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BIOCHEMICAL CONSEQUENCES OF POLYPLOIDY IN <u>MEDICAGO</u> (ALFALFA)

Вy

Carol Marie Schumann

A THESIS

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Department of Horticulture

ABSTRACT

BIOCHEMICAL CONSEQUENCES OF POLYPLOIDY IN MEDICAGO (ALFALFA)

Вy

Carol Marie Schumann

Gene dosage compensations are thought to have occurred in many polyploid plant species. In this study, protein levels in mature autotetraploid alfalfa germplasm were compared with those in newly induced tetraploids and with their wild diploid progenitor species. Levels of total buffer soluble protein (BSP) and activities of the specific proteins malic dehydrogenase (MDH), peroxidase (PX), and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC/O), were determined. Expressing these data relative to the DNA content of the various genotypes revealed that BSP levels were lower in mature tetraploids than in their diploid although levels of MDH and PX were not progenitors. significantly different. Recently induced tetraploids were not different from their progenitors. RuBPC/O activity was tightly correlated with BSP, suggesting that variation in BSP was largely due to variation in levels of this enzyme.

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INTRODUCTION

Polyploidy, a balanced increase in the entire haploid genome, is widely considered to have been a major force in plant evolution. Thirty to sixty percent of all higher plant species are believed to be of polyploid origin, and are thought to occur primarily via the fusion of unreduced gametes (Harlan and DeWet, 1975).

Despite the success of polyploids over the course of evolution, newly arisen polyploids are often poorly adapted to the environment and/or partially sterile due to meiotic irregularities (Stebbins, 1971). Physiologic and morphologic imbalances are also common. Cell volume approximately doubles with an increase from diploidy to tetraploidy, yet .nuclear and cell surface areas increase only by about sixty percent (Levin, 1983). Since messenger, ribosomal, and transfer RNAs must pass through pores in the nuclear membrane to reach the cytoplasm, the balance between nuclear membrane surface area and cell volume may be important in determining rates of protein synthesis (Cavalier-Smith. 1978). Processes mediated by membrane or cell wall bound enzymes are also disturbed (Albersheim, 1976; Weiss et. al., 1975). Such alterations in cell geometry could easily have a deleterious effect on the physiology of the plant.

There are two major types of polyploids. Allopolyploids are the products of unreduced gametes in inter-specific hybridizations, and autopolyploids are the products of unreduced gametes in intra-specific

hybridization. The most universal effect of polyploidy is an increase in cell size, which often translates to an increase in leaf and plant size (Stebbins, 1971). Other effects include alterations in enzyme activities and photosynthetic rates, and an increase in environmental stability. Polyploids are thought to have greater homeostasis than their related diploids because of higher levels of heterozygosity due to gene duplication (Gottlieb, 1982). Heterozygosity may persist in autopolyploids because inheritance, and may become of tetrasomic fixed in allopolyploids because of a preferential pairing of homologues from the same parental genome.

The presence of duplicate genes may release one of the genes from selective pressure, allowing it to accumulate mutations and evolve randomly (Ohno, 1970). If such genetic drift leads to an enzyme product having altered kinetic properties (i.e. different Km, temperature optima, or cofactor specificity), then the organism having that unique gene product might have an increased range of environmental conditions under which a given catalysis could occur. The presence of additional alleles at many loci could increase the homeostasis of the organism, thereby conferring an adaptive advantage.

Not all the duplicate genes in a polyploid, however, will diverge into something of functional and/or adaptive significance. When a gene is released from selective pressure and allowed to drift, it may accumulate mutations which make it no longer functional. Such mutations may

alter the catalytic site(s) of an enzyme rendering it inactive, or may alter the regulatory region of the gene inhibiting transcription. The activity of a duplicated gene may be rapidly lost if no physiologic advantage is conferred by higher levels of a given gene product. These events are termed dosage compensations. As compensations occur over the course of evolutionary time, the ancient polyploid will behave more like a diploid. Such diploidization may have been adaptive as a polyploid evolved in the wild, under resource limited conditions. Individuals maintaining the high metabolic load caused by an excess number of active genes may have been at a disadvantage, and compensation might have been favored by selection.

