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Genome plasticity during seed germination in *Festuca arundinacea*

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Abstract Feulgen/DNA cytophotometric determinations were carried out on early prophases in the meristems of seedlings obtained by germinating seeds of different accessions of *Festuca arundinacea* at 10°C, 20°C, or 30°C. Feulgen/DNA contents increased significantly with the increase in the temperature of seed germination. In each accession, the greater the increase in absorption in seedlings obtained at 30°C, the lower the absorption in seedlings obtained at 10°C. In contrast, Feulgen/DNA contents did not undergo changes when the temperature was altered at developmental stages other than seed germination. The results of molecular hybridizations (slot blots) indicated that the redundancy of repeated DNA sequences belonging to two families having $C_o t$ ranges of $0–2 \times 10^{-1}$ and $2 \times 10^{-1} - 2 \times 10^{0}$, respectively, was significantly higher in the genome of seedlings obtained at 30°C than in that of seedlings obtained at 10°C. When centrifuged to equilibrium in CsCl density gradients, the DNA extracted from seedlings obtained at 30°C formed a heavier and a lighter shoulder with buoyant densities of 1.707 g/ml and 1.692 g/ml, respectively, in addition to the main band (1.701 g/ml). Only a less apparent shoulder banding at 1.706 g/ml was formed by the DNA extracted from seedlings obtained at 10°C. After seed germination in the presence of [^{3}H]-thymidine for 24 h at 30°C, most of radioactivity was found in the guanine + cytosine- or adenine + thymine-enriched DNA fractions, which formed the two shoulders in the density profile. In contrast, only guanine + cytosine-enriched fractions, which formed the heavier shoulder, were preferentially labelled in the DNA from seedlings obtained at 10°C. These results prove that fluid domains do exist in the nuclear DNA of *F. arundinacea*. These DNA domains are capable of rapid, quantitative alterations, which represent the direct responses of the genome to developmental and environmental stimuli. Seed germination appears to be a limited, specific period in development within which the adaptive response to temperature variations can be put into effect.

Key words *Festuca arundinacea* · Intraspecific genomic changes · Repeated DNA sequences · Environmental adaptation · Plant development

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Introduction

There is a growing consensus of opinion at the present time that changes in the basic genome size may be not restricted to species divergence. By the mid-1980s, many reports of intraspecific variations in the amount of nuclear DNA had been published (Bennett and Leitch 1995, and references therein). It has been shown in several species that these variations are due to changes in the redundancy of repeated sequences, which alter the relative proportions of fractions of the genome and hence its organization (Ceccarelli et al. 1995). Thus, alterations in the genome size and organization within species may represent anything but exceptions, particularly in plants. Recent findings in different species have shown that these genomic changes can affect certain phenotypic characters at the cellular and organismal level (Cavallini et al. 1993; Ceccarelli et al. 1993; Natali et al. 1993; Minelli et al. 1996). Therefore, while helping to explain the extraordinary intraspecific

plasticity at various levels that is a common feature of plants, genomic alterations within species may be of special importance in vegetable organisms on account of their way of development and immobility, which demand adaptive responses to the environment.

Variations in genome size and organization have been observed within hexaploid *Festuca arundinacea*. Highly significant differences in the basic amount of nuclear DNA (up to 32.3%) occur between natural populations of this species scattered along the Italian peninsula. These differences are due to alterations in the redundancy of repeated DNA sequences, and particularly of certain families thereof. The genome sizes of the populations correlate positively with the mean temperature during the year at the stations and negatively with their latitudes. These correlations suggest that these intraspecific variations in the amount of nuclear DNA may play a role in environmental adaptation (Ceccarelli et al. 1992).

The findings obtained in *F. arundinacea* raise several questions. One of these relates to a fundamental problem of evolution. Are the genomic changes the result of a selection process, or can they be produced by direct responses of the genome to the environmental stimulus (Nagl 1990)? On account of this problem, and with the aim of further characterizing the genomic plasticity observed in *F. arundinacea*, we carried out further research. The results obtained are reported in this paper.

