Phenotypic and Transcriptomic Changes Associated with Potato Autopolyploidization

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ABSTRACT

Polyploidy is remarkably common in the plant kingdom and polyploidization is a major driving force for plant genome evolution. Polyploids may contain genomes from different parental species (allopolyploidy) or include multiple sets of the same genome (autopolyploidy). Genetic and epigenetic changes associated with allopolyploidization have been a major research subject in recent years. However, we know little about the genetic impact imposed by autopolyploidization. We developed a synthetic autopolyploid series in potato (*Solanum phureja*) that includes one monoploid (1x) clone, two diploid (2x) clones, and one tetraploid (4x) clone. Cell size and organ thickness were positively correlated with the ploidy level. However, the 2x plants were generally the most vigorous and the 1x plants exhibited less vigor compared to the 2x and 4x individuals. We analyzed the transcriptomic variation associated with this autopolyploid series using a potato cDNA microarray containing ~9,000 genes. Statistically significant expression changes were observed among the ploidies for ~10% of the genes in both leaflet and root tip tissues. However, most changes were associated with the monoploid and were within the 2-fold level. Thus, alteration of ploidy caused subtle expression changes of a substantial percentage of genes in the potato genome. We demonstrated that there are few genes, if any, whose expression is linearly correlated with the ploidy and can be dramatically changed because of ploidy alteration.
INTRODUCTION

Polyploidization events occur frequently during plant evolution. The most popular estimate of the proportion of polyploids in angiosperms is ~70% (MASTERSON 1994). However, recent genomic investigations have revealed that many classic diploid plant species have polyploid origins (BLANC and WOLFE 2004b; CUI et al. 2006; GAUT and DOEBLEY 1997; PATERSON et al. 2004; YU et al. 2005), indicating the near ubiquity of polyploidy throughout the evolutionary history of the plant kingdom. This ubiquity implies that polyploidy confers selective advantages over diploidy, which are often manifested in enhanced vigor of polyploid phenotypes. Potential selective advantages, such as increased heterozygosity, novel variation, and allelic sub-functionalization, have been widely discussed (ADAMS et al. 2003; ADAMS and WENDEL 2005; BLANC and WOLFE 2004a; CARPUTO et al. 2003; CHEN and NI 2006; COMAI 2005; LEITCH and BENNETT 1997; OSBORN et al. 2003; SOLTIS et al. 2004; UDALL and WENDEL 2006; WENDEL 2000).

Polyploids originate from either sexual reproduction via 2n gametes or somatic chromosome doubling. By traditional definition, there are two forms of polyploidy: allopolyploidy and autopolyploidy. These terms are often used to imply the mode of polyploid formation, but more accurately describe the degree of similarity between the subgenomes in polyploids. Allopolyploids have distinct subgenomes and typically originate from interspecific hybridization between divergent progenitor species. Autopolyploids have (nearly) identical subgenomes, and typically originate from intraspecific hybridization (or self-fertilization through 2n gametes) or somatic chromosome doubling. Allo- and autopolyploids have traditionally been distinguished by modes of chromosome pairing and inheritance, with allopolyploids exhibiting
bivalent pairing and disomic inheritance and autopolyploids exhibiting multivalent pairing and polysomic inheritance.

A number of well known polyploid plants of agricultural interest are classical allopolyploids, which include important crops such as bread wheat (2n=6x=42) and cotton (2n=4x=56). Studies of genetic and epigenetic changes associated with polyploidization have been focused mostly on newly synthesized allopolyploid materials (ADAMS et al. 2004; ALBERTIN et al. 2006; COMAI et al. 2000; HE et al. 2003; KASHKUSH et al. 2002; KASHKUSH et al. 2003; LIU et al. 1998; LUKENS et al. 2006; MADLUNG et al. 2002; MADLUNG et al. 2005; OZKAN et al. 2001; SKALICKA et al. 2005; SONG et al. 1995; WANG et al. 2006; WANG et al. 2004). However, in allopolyploids, ploidy level per se is difficult to tease apart from many other variables, such as diverged suites of regulatory factors from different genomes. For instance, investigations in maize indicate that gene expression is altered more by genome hybridization than by genome ploidy changes (AUGER et al. 2005). The effect of ploidy per se can only be assessed among a series of homozygous plants at different ploidy levels. There have been relatively few studies dedicated to elucidating the consequences of autopolyploidization on gene expression (ALBERTIN et al. 2005; AUGER et al. 2005; GALITSKI et al. 1999; GUO et al. 1996; STORCHOVA et al. 2006; WANG et al. 2006). Thus, the genetic impact imposed by ploidy alteration remains elusive.

We sought to identify a plant system in which the changes in gene expression are associated only with ploidy. The genus Solanum appeared to be an excellent choice due to its exceptional tolerance of ploidy manipulations. This genus includes a wide array of wild and domesticated diploid, tetraploid, and hexaploid accessions, many of which are closely related (HAWKES 1990). Meiotic mutants leading to 2n pollen and 2n eggs are prevalent in Solanum...
species, which could explain the repeated polyploidization events associated with several Solanum species (CAMADRO and PELOQUIN 1980; IWANAGA and PELOQUIN 1982; PELOQUIN et al. 1999). Furthermore, the cultivated potato, Solanum tuberosum (2n=4x=48), has been defined as a classic autopolyploid based on its tetrasomic inheritance (CARPUTO et al. 2003; GRANT 1981). Genetic manipulation via meiotic mutants associated with 2n gamete formation has played a more significant role in breeding of potato than in any other crop (PELOQUIN et al. 1999; PELOQUIN et al. 1989). Thus, potato provides an excellent model system for autopolyploidy studies.

