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Effects of polyploidy on photosynthetic properties and anatomy in leaves of *Phlox drummondii*

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Abstract. Polyploidy affects photosynthesis by causing changes in morphology, anatomy and biochemistry. However, in newly developed polyploids, the genome may be unstable. In this study, diploid (2×) and synthetic autotetraploids in initial (4×-C_0) and 11th generations (4×-C_{11}) of *Phlox drummondii* Hook were used to study the effects of chromosome doubling and genome stabilisation on leaf photosynthesis and anatomical properties. The light-saturated photosynthetic rate on a leaf area basis at 360 μmol CO_2 mol^{-1} air (A_{360}) was highest in 4×-C_{11} leaves, intermediate in 4×-C_0 leaves, and lowest in 2× leaves. Rubisco amounts, CO_2-saturated photosynthetic rate at 1200 μmol CO_2 mol^{-1} air at PPFD of 1000 μmol m^{-2} s^{-1} (A_{1200}, representing the capacity for RuBP regeneration), cumulative surface areas of chloroplasts facing intercellular spaces (S_c), all expressed on a leaf area basis, were all higher in 4× leaves than in 2× leaves, and stomatal conductance (g_s) at 360 μmol CO_2 mol^{-1} air was only higher in the 4×-C_{11} leaves. A_{360} for the 4×-C_{11} leaves was greater than that in the 4×-C_0 leaves despite having similar amounts of Rubisco. This was presumably associated with a greater RuBP regeneration capacity, as well as an increase in S_c and g_s, which would increase the CO_2 concentration of Rubisco. These results indicate that the higher rate of photosynthesis in 4×-C_{11} leaves was not an immediate outcome of chromosome doubling; rather, it was due to adjustment and adaptation during the process of genome stabilisation.

Additional keywords: autopolyploidy, photosynthesis, Rubisco, stomatal conductance.

Introduction

Polyploidy is an important factor underlying the evolution of flowering plants (Stebbins 1950, 1971), inducing various changes in the plants (Naggle 1946; Frydrych 1970; Levin 1983; Ramsey and Schemske 2002). Wang *et al*. (2005) suggested that many plant species, including cultivated rice, that are apparently diploids, have experienced genome duplication and that polyploidisation is still an ongoing process. Besides genome changes, phenotypic variation, such as flowering time variation, can occur rapidly after polyploidy formation (Schranz and Osborn 2000). It has been suggested that polyploid plants are more successful in adverse climatic conditions, have better colonising ability, and occur in more diverse habitats than their diploid progenitors (Thompson and Lumaret 1992; Soltis and Soltis 2000; Bretagnolle and Thompson 2001). Despite the abundance of polyploids in nature, only a few of the many new polyploids formed in any species would have been able to possess/accumulate the right combination of characters for better adaptability. Most of the phenotypic differentiations, such as cell size and organ size, occur immediately after polyploidisation (Wendel 2000; Osborn *et al.* 2003). However, newly formed polyploids are often unstable and rapid changes take place in the next generations. Recent studies suggest that non-Mendelian mechanisms operate in creating variations and stabilisation in the early generations following polyploidisation (Liu and Wendel 2002; Schranz and Osborn 2004, reviewed in Matzke *et al*. 1999; Comai 2000). To clarify the mechanisms of the adaptive advantages of polyploidy, we need to identify physiological traits that contribute to the success of polyploid plants. Enhanced photosynthetic capacity is a prime candidate that leads to the

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success of polyploids because it would improve dry matter production and growth of the plants and, in many plants, reproductive success is dependent on plant size or biomass (Silvertown and Lovett Doust 1993).

Various effects of polyploidy on the photosynthetic rate per unit leaf area have been reported (for a review see Warner and Edwards 1993). Some polyploid plants showed higher photosynthetic rates per unit leaf area than diploid plants [e.g. *Trifolium repens* L., (Stålfelt 1943) cited in Bjurman (1959); *Thalictrum alpinum* L., Mooney and Johnson 1965; *Festuca arundinacea* Schreb, Byrne *et al*. 1981; *Panicum virgatum* L., Warner *et al*. 1987], and other polyploids had similar [e.g. *Viola adunca* J. E. Smith, (Mauer *et al*. 1978); *Medicago sativa* L., (Pfeiffer *et al*. 1980); *Pennisetum americanum* L., (Warner and Edwards 1988)] or lower [e.g. *Ribes satigrum* (F_1 hybrid between *R. sativum* (Rchb.) Syme and *R. nigrum* L.), (Bjurman 1959); *Triticum aestivum* L., (Watanabe *et al*. 1997)] photosynthetic rates compared with the diploids. There are two possible reasons why it is difficult to generalise the effects of polyploidy on photosynthesis. First, as Warner and Edwards (1993) have pointed out, the photosynthetic rates per unit leaf area are affected by anatomical and biochemical changes. Polyploidy generally increases the gene dosage, protein amount and enzyme activities per cell. It also increases mesophyll cell volume and leaf thickness. However, the extent of the increases is different depending on nature of polyploidy (see below) and on plant species. Second, different types of polyploid plants, auto- and allopolyploids, have been used to study the effect of polyploidy on photosynthesis. Allopolyploids are formed by the union of genetically distinct chromosome sets, mostly from different species, and autopolyploids are originated by multiplication of a single genome such that all the chromosomes come from the same species. Therefore, allopolyploids show the effects of chromosome multiplication as well as hybridisation. In contrast, autopolyploids show the effects of chromosome doubling *per se*. In addition, some studies used natural polyploids, and others used synthetic ones. The precise backgrounds of natural polyploids are mostly unclear. In contrast, progenitors of the synthetic polyploids are known.

