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The nucleus of differentiated root plant cells: modifications induced by arbuscular mycorrhizal fungi

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SUMMARY

The nuclei of plant cells show marked differences in chromatin organisation, related to their DNA content, which ranges from the type with large strands of condensed chromatin (reticulate or chromonematic nuclei) to one with mostly decondensed chromatin (chromocentric or diffuse nuclei). A loosening of the chromatin structure generally occurs in actively metabolising cells, such as differentiating and secretory cells, in relation to their high transcriptional activity. Endoreduplication may occur, especially in plants with a small genome, which increases the availability of nuclear templates, the synthesis of DNA, and probably regulates gene expression.

Here we describe structural and quantitative changes of the chromatin and their relationship with transcription that occur in differentiated cells following an increase of their metabolism. The nuclei of root cortical cells of three plants with different 2C DNA content *(Allium porrum*, *Pisum sativum* and *Lycopersicon esculentm)* and their modifications induced by arbuscular mycorrhization, which strongly increase the metabolic activity of colonised cells, are taken as examples.

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INTRODUCTION

Angiosperms show considerable variation in genomic DNA content: 1C-values can differ more than 600-fold, ranging from less than 0.2 pg in *Arabidopsis thaliana* to 127.4 pg in *Fritillaria assyriaca* (Bennet and Leitch 1995). Much of the diversity in the nuclear genome size does not seem to be correlated with the complexity of the organisms (C-value paradox – Thomas, 1971), as variations of the C-value involve an increase of the proportion of repeated DNA sequences. Gymnosperms and some Monocotyledons contain an enormously high amount of DNA, and repeated sequences may account for even more than 90% of the nuclear DNA content in the Angiosperms with the largest C-value (Flavell, 1980). However, every organism, even those with a small genome, has a spectrum of repeated DNA components. Many of them are presumably non-genic and not transcribed (Bennet and Leitch 1995) and are probably involved in the spatial distribution of the chromosomes in the interphasic nucleus (Hemleben, 2000).

The nuclei of plant cells show marked differences in chromatin organisation which seems to depend on the different amounts of repetitive and genomic DNA. Chromatin structure, as shown by electron microscopy, ranges from the type observed in

reticulate or chromonematic nuclei, where strands of condensed chromatin form a reticulum and fill the entire nuclear volume, to that of nuclei with mostly decondensed chromatin and few patches of condensed chromatin, the chromocentres, associated to the nucleolus and nuclear envelope (diffuse or chromocentric nuclei) (Jordan *et al.*, 1980; Nagl, 1985; Vazquez-Nin *et al.*, 1992; Moreno Diaz de la Espina *et al.*, 1992). However, chromocentres are not an exclusive feature of the latter type of nuclei and they may be present independently of the degree of chromatin condensation (Barlow, 1977). With few exceptions, chromocentric nuclei have a DNA content of lower than 5 pg, while reticulate nuclei are typical of plants with a high DNA content (Barlow, 1977).

Studies carried out on hybrid plants of barley showed that pairs of homologue chromosome arms may show similar patterns of coiling or condensation, regardless of their physical separation in the nucleus. Therefore, condensation is probably a property of chromatin structure related to genes or to DNA sequences, which include protein binding and nuclear scaffold attachment sites (Heslop-Harrison *et al.*, 1993). Nevertheless, other factors, such as cell cycle stage, nuclear volume and mitotic chromatin length appear to interrelate and determine the nucleus type (Moreno Diaz de la Espina *et al.*, 1992).

Despite of the above mentioned differences in the chromatin organisation of plants, it has long been believed that all the nuclei of mature cells of a given plant species exhibit the same amount of condensed chromatin, independent of their transcriptional activity, with the exception of some highly specialised cells such antipodal cells or microsporocytes (Nagl *et al.*, 1983). By contrast, mammalian nuclei show prominent differences in chromatin condensation. These display patterns of condensed chromatin which are not species-specific but are characteristic of precise stages of development and definite tissues and cells, and are related to templates and gene activity (Nagl 1985 and references therein). The misleading interpretation in plants probably depends on the fact that only a small percentage of plant DNA represents genes in the active state, while most of the DNA is composed of noncoding and repetitive sequences (Nagl 1985). Thus, the conformational changes linked to nuclear activity, which may concern only the active fraction of chromatin, are not always easily visible; however, they do occur in plants so that modulation of the nuclear structure could provide a sensitive method for the control of a gene expression (Heslop-Harrison *et al.*, 1993). Chromatin structure varies in the different tissues of the maize root apex, with a close relation existing between RNA synthesis, nuclear chromatin structure and plant cell size (Baluska and Kubica 1992). A loosening of the chromatin structure generally occurs in actively metabolising cells, such as differentiating cells (Darzynkiewicz *et al.*, 1984, 1985; Biradar and Rayburn, 1994), secretory cells (e.i. *Mentha x piperita* glandular trichomes, Berta *et al.*, 1993) and in drug-treated cells (Evenson *et al.*, 1985).

