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Peculiarities in PMC Meiosis of *Pisum sativum*

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Summary. The general outline of meiotic prophase proposed by Wilson in 1925 included three stages which usually are missing in the description of meiosis today: (1) synizesis (= the synaptic stage in plants), (2) the diffuse stage, which follows the pachytene and (3) the second contraction, following the diffuse stage. The last two stages were regarded by Wilson as being very variable and could even be lacking. These stages, described by early plant cytologists later became regarded as fixation artefacts and after the introduction of the squash technique were either misinterpreted and/or forgotten.

However, all three stages are easily recognised in acetic-ethanol fixed preparations of plant meiosis and are demonstrated in *Pisum sativum* meiosis. *Pisum* meiosis differs from the usual plant meiosis by: (1) the peculiar timing of the late prophase, (2) the existence of extrachromosomal fibrillar structures appearing at synizesis and staining by Feulgen – Light Green, and (3) the appearance of multiple nucleoli in the late prophase. The possibility that these nucleoli and fibrillar structures are of relevance in relation to gene amplification is discussed.

Key words: *Pisum sativum* – Meiotic prophase stages – Multiple nucleoli – Gene amplification

Introduction

Pisum, the famous experimental plant of Mendel, is today one of the genetically best analysed organisms. *Pisum* has 14 chromosomes, and tentative chromosome maps with a great number of identified loci exist for all seven chromosome pairs. A great number of genetically well-analysed stocks are available and stocks with high fertility are of economical value. However, although the chromosomes are of appropriate size for cytological studies *Pisum* meiosis has only been studied by a few cytologists and, in fact, this area has been almost neglected during the last few years. The reason for this seems to be the difficulty to interpret the meiotic prophase and to analyse the meiotic events in *Pisum*.

We have conducted a close cytological investigation on *Pisum* meiosis for two reasons. Firstly, considering the peculiarities of *Pisum* meiosis recorded in the literature, it seemed to be of interest to re-analyse the progress of meiotic prophase in the light of the results that we have obtained on other material, particularly concerning the progress of chromosome condensation (Klášterská 1977; Klášterská and Ramel 1979, 1980a, 1980b). Secondly, and more important, a number of strains of *Pisum* with mutations affecting meiosis are available and these mutants may offer unique possibilities to analyse the progress of meiosis in a new way. The present report, therefore, constitutes some background data on normal meiosis in *Pisum* for further investigation of meiotic mutants.

In the course of the present survey of meiotic prophase of *Pisum* some interesting pecularities have been recorded, in particular the timing of the late prophase and the occurrence of multiple nucleoli.

Material and Methods

In *Pisum*, meiosis is strongly synchronised and one anther always includes only a short part of the meiotic prophase: leptotene + early synizesis (zygotene); early to late synizesis (zygotene to early pachytene); early to late pachytene; late pachytene + prediffuse diplotene + diffuse stage; diffuse stage + second contraction; second contraction + postdiffuse diplotene; postdiffuse diplotene to metaphase 1 or anaphase 1. In this way, the sequence of meiotic prophase stages is easily followed, without the danger of confusing one stage with another.

Buds from a standard line of *Pisum sativum* (L 110) were fixed in 1979 and in 1980 with acetic ethanol (1:3) and stored in 70% ethanol in the refrigerator.

For the preparation, one anther was dissected, heated and squashed in 45% acetic acid and frozen on dry ice. After removing the cover slip, the slides were transferred for some minutes to absolute ethanol and then stored in 70% ethanol. They were stained the

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same day by (1) a modified Giemsa procedure (see Klášterská and Ramel 1979) or (2) by Feulgen – Light Green, a specific technique for DNA and RNA structures: the slides were kept overnight in 10% formaldehyde, washed in running water (30 min), hydrolysed in nHCl at 60°C (7 min), stained with Schiffs reagens (2 h), rinsed in SO_2 water (3 × 10 min), washed in running water (20 min) and kept over night in Light Green alcoholic solution with picric acid. The next day the slides were differentiated in Na_2CO_3 alcoholic solution, transferred through absolute ethanol and xylol and subsequently mounted in DEPEX.