As mentioned above, some natural polyploids have higher photosynthetic rates than the related diploids, but other autopolyploids including synthetic ones show similar or lower photosynthetic rates per unit leaf area than the isogenic diploids. Both ploidy-dependent gene expression and repression were observed in isogenic *Saccharomyces cerevisiae* Hansen strains (Galitski *et al*. 1999), indicating involvement of such ploidy-dependent expression or repression in determining the photosynthetic capacity in plants. A recent proteomic study using synthetic autotetraploids of *Brassica oleracea* L., however, showed that chromosome doubling did not induce major differential regulations of gene expression in the leaves (Albertin *et al*. 2005). Hence, the following fundamental questions may be still relevant: Are higher rates of net photosynthesis attributed to polyploidisation itself, or to compound effects of polyploidisation and hybridisation? Does genome stabilisation after the polyploidisation contribute to the increase in the rate of net photosynthesis? To address the former question, it is necessary to uncouple the effects of chromosome multiplication from those of hybridity. To answer the latter one, it is necessary to compare autopolyploids at the initial and later generations with their diploid progenitors. Thus, synthesised autopolyploids are ideal materials.

Because polyploidy generally increases the mesophyll cell volume and leaf thickness, many studies have assessed relationships between the photosynthetic rates per unit leaf area and anatomical parameters that would affect the conductance for CO_2 diffusion from the substomatal cavity to the chloroplast stroma (g_i) (e.g. Romero-Aranda *et al*. 1997). Recently, the nature of g_i has been intensively studied (for reviews see Evans and Loreto 2000; Terashima *et al*. 2006). First, the internal conductance, g_i, is much smaller than previously thought and limits photosynthesis substantially. Second, anatomical factors, such as the cumulative surface area of chloroplasts facing the intercellular spaces per leaf area (S_c) and cell wall thickness, have been identified as important determinants of g_i on a leaf area basis. Because S_c and cell wall thickness represent the area for CO_2 dissolution and the path length for CO_2 diffusion in the liquid phase (in which diffusion of CO_2 is $\sim 10^4$ times slower than that in air), respectively, g_i is proportional to S_c and inversely proportional to cell wall thickness. Thus, analyses of the photosynthetic rate, S_c and cell wall thickness would reveal causal relationships between the photosynthetic rate and anatomical changes in the polyploids. However, such datasets have not been obtained for polyploid plants and a consensus concerning the effects of polyploidy on CO_2 diffusion has not been obtained.

The aims of this study were to (i) examine the effects of chromosome doubling on leaf photosynthesis and anatomical properties, and (ii) clarify how genome stabilisation after the chromosome doubling affects photosynthetic properties and leaf anatomy. In this study, we chose *Phlox drummondii* as an experimental material because *P. drummondii* has been used in a series of studies on the effects of polyploidy on plant metabolism (e.g. Levy 1976; Levin *et al*. 1979). In particular, Bazzaz *et al*. (1982) conducted an interesting study examining effects of polyploidy on leaf photosynthesis. They showed that most synthesised tetraploid *P. drummondii* cultivars had similar or lower photosynthetic rates per unit leaf area than their corresponding diploids, but, in one cultivar, the tetraploid had a higher photosynthetic rate. They concluded that the outcome of the chromosome doubling was dependent on the genetic background (strains) in *P. drummondii*. However, they measured only light-saturated photosynthetic rate and did not describe the generations of the synthetic tetraploids used in their experiment. We synthesised the autotetraploids of *P. drummondii* and maintained them for 11 generations. We also synthesised new autotetraploids from the same diploids. We examined photosynthetic properties and anatomical and biochemical features that would affect photosynthesis in the diploid and autotetraploid plants at the initial and 11th generations. Based on the data, we discuss the ecological advantages of polyploidy.

Materials and methods

Plant material

Phlox drummondii Hook (Polemoniaceae) is an annual ornamental plant. Seeds of *P. drummondii* [diploid (2×)] were collected locally in Delhi, India. Autotetraploid were raised by colchicine treatment (see below) from these seeds. The

diploid and autotetraploid at 11th generation (4×-C_{11}) were maintained through open pollination in an experimental garden in the University of Delhi. Their ploidy levels were checked in every generation by chromosomal counting. *P. drummondii* is originally allogamous and many bees visited our plants. However, our *P. drummondii* were not self-incompatible (P. Vyas, unpubl. data). The 2×-plants used in this study might not have been fully homozygous, but hybridisation with other varieties or introduction of new alleles might not have occurred because the plants were maintained in small groups.

The 2× and 4×-C_{11} seeds were sown in vermiculite in a growth room at Osaka University, Japan. Pots were maintained in the dark until the seedlings emerged (~5 days). When the seedlings reached the four-leaf stage, they were transplanted to larger pots (17.5 cm high, 10.5 cm diameter, one plant per pot) containing vermiculite. The day/night air temperatures were 22/18°C, and day length was 10 h. Photosynthetically active photon flux density (PPFD) was maintained at 200 μmol m^{-2} s^{-1}. The autotetraploid plants at zero generation (4×-C_0) were synthesised and were also maintained under the same growth conditions as for the 2× and 4×-C_{11} plants.

Induction of autopolyploidy

Diploid ($2n = 2\times = 14$) seedlings of *P. drummondii* at the cotyledonary stage were used for the induction of autopolyploidy. Small cotton swabs soaked in a colchicine solution (0.1% w/v) were placed on the shoot apex for 6–8 h. Care was taken to ensure the cotton swabs were wet by adding drops of the colchicine solution at regular intervals. After the treatment, the cotton swabs were removed and the seedlings were gently washed with water to remove the colchicine.

The flower buds of the shoots emerging from these seedlings were fixed in a solution of ethanol and glacial acetic acid for more than 12 h. Male meiosis at metaphase I or anaphase I was analysed to check the chromosome number.