Evidence of dosage compensation has been sought in a number of polyploid plant species, with varying results. Cukrova et al (1968) found lower photosynthetic rates in derived autotetraploid Datura stramonium than in the diploid type. Timco et al (1980) compared autotetraploid Ricinus communis that was derived from a diploid, and found it to be higher in esterase (EST) activity but equal in total protein. Guern and Herve (1980) compared aspartate transcarbamylase (ATC) activities in natural diploid. tetraploid, and hexaploid Hippocrepis comosa and found no differences. Roose and Gottlieb (1980) compared the recently formed allotetraploid Tragopogon miscellus to one of its diploid progenitors, and found no difference in levels of alcohol dehydrogenase (ADH) activity. Bazzaz et al (1982) compared photosynthetic rates in natural diploid

and autotetraploid <u>Phlox</u> <u>drummondii</u>. Tetraploids had lower rates than diploids among the cultivated types, but rates were equal among wild accessions.

Albuzio et al (1978) compared diploid and derived autotetraploid <u>Lycopersicon esculentum</u> for a range of enzymes. They found the tetraploid to be higher than the diploid in malate dehydrogenase (MDH), acid invertase (AI), glutamate dehydrogenase (GDH), nitrate reductase (NR), and acid phosphatase (AP); lower in peroxidase (PX), ribulose bisphosphate carboxylase/oxygenase (RuBPC/O), and glycolate oxygenase (GO); and equal to the diploid in neutral invertase (NI) and EST.

Studies cited above have compared values on a fresh doesn't take into account the weight basis, which differences in cell size between diploids and polyploids. Since compensation occurs at the cellular level, values should be expressed for a comparable number of cells to adequately examine the effects of polyploidy on enzyme production. Hoisington and Hancock (1981) reported both fresh weight and DNA values for two allotetraploid species of Hibiscus which had different ages. H. radiatus arose in this century, while <u>H. acetosella</u> arose thousands of years H. radiatus showed levels of ADH, MDH, leucine ago. aminopeptidase (LAP), and total protein equal to the averages of its diploid progenitors on a fresh weight basis, and equal to the sum of its progenitors on a DNA basis. However, levels of protein calculated on a fresh weight and a DNA basis were quite different in H. acetosella. Compared

to the average of its progenitors on a fresh weight basis, it showed higher levels of MDH and LAP, an equal level of protein, and a lower level of ADH. On a DNA basis, however, protein levels were lower than the sum of the progenitors. Expressing values on a DNA basis equalized species of different ploidy for differences in cell size. The lower protein value found in the old tetraploid <u>H. acetosella</u> compared to the recently formed <u>H. radiatus</u> was evidence of dosage compensation, but was not apparent without DNA data.

Many economically important crop species have evolved from ancient polyploids. Examples include peanut, oats, wheat, alfalfa, sweet potato, cotton, and tobacco. If dosage compensations had occurred prior to domestication of these crops, they may persist in the cultivated species. Since commercial growing conditions may be less resource limited than the conditions under which crops evolved, the compensations may no longer be adaptive. Hybridization of polyploid crop species with related raw polyploids may introduce uncompensated genes, resulting in higher enzyme levels per cell. An increase in the level and/or activity of a rate limiting enzyme may subsequently lead to an increase in yield. Additionally, an increase in the level of total protein in a forage crop would be of interest, since protein level is a critical nutritional characteristic (Sunberg et. al., 1982).

Cultivated <u>Medicago</u> <u>sativa</u> L. and its related species provide a system for investigating the adaptive consequences of autopolyploidy. Cultivated alfalfa is an autotetraploid

having a 2n chromosome number of 32. Its closest relatives are the diploid species (2n=16) <u>M. coerulea</u> Less. and <u>M.</u> <u>falcata</u> L. (Gunn et. al., 1978). <u>M. falcata</u> can also be found in the wild at the tetraploid level. All species having the same ploidy freely intercross, and are close enough genetically to be considered biotypes of the same species (Quiros, 1982).

Several studies have compared enzyme levels in diploid and induced autotetraploid M. sativa populations. Dunbier al (1975) showed two tetraploid populations to be lower et in glucose-6-phosphate dehydrogenase (G6PDH) activity per DNA than their respective diploid populations, and a third population to be equal to its diploid. Meyers et al (1982) showed levels of RuBPC/O and total protein per DNA to be equal among artificial populations at the two ploidy levels. However, neither of these studies included naturally occurring diploid and tetraploid alfalfa populations. The goal of this study was to determine whether dosage compensations have occurred in natural populations of autotetraploid <u>Medicago</u> <u>sativa</u>.

