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Genotype-specific changes associated to early synthesis of autotetraploids in wild potato species

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Abstract Polyploidy is an important factor in plant evolution that may trigger drastic genome reorganization and phenotypic differentiation. In the last decade, extensive studies have been carried to understand the consequences of allopolyploidization, where the effects of ploidy change may be confounded by interspecific hybridization. By contrast, less is known on autopolyploidization, which only involves doubling of homologous chromosomes. This study was undertaken to assess leaf anatomical modifications and gene expression changes occurring after doubling the somatic chromosome complement of diploid ($2n = 2x = 24$) potato species *Solanum commersonii* Dunal and *S. bulbocastanum* Dunal. Polyploidization did not induce qualitative changes in leaf structure and, for several leaf traits, anatomic modifications were stochastic. In addition, in both species a diploid superiority was generally observed, suggesting the occurrence of a high-ploidy syndrome. Expression change study was carried out on eight important cell cycle-regulatory genes in plant. It revealed a strong alteration of the expression patterns in the 4x genotypes with respect to the 2x parents. Changes often exceed the twofold, with no consistent trend towards up- or down regulation when comparing 2x vis-à-vis 4x. We discuss the possible relevance of epigenetic changes in controlling the expression of duplicated genes.

Keywords Chromosome doubling · Morpho-anatomical traits · *Solanum commersonii* · *Solanum bulbocastanum* · Cell cycle-regulatory genes

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Introduction

Estimates of the polyploid fraction amongst angiosperms are very high (Parisod et al. 2010a), suggesting that polyploidy may confer advantages over diploidy. Among benefits there are stronger heterosis, gene redundancy, phenotypic variation, loss of self-incompatibility and gain of asexual reproduction (reviewed in Comai 2005). In recent years, interest in deciphering the consequences of polyploidization has greatly increased. It has been found that ploidy changes may cause genomic, genetic and epigenetic rearrangements that can result in variation of phenotypic traits and, consequently, in the evolutionary success as well as in

the large use of polyploid plants (Udall and Wendel 2006; Yang et al. 2011). The effects of polyploidization at molecular level have been largely investigated in allopolyploids, where interspecific hybridization rather than genome doubling may have impacted genome and morphology. Newly synthesized allopolyploids often exhibited significant changes, such as loss/gain of DNA restriction sites, repatterning of gene expression, activation of transposable elements, and remodelling of DNA methylation (Madlung et al. 2002; Levy and Feldman 2004; Albertin et al. 2006; Parisod et al. 2010b). In contrast, less information is available in autopolyploids, which contain multiple sets of the same (or similar) genome(s) and, therefore, represent ideal materials for such studies. Evidences exist for a lack of general genome restructuring, limited gene expression changes and proteome alteration following genome doubling (Albertin et al. 2005; Pignatta et al. 2010; Allario et al. 2011). A recent treatment by Tayalé and Parisod (2013) provides an overview of reported genome changes occurring after polyploidization.

It is generally believed that polyploids exhibit distinctive phenotypic traits such as larger leaf and cell size, thicker stems, increased stress tolerance and enlarged reproductive structures (for a review see Ramsey and Schemske 2002). However, a number of studies have not revealed any phenotypic superiority of polyploids with respect to their diploid parents. In a recent paper by Chae et al. (2013), for example, autotetraploids of *Miscanthus* species displayed a lower plant height and stem diameter than their diploid parents. Similarly, autotetraploid *Citrus limonia* showed a number of anatomic traits (e.g. thickness of leaf cuticle, diameter of leaf central vein, root xylem cell area) significantly superior or similar in diploids compared to polyploids (Allario et al. 2011); analogous contrasting results are reported also in gene expression studies. In autotetraploid *Arabidopsis thaliana* Yu et al. (2010) found that hundreds of genes were differentially transcribed in tetraploids of the ecotype Columbia, whereas tetraploids of the ecotype L*er* had an almost diploid expression profile. The same authors did not find genes consistently responding to polyploidization. Recently, Li et al. (2012) reported that in *Arabidopsis* some cell cycle regulating genes (e.g. *ICK1*, *ICK2*) were consistently over expressed due to polyploidization. In light of these contradictory results, analysis of more species may help to better determine if superiority in response to polyploidization is a rule or an exception and if there are specific genes responding to polyploidization in plants.