Leaf properties and anatomy

Small leaf pieces (3 mm × 1 mm) were cut from the middle region and fixed in a phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde overnight at 4°C. The samples were post-fixed in 2% osmium tetroxide in a 25 mM phosphate buffer at pH 7.2 for 3 h, dehydrated in acetone and propylene oxide series and embedded in Spurr's resin (Spurr 1969). Transverse sections and paradermal sections of the palisade tissues were cut on an ultramicrotome (Reichert Ultracut S, Leica, Vienna, Austria) and stained by 0.5% toluidine blue. The micrographs of the sections were taken and scanned. Scanned images were used for measurements. For electron microscopy, the ultra-thin sections were stained by uranyl acetate (Stempak and Ward 1964) and lead citrate (Reynolds 1963) and mounted on formvar-coated grids. The sections were examined under a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan). Electron micrographs were taken at ×3000. We analysed at least three images from one leaf and three leaves were examined for each of the 2×, 4×-C_0 and 4×-C_{11} plants.

Total leaf thickness (L), mesophyll thickness (excluding both epidermis) (T_{mes}) and section width (W) were measured using NIH Image software (National Institute of Health). The total cross sectional area of the intercellular air spaces and the cross sectional area of mesophyll cells (A_{mes}) were measured. The length and width of spongy and palisade tissue cells were also measured. The volume of mesophyll cells per unit leaf area (V_{mes}) was estimated as $V_{mes} = A_{mes}/W$ (Syvertsen *et al*. 1995).

The total perimeter length of mesophyll cells facing the intercellular spaces (L_{mes}) and total perimeter length of chloroplasts facing the intercellular spaces (L_c) were measured on the micrograph of the transverse section. The cumulative surface area of the mesophyll cells facing the intercellular spaces was calculated as $S_{mes} = L_{mes} \times F/W$, where F is the curvature correction factor. The cumulative surface area of chloroplasts facing the intercellular spaces (S_c) was calculated as $S_c = S_{mes} \times L_c/L_{mes}$, where L_c is the perimeter length of chloroplasts facing the intercellular spaces (Syvertsen *et al*. 1995; Miyazawa and Terashima 2001). The curvature correction factor, F, was calculated separately for spongy and palisade tissue cells according to Thain (1983). The F-value for 2×, 4×-C_0 and 4×-C_{11} of the spongy tissue cells was 1.18, and those for the palisade tissue cells were 1.41, 1.38 and 1.38, respectively.

The number of cells per unit leaf area was counted from the paradermal sections of the palisade tissue. For cell wall thickness measurements, electron micrographs of the paradermal sections of the palisade tissue at ×3000 magnification were analysed using NIH Image.

Gas exchange measurements

The CO_2 exchange rate was measured in fully expanded attached leaves using a portable gas-exchange system (LI-6400, Li-Cor, Lincoln, NE, USA) with a cuvette enclosing the leaf area of 6 cm^2 at different PPFDs. The light was provided by an external halogen lamp (150 W, Type 6423, Philips, Hamburg, Germany) via an optical fibre with a rectangular light emitter. The plant was first kept in the dark for 30 min and the dark respiration rate in the leaf was measured. Next, the PPFD was varied from low to saturating levels to obtain a light response curve of photosynthesis. The CO_2 concentration in the ambient air was maintained at 360 μmol CO_2 mol^{-1} air. The leaf temperature and vapour pressure deficit were maintained at 22 ± 2°C and less than 1 kPa. All gas exchange parameters including stomatal conductance (g_s) were calculated according to von Caemmerer and Farquhar (1981).

Dependence of the photosynthetic rate on the intercellular CO_2 concentration (A-C_i response) was also analysed. We first measured the rate of photosynthesis at an ambient CO_2 of 360 μmol mol^{-1}, lowered CO_2 concentration to 50 μmol mol^{-1} and then increased to 1200 μmol mol^{-1}, keeping the other conditions similar to those for the light response curve. To minimise CO_2 leakage through the slits between the sealing pads of the cuvette, we routinely used a laboratory-made skirt with which the air, once exhausted, was blown to the slits from the outside (Miyazawa and Terashima 2001). The PPFD was maintained at 1000 μmol m^{-2} s^{-1}. The plants used for the measurements of A-C_i responses were grown as mentioned earlier but different from those used for other measurements.

Absorptance of the leaf was measured using a laboratory-made integrating sphere, and a quantum sensor (LI-190SA,

Li-Cor) attached on the side of the integrating sphere according to Funayama and Terashima (1997).

After the photosynthetic measurements, leaves were oven-dried at 70°C for 48 h to estimate leaf dry weight per unit area (LMA).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM 101, Walz, Effeltrich, Germany). The leaf, dark-adapted for at least 30 min, was initially exposed to the weak, modulated measuring beam to assess the initial minimal fluorescence yield (F_0), and then a strong flash was given for 1 s to assess the maximal fluorescence level with the PSII reaction centre closed (F_m). The maximal photochemical efficiency (F_v/F_m) was calculated.

Rubisco estimation

The amount of Rubisco was measured according to Makino *et al*. (1986). The frozen leaf discs (0.785 cm^2) were ground to powder in liquid nitrogen and homogenised in 400 µL of an extraction buffer containing 100 mM Na-phosphate buffer (pH 7.0), 1% (v/v) 2-mercaptoethanol, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.1% (v/v) Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone. The extract was centrifuged and the supernatant was used for the determination of Rubisco. Rubisco was separated by polyacrylamide gel electrophoresis with 12.5% resolving gel and 4.75% stacking gel (Laemmli 1970). The gel was stained with Coomassie Brilliant Blue R-250. The amount of Rubisco large subunit was determined spectrophotometrically with a gel densitometer (FD-A-IV, Fujiox, Tokyo, Japan).

