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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 (1*x*) clone, two diploid (2*x*) clones, and one tetraploid (4*x*) clone. Cell size and organ thickness were positively correlated with the ploidy level. However, the 2*x* plants were generally the most vigorous and the 1*x* plants exhibited less vigor compared to the 2*x* and 4*x* individuals. We analyzed the transcriptomic variation associated with this autopolyploid series using a potato cDNA microarray containing ∼9000 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 twofold 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.

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 (Gaut and Doebley 1997; Blanc and Wolfe 2004b; Paterson *et al.* 2004; Yu *et al.* 2005; Cui *et al.* 2006), 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 subfunctionalization, have been widely discussed (Leitch and Bennett 1997; Wendel 2000; Adams *et al.* 2003; Carputo *et al.* 2003; Osborn *et al.* 2003; Blanc and Wolfe 2004a; Soltis *et al.* 2004; Adams and Wendel 2005; Comai 2005; Chen and Ni 2006; Udall and Wendel 2006).

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

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(Song *et al.* 1995; Liu *et al.* 1998; Comai *et al.* 2000; Ozkan *et al.* 2001; Kashkush *et al.* 2002; Madlung *et al.* 2002, 2005; He *et al.* 2003; Kashkush *et al.* 2003; Adams *et al.* 2004; Wang *et al.* 2004, 2006; Skalicka *et al.* 2005; Albertin *et al.* 2006; Lukens *et al.* 2006). 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 (Guo *et al.* 1996; Galitski *et al.* 1999; Albertin *et al.* 2005; Auger *et al.* 2005; 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 on the basis of its tetrasomic inheritance (Grant 1981; Carputo *et al.* 2003). Genetic manipulation via meiotic mutants associated with $2n$ gamete formation has played a more significant role in breeding of potato than in any other crops (Peloquin *et al.* 1989, 1999). 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, *S. 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 affect 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 ($\frac{1}{2}$ *S. stenotomum*, $\frac{3}{16}$ *S. phureja*, $\frac{3}{16}$ *S. tuberosum*, $\frac{1}{8}$ *S. chacoense*). An F_1 selection derived from this cross was backcrossed to its DM parent. We selected a backcross seedling on the basis of 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 $2x$R3 and $2x$R5, respectively. Therefore, the O37 $1x$, $2x$R3, $2x$R5, 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 Biotron facility. Plants were grown under a 15/9-hr-day/night cycle with a 23.5/15.5° day/night temperature regime. Relative humidity was held constant at 50%. Following shoot emergence, plants were given watering treatments with 0.5× strength Hoagland solution twice daily. Terminal leaflet length was measured repeatedly for 17 days starting 20 days after planting. One leaflet from three different plants was measured for each of the O37 $1x$, $2x$R3, $2x$R5, 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 (Szymanski and Marks 1998; Downes *et al.* 2003). 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 predetermined 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 followed published methods (Kang *et al.* 2001). All material was embedded in Spurr's resin and polymerized at 70°. 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 fluorescence *in situ* hybridization (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 10 expanding terminal leaflets of each O37 plant genotype were pooled for nuclei isolation; 1*x* leaflets were collected at 10–20 mm in length and 2*x*R3, 2xR5, and 4*x* 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 1*x* plants; 15–25 mm in length for 2*x*R3, 2*x*R5, and 4*x* plants) were flash frozen in liquid nitrogen 7 hr 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, Carlsbad, CA), with the following modifications. The aqueous TRIzol:chloroform extractions were precipitated with 0.5 vol isopropanol, 0.5 vol 1.2 m sodium chloride, and 0.8 m sodium citrate solution. Purified RNA samples were treated with 2.5 units DNase I (Ambion, Austin, TX) and incubated at 37° for 15 min, and then extracted with phenol:chloroform. Purified RNA samples were quantified and qualified using the Nanodrop spectrophotometer with version 2.5.3 software (Nanodrop Technologies, Montchanin, DE) and agarose gel electrophoresis analyses, respectively.

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). Roots were stored in RNALater reagent for up to 7 days at 4°. Opaque root tip meristematic regions were subsequently harvested with a fine forceps for RNA extraction. The Plant Mini RNeasy kit (QIAGEN, 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 vol ethanol, 0.1× sodium acetate, and 1 μl pellet paint NF coprecipitate (EMD Biosciences, San Diego). Purified RNA samples were quantified using the Nanodrop spectrophotometer with version 2.5.3 software.