Endopolyploidy, a very common phenomenon in plants (D'Amato, 1998), is developmentally regulated according to the age, position and function of the tissue within the plant (De Rocher *et al.*, 1990; Galbraith *et al.*, 1991), and only in some families, especially *Compositae* and *Umbelliferae*, does tissue differentiation occur in a diploid state (D'Amato, 1998). According to some authors, increases in ploidy levels are related to increases in cell size (Galbraith *et al.*, 1991; Melaragno *et al.*, 1993). Plant cells with small genomes (among which polyploidy is more common) may increase the number of copies of their "useful" genes more simply by redoubling their entire genome rather than by selective gene amplification (Galbraith *et al.*, 1991).

Polyploidization is also involved in the regulation of gene expression. During replication, the structure of both active and repressed chromatin is disrupted, and the entire nuclear organization can be rearranged after each replication event (Baluska *et al.*, 1995; Wolffe, 1991). In addition, ploidy is able to control gene expression, inducing or repressing genes in relation to the number of chromosome sets (Galitski *et al.*, 1999; Mittelsten Scheid *et al.*, 1996).

Some evidence exists that somatic polyploidization in plants is inversely related to genome size (De Rocher *et al.*, 1990): multiplication of the DNA content is very important for the differentiation of plants with small genomes. Infact, it has been suggested that certain specialised cells require a minimal mass of nuclear DNA in order to maintain specific regulatory and functional states (Galbraith 1991), and that there is a direct proportionality between nuclear DNA level, cell size (Melaragno *et al.* 1993) and the number of

plastids and mitochondria (Galbraith 1991; D'Amato 1998). These considerations indicate that nucleus structure and ploidy can undergo modifications related to changes in cell metabolic activity depending, at least in part, on the nuclear DNA content of 2C nuclei. In order to study structural and chromatin changes and their relations with transcription in differentiated cells, we have analysed the changes induced by arbuscular mycorrhizal colonization on the nuclei of root cortical cells, which strongly increase their metabolic activity following hyphal penetration.

Arbuscular mycorrhizae are stable, mutually beneficial, plant-fungus associations in which the fungus develops in two distinct functional phases: the intraradical phase with intercellular hyphae and specialized intracellular structures, arbuscules, involved in exchange of substances between the two partners; the extraradical phase, extending from the root into the soil, which channels water and minerals, mainly phosphorus, to the plant. Carbon compounds are supplied by the plant to the fungus. The result is improved growth of the plant, and the completion of the life cycle of the fungus. During arbuscule formation, cortical cell size increases and host cell walls undergo a significant reduction in thickness (A. Fusconi unpublished results), amyloplasts disappear, the central vacuole fragments and the cytoplasm volume and number of organelles increase. The configuration of microfilaments changes from a loose cortical and perinuclear network in uninfected cells to a dense reticulum surrounding the branches of the arbusculus and the nucleus (Fig. 1). The microtubules cross the cytoplasm and bridge the fungal branches, perhaps creating a rigid structure around the fungus as a complement to the deposition of new cell wall components (Genre and Bon-