In this study, we have attempted to isolate the variable of ‘ploidy’ in generating phenotypic and gene expression changes in a Solanum autopolyploid system. We used a synthetic autopolyploid series of plant materials from a clonally propagating potato species, Solanum phureja. These plant materials did not experience any meiotic or gametophytic developmental stages during propagation and were therefore not subjected to the genomic and epigenetic changes that can be generated through successive generations, nor were the duplicated genotypes subject to selective forces. Observed changes in the relative expression of given genes were thus more likely attributed to ploidy per se and the factors therein, such as nuclear dosage and ploidy-driven cellular modifications that may effect cell size, division rates or organellar composition. Furthermore, we have investigated this topic in both the principally undifferentiated cells of plant root tips and the principally differentiated cells of plant terminal leaflets. The objectives of this study were to define the patterns of gene expression that accompany changes in ploidy, and to identify any genes that exhibit ploidy-dependent expression patterns.
MATERIALS AND METHODS

Development of the O37 autopolyploidy series

A homozygous doubled monoploid (DM) derived by anther culture followed by leaf disk regeneration of adapted diploid *S. phureja* clone BARD 1-3 was outcrossed to a diploid complex hybrid (1/2 *S. stenotomum*, 3/16 *S. phureja*, 3/16 *S. tuberosum*, 1/8 *S. chacoense*). An F₁ selection derived from this cross was backcrossed to its DM parent. We selected a backcross seedling based on vigor and tuber yield, and then applied anther culture to produce the O37 1x monoploid used in this study. Diploid and tetraploid regenerants of monoploid O37 were produced by leaf disk regeneration in tissue culture (Hulme et al. 1992; Paz and Veilleux 1999). Two 2x diploid regenerants and one 4x tetraploid regenerant were recovered and subjected to flow cytometry for ploidy estimation. The diploid regenerants were named 2xR3 and 2xR5, respectively. Therefore, the O37 1x, 2xR3, 2xR5 and 4x plants used in this study are isogenic. Initially several autopolyploidy series derived from different monoploids were screened by chromosome counting. At least one of the plants in most of the autopolyploidy series was found to be an aneuploid. Only the O37 series was used for morphological and gene expression studies.

Plant growth and anatomical analysis

The plant materials were moved from tissue culture to greenhouse pots and clonally propagated for several generations via tubers. Plants were planted from tubers and grown in a walk-in growth chamber at the University of Wisconsin-Madison Biotron facility. Plants were grown under a 15/9 h day/night cycle with a 23.5°C/15.5°C day/night temperature regime. Relative humidity was held constant at 50%. Following shoot emergence, plants were given watering
treatments with 0.5x strength Hoagland solution twice daily. Terminal leaflet length was measured repeatedly for 17 d starting 20 d after planting. One leaflet from three different plants was measured for each of the O37 1x, 2xR3, 2xR5 and 4x plants. Growth curves of leaflet length were plotted over time and compared to identify comparable growth stages among the plants.

Microscopic nuclear endoreduplication and stomatal size assessments were performed on terminal leaflets during early growth. Staining with 4’,6-diamidino-2-phenylindole (DAPI) and visualization of epidermal cells essentially followed published protocols (DOWNES et al. 2003; SZYMANSKI and MARKS 1998). Preparation of epidermal peels for stomatal cell size analysis was performed as described (SZYMANSKI and MARKS 1998). For transmission electron microscopy (TEM) analysis, only leaflets in their pre-determined exponential growth range (10-20 mm in length for 1x plants; 15-25 mm in length for 2x and 4x plants) were harvested. TEM fixation and microscopy procedures essentially follow published methods (KANG et al. 2001). All material was embedded in Spurr’s resin and polymerized at 70°C. Samples were sectioned for TEM using a Reichert-Jung Ultracut-E Ultramicrotome and contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% EtOH. Ultrathin sections were observed with a Philips CM120 electron microscope and images were captured with a Mega View III side mounted digital camera.

**Chromosome counts and flow cytometry**

Metaphase chromosome preparation and FISH analysis were performed as described (CHENG et al. 2001). Flow cytometry methods followed published protocols (ARUMUGANATHAN and EARLE 1991). Plant growth conditions and leaflet tissue sampling for flow cytometry analyses
followed the same methodology used to generate the tissue samples for gene expression analysis (see “RNA isolation” section below). Approximately ten expanding terminal leaflets of each O37 plant genotype were pooled for nuclei isolation; 1x leaflets were collected at 10-20 mm in length and 2xR3, 2xR5, and 4x leaflets were collected at 15-25 mm in length. Soybean cv. Belle nuclei from fully expanded leaflets were used as an internal reference for flow cytometric analyses.

**RNA isolation**

For RNA analyses, three independent O37 series biological replicates were grown sequentially in the same growth chamber; the plant growth and tissue sampling were consistent across ploidy genotypes and biological replicates. Each biological replicate consisted of 6-8 plants of each ploidy genotype, with two tubers planted per pot. At 20 days after planting, young terminal leaflets at comparable growth stages (10-20 mm in length for 1x plants; 15-25 mm in length for 2xR3, 2xR5 and 4x plants) were flash-frozen in liquid nitrogen 7 h following light onset in the growth chamber. All healthy terminal leaflets of the appropriate growth stage were collected and pooled for each genotype. Approximately two leaflets were collected per plant, resulting in a total of ~12-16 leaflets per pooled sample. Total RNA from each ploidy genotype was isolated by TRIzol extractions according to manufacturer instructions (Invitrogen Corp, Carlsbad, CA), with the following modifications. The aqueous TRIzol:chloroform extractions were precipitated with 0.5 volume isopropanol and 0.5 volume 1.2 M sodium chloride, 0.8 M sodium citrate solution. Purified RNA samples were treated with 2.5 U DNase I (Ambion Inc., Austin, TX) and incubated at 37°C for 15 min, then extracted with phenol:chloroform. Purified RNA samples were quantified and qualified using the Nanodrop spectrophotometer with version 2.5.3 software.
Two independent O37 series biological replicates were sequentially grown for root tip RNA isolation. Plant growth conditions were the same as those described above. Root tip samples, however, were harvested 7 days following planting; therefore there were no watering treatments given to these plants. Roots were harvested and immediately submerged in RNALater solution (Ambion Inc., Austin, TX). Roots were stored in RNALater reagent for up to 7 days at 4°C. Opaque root tip meristematic regions were subsequently harvested with a fine forceps for RNA extraction. The Plant Mini RNeasy kit (Qiagen Corp., Valencia, CA) was used to extract root tip total RNA. RNA samples were then DNase I treated as above, phenol:chloroform extracted, and then precipitated with 2.5 volume ethanol, 0.1 X sodium acetate, and 1 µl pellet paint NF co-precipitate (EMD Biosciences, Inc., San Diego, CA). Purified RNA samples were quantified using the Nanodrop spectrophotometer with version 2.5.3 software.

