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Unscheduled apoptosis in meristematic plant cells is triggered via terminal deletions in artificially elongated chromosome arms

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Abstract Chromosomes elongated beyond a critical size by balanced rearrangements reduce the viability and fertility of field bean individuals. The severity of symptoms, ranging from growth retardation to early death of seedlings, increases with the length of the longest chromosome arm. This is paralleled by the incomplete separation of sister chromatids during nuclear division, resulting in chromatin connections between daughter nuclei which become disrupted by cell-wall formation and yield chromatid deletions detectable as micronuclei. By means of the TUNEL assay we show that, compared to the wild-type, a >6-fold higher number of meristematic cells of karyotypes with chromosome arms surpassing the limit of tolerance reveal apoptotic nuclei and are prone to die. Thus, terminal chromatid deletions apparently trigger unscheduled apoptosis. Extensive cell death in meristems is eventually responsible for reduced growth, disturbed development and reduced seed set. Differentiated root tissues and microspores did not reveal apoptotic nuclei.

Key words Chromosome elongation · Deletion · Mitosis · Disturbed development · Fertility · Apoptosis

Introduction

Apoptosis, or programmed cell death, represents a genetically controlled pathway for the elimination and 're-cycling' of 'unwanted' cells. It probably can be found in all multicellular organisms. Mediated by internal signals, scheduled apoptosis occurs at various developmental stages during plant tissue differentiation. Vascular cells, for example, die during xylogenesis (Mittler and Lam 1995). Similarly, cells of the calyptra (Wang et al. 1996 a), tapetum cells surrounding developing pollen grains, some cells of the anther wall during dehiscence (Goldberg 1993), cells of the transmitting tissue through which the pollen tubes grow (Wang et al. 1996 b), cells of the senescent carpel (Orzaez and Granell 1997), three of the four haploid megaspores (Bell 1996), cells of the barley aleurone (Wang et al. 1996 c) and probably also suspensor cells during embryo maturation (Yeung and Clutter 1979), are all prone to die via programmed cell death (for review see Greenberg 1996).

In addition, external stress factors such as pathogens and their toxins (Greenberg et al. 1994; Ryerson and Heath 1996; Wang et al. 1996 a) may induce programmed cell death in plants, a phenomenon termed unscheduled apoptosis (Havel and Durzan 1996). Chromosome aberrations and subsequent micronucleus formation are also presumed to cause cell lethality via apoptotic pathways (Havel and Durzan 1996). In this context, we have recently reported on a series of field bean karyotypes with intentionally elongated chromosome arms (Schubert and Oud 1997). When these arms were longer than half of the average extension of the spindle axis at telophase, progressive reduction of the fertility and viability of the carrier individuals was observed. This was paralleled by incomplete separation of sister chromatids of the longest chromosome arms during nuclear division, and subsequent disruption of chromatin fibers protruding from or connecting the daughter nuclei by cell-wall formation in root-tip meristems. This, in turn, yielded micronuclei at a frequency proportional to the chromosome arm length. The nuclei from which the micronuclei descend suffer from deletions and the corresponding

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cells presumably die, possibly through apoptosis. If the number of damaged meristematic cells surpass a threshold, the plants concerned reveal developmental disturbances. The first symptom is reduced seed yield. In general it holds true that the longer the chromosome arms of the corresponding karyotypes, i.e. the more that cells contain micronuclei, the higher is the proportion of individuals with slower growth, a reduced number of leaves and flowers, and early death of the seedlings, respectively.

One of the characteristic steps in apoptosis is extensive degradation of nuclear DNA prior to the disintegration of nuclei. This process can be efficiently detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling (TUNEL) assay, which labels 3′-OH ends of fragmented DNA (Gavrieli et al. 1992; Gorzyca et al. 1993; Wang et al. 1996 a).