Microarray hybridization and data analysis: The 10,000 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 that are termed "STM" for ***S. t****uberosum* **m**icroarray. 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 9029 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) 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 data sets 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 at http://www.genetics.org/supplemental/ 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 $\log_2$ fold changes. For example, gene X may have a 2*x*–1*x* *M*-value difference of 2; this indicates that gene X experiences a fourfold increase in expression in the 2*x* plant relative to the 1*x* plant. The *M*-value differences between the 2*x*–1*x* and 4*x*–2*x* 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: 1*x*–2*x*; 1*x*–4*x*; 2*x*–4*x*; 1*x*–2*x*R3; 1*x*–2*x*R5; 2*x*R3–4*x*; 2*x*R5–4*x*; 2*x*R3–2*x*R5. Each of the significant genes identified in the *F*-test was further defined by its 1*x*–2*x* and 2*x*–4*x* contrast *P*-values. Contrasts were determined to be significant at $P < 0.05$. The directionality of the difference was inferred on the basis of 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 ($1x > 2x > 4x$), 2*x* upregulated ($1x < 2x > 4x$), 2*x* downregulated ($1x > 2x < 4x$), 4*x* over 2*x* ($1x \approx 2x < 4x$), 2*x* over 4*x* ($1x \approx 2x > 4x$), 2*x* over 1*x* ($1x < 2x \approx 4x$), 1*x* over 2*x* ($1x > 2x \approx 4x$), and no pairwise difference ($1x \approx 2x \approx 4x$).

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 *S. 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 Figure 7, A and B, 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). All primers were designed on the basis of 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 were performed for the standard primer pairs for each biological replicate; two to three 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 (http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE5428).

RESULTS

Polyploid series O37 includes molecularly monomorphic 1*x*, 2*x*, and 4*x* 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 2*x*R3 and 2*x*R5, 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 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 at http://www.genetics.org/supplemental/) 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.

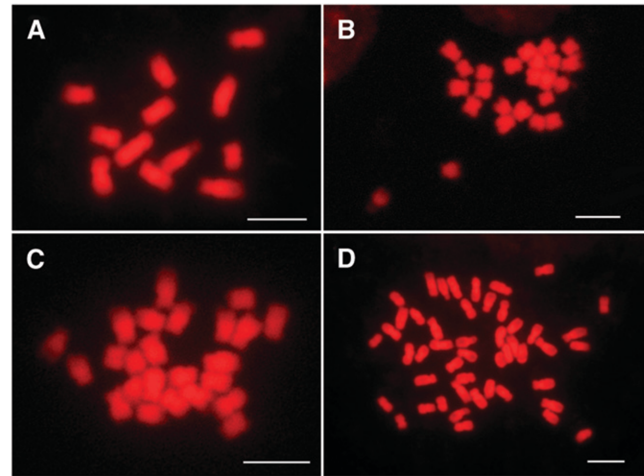

Figure 1.—Confirmation of the euploid chromosome number of the O37 series plants. Chromosomes were stained with 4′,6-diamidino-2-phenylindole and pseudo-colored in red. (A) Monoploid ($2n = 1x = 12$). (B) Diploid 2*x*R3 ($2n = 2x = 24$). (C) Diploid 2*x*R5 ($2n = 2x = 24$). (D) Tetraploid ($2n = 4x = 48$). Bars = 5 μm.

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 2*x*R3 and 2*x*R5 plants were more vigorous than the 1*x* and 4*x* plants and generated the greatest overall biomass; furthermore, the 4*x* plants were more vigorous than the 1*x* 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 2*x*R3 and 2*x*R5 plants, indicating that the tissue culture regeneration procedure had minimal effect on the plant phenotypes.