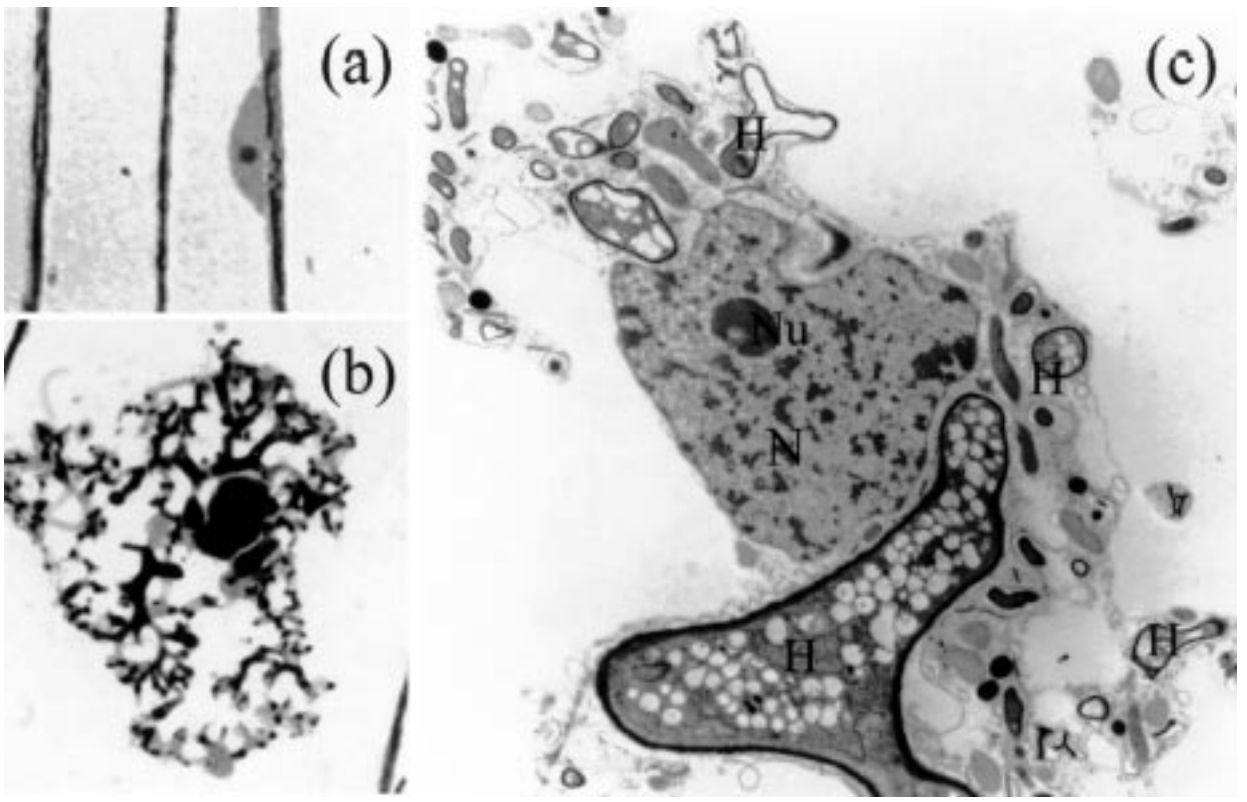


Fig. 1 - a-b, light microscopy, longitudinal sections of root cortical cells. **a** (1820x), non-colonised cells with a large vacuole filling most of the total cell volume, the nucleus is adjacent to the cell wall. **b** (760x), arbuscule hosting cell, the nucleus is in central position, entrapped by the arbuscular branches. **c** (5620x), T.E.M., arbuscule hosting cell, hyphae are surrounded by the host plasma membrane. H, arbuscular hyphae, N, nucleus; Nu, nucleolus.

fante, 1997). The plasma membrane invaginates and grows around the developing arbuscule, and its activities are modified with respect to those of the plasma membrane of uncolonized cells (e.g. increased ATPase activity). New wall components are contemporaneously deposited between fungal wall and host membrane, giving rise to a new compartment, the symbiotic interface of high molecular complexity (Balestrini *et al.*, 1997; Bonfante and Perotto, 1995).

This laboratory has studied the effect of mycorrhizal colonization in plants with different DNA content of their 2C nuclei: *Allium porrum* L. cv. Mostruoso di Carentan; *Pisum sativum* cv. Lincoln and cv. Frisson; and *Lycopersicon esculentum* cv. Early Mech. *A. porrum* and *P. sativum* possess reticulate nuclei (Fig. 2; Fig. 3a-d); however, the DNA content is much higher in *A. porrum* (about 45 pg, Bennet and Leich 1997) than in *P. sativum* (9.1 pg, Bennet and Leitch 1995). As a consequence, the nuclei of the two species present different degrees of chromatin condensation in the meristematic interphasic nuclei. The nucleus of *A. porrum* was initially classified as eureticulate and that of *P. sativum* as reticulate (Barlow 1977), by Delay (1947) who described four major types of nuclear chromatin organization: eureticulate, reticulate, semireticulate and areticulate, with a progressive reduction in the density of the chromatin reticulum. The nuclei of *L. esculentum* are classified as chromocentric, areticulate or diffuse according to Moreno Diaz de la Espina *et al.* (1992) since they have low DNA content (about 2 pg, Bennet and Leich 1995) and the chromatin reticulum typical of reticulate nuclei is not observed, even in the early G1 and late G2 periods, when chromatin appears more condensed (Fig. 3e-f).

Several flow cytometric and microscopic techniques have been applied to the above mentioned systems. Light and electron microscopy combined with immunocytochemistry have been used to study the state of the nuclei and their chromatin *in situ*. Flow cytometry was performed on nuclei extracted from the entire root system deprived of the apices and stained with saturating concentrations of the fluorescent dye DAPI to evalutate quantitative DNA changes (for details about the protocol see Lingua *et al.*, 2001). To evaluate chromatin accessibility, and therefore the degree of chromatin condensation, some samples were analysed after DNAse I treatment (Weintraub and Groudine, 1976), or stained with propidium iodide or with under-saturating DAPI concentrations (Mazzini *et al.*, 1983). Nuclei extracted from specific zones of tomato roots were also analysed (see later for details). Flow cytometry permits the analysis of a large number of nuclei in a short time, although being a destructive technique. For this reason, microfluorimetry has been applied to some root zones to get information on morphology and histology.

Two examples of reticulate nuclei, as influenced by arbuscular mycorrhizal infection, in differentiated root parenchyma cells: *Allium porrum* and *Pisum sativum* nuclei

In uninfected root parenchyma cells of *Allium porrum*, the nuclei, lenticular in shape, are in lateral position and show the chromatin organised in large patches, with a higher degree of condensation than in the meristem: during differentiation, there is a progressive increase in chromatin condensation in control roots (Berta and Squadrone, 1993). This is in agreement with the results of Biradar and Rayburn (1994), who showed significant differences in chromatin condensation among the diploid nuclei from different plant organs (Fig. 2a-b; Fig. 3a).

