482	0.22	2750	144	0.05	2782	225	0.08	2539	249	0.10	2015	183	0.09
	Sum	7560	1235	0.16	8480	462	0.05	9226	786	0.09	8410	811	0.10	6396	499	0.08
Hypocotyl	Ct				780	626	0.80	408	178	0.44	545	613	1.12	565	434	0.77
					1303	926	0.71	811	608	0.75				270	225	0.83
					997	611	0.61	683	409	0.60				278	202	0.73
	Sum				3080	2163	0.70	1902	1195	0.63	545	613	1.12	1113	861	0.77
	U9				1511	1802	1.19	999	740	0.74	898	1031	1.15	428	237	0.55
					1143	1209	1.06	900	609	0.68				496	249	0.50
					1047	820	0.78	1156	729	0.63				783	571	0.73
	Sum				3701	3831	1.04	3055	2078	0.68	898	1031	1.15	1707	1057	0.62
Root	Ct	475	528	1.11	257	498	1.94	156	470	3.01	316	482	1.53	443	655	1.48
		530	682	1.29	148	347	2.34	125	409	3.27	337	375	1.11	617	565	0.92
		321	407	1.27	267	495	1.85	127	397	3.13	252	327	1.30	266	204	0.77
	Sum	1326	1617	1.22	672	1340	1.99	408	1276	3.13	905	1184	1.31	1326	1424	1.07
	U9	314	514	1.64	387	886	2.29	136	554	4.07	135	190	1.41	367	810	2.21
		231	573	2.48	353	687	1.95	335	880	2.63	158	279	1.77	494	606	1.23
		199	483	2.43	146	419	2.87	255	766	3.00	339	561	1.65	386	916	2.37
	Sum	744	1570	2.11	886	1992	2.25	726	2200	3.03	632	1030	1.63	1247	2332	1.87

Numbers indicated the counts of nuclei