Materials and methods

Plant material

Seeds (caryopses) of *Festuca arundinacea*, collected from different natural populations, or of the cultivar 'Sibilla', were germinated on wet paper in petri dishes under sterile conditions at 10°C, 20°C, or 30°C, and the seedlings were grown at these temperatures for different durations of time in different combinations of experimental conditions. The seedlings, or embryos excised from seeds obtained from plants grown under experimental conditions, were fixed in ethanol-acetic acid 3:1 (v/v) or in 10% neutral formalin for cytophotometric analyses. Other seedlings were used for DNA extraction. A portion of these was fed with a 55.5 GBq/m*M* aqueous solution of [^{3}H]-thymidine (Radiochemical Centre, 925 GBq/m*M*) for different durations of time.

DNA cytophotometry

Root or shoot apices were squashed under a coverslip in a drop of 45% acetic acid after treatment with a 5% aqueous solution of pectinase (Sigma) for 1 h at 37°C. The coverslips were removed by the solid CO_2 method, and the preparations were Feulgen-stained after different hydrolysis durations in 1 *N* HCl at 60°C: 8 min for those made with materials fixed in ethanol-acetic acid, and 20 min for those made with formalin-fixed materials. After staining, the slides were subjected to three 10-min washes in SO_2 water prior to dehydration and mounting in DPX (BDH Chemicals). Since simultaneous processing was not possible due to the large number of preparations to be analyzed, squashes made with the root tips of a single plantlet of *Vicia faba* were concurrently stained for each group of slides and used as standards in order to make the results comparable. All notable differences in Feulgen/DNA absorption between preparations observed using the above method of comparison were further checked by analyzing preparations that were made again and processed all together. Feulgen/DNA absorptions in individual cell nuclei were measured, at the wavelength of 550 nm, using a Leitz MPV3 microscope photometer equipped with a mirror scanner.

DNA extraction

DNA was extracted from seedlings which were pulverized with a pestle and mortar in the presence of liquid nitrogen and homogenized in 5 ml of a pH 8.0 buffer containing 0.1 *M* TRIS-HCl, 0.005 *M* EDTA, 0.5 *M* NaCl and 0.01 *M* mercaptoethanol. DNA extraction and purification were performed as described by Maggini et al. (1978). The isolated DNA was then suspended in TE buffer (0.01 *M* TRIS-HCl pH 8.0 plus 0.001 *M* EDTA, pH 8.0) and stored at 4°C.

DNA fractionation

DNA was fractionated at different Cot values by first solubilizing it in 0.12 *M* Na phosphate buffer pH 7.0 and then shearing by sonication in an MSE sonicator at medium energy output for 5×5 s with 10-s intervals at 4°C. The DNA was then denatured for 10 min at 103°C, allowed to reassociate according to Britten et al. (1974) up to the desired Cot values, and fractionated by elution through a hydroxylapatite column equilibrated in the same buffer as above. Single-strand DNA was eluted with this same buffer, and reassociated sequences were recovered by elution with 0.5 *M* Na phosphate buffer.

For density gradient fractionation, CsCl was added to the purified DNA solubilized in water up to an initial density of 1.706 g/ml, and centrifugation was carried out in a Beckmann L5-65 ultracentrifuge at 20°C for 96 h at a speed of 25,000 rpm using the Ti65 rotor. The gradient was fractionated (1 ml per fraction), and absorbance was recorded at 258 nm by a Shimadzu UV-2101PC spectrophotometer. Radioactivity was counted in a Packard Tricarb scintillator after adding 5 ml of Insta-gel (Packard) to 50 µl of each DNA fraction.

DNA blotting

For slot DNA blots, 1-µg DNA samples were denatured by heating at 37°C for 10 min in 0.5 *M* NaOH, then neutralized by the addition of an equal volume of 2 *M* ammonium acetate. Scalar dilutions of DNA from 0.5 to 0.0625 µg were loaded on nylon filters (Hybond-N, Amersham) using a commercial slot-blotting apparatus (Minifold II, Schleicher and Schuell). The fractions of genomic DNA obtained by reassociating it at different Cot values as described above were used as probes after having been labelled with digoxigenin-11-dUTP by a DIG-DNA labelling kit (Boehringer). Hybridization was performed according to Sambrook et al. (1989). Filters were then washed sequentially in $2\times$, $1\times$, and $0.3\times$ SSC containing 0.05% SDS at 65°C, and hybridization was detected by a DIG-DNA detection kit (Boehringer). Filters were scanned in a Vernon PH1-type densitometer, and the tracings were used for quantitative determinations. As a control, all filters were rehybridized with *Pst*I maize alcohol dehydrogenase cDNA cloned into pBR322 vectors (Dennis et al. 1984) and labelled with a [^{32}P]-dCTP Random Primer Kit (Amersham). Filters were then autoradiographed and scanned as above.