In mycorrhizal *A. porrum* plants, nuclei are generally round, larger than in controls and in central position (Berta and Fusconi, 1998; Balestrini *et al.*, 1992). No difference is found in ploidy level between mycorrhizae and controls, and nuclear hypertrophy can be explained by chromatin decondensation (Fig. 3b) (Berta *et al.*, 1990).

The central position has been related to microtubule reorganization following mycorrhizal infection (Balestrini *et al.*, 1992); this is in line with the results of by Baluska *et al.* (1995) on the relationship between chromatin decondensation and microtubule reorganization after infection with the pathogenic fungus *Erisiphe graminis* in barley and defense gene activation. The same situation has been observed in suspension-cultured parsley cells inoculated with *Phytophthora infestans* (Gross *et al.*, 1993). In arbuscular mycor-

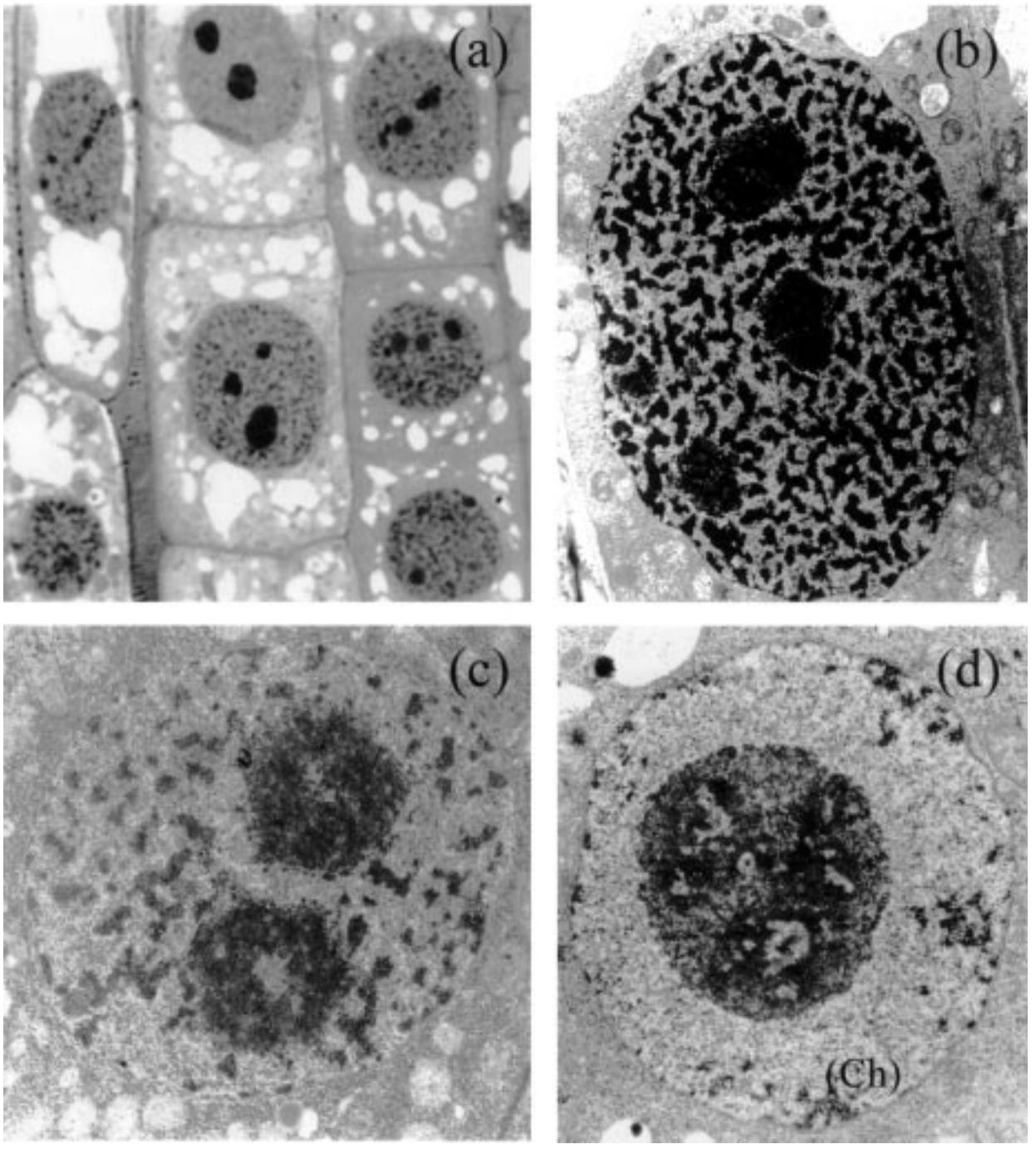
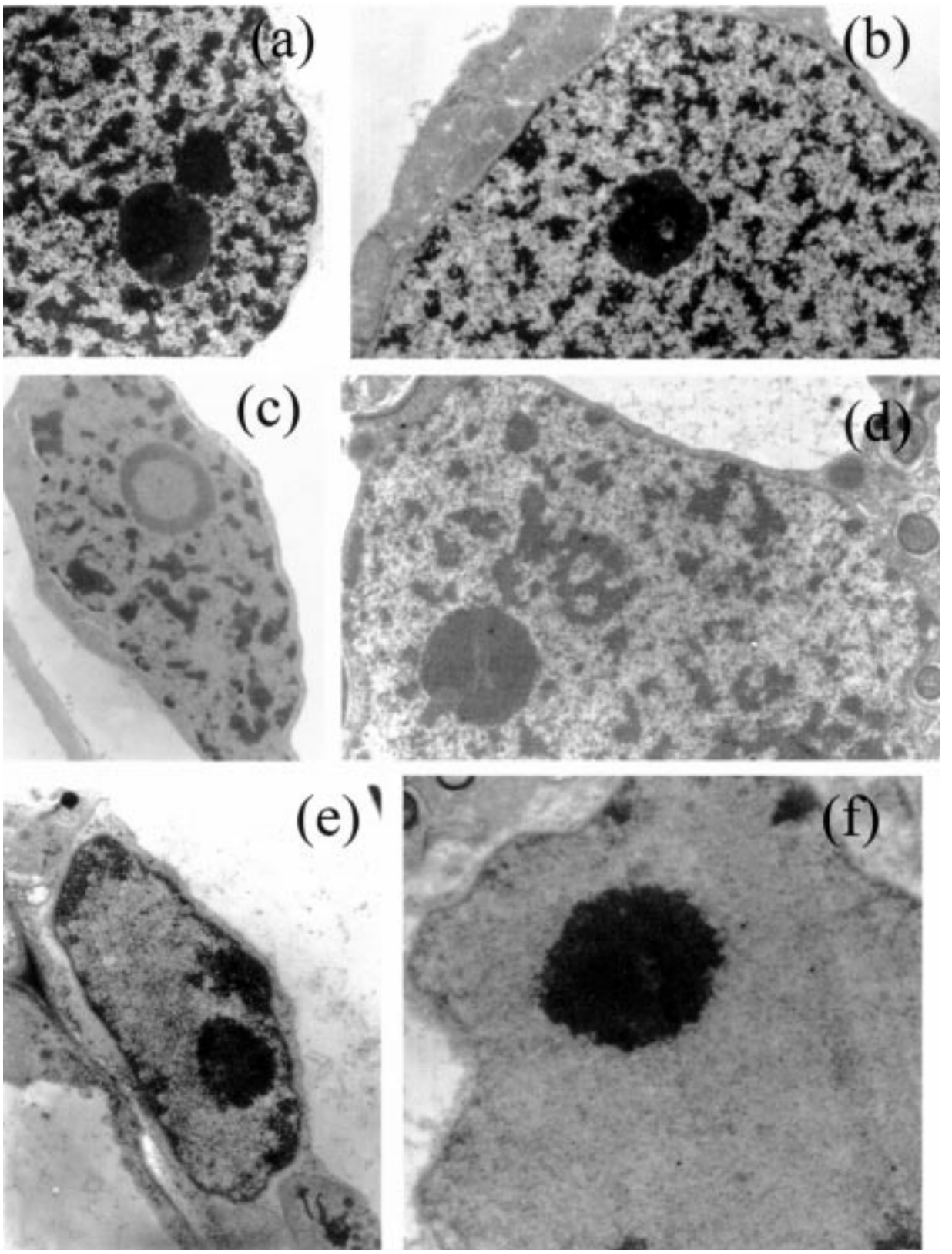