Microarray hybridization and data analysis

The 10K version 2 cDNA potato microarray developed at The Institute for Genomic Research (TIGR) was used for gene expression analyses. This array contains 15,264 cDNA clones spotted in duplicate from potato that have been gel and sequence verified as described in Rensink et al. (2005), resulting in 11,243 verified cDNA clones which are termed “STM” for Solanum tuberosum microarray. Some redundancy is present within the 11,243 verified clones and after assembly and clustering the 5’ and 3’ end sequences of the STM clones, along with all other available S. tuberosum ESTs and cDNAs, into the S. tuberosum Transcript Assembly
(http://plantta.tigr.org), the validated clones on the array could be collapsed into 9,029 genes. The annotations used in this study were from the December 5, 2005 update of the microarray annotation file (http://www.tigr.org/tdb/potato/microarray_comp.shtml). The relative expression of each gene per ploidy transcriptome was determined as described below.

Two-color microarray hybridizations were performed according to a loop design over the multiple biological replicates to compare RNA from different ploidy plant tissues. For leaflet tissue, 20 µg total RNA was annealed to random hexamers and reverse transcribed into amino-allyl modified cDNA; these cDNAs were then coupled to Cy-dye esters according to the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ). For root tips, total RNA was amplified using the SuperScript RNA amplification system according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA) to yield amplified antisense RNA (aRNA); these aRNAs were then amino-allyl labeled and dye-coupled as described above. Techniques used for microarray hybridization, microarray washing, and microarray scanning have been previously described (HEGDE et al. 2000; RENSINK et al. 2005).

Microarray data normalization essentially followed published protocols (RENSINK et al. 2005). The leaflet and root tip datasets were independently normalized and statistically analyzed. Prior to normalization, the values for the two on-slide replicates were averaged for each gene. The R/LIMMA package (SMYTH 2004) was used to normalize microarray data. Data were normalized within arrays using the ‘Loess’ parameter and between arrays using the default LIMMA parameter. The R/MAANOVA package (Wu et al. 2003) was used for statistical analyses. A mixed-effects model was designed to calculate M-values, perform F-tests, and perform t-tests between the different ploidies for each gene (see Supplemental Methods for details of experimental design and MAANOVA code used; data generated by these analyses are
available in Supplemental Table 1). M-values were calculated for each gene at each of the four genotypes. M-value differences among genotypes represent log2 fold-changes. For example, gene X may have a 2x-1x M-value difference of 2; this indicates that gene X experiences a 4-fold increase in expression in the 2x plant relative to the 1x plant. The M-value differences between the 2x-1x and 4x-2x genotypes were plotted for selected groups of genes using OriginPro 7.5 software.

F-test p-values and false discovery rate (FDR) Q-values (STOREY and TIBSHIRANI 2003) were calculated for each gene. Genes were determined significant at Q < 0.10. Contrast matrices were specified in this analysis such that t-statistics were generated for each meaningful contrast in this study: 1x-2x; 1x-4x; 2x-4x; 1x-2xR3; 1x-2xR5; 2xR3-4x; 2xR5-4x; 2xR3-2xR5. Each of the significant genes identified in the F-test was further defined by its 1x-2x and 2x-4x contrast p-values. Contrasts were determined to be significant at p < 0.05. The directionality of the difference was inferred based on the M-value difference for each contrast. Therefore, each gene could be classified by one of nine expression patterns: ploidy-upregulated (1x<2x<4x), ploidy-downregulated (1x>2x>4x), 2x-upregulated (1x<2x>4x), 2x-downregulated (1x>2x<4x), 4x over 2x (1x≈2x<4x), 2x over 4x (1x≈2x>4x), 2x over 1x (1x<2x≈4x), 1x over 2x (1x>2x≈4x), and no pair-wise difference (1x≈2x≈4x).

Gene ontology assignments were used to identify gene classes that were most frequently differentially expressed across ploidies. To assign gene ontologies, we searched a total of 44,468 (35.3 Mb) sequences of Solanum tuberosum Transcript Assembly (TA) Release 1 using blastx against go.pep, which is a collection of protein sequences with GO assignments of several selected eukaryotic organisms, including Arabidopsis thaliana, Drosophila melanogaster, Caenorhabditis elegans, Mus musculus, Plasmodium falciparum, and Schizosaccharomyces
*pombe*. Gene ontologies were transferred from matched go.pep protein to *S. tuberosum* TA sequences with a cut-off of E value ≤ -10 for the matches. GO terms were then converted to the Plant GOSlim terms (http://www.geneontology.org/GO.slims.shtml) with map2slim (http://www.geneontology.org/GO.slims.shtml#script). EST reads, as components of *S. tuberosum* TAs, subsequently inherited the GOSlim assignments from the TAs. Some genes have multiple GOSlim assignments. The GOSlim terms for molecular function and for structural component are listed in Figures 7A and 7B, respectively.