Results

Cytophotometry

Cytophotometric determinations of Feulgen absorptions of early prophases were carried out in the meristems of seedlings grown at different temperatures. The results obtained on materials fixed in ethanol-acetic acid were always comparable to those obtained on formalin-fixed materials. Significant differences were never observed between the Feulgen absorptions in the root and shoot meristems of one and the same plant (not shown).

Feulgen absorptions in seedlings obtained by germinating seeds of seven natural populations and of cv 'Sibilla' at 10°C, 20°C, or 30°C increased with increasing temperature. When the absorption values in seedlings obtained at 10°C were compared to those obtained at 30°C, the differences were always significant (Fig. 1). However, Feulgen absorptions did not increase with the increase in the temperature of seed germination to the same extent in all accessions. In each accession, the greater the increase in absorption in seedlings obtained at 30°C the lower the absorption in seedlings obtained at 10°C. Indeed, a highly significant, inverse correlation was found to exist between the absorption values in the latter seedlings and their increase in the former (Fig. 2).

Feulgen/DNA contents did not undergo changes when the temperature was altered at developmental stages other than seed germination. Indeed, Feulgen absorptions did not increase when seedlings obtained by germinating seeds at 30°C were grown at this temperature for 15 days instead of 5 days Feulgen absorptions did not differ either between seedlings grown for 15 days at 10°C or 30°C after seed germination at 10°C, or between seedlings grown at 10°C or 30°C after seed germination at 30°C (Fig. 3). Embryos in dry seeds of plants which had been grown and allowed to bear fruit at 10–15°C or 25–30°C after seed germination at 20°C showed comparable Feulgen/DNA contents. Indeed, Feulgen absorptions of DNA presynthetic (G_1) nuclei in their root apices were practically the same (14.94 ± 0.34 and 14.53 ± 0.61 arbitrary units, respectively).