Results

After the premeiotic interphase (Fig. 1 A), the chromosomes emerge as leptotene threads (Fig. 1 B) which successively assemble into the synizetic knot enclosing the nucleolus (Fig. 1 C-E, G). During this period, the homologues become paired (Fig. 1 D, E, G). At the same time, some fibrillar structures seem to be released from the chromosomes (Fig. 1 G, arrows) and appear as extrachromosomal structures, staining green with Feulgen – Light Green, in the nucleus (Fig. 1 D, E, arrows). The pachytene bivalents successively unravel and fill the hollow of the nucleus (Fig. 1 E, F, H). After the onset of the homologue separation (Fig. 2 A), the nucleus enters the diffuse stage (Fig. 2 B) which is followed by a configuration named by early cytologists the 'second contraction' (Fig. 2 C). The recontraction of the bivalents into the postdiffuse diplotene follows (Fig. 2 D, E). No typical diakinesis bivalents are ob-

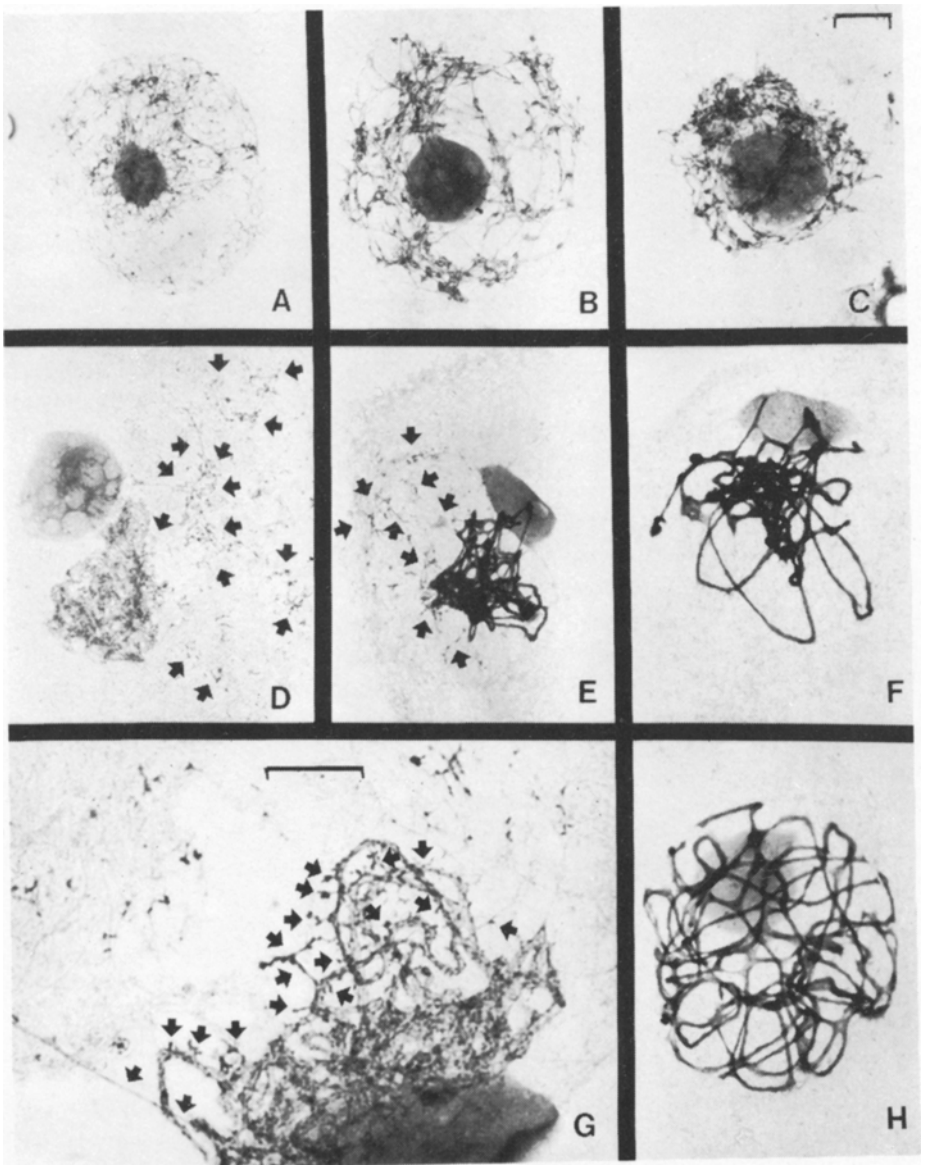

Fig. 1A-H. Sequence of meiotic prophase stages in *Pisum sativum.* **A** praemeiotic interphase; **B** leptotene; **C, D, E** synizetic contraction during which the homologues pair; **F** middle pachytene; **G** early pachytene (synizesis), detail: sites of the release of fibrillar structures (arrows), which stain green with Feulgen-Light Green; **H** late pachytene. The bar represents 10 μm