**Real-time PCR and Northern blot hybridization**

Total RNA isolated from terminal leaflet was converted to cDNA by reverse transcription using Superscript III Reverse Transcriptase enzyme and an oligo dT primer, according to the manufacturer’s instructions (Invitrogen Corp, Carlsbad, CA). All primers were designed based on potato EST data, available at http://plantta.tigr.org. Oligo 4.0 software (National Biosciences, Plymouth, MN) was used to design primers and assess primer quality. All real-time PCR reactions and subsequent amplicon melting curve analyses were performed using the DyNAmo SYBR Green qPCR kit on an Opticon 2 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA). The comparative Ct method (Livak and Schmittgen 2001) was used to determine the relative transcript abundance of each gene. Primer sets for actin 97 (BQ115827- BQ115828) and ubiquitin conjugating enzyme 2 (BQ112522- BQ112523) were used as standards. Primer sequences and cycling conditions have been described (Stupar 2005) and are available upon request.

Real-time PCR reactions were performed for each primer pair on all genotypes of all three leaf biological replications. Real-time PCR reactions was performed for the standard
primer pairs for each biological replicate; 2-3 technical replicate reactions were performed with each standard primer pair to increase the accuracy of each biological replicate measurement. The data from the two standard primers were averaged to generate a single standard value for comparative Ct analysis. Average expression changes and standard deviations for the three biological replicates were calculated for each primer pair. Northern blot analyses were performed essentially as described (STUPAR et al. 2006). Potato cDNAs for cyclin B-type (BQ115975- BQ115976) and expansin precursor (BQ515454- BQ515455) were used as probes on independent blots. A cDNA for actin 97 (BQ115827- BQ115828) was subsequently used as a probe to verify equal RNA loading on each blot.

Data Deposition
The microarray data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5428)

RESULTS
Polyploid series O37 includes molecularly monomorphic 1x, 2x, and 4x euploids
A monoploid potato clone O37 (2n=1x =12) was used to develop a polyploid series (PAZ and VEILLEUX 1999). Somatic leaf cells from the monoploid were exposed to leaf disk regeneration, which resulted in diploid (2n=2x=24) and tetraploid (2n=4x=48) regenerants initially characterized by flow cytometry (JOHNSON et al. 2001). Therefore, the resulting monoploid, diploid, and tetraploid plants were, in theory, isogenic and differed solely in genome size.

Polyploid series O37 included one monoploid, two diploids, and one tetraploid. The two diploids, named 2xR3 and 2xR5, respectively, were independent shoot regenerants.
Chromosome counting verified that the monoploid, diploids, and tetraploid contained 12, 24, and 48 chromosomes, respectively (Figure 1). We also conducted fluorescence in situ hybridization (FISH) analyses using 5S and 45S rDNA probes and a chromosome 8-specific bacterial artificial chromosome (BAC) clone. The number of FISH foci from these three probes matched the expected numbers of chromosome 1 (5S rDNA), chromosome 2 (45S rDNA) and chromosome 8 in the O37 series (data not shown). Randomly Amplified Polymorphic DNA (RAPD) (Supplemental Figure 1) and Amplified Fragment Length Polymorphism (AFLP) (data not shown) analyses displayed no polymorphism within the O37 series. These data further confirm that the O37 series plants differ from one another only in terms of ploidy.

The O37 series plants display phenotypic and cellular variation

The O37 plants were moved from the tissue culture and propagated via tubers in greenhouse and growth chamber environments. These plants had vigorous phenotypes (Figure 2) and produced both tubers and flowers. Clearly visible morphological differences were observed among the different ploidies. The 2xR3 and 2xR5 plants were more vigorous than the 1x and 4x plants and generated the greatest overall biomass; furthermore, the 4x plants were more vigorous than the 1x plants (Figure 2). These observations are similar to previous comparisons of synthetic autopolyploid series of potato (Uijtewaal et al. 1987), maize (Riddle et al. 2006) and other autopolyploidized species (Stebbins 1971). Organ thickness, especially in leaves, tended to increase with ploidy in the O37 series. However, the lower ploidy individuals tended to produce more lateral growth. Importantly, there was no obvious morphological difference between the 2xR3 and 2xR5 plants, indicating that the tissue culture regeneration procedure had minimal effect on the plant phenotypes.
Growth measurements were performed on the terminal leaflets of the growth chamber grown O37 series plants. The 2x and 4x terminal leaflets displayed more rapid growth than the 1x terminal leaflets, which is consistent with the differences in overall plant vigor. The 1x terminal leaflets 10-20 mm in length were developmentally comparable to 2x and 4x terminal leaflets 15-25 mm in length (STUPAR 2005); these growth stages were determined to be early and rapid phases of terminal leaflet growth. All further leaflet analyses of these plants were conducted at these specified developmental stages.

Terminal leaflets at the comparable developmental stages were assessed to determine whether the differentiated cell types exhibited the cellular differences typically attributed to ploidy. Stomatal cell size has been used as an established indicator of base plant ploidy, as these cells normally do not undergo nuclear endoreduplication (MELARAGNO et al. 1993). An increase in base ploidy is typically accompanied by an increase in stomatal guard cell size (MASTerson 1994). A microscopic investigation of series O37 stomatal cell sizes determined that the O37 stomatal guard cells increased in size proportionally with plant ploidy (data not shown), which is consistent with the observations made in comparisons of natural polyploid plants (MASTerson 1994). Additionally, transmission electron microscopy (TEM) of leaflet cross-sections showed that ground tissue cells also experience an increase in size across ploidies (Figure 3).