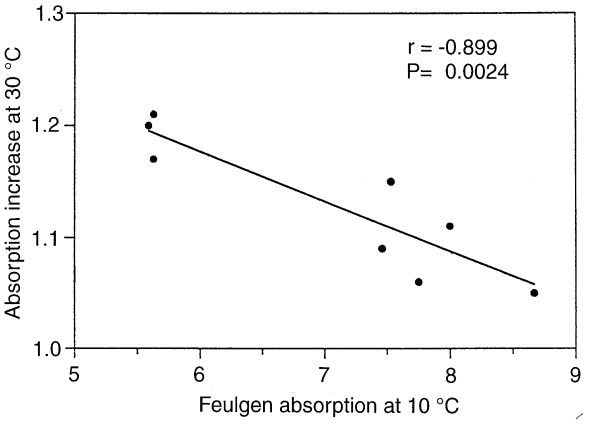


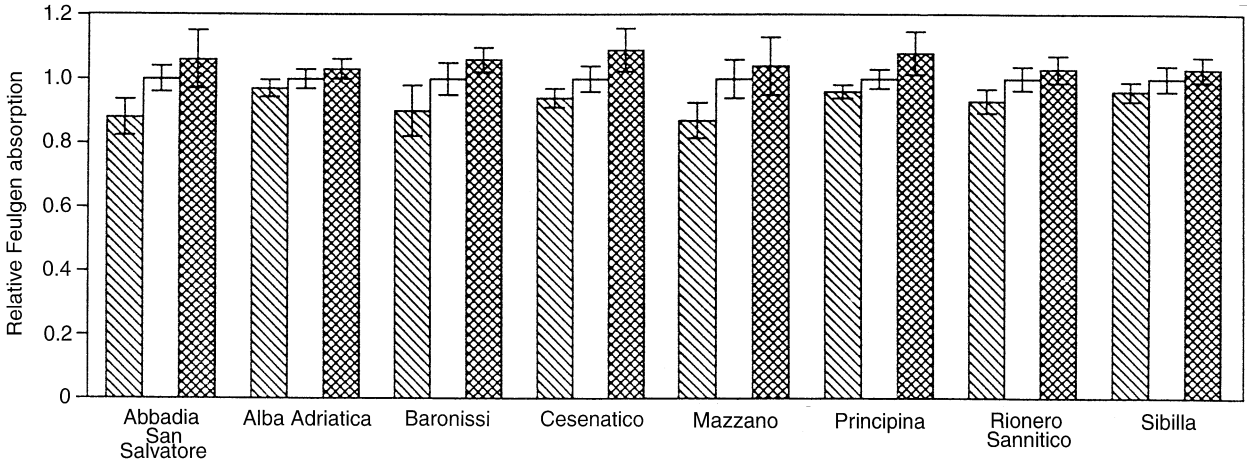
Fig. 2 Correlation, in the accessions studied, between the Feulgen absorptions in seedlings obtained at 10°C and the absorption increases in seedlings obtained at 30°C (ratios between the absorptions at 30°C and 10°C). The data given in Fig. 1 were used

Fig. 1 Mean Feulgen absorptions of early prophases (= 4C) in the meristem of 3-mm-long seminal roots of seedlings obtained by germinating seeds at 10°C (▨), 20°C (□), or 30°C (▩). Seeds collected from seven natural populations and from cv 'Sibilla' were used. The values, which were normalized (□ = 1) to facilitate comparisons, are means of those obtained by analyzing 20 prophases in each of 5 seedlings. Confidence limits at $P < 0.01$

Molecular hybridization

The results of molecular hybridization experiments provided evidence that changes in the redundancy of given DNA sequences actually occurred in the genomes of seedlings obtained from seeds germinated at different temperatures.

Three probes were obtained by fractionating the nuclear DNA of *F. arundinacea* on the basis of previous



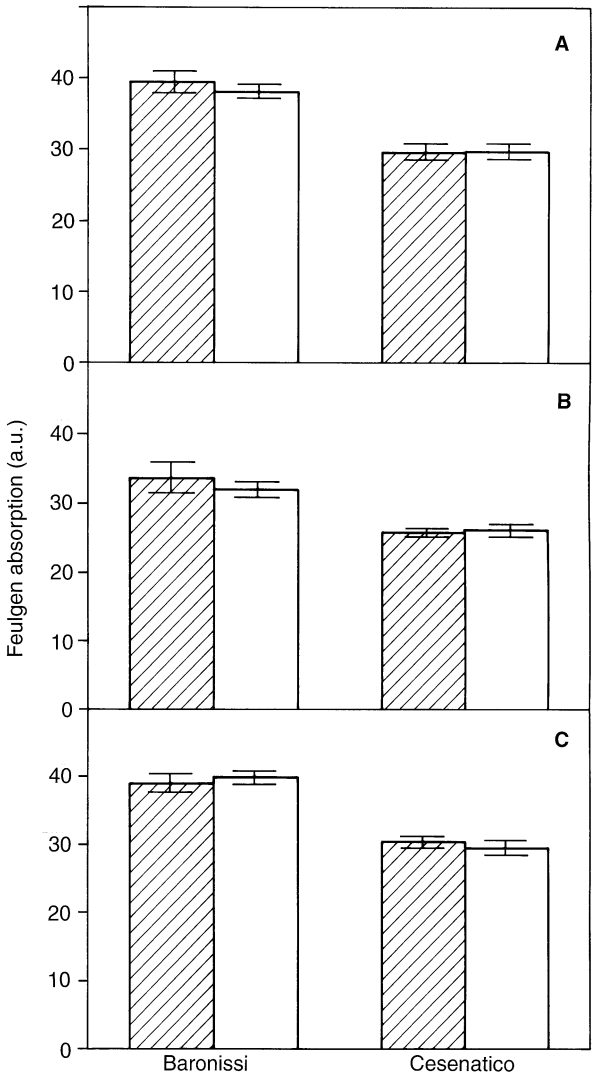


Fig. 3A–C Mean Feulgen absorptions of early prophases in the meristem of the seminal root of seedlings obtained and grown under different conditions. **A** Seeds germinated at 30°C and seedlings grown at this temperature for 5 days (▨) or 15 days (□); **B** Seeds germinated at 10°C and seedlings grown for 15 days at 10°C (▨) or 30°C (□); **C** Seeds germinated at 30°C and seedlings grown for 15 days at 10°C (▨) or 30°C (□). Seeds collected from the populations at Baronissi and Cesenatico were used. Each value is the mean of those obtained by analyzing 20 prophases in each of 5 seedlings. Confidence limits at $P < 0.01$

