Planta
https://doi.org/10.1007/s00425-018-3029-0

ORIGINAL ARTICLE

Ploidy and hybridity effects on leaf size, cell size and related genes expression in triploids, diploids and their parents in *Populus*

Yan Zhang[1] · Beibei Wang[1] · Shuaizheng Qi[1] · Mingliang Dong[1] · Zewei Wang[1] · Yixuan Li[1] · Siyuan Chen[1] · Bailian Li[1,2] · Jinfeng Zhang[1]

Received: 31 July 2018 / Accepted: 5 October 2018

Abstract

Main conclusion **Cell-size enlargement plays a pivotal role in increasing the leaf size of triploid poplar, and polyploidization could change leaf shape. *ABP1* was highly expressed in triploid plants and positively related to cell size.**

In the plant kingdom, the leaf is the most important energy production organ, and polyploidy often exhibits a "Gigas" effect on leaf size, which benefits agriculture and forestry. However, little is known regarding the cellular and molecular mechanisms underlying the leaf size superiority of polyploid woody plants. In the present study, the leaf area and abaxial epidermal cells of diploid and triploid full-sib groups and their parents were measured at three different positions. We measured the expression of several genes related to cell division and cell expansion. The results showed that the leaf area of triploids was significantly larger than that of diploids, and the triploid group showed transgressive variation compared to their full-sib diploid group. Cell size but not cell number was the main reason for leaf size variation. Cell expansion was in accordance with leaf enlargement. In addition, the leaf shape changes in triploids primarily resulted from a significant decrease in the leaf ratio of length to –width. *Auxin-binding protein 1* (*ABP1*) was highly expressed in triploids and positively related to leaf size. These results enhanced the current understanding that giant leaf is affected by polyploidy vigor. However, significant heterosis is not exhibited in diploid offspring. Overall, polyploid breeding is an effective strategy to enhance leaf size, and *Populus*, as an ideal material, plays an important role in studying the leaf morphological variations of polyploid woody plants.

Keywords Allopolyploidization · Epidermal cell expansion · Gigantic organ · Hybridization · Leaf width

Abbreviations

ABP1	Auxin-binding protein 1
BB	BIG BROTHER
CYCD3;1	Cyclin D3;1
DG	Diploid group
FP	Female parent
MP	Male parent
TG	Triploid group

Introduction

There have been several studies on the organ sizes of plants, as seeds, fruits and stems are necessities for humans. These organs acquire biological energy through photosynthesis in leaves (Barber 2009; Zhu et al. 2010); thus, the leaf is the energy production factory of plants (Gonzalez et al. 2012). Additionally, leaf size and shape influence gas exchange and transpiration in plants (Jarvis and Slatyer 1970; Tsukaya

Yan Zhang and Beibei Wang have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-3029-0) contains supplementary material, which is available to authorized users.

✉ Jinfeng Zhang
zjf@bjfu.edu.cn

[1] Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, National Engineering Laboratory for Tree Breeding, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants of Ministry of Education, Key Laboratory of Forest Trees and Ornamental Plants Biological Engineering of State Forestry Administration, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China

[2] Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, NC 27695, USA

Published online: 16 October 2018

2005). Leaf size is controlled by environmental and genetic factors (Cookson et al. 2006; Kondorosi et al. 2000). Such mechanisms as shade avoidance or drought resistance could help plants in adapting to stressful environments, but these strategies are often accompanied by reductions in leaf size (Robson et al. 1993; Lecoeur et al. 1995).

On the cytological and molecular levels, the final leaf area is majorly controlled by the cell size and cell number (Krizek 2009; Gonzalez et al. 2012). Generally, the conversion of the leaf primordium into a mature leaf can be divided into two phases: cell proliferation and cell expansion (Huang et al. 1998; Donnelly et al. 1999; Hu et al. 2003). The relationship between the two phases is complex and common existence during transition phase rather than independent (Tsukaya 2002; Beemster et al. 2003), and recent research is more inclined to this view that small leaf primordium to a mature leaf is controlled by at least five or six distinct processes (González et al. 2012; Kalve et al. 2014; González and Inzé 2015). González et al. (2012) described that the small leaf primordium to a mature leaf is controlled by five phases: (1) an initiation phase, (2) a general cell division phase, (3) a transition between division and expansion phase, (4) a cell expansion phase, (5) and a cell differentiation phase (González et al. 2012). Kalve et al. (2014) made a point that endoreduplication is one of the distinct phases in leaf growth too. At least five elements influence the final leaf size: (i) the number of cells recruited from the meristem to the primordium; (ii) the rate of cell division; (iii) the extent of cell proliferation; (iv) cell expansion; and (v) the duration of meristemoid division (González et al. 2012). Such as plant hormone, sugar, peptide, protein, and microRNA are important factors to influence leaf area (Kalve et al. 2014), and numerous key genes on leaf growth development also are identified. Because cell division and expansion play more important roles in leaf size controlling, molecular research is more focused on them.

Commonly, cell proliferation occurs during the early stages prior to cell expansion. Cell division determines the final leaf size (Korner et al. 1989; Krizek 2009; González et al. 2012), and the rate and duration of cell division are determining factors for the final leaf size. Previous studies have identified some genes that play important roles in cell proliferation. *Cyclin D3;1* (*CYCD3;1*) positively promotes cell division, resulting in increased cell number, as well as the conversion from cell production into cell expansion (Dewitte et al. 2007). Small changes in *BIG BROTHER* (*BB*) gene expression can alter organ size (Disch et al. 2006), and the function of *DA1* is similar to that of *BB*, which negatively regulates the duration of cell proliferation in leaves (Disch et al. 2006; Li et al. 2008). Cell expansion could affect the final leaf area (Sugimoto-Shirasu and Roberts 2003; González et al. 2012). Similarly, *DELLA, ARGOS-like protein* (*ARL*) and *Auxin-binding protein 1* (*ABP1*) play vital roles in cell enlargement (Jones et al. 1998), and the above-mentioned genes related to cell division and cell size are regulated through the associated hormones, such as brassinosteroid (Nakaya et al. 2002), auxin (Hu et al. 2003), gibberellin (Huang et al. 1998; Coles et al. 1999; Gonzalez et al. 2010), and cytokinin (Holst et al. 2011), which influence final leaf size. However, the molecular basis for this correlation remains elusive.

From cultivation techniques to genetic breeding, there is considerable interest in improving the organ size of agriculture and forestry products. Polyploidization is generally recognized as an effective strategy to improve organ size (Sattler et al. 2016). In plants, compared with cell division, more profit is acquired from cell expansion (Sugiyama 2005). Chromosome doubling is often associated with an increase in cell size (Sugimoto-Shirasu and Roberts 2003). In plants, such as *Arabidopsis* (Melaragno et al. 1993), *Triticum* (Del Blanco et al. 2000), and *Malus domestica* (Xue et al. 2015), there is a strong correlation between final leaf area and final cell size in polyploidy. However, the ploidy level, such as octaploidy *Arabidopsis* (Tsukaya 2008) and tetraploid apple (Hias et al. 2017), are not always directly related to leaf or cell size (Roeder et al. 2012).