served in the late prophase: the chromosomes are associated in configurations of different appearance and interconnected in different ways, and a great number of nucleoli appear in the nucleus before the breakdown of the nuclear membrane (Fig. 2 F, 3 A-C, arrows). With Feulgen – Light Green staining, the chromosomes and their interconnections are red (DNA; Fig. 3 B thick arrows) and the nucleoli green (RNA; Fig. 3 B thin arrows). During prometaphase (Fig. 3 D-F) the chromosomes concentrate toward the middle of the cell (this configuration was called the 'third contraction' by the early cytologists (Sharp 1926; Fig. 3 E) and the nucleoli disappear. Successively, 7 distinct bivalents appear in the metaphase 1 plate. They are either free (Fig. 3 G) or sometimes they may still be interconnected (Fig. 3 H, I), but the interconnections disappear before anaphase separation.

Discussion

The early general outlines of meiosis (Wilson 1925; Sharp 1926) included three stages, usually missed in the meiotic outlines of today: synizesis (= the time of homologue synapsis), the diffuse stage and the second contraction. The last two stages, which follow pachytene, are variable and they can be missing. By the end of the twenties these three stages had became regarded as 'especially sensitive to the fixation' and were eliminated from descriptions of plant meiosis as 'fixation artefacts'. This mistake was caused by a lack of understanding of the real structure of chromosomes, which was not recognised until electron-microscopical pictures were available. The early cytologists regarded chromosomes as compact bodies, which have the ability to change their size as an amoeba, and with such a concept

