# Triploidy in callus culture of *Vicia faba* L. investigated by the Giemsa C-banding technique[1]

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*Summary.* Callus tissue of *Vicia faba* has been grown from explants of immature embryos. The karyological analysis by the Giemsa C-banding technique has shown triploid and tri-aneuploid mitoses, all with an extra chromosome and with structurally altered karyotypes. Estimates of the amounts of interphase heterochromatin (chromocentres) indicate endopolyploidy up to 12 n.

*Vicia faba* [2n = 12], a classical experimental plant in nuclear cytology, has been studied extensively in recent years by various chromosome banding techniques[3-11]. Using the Giemsa C-band method, the heterochromatin has been mapped, allowing the identification of all 6 chromosome pairs. We have succeeded in applying the Giemsa C-staining method to this species when grown as tissue in vitro, with the ultimate aim to analyse chromosome behaviour in culture. The investigation of tissue cultures of *Vicia faba* by classical karyological techniques has shown the feasibility of stabilizing diploid and polyploid subpopulations for a period of 4 years with remarkably few structural changes of chromosomes[12-14].

*Material and methods.* We succeeded in obtaining callus tissue of *Vicia faba* L. by culturing 3-5-mm-long explants from immature embyos after the radicle tip and the plumule had been removed from them. The explants were inoculated on Murashige-Skoog[15] complete medium solidified with 0.9% agar containing 30 g glucose, 0.5 mg 2,4-D and 0.01 mg kinetin per l. The cultures were kept at 26±1 °C in artificial light [day-light fluorescent tubes TEŽ-Zagreb, 40 W, 220 V, 6500 °K 16h light/8 h dark daily] at a lighting intensity of about 500 lx. The callus obtained was subcultured on the same medium every 8 weeks for more than 2 years, always showing a reasonably rapid growth. All karyological analyses were done on one and the same strain of callus tissue in various subcultures and test tubes. From the 7th to the 11th passage, the chromosome complement of the cultures was investigated by the Giemsa C-banding procedure as developed by Marks[16] for pollen mother cells. Working on the assumption that the dark chromocentres in the interphase nuclei of Giemsa C-stained preparations correspond to the C-bands of chromosomes[16-21], the nuclei of callus tissue have been separated by appropriate heavy squashing[21], and the chromocentres from each interphase nucleus have been carefully drawn by means of a camera lucida on a separate semitransparent foil. An estimation of the amount of the chromocentres per nucleus was obtained from these drawings by means of the VP-8 Image Analyser ISI [Lawrence, Kansas, USA] by determining the area of dark chromocentres per nucleus as the percentage of the total screen area.

*Results and discussion.* C-band karyotype studies have been made on a limited number of cells, from the 7th to the 11th subculture - 34 karyotypes in all - because dividing cells were rather rare. All the metaphases investigated were on the triploid level [2 n = 3 x = 18], and some were also aneuploids, mostly 18±1. In each nucleus there is an extra chromosome, which obviously does not belong to any of the homologue groups [figure 1]. The extra chromosome seems to be a duplicated segment, which should contain a centromere, since it has not been lost from callus cells in 2 years. Also, in all nuclei, various structural changes, including translocations and inversions, have been found as evidenced by alterations in chromosome morphology and Giemsa C-banding patterns. The number and the type of structural changes, as well as aneuploidy [±1], vary from cell to cell, even within samples from the same test tube. It seems, however, that some homologues are generally more stable than others. We have noticed the majority of structural changes on metacentrics and in 2 groups of subtelocentrics, while the extra chromosome appears to be very stable.

Such a high degree of chromosome structural changes has not been observed by other authors[13,14] using a classical chromosome staining technique. Infraspecific variation in the C-banding patterns is known to occur both within individual plants as well as between samples[17-22]. Also, in *Vicia faba*, C-band polymorphisms have been demonstrated, although on a very small scale[9]. These normally occurring variations of C-banding patterns are, however, stable

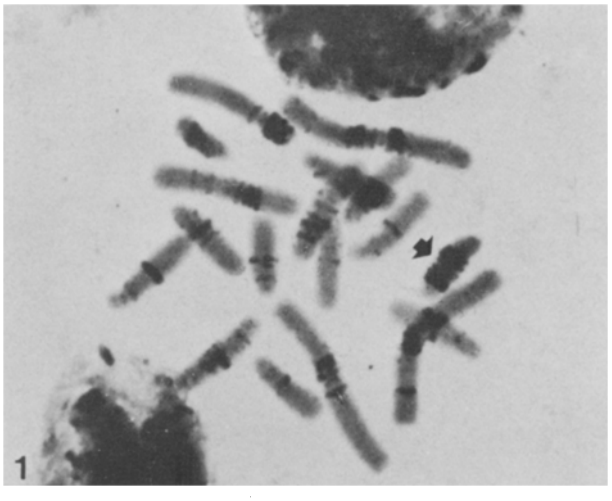


Fig. 1. Giemsa C-banded hypotriploid metaphase of *Vicia faba* callus culture with an extra chromosome (arrowed).