Fig. 2 - a (2740x), light microscopy, meristematic cells of a root apex of *Allium porrum* L. The cytoplasm has little vacuoles and the nuclei are centrally localized in the cells. **b-d**, T.E.M. of meristematic interphase nuclei. **b** (5240x), reticulate nucleus of *A. porrum* characterized by a coarse network of strands of condensed chromatin, interspersed with regions of fine chromatin fibrils. **c** (5010x), Reticulate nucleus *of Pisum sativum*. The chromatin organization is similar to that of *A. porrum* even if the proportion of condensed chromatin is lower, in relation to the lower DNA content. **d** (10880x), chromocentric nucleus of *Lycopersicon esculentum*. The chromatin is mostly decondensed with homogeneous appearance, dense patches of chromatin (chromocentres, Ch) are associated to the nucleus envelope.


(a)
(b)
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rhizae, defense genes are only weakly and transiently activated, but the expression of many other genes as consequence of the infection is enhanced (Gianinazzi *et al.*, 1996), or induced (Tahiri-Alaoui and Antoniw, 1996). Moreover, a reorganization of cytoskeleton following fungal penetration has been shown in mycorrhizal tobacco (Genre and Bonfante, 1997). The mechanisms may, therefore, be the same, and it has been suggested by Baluska *et al.* (1995) that the cytoskeleton plays an important role in translating extracellular messages into endocellular responses.