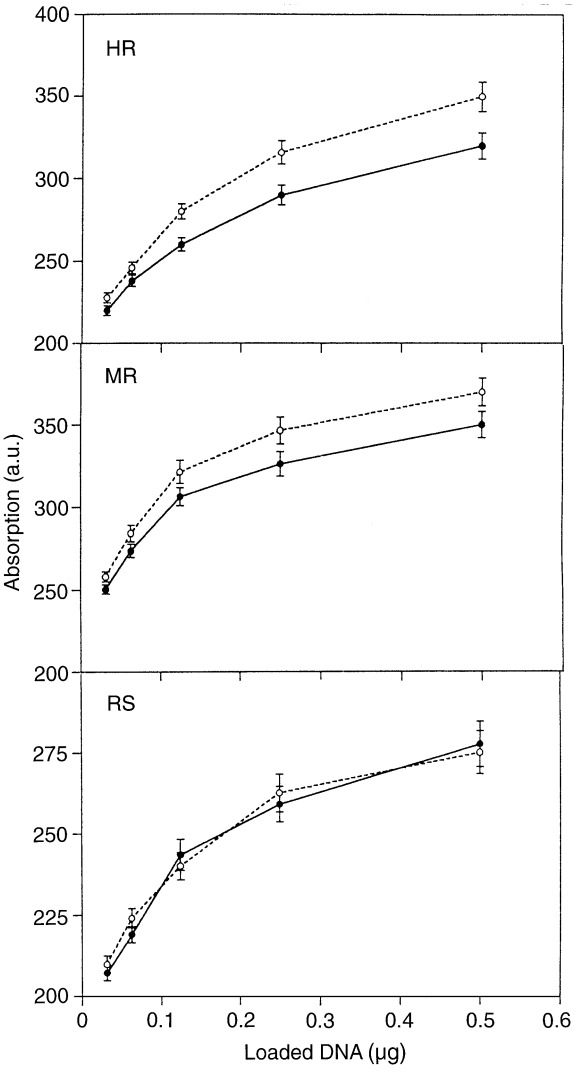


Fig. 4 Densitometric absorptions of slot-blot filters loaded with different amounts of genomic DNA from seedlings of cv 'Sibilla' obtained by germinating seeds at 10°C (●——●) or 30°C (○- - -○). Filters were probed with the DNA fraction that reassociated within an equivalent Cot of 2×10^{-1} (HR), the fraction having a Cot range of $2 \times 10^{-1} - 2 \times 10^{0}$ (MR), or the remaining sequences (RS). Each value is the mean of those obtained in six repetitions, three for each of two DNA extractions. Confidence limits at $P < 0.01$

findings obtained by studying its reassociation kinetics (Ceccarelli et al. 1992). One fraction represented highly repeated sequences (HR; reassociation within an equivalent C_0t of 2×10^{-1}); a second one represented medium repeated sequences (MR; C_0t range $2 \times 10^{-1} - 2 \times 10^{0}$); and a third fraction consisted of the remaining sequences in the genome (RS). The results of slot-blot hybridizations carried out using these DNA probes showed that both HR and MR hybridized to a larger extent to the genomic DNA from seedlings of cv 'Sibilla' obtained at 30°C than to that from seedlings obtained at 10°C, whereas no difference was found between the two DNAs when RS were hybridized (Fig. 4). Figure 5 provides a comparison of the levels of hybridization at saturation. It can be seen that the difference in the redundancy of repeated sequences between the genomes of seedlings obtained at 10°C or 30°C was particularly large in the case of the HR family.

