Nuclear DNA Contents of *Pisum* Genotypes Grown *in vivo* and *in vitro*

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Received March 11, 1972

Summary. The nuclear DNA content of prophase nuclei in root tips of two cultivars and two primitive lines of *Pisum sativum* and of *Pisum fulvum* have been determined, using a scanning microdensitometer. The nuclear DNA contents differed significantly between the genotypes investigated but there was no correlation with their supposed phylogenetic positions.

A loss of 73% of the DNA from cells of aseptically cultured excised pea roots has been recently reported (Abbott, 1971). In marked contrast to this claim, our measurements of the nuclear 4C DNA content of root tip meristematic cells have shown that there is no significant loss in excised roots compared with attached roots.

Introduction

In this report we describe our studies to determine the extent to which changes in nuclear DNA content have occurred during the course of the recent evolution, selection and improvement of a crop plant. An increase might indicate for example that the duplication or amplification of certain DNA sequences accompanies such a progression from primitive form to modern cultivar. As a first step in such an analysis, we have compared the nuclear DNA content of two primitive forms and two modern cultivars of *Pisum sativum* and of *P. fulvum*; if significant differences were found then further analysis of the changes could be undertaken using density gradient and molecular hybridisation techniques.

In contrast to the widely established interspecific differences in nuclear DNA contents of plant species there is little published data for intraspecific DNA variation. In *Picea glauca* and *Pinus banksiana*, the DNA per cell in different genotypes has been shown to differ by a factor of up to 1.6 (Miksche, 1968) and comparable differences in nuclear DNA content were later observed within *Picea sitchensis* (Miksche, 1971). Significant intraspecific differences in DNA content per cell have recently been reported also in *Vicia villosa*, *V. sativa* and *V. benghalensis* (Chooi, 1971).

We have also examined the problem of the stability of the genome of *P. sativum* in another context. Recently, Abbott (1971) claimed that
the DNA content of cells of excised pea roots cultured in vitro was only 27% of that found in cells of attached control roots. On the basis of his data, Abbott (1 c.) suggested that "there is excess nucleic acid in whole plant tissues above that required for transfer of genetic information necessary for normal growth and differentiation of root cells". This suggestion has such important implications regarding the nature of the genome and the fate of cells grown in tissue culture systems, that they need confirmation.

Materials and Methods

Aseptic Germination of Seeds. Selected seeds of the five pea lines (Table 1) were surface sterilized (70% ethanol, 10 min; washed in sterile distilled water; NaOCl c. 1.5 w/v available chlorine, 20–30 min; washed in sterile distilled water), and aseptically soaked for 24–28 h in aerated water at room temperature in the dark. In lines JI 224, 225, 261 the seed coat was partly removed in order to facilitate the soaking process. Individual seeds were grown at 23–25°C in the dark in sterile Pyrex test tubes containing a moist mixture of vermiculite and peat (9:1). Five plants of each line were chosen at random and used for the DNA measurements. Primary roots were fixed overnight in freshly prepared ethanol-acetic acid (3:1) in the refrigerator. They were subsequently washed and stored in 90% ethanol for two days before use.

Aseptic Culture of Excised Roots. For the in vitro culture of excised roots (treatment a) seeds of Chemin Long (JI 296) were germinated as above, and 10 mm root tips cut off and transferred into stoppered 100 ml conical flasks containing 25 ml of a nutrient medium (Abbott, 1968). The flasks were kept in the dark at 25°C on a rotator. A batch of seedlings for attached roots (treatment b) was kept under identical conditions, but with the shoots exposed to light (16 h day). After 7 1/2 days, uniformly growing excised roots were subcultured into new medium using again the terminal 10 mm. Five plants of treatment b which had developed many laterals were also transferred into new nutrient medium. Both treatments were allowed to grow for a further 5 days. As an additional control (treatment c) one batch of seedlings was left in the germination-tubes in vermiculite/peat and fixed one day later. Roots of all treatments were fixed in ethanol-acetic (3:1) for 4 h, post-treated overnight with absolute ethanol and stored in 90% ethanol in the refrigerator for 13 days (a and b) or 24 1/2 days respectively (c).

Preparation of the Root Meristems and Microdensitometry of Feulgen-DNA. Within an experiment, all roots were treated simultaneously and in an identical manner. Roots were transferred stepwise from 90% ethanol to distilled water prior to hydrolysis in 5 N HCl at 20°C for 50 min (Fox, 1969). They were then washed twice in distilled water and stained in freshly prepared Schiff’s reagent for 2 h at 20°C. After staining, the roots were washed immediately in three changes of SO2-water of 10 min each, washed several times and subsequently stored in distilled water in the refrigerator until preparation. Squash preparations in 40% acetic acid were made from the terminal 1 to 2 mm of the root tip meristem, and mounted in neutral Canada-Balsam. All slides were randomized and coded prior to measurements.