Ultrastructural results, in addition to an increased accessibility of DNA to DAPI at undersaturating concentration (Berta *et al.*, 1990), and to other fluorochromes such as propidium iodide (unpublished results) and auramine O (Berta *et al.*, 1991), or to the enzyme DNase I (Lingua *et al.*, 1999), consistently reveal a dramatic and functional change of the nuclei following the infection process: chromatin is significantly more decondensed than in controls, and decondensation is correlated with the presence of the fungus (Fig. 4) (Berta *et al.*, 1990; Berta and Fusconi, 1998). This has been confirmed by immunocytochemistry using a monoclonal antibody that mostly binds to the condensed chromatin: a significant decrease in gold-labelling intensity over the nuclei of mycorrhizal root cells, especially those containing arbuscules, has been observed (Balestrini *et al.*, 1992). Moreover, mycorrhizal plant nuclei labelled with an anti-histone antibody and analysed by flow cytometry, showed a lower content of this histone, whose importance in chromatin condensation is generally recognized, in comparison to controls (Lingua *et al.*, in press).

Chromatin decondensation of mycorrhizal plant nuclei can be explained by an increase in transcription: autoradiography and ^{3}H-Uridine uptake showed a higher intensity of labelling in cortical cell nuclei of infected leek plants, confirming that dispersed chromatin is generally the active fraction (Berta *et al.*, 1996). In addition, the number of senescent nuclei (chromatolytic or pyknotic) was significantly lower in mycorrhizae, as shown by flow cytometry and TUNEL assays (Lingua *et al.*, 1999).

In *P. sativum*, root cortical cell nuclei of mycorrhizal plants show the same modifications observed in *A. porrum*, i.e. hypertrophy, round shape, chromatin decondensation (Sgorbati *et al.*, 1993) (Fig. 2c-d; Fig. 3c-d). No variation in ploidy has been detected. Results obtained with a pea mutant resistant to mycorrhizal infection support the correlation between chromatin decondensation and the presence of the fungus found in *A. porrum*: in these mutants, where the presence of the fungus is limited to the surface of root epidermal cells (Duc *et al.*, 1989), chromatin decondensation, measured as intensity of fluorescence after DAPI staining at undersaturating concentration, was considerably lower (20%) than in the wild type, whose cortical parenchyma cells were highly infected. Chromatin decondensation observed in mycorrhizal pea nuclei does not itself demonstrate higher transcription, but their DNase sensitivity is very close to that of nuclei of pea embryos during germination (Sgorbati *et al.*, 1993), when cells are transcriptionally very active (Grellet *et al.*, 1977).

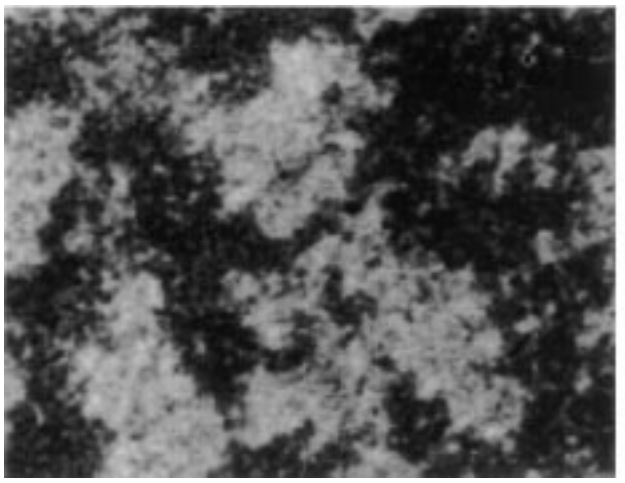
An example of diffuse nucleus as influenced by arbuscular mycorrhizal infection in differentiated root parenchyma cells: *Lycopersicon esculentum*

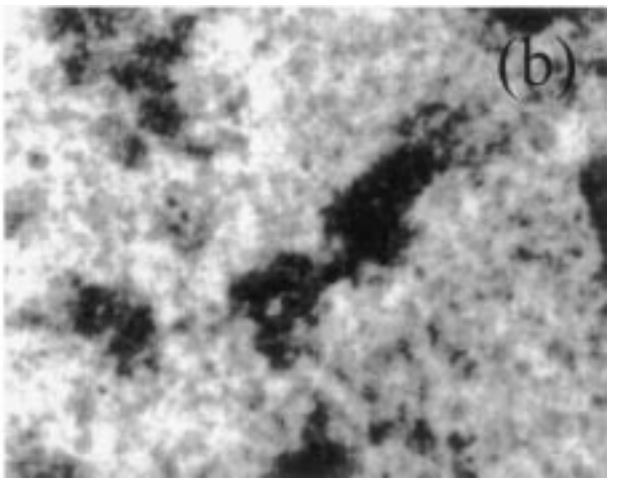
As previously reported, the nuclei of tomato are of the diffuse type. In control, non-mycorrhizal plants, cortical cell nuclei are generally active (i.e. their structure resembles that of meristem cell nuclei) and round or lenticular in shape. Chromatolysis is linked with an increase in the nuclear size, while pyknotic nuclei have reduced size (Berta *et al.*, 2001).