Gradient ultracentrifugation

Evidence for the occurrence of preferential DNA syntheses in germinating embryos of *F. arundinacea* was

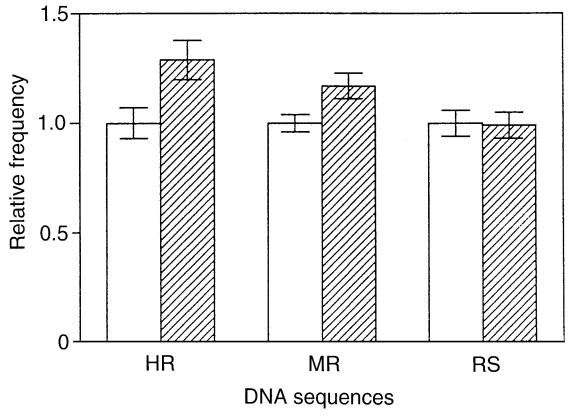


Fig. 5 Histograms representing the means of the values obtained by scanning densitometrically slot-blot filters loaded with genomic DNA from seedlings of cv 'Sibilla' obtained by germinating seeds at 10°C (□) or 30°C (▨). The filters were probed at saturation, in six repetitions (three for each of two different DNA extractions), with different fractions of DNA as in Fig. 4. The values were normalized (□ = 1) to facilitate comparisons. Confidence limits at $P < 0.01$

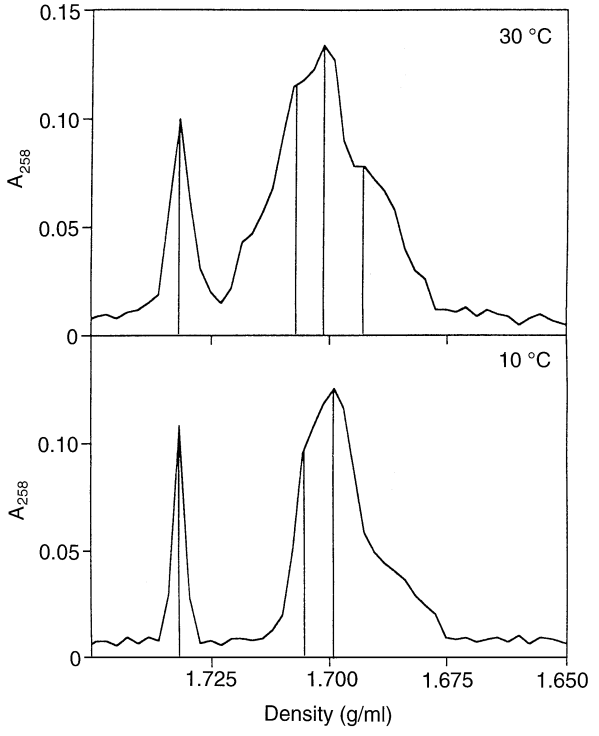


Fig. 6 Ultracentrifugation in CsCl gradients of the DNA extracted from seedlings obtained by germinating seeds of the cv Sibilla at 10°C or 30°C. The density marker is *Micrococcus lysodeikticus* DNA (1.731 g/ml)

attained by studying their DNA through density gradient fractionation.

When centrifuged to equilibrium in a CsCl density gradient, the DNA from seedlings obtained at 30°C formed a heavier and a lighter shoulder with buoyant densities of 1.707 g/ml and 1.692 g/ml, respectively, in addition to the main band with a buoyant density of 1.701 g/ml. On the contrary, only a less apparent shoulder banding at 1.706 g/ml was formed, in addition to the main band (1.699 g/ml), by the DNA from seedlings obtained at 10°C (Fig. 6).

After seed germination at 30°C in the presence of [^{3}H]-thymidine, most of the radioactivity was found in the DNA fractions enriched in guanine + cytosine or adenine + thymine, which formed the two shoulders in the density profile. An indication that extra DNA synthesis started very early during seed germination was the observation that [^{3}H]-thymidine incorporation as above described was already apparent 24 h after seed imbibition (Fig. 7). [^{3}H]-thymidine incorporation in the DNA from seedlings obtained at 30°C and in that from seedlings obtained at 10°C was compared. A somewhat unexpected result was that preferential labelling of certain fractions also occurred in the DNA from the latter seedlings. However, when seeds germinated at 10°C, only guanine + cytosine-enriched sequences that formed the heavier shoulder in the density profile underwent extra synthesis (Fig. 8).