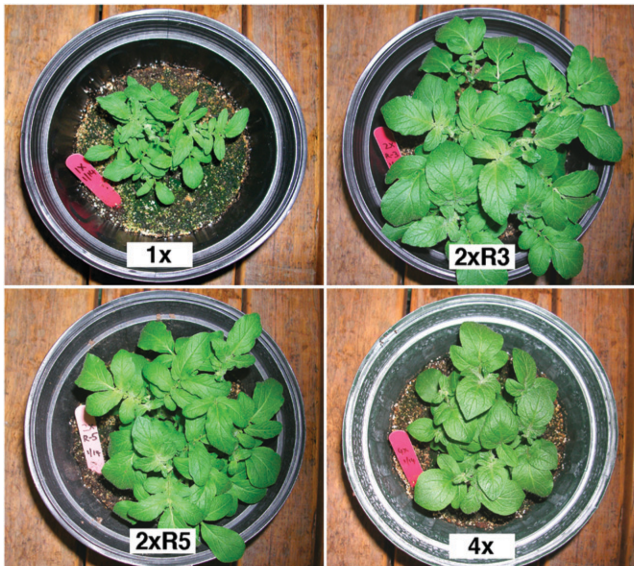

FIGURE 2.—Plant phenotypes of the O37 series at 20 days after planting.

Growth measurements were performed on the terminal leaflets of the growth chamber-grown O37 series plants. The 2*x* and 4*x* terminal leaflets displayed more rapid growth than the 1*x* terminal leaflets, which is consistent with the differences in overall plant vigor. The 1*x* terminal leaflets 10–20 mm in length were developmentally comparable to 2*x* and 4*x* 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 exhibit 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 1*x*, 2*x*, and 4*x* 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 G_2 stage of the cell cycle. The 2*x* peak of the 1*x* plant was relatively larger than the 4*x* peak of the 2*x* plants and the 8*x* peak of the 4*x* plant (Figure 4), suggesting that the 1*x* plant may have a greater tendency toward endoreduplication or spontaneous chromosome doubling. Additionally, the 1*x* plant shows a small peak at 4*x*; however, no analogous 8*x* peak was observed in the 2*x* 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 1*x*, 2*x*R3, 2*x*R5, and 4*x* 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, and 3) and two biological replicates for root tip (replications 1 and 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 at http://www.genetics.org/supplemental/). Approximately 10.5% of the genes (948 of the 9029 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 9029) 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 $\log_2$ *M*-value differences between 2*x*–1*x* and 4*x*–2x ploidies, respectively (the 2*x* category represents the average value between the 2*x*R3 and

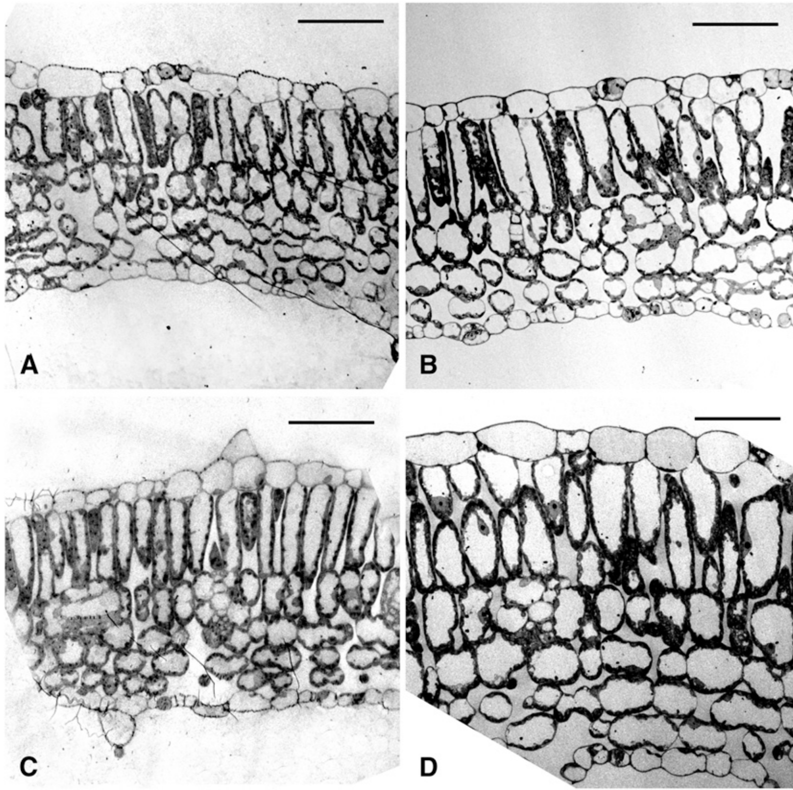

Figure 3.—Transmission electron microscopy images of leaflet cross sections of O37 plants. (A) 1*x*. (B) 2*x*R3. (C) 2*x*R5. (D) 4*x*. Cell sizes generally increased with ploidy. Bars, 50 μm.