**Endoreduplication of the O37 series plants**

Significant variation of endoreduplication and/or spontaneous chromosome doubling in the O37 series leaf tissue would impact the gene expression profiling experiments. We estimated the overall rates of leaflet nuclear endoreduplication (or spontaneous chromosome doubling) using flow cytometry. The 1x, 2x, and 4x plants each exhibited two major flow cytometry peaks
(Figure 4); the first peak was consistent with the base ploidy and the second peak appeared to be twice the base ploidy. It is unknown what proportion of the double-ploidy cells was endoreduplicated and what proportion was simply in the G2 stage of the cell cycle. The 2x peak of the 1x plant was relatively larger than the 4x peak of the 2x plants and the 8x peak of the 4x plant (Figure 4), suggesting that the 1x plant may have a greater tendency toward endoreduplication or spontaneous chromosome doubling. Additionally, the 1x plant shows a small peak at 4x, however no analogous 8x peak was observed in the 2x plants (data not shown). However, the O37 series plants maintain sizeable cell ploidy differences relative to one another in the differentiated terminal leaflets during exponential growth. The flow cytometry analyses indicated that nuclear endoreduplication should not be a major problem in comparing the O37 plants, as the materials still maintained large nuclear differences in the sampled differentiated leaflet tissue.

**Ploidy-related expression differences in terminal leaflet and root tip tissues**

Microarray hybridizations were performed using RNA isolated from terminal leaflet and root tip tissues for the 1x, 2xR3, 2xR5 and 4x plants (see Materials and Methods). Due to its low yield, RNA from root tips was amplified prior to labeling and hybridization. The arrays were hybridized in a loop design across three biological replicates for terminal leaflet (replications 1, 2, 3) and two biological replicates for root tip (replications 1, 2) tissues, respectively (Figure 5). This experimental design and methodology treat the total transcriptional output between ploidy levels as equal, thus allowing us to investigate the effect of ploidy on a per genome basis. Gross differences in overall mRNA accumulation between ploidy levels are not resolved using this microarray approach.
ANOVA applying a mixed-effects model was used to identify genes that were significantly differentially expressed among the different ploidy levels in the leaflet and root tip tissues, respectively. Applying stringent statistical thresholds (ANOVA false discovery rate (FDR) Q-value significance of < 0.10), ~20% of the genes exhibited a significant change in expression in at least one of the two tissues across the different ploidies (Supplemental Table 1). Approximately 10.5% of the genes (948 of the 9,029 genes present on the array) were identified to have a significant change in expression in the leaflet analysis. Similarly, ~10.6% of the genes (955 of 9,029) were identified to have a significant change in the root tip analysis.

To analyze overall expression changes between the different ploidies, we plotted the average expression ratios (M-values) between ploidies against each other (Figure 6). The values were plotted as log₂ M-value differences between 2x-1x and 4x-2x ploidies, respectively (the “2x” category represents the average value between the 2xR3 and 2xR5 plants). Thus, a value of ‘1’ along either axis in this plot represents a 2-fold change and a value of ‘2’ represents a 4-fold change between the ploidies plotted. The majority of differentially expressed genes exhibited less than 2-fold changes in either the 2x-1x or the 4x-2x ploidy comparisons for both tissue types (Figure 6).

The leaflet and root tip datasets generated similar numbers of differentially expressed genes; however, only a small portion of these genes was significant in both tissue types. The proportion of genes significant in both tissue types (~1.4% of all genes on the array) was only slightly greater than the number of genes expected to be significant in both tissues at random (10.5% x 10.6% = 1.11%). We considered the possibility that the microarray may include numerous probes with tissue-specific expression, thus contributing to the non-correspondence of differentially expressed gene lists in the leaflets and root tips. To address this, we compared the
tissue-specific expression levels for the significant and non-significant gene groups from the leaflet and root tip microarray datasets (Supplemental Figure 2). In general, the leaflet and root tip data showed similar relative expression levels for each gene list. The 948 genes identified as significant in the leaflet study displayed greater microarray expression signals than non-significant genes, indicating that changes among the ploidies were more frequently detected for highly expressed genes in the leaflets. However, these 948 genes also displayed greater expression levels in the root tip dataset, in which the vast majority was not differentially expressed. The 955 genes identified as significant in the root tip study displayed expression levels similar to non-significant genes, in both leaflet and root tip tissues. Collectively, our data indicate that the non-correspondence of the leaflet and root tip significant gene lists was not a consequence of tissue-specific genes represented on the microarray.

Gene Ontology (GO) slim terms were assigned to all genes on the array for 27 molecular function and 27 structural component categories (Figure 7). These GO assignments were used to identify gene classes that were most frequently differentially expressed across ploidies. The gene ontologies of differentially expressed genes from the leaflet and root tip datasets were compared with the gene ontologies for all 9,029 genes on the array. In general, the ontology distribution of differentially expressed genes in the root tip dataset reflected the ontology distribution of the array for both molecular function and structural component (Figure 7). The leaflet dataset also reflected the array ontology distribution for most ontology groups, with some notable exceptions. The ‘molecular function’ ontologies for nucleic acid binding and structural molecule activity (Figure 7A) and the ‘structural component’ ontologies for cytosol and ribosome (Figure 7B) showed higher proportions of differentially expressed genes in leaflet tissues than would be expected based on the proportion of these classes on the array. The most
obviously altered gene group in the leaflet data was a ploidy-upregulation of ribosomal protein genes (Supplemental Figure 3; Supplemental Table 1); a careful examination of the leaflet data further revealed a collective ploidy-upregulation of histone encoding genes (Supplemental Table 1).