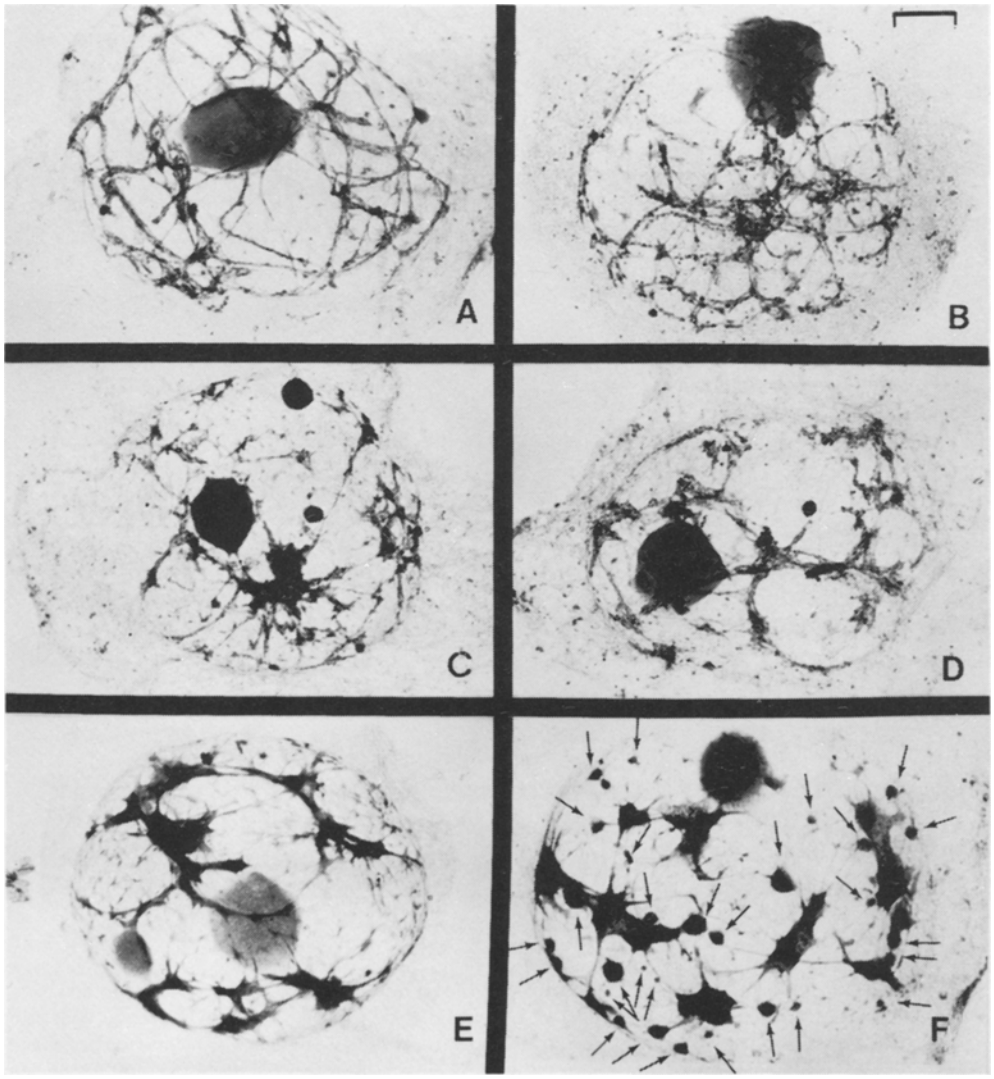

Fig. 2A-F. Sequence of meiotic prophase stages in *Pisum sativum*. **A** the onset of the diplotene separation of bivalents; **B** diffuse stage; **C** second contraction; **D, E** post-diffuse diplotene; **F** late prophase, the appearance of multiple nucleoli (arrows). The bar represents 10 μm

it was impossible to understand the postpachytene events. Lewitsky, (1927) for one, compared the effect of the fixative on the chromosomes at synizesis and postpachytene stages to the reaction of amoebae to rough environmental conditions which make them collapse or burst. As already discussed in our earlier papers (Klášterská 1977; Klášterská and Ramel 1980a, 1980b), the description of meiosis published by Wilson (1925) and by Sharp (1926) is closer to reality and might be rehabilitated as a general outline of meiosis.

The appearance of synizesis and of the successive untwisting of pachytene bivalents may be due to the attachments of the telomeres to a limited part of the nuclear membrane during zygotene and to the movement of chromosomal ends on the nuclear membrane taking place during early to late pachytene. Also, the second contraction may possibly reflect the regulation of the chromosomal movements necessary for interconnecting the postpachytene bivalents (for discussion see Klášterská and Ramel 1980b).

The published observations on *Pisum* prophase are incomplete and confused. The existence of synizesis was reported as early as 1903 by Cannon, but in later descriptions only Atabekova (1959) men-