Synthetic autopolyploids represent excellent materials for comparative genetic and phenotypic studies to understand the effects of genome doubling. The main objectives of this research were to determine the extent of (1) leaf anatomical modifications and (2) changes in the expression of selected cell-cycle regulating genes previously reported to respond to polyploidization (Li et al. 2012) in a set of independent autotetraploids obtained from two diploid ($2n = 2x = 24$) potato species employed in breeding, *Solanum commersonii* Dunal and *S. bulbocastanum* Dunal. This study extends and complements an earlier report by Aversano et al. (2013) underlying how autotetraploids produced from these species were epigenetically different.

Materials and methods

Plant material

Synthetic autotetraploids were generated through oryzalin treatments as previously described (Caruso et al. 2011). Here we used two independent tetraploids (cmm24 and cmm30) derived from clone cmm1T of *S. commersonii* ($2n = 2x = 24$) and four independent tetraploids (blb10, blb22, blb25 and blb26) produced from blb1c, a diploid ($2n = 2x = 24$) clone of *S. bulbocastanum*. To collect material for morpho-anatomical and molecular analyses, five plants per genotype were grown in pots in a temperature-controlled greenhouse (20–25 °C), under natural light and according to a fully randomized design. Plants were watered two–three times a week, depending on water demand.

Morpho-anatomical analysis

Morphological and anatomical analyses were performed on diploid cmm1T and its tetraploids cmm24 and cmm30, and on diploid blb1c and its tetraploids blb10, blb22, blb25 and blb26. For each genotype, 15–20 fully expanded leaves from five plants were collected and scanned. The digital images were previously analysed to measure leaf area (Aversano et al. 2013), which was re-examined here to calculate

the ratio between the mean leaf area of each polyploid and that of the corresponding diploid. For anatomical analyses, three fully expanded leaves of each genotype were collected at the same phenological stage (before flowering) on the same date. Leaves were fixed in a solution of FAA (40 % formaldehyde: glacial acetic acid: 50 % ethanol, 5:5:90 by volume) for several days to stop all metabolic cellular processes.

Leaf sub-samples of about 5 mm × 5 mm were dissected from the middle part of the lamina avoiding the main vein. Sub-samples were dehydrated in an ethanol series up to 90 % (Jensen 1962) and embedded in JB4® acrylic resin (Polysciences Europe, Eppelheim, Germany). Semi-thin cross-Sections (5 µm thick) were cut by a rotative microtome and collected on glass slides. Several sections per sample were stained with 0.5 % Toluidine Blue (Feder and O'Brien 1968), mounted with Canadian Balsam and observed under a transmitted light microscope (BX 60, Olympus). Digital micrographs of the leaf sections were obtained with a digital camera (CAMEDIA C4040, Olympus) at various magnifications. Images were analysed with AnalySIS 3.2 (Olympus), a software program devised to quantify anatomical features. The following parameters were measured to characterise the various tissues: (a) thickness of palisade and spongy parenchyma (PPT and SPT respectively); (b) cell area (CA) and shape in upper epidermis (E), palisade and spongy parenchyma (PP and SP respectively). Cell shape was characterised as: (i) aspect ratio (AR—maximum width/height ratio of a bounding rectangle for the cell, defining how it is elongated), and (ii) convexity (CO—the fraction of the cell surface area and the area of its convex; a turgid cell has a maximum value of 1). These shape indexes are used as indicators of the cell differentiation and health status (De Micco et al. 2008; Van Buggenhout et al. 2008).

Molecular analysis

To verify the genome integrity of genotypes studied, fully developed young leaves of diploid progenitors and synthetic tetraploids were collected from individual plants grown in greenhouse. From each genotype, DNA was isolated from a leaf pool of three different plants using the DNeasy Plant mini kit (Qiagen, Valencia, USA) following the manufacturer's instructions. DNA quality and integrity were checked by gel electrophoresis and spectrophotometric assay. A total of 12 primers were used to amplify DNA (Online Resource 1) as described in Scarano et al. (2002). In brief: each 25-µl amplification reaction consisted of 1 × reaction buffer with 3 mM $MgCl_2$, 0.2 mM of each dNTP, 1,2 µM of ISSR primer, 1 unit of Taq DNA polymerase Recombinant (Life Technologies, Carlsbad, USA), and 20 ng of genomic DNA. PCR was carried out using the following cycling profiles: 4 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at annealing temperature (Ta), and 2 min at 72 °C, with a final extension step of 10 min at 72 °C. Amplification products were separated on a 1.5 % agarose gel containing 1 × TBE buffer (0.045 M Tris–borate, 0.001 M EDTA) and 0.5 µg/ml ethidium bromide for 30 min at 50 V and 4 h at 100 V. DNA samples from each genotype were analyzed in triplicate to provide technical replicates.