MATERIALS AND METHODS

PLANT MATERIAL

Seed of all isogenic populations was kindly supplied by Dr. E. T. Bingham, Agronomy Dept., Univ. Wisc., Madison, Wisc, 53706. Seed lines based on natural collections were obtained from the USDA North Central Regional Plant Introduction Station, Ames, Iowa. Seed of the cultivar 'Vernal' was obtained locally. Accession numbers and their origins are listed in Table 1.

The two sets of isogenic populations were derived in different ways. W70-22 is the product of crosses between accessions of cultivated diploid M. sativa and wild diploid M. falcata. Progeny from initial crosses were allowed to intercross for several generations to develop a diploid population of maximum variability. W71-42 is the product of 4x-2x and 2x-4x crosses between the same accessions of M. sativa and M. falcata as those used to develop W70-22. Tetraploids resulting from these were then crosses intercrossed to develop a population that was highly heterozygous (Bingham, 1975).

Wisol-2x and Wisol-4x populations originated from eight diploid parents. Vegetative clones of each diploid were artificially doubled by colchicine treatment. The parents at each ploidy were then combined via a double-double cross to produce isogenic populations at both the diploid and the tetraploid level (Pfeiffer et al, 1980).

	Ploidy	Origin
Natural Accessions		
299051	2x	USA (MN)
172989	2x	Turkey
262532	2 x	Israel
235021	2 x	Germany
251830	2 x	Austria
239953	4 x	Algeria
277489	4 x	Spain
172984	4 x	Turkey
251205	4 x	Yugoslavia
172983	4 x	Turkey
Artificial Populations		
W70-22 (5)	2 x	Univ. Wisc.
W71-42 (5)	4 x	Univ. Wisc.
Wiso-2x (5)	2 x	Univ. Wisc.
Wiso-4x (5)	4 x	Univ. Wisc.
Cultivar		
Vernal	4 x -	USDA

Table 1. Summary of alfalfa plant material.

EXPERIMENTAL PROCEDURES

Seeds were germinated in a peat:perlite(1:1) soil mix in 4.75 cm cell packs in September, 1982 in the greenhouse at Michigan State University, East Lansing, Michigan, 48824. Six weeks after germination, seedlings were transplanted into 20 cm plastic pots using the same soil mix. Five different accessions of natural diploids and natural tetraploids were selected for use in the study. Five individuals from each of the artificial populations and the cultivar 'Vernal' were also chosen. A11 plants were arranged and maintained in a completely randomized design in the greenhouse, and watered and fertilized as needed. Sixteen hour supplimental lighting of 120 μ mols⁻¹m⁻² was provided during the winter months by high intensity sodium lamps.

Ploidies of all plants were verified by chromosome counts of root tips. Root tips were collected in distilled water, given a 24 hour cold treatment (4°C), and fixed in cold Farmers solution (ethanol:acetic acid, 3:1). Root tips were then washed, hydrolyzed in 1N HCl at 60°C for 20 minutes, placed in Feulgans solution for 15 minutes, and squashed in aceto-carmine. Counts were made using a Zeiss phase contrast microscope, model #4255696.

Plant material was harvested from vigorously growing shoots in July, September and November of 1984. One gram of tissue from fully expanded leaves was ground in 9 ml cold

buffer (50mM Na-phosphate, pH 7.5, 10mM EDTA) using a Tissumizer, model #SDT-1810 (Tekmar Co.). Aliquots of the homogenate were removed and frozen for later buffer soluble protein (BSP) and DNA determinations. The remaining homogenate was centrifuged in a Sorvall RC2-B refrigerated centrifuge, using the SS-34 rotor, at 5000 rpm for 20 minutes. The supernatent was decanted and used for enzyme activity determinations.

Malic dehydrogenase (MDH, E.C. 1.1.1.37) activity was determined by the decrease in absorbance at 340 nm as NADH was oxidized in the conversion of oxaloacetate to malate (Worthington, 1977). One unit of MDH activity will convert 1 μ mole/min OAA and B-NADH to malate and B-NAD⁺. A standard curve was generated using Sigma #M9004 MDH. Peroxidase (PX, E.C. 1.11.1.7) activity was determined by an increase in nm as o-dianisidine was oxidized by absorbance at 460 hydrogen peroxide (Worthington, 1977). One unit of PX activity will form one mg purpurogallin in 20 sec. from pyrogallol at pH 6.0 and 20°C. A standard curve was generated using Sigma #P8000 PX. Both activities were measured in a Beckman dual beam spectrophotomoter, model #24, with a Linear strip chart recorder, model #H5232X, attached.