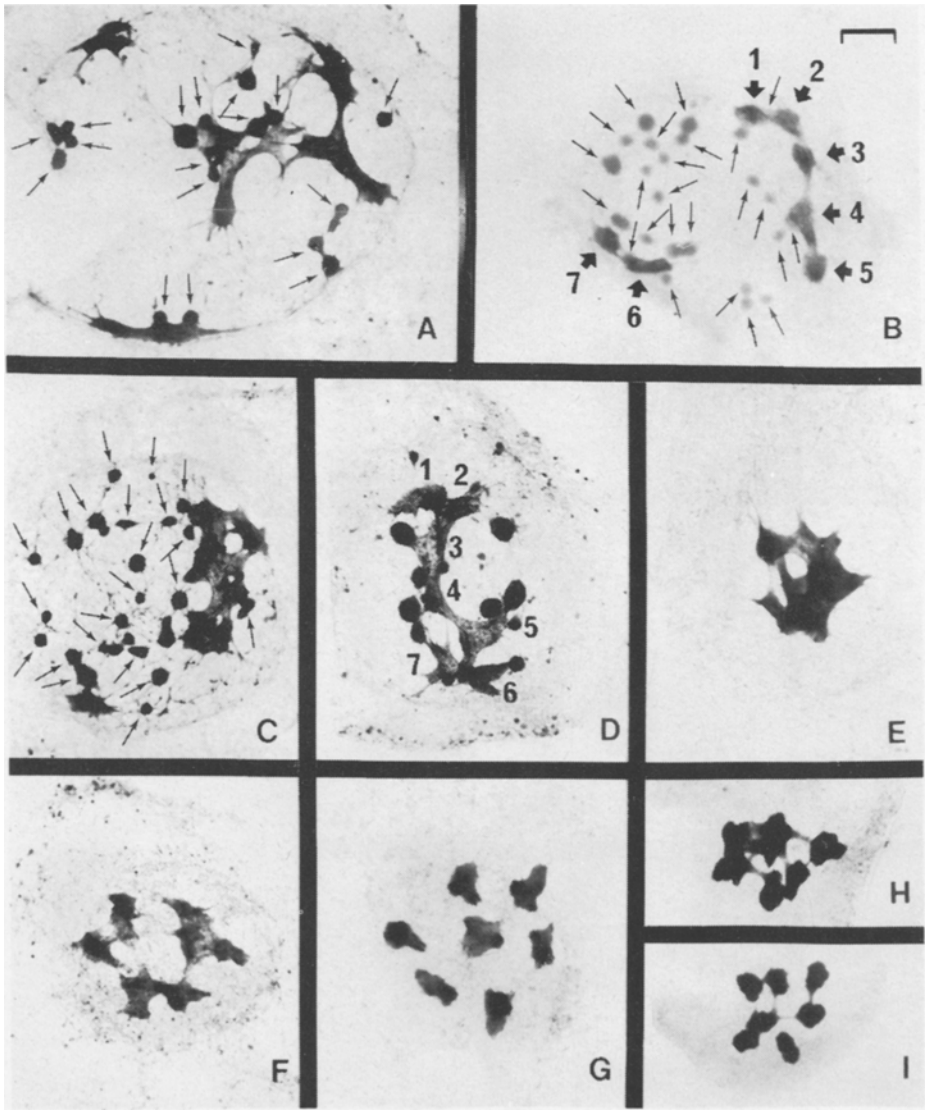

Fig. 3A-I. Sequence of meiotic prophase stages in *Pisum sativum.* **A, B, C** late prophase: the bivalents are associated in various configurations, often in connection with multiple nucleoli (arrows); **B** late prophase stained with Feulgen – Light Green: the 7 bivalents and their interconnections are stained red (big arrows), the multiple nucleoli, green (small arrows); **D** prometaphase, the nuclear membrane has already disappeared, the nucleoli still persist, the 7 bivalents are not separate structures; **E** prometaphase ('third contraction') the multiple nucleoli have disappeared, the bivalents cannot be discerned as separate structures; **F** prometaphase, the bivalents start to appear as separate entities; **G** metaphase 1 with 7 distinct bivalents; **H, I** metaphase 1 with some persisting interconnections. The bar represents 10 μm

tioned its occurrence, with the comment, that some cytologists interpret this configuration as a fixation artefact, whereas others regard it as a regular stage appearing after leptotene. Pachytene is easily stained and recognised, but the bivalents are too long and without distinct chromomeres and therefore the pairing cannot be analysed (Lamm 1976). The postpachytene nuclei show a marked growth (Cooper 1938, Atabekova 1959). Håkansson (1931) regarded the postpachytene stages – in agreement with the common opinion – as 'especially sensitive to the fixation'.

The descriptions of the late prophase are confusing and contradictory. Whereas some cytologists reported 7 contracted bivalents at diakinesis (Håkansson 1931; Cooper 1938; Atabekova 1959; Kaul personal communication), others found the chromosomes at the late prophase indistinct and diffuse (Morrison and Lin 1955) or consisting of a net of clumped and thread-like portions (Klein 1972). Klein analysed meiosis in many *Pisum* lines and mutants with the conclusion that the late prophase in *Pisum* meiosis is as a rule indistinct, without clearly defined bivalents. He found only one exception with fully contracted and analysable diakinesis bivalents, in a sterile *Pisum* mutant (Klein 1971). Gottschalk and Baquar (1971) concluded, that '... all stages of the meiotic prophase are highly ambiguous. Therefore, the earliest reliable results can be obtained from metaphase 1 and we are obliged to reconstruct the pachytene situation from the findings in metaphase 1 ...'.