The expression study was carried out using fully expanded young leaves at the pre-flowering stage. We focused on eight genes belonging to key classes of cell cycle regulatory molecules: cyclin-dependent kinases (*CDKs*) and cyclin-dependent kinase inhibitors (*ICK*). RNA was isolated from a 100 mg leaf pool of three different plants/genotype obtained by homogenizer (TissueLyzer by Qiagen) using TRIZOL reagent (Life Technologies). RNA extraction was performed following TRIZOL Life Technologies instruction manual. The concentration and the purity degree of extracted RNAs were estimated using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The quality and the integrity of RNA were checked after electrophoresis 1 µg of RNA samples on 1 % agarose gel stained with SYBR® Safe (Life Technologies). Complementary DNA was synthesized from DNase-treated total RNA using the SuperScript® III Reverse Transcriptase (Invitrogen™). Real-Time RT-PCR experiments were carried out in biological triplicates using different RNA samples extracted from three independent plants. The Qiagen 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Valencia, USA) and the ABI PRISM 7900HT Instrument (Life Science) were used to quantify transcripts. Gene-specific primers were designed for the target genes as well as the *aprt* housekeeping gene transcript (Online Resource 2). Each reaction comprised 300 nM of each primer and cDNA synthesized from 1 µg of total RNA (three replicates for each reaction) in a total volume of 15-µl and began with a 50 °C hold for 2 min and a 95 °C hold for 10 min followed by 40 cycles at 95 °C for

30 s, 55 °C for 30 s, and 72 °C for 20 s. Data acquisition were performed during the combined annealing/extension step. A melting curve analysis of the PCR products was produced to verify their specificity and identity. Results were then analyzed using the ABI PRISM 7900HT Sequence Detection System Version 2.1 (SDS 2.1). Relative quantification was measured using the Comparative Ct (Threshold Cycle) Method (Livak and Schmittgen, 2001). The endogenous control gene (*aprt*) was used to normalize the cDNA of each sample. The relative quantity (RQ) was expressed as fold change (FC) and calculated as follows:

1. $\Delta Ct = Ct\ (sample) - Ct\ (endogenous\ control)$.
2. $\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (calibrator)$.
3. $FC = 2^{-\Delta\Delta Ct}$ (normalized fold change relative to calibrator), where 2 represents doubling of the amount of the product of amplification after each PCR cycle and the calibrators were cmm1T and blb1C for *S. commersonii* and *S. bulbocastanum* tetraploids, respectively.

Results

Morpho-anatomical analysis

Polyploidization induced either an increase or a decrease in the area of leaf lamina (data not shown). In both cmm24 and cmm30 a reduction of leaf lamina expansion was observed ($LA_{cmm24}/LA_{cmm1T} = 0.505$, $LA_{cmm30}/LA_{cmm1T} = 0.901$). By contrast, a significant increase in leaf lamina expansion was found in blb10 and blb22, which presented a ratio of their leaf area over that of blb1c equal to 1.08 and 1.22 respectively. In blb25 and blb26, a reduction was detected ($LA_{blb25}/LA_{blb1c} = 0.906$; $LA_{blb26}/LA_{blb1c} = 0.819$). Polyploidization did not induce qualitative changes in leaf structure (Fig. 1). In both *S. commersonii* (Fig. 1a, c, f) and *S. bulbocastanum* (Fig. 1b, d, e, g, h), leaf lamina was characterized by a dorsiventral structure with one layer of upper and lower epidermis, and a mesophyll made of a layer of palisade cells and multiple layers of spongy parenchyma. In general, mesophyll of *S. commersonii* showed a less dense structure with higher incidence of intercellular spaces than *S. bulbocastanum*; such a tendency was maintained unaltered after polyploidization. In *S. commersonii*, for 9 out of 11 morpho-anatomical traits significant differences were detected between 2x and 4x genotypes, suggesting a phenotypic effect of polyploidization (Table 1). It is worth noting that the 4x derivatives (Fig. 1c, f) did not show superiority compared to their 2x parent cmm1T (Fig. 1a). A stochastic behaviour was recorded in terms of palisade parenchyma thickness (PPT), spongy parenchyma thickness (SPT) and CA of both parenchyma tissues (PPCA, SPCA). Indeed, the thickness and CA of both tissues was significantly decreased after polyploidization in cmm24, while resulted unaltered in cmm30 (Fig. 1a, c, f). Only in epidermis, cells were consistently larger and more turgid (significantly higher convexity) in the 2x parent than in the 4x genotypes. In the spongy parenchyma, an increase in cell elongation and turgidity (higher values of aspect ratio and convexity) was significant in cmm24 (Fig. 1c). Cmm30 (Fig. 1f) was the tetraploid that displayed the highest number of traits (8 out of 11) for which no differences were found when compared to diploid cmm1T (Fig. 1a).