The reason why increases in ploidy level increase cell volume remains unknown (Tsukaya 2008). However, over the past several decades, there has been progress in this area. There is some evidence that somatic polyploidy, via endoreduplication, is also implicated in cell-size control. Endoreduplication occurs when a cell undergoes DNA duplication without cell division, representing a modified version of the cell cycle that lacks the M phase (Tsukaya 2008). Endoreduplication can be detected in a majority of polyploid plant tissues (Galbraith et al. 1991), resulting in a doubling of the quantity of DNA (Melaragno et al. 1993; Sugimoto-Shiras and Roberts 2003). The DNA content is positively correlated with leaf size (Beaulieu et al. 2008), and polyploidy can change leaf shape (Thao et al. 2003; Tang et al. 2010). Remarkable variations related to leaf size heterosis are shown in *Arabidopsis*, and heterosis does not always occur in hybrids (Miller et al. 2012; Groszmann et al. 2014). Although the molecular mechanism of ploidy and hybridity remains elusive, with the rapid development of molecular biology, a number of genetic and epigenetic changes have been identified in hybrid diploid and polyploidy plants. The gene expression of polyploid plants was significantly different from that of diploid plants as a result of molecular effects, including DNA methylation, histone acetylation, RNA interference, and dosage compensation (Chen 2013).

Populus is one of the most important commercial and ecological trees, and these plants are internationally popular, especially in developing countries, as these trees provide fuel wood, wood, fiber, and other forestry products and play pivotal roles in sustainable development (Isebrands

and Richardson 2014). In addition, the complete genome of *Populus* has been sequenced (Tuskan et al. 2006), showing a deeper molecular basis compared with other tree species. Polyploidy breeding of poplars has also significantly progressed (Zhu et al. 1995; Xi et al. 2012; Guo et al. 2017). Thus, poplar is an ideal material to study leaf area and cell size and the mechanisms underlying these traits. Although, Liao et al. (2016) confirmed that the leaf size of triploid *Populus* was significantly higher than that of diploid plants, the cell size and gene expression in the offspring of different ploidy levels and their parents have not been reported in woody plants.

In this study, leaf area, leaf cell size and other leaf phenotypic indices of different ploidy levels of a *Populus* progeny population and their parents were measured at different positions. In addition, the expression of genes related to leaf area was quantified. Finally, the contribution of heterozygosity and ploidy was evaluated. The present study aims to shed light on *Populus* leaf-area variations between different ploidy levels.

Materials and methods

Ploidy level determination

Vigorously growing leaves of all genotypes were collected and the ploidy level was analyzed by Cyflow Ploidy Analyser (Partec, Görlitz, SN, Germany). Ploidy level determination was according to the methods described in Zhang et al. (2017).

Plant materials and growth conditions

One-year-old full-sib diploid and triploid families were obtained by hybridization [(*P. simonii* × *P. nigra* var. *Italica*) × (*P.* × *'popularis'*)], and an artificial triploid family was acquired from female catkins treated with high temperature after pollination for 66 h, as previously described (Guo et al. 2017). Full-sib diploid and triploid groups, each containing 10–12 genotypes, respectively, and their female parent, *P. simonii* × *P. nigra* var. *italica* and male parent, *P.* × '*popularis*' were used in the present study. To acquire enough clones for each genotype, all materials were derived from tissue culture propagation, and in vitro plantlets were rooted in half-strength MS medium supplemented with 0.2 mg/L indole butyric acid (IBA), 0.04 mg/L naphthaleneacetic acid (NAA), 5.5 g/L agar, and 30 g/L sucrose, The pH was adjusted to 5.8 prior to autoclaving. After acclimatization, in vitro-rooted plantlets were transplanted into plastic pots (bottom diameter 25 cm, top diameter 30 cm, height 30 cm) on the same day, containing a 1:2:1:1 sterilized mixture (autoclaving, 121 °C, 30 min) of garden soil, peat, perlite, and vermiculite. Additionally, the plantlets that were maintained in a greenhouse at 25 ± 5 °C with a natural photoperiod, in vitro propagating system and acclimatization method were generated as previously described (Zhang et al. 2018). Overall, 6–8 1-year-old clones of each genotype were propagated in the present study, and the materials were obtained on July 15, 2017 for RNA extraction, leaf and epidermal cell measurement; the materials performed fast growing in July–August. In a previous study, we found that the leaves, from top to bottom of a tree, at the sixth position reached maturity; thus, in the present study, to examine leaf development, we selected leaves at the second, fourth, and sixth positions.

Leaf area and petiole length measurement

Leaf area and the petiole length at the second, fourth, and sixth positions of each genotype were measured and three biological replicates were obtained in the present study. The images of the leaves were recorded by digital camera, and 1 × 1 cm grid paper was used as the background of each picture. Leaf area was measured by ImageJ (http://rsb.info.nih.gov/ij/) and petiole length was measured with a ruler.

Abaxial epidermal cell size and number measurement

Abaxial epidermis leaves at the second, fourth, and sixth positions of each genotype were measured. Fresh leaves were placed into FAA fixation solution (38% formaldehyde: glacial acetic acid: 70% ethanol, 1:1:18, by vol.) 3 h, and stored in 70% ethanol at 4 °C. Fixed leaves were washed three times with water and dried on filter paper. The base, middle and tip of each leaf were randomly selected, observed and photographed by a microscope with a 100 × oil immersion lens (Olympus, Tokyo, Japan), and the epidermal cells were measured by ImageJ. A total of 20–30 cells were randomly measured for each leaf, and this procedure was repeated in three similar type leaves. The epidermal cell number: the mean leaf area/the mean epidermal cell size.

RNA extraction

Three biological replicates were used for total RNA extraction (for diploid and triploid groups, 10–12 leaf tissues with same weight from each genotype were pooled into one group), and all materials were immediately frozen in liquid nitrogen until RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for gene expression analysis, followed by DNase I (Ambion, Austin, TX, USA) treatment to remove genomic DNA. The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific,

Wilmington, DE, USA), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide.

qRT-PCR analysis

The expression of six genes was validated using qRT-PCR. The cDNA was synthesized using 0.5 µg RNA, 2 µL of 4 × gDNA Wiper Mix, add Nuclease-free H_2O to 8 µL. Following the addition of 2 µL of 5 × HiScript II Q RT SuperMix IIa, the reactions were performed on a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using LightCycler® 480 II Real-time PCR Instrument (Roche, Basel, BS, Swiss) with a 10 µL PCR reaction mixture that included 1 µL of cDNA, 5 µL of 2 × QuantiFast® SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany), 0.2 µL of forward primer, 0.2 µL of reverse primer and 3.6 µL of nuclease-free water.

Homologous sequences of *CYCD3;1*, *BB*, *DA1*, *ABP1*, *ARGOS-like protein* (*ARL*) and *DELLA* in *Populus* were identified by (https://www.ncbi.nlm.nih.gov/) and (https://phytozome.jgi.doe.gov/). Sequence ID numbers and the primer sequences are listed in Supplemental Table S1. The 18s rRNA was used as the endogenous reference gene, and the relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Statistical analyses

The significance differences among treatments were evaluated at $P = 0.05$ by Duncan's multiple range test, performed by using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) after passing the homogeneity test. Histograms and line graphs were performed using Microsoft Excel 2010 software (Microsoft Corp., Redmond, WA, USA).