2*x*R5 plants). Thus, a value of 1 along either axis in this plot represents a twofold change and a value of 2 represents a fourfold change between the ploidies plotted. The majority of differentially expressed genes exhibited less than twofold changes in either the 2*x*–1*x* or the 4*x*–2*x* ploidy comparisons for both tissue types (Figure 6).

The leaflet and root tip data sets 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 × 10.6 = 1.11%). We considered the possibility that the microarray may include numerous probes with tissue-specific expression, thus contributing to the noncorrespondence 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 nonsignificant gene groups from the leaflet and root tip

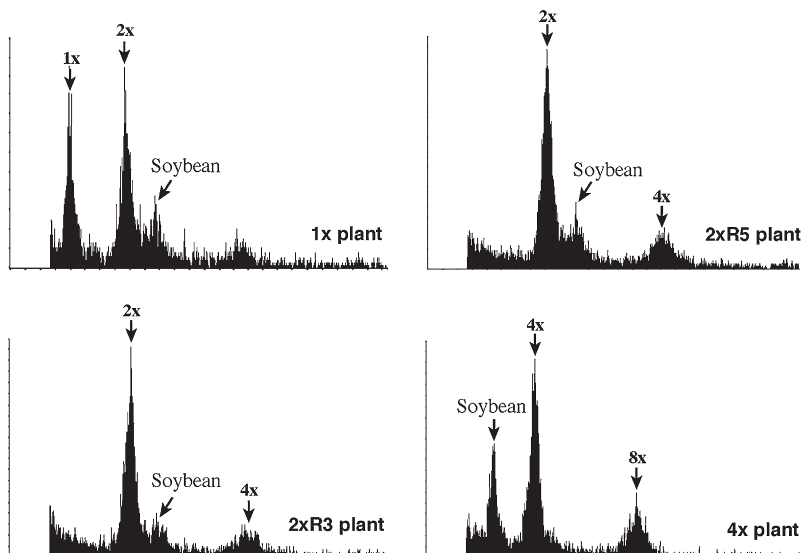

Figure 4.—Flow cytometric analyses of the O37 series plants. The peaks were derived from leaflet nuclei samples for each genotype. The peak from the soybean reference is labeled "Soybean" in each graph. Note that the scales of the graphs were modified and the 8*x* peak is shown only for the 4*x* plant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

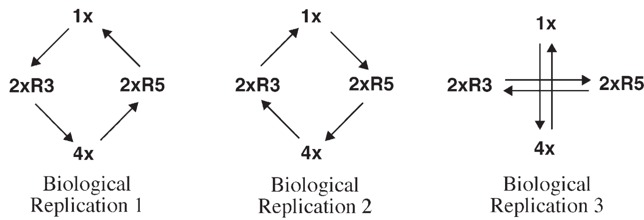

Figure 5.—Experimental design of ploidy microarray comparisons, independently used for both the leaflet (biological replicates 1–3) and root tip (biological replicates 1–2) microarray analysis. The arrows indicate the dye assignments on each slide; the arrow ends indicate Cy3 and the blunt ends indicate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