Buffer soluble protein (BSP) was determined by the Bensadoun et al (1976) modification of the procedure of Lowry et al (1951). Previously frozen plant homogenates were thawed for use in the assay. A 50 μ l aliquot was added to 1.5 ml dH₂O and 25 μ l 1% Na-deoxycholate, and incubated

for 15 minutes. at room temperature Protein was precipitated by the addition of 500 μ l 24% trichloroacetic acid and centrifugation at $2000 \times g$ for 30 minutes. The supernatent was discarded and the pellet dissolved in 1.5 ml Lowry reagent (2.0% Na₂CO₃, 0.02% Na-tartrate, 0.4% NaOH, 0.005% CuSO_{4.H2}0). After 10 minutes, 300 µl 0.5N Folin-Ciocalteau reagent was added. Absorbance at 660 nm was #24 later, using recorded 30 minutes a Beckman spectrophotometer. Conversion from absorbance units to μg protein was done using a standard curve generated from Bovine Albumin, fraction V (Sigma Chemical Co.).

DNA determinations were made by the method of Baer et al (1982). Previously frozen plant homogenates were thawed and diluted with an equal volume of 4M NaCl. Samples were then extracted twice with an equal volume of chloroform. Aqueous and organic phases were separated by low speed centrifugation (5 minutes at $1000 \times g$), and organic phases discarded. The remaining clear aqueous supernatent was used for DNA determinations. Fluorescence was measured with an SLM-Aminco Bowman spectro-fluorimeter, model #FA-537, with excitation and emission wavelengths set at 350 nm and 450 respectively. Three ml of a fluorescent dye solution nm, (100 ng DAPI/ml, 10mM Tris HCl pH 7.0, 10mM EDTA) were pipetted into a cuvette. Fluorescence was measured after the successive addition of four 10 µl aliquots of supernatent, followed by four 10 μ l aliquots of calf thymus internal standard. DNA. 25 ug/ml, as an Plotting fluorescence against cumulative aliquot volume yielded two

straight lines, one for the plant sample and one for the standard, with different slopes. Concentration of DNA in the plant sample was calculated using the known concentration of the DNA standard and the slopes of the two lines.

Ribulose bisphosphate carboxylase/oxygenase (RuBPC/O, E.C. 4.1.1.39) activity and quantity were determined in September 1984 by the procedure of McCurry et al (1981). Plant material was ground by hand in a mortar and pestle in cold buffer (20mM MgCl₂, 10mM DTT, 1mM EDTA, 1mM PMSF, 2% PVPP in 50mM Bicine KOH, pH 8.2), 0.5 g/10 ml. The slurry was then filtered through 4 layers of cheesecloth and centrifuged in an Eppendorf microfuge, model #5414, for 2 minutes. A 100 µl aliquot of enzyme extract was activated at 30°C for 30 minutes in 20mM MgCl₂, 10mM KH $^{14}CO_3$ (0.1 μ Ci/ μ mol), 0.2mM EDTA, and 1mM DTT in 100 mM Bicine KOH, pH 8.2. The activity assay was performed in the same buffer as the activation of the enzyme, but with the addition of 0.5mM ribulose bisphosphate. A 50 μ l aliquot of activated enzyme was added to 450 μ l assay buffer, and allowed to run for 30 seconds. The reaction was terminated with the addition of 200 μ l of 2N HCl. Samples were dried at 80°C to evaporate ¹⁴C⁰₂, unfixed and radioactivity determined by liquid scintillation counting. All assays were performed in triplicate.

Quantitation of RuBPC/O was done by irreversable binding of the labelled transition state analogue 2carboxyarabinitol 1,5-bisphosphate (CABP). One ml of activated enzyme was incubated with 10.25 ml CABP solution $(0.1 \text{mM} \ ^{14}\text{CABP}, \ ^{1}\text{mM} \ ^{1}\text{NaHCO}_3, \ ^{2}\text{OmM} \ ^{M}\text{gCl}_2$ in 50mM Bicine KOH, pH 8.2) at 30°C for 30 minutes. An equal volume of 40% polyethylene glycol (PEG) 4000 in Bicine buffer was added to precipitate the enzyme complex. The precipitate was collected by centrifugation at 27000 x g for 10 minutes, and washed twice with 20% PEG. The precipitate was then dissolved in 1 ml dH_20 and split into 2 vials for liquid scintillation counting.

