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FLOW CYTOMETRIC ASSESSMENT OF NUCLEOTYPE VARIABILITY AND ITS EVOLUTIONARY IMPLICATIONS

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Since the discovery that higher organisms have vast amounts of nuclear DNA, much more than is necessary to serve coding functions, scientists have attempted to elucidate why this DNA exists. Hypotheses as to the role of this noncoding DNA have ranged from regarding it as useless junk or selfish DNA to speculation that these sequences are critical to gene regulation in the Metazoa. One of the most popular theories with respect to the function of these sequences is the nucleotypic theory.

Coined by Dr. M. Bennett in 1972, the term nucleotype refers to the effect of noncoding DNA (at the cellular or organismal level) because of its mere physical presence and independence of any base sequence information. For example, plant cells with more DNA would be expected to have a larger nuclear volume to hold this extra DNA. These same cells also would have a larger cell volume to contain the larger nucleus. To further illustrate the wide-ranging effects of the nucleotypic theory, if cells with the larger volumes to accommodate larger nuclei were photosynthetically active, these cells also would be expected to contain more chloroplasts. Thus, adding or deleting noncoding DNA sequences, in theory, could have a significant impact on photosynthesis in higher plants.

In order to have the greatest impact in an evolutionary sense, the total amount of nuclear DNA (or genome size) must fluctuate below the species level. The amount must vary between populations within a species and, ultimately, within populations. Five questions need to be addressed to determine the significance of genome size (and nucleotype) in evolution. 1) Does intraspecific variation in genome size exist in plants? 2) If this genome size variation exists, is it associated with nucleotypic responses? 3) Do specific genome sizes appear to be of adaptive significance? 4) Can the genome size of a plant be affected by selection? and 5) Can the total nuclear DNA content of a plant be a predictor of plant performance in a given environment? Before any of these questions can be addressed, one has to have the means to detect significant, though potentially small, intraspecific DNA variability.

Several methods are available for determining the total nuclear DNA content of an organism. These methods include, but are not limited to, chemical analysis, fluorimetry, densitometry, and flow cytometry. Although all methods of DNA analysis have their advantages and disadvantages, flow cytometric analysis seems to have a clear advantage over all the others. Because of rapid and accurate measurements from large sample sizes, flow cytometry is becoming increasingly popular. When using the other methods, one is faced with at least one, if not two, major problem(s). Either the number of nuclei and their position in the cell cycle must be estimated, or the sample size of nuclei analyzed per individual is rather low (< 100). With flow cytometry over, 5,000 nuclei can be analyzed in less than 60 seconds. With this large number of samples, an accurate determination can be made of the number of nuclei at each cell cycle stage.

The major obstacle in using flow cytometry is the ability to obtain large numbers of plant cells or intact nuclei in a suspension. This can be accomplished by exposing a particular tissue to cell wall-degrading enzymes to form a protoplast. These intact protoplasts then can be passed through a flow cytometer, and the DNA data collected on a cellular basis. Alternatively, the tissue can be sheared mechanically to release the nuclei in a nuclear stabilization buffer. Both methods have been used successfully in plants.

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When using flow cytometry, a wide variety of fluorochromes are available. Flow cytometric analysis is very dependent on DNA fluorochromes. These fluorochromes bind to DNA and when the fluorochrome-DNA complex is excited with a light source of proper wavelength, the complex gives off a light (or fluoresces) at a specific wavelength depending on the fluorochrome used. Several types of fluorochromes can be used. Most of the DNA fluorochromes used in flow cytometry can be divided into three classes: 1) intercalating fluorochromes such as propidium iodide (PI), 2) A–T-binding dyes such as Hoescht 33258, and 3) G–C-binding dyes such as mithramycin. The majority of plant analysis is accomplished with either PI or DAPI, an A–T-binding dye. New, more sensitive fluorochromes are being synthesized continually and evaluated for their usefulness in plant flow cytometry.