In our pictures on the *Pisum* prophase, all stages described by Wilson (1925) and Sharp (1926) but missed in the later conventional outlines of meiosis, are easily identified: a distinct synizesis during which the homologues pair (Fig. 1 C, D, E, G), a prominent diffuse stage (Fig. 2 B) and a second contraction (Fig. 2 C). Besides, a peculiar timing of the late prophase, an unusual appearance of multiple nucleoli in the late prophase and some extrachromosomal fibrillar structures staining with Feulgen – Light Green were noted in the *Pisum* meiotic prophase.

a) The Peculiar Timing of the Late Prophase

The stages from leptotene to the early postdiffuse diplotene agree with the appearance of the same stages in other plants with a prominent diffuse stage and second contraction (*Najas, Turritis, Alliaria* – Klášterská and Ramel 1979, 1980a and unpublished results), but in *Pisum* the breakdown of the nuclear membrane (= the end of the prophase) starts earlier, before the bivalents are fully contracted and before the interconnections disappear. The ends of the late pachytene bivalents are as a rule free in plant meiosis, whereas the bivalents reappearing after the diffuse stage or second contraction are temporarily interconnected by threads which usually disappear before diakinesis (Klášterská 1978; Klášterská and Ramel 1979, 1980a, 1980b and unpublished results). In *Pisum*, these interconnections (which are DNA, as they stain with Feulgen) often persist up to the prometaphase or still later (Fig. 3 H, I). They may give rise to metaphase associations which look like multivalents, but there is no regular pattern in their occurrence. We observed such associations in some mutants and in a tetraploid (unpublished observations). Because of the lack of any alternative explanation, these multivalent-like configurations, observed in different *Pisum* plants and mutants, are often regarded as true multivalents due to translocations (Gottschalk and Milutinović 1970; Gottschalk and Baquar 1973). But as the pattern of chromosome associations in the metaphase 1 of these plants is highly variable, a definite configuration is not possible to attribute to them. Moreover, the fertility of such plants may be surprisingly high, which led to the speculations about some kind of 'repair mechanism' which 'helps in normalizing the course of meiosis' (Gottschalk and Baquar 1973). With such difficulties it is not surprising, that plant cytologists have avoided studies on *Pisum* meiosis.

b) The Occurrence of the Multiple Nucleoli and of the Extrachromosomal Fibrillar Structures

Multiple nucleoli were observed in the late prophase of all seven different lines of *Pisum sativum* and in a standard line of *Pisum arvense* under investigation. It is very amazing that there are no records in the *Pisum* literature of this characteristic occurrence of nucleoli, with exception of a note by Gottschalk and Petrini (1965), that '... during passage from late prophase to metaphase 1 normal dissolution of all nucleoli present occurs ...'. The existence of multiple nucleoli was reported in the mutant with the supercontraction of chromosomes (i.e. with diplotene and diakinesis bivalents of a normal appearance – Klein 1972) but in this plant, the nucleoli did not disappear during prometaphase but persisted through whole meiosis. Only Lamm (personal communication) observed similar multiple nucleoli in his preparations. Håkansson and Levan (1942) only occasionally found one or two accessory nucleoli at diakinesis, whereas Kaul (personal communication) observed only regular nucleolar behaviour in *Pisum* meiosis.

Similar multiple nucleoli were observed in *Rosa* (Klášterská and Natarajan 1974). Extra nucleoli, released from the nucleolar organizing regions (NORs) in the form of chains were reported in pachytene and diplotene of *Lilium* (Williams et al. 1973).

Nucleoli are known to appear in connection with the nucleolus organising regions (NORs) which are definite regions in specific chromosomes characteristic for every genome. A specific replication of the NORs (= gene amplification) is known from the oocyte meiosis of some insects and amphibians (Gall 1968, 1969) and is supposed to be widespread (Gall 1969). This selective DNA synthesis takes place at a long lasting pachytene. The extra DNA becomes free in the form of minute granules (Gall 1968) or as a network of fine fibrils (Bier et al. 1967) and is involved in the production of multiple nucleoli during diplotene and diakinesis.

It is tempting to see some parallels between these reports on the one hand and the occurrence of fibrillar structures and multiple nucleoli at the same stages of *Pisum* meiosis

on the other. The main difference is the timing of these stages, as meiosis in PMCs of *Pisum* probably only takes some days or less.

The nature of the extrachromosomal fibrillar structures observed at early prophase in *Pisum* and the way they are released are not known so far. They stain specifically as RNA, but it is necessary to study their nature, origin and fate with more exact techniques, before any suggestions can be made. Also the function of the nurse cells (tapetum) may be important in this connection.