In the root system, deprived of the apices, of control plants, we could detect nuclei with three different ploidy levels, corresponding to 2C, 4C and 8C DNA content (Fusconi *et al.*, 1997; Berta *et al.*, 2001; Tahiri-Alaoui *et al.*, manuscript submitted). The 4C nuclei were by far the largest population.

Analysing nuclei from the cortex (the tissue where arbuscular mycorrhizal colonization takes place) of roots at 3.5 and 6 cm away from the root tip by static cytometry, a very small (or nil) percentage of 8C nuclei was measured, while the 4C population was

Fig. 3 - T.E.M. Nuclei of differentiated cortical cells of controls (left) and mycorrhizas (right). **a** (9170x), **b** (7910x), *Allium porrum*; **c** (10490x), **d** (9090x), *Pisum sativum*; **e** (12270x), **f** (10830x), *Lycopersicon esculentum*. In the three nucleus types the proportion of condensed chromatin is reduced by colonisation, in relation to the higher transcriptional activity.

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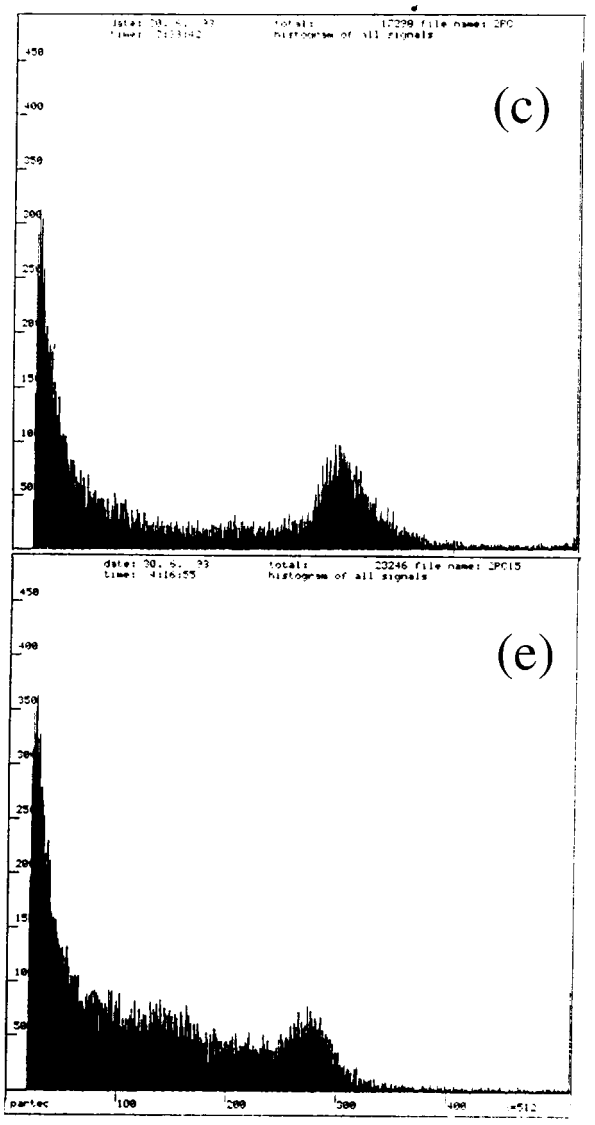
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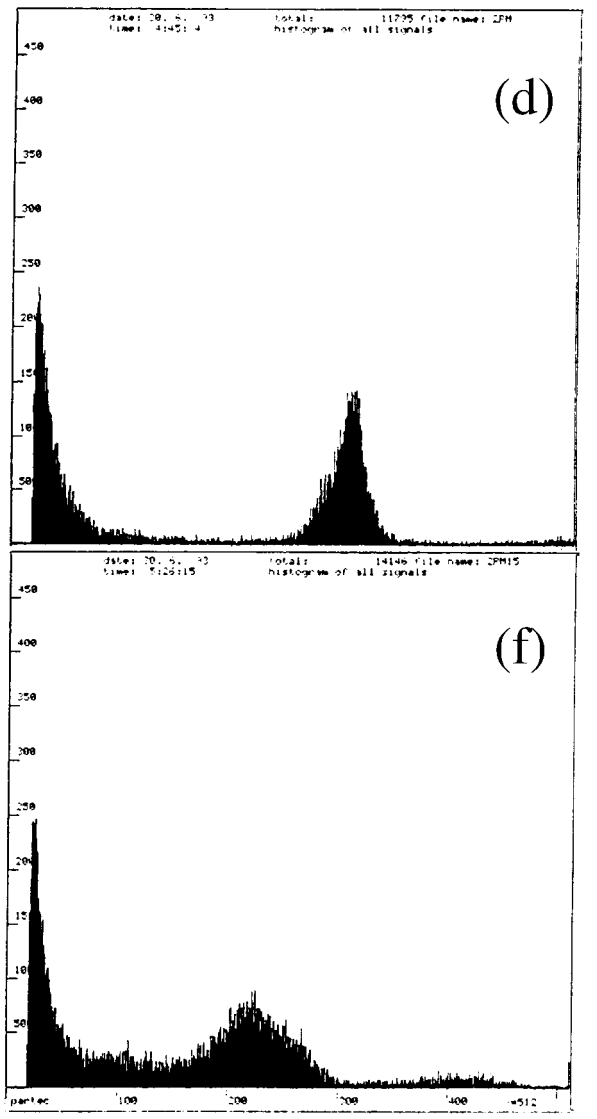
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the most abundant one (Berta *et al.*, 2001; Tahiri-Alaoui *et al*., manuscript submitted).