Northern blot hybridization and real-time PCR were used to validate the leaflet microarray data for a subset of genes (Figure 8). Although it is difficult to validate small changes in expression levels, the real-time PCR data and Northern blot hybridizations confirmed the general trends observed in the microarray data. For instance, the microarray data indicated that the cyclin B-type gene (GenBank accessions BQ115975- BQ115976) was ploidy-upregulated and the expansin precursor gene (BQ515454- BQ515455) was ploidy-downregulated. These results were supported by both Northern blot and real-time PCR analyses (Figure 8).

Expression differences between individual ploidy levels
The distributions in Figure 6 suggest that many genes may have expression differences between the 1x and 2x genotypes, but not as many genes have differences between the 2x and 4x genotypes. Pair-wise contrasts were performed to estimate the expression differences between individual ploidy levels in both leaflet and root tip datasets (see Materials and Methods). The distributions of p-values for these contrasts are shown in Figure 9. The 1x-2x contrast distributions skewed towards significant p-values, indicating that more genes exhibited differential expression between the 1x and 2x ploidies in both tissue types (Figures 9A and 9D). The 2x-4x contrast distributions also skewed towards significant values, however the effect was less dramatic than in the 1x-2x contrasts (Figures 9B and 9E). These data indicate that gene
expression in leaflets and root tips was more affected by a change from 1x to 2x than from 2x to 4x. Importantly, the distributions of p-values from the 2xR3-2xR5 contrasts were relatively flat in both tissue types (Figures 9C and 9F). The flat distributions indicate that the 2xR3 and 2xR5 genotypes do not generate major differences in gene expression. Therefore, technical factors, such as the leaf-disk regeneration procedure or technical variation in the microarrays, did not likely contribute significantly to the changes observed in the 1x-2x and 2x-4x contrasts.

The p-value distributions were further used to better define the expression patterns among all ploidies (see Methods for statistical details). The significant genes from ANOVA were each classified according to one of nine possible patterns based on pair-wise contrasts (Table 1). Except for the 1x<2x>4x and 1x≈2x≈4x categories, the frequency of each expression pattern was remarkably similar for the leaflet and root tip datasets (Table 1). The majority of the differentially expressed genes in both tissue types (~60% from the leaflet dataset and ~56% from the root tip dataset) displayed significant differences only between 1x and 2x genotypes (1x<2x≤4x and 1x>2x≥4x patterns). Respectively, only ~12% and ~8% of the differentially expressed genes displayed either completely ploidy-upregulated (1x<2x<4x) or ploidy-downregulated (1x>2x>4x) patterns. None of the ploidy-upregulated genes (1x<2x<4x) displayed matching patterns across tissue types. Furthermore, only one ploidy-downregulated gene (1x>2x>4x) displayed matching patterns in both leaflets and root tips; this gene (BQ504923- BQ504924) has high sequence similarity to the rice gibberellin receptor GIB1 (UEGUCHI-TANAKA et al. 2005). This gene displayed only a modest decrease across ploidies, approximately 1.5 fold.
DISCUSSION

We observed an interesting relationship between overall plant vigor and cell size in the O37 series plants. The 2x plants were most vigorous and generated the greatest biomass, such that 1x<2x<4x. However, microscopic analyses clearly showed that cell and nuclear sizes were correlated with ploidy, such that 1x<2x<4x. Based on the phenotypic observations, we were interested in identifying O37 gene expression profiles that matched either cell-size-like ploidy-dependent patterns (1x<2x<4x or 1x>2x>4x) or vigor-like ploidy-nonlinear patterns (1x<2x>4x or 1x>2x<4x). However, expression profiles with these patterns only occurred in a few genes. Both leaflet and root tip tissues generated numerous expression changes between the 1x and 2x ploidies, but significant changes between 2x and 4x ploidies were far less frequent. Consequently, the majority of differentially expressed genes exhibited patterns that were significantly different between the 1x and 2x levels, but not statistically different at the 2x and 4x levels (1x<2x<4x or 1x>2x<4x) (Table 1). The global microarray patterns were consistent with the fact that the 1x plants were most phenotypically different from the 2x and 4x plants. Although phenotypic differences can be observed between the 2x and 4x plants, both developed much larger and more vigorous tissues than the 1x individuals.

Phenotypic superiority of diploids

Natural autopolyploids and autopolyploids developed for agricultural purposes are often more vigorous and larger in size than their diploid relatives, indicating a fitness advantage associated with higher ploidy. However, this does not seem to be true for synthetic autopolyploids. A recent study in yeast *S. cerevisiae* observed this phenomenon in an evolutionary context when isogenic 1x, 2x and 4x yeast strains all converged towards diploid DNA levels over the course of
1,766 mitotic cell divisions (GERSTEIN et al. 2006). Further experimental evidence indicated that selection on genome size drove this convergence (GERSTEIN et al. 2006). The exact mechanism of diploid superiority was unclear, however the authors speculated that selection over evolutionary time has optimized organismal function at the diploid level, the historical ploidy state for this species (GERSTEIN et al. 2006). Another recent study in yeast found that ploidy differences had little effect on gene expression, but important molecular phenotypic differences were still observed (STORCHOVA et al. 2006). The authors identified several yeast genes with ‘ploidy-specific lethality’. Deletion of such genes is not lethal in haploid or diploid yeast strains, but causes lethality in triploids or tetraploids (LIN et al. 2001; STORCHOVA et al. 2006). Most of the ploidy-specific lethality genes are associated with genomic stability, including homologous recombination, sister chromatid cohesion, and mitotic spindle function (STORCHOVA et al. 2006). Interestingly, the authors observed that tetraploid yeast appeared to be more susceptible to defects associated with the function of some of these genes. However, these genes displayed similar transcriptional and protein levels between wild-type diploids and tetraploids (STORCHOVA et al. 2006).