Chlorophyll estimation

Leaf discs (0.785 cm^2) were sampled avoiding the major veins and placed in 3 mL of *N*,*N*-dimethylformamide (DMF) solution in the dark at 4°C overnight. Chlorophyll content and chlorophyll *a*/*b* ratio were estimated spectrophotometrically using the equations by Porra *et al*. (1989).

Statistical analysis

All data were statistically analysed according to Sokal and Rohlf (1995). Differences of leaf anatomical and photosynthetic properties between diploid and C_0 and C_{11} tetraploids were tested with Tukey–Kramer's multiple comparison test at $P < 0.05$. Relationships between A_{360} and leaf characteristics were examined using linear regression analysis.

Results

Autotetraploid plants

Colchicine-treated plants were examined at their meiotic stage. The plants with 2*n* chromosome number of 28 were selected as autotetraploids. The autotetraploid plants showed 2–12 bivalents and one to six quadrivalents at metaphase I. The autotetraploid plants clearly showed 14:14 chromosome distribution at anaphase I (inset images in Fig. 1). The present colchicine treatment caused autotetraploidy in ~16% of the plants.

Fig. 1. Diploid (2×) and autotetraploid at 11th generation (4×-C_{11}) of *Phlox drummondii*. Chromosomes in *P. drummondii* (inserted images). Scale bar indicates 10 µm.

Morphology of plants

Seeds of both 4×-C_0 and 4×-C_{11} germinated more slowly than those of 2×. Growth of 4× seedlings was slower, but the 4× plants appeared more robust and healthier even at the seedling stage. The leaves, flowers, seeds of both 4× plants were larger than those of 2× plants (Fig. 1). The leaves of 4× plants were larger, thicker, and greener than those of 2× plants. The flowering period was longer in both 4× plants than in the 2× plants.

Leaf anatomical characteristics

Figure 2*a* shows cross sections of the leaves. Stomata were located on both surfaces in all the leaves. The mesophyll cells (both the palisade and spongy tissue cells) were larger in 4×leaves than in 2× leaves. Leaves were thicker in both 4× plants than in 2× plants. The paradermal sections clearly demonstrated differences in the cell arrangement (Fig. 2*b*). In 2× leaves, the palisade tissue cells were sparsely arranged. The cells in 4×-C_0 leaves were also sparse, although those in 4×-C_{11} leaves were more compact (Fig. 2*b*). The palisade tissue cell number per unit leaf area was smaller in both 4× leaves than in 2× leaves (Table 1). Between the 4× leaves, 4×-C_{11} leaves had more cells than 4×-C_0 leaves.

As expected from the thicker leaves with compact cell packaging, the cumulative surface area of mesophyll cells facing the intercellular spaces (S_{mes}) was greatest in 4×-C_{11} leaves, and 4×-C_0 and 2× leaves had almost similar values (Table 1). Cumulative surface area of chloroplasts facing the intercellular spaces per leaf area (S_c) was much greater in 4×-C_{11} leaves than in 4×-C_0 leaves, and 2× leaves showed the smallest value (Table 1). The difference in S_c between the 4× leaves was attributed to the difference in mesophyll surface area (S_{mes}), because S_c/S_{mes} ratio did not differ significantly between 4×-C_0 and 4×-C_{11} leaves. S_{mes} in 2× leaves was comparable with that in 4×-C_0 leaves, but the ratio of S_c/S_{mes} was lowest in 2×, which resulted in the lowest S_c in 2× leaves. Cell wall was thickest in 4×-C_{11} leaves and those in 2× and 4×-C_0 leaves were comparable (Table 1).

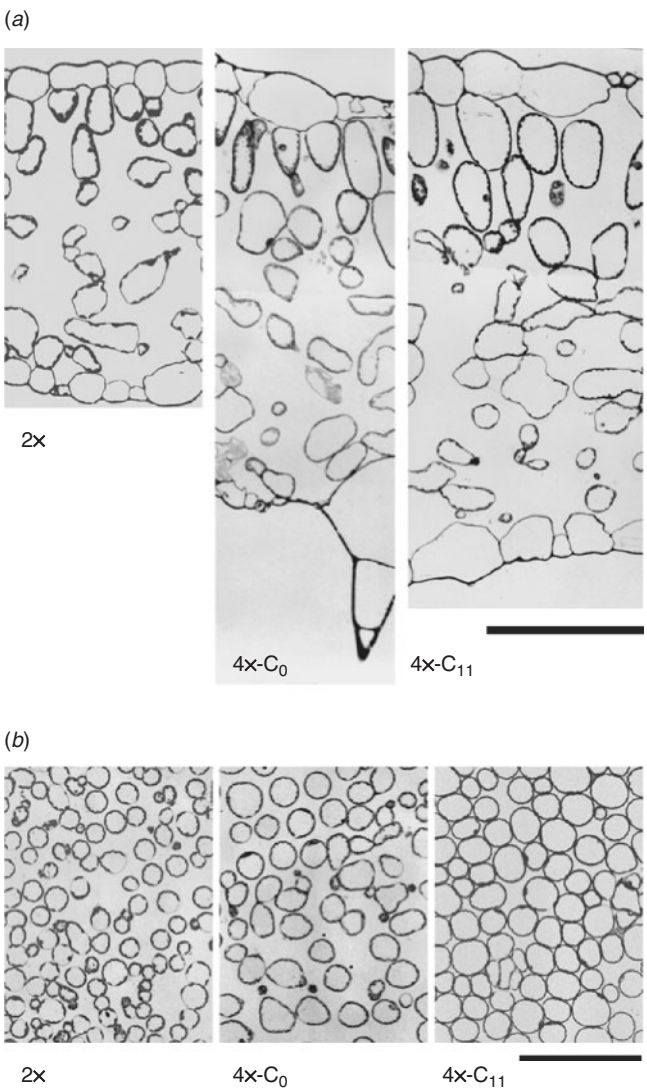

Fig. 2. Cross sections (*a*) and paradermal sections (*b*) of leaves in diploid (2×) and autotetraploid at initial (4×-C_0) and 11th generations (4×-C_{11}) of *P. drummondii*. Scale bars denote 100 μm (*a*) and 200 μm (*b*).