As far as *S. bulbocastanum* is concerned, morpho-anatomical data are reported in Table 2. For most traits, significant differences were found between 2x blb1C (Fig. 1b) and its 4x derivatives (Fig. 1d, e, g, h). As in *S. commersonii*, a consistent superiority of 4x genotypes compared to their 2x progenitor was never detected and most changes were stochastic. In both blb10 and blb22 (Fig. 1d, e), polyploidization induced a significant reduction in CA and aspect ratio in epidermis and palisade parenchyma (ECA, EAR, PPCA, PPAR), but a significant increase in CA in spongy parenchyma (SPCA). Blb25 (Fig. 1g) showed significantly lower values of CA than the 2x only in palisade parenchyma (PPCA).

In the mesophyll, polyploidization either induced the formation of more elongated cells (higher aspect ratio), or maintained cell shape unaltered. Finally, apart from epidermal cells, an increased turgidity (higher convexity values) was found in blb26 4x genotype in the palisade cells (Fig. 1h) and in three tetraploids (blb10, blb22 and blb26) in the spongy cells (Fig. 1d, e, h). The significantly higher values of convexity in the spongy cells of most tetraploids indicated a better turgidity status of the 4x plants than the diploids.

Molecular analysis

Twelve ISSR primers were selected to test genome integrity/stability. We were able to detect a total of 161

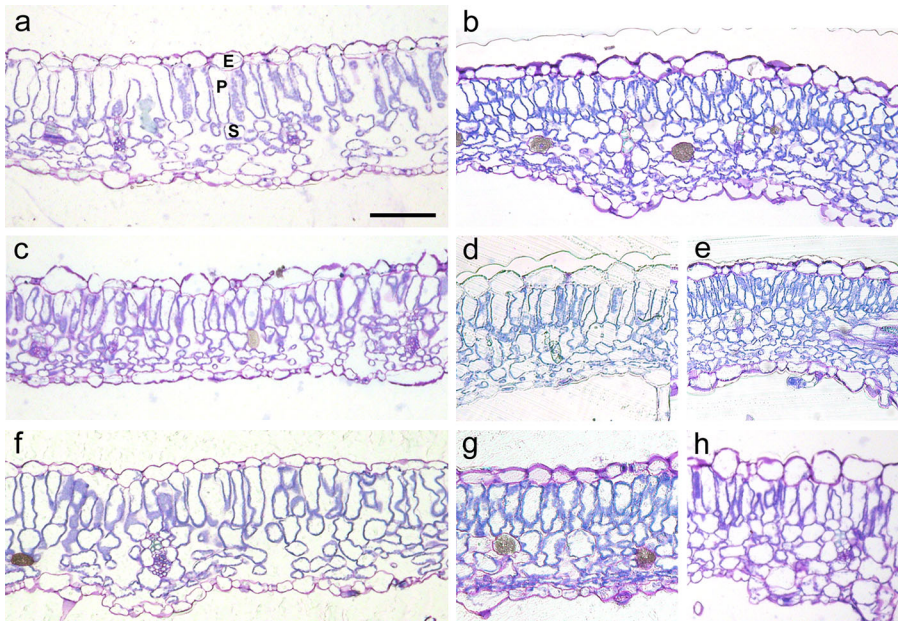

Fig. 1 Light micrographs of cross sections of leaves belonging to 2x *S. commersonii* (cmm1T) and *S. bulbocastanum* (blb1c) and their 4x derivatives. **a**: cmm1T; **b**: blb1c; **c**: cmm24; **d**: blb10; **e**: blb 22; **f**: cmm30; **g**: blb25; **h**: blb26. *E* epidermal cells; *P* palisade parenchyma cells, *S* spongy parenchyma cells. Images are all at the same magnification; bar is 100 μm

Table 1 Results (mean ± SE) on morpho-anatomical traits of *S. commersonii* synthetic tetraploids and the diploid parent they derived from (cmm1t)