Here we report on the occurrence and frequency of apoptotic nuclei in tissues of faba bean individuals of the wild-type as well as in karyotypes characterized by chromosome arms longer than half the length of the average spindle extension at telophase (> 14 μm). In parallel with the formation of micronuclei, an increased frequency of apoptotic nuclei was found in root-tip meristems, as compared to the wild-type, but not in differentiated tissues of lines with reconstructed karyotypes.

Materials and methods

Lateral roots of faba bean seedlings of the wild-type or of the homozygous karyotype $\overline{\mathrm{FHE}}$ (see Fig. 1) germinated between wet filter paper and grown on running tap water were fixed overnight in ethanol : glacial acid, 3 : 1, washed in Na-citrate buffer 0.01 M, pH 4.5, treated for 30 min in 1% cellulase/pectinase at 37°C and squashed by the dry ice method. For the detection of apoptotic DNA degradation on slides by the TUNEL assay, we used the Apop Tag$^{\mathrm{TM}}$ in situ apoptosis detection kit (Oncor S 71 10-kit) according to the manufacturer's instructions and a Zeiss Axioskop fluorescence microscope with an appropriate filter combination for simultaneous transmission of FITC (antibody) and propidium-iodide (counterstain) fluorescence.

Longitudinal sections from lateral roots (fixed as above) were made of wild-type and $\overline{\mathrm{FHE}}$ plants. After embedding the roots in paraffin, 7–10 μm sections were cut. The sections were washed in xylene and used for the detection of apoptosis as above.

Anthers (wild-type or heterozygous for karyotype $\overline{\mathrm{HE}}$, Fig. 1) were fixed for 1 h and squashed in 45% acetic acid. The youngest anthers containing microspores were selected for the TUNEL assay.

Results and discussion

Via terminal deletions, too-long chromosome arms lead to apoptosis in somatic meristems with subsequent developmental disturbances

The size of the longest chromosome arm of the field bean karyotype $\overline{\mathrm{FHE}}$ (Fig. 1) amounts to 14.1 μm (approximately 21.7% of the metaphase genome length), due to the balanced combination of three primary translocations, and is more than 1.3-fold ($> 5\%$ of the metaphase genome) larger than the longest arm of the wild-type. While individuals of a line with a slightly shorter chromosome arm (21.3% of the metaphase genome) were previously shown to develop normally, carriers of the homozygous $\overline{\mathrm{FHE}}$ karyotype revealed retarded growth, diminished fertility (about 1 seed/plant) and reduced viability (Schubert and Oud 1997). Root tips of this phenotype showed incomplete separation of the sister chromatids of the longest chromosome arms in 39% of dividing cells, inspite of prolonged telophase duration, and at least 3.5% of the meristematic cells having micronuclei with a size of 3– $> 8\%$ of the genome. Such phenomena were not observed during comparative confocal microscopic studies of the wild-type. Since *Vicia faba* does not tolerate deletions of 1% or more of its genome (Schubert and Rieger 1990), we presumed that cells from which the micronuclei descend die and cause the observed phenotypes when 3.5% or more of the meristematic cells are concerned. Therefore, squash preparations of root-tip meristems of the wild-type and of karyotype $\overline{\mathrm{FHE}}$ were submitted to the TUNEL assay for the detection of extensive DNA degradation in meristematic nuclei. As a positive control squashed wild-type meristems were pre-treated for 30 min with DNase (100 μg/ml PBS, pH 7.5). This resulted in strong labelling of all nuclei (Fig. 2 A). No labelling of nuclei was obtained when TdT was omitted (Fig. 2 B). In the presence of TdT, only 1.7% of the 1343 scored wild-type nuclei were clearly labelled (Fig. 2 C). The same procedure resulted, on average, in strong label over 10.4% of 3473 nuclei in three slides (8.1, 8.1 and 17.4% in the individual slides) prepared from two seedlings of the $\overline{\mathrm{FHE}}$ karyotype (Fig. 2 D). Also the extent of sterility and other developmental disturbances varied between homozygous $\overline{\mathrm{FHE}}$ individuals (one of the two homozygous seedlings obtained in 1995 grew up and yielded 14 seeds; in 1996 two of these did not germinate, eight plants died before flowering and four produced a single seed each).