The discrepancies in the data on the late prophase and on the nucleolar behaviour reveal an unusual variability in the prophase events to which we lack any explanations. It is possible that these discrepancies may reflect an adaptation to different climatic or other conditions which would explain the different observations performed in India by Kaul and in Germany by Gottschalk and by Klein. However this remains at present in the realm of speculations.

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Literature

Atabekova, A.J. (1959): Cytologische Untersuchungen an der Kulturerbse *Pisum sativum*. Biol. Zbl. 3, 424-438

Bier, K.; Kunz, W.; Ribbert, D. (1967): Struktur und Funktion der Oocytenchromosomen und Nukleolen sowie der Extra-DNS während der Oogenese panoistischer und meroistischer Insekten. Chromosoma 23, 214-254

Cannon, W.A. (1903): Studies in plant hybrids: the spermatogenesis of hybrid peas. Bull. Torrey Bot. Club 30, 510-543

Cooper, G. (1938): Cytological investigations of *Pisum sativum*. Bot. Gaz. 99, 584-591

Gall, J.G. (1968): Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis. Proc. Nat. Acad. Sci. (Wash.) 60, 553-560

Gall, J.G. (1969): The genes for ribosomal RNA during oogenesis. Genetics (suppl.) 61, 121-132

Gottschalk, W.; Baquar, S.R. (1971): Desynapsis in *Pisum sativum* induced through gene mutation. Can. J. Genet. Cytol. 13, 138-143

Gottschalk, W.; Baquar, S.R. (1973): Highly unstable translocations induced through irradiation in *Pisum*. Cytologia 38, 327-336

Gottschalk, W.; Milutinović, M. (1970): Die Analyse hochgradiger Chromosomenverkettungen in der Meiosis von *Pisum sativum*. Caryologia 23, 473-488

Gottschalk, W.; Villabos-Petrini, R. (1965): The influence of mutant genes on chiasma formation. Cytologia 30, 88-97

Håkansson, A. (1931): Über Chromosomenverkettung in *Pisum*. Hereditas 15, 17-61

Håkansson, A.; Levan, A. (1942): Nucleolar conditions in *Pisum*. Hereditas 28, 436-440

Klášterská, I. (1977): The concept of the prophase of meiosis. Hereditas 86, 205-210

Klášterská, I. (1978): Structure of eukaryotic chromosomes: The differences between mammalian (mouse), grasshopper (*Stethophyma*) and plant (*Rosa*) chromosomes as revealed at the diffuse stage of meiosis. Hereditas 88, 243-253

Klášterská, I.; Natarajan, A.T. (1974): Cytological studies of the genus *Rosa* with special reference to the section *Caninae*. Hereditas 76, 97-108

Klášterská, I.; Ramel, C. (1979): Prophase of plant meiosis: sequences and interpretation of stages. Genetica 51, 15-20

Klášterská, I.; Ramel, C. (1980a): Sequence and interpretation of meiotic prophase stages in PMCs of *Najas marina* (*Najadaceae*). Hereditas 92, 171-175

Klášterská, I.; Ramel, C. (1980b): Meiosis in PMCs of *Arabidopsis thaliana*. Arabidopsis Inf. Serv. 17, 1-10

Klein, H.D. (1971): Eine *Pisum* Mutante mit zahlreichen meiotischen Störungen. Cytologia 36, 15-25

Klein, H.D. (1972): Genetic control of chromosome morphology. Pisum Newsl. 4, 12-13

Lamm, R. (1976): Technical hints for chromosome studies in *Pisum*. Hereditas 84, 235-237

Lewitsky, G.A. (1927): Die Bildung bivalenter Chromosomen in der Gonogenese von *Beta vulgaris L.* Planta 3, 100-114

Morrison, J.W.; Lin, S.C. (1955): Chromosomes and nucleoli in *Pisum sativum*. Nature 175, 343-344

Sharp, L.W. (1926): An Introduction to Cytology. 2. ed. New York: McGraw-Hill

Williams, E.; Heslop-Harrison, J.; Dickinson, H.G. (1973): The activity of the nucleolus organising region and the origin of cytoplasmic nucleoloids in meiocytes of *Lilium*. Protoplasma 77, 79-93

Wilson, E.B. (1925): The Cell in Development and Heredity. 3. edition. New York: McMillan

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