Flow cytometric analysis now allows for routine estimates of 3–5 % genome size variation among plants or plant populations. The ability to detect these small differences is confirmed cytologically using the aneuploids of various plant species, including wheat and maize. Flow cytometry possesses the sensitivity and efficiency to probe intraspecific or intrapopulation variation of DNA content in plants. It should be noted that using flow cytometry to measure DNA content is only one application of a very powerful tool in plant cytogenetics. Two excellent texts where the full potential of flow cytometry to cytogenetics can be examined are: 1) Flow Cytometry and Sorting, edited by MR Melamed, T Lindmo, and ML Mendelsohn, John Wiley &Sons, Inc. and 2) Flow Cytogenetics, edited by JW Gray, Academic Press. With the method of determining intraspecific DNA content established, the five questions concerning the role of the nucleotype in evolution now can be addressed.

DOES INTRASPECIFIC VARIATION IN GENOME SIZE EXIST IN PLANTS?

Originally, it was thought that each species had its specific genome size and that it is constant within a given species. Thus, a haploid genome was defined as having a 1C (C = constant) nuclear DNA amount. A 2C DNA amount would be the amount of DNA contained within a G1 diploid nucleus. Soon after the consistency of species genome size was proposed, doubts were raised as to whether this hypothesis was true. Several investigators combined genome size data from laboratories worldwide. The accumulation of data showed that when plants of the same species were examined in different laboratories, large DNA differences could occur. Controversy ensued when those that believed in the consistency hypothesis stated that the observed differences could be attributed to the use of different techniques, instrumentation, etc., among the different laboratories. Other scientists stated that, although differences did exist among laboratories, the results across laboratories were comparable and that real differences in DNA content within a species did exist. Over time, data from within the laboratories began to indicate that populations within a species indeed did have different DNA contents. Various reports in the literature now support the argument against the constancy of the genome size within a species.

Maize is an example of a plant species for which intraspecific DNA content is well documented. Maize is a diploid organism with 2n = 20 chromosomes. Overall, the average amount of DNA per 2C (diploid) nucleus is approximately 5.5 picograms (pg). The genome size of over 400 maize lines has been determined. Within inbred lines, a 26 % variation was observed in nuclear DNA content, with the genome sizes ranging from 4.7 to 5.9 pg of DNA/2C nucleus. In all inbred lines examined to date, only 20-chromosome plants have been observed. When open pollinated lines are considered, the genome sizes range from 4.9 to 7.0 pg of DNA/2C nucleus, a 43 % variation. Several of the higher DNA plants were observed to have extra (B) chromosomes. However, when the lines containing these B chromosomes are removed from the analysis, the observed DNA variation still exceeds 37 % . In total, when the data from all types of maize are combined, a variation of over 56 % is observed in total nuclear DNA content.

Cytological observations confirm the fluctuating DNA contents in maize. In 1926, McClintock first described knob-like structures on maize pachytene chromosomes. These structures have become important morphological markers on the maize chromosomes. Maize lines are known to vary with respect to the number and size of these large heterochromatic areas along the chromosomes. Maize lines may have no knobs or up to 20 large heterochromatic knobs. These knobs have been shown to contain a 185 bp repetitive DNA sequence. As the number of knobs increases, the amount of this repetitive sequence also increases. This being the case, one would expect maize lines with low numbers of the knob structures to have less total DNA than those lines with larger numbers. On average, this is exactly what occurs. Therefore, intraspecific DNA content variation is clearly documented and cytologically confirmed in maize.

IS GENOME SIZE VARIATION ASSOCIATED WITH NUCLEOTYPIC RESPONSES?