Fresh plant extracts were subjected to starch gel electrophoresis in September, 1984. Gel buffer was composed of 270 ml 50mM Tris citrate, pH 8.3 and 30 ml 30mM lithium borate, pH 8.3. Tray buffer was 30mM lithium borate, pH 8.3 (Scandalios, 1969). Fully expanded, vigorously growing leaves were ground on ice in a mortar and pestle with several drops cold extraction buffer (50mM Na-phosphate, pH 7.5, 10mM EDTA). Extracts were absorbed onto 4x8 mm filter paper wicks and placed into a chilled 10% (w/v) starch gel. Gels were run in the cold at 10 watts power for 5 hours, then sliced and stained for PX (Gottlieb, 1973) and MDH (Scandalios, 1969) activity.

RESULTS AND DISCUSSION

Differences in cell size between genotypes within a ploidy and across different ploidies can create difficulties in measuring dosage compensation on the basis of fresh weight, since the number of cells per sample will vary. Cells of the same ploidy, however, will have approximately the same amount of DNA regardless of cell size, and an autotetraploid cell will have approximately twice as much DNA as its diploid progenitor (if large scale deletions have not occurred). Expressing enzyme and protein data on a DNA basis, therefore, removes the effect of cell size differences and allows valid comparisons to be made across ploidy. For this reason, DNA content per gram fresh weight was determined at each sampling time, and subsequent data expressed on the basis of total DNA. Equal ratios of protein to DNA in genetically similar diploid and tetraploid plants suggests equivalent levels of gene activity and an absence of dosage compensation in the tetraploid. Since determinations of total DNA include organelle as well as nuclear DNA, differences in organelle DNA content between ploidies could alter the results. However, Bowman (1986) reports a constant percentage of chloroplast DNA at three levels of ploidy in Triticum and Aegilops species.

No significant differences (p<.05) were found in either set of 2x/4x isogenic artificial populations grown in the greenhouse for BSP, PX or MDH activity on a fresh weight basis or per unit DNA. The absence of differences on a DNA

basis between these populations at different ploidies suggests an absence of compensation in raw autopolyploids.

Similarly, no significant differences were found between natural diploids and tetraploids grown in the greenhouse for BSP or MDH activity on a fresh weight basis, although diploids were significantly higher in PX (Table 2). However, natural diploids had significantly more total protein per unit DNA than natural tetraploids on sample date 3 and over all dates. The natural diploids were also higher in PX activity per unit DNA on sample dates 1 and 2 (Table 3).

Meyers et al (1982) describe differences in responce of isogenic diploid and tetraploid alfalfa populations between greenhouse and growth chamber grown plants. Among plants grown in a growth chamber, soluble protein levels were similar across ploidy. However, among plants grown in the greenhouse, diploids had higher levels. Although we did not observe differences between artificials under greenhouse conditions, our differences between the natural populations could have been due to a similar environmental effect. Possible causes include wider temperature, humidity, and light fluctuations in the greenhouse than are commonly found in the growth chamber. To test this, we cut back the mature plants and placed them in a growth chamber during regrowth $(500 \ \mu mo) \ s^{-1}m^{-2}$, 16 hr. photoperiod, 25°C day temp., 20°C at night). Mature leaves were harvested after four weeks, and BSP and DNA determined. Absolute values for diploids and tetraploids were different than for greenhouse grown

Table 2. Natural diploid and natural tetraploid alfalfa populations compared for buffer soluble protein (μg BSP/g. fresh weight), malic dehydrogenase (units MDH/g. fresh weight), and peroxidase (milliunits PX/g. fresh weight).

		BSP	MDH	PX
Date 1	2 x	13.6	0.11	5.1
	4 x	11.2	0.09	3.5
Date 2	2 x	11.4	0.34	2.5
	4 x	13.3	0.35	1.8
Date 3	2 x	20.0	0.84	2.5
	4 x	21.6	0.89	0.3
Combined	2 x	15.0	0.43	2.7*
	4 x	14.8	0.44	1.9

t statistic significant at the 5% level.