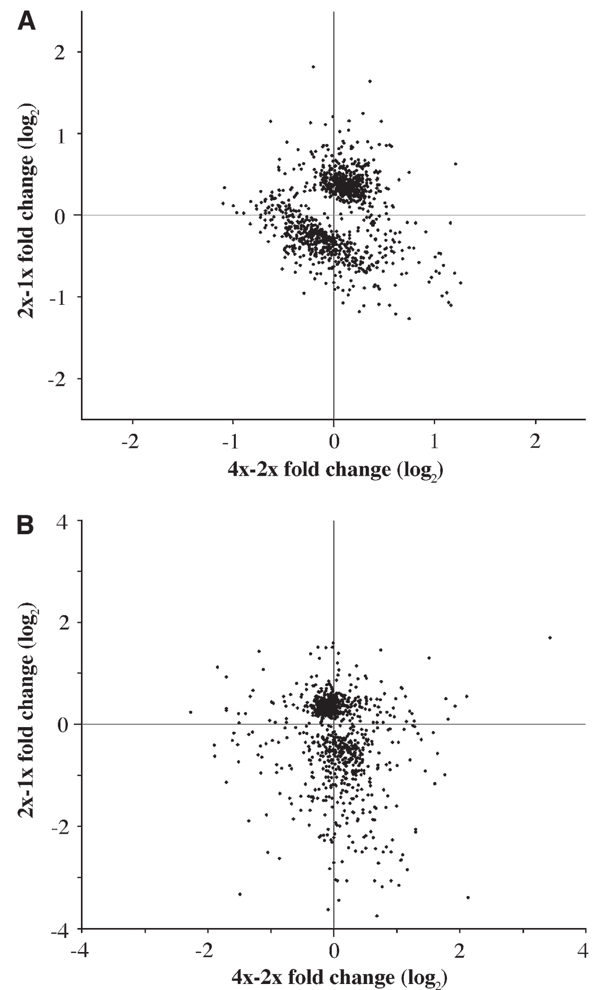

Figure 6.—Plot of *M*-values for $2x$–$1x$ and $4x$–$2x$ differences in leaflets (A) and root tips (B). Values for only the 948 and 955 respective genes that displayed significant expression changes across the genotypes were plotted (ANOVA $Q < 0.10$). The *y*-axis plots the $2x$–$1x$ $\log_2$ fold change and the *x*-axis plots the $4x$–$2x$ $\log_2$ fold change. Genes that plot into the top right quadrant exhibit expression changes in a ploidy-upregulated trend. Genes in the bottom left quadrant exhibit a ploidy-downregulated trend. Genes in the top left and bottom right quadrants exhibit $2x$-upregulated and $2x$-downregulated trends, respectively.

microarray data sets (supplemental Figure 2 at http://www.genetics.org/supplemental/). 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 nonsignificant 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 data set, 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 nonsignificant genes, in both leaflet and root tip tissues. Collectively, our data indicate that the noncorrespondence 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 data sets were compared with the gene ontologies for all 9029 genes on the array. In general, the ontology distribution of differentially expressed genes in the root tip data set reflected the ontology distribution of the array for both molecular function and structural component (Figure 7). The leaflet data set 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 on the basis of 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 at http://www.genetics.org/supplemental/); 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 accession 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).