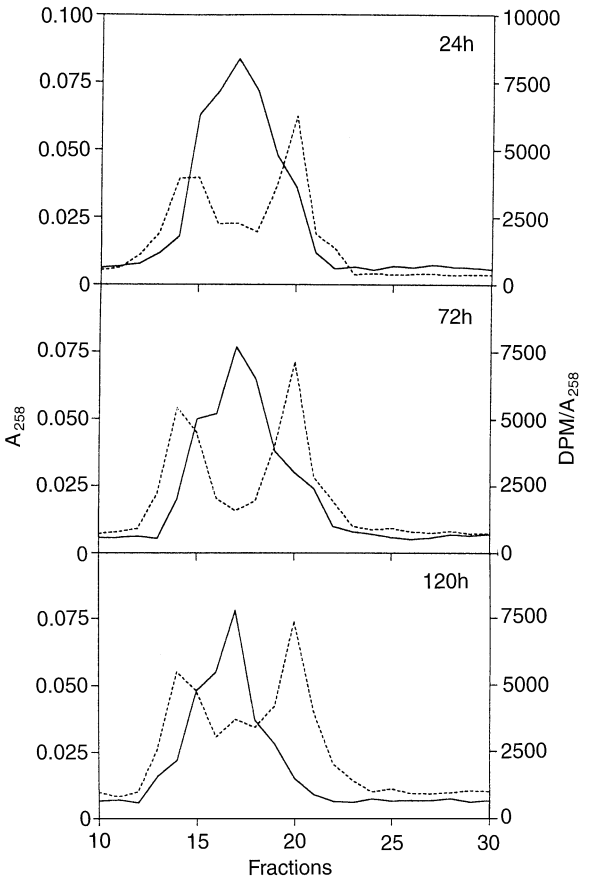


Fig. 7 Optical density (——) and ratio between disintegrations per minute and optical density (- - - -) in fractions obtained by CsCl density gradient ultracentrifugation of the DNA extracted from seedlings of cv 'Sibilla'. The seeds were germinated at 30°C and the seedlings were grown at this temperature for different durations of time in a 55.5 GBq/m*M* aqueous solution of [^{3}H]-thymidine

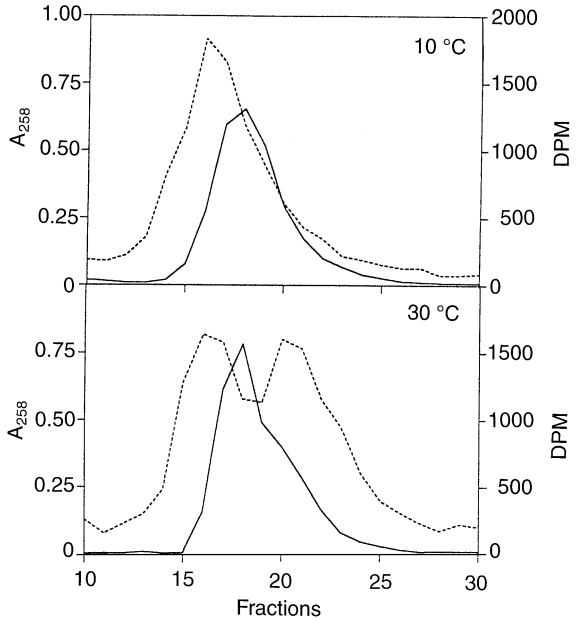


Fig. 8 Optical density (——) and radioactivity (- - - -) in fractions obtained by CsCl density gradient ultracentrifugation of the DNA extracted from seedlings of cv 'Sibilla' at the developmental stage when the first foliage leaf was 7–9 cm in height. The seeds were germinated and the seedlings were grown at 10°C or 30°C in a 55.5 GBq/m*M* aqueous solution of [^{3}H]-thymidine

Discussion

In line with previous findings (Ceccarelli et al. 1992), our present results prove that adaptable, fluid domains do exist in the nuclear DNA of *F. arundinacea*. These DNA domains are capable of rapid, quantitative variations, which represent direct responses of the genome to developmental and environmental stimuli. Data obtained using different methods of investigation have shown that the resumption of embryo development with seed germination is accompanied by extra synthesis of G+C-enriched DNA sequences and that heat-induced preferential synthesis of other repeated sequences which are enriched in A+T base pairs may occur at this developmental stage.