The percentage (10.4%) of apoptotic nuclei in $\overline{\mathrm{FHE}}$ meristems was higher than that of cells with micronuclei (3.5%) – the latter figure is certainly an underestimation since only micronuclei larger than 1 μm in diameter were scored and some of these might have been hidden behind the nucleus – but lower than that of mitotic configurations with incompletely separated chromatids (39%). Therefore, it seems reasonable to assume that too-long chromosome arms may cause unscheduled apoptosis in a significant fraction of meristematic cells due to the deletions resulting from incomplete chromatid separation during nuclear division and subsequent interference with the formation of the new cell wall. Although mutagen exposure may also

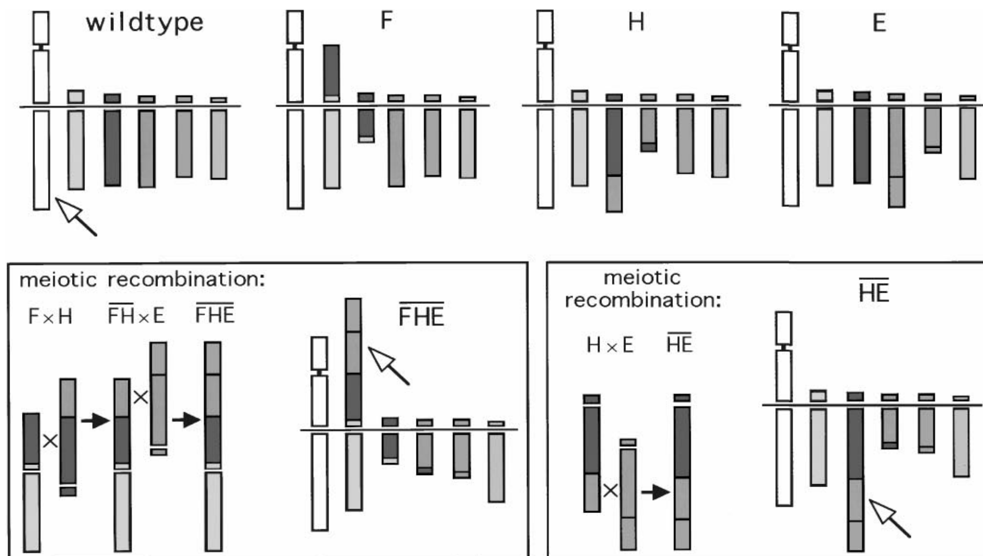

Fig. 1 Schematic presentation of the haploid chromosome sets of the wild-type and the translocation karyotpyes F, H and E of the field bean (above). Subsequent meiotic cross-overs between partially homologous translocated chromosomes of heterozygotes from the crosses F × H and $\overline{\text{FH}}$ × E (bottom left) resulted in the karyotype $\overline{\text{FHE}}$. Cross-overs between the corresponding chromosomes of karyotypes H and E resulted in the karyotype $\overline{\text{HE}}$ (bottom right). The longest arms of wild-type and of karyotypes $\overline{\text{FHE}}$ and $\overline{\text{HE}}$ are marked by *arrows*. The fertility and viability of plants with these karyotypes are severely impaired due to incomplete separation of the longest chromatids during mitosis and subsequent loss of DNA (modified after Schubert and Oud 1997)