Trait	Cmm 1T		Cmm 24		Cmm 30	
PPT, μm	109.81 ± 2.57	a	91.97 ± 3.51	b	109.12 ± 4.56	a
SPT, μm	112.28 ± 5.49	a	96.45 ± 2.97	b	112.78 ± 3.34	a
ECA, μm^2	1020.35 ± 133.50	a	666.36 ± 96.72	b	600.01 ± 67.78	b
EAR	2.18 ± 0.10	a	2.03 ± 0.06	a	1.57 ± 0.12	b
ECO	0.919 ± 0.01	a	0.870 ± 0.01	b	0.883 ± 0.01	b
PPCA, μm^2	2360.63 ± 141.64	a	1358.53 ± 93.75	b	2145.13 ± 171.76	a
PPAR	3.33 ± 0.09	a	3.45 ± 0.09	a	3.24 ± 0.01	a
PPCO	0.939 ± 0.02	a	0.924 ± 0.01	a	0.929 ± 0.01	a
SPCA, μm^2	482.35 ± 55.26	a	338.05 ± 33.49	b	501.53 ± 56.38	a
SPAR	1.63 ± 0.06	b	1.94 ± 0.12	a	1.63 ± 0.07	b
SPCO	0.895 ± 0.01	b	0.919 ± 0.01	a	0.895 ± 0.01	b

For each trait, means denoted by the same letter did not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test

PPT Palisade parenchyma thickness, *SPT* spongy parenchyma thickness, *ECA* cell area of epidermis, *EAR* aspect ratio of epidermis cells, *ECO* convexity of epidermis cells, *PPCA* cell area of palisade parenchyma, *PPAR* aspect ratio of palisade parenchyma cells, *PPCO* convexity of palisade parenchyma cells, *SPCA* cell area of spongy parenchyma, *SPAR* aspect ratio of spongy parenchyma cells, *SPCO* convexity of spongy parenchyma cells

alleles in diploid *S. commersonii* and its tetraploids and 139 alleles in diploid *S. bulbocastanum* and its tetraploids (Online Resource 1). Among tested loci, ISSR 6 and ISSR 16 were the most polymorphic for *S. bulbocastanum* genotypes, both producing 17 bands. ISSR 10 showed 22 polymorphic bands in

Table 2 Results (mean ± SE) on morpho-anatomical traits of *S. bulbocastanum* synthetic tetraploids and the diploid parent they derived from (blb1c)

Trait	Blb1c		Blb10		Blb22		Blb25		Blb26	
PPT, µm	83.65 ± 1.9	a	81.95 ± 3.08	ab	85.28 ± 3.94	a	72.61 ± 2.48	b	78.96 ± 3.87	ab
SPT, µm	93.41 ± 4.11	ab	83.49 ± 3.58	ac	97.48 ± 5.05	a	75.59 ± 4.37	c	79.85 ± 4.36	c
ECA, μm^2	1307.68 ± 134.39	a	931.35 ± 116.2	b	854.14 ± 112.08	b	1126.39 ± 95.74	ab	1444.14 ± 165.84	a
EAR	2.08 ± 0.137	a	1.75 ± 0.067	b	1.70 ± 0.064	b	1.82 ± 0.110	ab	1.80 ± 0.113	ab
ECO	0.895 ± 0.009	a	0.904 ± 0.013	a	0.897 ± 0.013	a	0.899 ± 0.014	a	0.926 ± 0.006	a
PPCA, μm^2	1122.69 ± 95.51	a	886.77 ± 49.94	b	733.91 ± 37.65	b	920.55 ± 68.4	b	1158.5 ± 66.36	a
PPAR	2.93 ± 0.113	b	3.34 ± 0.151	a	3.58 ± 0.123	a	3.25 ± 0.146	ab	3.57 ± 0.132	a
PPCO	0.819 ± 0.021	bc	0.838 ± 0.018	b	0.793 ± 0.018	c	0.813 ± 0.018	bc	0.875 ± 0.014	a
SPCA, μm^2	524.96 ± 48.21	b	931.35 ± 116.2	a	854.14 ± 112.08	a	431.35 ± 42.45	b	561.04 ± 52.89	b
SPAR	1.89 ± 0.090	a	1.75 ± 0.067	a	1.70 ± 0.064	a	1.64 ± 0.078	a	1.64 ± 0.067	a
SPCO	0.875 ± 0.015	b	0.912 ± 0.019	a	0.902 ± 0.021	a	0.870 ± 0.020	b	0.916 ± 0.012	a