Other leaf anatomical characteristics are shown in Table 1. The leaf dry mass per unit area (LMA) in 4×-C_0 and 4×-C_{11} leaves were greater than that in 2× leaves by 56 and 81%, respectively. Mesophyll thickness (T_{mes}) was greatest in 4×-C_0 leaves, followed closely by 4×-C_{11} leaves and then by 2× leaves. Mesophyll cell volume (V_{mes}) was greatest in 4×-C_{11} leaves, closely followed by 4×-C_0 leaves and was smallest in 2× leaves. We note that standard deviation of V_{mes} for 4×-C_0 leaves was extremely large, because V_{mes} varied among the 4×-C_0 leaves.

Leaf photosynthetic properties

Figure 3 compares dependency of the rate of photosynthetic CO_2 assimilation on PPFD. Light-saturated photosynthetic rate at 360 μmol CO_2 mol^{-1} air (A_{360}) was highest in the leaves of 4×-C_{11} leaves and lowest in 2× leaves (Table 2). A_{360} in the 4×-C_{11} leaves was more than twice (218%) of that in 2× leaves. The light saturation levels also increased in 4×-C_{11} (1200 μmol m^{-2} s^{-1}) and 4×-C_0 leaves (1000 μmol m^{-2} s^{-1}) from ∼700 μmol m^{-2} s^{-1} in 2× leaves. The initial slope of the light-response curve on an incident PPFD basis was significantly higher in 4×-C_{11} leaves than in 2× leaves (Fig. 3; Table 2). 4×-C_0 leaves showed an intermediate value, but neither the difference between 4×-C_{11} and 4×-C_0 leaves nor that between 2× and 4×-C_0 leaves was statistically significant. The variation in the initial slope of the light-response curve was partly attributed to differences in light absorptance among the leaves (Table 2).

Figure 4 compares the photosynthetic rate (*A*)-intercellular CO_2 concentration (C_i) relationships. The CO_2-saturated photosynthetic rate at PPFD of 1000 μmol m^{-2} s^{-1} at 1200 μmol CO_2 mol^{-1} air (A_{1200}) were 2.5 and 1.7 times that in 2× leaves for the 4×-C_{11} and 4×-C_0 leaves, respectively. The initial slope of A-C_i curve in 4×-C_{11} leaves was slightly higher than that in 4×-C_0 leaves, and significantly higher than that in 2× leaves. The curves attained the asymptote levels at lower C_i in 4×-C_0 leaves than in 4×-C_{11} leaves, suggesting that the capacity for the RuBP regeneration relative to that for the RuBP carboxylation was lower in 4×-C_0 leaves.

Photosynthetic properties in leaves of 2×, 4×-C_0 and 4×-C_{11} are summarised in Table 2. The stomatal conductance (g_s), which usually highly correlated with A_{360}, was highest in 4×-C_{11} leaves. 2× and 4×-C_0 leaves showed similar g_s. The amount of Rubisco per leaf area in 4×-C_{11} leaves was slightly lower than that in 4×-C_0 leaves, but significantly higher than that in 2× leaves. The ratio of the initial slope of the CO_2 response curve to A_{1200}, which is an indicator of the *in vivo* balance between the capacities for RuBP carboxylation and RuBP regeneration, was lowest in 4×-C_{11} leaves (0.0027), intermediate in 2× leaves (0.0033) and highest in 4×-C_0 leaves (0.0036). The amount of chlorophyll $a+b$ (chl $a+b$) was highest in 4×-C_{11} leaves and the differences between 4×-C_0 and 2× leaves were not significant. Leaf absorptance differed reflecting chl $a+b$. The maximal photochemical efficiency of PSII, F_v/F_m, was not different among the leaves. Dark respiration rate was higher in both 4× leaves than in 2× leaves.

The dependences of A_{360} on S_{mes}, S_c, g_s and Rubisco amount are shown in Fig. 5. For the leaf anatomical properties, the determination coefficient was greater for the dependence on S_c than that on S_{mes}. The stomatal conductance (g_s) and Rubisco amount are usually correlated with A_{360}. In the present study, the dependence of A_{360} on g_s was strong, but that on Rubisco amount was weak. As mentioned above, in 4×-C_0 leaves, the ratio of the initial slope to A_{1200} was highest. All these features indicate that the 4×-C_0 leaves have more Rubisco for their capacity for RuBP regeneration.

A_{360} on various unit bases are compared (Table 3). A_{360} per unit leaf dry weight and per Rubisco were highest in 4×-C_{11} leaves, and those in 4×-C_0 and 2× leaves were comparable. A_{360} on chl basis was higher in 4×-C_0 leaves than in 4×-C_{11} and 2× leaves by ∼26%.

Discussion

The purpose of this study was to assess the effects of chromosome doubling on leaf photosynthesis and how the

Table 1. Leaf anatomical properties in diploid (2×) and autotetraploid at initial (4×-C_0) and 11th generations (4×-C_{11}) of *Phlox drummondii*

Values are mean ± s.d. for number of data in column *n*. Different letters within a row indicate statistical difference at 5% level (Tukey–Kramer's multiple comparison test, $P < 0.05$). V_{mes}, volume of mesophyll cell per unit leaf area; T_{mes}, mesophyll thickness; S_{mes}, cumulative surface area of mesophyll cells facing intercellular space per unit leaf area; S_c, cumulative surface area of chloroplasts facing intercellular space per unit leaf area