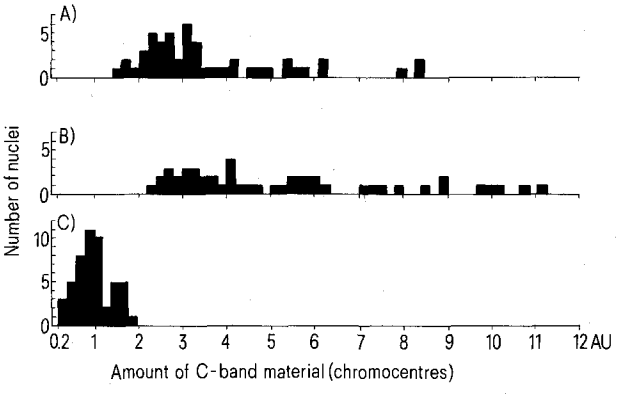


Fig. 2. Total area of Giemsa C-material (chromocentres) in heavily squashed nuclei of *Vicia faba* expressed in arbitrary units (AU) as a percentage of the total screen area of the image analyser. *A* Root tips of intact plant; *B* the 9th passage, and *C* the 11th passage of callus culture.

within individuals, and the extent of variation is much smaller than the differences we have found in callus culture[23].

As the existence of degrees of polyploidy higher than triploidy can be expected within the callus tissue grown in vitro, we have tried to determine the degree of polyploidy by estimating the content of heterochromatin per interphase nucleus by means of the PV-8 Image Analyser. The results [figure 2] show that there is no perceivable variation of heterochromatin contents in root tip cells of intact plants, the total area of chromocentres measuring 0.2–1.9%, whereas in callus culture there are considerable variations in the 9th and 11th passage, the corresponding figures being 2.2–11.4%, and 1.4–8.5% respectively. Considering that the root tips of the intact plant have diploid nuclei, and excluding the possibility of differential heterochromatin replication, in the callus tissue the ploidy levels of non-dividing nuclei are estimated to reach values as high as 12n. Detailed studies are in progress on the C-band karyotypes of different *Vicia faba* callus lines and their sources, to define the nature of the observed extra chromosome and of the karyotypic alterations.

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## Reoxidation of reduced hen egg white lysozyme fragment 1–123

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*Summary.* The reactivation of reduced lysozyme, whose 6 COOH-terminal amino acid including cysteine 127 were cut off, was studied. The results show that the disulfide bridge I–VIII as well as the COOH-terminal hexapeptide do not play a decisive role in the acquisition of the native 3-dimensional structure of the enzyme.

It is generally accepted that the conformation adopted by a polypeptide chain is a direct function of its amino acid sequence. Numerous studies including those on insulin, chymotrypsin, ribonuclease and staphylococcal nuclease have suggested that the entire amino acid sequence of the protein is required for folding from a disordered state to a native-like conformation[2–9].

In the case of hen egg white lysozyme, however, the COOH-terminal dipeptide, Arg-Leu, was recently proved to be not essential for the acquisition of the native 3-dimensional structure of this enzyme[10]. Moreover, it was suggested that the formation of disulfide I–VIII, linking cysteinyl residues 6 and 127, one of the last events of the reoxidation process, is accompanied by only minor effects on the biological activity as well as on the 3-dimensional structure[11].

Checking such a role for cysteinyl residue 127 and for the COOH-terminal amino acid sequence of lysozyme, has been made possible by the discovery of carboxypeptidase Y from *Saccharomyces cerevisiae*. This enzyme conserves its proteolytic activity in denaturing media such as 6 M urea[12]. The study of the refolding of reduced lysozyme cut off between residues 122 and 123 is reported in this communication.

*Material and methods.* Dithiothreitol (lot 124C-0218), Tris base (lot 104C-5000), 2-mercaptoethanol (lot 32C-0170) and an aceton powder of *Micrococcus Luteus* (lot 128C-1980) were provided by Sigma. Hen egg white lysozyme was purchased from Boehringer and 5,5′-dithiobis(2-nitrobenzoic acid) from Fluka. Lysozyme was further purified by means of a chromatography on Biorex-70 according to Johnson et al.[10]. Carboxypeptidase Y was purified from *Saccharomyces cerevisiae* according to previously described procedures[12,13]. Urea and cuppric chloride were provided by Baker. Only freshly prepared solutions of deionized urea were used. All the other chemicals were of the best grade available. Reduction of lysozyme and regeneration of the reduced protein were performed according to Ristow and Wetlaufer[14].

The removal of the COOH-terminal hexapeptide from reduced lysozyme was performed by incubating the protein (2 mg/ml) in a 0.1 M acetate buffer containing 2 M urea, 10 mM 2-mercaptoethanol and 10 mM EDTA at pH 5.5 in the presence of carboxypeptidase Y (1% enzyme to substrate protein). Digestion proceeded for 5 h at room temperature and was stopped by adjusting the pH of the reaction mixture to 2 with 6 M HCl. Amino acid analysis revealed that the terminal hexapeptide COOH-Leu-Arg-Cys-Gly-Arg-Ile had been quantitatively removed from lysozyme. As a consequence, the new derivative was named lysozyme fragment 1–123. It was desalted on a Sephadex G-25 column and lyophilized.