For each trait, means denoted by the same letter did not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test

PPT Palisade parenchyma thickness, *SPT* spongy parenchyma thickness, *ECA* cell area of epidermis, *EAR* aspect ratio of epidermis cells, *ECO* convexity of epidermis cells, *PPCA* cell area of palisade parenchyma, *PPAR* aspect ratio of palisade parenchyma cells, *PPCO* convexity of palisade parenchyma cells, *SPCA* cell area of spongy parenchyma, *SPAR* aspect ratio of spongy parenchyma cells, *SPCO* convexity of spongy parenchyma cells

S. commersonii, resulting to be the most selective for this group of plants. No polymorphisms between the tetraploids and the diploid progenitors they derived from were found, revealing that structural changes due to chromosome doubling *per se* did not affect the genome of both species at analyzed loci.

A set of eight different cell cycle genes was selected to study gene expression alteration linked to polyploidy. Most of genes were differentially expressed in 4x vis-à-vis their relative 2x parent assuming a 2 FC threshold. Figure 2a shows the expression profile in *S. commersonii* tetraploids analysed. A general down regulation of cell cycle genes was found. In cmm24 six out of eight genes were differentially expressed, with *ICK7* being the most down regulated one (− 7 FC) and *CDK-B2* the only up regulated (2.05 FC) cell cycle gene. In cmm30, four out of eight genes were differentially expressed, with *ICK* and *CDK-C* up regulated and *ICK7* and *CDK-B2* down regulated. As for gene relative expression profiling in 4x of *S. bulbocastanum* vs. 2x blb1c, results are reported in Fig. 2b. After polyploidization, blb10 and blb22 showed a general down regulation of cell cycle genes. In the former *CDKp34* and *CDK-B1* were down regulated by a − 3.9 and − 2.58 FC factor, respectively. In the latter, together with *CDKp34* and *CDK-B1*, also *CDK-C1* and *ICK7* were down regulated (− 2.2 FC and − 3.2 FC, respectively). An opposite trend was observed in blb25 and blb26, with most of the differentially expressed genes being up regulated. In these tetraploids, six out eight genes were differentially expressed with a 2 FC threshold. *CDK-B2* and *CDKp34* were the highest up- and down regulated genes in blb25, respectively. In blb26 all genes were up regulated and among them *CDK-C* was expressed 9 times more after polyploidization.

Discussion

In general, polyploids are reported to be more vigorous and to show larger organs than their diploid counterpart. However, the results reported in our study confirm that this is not a universal phenomenon. For several traits we observed a diploid superiority and in both species studied we never found a trait for which all tetraploids displayed a consistently higher value than the diploid progenitor. This lack of polyploid phenotypic superiority is in line with some previously published papers. In *S. phureja*, Stupar et al. (2007) observed that, although 2x and 4x genotypes had