Results

Leaf area, cell size and number variation in different ploidy levels family

The ploidy levels of each genotype have been determined by flow cytometry before doing cytological and molecular examination (Fig. 1). Leaf area increased with the increasing leaf location (Fig. 2). The leaf area of the triploid group (TG) was significantly higher than that of other groups at all leaf locations, reaching 2915.8 mm^2 at the sixth leaf position. And the leaf area of the diploid group (DG) at the sixth leaf position was higher than that of the male parent (MP) but lower than that of the female parent (FP). On the cellular level, like leaf area, cell size increased with the increasing leaf location too (Fig. 3). The cell size of the TG was significantly higher than that of other groups at all leaf locations, reaching 1003.6 μm^2 at the sixth leaf position (Fig. 4a–c). Except for the FP, the leaf area was positively related to increasing cell size (Fig. 4a–c), and the cell size of FP was smaller than that of MP, whereas the leaf area was higher than those of MP and DG, as the cell number of FP was much more than that of MP (Fig. 4d). In addition, the cell number of MP was significantly lower than that of other plants. The cell numbers of DG and TG were between those of MP and FP. The cell numbers of each group remained stable from the second to the sixth positions (Fig. 4d). For petioles, at the second position, the length in the triploid group was significantly higher than those in other groups (Fig. 5a). Although, petioles length in the TG was slightly higher than that in other groups, in the fourth and the sixth positions, no dramatic difference was observed in all groups (Fig. 5b, c), and the relevance between petioles length and leaf area was low. Thus, the leaf area of triploid was significantly higher than that of diploid. The leaf area of diploid offspring was between those of maternal parent and paternal parent and did not show obvious heterosis. Cell

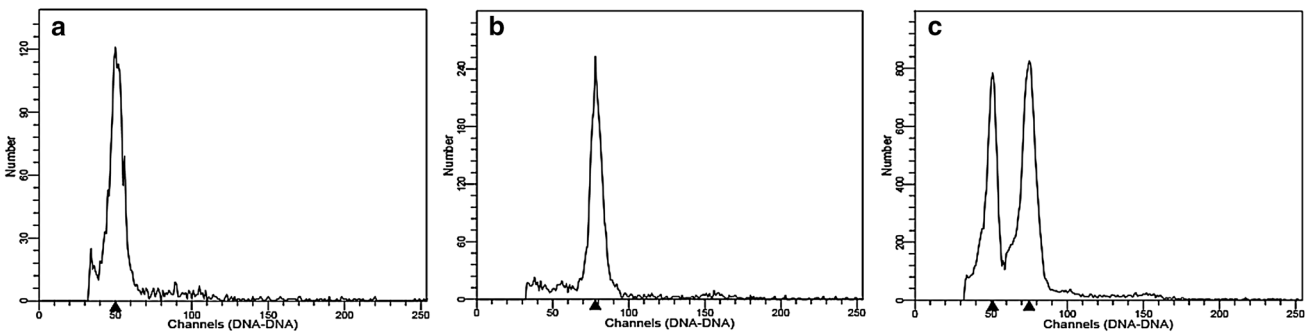

Fig. 1 Histograms of flow cytometric analysis. **a** Diploid, **b** triploid, **c** diploid and triploid

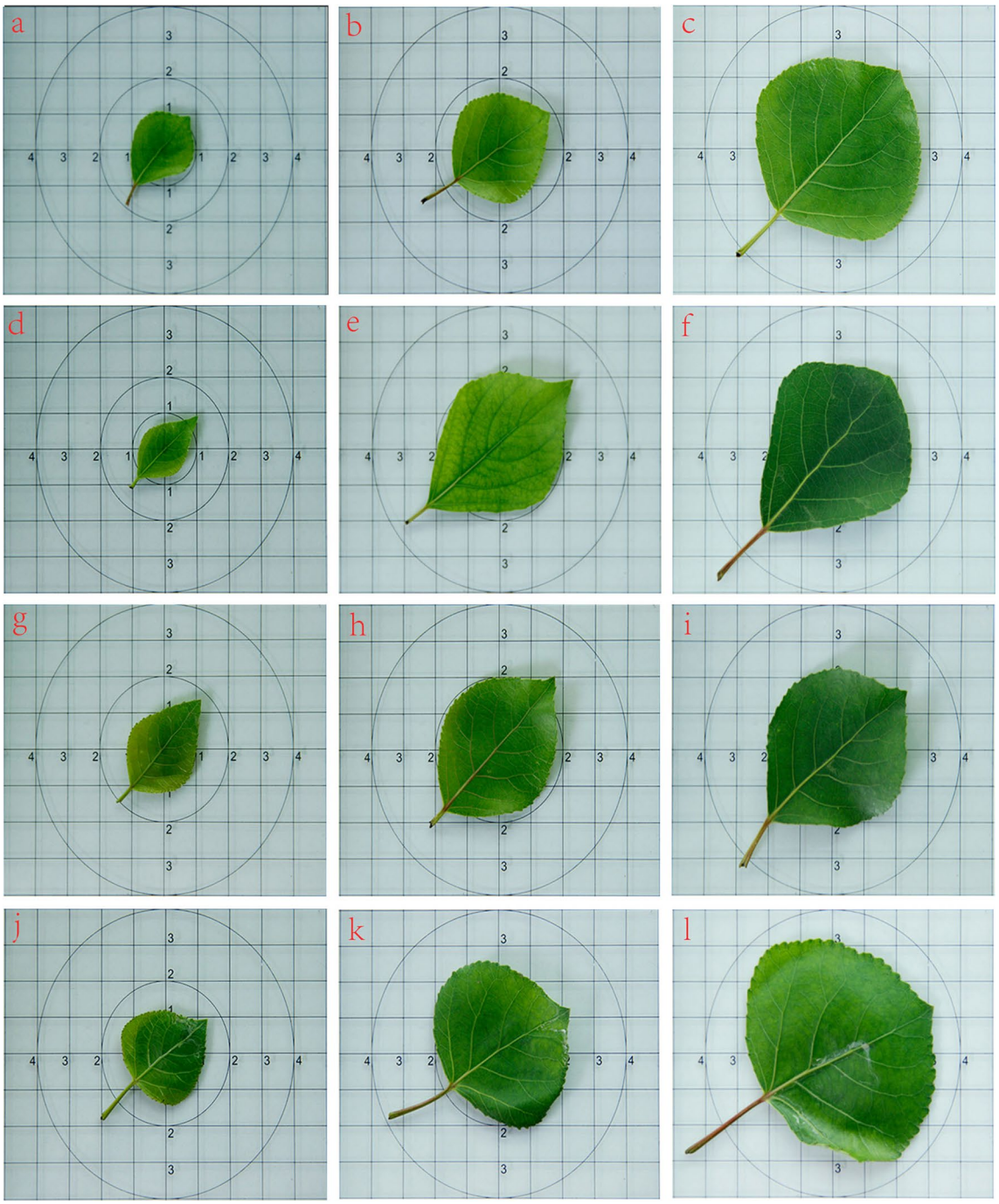

Fig. 2 Morphology of leaf at different leaf positions of female parent (*P. simonii* × *P. nigra* 'Italica'), male parent (*P.* × '*popularis*') and their diploid and triploid progenies, respectively. **a–c** Female parent (FP) at second, fourth and sixth leaf position, respectively. **d–f** Male parent (MP) at second, fourth and sixth leaf position, respectively. **g–i** Diploid group (DG) at second, fourth and sixth leaf position, respectively. **j–l** Triploid group (TG) at second, fourth and sixth leaf position, respectively. Each square area: $1 \times 1 = 1$ cm^2

expansion was in accordance with leaf enlargement. Cell size but not cell number was the main reason for leaf size increasing after cellular analysis. These results indicated that polyploidization plays a vital role in increasing the leaf and cell area in *Populus* from the second to the sixth