The phenotypic superiority of diploids has also been previously reported in synthetic autopolyploidy series of plants (RIDDLE et al. 2006; UIJTEWAAL et al. 1987). The results in our study are consistent with these findings, as 2x plants were more vigorous than 1x or 4x isogenic strains. Additionally, our flow cytometry data indicated that the 1x plants were showing greater levels of endoreduplication or spontaneous chromosome doubling than the 2x or 4x plants, perhaps compensating for a suboptimal ploidy by increasing the proportion of 2x cells. It is possible that some of the differential expression patterns observed in the 1x plants may be related to these increased levels of 2x cells.
It is curious, however, that the 2x and 4x plants have similar expression patterns, yet the 2x plant appears phenotypically superior. Higher ploidy plants may experience fitness costs as a consequence of elevated DNA content and replication, increased cell sizes, or mitotic difficulties (COMAI 2005), but these fitness costs may not be associated with large changes in gene expression. However, autopolyploid species may experience fitness advantages if they are able to exploit the increased potential for heterozygosity that polyploidy affords (COMAI 2005). This may help explain why autopolyploid plants tend to be outcrossing species, as this mode of reproduction may accelerate the accumulation of heterozygous loci following neoautopolyploid formation.

**Ploidy-related gene expression phenomena**

Higher ploidy cells are larger and would be expected to have a higher level of transcription than lower ploidy cells. Therefore, the gross expression of most genes would be expected to increase linearly with ploidy on a "per cell" basis. However, the methodologies used in this study correct for the differences in overall transcript abundances among ploidies. The genes identified as differentially expressed in this study represent genes in which expression abundance changes "per transcriptome" among ploidies. These genes also likely deviate from the "per cell" linear trend described above.

Some classes of genes identified as differentially expressed in the leaflet portion of our study encode critical components of cellular infrastructure, namely ribosomes and histones. We speculate that these genes were upregulated in the leaflets of higher ploidy plants as a consequence of larger cell sizes and/or changes in cell division rates. These gene families were not found to be consistently upregulated in the undifferentiated root tip tissues of higher ploidy
plants. We speculate that the differing physiological conditions and differing degree of cellular differentiation and endoreduplication of the leaflet and root tip tissues caused different sets of genes to respond to alterations in ploidy in each tissue.

However, considering the broad range of phenotypes observed in the O37 series, perhaps the biggest surprise of this study was that relatively few genes showed dramatic expression level changes among the different ploidies. In a previous microarray-based study of an autopolyploid series in yeast (*Saccharomyces cerevisiae*, 1x, 2x, 3x, and 4x) a total of 17 “ploidy-regulated” genes were reported, including some genes exhibiting greater than 10-fold expression differences among different ploidies (GALITSKI et al. 1999). Some of these genes were believed to control ploidy-dependent cell type differences (GALITSKI et al. 1999). We did not find such “ploidy-regulated” potato genes. The significant genes in our study showed much smaller changes than those reported in yeast, and genes with expression levels directly or inversely proportional to ploidy were rare. Instead, we found that approximately 10% of the ~9,000 potato genes exhibit low levels of expression changes after ploidy alteration. Perhaps a more extensive potato microarray platform will be able to detect genes with greater expression changes than those identified in this study.

The altered monoploid expression patterns observed in our study are consistent with the odd-ploidy effect phenomenon first observed by Guo et al. (1996) in an autopolyplloid maize series (1x, 2x, 3x, and 4x). Guo et al. (1996) measured the transcription level of 18 genes using Northern blot hybridization. Expression of most genes increased with ploidy, but some showed an inverse relationship to ploidy (GUO et al. 1996). The expression level changes for most genes were within a 2-fold range. However, some genes showed significantly greater expression level changes in monoploid and triploid plants. For example, the sucrose synthase (*Sus 1*) gene
showed similar expression levels in diploid and tetraploid plants. However, compared to the
diploid, Sus 1 expression was 2.9- and 6.7-fold higher in monoploid and triploid plants,
respectively. Several other maize genes showed similar but less severe expression level changes
in monoploid and/or triploid plants (Guo et al. 1996). In our study, we observed a greater
divergence of global gene expression in the monoploid. However, we do not have access to an
isogenic 3x individual to confirm these observations as odd-ploidy effects.

Questions remain about the differences in gene expression regulation following
autopolyploid and allopolyploid formation. Genetic and epigenetic changes associated with
newly synthesized allopolyploids have been well documented in a number of recent studies
(Adams et al. 2004; Albertin et al. 2006; Comai et al. 2000; He et al. 2003; Kashkush et al.
2002; Lukens et al. 2006; Madlung et al. 2002; Wang et al. 2004). Qualitative gene
expression changes, such as complete gene silencing and derepression, have been commonly
observed in allopolyploidy systems. In contrast, the results from the present study as well as
several previous investigations indicate that newly synthesized autopolyploids undergo much
less gene expression changes (Albertin et al. 2005; Wang et al. 2006). A recent microarray-
based study on both allopolyploids and autopolyploids derived from A. thaliana clearly
demonstrated that newly synthesized allopolyploids experience far greater global gene
expression changes than do newly synthesized autopolyploids (Wang et al. 2006). Gene
expression changes with gene-silencing phenomena in particular are more prevalent in
allopolyploids, as homoeologous alleles may complement silenced orthologs. Additionally, such
changes are potentially advantageous in allopolyploids as it may allow for the rapid sub-
functionalization of homoeologous alleles (Adams et al. 2003). In autopolyploids, dramatically
altering the expression of any allele would be more likely to affect the organism, as there is no potential for homoeologous complementation or advantageous sub-functionalization.