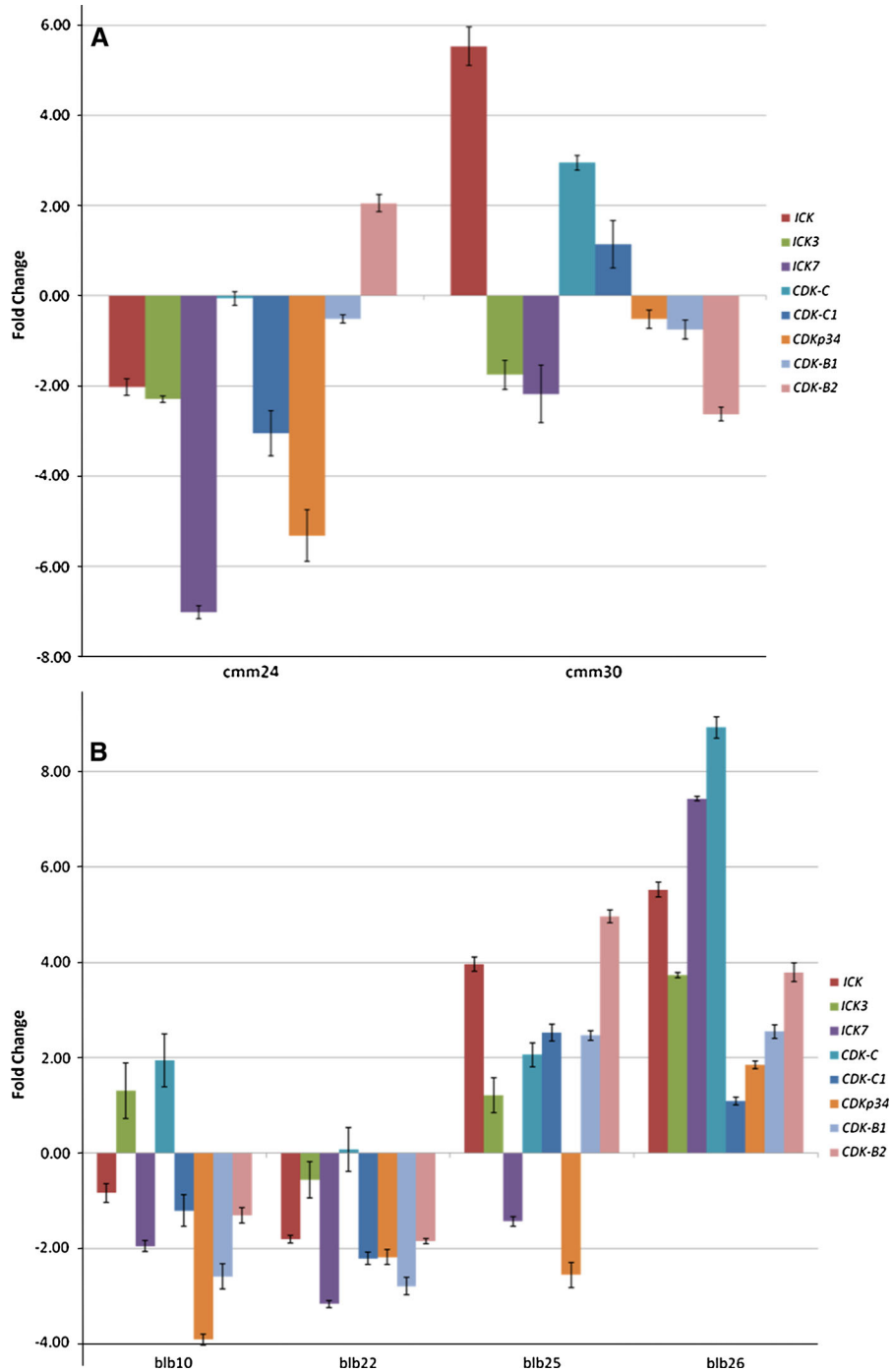

Fig. 2 Gene expression of three cyclin-dependent kinase inhibitor genes (ICK, ICK3 and ICK7) and five cyclin-dependent kinases (CDK-C, CDK-C1, CDKp34, CDK-B1 and CDK-B2). Data are presented as mean ± SD of three biological replications **a** Fold Change for each *S. commersonii* tetraploid (cmm24 and cmm30) vis-à-vis the diploid parent cmm1t. **b** Fold Change for each *S. bulbocastanum* tetraploid (blb10, blb22, blb26 and blb26) vis-à-vis the diploid parent blb1c

similar gene expression patterns, the 2x plants were phenotypically superior. Similarly, Nakadozono et al. (2007) in *E. ulmoides* and Anssour et al. (2009) in *Nicotiana attenuata* and *N. obtusifolia* did not observe larger leaves in 4x plants compared to 2x. To explain these results, Hessen et al. (2010) suggested that polyploidy can enhance growth in certain conditions, but can also be associated with increased material costs for producing and maintaining DNA and with high demand of phosphorous for ribosomes and

nitrogen for proteins. Lack of superiority of polyploids compared to diploids may also be explained on the basis of time required for adjustment, adaptation and evolution after chromosome doubling. In *Phlox drummondii,* Vyas et al. (2007) found that later generations had higher leaf photosynthesis rates than newly synthesized autotetraploids. The findings of our study also suggested that most of phenotypic responses to polyploidization were species and genotype-dependent. Indeed, comparing the two species, we observed that in *S. bulbocastanum* there were 4x genotypes with more expanded leaf lamina and larger CA in the spongy parenchyma than 2x blb1c. No general tendency towards the increase or decrease in the various measured parameters was found. By contrast, as far as leaf or cell size is concerned, in *S. commersonii* we never found a 4x genotype superior to its 2x parent. In this species, one of the tetraploids showed lower values of leaf area and cell size in all the tissues, as if the whole organ has been miniaturized. The formation of smaller cells is in agreement also with the formation of a smaller number of narrower vessels found in the same 4x with respect to the 2x parent (Aversano et al. 2013). This suggests the occurrence of an overall harmonization of all morpho-anatomical parameters. Considering that morphological and physiological traits are generally balanced to achieve a coordination of the hydraulic and photosynthetic behaviour (Aasama et al. 2001; De Micco et al. 2011), this harmonization likely results also in adjustments of the physiological processes. The occurrence of very high values of convexity, indicating high cell turgidity, supports the hypothesis of a good hydraulic control in all tetraploids. Our DNA analysis suggested that structural changes due polyploidization were not present and previous papers reported that the genomic shock was induced by polyploidization itself and not by the method used for chromosome doubling (Ozkan et al. 2001; Martelotto et al. 2005). Therefore, we hypothesize it is unlikely that residual oryzalin effects have affected our findings. Comparisons of 4x and 2x plants deriving from the same oryzalin-treated parent will help to further elucidate this aspect.