The Feulgen-DNA (arbitrary units) was determined as integrated density using the Vickers M 85 Scanning Microdensitometer. Because the nuclei were darkly stained an off-peak-wavelength (507 nm) had to be chosen. All measurements were performed on nuclei judged to be about in mid prophase (4C values). The
average of two determinations per nucleus was used, each determination consisting of two readings, one for the background and one for the specimen. Much effort was made to choose a background area that included about the same amount of cytoplasm and cell wall as the reading of the prophase nucleus and only the central part of the available field was used for all measurements. Seventeen nuclei per individual (Tables 1 and 2) or ten nuclei per individual respectively (Table 3) and five individuals per line or treatment were employed.

Results

1. The DNA Content of Different Pisum Genotypes

Table 1 summarises the 4C nuclear Feulgen DNA values for two commercial cultivars, one primitive cultivar, one weed pea (all *P. sativum*) and a closely related wild species, *P. fulvum*.

The 5 genotypes show significant differences in DNA content (*P* < 0.001, Table 2). The variance ratio for the differences between individuals within lines is also highly significant (*P* < 0.001, Table 2), showing that the randomly chosen individuals within a line are not entirely homogeneous. If the analysis of variance is repeated with the omission of the *P. fulvum* data the remaining four *P. sativum* lines are still significantly different (0.01 < *P* < 0.025) and again highly significant differences exist between individuals within lines.

Table 1. The 4C nuclear Feulgen-DNA content of wild and cultivated *Pisum* lines

<table>
<thead>
<tr>
<th>John Innes accession number</th>
<th><em>Pisum</em> line</th>
<th>4C nuclear Feulgen-DNA (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean</td>
</tr>
<tr>
<td>JI 296</td>
<td><em>P. sativum</em> var. Chemin long, cultivar</td>
<td>80.22</td>
</tr>
<tr>
<td>JI 557</td>
<td><em>P. sativum</em> var. Aurora, cultivar</td>
<td>81.83</td>
</tr>
<tr>
<td>JI 225</td>
<td><em>P. sativum</em> ssp. abyssinicum, primitive cultivar from Ethiopia</td>
<td>85.66</td>
</tr>
<tr>
<td>JI 261</td>
<td><em>P. sativum</em>, weed from Turkey</td>
<td>85.12</td>
</tr>
<tr>
<td>JI 224</td>
<td><em>P. fulvum</em>, weed from Jerusalem</td>
<td>78.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> The standard deviation is derived from the 5 means of the 5 replicate seedlings within a line.

2. Nuclear DNA Content of Excised Pea Roots Cultured in vitro

The mean Feulgen DNA values of prophase nuclei in root-tip meristems of the three treatments were compared and the results are summarised in Table 3.
Table 2. Analysis of variance of the DNA values for the five *Pisum* lines described in Table 1

<table>
<thead>
<tr>
<th>Item</th>
<th>df</th>
<th>MS</th>
<th>VR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Between lines</td>
<td>4</td>
<td>886.7110</td>
<td>7.5166***</td>
</tr>
<tr>
<td>2. Between individuals within lines</td>
<td>20</td>
<td>117.9655</td>
<td>12.5578***</td>
</tr>
<tr>
<td>3. Between nuclei within individuals</td>
<td>400</td>
<td>9.3938</td>
<td>—</td>
</tr>
</tbody>
</table>

Omitting the *P. fulvum* values:

<table>
<thead>
<tr>
<th>Item</th>
<th>df</th>
<th>MS</th>
<th>VR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Between lines</td>
<td>3</td>
<td>581.0291</td>
<td>4.1809*</td>
</tr>
<tr>
<td>2. Between individuals within lines</td>
<td>16</td>
<td>138.9716</td>
<td>15.0831***</td>
</tr>
<tr>
<td>3. Between nuclei within individuals</td>
<td>320</td>
<td>9.2137</td>
<td>—</td>
</tr>
</tbody>
</table>

* significant at the 5% level; *** significant at the 0.1% level.

Table 3. The 4C nuclear Feulgen-DNA content of cultured excised and attached pea roots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4C nuclear Feulgen-DNA (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
</tr>
<tr>
<td>(a) excised main roots cultured <em>in vitro</em></td>
<td>73.07</td>
</tr>
<tr>
<td>(b) attached lateral roots from seedlings grown under the same conditions as (a)</td>
<td>68.18</td>
</tr>
<tr>
<td>(c) attached main roots from seedlings grown in vermiculite/peat</td>
<td>77.53</td>
</tr>
</tbody>
</table>

* The standard deviation is derived from the 5 means within a treatment.

The mean DNA content of cells of excised roots, (a), was higher than that of the attached laterals, (b), but lower than the mean of the attached main roots, (c). An analysis of variance revealed significant differences between treatments (df 2,12; VR 10.254**, $P<0.01$) and again highly significant differences between individuals within treatments (df 12, 135; VR 11.835***, $P<0.001$).