The genetic and epigenetic instability associated with newly synthesized allopolyploids can be attributed to many potential factors, including dosage-regulated gene expression, nucleocytoplasmic interactions, homoeologous recombination, and other downstream factors associated with the merger of two subgenomes. Expression of the homoeologous genes in the new allopolyploids may be regulated by diverged regulatory elements associated with different subgenomes. Reconciling diverged regulatory pathways may play a significant role in driving the genetic and epigenetic changes observed in newly synthesized allopolyploids (Chen and Nl 2006; Comai 2005; Osborn et al. 2003). Autopolyploids derive all of their alleles from within a single species and therefore may experience fewer alterations in their regulatory networks, resulting in fewer gene expression changes in newly synthesized autopolyploids.

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Table 1. Patterns of gene expression in differentially expressed genes

<table>
<thead>
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<th>Pattern</th>
<th>Genes from leaflet</th>
<th>Genes from root tips</th>
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<td>5.9%</td>
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<td>3.6%</td>
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<td>13.3%</td>
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</tr>
<tr>
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<td>23.9%</td>
</tr>
<tr>
<td>1x≈2x≈4x</td>
<td>2.5%</td>
<td>1.6%</td>
</tr>
</tbody>
</table>

1Based on 1x-2x and 2x-4x pair-wise contrasts. < and > indicate that the contrast was significant at p < 0.05; the directionality was inferred by the M-value differences between ploidies. ≈ indicates that the pair-wise contrast was not significant at p < 0.05.

2Indicates the percentage of genes that fit each pattern from the 948 genes identified as significant in the MAANOVA test (FDR Q < 0.10).

3Indicates the percentage of genes that fit each pattern from the 955 genes identified as significant in the MAANOVA test (FDR Q < 0.10).
Figure Legends

**Figure 1.** Confirmation of the euploid chromosome number of the O37 series plants.

Chromosomes were stained with 4’,6-diamidino-2-phenylindole and pseudo-colored in red. (A) Monoploid (2n=1x=12). (B) Diploid 2XR3 (2n=2x=24). (C) Diploid 2XR5 (2n=2x=24). (D) Tetraploid (2n=4x=48). Bars = 5 µm.

**Figure 2.** Plant phenotypes of the O37 series at 20 days after planting.

**Figure 3.** Transmission electron microscopy images of leaflet cross-sections of O37 plants. (A) 1x. (B) 2xR3. (C) 2xR5. (D) 4x. Cell sizes generally increased with ploidy. Bars = 50 µm.

**Figure 4.** Flow cytometric analyses of the O37 series plants. The peaks were derived from leaflet nuclei samples for each genotype. The peak from the soybean reference is labeled “Soybean” in each graph. Note: the scales of the graphs were modified and the 8x peak is shown only for the 4x plant nuclei (no 8x peak was observed in the 1x and 2x samples).

**Figure 5.** Experimental design of ploidy microarray comparisons, independently used for both the leaflet (biological replicates 1-3) and root tip (biological replicates 1-2) microarray analysis. The arrows indicate the dye assignments on each slide; the arrow ends indicate Cy3 and the blunt ends indicate Cy5.

**Figure 6.** Plot of M-values for 2x-1x and 4x-2x differences in leaflets (A) and root tips (B). Values for only the 948 and 955 respective genes that displayed significant expression changes.
across the genotypes were plotted (ANOVA Q < 0.10). The y-axis plots the 2x-1x log₂ fold-change and the x-axis plots the 4x-2x log₂ fold-change. Genes that plot into the upper-right quadrant exhibit expression changes in a ploidy-upregulated trend. Genes in the lower-left quadrant exhibit a ploidy-downregulated trend. Genes in the upper-left and lower-right quadrant exhibit 2x-upregulated and 2x-downregulated trends, respectively.

**Figure 7.** Distribution of differentially expressed genes among gene ontologies. (A) The distribution of molecular function gene ontologies of all genes on the microarray (blue bars) is compared with the distribution of ontologies from the differentially expressed genes from leaflet (green bars) and root tip (yellow bars) tissues. The GO terms are ordered on the graph from highest frequency on the array (left) to lowest frequency on the array (right). GO terms “nucleic acid binding” and “structural molecule activity” show enrichment in the number of differentially expressed genes in the leaflet tissue. (B) The distribution of structural component gene ontologies on the microarray is compared with the distribution of ontologies from the differentially expressed gene lists, as in A. Gene ontology terms “cytosol” and “ribosome” show significant enrichment in the number of differentially expressed genes in the leaflet tissue.

**Figure 8.** Northern blot hybridization and real-time PCR validation of leaflet microarray data. (A) Northern blot validation of the microarray results for a cyclin-B type EST (BQ115975-BQ115976) and an expansin precursor EST (BQ515454-BQ515455). (B) Real-time PCR validation of leaflet microarray data for 11 genes. Real-time PCR data fold-change (the error bars represent the standard deviation across the three biological replicates). The 11 genes are each presented as a cluster of three, representing (from left to right) the 1x expression level, 2x
expression level, and 4x expression level. The genes are further grouped according to putative annotation. The first four genes are ribosomal protein genes (GenBank accession numbers BQ518779-BQ518780, BQ115191-BQ115192, BQ112187-BQ112188, BQ515383-BQ515384), the next four genes encode histones (BQ121261-BQ121262, BQ505673, BQ514906-BQ514907, BQ516716-BQ516717), the next gene encodes a cyclin B-type (BQ115975-BQ115976), and the final two genes are ploidy-downregulated (BQ114667- BQ114668: systemic acquired resistance-related protein SRE1a; BQ515454- BQ515455: expansin precursor). Microarray expression data and specific annotations for each gene are given in Supplemental Table 1.

**Figure 9.** P-value distribution for leaflet and root tip microarray pair-wise contrasts. (A) Leaflet contrast 2x-1x. (B) Leaflet contrast 4x-2x. (C) Leaflet contrast 2xR3-2xR5. (D) Root tip contrast 2x-1x. (E) Root tip contrast 4x-2x. (F) Root tip contrast 2xR3-2xR5.