To test whether the response to genome doubling was dependent on the genotype used also from the transcriptomic standpoint, we profiled the expression of eight genes involved in the cell cycle. Three of them were annotated on the potato genome consortium (Potato Genome Sequencing Consortium 2011) as ICK and the rest as CDKs. Expression analysis revealed a strong alteration of the expression patterns in the 4x with respect to the respective 2x parents. Changes in tetraploids often exceed the twofold and there was not a consistent trend towards up- or down regulation comparing 2x vs. 4x. In a corn ploidy series, a pioneering paper by Guo et al. (1996) reported that expression of most genes analyzed increased with polyploidization, whereas some others showed an opposite trends. Genes that were positively or negatively modulated by autopolyploidization have been reported also by Yao et al. (2011) comparing 2x, 4x and 6x corn lines. Yu et al. (2010) confirmed a genotype-specific gene expression change in analysing three selected genes in *A. thaliana* ecotypes. It should be pointed out that for both species we used independent synthetic tetraploids, whereas in several other studies comparisons were made between a single tetraploid vis-à-vis its diploid parent (Stupar et al. 2007; Shcherban et al. 2008; Lavania et al. 2012; Cohen and fait A, Tel-Zur N, 2013; Zhang et al. 2014). This allowed us to show that phenotypic as well as gene expression changes were often stochastic. The finding that the various tissues do not respond uniformly to polyploidization suggests that their formation and differentiation is not regulated by common processes as also happens for other functions that are regulated in a tissue-autonomous manner (Kozuka et al. 2011). Interestingly, both inhibitors and CDKs were either up- or down regulated in the same tetraploid, suggesting a post-translation regulation of CDKs realized through the physical interaction with ICK (Blomme et al. 2013). The similar expression pattern between inhibitors and the relative CDKs also suggests that the cell cycle timing and the cell division parameters were not modified between diploid and tetraploid plants and consequently a transcriptional adjustment of cell-cycle genes was triggered. Similarly, Sugiyama (2005) observed no significant differences for cell cycle parameters analysing 2x and 4x cultivars of *Lolium*. In both species we analysed, a contrasting pattern of expression between genotypes was observed. This was particularly evident in *S. bulbocastanum*, where blb10 and blb22 displayed a general trend towards down regulation, whereas blb25 and blb26 showed over expression of cell cycle genes. This result seems in line with some morpho-anatomic data such as CA of epidermis and spongy parenchyma,

for which the same two groups (blb10/blb22 and blb25/blb26) could be identified. These morphological modifications have been already reported to be affected by the expression of cell cycle genes, and in particular by CDK type A (here named CDKp34) and type B (Boudolf et al. 2004; Blomme et al. 2013). The expression data of the current study also seems consistent with epigenetic data previously reported on the same genotypes by Aversano et al. (2013). The authors found that, following polyploidization, blb10 and blb22 were those with the highest hypermethylation frequency, whereas blb25 and blb26 were those with the strongest hypomethylation frequency. Future research will be carried out to gain new insights on the epigenetic consequences of polyploidization on cell regulation mechanisms.

In conclusion, after polyploidization a great variability was observed at the morpho-anatomic and expression level in synthetic 4x vis-à-vis their 2x parents. Most of responses to polyploidization were species and genotype-dependent, as already suggested in corn (Riddle et al. 2006), and in several cases we determined a phenotypic superiority of 2x progenitors with respect to 4x genotypes. Since epigenetic phenomena are usually involved in the early events of polyploid formation (Li et al. 2012), we can hypothesize that the expression of duplicated genes might be regulated by epigenetic mechanisms causing the lack of 4x superiority we observed and the stochastic pattern of changes at both phenotypic and expression level.

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