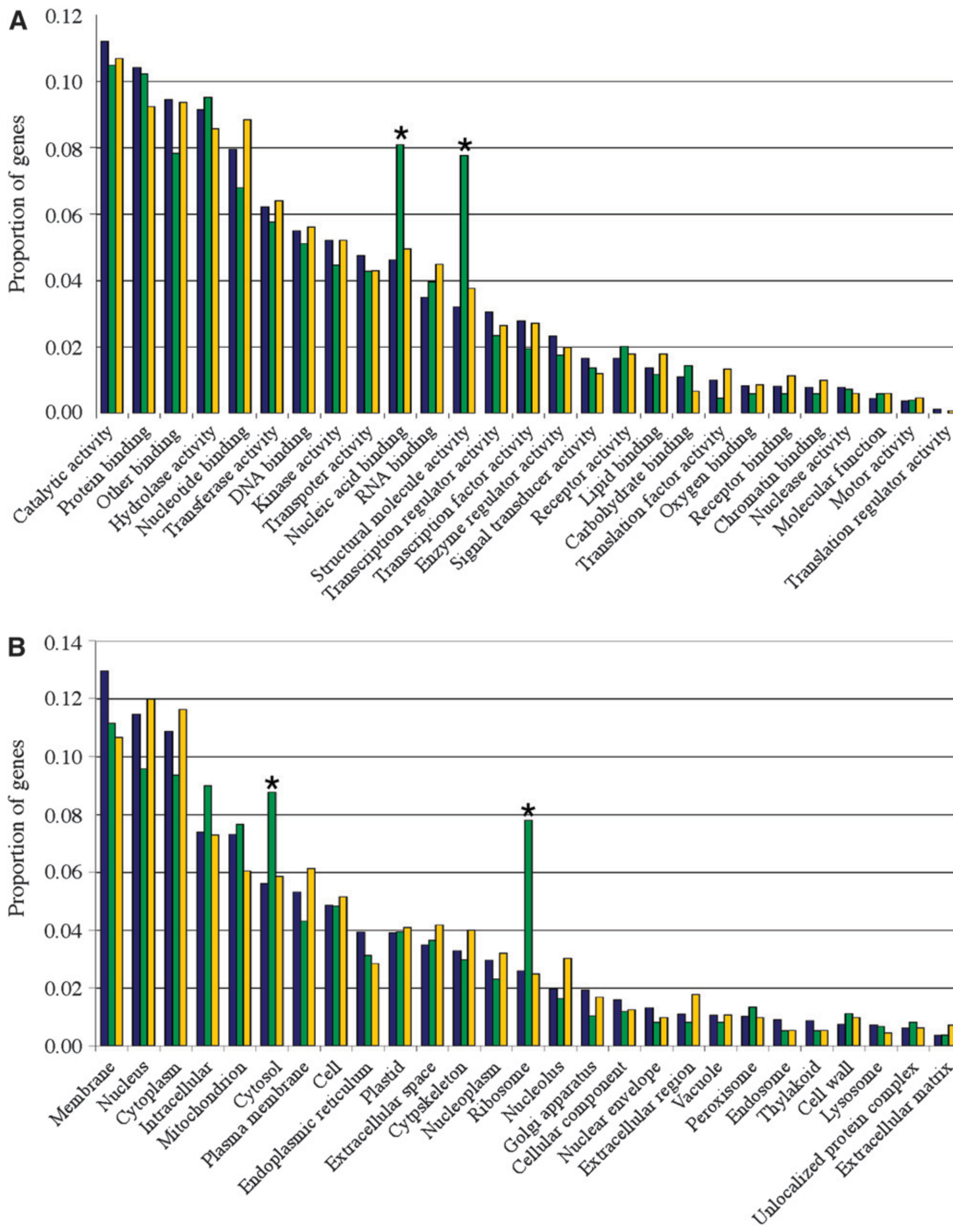

FIGURE 7.—Distribution of differentially expressed genes among gene ontologies. (A) The distribution of molecular function gene ontologies of all genes on the microarray (blue bars) is compared with the distribution of ontologies from the differentially expressed genes from leaflet (green bars) and root 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.

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. Pairwise contrasts were performed to estimate the expression differences between individual ploidy levels in both leaflet and root tip data sets (see MATERIALS AND METHODS). The distributions of *P*-values for these contrasts are shown in Figure 9. The $1x$–$2x$ contrast distributions skewed toward significant *P*-values, indicating that more genes exhibited differential expression between the $1x$ and $2x$ ploidies in both tissue types (Figure 9, A and D). The $2x$–$4x$ contrast distributions also skewed toward significant values; however, the effect was less dramatic than in the $1x$–$2x$ contrasts (Figure 9, B and E). 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 $2x$R3–$2x$R5 contrasts were relatively flat in both tissue types (Figure 9, C and F). The flat distributions indicate that the $2x$R3 and $2x$R5 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 MATERIALS AND METHODS for statistical details). The significant genes from ANOVA were each classified according to one of nine possible patterns based on pairwise contrasts (Table 1). Except for the $1x < 2x > 4x$ and $1x \approx 2x$

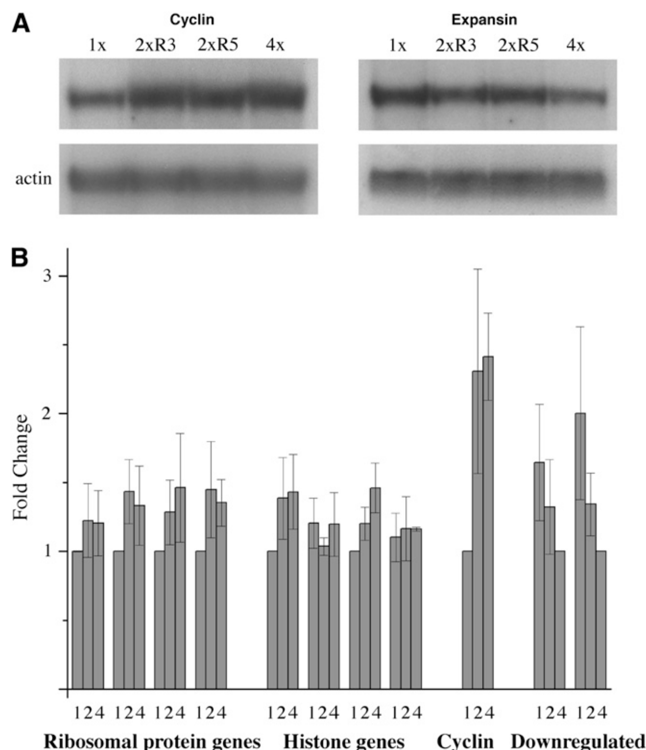

FIGURE 8.—Northern blot hybridization and real-time PCR validation of leaflet microarray data. (A) Northern blot validation of the microarray results for a cyclin-B type EST (BQ115975–BQ115976) and an expansin precursor EST (BQ515454–BQ515455). (B) Real-time PCR validation of leaflet microarray data for 11 genes (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 nos. 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 at http://www.genetics.org/supplemental/.