	2×	4×-C_0	4×-C_{11}	*n*
Leaf dry mass per area ($g\,m^{-2}$)	16.0 ± 0.3a	24.5 ± 1.3b	28.6 ± 5.4b	3
V_{mes} ($mm^3\,mm^{-2}$)	39.8 ± 8.2a	63.4 ± 30.1ab	67.2 ± 10.1b	3–5
T_{mes} (μm)	112.3 ± 34.2a	178.5 ± 3.5a	173.6 ± 26.3a	4
Palisade cell number per unit leaf area (mm^{-2})	1064 ± 151a	467 ± 49b	688 ± 71c	8–10
S_{mes} ($m^2\,m^{-2}$)	14.64 ± 0.94a	14.65 ± 1.15a	20.28 ± 1.7b	3
S_c ($m^2\,m^{-2}$)	8.54 ± 0.92a	11.62 ± 0.09b	18.18 ± 1.73c	3
S_c/S_{mes}	0.58 ± 0.03a	0.80 ± 0.06b	0.90 ± 0.04b	3
Cell wall thickness (μm)	0.12 ± 0.02a	0.14 ± 0.02a	0.25 ± 0.02b	3–5

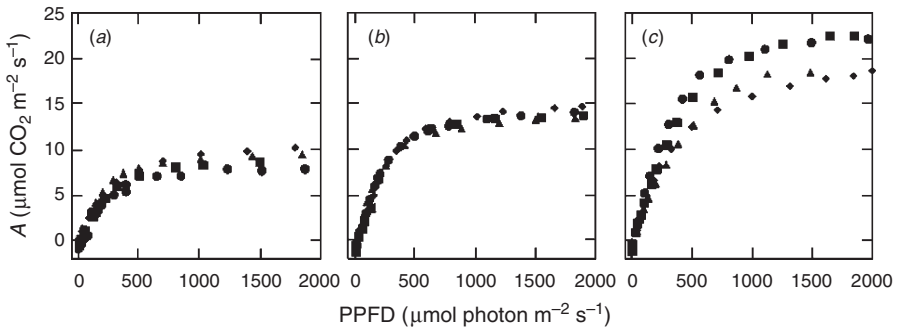

Fig. 3. Light-response curves of photosynthesis at 360 μmol CO_2 mol^{-1} air of leaves in diploid (2×, (*a*)) and autotetraploid at initial (4×-C_0, (*b*)) and 11th generations (4×-C_{11}, (*c*)) of *P. drummondii*. Different symbols denote different leaves.

photosynthetic properties of the synthesised polyploid plants change with generation. The comparison of 2×, 4×-C_0 and 4×-C_{11} leaves illustrated interesting features: light-saturated photosynthetic rate at the CO_2 concentration of 360 μmol mol^{-1} air (A_{360}) was greatest in 4×-C_{11} leaves, intermediate in 4×-C_0 leaves and lowest in 2× leaves.

Effects of polyploidy on leaf anatomy and photosynthesis

The leaves in 4× *P. drummondii* were larger and thicker (Figs 1, 2). Although the cell number per unit leaf area decreased, T_{mes}, V_{mes} and mesophyll cell size increased in 4× leaves (Table 1; Fig. 2). Negative correlations between the photosynthetic rate and mesophyll cell size have often been reported (Dunstone and Evans 1974; Romero-Aranda *et al*. 1997). They attributed the decrease in the photosynthetic rate to the decrease in surface area per unit cell volume for gas exchange. However, in the present study, S_{mes} increased in 4× leaves in spite of large mesophyll cells because T_{mes} increased markedly. Thick mesophyll tends to impose greater resistances to CO_2 diffusion in the intercellular spaces. However, the leaves of the present *P. drummondii* were amphistomatous and the tissue density was not very high. Thus, CO_2 flux inside the intercellular spaces would not be an important limiting factor in 4× *P. drummondii* leaves (Terashima *et al*. 2001).

The 4×-C_{11} leaves had greater S_c than 2× and 4×-C_0 leaves (Table 1). In 2× and 4×-C_0 leaves, the cell surfaces facing the intercellular spaces were not totally covered by the chloroplasts, leaving vacant areas that would not be effectively used for gas exchange. Thus, S_c is a more important factor than S_{mes} in considering internal CO_2 conductance in leaves (Evans and Loreto 2000; Oguchi *et al*. 2003; Terashima *et al*. 2006).

The cell wall was considerably thicker in 4×-C_{11} leaves than in 2× and 4×-C_0 leaves (Table 1). Thicker cell walls impose greater resistance to gas diffusion. Considering the data of S_c and cell wall thickness and given that g_i is proportional to S_c and inversely proportional to cell wall thickness, it is highly likely that the lowering of g_i by the thick cell walls was counterbalanced by the increase in S_c in 4×-C_{11} leaves. Unfortunately, we did not measure g_i with the same materials used in the present study. However, we measured g_i in a separate experiment using the 2× and 4×-C_{11} leaves grown under similar conditions by the concurrent measurements of gas exchange and stable carbon isotope analyses, the most reliable method for g_i determination (Evans and Loreto 2000). Internal conductances were 0.16 ± 0.026 in 2× leaves and 0.18 ± 0.038 mol $m^{-2}\,s^{-1}$ in 4×-C_{11} leaves (mean ± s.d., $n = 3$)

Table 2. Leaf photosynthetic properties in diploid (2×) and autotetraploid at initial (4×-C_0) and 11th generations (4×-C_{11}) of *Phlox drummondii*

Values are mean ± s.d. for number of data in column *n*. Different letters within a row indicate statistical difference at 5% level (Tukey–Kramer's multiple comparison test, $P < 0.05$). A_{360}; light-saturated photosynthetic rate at 360 μmol CO_2 mol^{-1} air; R_d; dark respiration rate