Having documented the existence of intraspecific genome size variation in maize, the next question can now be asked. Do nucleotypic responses occur in maize? To answer this question, let's look at the maize epidermal guard cells. The guard cells in maize lines, ranging in genome size from 4.8 to 5.2 pg/2C nucleus, were measured and their chloroplast numbers determined. According to the nucleotypic theory, lines with the higher DNA amount should have larger cells. This is the case in maize. The cell sizes ranged from 41 to 47 microns, with a significant positive correlation between genome and guard

cell size. When the chloroplast number per guard cell was correlated with genome size, a significant positive correlation was observed again. Both of these results are consistent with the nucleotypic theory and substantiate the fact that intraspecific genome size variation can, and does, influence cellular parameters in maize.

DO THESE RESPONSES APPEAR TO BE OF ADAPTIVE SIGNIFICANCE?

Because nucleotypic responses occur in maize, the question can be asked if these responses appear to be of adaptive significance. With the first reports of chromosomal and genome size variations in maize, it was speculated that maize lines adapted to higher altitudes would have less DNA than maize lines adapted to lower altitudes. This speculation was based on the observation that in open-pollinated varieties of Mexican maize, lines grown at high altitudes tended to have fewer knob structures (described above) located on their chromosomes than those lines grown at lower altitudes. As stated previously, knobs contain a repetitive DNA element, thus, the amount of this element would increase with increasing knob number and result in an overall increase in total nuclear DNA content.

When this hypothesis was tested in the southwestern United States, i.e., Indian maize lines from New Mexico, a negative correlation between genome size and altitude was observed. This was in agreement with the Mexican maize studies. However, a major problem was encountered when attempts were made to determine the environmental factors associated with the correlation . Several environmental factors are associated with increasing altitude. These factors include, but are not limited to, temperature, light intensity, growing season, and UV radiation. There was no way to correlate the observed changes with any specific environmental factor.

In order to gather more data, several Indian populations from Arizona were analyzed. The altitude of adaptation extended lower than those of the New Mexico lines, and an unexpected result was obtained from these lines. The lines adapted to the lower elevations had less DNA than those from higher elevations. In fact, the DNA content of the low altitude, Arizona populations was approximately the same as the DNA content of the high altitude, New Mexico populations. The Arizona and New Mexico populations adapted to similar intermediate altitudes had approximately the same, larger, genome sizes. Could some environmental factor explain the results obtained in the Arizona and New Mexico studies and be compatible with the original observations in the Mexican populations?

In a comparison of the growing conditions at the lower altitudes in Arizona with the conditions at the higher altitudes in New Mexico, an interesting observation was noted. The growing seasons at both of these elevations were rather short. At higher altitudes in New Mexico, the time between the earliest possible planting date and the first frost is relatively short. Therefore, the plant must adapt by developing early maturity. At the lower elevations, the time from the earliest planting date to the time when high temperatures become critical for maize growth and development is also short. Comparison of these two potential growing seasons (lower vs. higher altitudes) shows that they are remarkably similar. Because plants at either of these elevations would be adapted to a short growing season, the nucleotypic hypothesis would predict that both would have less DNA than similar lines adapted to a less constrained environment. The reasoning behind this prediction is that cells with less DNA undergo cell division at a more rapid rate. Plants adapted to shorter growing seasons would need a short minimum-generation time. Cells in these plants would have to divide and rapidly reach maturity.

Because the observations in maize are in agreement with the nucleotypic theory, an adaptive significance must be associated with a nucleotypic response. With respect to the original Mexican populations, those adapted to lower altitudes are not subjected to the shortened growing season of the desert conditions of the southwest United States. The high altitude, Mexican, maize populations are grown under short-season conditions. Thus, the results from the Mexican populations are consistent with the environmental hypothesis formulated from the southwestern United States, Indian maize populations.

CAN SELECTION RESULT IN AN ALTERED DNA CONTENT OF A POPULATION?