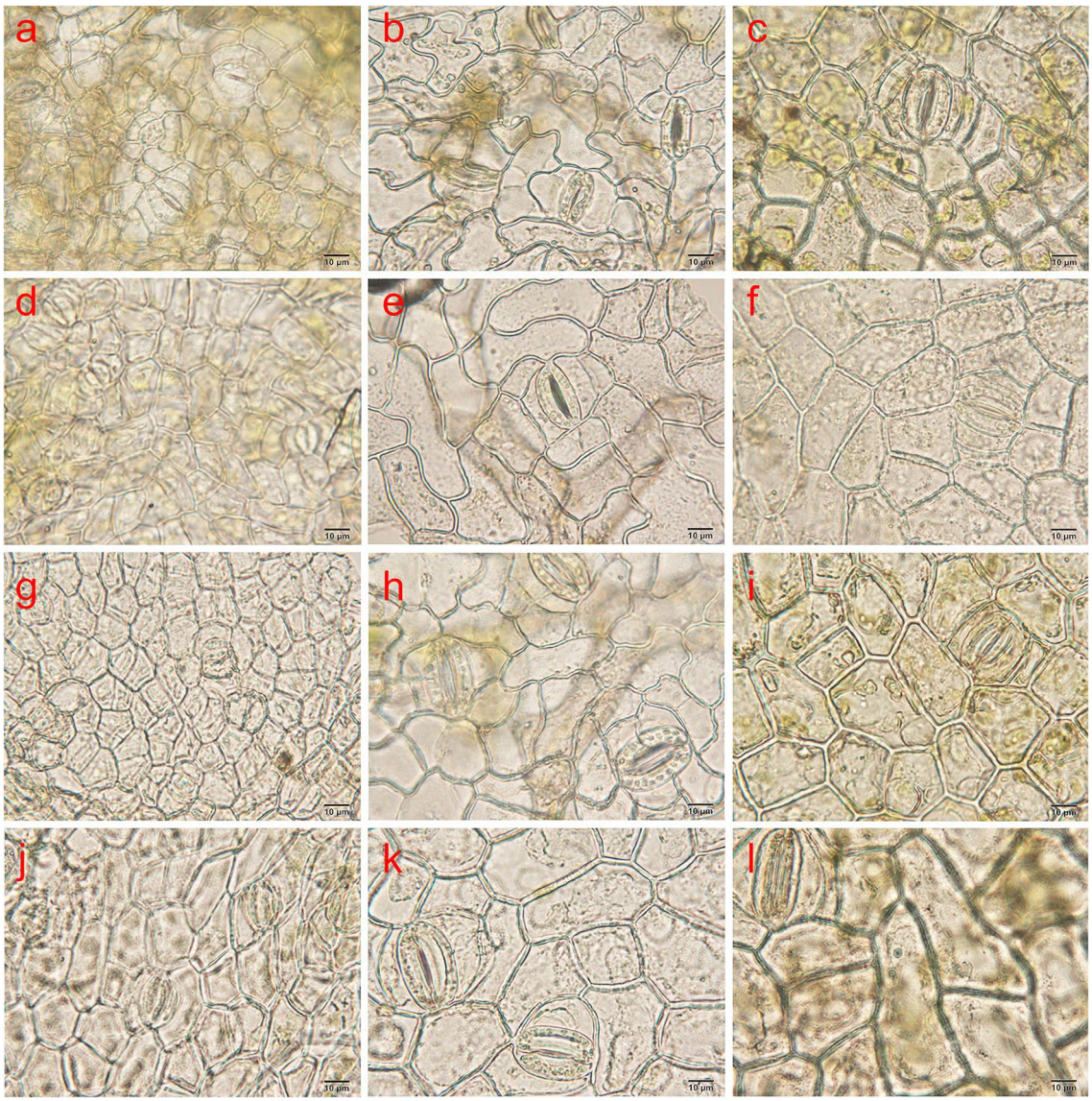

Fig. 3 Morphology of abaxial epidermal cell at different leaf position of female parent (*P. simonii*×*P. nigra* 'Italica'), male parent (*P.*×'*popularis*') and their diploid and triploid progenies, respectively. **a–c** Female parent (FP) at second, fourth and sixth leaf position, respectively. **d–f** Male parent (MP) at second, fourth and sixth leaf position, respectively. **g–i** Diploid group (DG) at second, fourth and sixth leaf position, respectively. **j–l** Triploid group (TG) at second, fourth and sixth leaf position, respectively. Each bar = 10 µm

position, and the cell numbers may be determined by the two parents together.

Leaf shape changed after suffering polyploidization

In the present study, based on the shape (Fig. 2) and morphological measured data (Fig. 5d–f), we observed that leaf length and width increased with increasing leaf position. Compared with the narrow rhomboid oval shape of the parents, the leaf shape of TG changed to a triangle shape (Fig. 2). Except for the leaf length of diploids, the length of the second leaf was higher than that for TG, and TG showed higher leaf length and wider leaf width than those in the other groups. In the sixth position, the average leaf length of the triploid group was 6.80 cm, and the width was 5.78 cm. The leaf blade length–width ratio of the triploid group, near 1:1, was significantly lower than that of the other groups (Fig. 5d–f). In general, except for the TG, FP, MP and DG, there were no significant differences in each group. The trend of the blade length–width ratio in the four groups remained stable in the leaves, and this result was basically stereotyped at the second position (Figs. 2, 5d–f). Thus, the results of the present indicated