$> 4x$ categories, the frequency of each expression pattern was remarkably similar for the leaflet and root tip data sets (Table 1). The majority of the differentially expressed genes in both tissue types (~60% from the leaflet data set and ~56% from the root tip data set) displayed significant differences only between $1x$ and $2x$ genotypes ($1x < 2x \approx 4x$ and $1x > 2x \approx 4x$ 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, ~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 $1x < 2x < 4x$. On the basis of 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 occurred in only 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 ($1x < 2x \approx 4x$ or $1x > 2x \approx 4x$) (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 *Saccharomyces cerevisiae* observed this phenomenon in an evolutionary context when isogenic $1x$, $2x$, and $4x$ yeast strains all converged toward diploid DNA levels over the course of 1766 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

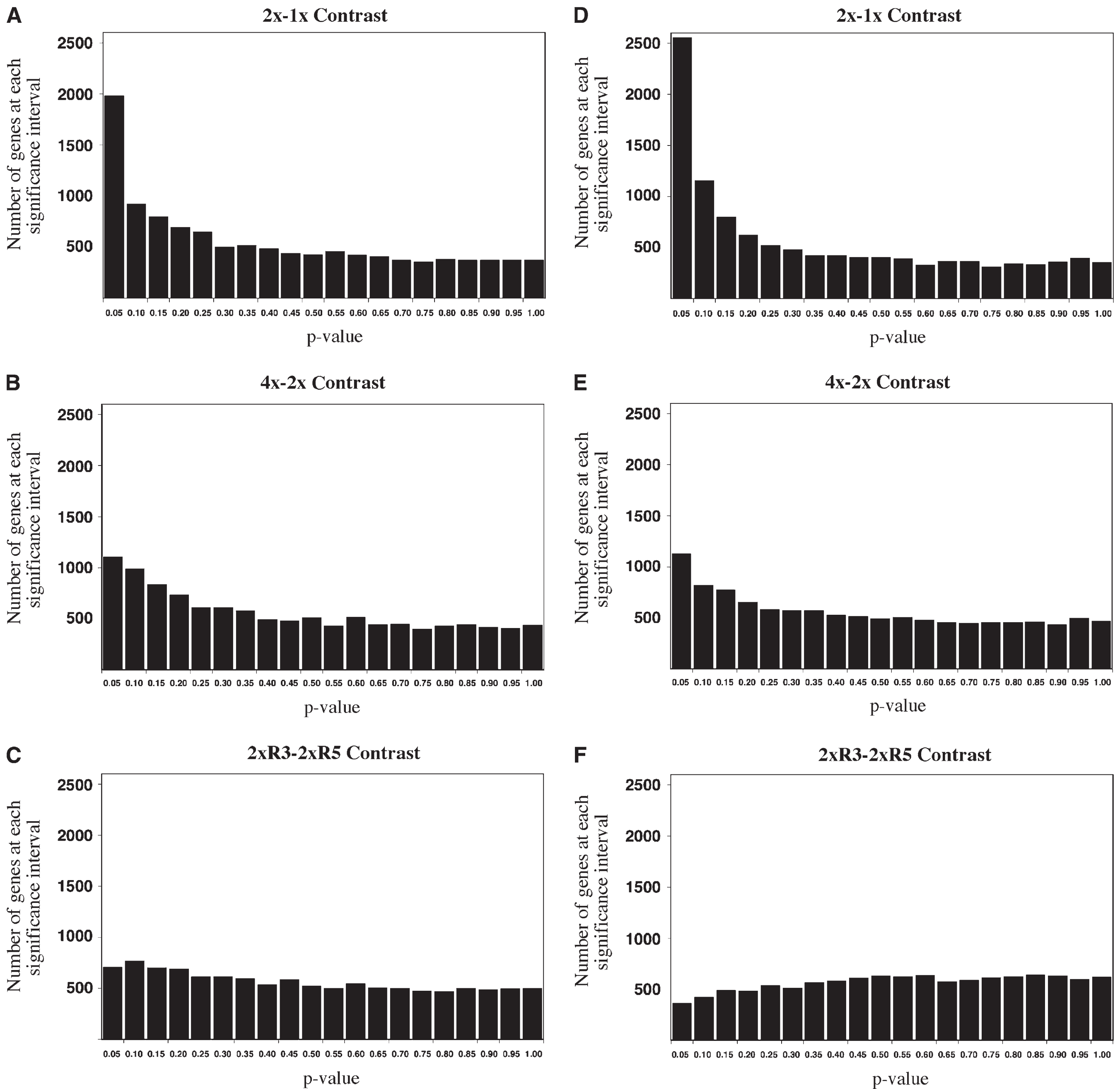

Figure 9.—*P*-value distribution for leaflet and root tip microarray pairwise contrasts. (A) Leaflet contrast 2*x*–1*x*. (B) Leaflet contrast 4*x*–2*x*. (C) Leaflet contrast 2*x*R3–2*x*R5. (D) Root tip contrast 2*x*–1*x*. (E) Root tip contrast 4*x*–2*x*. (F) Root tip contrast 2*x*R3–2*x*R5.

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 (Uijtewaal *et al.* 1987; Riddle *et al.* 2006). The results in our study are consistent with these findings, as 2*x* plants were more vigorous than 1*x* or 4*x* isogenic strains. Additionally, our flow cytometry data indicated that the 1*x* plants were showing greater levels of endoreduplication or spontaneous chromosome doubling

TABLE 1

Patterns of gene expression in differentially expressed genes

Pattern[a]	Genes from leaflet[b] (%)	Genes from root tips[c] (%)
$1x < 2x < 4x$	5.9	4.8
$1x > 2x > 4x$	6.1	3.6
$1x < 2x > 4x$	2.1	13.3
$1x > 2x < 4x$	6.4	10.6
$1x \approx 2x < 4x$	5.4	5.7
$1x \approx 2x > 4x$	12.2	4.5
$1x < 2x \approx 4x$	35.8	31.9
$1x > 2x \approx 4x$	23.7	23.9
$1x \approx 2x \approx 4x$	2.5	1.6

[a] Based on 1*x*–2*x* and 2*x*–4*x* pairwise contrasts. $<$ and $>$ indicate that the contrast was significant at $P < 0.05$; the directionality was inferred by the *M*-value differences between ploidies. $\approx$ indicates that the pairwise contrast was not significant at $P < 0.05$.