If the nucleotype of an organism is to be involved in adaptation, the genome size of the organism would have to respond to selection pressures. Could selection result in an altered DNA content of a population? To answer this question, let's look at the analysis of a photoperiod-insensitive, maize composite from South Africa. The original composite was subjected to six cycles of selection for earliness. Between the original, C_0 population and the most advanced cycle, C_6 population, a 14-day decrease in days-to-flowering was observed. When the genome sizes of 100 plants of the two populations were analyzed, an interesting trend was noted. In the C_0 population, the nuclear DNA content ranged from 5.2 to 6.0 pg/2C nucleus with a mean of 5.6 pg/2C. In the C_6 population, the 2C nuclear DNA content ranged from 5.2 to 5.8 pg with a mean of 5.4 pg. However, the most interesting trend was observed when the frequency distribution of each population was analyzed. In the original C_0 population, 50 of the plants were observed to have a genome size over 5.7 pg/2C nucleus. In the C_6 population, only 11 plants had a 2C genome size over 5.7 pg. The selection for earliness resulted in a shift in the population distribution. Those plants in the C_0 population with the larger genome sizes appeared to be selected against. This population shift and the decrease in mean nuclear DNA

content in the C_6 population strengthens the evidence for the nucleotypic theory.

The nucleotypic theory would predict that, as one selects for minimum generation time, i.e., earliness, the total nuclear DNA decreases. This would be due to the effect of bulk DNA on parameters associated with growth rate, cell size, and doubling time. In the South African maize populations, plants that had the largest genome size were selected against, because their nucleotype was adapted to longer generation times. Therefore, the nucleotype of maize plants is subject to selection pressures, and selection at the level of total nuclear DNA content can occur.

CAN THE NUCLEOTYPE BE A PREDICTOR OF PLANT PERFORMANCE IN A GIVEN ENVIRONMENT?

The last question to be answered is: Can the nucleotype be a predictor of plant performance in a given environment? In other words, can the genome size be a useful indicator of plant performance? To answer this question, let's return to the Arizona and New Mexico Indian lines previously discussed. These populations significantly differ with respect to genome size. These populations were planted in replicated field plots at the University of Illinois Agronomy-Plant Pathology South Farm, Urbana, IL. Various growth and yield parameters were analyzed, and all were correlated negatively with genome size. The most striking correlations were between genome size and plant height at 60 days after planting (DAP), genome size and number of leaves at 60 DAP, genome size and ear weight per plant, and genome size and seed weight per plant. In all cases, the lower genome-size populations performed better than the higher genome-size populations at the Urbana, IL, location.

When one examines the genome sizes of the maize lines adapted to Illinois growing conditions, one finds them to be comparable to the low genome sizes observed in the southwestern Indian maize populations. Thus, a specific genome size appears to be adapted to this particular location. It should be noted that these results cannot be extrapolated back to the areas where the southwestern maize lines originated. It seems unlikely that similar correlations would occur under different environmental conditions. Each environment could have its own optimum nucleotype. However, this does not negate the negative correlation observed between varying growth and yield parameters and genome size in maize. In fact, it would further strengthen the role of the nucleotype in plant performance.

CONCLUSIONS

In conclusion, variation in intraspecific nuclear DNA content has been documented clearly in plants. Flow cytometry has proven to be an invaluable tool in the analysis of genome size in plants. These genome size variations are associated with nucleotypic responses and appear to be one component in adaptation to specific environments. Selection can, and does, affect the genome size of populations. Specific environments do favor certain nucleotypes. All evidence to date supports the role of the nucleotype in plant adaptation. The maize genome has a predisposition for DNA fluctuations. These fluctuations result in nucleotypic responses that then are acted on by selection. Therefore, we can no longer ignore the role of the nucleotype in plant adaptation and evolution. Most studies have concentrated on how evolution acts on coding genes and ignored the "junk" DNA. It is now apparent that this DNA is not junk and plays a significant role in plant adaptation and evolution.

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QUESTIONS

Birchler: Some studies have been done on specific repetitive elements that change in the F_1. Have you looked at any F_1s?