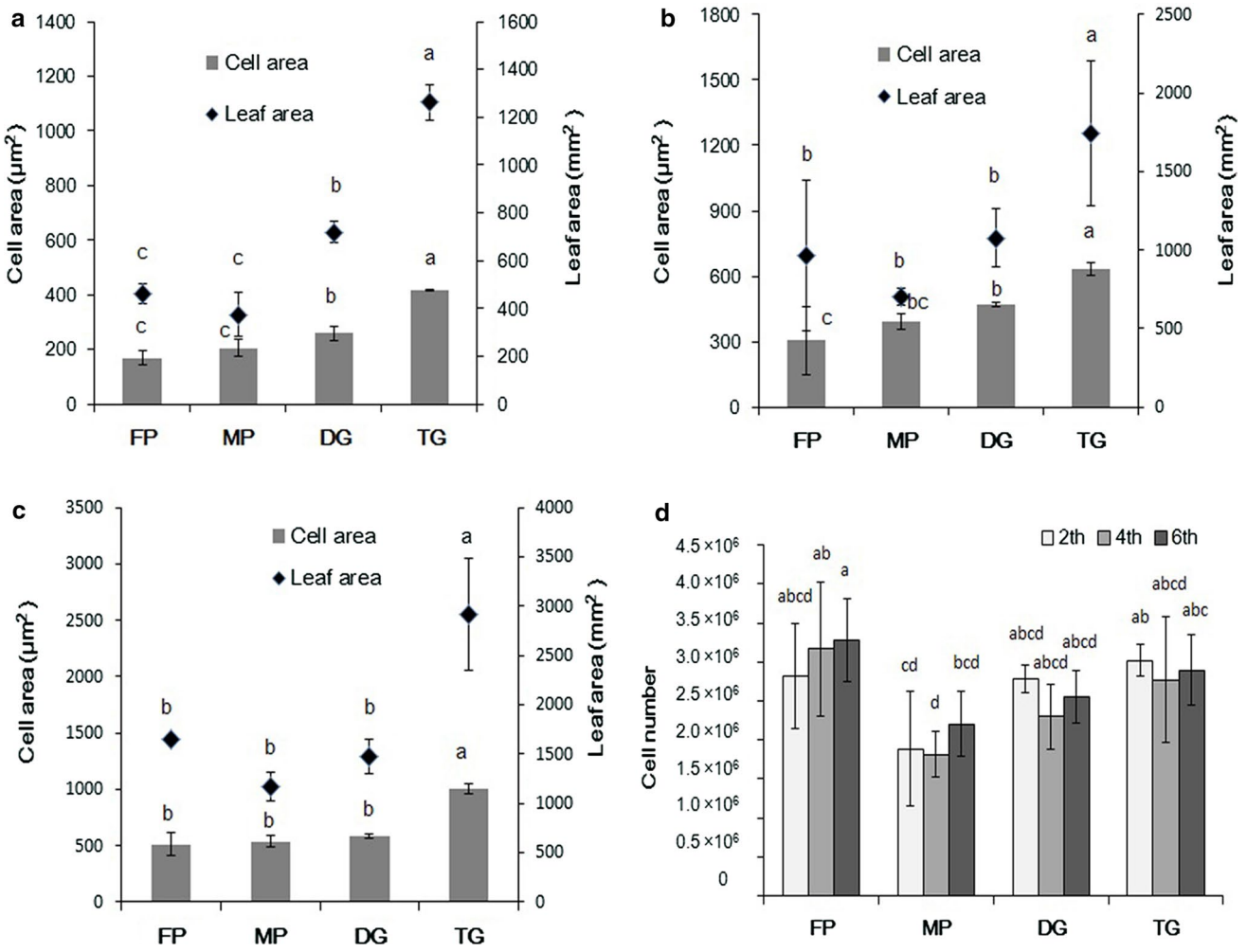

Fig. 4 Leaf area, cell area and cell number per leaf of female parent (*P. simonii*×*P. nigra* 'Italica'), male parent (*P.*×'*popularis*') and their diploid and triploid progenies, respectively. **a–c** Leaf and cell area of female parent (FP), male parent (MP), diploid group (DG) and triploid group (TG) at second, fourth and sixth leaf position, respectively. **d** Cell number per leaf of female parent (FP), male parent (MP), diploid group (DG) and triploid group (TG) at second, fourth and sixth leaf position, respectively. Each value is the mean of three biological replicates ± SD. Different lowercase letters indicate significant differences among leaf, cell area and cell number per leaf as, respectively, determined by Duncan's test ($P \leq 0.05$)

that polyploidization, rather than hybridization, might be the major reason for changes in leaf shape.

Gene expression at different ploidy levels

The relationship variations in the expression of genes related to leaf size among different ploidy levels were measured in the present study. Three genes related to cell division and two genes related to cell expansion were validated by qRT-PCR. *CYCD3;1* expression at the second position was higher than that at the fourth and sixth positions for the four groups, and FM and TG showed higher *CYCD3;1* expression than the other groups, and this effect was positively correlated with cell number (Fig. 6a). Similar to the cell numbers, the expression *CYCD3;1* in the TG was lower than that in the FP, but higher than that in the DG, and the expression *CYCD3;1* in the TG and DG was between that in the FP and MP. These results showed that *CYCD3;1* expression may be determined by the two parents together. *DA1* and *BB* showed similar expression trends of gradually increasing with increasing leaf location and were not directly related to cell number and leaf area (Fig. 6b, c). The expression in the FP and triploid group at the second position was not lower than that in other groups, whereas those genes were commonly considered to play roles in cell proliferation and were related to cell number and leaf area.

For *DELLA* (Fig. 6e), no significant difference was observed at the three positions. *ABP1* expression (Fig. 6d) in

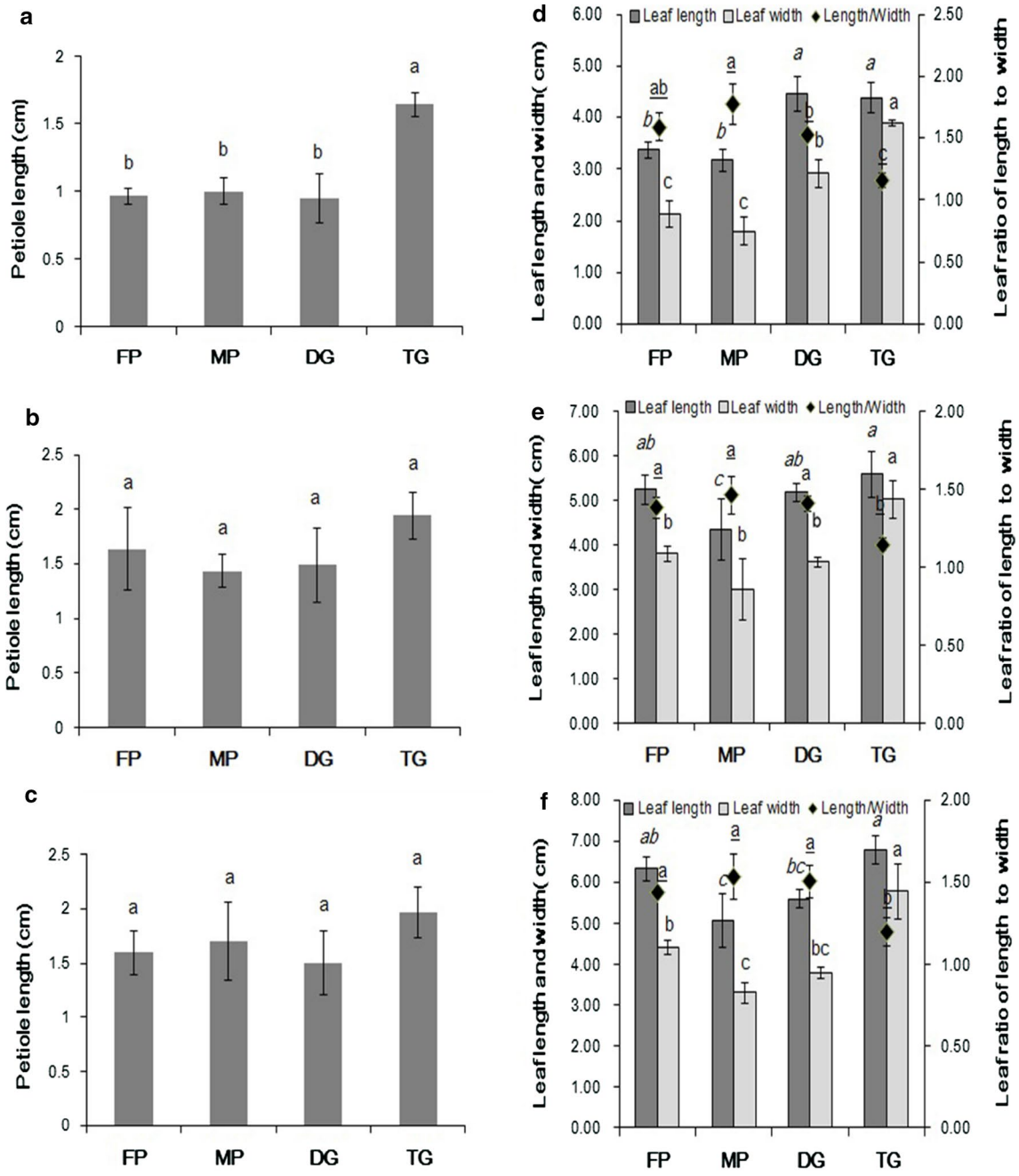

Fig. 5 Petiole length, leaf length, leaf width and leaf ratio of length to width of female parent (*P. simonii*×*P. nigra* 'Italica'), male parent (*P.*×'*popularis*') and their diploid and triploid progenies, respectively. **a–c** Petiole length of female parent (FP), male parent (MP), diploid group (DG) and triploid group (TG) at second, fourth and sixth leaf position, respectively. **d–f** Leaf length, leaf width and leaf ratio of length to width of female parent (FP), male parent (MP), diploid group (DG) and triploid group (TG) at second, fourth and sixth leaf positions, respectively. Each value is the mean of three biological replicates ± SD. Different lowercase letters indicate significant differences among leaf length, leaf width and leaf ratio of length to width as, respectively, determined by Duncan's test ($P \leq 0.05$)

the TG was higher than that in the other groups at the fourth and sixth positions, and except for the FP, *ABP1* expression peaked at the fourth position, and this *gene* expression was positively correlated with cell size.