Table 3. Natural diploid and natural tetraploid alfalfa populations compared for buffer soluble protein (μ g BSP/ μ g DNA), malic dehydrogenase (units MDH/ μ g DNA), and peroxidase (milliunits PX/ μ g DNA).

		BSP	MDH	PX
Date 1	2 x	21.2	0 39	7.4*
	4 x	14.4	0.12	4.7
Date 2	2x	18.9	0.57	4.0*
	4x	14.1	0.39	2.0
Date 3	2 x	57.4*	3.33	19.0
	4 x	39.1	5.58*	18.1
Combined	2 x	32.5**	1.43	10.1
	4 x	22.5	2.05	8.6

* t statistic significant at the 5% level.

** t statistic significant at the 1% level.

plants, however the trends remained the same (Table 4). This result indicates that the difference we observed between ploidies in natural populations was probably not an artifact of environment.

The significant differences in BSP and PX per DNA found between ploidies in natural accessions suggest that dosage compensation has occurred over evolutionary time in the tetraploids. Of course, this conclusion assumes that the sampled diploids are representative of the progenitors of the original polyploids. We can not be sure of this, but have tried to minimize any potential bias by sampling genotypes from a wide geographical range.

The differences in PX activity per DNA between ploidies may have been due to regulatory rather than structural genes since PX allozymes found in diploid alfalfa are also found in tetraploids (Quiros, 1979). Four PX loci have been described in alfalfa. Loci PX1, PX2, and PX3 run anodally during starch gel electrophoresis, and PX4 runs cathodally (Quiros and Morgan, 1981; Quiros, 1982). Although our cathodal banding pattern was more complex than others have described, the anodal pattern was consistant with a three locus, multi-allelic model and all PX loci appeared to be expressed in both 2x and 4x natural populations (Figure 1). PX2 activity was missing in some natural tetraploid individuals, but enzyme activity at this locus was also absent in two diploids. PX2 activity was also missing in several individuals from the W70-22 and W71-42 (Figure 2), and was completely absent in the Wiso-1 2x/4x populations Table 4. Buffer soluble protein ($\mu g/\mu g$ DNA) in natural diploid and tetraploid alfalfa accessions grown under greenhouse and growth chamber conditions.

	Greenhouse	Growth Chamber
Diploids		
235021	34.3	48.1
172989	29.2	35.4
262532	37.7	43.9
Mean	33.7	42.5
Tetraploids		
25 1205	23.9	26.2
172983	22.8	26.5
239953	25.3	30.9
Mean	24.0	27.9













(Figure 3).

Not all loci appear to be compensated in the natural tetraploids. MDH activity in the 2x and 4x populations differed on only one date, and then the tetraploids were higher than the diploids (Table 3, date 3). Perhaps higher levels of specific enzymes such as MDH are beneficial in alfalfa, or mutations have not yet arisen which lower its activity. MDH isozymes could not be resolved into sharp bands using several different electrophoretic buffer systems, so qualitative differences could not be determined. Qualitative differences in BSP were also not determined.

Dosage compensations can occur at several different levels in the cell. Carlson (1972) studied a number of enzymes in an aneuploid series of <u>Datura</u>, and saw a direct relationship between gene dosage and enzyme activity. He suggested that transcription rates of structural genes are constant and independant of gene number, so an increase in gene dose results in an increase in gene product. Since regulatory as well as structural genes are increased in a polyploid (Becak, 1969; DeMaggio & Lambrukor, 1974; Wolf et al, 1977), subsequent modifications in promoter regions of individual structural genes could cause changes in transcription rates. Translational and post-translational events can also influence levels of gene products. Availability of mRNAs, tRNAs, amino acyl-tRNA synthetases, or ribosome subunits could limit translation (Bryant, 1976). Post-translational events can limit function and expression of a protein. These include availability of prosthetic

groups, presence of inhibitors, and changes in protein structure due to altered hydrogen bonding or disulfide bridges (Bryant, 1976: Ohno. 1970). That naturally occurring alfalfa tetraploids showed lower levels of total protein than the diploids suggests that regulatory or pretranslational compensations exist, because a translated but inactive protein would still be detected when measuring BSP. not known if there have been other types It is of compensations.