[b] The percentage of genes that fit each pattern from the 948 genes identified as significant in the MAANOVA test (FDR $Q < 0.10$).

[c] The percentage of genes that fit each pattern from the 955 genes identified as significant in the MAANOVA test (FDR $Q < 0.10$).

than the 2*x* or 4*x* plants, perhaps compensating for a suboptimal ploidy by increasing the proportion of 2*x* cells. It is possible that some of the differential expression patterns observed in the 1*x* plants may be related to these increased levels of 2*x* cells.

It is curious, however, that the 2*x* and 4*x* plants have similar expression patterns, yet the 2*x* 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 out crossing 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 autopolyploidy series in yeast (*S. cerevisiae*, 1*x*, 2*x*, 3*x*, and 4*x*) 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 ~10% of the ~9000 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 autopolyploid maize series (1*x*, 2*x*, 3*x*, and 4*x*). 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 twofold 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 (Comai *et al.* 2000; Kashkush *et al.* 2002; Madlung *et al.* 2002; He *et al.* 2003; Adams *et al.* 2004; Wang *et al.* 2004; Albertin *et al.* 2006; Lukens *et al.* 2006). 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 fewer 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 homeologous alleles may complement silenced orthologs. Additionally, such changes are potentially advantageous in allopolyploids as it may allow for the rapid subfunctionalization of homeologous 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 homeologous complementation or advantageous subfunctionalization.

The genetic and epigenetic instability associated with newly synthesized allopolyploids can be attributed to many potential factors, including dosage-regulated gene expression, nucleo-cytoplasmic interactions, homeologous recombination, and other downstream factors associated with the merger of two subgenomes. Expression of the homeologous 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 (Osborn *et al.* 2003; Comai 2005; Chen and Ni 2006). 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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