Rayburn: I didn't show what we actually did because I didn't think I would have time, but at some point, we made crosses between lines with different DNA contents. In this particular hybrid, here is the midparent, and here is the DNA content of the hybrid. It was right on the midparent. We have looked at other DNA. We have looked at other hybrids. This is the midparent of the hybrid, and we have an increase in DNA content over the hybrid in all plants. So, what I would say is yes, but it is not in all hybrids. When you make some hybrids, you get an increase in the DNA content. When you make other hybrids, you get the DNA that you expected in the midparent. And we actually looked at this in reference to some lines that John Dudley had at the University of Illinois, where he actually had heterosis per yield calculated as a number. And those lines that gave low heterosis were the ones that gave this type of response. With lines that give low heterosis, you get a non-Mendelian type of inheritance of DNA. With lines that give high heterosis, you get the normal inheritance of DNA in the F_1. So yes, our data would completely indicate that this type of inheritance would happen.

Phillips: On one of your first slides, you had the chromosome DNA content of rye. It seemed to be basically, negatively correlated with size, assuming, I guess, that the numbers from 1 to 7 were large to small. But your DNA content was mostly in the other direction.

Rayburn: Well, that particular study with rye was done with the fluorochrome PI. And PI is inaccessible to heterochromatin. It was just easier in wheat. There are two types of chromosomes in rye, those that have C^+N^+ bands and those that have C^+N^- bands. When we separate those chromosomes out that have C^+N^- bands, you see a nice correlation between chromosome size and DNA content. The C^+N^+ bands are where that correlation starts falling off. And what we think is happening is there is something, a protein or a particular structure that prevents the PI from getting into the heterochromatin of C^+N^+.

Phillips: One other remark. If you want to have some variation in the nucleolus organizer heterochromatin, look in Illinois reverse high protein lines. It looks like a piece of sausage lying right on top of the nucleus. It has lots of ribosomal genes.

Rayburn: We'll go after that, thank you.

BS Gill: Just to follow Ron Phillips' comment; I don't think those rye chromosomes are arranged based on size.

Rayburn: That's right. They are not by size. What we did was, we actually took that data from the standard karyotype. We could have arranged them by size.

Sharma: Lane, about your comment on the ratings of the DNA content, seed size, and generation times of different populations. How do these three things fit together?

Rayburn: This is something I think you have to be very careful of and I think was one of the problems when nucleotype was first looked at. That is why we stuck with southwestern open pollinated populations. There is a very harsh selection for short generation time in the lower altitudes and the higher altitudes. This would be very important for selection. If you took populations of corn

lines that were in the Midwest, there might be another overriding factor. Chloroplast number could be more important. This would result in a different nucleotypic response. So I don't think you can put it all together and expect to get a big picture. It depends upon the adaptation in a given area. I think that is why it is important that we selected those southwestern lines, because of the precisely defined information about the weather in those regions. We had a source of data to associate with the nucleotypic response. I don't think you can lump everything. Because, if you look back at the original data for the Mexican corn line, when you look at altitude, knobs decrease when you go up in altitude, and you get the same response when you look at the southwestern New Mexican lines. If we had looked only in the New Mexico lines, we never would have noticed that this correlation was different in the area of the southwestern US. So, I guess I wouldn't expect it to be the same in every population.

Sharma: If the plant is allowed to grow slowly over a longer time, it has a lot less seed weight hasn't it? Compared to when the plant has to grow very quickly.

Rayburn: Well again, we had different lines in here and so some of them grew for very long times, some might have been actually too long. You might run into them having too long a growing season in Illinois. And who knows what it would be if they were growing in Arizona. There are a lot of things missing in here. I will talk about it in the paper, but only allude to it here. Yes, that could be a factor.

Phillips: On seed size, it may be more related to the amplification of DNA during endosperm development than it is to base level of DNA, so that's going to do something.

Rayburn: Yes, we are going to go back and look at that and check what happens. That could be going on as well.