	2×	4×-C_0	4×-C_{11}	*n*
Absorptance	0.73 ± 0.13a	0.83 ± 0.05ab	0.87 ± 0.04b	5–7
A_{360} (μmol CO_2 m^{-2} s^{-1})	9.1 ± 1.2a	13.5 ± 1.2b	19.9 ± 1.9c	5–10
Initial slope of light-response curve	0.032 ± 0.004a	0.046 ± 0.005ab	0.051 ± 0.010b	3–4
Stomatal conductance (mmol m^{-2} s^{-1})	0.16 ± 0.01a	0.21 ± 0.03a	0.49 ± 0.06b	3
F_v/F_m	0.781 ± 0.010a	0.790 ± 0.021a	0.792 ± 0.003a	2–4
Rubisco (g m^{-2})	0.59 ± 0.07a	0.92 ± 0.11b	0.85 ± 0.05b	3
Chl $a + b$ (mmol m^{-2})	0.25 ± 0.003a	0.29 ± 0.03a	0.53 ± 0.06b	3
R_d (μmol CO_2 m^{-2} s^{-1})	0.50 ± 0.28a	1.16 ± 0.13ab	1.11 ± 0.22b	3–4

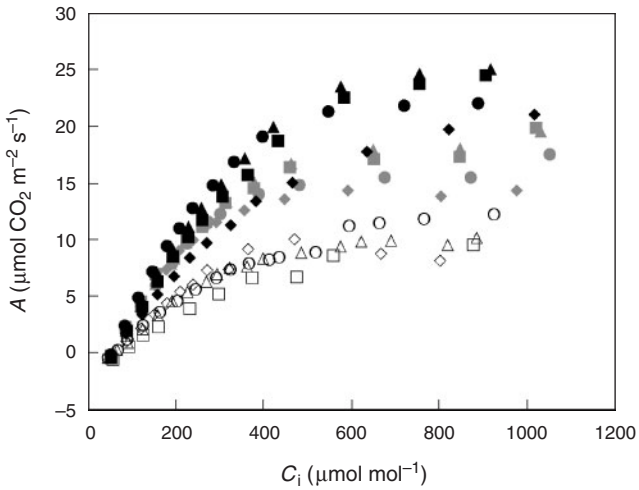

Fig. 4. Light-saturated photosynthesis (*A*)-intercellular CO_2 concentration (C_i) curves of leaves in diploid (2×, open symbols) and autotetraploid at initial (4×-C_0, grey symbols) and 11th generations (4×-C_{11}, closed symbols) of *P. drummondii*. Different symbols denote different leaves. CO_2-saturated photosynthetic rate at PPFD of 1000 μmol m^{-2} s^{-1} at 1200 μmol CO_2 mol^{-1} air (A_{1200}) are 10.0 ± 1.7 in 2× leaves, 16.4 ± 1.6 in 4×-C_0 leaves and 23.9 ± 1.6 in 4×-C_{11} leaves (average ± s.d., μmol CO_2 m^{-2} s^{-1}); Initial slopes of A-C_i curves are 0.033 ± 0.005 in 2× leaves, 0.059 ± 0.002 in 4×-C_0 leaves and 0.064 ± 0.011 in 4×-C_{11} leaves (average ± s.d., mol air m^{-2} s^{-1}).

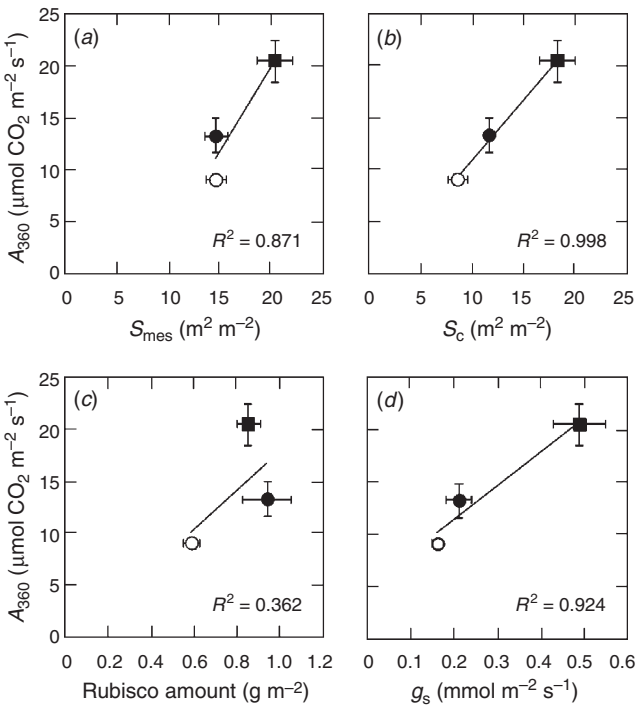

Fig. 5. Relationships between light-saturated photosynthetic rate at 360 μmol CO_2 mol^{-1} air (A_{360}) and S_{mes} (*a*), S_c (*b*), Rubisco amount (*c*) and stomatal conductance (*d*) of leaves in diploid (2×, ○) and autotetraploid at initial (4×-C_0, ●) and 11th generations (4×-C_{11}, ■) of *P. drummondii* Bars indicate s.d.

(Terashima *et al*. 2006). These values strongly supported the above prediction that the adverse effect of the thickened cell wall on g_i was counterbalanced by the large S_c in 4×-C_{11} leaves.

Effects of polyploidy on leaf biochemical components and photosynthesis

Leaves of 4×-C_{11} leaves had less Rubisco per unit area than 4×-C_0 leaves, and both of them had more Rubisco than 2× leaves. Leech *et al*. (1985) also observed lower Rubisco amount in established hexaploids than in synthesised hexaploids of *Triticum* and speculated that a longer period of adaptation and selection might have caused the reduction of Rubisco.