Duncan's multiple-range test (Duncan, 1955) is a method of making comparisons among treatment means, and it is possible to determine which of the 3 differences among the 3 treatment means are significant. The results of the 1% multiple-range test are summarised below. The two means underscored by the same line are not significantly different.
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Discussion

1. **Nuclear DNA Content of the Five Pea Genotypes.** The values for the nuclear DNA content of the five pea lines indicate that there are significant differences between them. However, the variation of nuclear DNA content was rather small—differing by a maximum factor of 1.07 for *P. sativum* and when including *P. fulvum* of 1.10. There is no correlation between nuclear DNA content and the supposed phylogenetic relationship of the pea lines investigated. *P. fulvum* can be readily crossed with *P. sativum* when the former is used as male parent and the justification for considering them as separate species is a matter of discussion.

Few other estimates have been made of intra-specific variation in nuclear DNA. In five cultivated *Hordeum vulgare* varieties, no significant differences in the 4C nuclear DNA content were observed (Bennett and Smith, 1971) and when 17 wild *Hordeum* species were included, 7 of which had polyploid genotypes, the basic DNA content in *Hordeum* was found to be similar. Varietal differences were however observed in linseed but not in flax (Evans, 1968). The three conifer species, *Picea glauca*, *P. sitchensis* and *Pinus banksiana*, showed a remarkable intra-specific variation in nuclear DNA content (Miksche, 1968, 1971), and for two of these species a correlation was detected between DNA per cell and the geographical origin of the seeds. Again in *Vicia* (Chooi, 1971), highly significant variation in DNA content per cell was established between taxonomically synonymous species and between subspecies.

The variation in DNA content between the various *Pisum* genotypes could be due to technical shortcomings in the method used, though we consider this unlikely in view of the care taken to undertake all steps simultaneously with wholly randomised designs. If genuine, they are not attributable to changes in chromosome number, but must either reflect gross chromosomal duplications and deletions of the kind known in inter-specific variation (Rees *et al.*, 1966), or more subtle variations in degrees of amplification of certain DNA sequences. One other aspect of the DNA variation in peas is worth noting. There is a high degree of heterogeneity among individuals within a line (Tables 1 and 2), which exceeded in some cases the differences between the line means. This in itself would suggest the use of more than five seedlings in each line for similar experiments in order to overcome this variability. However, similar results have been reported by several authors (e.g. John and Hewitt, 1966; Miksche, 1971). This could be attributed to some technical artifact although the possibility of real differences between individuals is not excluded.

2. **Nuclear DNA Content in Excised Pea Roots.** In the experiments described by Abbott (1971) the DNA content of cells from excised
pea roots was claimed to be only 27% of the DNA per cell found in attached roots. However, we have re-examined the data in his Tables 1 and 2 for total cell number, total DNA content and average DNA per cell and find on the basis of the published results, that the correct ratio of DNA per cell is 1.8:1 for attached to excised roots, instead of 4:1 as claimed; in his data therefore the correct content of DNA per cell in cultured root cells is thus 56% of the amount in attached roots. In spite of this arithmetic error there remains a marked difference between the two DNA values.

As seen in Table 3 our data are at variance with these observations; the nuclear 4C Feulgen DNA content in pea root tip meristems of excised cultured roots was found to be 7.2% above the value for attached laterals grown under identical conditions, treatment (b), and 5.8% below the value for attached main roots grown in vermiculite-peat, treatment (c). We can conclude therefore that there is no significant *in vitro* loss or increase of nuclear DNA content in cultured roots (Duncan test, 1% level), though there is a significant difference between treatments (b) and (c). This difference may be real or demonstrate the limits of the method used. It is well known in Feulgen-densitometry that care must be taken to standardize all experimental conditions (e.g. Garcia, 1970); in the present experiment the roots in the three treatments were of differing ages, due to their differential growth rates, and the growth medium in (a) and (b) was not the same as in (c). The differences seen in Table 3 are thought to be mainly methodological and do not detract from the results of the first investigation (Tables 1 and 2) where all pea lines were completely randomised and grown, prepared and measured under identical conditions.

There are several possible explanations for Abbott’s results which are related to the method he used for determining average DNA content. The “10 mm root tip” he used probably contains in addition to the meristematic cells, many differentiated cells; the latter are known to have a higher DNA content in some instances (Swift, 1950; McLeish and Sunderland, 1961). Should the organisation of the terminal root segment be different in those cultured *in vitro* and grown *in vivo*, then there could well be different proportions of meristematic and differentiated cells in the two instances. As a result the mean DNA content per cell would differ. Secondly the mean nuclear DNA content even within the meristem could be changed simply by having a change in the mitotic cycle. For example, if one particular cultural condition led to an increase in the percentage of nuclei in G1, with the 2C amount of DNA, then the average DNA per cell would be lower. There remains the possibility that the DNA per cell in the pea variety “Sutton’s Onward” used by Abbott does decrease by 44% when roots are cultured
in vitro; however, until the above two and other similar technical explanations are eliminated, there is no reason for invoking the concept that substantial loss of DNA occurs in plant cells grown in vitro—a conclusion completely at variance with our experience in Pisum, and also in a range of Asparagus tissues cultured in vitro (Davies, unpublished).

The senior author wishes to thank the Schweizerischer Nationalfonds for financial support. The cooperation of Dr. A. E. Arthur in the statistical analyses is gratefully acknowledged.

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