Discussion

Cell size and cell number determine organ size in model plants, from the meristem to mature leaves. Patterns related to cell proliferation and expansion in polyploids are different from those in diploids, and this phenomenon in woody

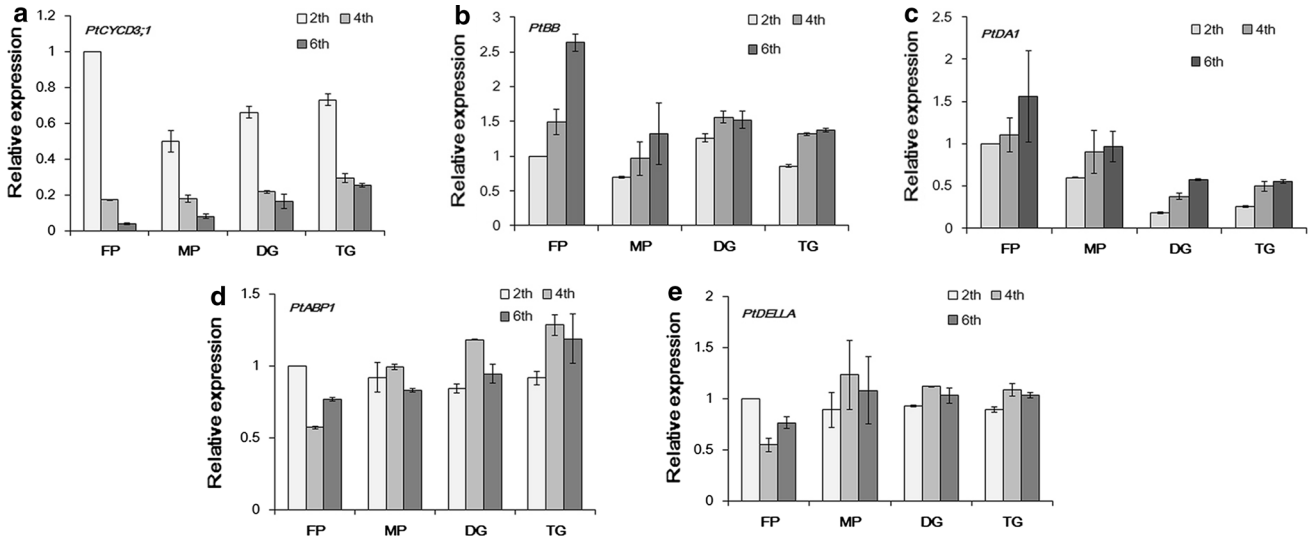

Fig. 6 Expression of *CYCD3;1* (**a**), *DA1* (**b**), *BB* (**c**), *ABP1* (**d**), *DELLA* (**e**) and *ARL* (**f**) are measured by real-time qRT-PCR in female parent (*P. simonii* × *P. nigra* 'Italica'), male parent (*P.* × '*popularis*') and their diploid and triploid progenies, respectively. Each group contains 10–12 pooled genotypes. Each value is the mean of three biological replicates ± SD (*ACT1* is used as a control)

plants is elusive. In this study, we examined the relationship between cellular and leaf morphological variations of triploid poplar and characterized the molecular base underlying these variations. In the present study, we selected three leaf positions to reflect leaf development, and measured cell size, collected data on leaf morphology and calculated the cell number. Cell expansion plays a more important role in increasing triploid leaf area and triploid groups may enhance cell expansion ability to create bigger leaves. Polyploidization can alter the leaf shape, and the expression patterns of the genes related to cell division and expansion were both similar and different from those of other species. However, compared with triploids, hybrid diploids did not show significant heterosis. Overall, the method of inducing triploid poplar is an effective strategy to enhance leaf size, and *Populus* can be used as an ideal material to study leaf morphological variations of polyploidy in woody plants.

Superiority of triploid leaf size positively related to cell-size increasing

Leaf area is controlled by a variety of mechanisms (Kondorosi et al. 2000; Cookson et al. 2006). In the present study, we studied the influence of cell size and cell number on leaf area. The triploid plants exhibited an advantage of cell size, resulting in increasing leaf area. In mature leaves (the sixth position), hybrid diploid plants showed no significant differences from their parents; thus, the ploidy effect, rather than hybridization, may be the major reason that cell size markedly increases. Similar results have been reported in *A. thaliana* hybrids (Miller et al. 2012), and in *Lolium*, tetraploid cultivars showed longer leaves than diploids, mainly due to their longer mature cells (Sugiyama 2005). Why chromosome doubling is consistently followed by cell-size increasing currently remains unknown, and additional studies focused on genome size and evolution adaption are needed. Several reports have shown that the cell size is robustly associated with the DNA content, as the genome size of polyploids is larger than that of diploids (Kondorosi et al. 2000; Jovtchev et al. 2006; Beaulieu et al. 2008). Moreover, changes in the genome size could alter the cell and stomata density, which eventually influence the efficiency of water and nutrient use. Thus, increasing genome size may strengthen plant resistance and vigor, and subsequently improve the chances of survival during evolution (Hetherington and Woodward 2003; Beaulieu et al. 2008).

In the present study, hybrid triploids, rather than their full-sib diploids, inherited the cell number vigor of the female parent. Therefore, in the present study, the cell number was not significantly decreased, consistent with the findings of Sugiyama (2005), showing that the cell number of tetraploids was not significantly different from that of diploids. Similarly, there are no studies showing evidence of increasing cell number in polyploids. Thus, in the present study, increasing cell size likely resulted in the enhanced leaf size in triploids. The opposite conclusion is reported in animals, suggesting that increased cell size is followed by limited proliferation and a decrease in cell number after chromosome doubling (Conlon and Raff 1999; Day and Lawrence 2000; Ganem and Pellman 2007), and the size of the organism does not increase. Additionally, in higher ploidy plants, such as octaploid *Arabidopsis*, larger cell size

was accompanied by a lower cell number (Levan 1939). In addition, does higher ploidy level improve the leaf area in plants? The answer is no. For example, the leaf area of some octaploids was much smaller than that of diploids (Levan 1939; Tsukaya 2008), and although the cell size in octaploids was larger than that in diploids (Tsukaya 2008), this result also indicated that cell size is not a unique key factor directly proportional to leaf area.

Polyploidization alters leaf shape in *Populus*

Remarkable variations in leaf shape among different ploidy levels have been reported in many species, such as hybrid cassava (Nassar 2006), olive (Rugini et al. 1996) and *Alocasia micholitziana* (Thao et al. 2003). In the present study, the leaf shape of triploids was significantly different from that of diploids; particularly, the ratio of leaf length to –leaf width was significantly lower than that of diploids. Leaf width is considered a key factor determining leaf area, and a significant difference was observed between species and ploidy (Sugiyama 2005), consistent with the results obtained for *Alocasia micholitziana* (Thao et al. 2003). In the present study, the increase in the leaf width rate of triploids was higher than that of the leaf length growth rate, which may be the reason for the significant change in the leaf shape of triploid plants.