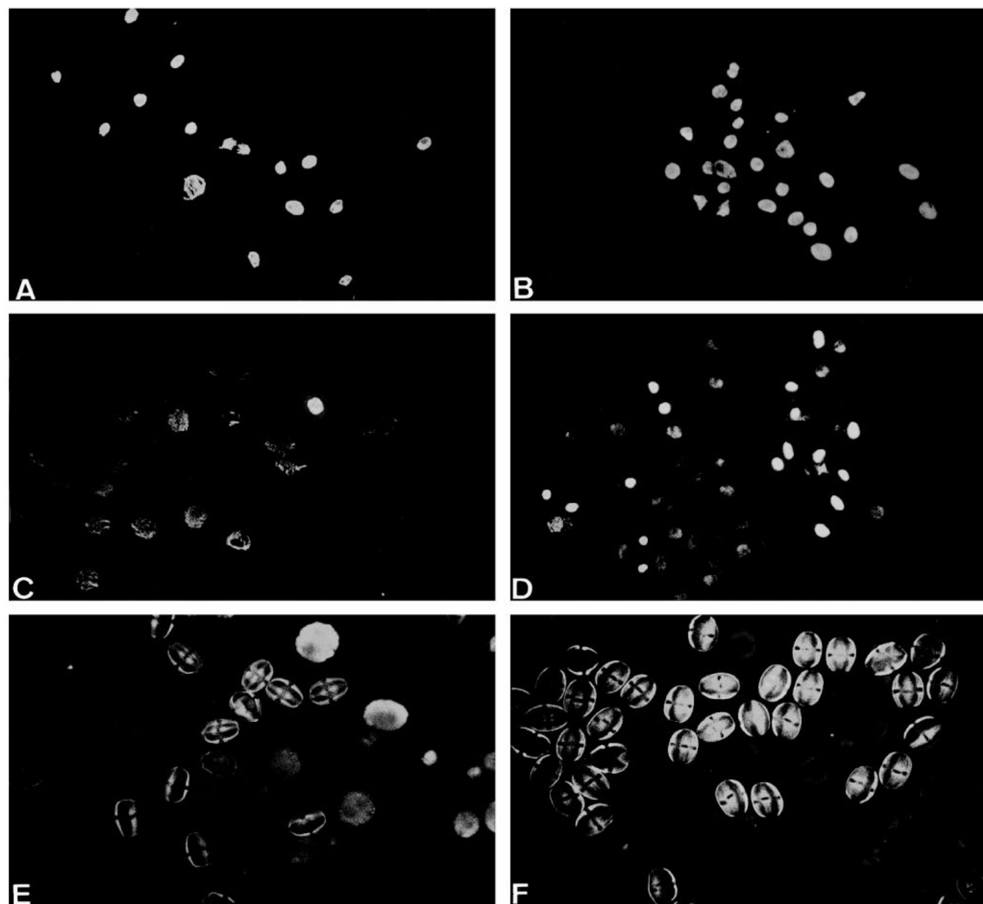

Fig. 2A–F Detection of apoptotic nuclei in field bean root-tip meristems and young anthers by the use of the TUNEL assay. **A** Meristematic wild-type nuclei pretreated with DNase (100 μg/ml, 30 min at room temperature) as a positive control (labelled yellow by fluorescein-conjugated antidigoxigenin antibodies); **B** wild-type nuclei treated without terminal deoxynucleotidyltransferase as a negative control (red); **C** intact wild-type cells (red) and a single apoptotic nucleus (yellow); **D** meristematic $\overline{\text{FHE}}$ cells with a high proportion of apoptotic nuclei (yellow); **E** wild-type microspores with intact nuclei (note the areas not covered by fluorescing parts of the exine do not show yellow flourescence) surrounded by tapetum nuclei of which some are apoptotic; **F** microspores of plants heterozygous for karyotype $\overline{\text{HE}}$ with intact nuclei surrounded by non-apoptotic tapetum nuclei (counterstaining with propidium iodide). Magnification: 1000 ×

induce apoptosis (for a review see Harms-Ringdahl et al. 1996), it is difficult to prove whether a certain type(s) of chromosome aberration(s) and/or other effects of the treatment elicit apoptosis.