The Rubisco amount was slightly higher but A_{360} on a leaf area basis was lower in 4×-C_0 leaves than in 4×-C_{11} leaves (Table 2). Thus, A_{360} on Rubisco basis was much lower in 4×-C_0 leaves than in 4×-C_{11} leaves (Table 3). The ratio of the initial slope of the CO_2 response curve to A_{1200} suggests that A_{360} was not limited by the RuBP carboxylation but rather by the RuBP regeneration in 4×-C_0 leaves. In other words, in 4×-C_0 leaves, the capacity for RuBP regeneration was not enough for the amount of Rubisco, and thus the rate of RuBP carboxylation at the ambient C_a of 360 μmol CO_2 mol^{-1} air was lower than the rate expected from the capacity of RuBP carboxylation. Moreover, the low A_{360} would be also attributed to the smaller S_c in 4×-C_0 leaves. Because S_c is a measure of available spaces

Table 3. Light-saturated photosynthetic rate at 360 μmol CO_2 mol^{-1} air (A_{360}) on different unit basis in leaves of diploid (2×) and autotetraploid at initial (4×-C_0) and 11th generations (4×-C_{11}) of *P. drummondii*

Percentage in parenthesis denotes percentage increase in the value to that in 2× plants

A_{360}	2×	4×-C_0	4×-C_{11}
Leaf area basis (μmol CO_2 m^{-2} s^{-1})	9.1	13.5 (+48%)	19.9 (+119%)
Dry weight basis (μmol CO_2 g^{-1}DW s^{-1})	0.56	0.54 (−4%)	0.71 (+27%)
Rubisco basis (μmol CO_2 g^{-1} rubisco s^{-1})	15.4	14.3 (−7%)	23.4 (+52%)
Chl basis (μmol CO_2 mol^{-1} chl s^{-1})	36	45 (+25%)	37 (+2%)

for gas exchange and is generally correlated well with g_i, the smaller S_c in 4×-C_0 leaves would limit A_{360} through suppressing g_i and thus a lower CO_2 concentration in the chloroplast stroma (C_c). Taken together, the high Rubisco amount would lead to the proportional enhancement of A_{360} in 4×-C_{11} leaves, but not in 4×-C_0 leaves, because the increase in the Rubisco content was accompanied neither by the appropriate increase in RuBP regeneration capacity (A_{1200}) nor by that in S_c in 4×-C_0 leaves. The smaller g_s contributed to maintain lower C_i in 4×-C_0 plants than in 4×-C_{11} plants ($C_i = 257$ and 295 μmol CO_2 mol^{-1} air in 4×-C_0 and 4×-C_{11} leaves, respectively), which also lowered A_{360} in 4×-C_0 leaves.

In contrast, the lowest A_{360} in 2× leaves would be attributed not only to lower C_i (269 μmol CO_2 mol^{-1} air) caused by small g_s, but also to the smaller Rubisco content and S_c.

Effects of polyploidy on leaf photosynthetic properties: underlying mechanisms and ecological significance

In this study, we observed the biochemical and anatomical changes in the newly synthesised polyploids (4×-C_0), polyploid plants at later generation (4×-C_{11}) and original diploid plants. We clearly showed that A_{360} increased immediately after chromosome doubling and continued to increase at later generations. Chromosome doubling enhanced A_{360} through increases in S_c and Rubisco content accompanied by enlargement of cell size and increased cell constituents. However, in 4×-C_0 leaves, the extent of the change in each component was unbalanced. For example, the amount of Rubisco increased to a great extent, but the amount of chl, one of the representative components for thylakoid reactions, and g_s increased to the lesser extents (Table 2). On the other hand, during stabilisation processes within 10 generations, it is probable that the cell size and many other characteristics had been adjusted to optimise the cell functions, as pointed out by Butterfass (1989). In particular, adjustment of the balance between RuBP carboxylation and regeneration, increased g_s and S_c, played crucial roles in the enhancement of A_{360} in 4×-C_{11} leaves.

Warner and Edwards (1993) summarised that raised polyploids have similar or lower photosynthetic rates than their diploids although naturally occurring polyploids do have higher rates. A change in chromosome number induces genome instability in newly developed polyploids. In our study, the later generation (4×-C_{11}) plants have higher leaf photosynthetic rates compared with the newly synthesised autotetraploid (4×-C_0) plants. This indicates that higher rates of net photosynthesis were not only an immediate result of chromosome doubling; rather, they were due to long-term adjustment, adaptation and evolution after chromosome doubling. Leaf construction costs can be assessed by expressing photosynthesis per unit investment in leaf dry weight or soluble leaf protein (mainly Rubisco) (Givnish 1987), and, thus, A_{360} on a dry weight basis or Rubisco basis may be more useful in evaluating the benefits of photosynthetic properties in carbon balance than A_{360} on a leaf area basis. 4×-C_{11} leaves have higher A_{360} not only on a leaf area basis, but also on a dry weight basis and Rubisco basis than 4×-C_0 and 2× leaves. However, 4×-C_0 leaves have higher A_{360} on a leaf area basis, but lower A_{360} on a dry weight basis and Rubisco basis than 2× leaves (Table 3). These results suggest that 4×-C_{11} plants may have an advantage over 4×-C_0 and 2× plants in carbon balance. Thus, the enhancement in leaf photosynthesis in 4×-C_{11} plants would contribute to ecological advantages such as whole-plant growth and competitive ability.

All the polyploids formed in a species are not expected to follow the same path. As Bazzaz *et al*. (1982) noted, genetic background has a profound effect on the photosynthetic properties of polyploids. In this study, the polyploids photosynthetically performed better especially in the later generations. It is still unclear whether these photosynthetic changes are related to autopolyploidy *per se*, or are attributed to genetic variability that resulted from the fixation of different alleles. In either case, the present results may give insights into how natural polyploids perform better than newly synthesised polyploids. Further genetic, biochemical and molecular studies may clarify the mechanisms for polyploid adaptation and evolution that happen in the 'real world.'

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