The ratio of leaf length to the width of diploid offspring was not significant compared with that of their parents. Thus, the ploidy effect, rather than hybridization, may be the key factor altering leaf shape. Interestingly, the ratio of leaf length to leaf width was not significantly different at the second, fourth, and sixth leaf positions of each group; thus in the cell expansion phase, the proportionality of leaf length and width was well contained. Kessler and Sinha (2004) reported the characteristics of leaf formation in early leaf development. Similar to leaf size formation, the leaf shape also results from the coordination of multiple hormones involved in cell division and cell expansion, and several genes, including *CLAVATA1*, *CLAVATA3*, and *WUSCHEL*, have been implicated in the regulation of leaf shape formation. Whether polyploids show modifications of those genes and related proteins remains unclear.

Relationship between genes expression and leaf size

In the present study, we selected three genes, *ABP1* and *DELLA*, associated with auxin, gibberellin and brassinolide, respectively. Although *DELLA*, *DA1* and *BB1* have been confirmed to influence leaf size in *Arabidopsis* (Peng et al. 1997; Disch et al. 2006; Li et al. 2008), in the present study, a close correlation was not observed in this *Populus* species. The overexpression of *ABP1* increased cell size, while the repression of *ABP1* in *Arabidopsis* suppressed leaf expansion (Braun et al. 2008). In the present study, the expression of *ABP1* in the triploid group was significantly higher than that in the diploid group at the fourth or sixth leaf position. This study is the first to report that polyploid cell size was positively associated with *ABP1*. Polyploidization alters genetic and epigenetic status; thus, we proposed that gene dosage effects, methylation, sRNA interference and silencing and histone modifications might work on *ABP1* gene or ABP1 protein.

CYCD3 plays an important role in determining cell number in developing lateral plant organs by controlling the G1/S transition, and contributes to the alternative event of cell production to cell expansion in these organisms (Planchais et al. 2004; Dewitte et al. 2007; De Jager et al. 2009). In the present study, at the second position, the gene expression of *CYCD3* was positively correlated with cell number, and the expression of *CYCD3;1* was markedly decreased at the fourth and sixth leaf positions, as the high expression of *CYCD3;1* is primarily observed in early development stages (Dewitte et al. 2007). Thus, we propose that studying the meristem at initial stages may provide additional information on cell proliferation. Recently, quantitative, dynamic, and multifactorial trait regulated by numerous genetic factors, and both of them participated in regulated leaf size (González and Inzé 2015). At present, more studies were focused on molecular cell division and cell expansion.

Unlike autopolyploidy, allopolyploidy not only exhibits a ploidy effect but also merges with hybridization effects (Miller et al. 2012). In a previous study, high-temperature-treated female catkins for chromosome doubling at 66 h after fertilization (Guo et al. 2017) showed that this triploid induction mechanism may involve post-meiotic restitution (PMR) produced through the embryo sac by high-temperature treatment. Two of the three genomes of triploids were derived from the female parent. Thus, the parent of origin effects or dosage effects may affect triploid leaf area and these two phenomena are influenced by differential parental contributions (Birchler et al. 2001; Miller et al. 2012; Yao et al. 2013). In the future, different materials can be used to study parent of origin and dosage effects in polyploid poplars, such as triploids in which two of the three genomes are derived from male parent, or allotetraploids of *Populus*.

Conclusion

To the best of our knowledge, this study is the first comprehensive report describing the relationship between leaf area and cell size and number at three leaf positions of different ploidy levels of a fullsib *Populus* family. Based on these findings, we suggested that dramatic cell size increases and leaf shape changes result from polyploidization. In the

present study, *CYCD3;1* positively regulated cell number in the cell-division phase, and significant increases in cell size in the triploid group were mediated through *ABP1* during the cell expansion phase. Overall, polyploid breeding is an effective method to increase cell size and thereby increase leaf area. Studies at the cellular and molecular level can further reveal the secrets of gigantic leaf in polyploid plants. Furthermore, *Populus* can be used as an ideal material to study leaf morphological variations of polyploids in woody plants.

Author contribution statement JZ and BL guided the research. YZ, JZ and BL conceived and designed the research. YZ, BW, MD and ZW completed the work, including in vitro subculture, plant acclimatization and material cultivation, RNA extraction and qRT-PCR analysis. YZ, YL and SC performed the leaf and cell-size measurements; YZ analyzed the data and drafted the manuscript. All authors read and approved the manuscript.

Acknowledgements This work was supported by Major Science and Technology Special Project of Xuchang, Henan province, China (20170112006), Medium and Long Scientific Research Project for Young Teachers in Beijing Forestry University (2015ZCQ-SW-02), the Project of National Natural Science Foundation of China (31370658) and "948" Project of China (2014-4-59).

References

Barber J (2009) Photosynthetic energy conversion: natural and artificial. Chem Soc Rev 38:185–196

Beaulieu JM, Leitch IJ, Patel S, Pendharkar A, Knight CA (2008) Genome size is a strong predictor of cell size and stomatal density in angiosperms. New Phytol 179:975–986

Beemster GTS, Fiorani F, Inzé D (2003) Cell cycle: the key to plant growth control? Trends Plant Sci 8:154–158

Birchler JA, Bhadra U, Bhadra MP, Auger DL (2001) Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndromes, and quantitative traits. Dev Biol 234:275–288

Braun N, Wyrzykowska J, Muller P, David K, Couch D, Perrot-Rechenmann C, Fleming AJ (2008) Conditional repression of AUXIN BINDING PROTEIN 1 reveals that it coordinates cell division and cell expansion during postembryonic shoot development in *Arabidopsis* and tobacco. Plant Cell 20:2746–2762

Chen ZJ (2013) Genomic and epigenetic insights into the molecular bases of heterosis. Nat Rev Genet 14:471–482

Coles JP, Phillips AL, Croker SJ, García-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant J 17:547–556

Conlon I, Raff M (1999) Size control in animal development. Cell 96:235–244

Cookson SJ, Radziejwoski A, Granier C (2006) Cell and leaf size plasticity in *Arabidopsis*: what is the role of endoreduplication? Plant Cell Environ 29:1273–1283

Day SJ, Lawrence PA (2000) Measuring dimensions: the regulation of size and shape. Development 127:2977–2987

De Jager SM, Scofield S, Huntley RP, Robinson AS, den Boer BGW, Murray JAH (2009) Dissecting regulatory pathways of G1/S control in *Arabidopsis*: common and distinct targets of CYCD3;1, E2Fa and E2Fc. Plant Mol Biol 71:345–365

Del Blanco IA, Rajaram S, Kronstad WE, Reynolds MP (2000) Physiological performance of synthetic hexaploid wheat-derived populations. Crop Sci 40:1257–1263

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V, Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell. proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Natl Acad Sci USA 104:14537–14542

Disch S, Anastasiou E, Sharma VK, Laux T, Fletcher JC, Lenhard M (2006) The E3 ubiquitin ligase BIG BROTHER controls *Arabidopsis* organ size in a dosage-dependent manner. Curr Biol 16:272–279

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Dev Biol 215:407–419

Galbraith DW, Harkins KR, Knapp S (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol 96:985–989

Ganem NJ, Pellman D (2007) Limiting the proliferation of polyploid cells. Cell 131:437–440

González N, Inzé D (2015) Molecular systems governing leaf growth: from genes to networks. J Exp Bot 66:1045–1054

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiol 153:1261–1279

Gonzalez N, Hannes VH, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends Plant Sci 17:332–340

Groszmann M, Gonzalez-Bayon R, Greaves IK, Wang L, Huen AK, Peacock WJ, Dennis ES (2014) Intraspecific Arabidopsis hybrids show different patterns of heterosis despite the close relatedness of the parental genomes. Plant Physiol 166:265–280