The low number of apoptotic nuclei in wild-type meristems could be due to contamination of the preparations by a few apoptotic cells of the root cap (Wang et al. 1996 a), although the calyptra was removed prior

to squashing of the meristems; alternatively a small number of apoptotic cells might occur in wild-type meristems for unknown reasons.

Within longitudinal sections from lateral roots of the $\overline{\text{FHE}}$ karyotype the nuclei of cells of the differentiation zone behind the meristem did not reveal any label. Therefore, in agreement with data obtained from other material (Wang et al. 1996 a), apoptotic nuclei were no longer present in differentiated tissues. However, if a substantial proportion of cells is involved, cell death can be one reason for the retardation of growth and development and possibly also for the incomplete development of tissues responsible for the nutrition of developing seeds.

What is the relationship between too-long chromosome arms and reduced seed formation?

Homozygous $\overline{\text{FHE}}$ plants are characterized on average by very low fertility. Even heterozygous $\overline{\text{FHE}}$ plants revealed reduced fertility. Gametes of the $\overline{\text{FHE}}$ karyotype contributed about 20% to the sparse progeny (97 individuals, 31 heterozygous and 3 homozygous for karyotype $\overline{\text{FHE}}$) of these heterozygous plants. Within the small progeny that was obtainable from plants heterozygous for karyotype $\overline{\text{HE}}$ with an even longer chromosome arm (24.4% of the metaphase genome length), the $\overline{\text{HE}}$ karyotype is transmitted according to Mendelian expectation. From only 1 out of 23 heterozygous $\overline{\text{HE}}$ plants four seeds were obtained and four gametes of the $\overline{\text{HE}}$ karyotype were involved. One of the four seedlings which died soon after germination was homozygous for the $\overline{\text{HE}}$ karyotype, showing that this karyotype is transmissible by male and female gametes. This, together with the observation that only 2.8% of the tetrads in heterozygous $\overline{\text{HE}}$ plants showed abnormalities (one of the spores degenerated or a fifth spore occurred with a micronucleus), indicates that for karyotype $\overline{\text{HE}}$ (male) meiosis-II and gametophytic mitoses are not the bottle necks responsible for the highly diminished offspring and that the ratio of chromosome arm length to spindle extension is probably not higher in these tissues than in sporophytic mitoses. This assumption gains further support from the results of the TUNEL assay with squashed anthers containing microspores. Neither microspores of wild-type nor of heterozygous $\overline{\text{HE}}$ plants revealed apoptotic nuclei although nuclei of the neighbouring tapetal cells were occasionally strongly labelled (Fig. 2E, F). However, nuclei of microspores pre-treated with DNase were strongly labelled (data not shown). The easiest explanation seems to be that limited seed set in karyotypes with too-long chromosome arms may not only be caused by disturbed meiotic and post-meiotic divisions but also by insufficiently developed maternal supportive tissue, the developing seed coat, which is required for appropriate nutrition of the embryo. Due to the late differentiation of this tissue there is a high risk of many disturbed cell divisions, and subsequent cell death, at this stage. This could eventually be responsible for frequent seed abortion in lines with chromosomes slightly longer than half of the telophase spindle axis. Interestingly, plants heterozygous for karyotype $\overline{\text{T140E}}$ with a chromosome arm comprising 26% of the metaphase genome, i.e. being longer than the longest ones of karyotypes $\overline{\text{FHE}}$ and $\overline{\text{HE}}$, no longer contributed gametes of a $\overline{\text{T140E}}$ karyotype to their progeny. Therefore, the sterility of plants which contain karyotypes with chromosomes of that length could be caused by a further increase of both disturbed sporophytic mitoses and incomplete chromatid segregation during meiosis and the gametophytic mitoses. Anthers of heterozygous $\overline{\text{T140E}}$ plants could not be studied since no flowering plants were available.

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