Natural diploids had higher protein levels than either artificial diploid population. It is possible that natural diploids have substantial amounts of heterotic and epistatic interactions which account for the high protein levels. Intercrossing many accessions to create the artificial coadapted disrupted complexes. populations may have resulting in lower protein levels in the artificials. It is also possible that high activity genes have been favored by selection in diploids in the wild, rather than tetraploids being compensated. When tetraploids first appeared and became established, perhaps only the most fit diploids were able to compete successfully. Natural tetraploids may not have actually undergone compensation, but rather are able to maintain individuals with a wider range of gene activities. The tetraploid population mean might then be lower than that of natural diploids. This hypothesis would also account for the observed lack of difference in protein levels between natural and artificial tetraploids. If mature tetraploids undergone substantial dosage compensation, it might be had

expected that they would have lower levels of protein than recently induced tetraploids, even if the two populations were genetically dissimilar.

Although selection "up" for diploids rather than selection "down" for tetraploids cannot be classified as dosage compensation in the strictest sense, the evolutionary result may be the same. That is, many null or low activity alleles have been eliminated from the diploid may populations but not from the tetraploids. While it is thought that a significant amount of hybridization between ploidies occurs in the wild, it generally occurs in only one direction (diploid to tetraploid via unreduced gametes). So while tetraploids may aquire some high activity genes from these hybridizations, they might still carry low activity alleles not found in the diploids. Their tetrasomic inheritance patterns would produce very few homozygous individuals. If this is the case, vigorous wild diploids may be a better source of high activity genes for plant breeders to incorporate into commercial varieties than tetraploids. Additionally, transfering the genes via unreduced gametes would preserve any heterotic or epistatic interactions that may exist.

Much of the observed variation in total protein was probably due to variation in RuBPC/O, the key enzyme involved in photosynthetic carbon fixation. RuBPC/O is the most abundant protein found in higher plants, comprising up to 50% of total soluble protein in many species (Kung, 1976; Jensen and Bahr, 1977). RuBPC/O activity was significantly

correlated with BSP in both diploid and tetraploid alfalfa populations (Figure 4) and the 14CABP binding assav indicates that 38-64% of the BSP was RuBPC/O (Table 5). The tight association of RuBPC/O with BSP, plus the significant portion of total protein that it comprises, suggests that a large part of the variation observed for BSP is accounted for by variation in the carboxylase enzyme. Since only one of the two types of RuBPC/O subunits is nuclearly encoded. this enzyme will be uniquely affected by polyploidy. Although synthesis of both the nuclear encoded small subunit (SSU) and the plastid encoded large subunit (LSU) must be somewhat coordinated, the extent of the coordination is not known. Existing variation for RuBPC/O in alfalfa at both ploidies may be useful in further understanding the regulation of this enzyme.



Table 5. Quantification of RuBPC/O protein by 14CABP binding (RuBPC/O and BSP in $\mu g/\mu g$ DNA).

Genotype	Ploidy	RuBPC/O	BSP	%RuBPC/0
299051	2 x	15.6	26.3	59
172989	2 x	15.7	16.8	59
262532	2× •	21.8	35.4	62
W70-22	2 x	14.3	38.0	38
239953	4×	12.9	20.6	63
277489	4 x	13.2	28.6	46
W71-42	4 x	14.7	33.3	44
Vernal	4 x	27.3	42.4	64

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Accession	Ploidy	Date 1	Date 2	Date 3	Mean
299051	 2x	12.7	20.4	65.8	33.0
172989	2 x	17.5	15.3	54.7	29.2
262532	2 x	20.6	20.1	72.3	37.7
235021	2 x	35.7	16.9	50.2	34.3
251830	2 x	19.3	21.7	43.9	28.3
239953	4 x	10.2	13.0	40.8	21.3
277489	4 x	17.2	9.4	58.2	28.3
172984	4 x	12.5	10.1	35.3	19.3
25 1205	4 x	10.5	28.8	32.4	23.9
172983	4 x	21.7	9.0	28.6	19.8

Appendix 1. Buffer soluble protein levels in natural accessions of alfalfa (μg BSP/ μg DNA).

Appendix 2. Mean values of BSP (μ g/ μ g DNA)

Genotype	Date 1	Date 2	Date 3	Total
Natural 2x	21.16	18.88	57.38	32.50
Natural 4x	14.42	14.06	39.06	22.52
W70-22	21.40	15.92	33.36	23.56
W71-42	22.10	12.62	31.02	21.90
Wisol-2x	18.48	14.80	30.95	21.40
Wisol-4x	15.36	15.98	32.34	21.22
Vernal	19.10	15.78	27.42	20.76