As far as the latter DNA amplification is concerned, *F. arundinacea* may be added to other species in which genome alterations in response to environmental stress have been documented (Cullis 1990). Thus, our findings represent a further piece of evidence against the view, which is still largely accepted, that the effect of the environment on an organism is as a rule physiological, with the genome remaining unchanged. In this connection, it seems worth stressing that the more the DNA content increases in seedlings obtained at 30°C, the lower is its amount in seedlings obtained at 10°C (Fig. 2). This observation suggests the existence, in the species we studied, of optimal genome sizes for seed germination and plant development at given temperatures.

The biochemical data confirm those obtained by cytophotometry and rule out the possibility that the shifts in Feulgen absorptions in seedlings obtained at higher temperatures are due to changes in the structure of the chromatin and/or in its protein component (Mello and Vidal 1980) owing to the binding of heat shock proteins (Nover 1984). This possibility appeared to be remote even before the present investigation since it was known that plants synthesize heat shock proteins after seed germination also (Cremonini et al. 1992), while Feulgen absorptions do not change in our material when the temperature is raised after this stage in development (Fig. 3). Apart from this, Cremonini et al. (1992) showed that the exposition of *Vicia faba* seedlings to high temperatures did not affect Feulgen absorptions, even if the pattern of nuclear proteins and the eu/heterochromatin ratio were altered. Therefore, our cytophotometric findings appear reliable in indicating that the heat-induced amplification of DNA sequences that can occur during early seed germination is not feasible at any other developmental stage. The adaptation of *Sorghum bicolor* to salinity may represent another example of the existence of a limited time in development within which an adaptive response to environmental stress can be put into effect. Indeed, adaptation only occurs when exposure of the plants to NaCl is initiated between 5 and 10 days following germination. In this instance, it is still unknown whether genomic changes are involved; however, salt tolerance has been shown to be heritable (Amzallag et al. 1993).

Our results also suggest that amplification of G+C-enriched DNA sequences occurs during early seed germination independently of the temperature at which it takes place (Fig. 8). Rapid genomic changes have been suggested to characterize different developmental processes and stages in many species (Cionini 1989; Bassi 1990; Nagl 1990). In particular, other findings obtained in graminaceous species indicate that seed germination may be a step in development during which fluid DNA domains are particularly prone to variations. Alterations in the redundancy of ribosomal cistrons and other sequences have for some time been reported in the resting DNA during the early germination of wheat embryos (Chen and Osborne 1970). The synthesis of DNA sequences not related to either reduplication or repair has been found to occur in germinating cereal seeds (Bucholc and Buchowicz 1992, and references therein). In *Dasypyrum villosum*, seed germination is accompanied by the amplification of DNA sequences, some of which have been shown to be highly repeated (Frediani et al. 1994).

Repeated, in particular highly repeated, sequences are involved in the alterations in the nuclear DNA that we observed (Figs. 4 and 5). There is now stringent evidence for a dominant role of non-coding DNA in

rapid genomic changes which may occur during development and in response to environmental stimuli Bassi 1991). By producing changes either in the regulation of structural genes or in the nucleotype (Bennett 1985), intraspecific alterations of sequence redundancy in repetitive fractions of the genome can affect developmental dynamics and phenotypic characters at the cellular and organismal level (see Introduction). Indeed, mitotic cycle time, seed germination power, early growth rate of the seedlings, height of the culm and other quantitative characters of the plant, and flowering time actually differ in *F. arundinacea* plants with differing genome sizes (Ceccarelli et al. 1993). The ability to carry out alterations in the nuclear genome and then in the nucleotype may represent an evolutive character which allows this species optimal growth dynamics in relation to different developmental stages and environmental conditions, and may contribute to explain its distribution in a wide geographical area (North Africa, West Central Asia, most of Europe, Iceland and the Faeroes and Spitsbergen Islands; Markgraf-Dannenberg 1980).

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