Guo LQ, Xu WT, Zhang Y, Zhang JF, Wei ZZ (2017) Inducing triploids and tetraploids with high temperatures in *Populus* sect. *Tacamahaca*. Plant Cell Rep 36:313–326

Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. Nature 424:901–908

Hias N, Leus L, Davey MW, Vanderzande S, Van Huylenbroeck J, Keulemans J (2017) Effect of polyploidization on morphology in two apple (*Malus* × *domestica*) genotypes. Hortic Sci 44:55–63

Holst K, Schmülling T, Werner T (2011) Enhanced cytokinin degradation in leaf primordia of transgenic *Arabidopsis* plants reduces leaf size and shoot organ primordia formation. J Plant Physiol 168:1328–1334

Hu YX, Xie Q, Chua NH (2003) The Arabidopsis auxin-inducible gene *ARGOS* controls lateral organ size. Plant Cell 15:1951–1961

Huang S, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. Plant Physiol 118:773–781

Isebrands JG, Richardson J (2014) (eds) Poplars and willows: trees for society and the environment. In: The Food and Agriculture Organization of the United Nations and CABI, Rome, pp 1–7

Jarvis PG, Slatyer RO (1970) The role of the mesophyll cell wall in leaf transpiration. Planta 90:303–322

Jones AM, Im KH, Savka MA, Wu MJ, DeWitt NG, Shillito R, Binns AN (1998) Auxin-dependent cell expansion mediated by overexpressed auxin-binding protein 1. Science 282:1114–1117

Jovtchev G, Schubert V, Meister A, Barow M, Schubert I (2006) Nuclear DNA content and nuclear and cell volume are positively correlated in angiosperms. Cytogenet Genome Res 114:77–82

Kalve S, De Vos D, Beemster GTS (2014) Leaf development: a cellular perspective. Front Plant Sci 5:362. https://doi.org/10.3389/fpls.2014.00362

Kessler S, Sinha N (2004) Shaping up: the genetic control of leaf shape. Curr Opin Plant Biol 7:65–72

Kondorosi E, Roudier F, Gendreau E (2000) Plant cell-size control: growing by ploidy? Curr Opin Plant Biol 3:488–492

Korner C, Pelaez MRS, John P (1989) Why are bonsai plants small? A consideration of cell size. Funct Plant Biol 16:443–448

Krizek BA (2009) Making bigger plants: key regulators of final organ size. Curr Opin Plant Biol 12:17–22

Lecoeur J, Wery J, Turc O, Tardieu F (1995) Expansion of pea leaves subjected to short water deficit: cell number and cell size are sensitive to stress at different periods of leaf development. J Exp Bot 46:1093–1101

Levan A (1939) Tetraploidy and octaploidy induced by colchicines in diploid *Petunia*. Hereditas 25:109–131

Li Y, Zheng LY, Corke F, Smith C, Bevan MW (2008) Control of final seed and organ size by the DA1 gene family in *Arabidopsis thaliana*. Genes Dev 22:1331–1336

Liao T, Cheng SP, Zhu XH, Min Y, Kang XY (2016) Effects of triploid status on growth, photosynthesis, and leaf area in *Populus*. Trees 30:1137–1147

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Melaragno JE, Mehrotra B, Coleman AW (1993) Relationship between endopolyploidy and cell size in epidermal tissue of Arabidopsis. Plant Cell 5:1661–1668

Miller M, Zhang C, Chen ZJ (2012) Ploidy and hybridity effects on growth vigor and gene expression in *Arabidopsis thaliana* hybrids and their parents. G3 2:505–513

Nakaya M, Tsukaya H, Murakami N, Kato M (2002) Brassinosteroids control the proliferation of leaf cells of *Arabidopsis thaliana*. Plant Cell Physiol 43:239–244

Nassar NMA (2006) Chromosome doubling induces apomixis in a *cassava* × *Manihot anomala* hybrid. Hereditas 143:246–248

Peng JR, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The *Arabidopsis GA1* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev 11:3194–3205

Planchais S, Samland AK, Murray JAH (2004) Differential stability of *Arabidopsis* D-type cyclins: CYCD3;1 is a highly unstable protein degraded by a proteasome-dependent mechanism. Plant J 38:616–625

Robson PRH, Whitelam GC, Smith H (1993) Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiol 102:1179–1184

Roeder AHK, Cunha A, Ohno CK, Meyerowitz EM (2012) Cell cycle regulates cell type in the *Arabidopsis* sepal. Development 139:4416–4427

Rugini E, Pannellig G, Ceccarellim M, Muganu M (1996) Isolation of triploid and tetraploid olive (*Olea europaea* L.) plants from mixoploid cv. 'Frantoio' and 'Leccino' mutants by in vivo and in vitro selection. Plant Breed 115:23–27

Sattler MC, Carvalho CR, Clarindo WR (2016) The polyploidy and its key role in plant breeding. Planta 243:281–296

Sugimoto-Shirasu K, Roberts K (2003) ''Big it up'': endoreduplication and cell-size control in plants. Curr Opin Plant Biol 6:544–553

Sugiyama S (2005) Polyploidy and cellular mechanisms changing leaf size: comparison of diploid and autotetraploid populations in two species of *Lolium*. Ann Bot 96:931–938

Tang ZQ, Chen DL, Song ZJ, He YC, Cai DT (2010) In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. Plant Cell Tissue Organ Cult 102:213–220

Thao NTP, Ureshino K, Miyajima I, Ozaki Y, Okubo H (2003) Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. Plant Cell Tissue Organ Cult 72:19–25

Tsukaya H (2002) The leaf index: heteroblasty, natural variation, and the genetic control of polar processes of leaf expansion. Plant Cell Physiol 43:372–378

Tsukaya H (2005) Leaf shape: genetic controls and environmental factors. Int J Dev Biol 49:547–555

Tsukaya H (2008) Controlling size in multicellular organs: focus on the leaf. PLoS Biol 6:e174. https://doi.org/10.1371/journal.pbio.0060174

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I et al (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313:1596–1604

Xi XJ, Li D, Xu WT, Guo LQ, Zhang JF, Li BL (2012) 2n egg formation in *Populus* × *euramericana* (Dode) Guinier. Tree Genet Genomes 8:1237–1245

Xue H, Zhang F, Zhang ZH, Fu JF, Wang F, Zhang B, Ma Y (2015) Differences in salt tolerance between diploid and autotetraploid apple seedlings exposed to salt stress. Sci Hortic 190:24–30

Yao H, Gray DA, Auger DL, Birchler JA (2013) Genomic dosage effects on heterosis in triploid maize. Proc Natl Acad Sci USA 110:2665–2669

Zhang Y, Wang ZW, Qi SZ, Wang XQ, Zhao J, Zhang JF, Li BL, Zhang YD, Liu XZ, Yuan W (2017) *In vitro* tetraploid induction from leaf and petiole explants of hybrid sweetgum (*Liquidambar styraciflua* × *Liquidambar formosana*). Forests 8:264. https://doi.org/10.3390/f8080264

Zhang Y, Wang BB, Guo LQ, Xu WT, Wang ZW, Li BL, Zhang JF (2018) Factors influencing direct shoot regeneration from leaves, petioles, and plantlet roots of triploid hybrid *Populus* sect. *Tacamahaca*. J For Res 29:1533–1545

Zhu ZT, Lin HB, Kang XY (1995) Studies on allotriploid breeding of *Populus tomentosa* B301 clones. Sci Silvae Sin 31:499–505

Zhu XG, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. Annu Rev Plant Biol 61:235–261