Colonization of the root by the arbuscular mycorrhizal fungus *Glomus mosseae* triggers various modifications in the nuclei of tomato plant. First of all, the nuclei of mycorrhizal plants are larger than those of control plants, in agreement with the data obtained in the past in many different mycorrhizal systems (Williams, 1966; Williamson and Hadley, 1969; Holley and Peterson, 1979; Carling and Brown, 1982; Bonfante-Fasolo, 1984; Toth and Miller, 1984; Barroso and Pais, 1990; Berta *et al.*, 1990; Sgorbati *et al.*, 1993). Chromatin decondensation occurs and can be observed by TEM, although it is less evident than in nuclei with reticulate or semi-reticulate structure (Fig. 3e-f). The shape of the nuclei is often lobed and again the proportion of nuclei with senescing appearance (chromatolytic or pyknotic) is very small. The mean diameter is larger in these senescing nuclei too, if compared with the same type of nuclei from control plants (Berta *et al.*, 2001).

Flow cytometry indicates the presence of three nuclear populations, as in control plants, but the proportion of nuclei in the three groups is heavily affected by the symbiosis (Lingua *et al.*, 1996; Fusconi *et al.*, 1997; Berta *et al.*, 2001; Tahiri-Alaoui *et al.*, manuscript submitted). Analyses of the nuclei extracted from the whole root system (excluding the apices) of 60-day-old plants showed a significant increase in the percentage of diploid and octoploid nuclei (Berta *et al.*, 2001).

Static cytometry (again considering zones at 3.5 and 6 cm from the root apex) confirmed the increase of diploid and octoploid nuclei in mycorrhizal plants (Berta *et al.*, 2001; Tahiri-Alaoui *et al.*, submitted) but, most importantly, it allowed the analysis of the nuclei of arbuscule-containing cells. This showed that octoploid nuclei were present in more than 90% of the arbuscule-containing cells at 3.5 cm (and in more than 40% of the arbuscule-containing cells at 6 cm) from the root tip (Berta *et al.*, 2001; Tahiri-Alaoui *et al.*, submitted).

These results established the relationship, for the first time, between polyploidization and arbuscular mycorrhizal colonization, similarly to what happens in orchid mycorrhiza (Williamson and Hadley, 1969), and in contrast with the previous reports on ploidy of plants colonized by glomalean fungi (Berta *et al.*, 1990; Sgorbati *et al.*, 1993). Increased nucleus and cell size, as well as chromatin decondensation, could also be related to high metabolic activity, a hypothesis which is consistent with our observations of increased respiratory activity (Berta *et al.*, 2001) and with the findings by Gianinazzi-Pearson *et al.* (1996) of an increased number of mitochondria and plastids during plant cell development, especially in arbuscule-colonized cells.

Following AM colonization, many tomato genes are induced or repressed (Tahiri-Alaoui and Antoniw, 1996); it is possible that some of them are regulated by the mycorrhiza-dependent change of ploidy occurring in the arbuscule-colonized cells. Conversely, it is also possible that some of the newly induced genes could be involved in the formation of polyploid cells; for example, the expression of a cullin gene has recently been reported in mycorrhizal tomato roots and the comparison with genes belonging to the same family suggests a possible role in the control of DNA synthesis (Tahiri-Alaoui *et al.*, manuscript submitted).

CONCLUSIONS

The reports by Berta *et al.* (1990) and Sgorbati *et al.* (1993) have described how nuclear hypertrophy in mycorrhizal pea and leek roots was due to chromatin decondensation, excluding polyploidization. In tomato roots colonized by *Glomus mosseae*, ploidy variations clearly contribute to increase the mean diameter of nuclei, especially in arbuscule-colonized cells. Nevertheless, chromatin condensation is still a very important factor. Unpublished data (Lingua and Berta, manuscript in preparation) show that nucleus diameter, in tomato root cells, does not depend on DNA content alone, and that chromatin condensation plays a central role in the regulation of this parameter.

Fig. 4 - a-b (both 44280x), T.E.M. Nuclear chromatin in Allium porrum L. **a**, non-mycorrhizal nucleus with highly condensed chromatin. **b**, mycorrhizal cell nucleus, with very decondensed chromatin. **c-f**, flow cytometry, after DAPI staining. **c**, control nuclei stained with DAPI; **d**, mycorrhizal nuclei stained wih DAPI; **e**, control nuclei treated with Dnase I for 15 min and DAPI stained; **f**, mycorrhizal nuclei treated with Dnase I for 15 min and DAPI stained. The main peak is digested more quickly in mycorrhizal nuclei, suggesting more accessible (i.e. decondensed) chromatin.

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