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Two-dimensional spreads of synaptonemal complexes from solanaceous plants

III. Recombination nodules and crossing over in *Lycopersicon esculentum* (tomato)

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Abstract. By using serial sectioning and a new hypotonic bursting technique on primary microsporocytes of tomato (*Lycopersicon esculentum*), relatively large numbers of recombination nodules (RNs) are observed on the synaptonemal complexes forming during zygonema. In pachynema most, but not all, of these RNs are lost. If RNs represent sites of potential crossing over during zygonema and sites of actual crossing over during late pachynema, the observed temporal and spatial distribution of RNs may provide answers for some classic cytogenetic questions such as: how is at least one crossover per bivalent assured? How are crossovers localized? What is the basis for positive chiasma interference?

Introduction

Recombination nodules (RNs) are spherical to ellipsoidal structures about 100 nm in their longest dimension that lie on the central element of the synaptonemal complex (SC) (Carpenter 1979a; von Wettstein et al. 1984). In her initial description of RNs in *Drosophila melanogaster*, Carpenter (1975) suggested that they are located at the sites of crossing over and comprise the essential machinery for forming a crossover. In support of this hypothesis, when the number and distribution of RNs has been determined by serial sectioning and three-dimensional reconstruction of pachytene nuclei, a close correlation with the number and distribution of chiasmata and crossover events is observed (Carpenter 1979a; von Wettstein et al. 1984). Furthermore, in male *Bombyx mori* and in *Coprinus cinereus*, RNs seem to be retained at sites where chiasmata are formed (von Wettstein et al. 1984), and in female *D. melanogaster*, two mutations causing reduced recombination are correlated with reduced numbers of RNs (Carpenter 1979b). However, RNs do not necessarily result in crossing over since they have been reported on central region material associated with unsynapsed axial cores in triploid *Coprinus cinereus* (Rasmussen et al. 1981) and on SCs involved in non-homologous synapsis (Hobolth 1981). Another mutation in *D. melanogaster* reduces recombination but not the number of RNs (Carpenter 1979b). From this evidence, it seems likely that RNs, like SCs, are associated with normal meiosis and are necessary but not sufficient prerequisites for the high levels of crossing over characteristic of meiosis.

Most current models for recombination at the molecular level include an intermediate that, through isomerization, has an equal probability of resulting in a crossover (reciprocal exchange) or of being resolved without crossing over (Holliday 1977; Radding 1978). In either case the recombination intermediate permits gene conversion in its vicinity. One problem with relating RNs to these models is that in organisms on which quantitative studies of RNs have been performed, usually less than an average of four RNs per SC have been observed from zygonema through pachynema (von Wettstein et al. 1984). If each RN forms a recombination intermediate that has a 50:50 chance of causing a crossover, a significant proportion of bivalents should have no crossovers or chiasmata, e.g., $\geqq(0.5)^4$. This proportion may be even higher since it has been observed in certain fungi that less than the expected 50% of the conversion events are associated with crossing over (Klein 1984). Because crossing over and chiasma failure are much less frequently observed (Holm and Rasmussen 1980; Holm et al. 1981; Jones 1967), there appears to be a means of practically guaranteeing at least one crossover and chiasma per bivalent. Given an RN, this problem could be resolved by genetic control of isomerization so that the presence of an RN assures a crossover (Carpenter 1984). Two mutations in *D. melanogaster* that reduce crossing over to 8% of that in the wild type while leaving the amount of gene conversion unaffected imply the possibility of such control (Carpenter 1982; Carpenter 1984). However, this would not solve the problem because it only replaces an unknown mechanism for guaranteeing a crossover with an unknown mechanism for guaranteeing an RN on each bivalent.

Quantitative analyses of RNs have been reported for only three plant species (Abirached-Darmency et al. 1983; Kehlhoffner and Dietrich 1983; Hobolth 1981). The samples were small due, at least in part, to the labor intensive requirement of three-dimensional reconstruction of pachytene nuclei from serial thin sections. While the Counce and Meyer (1973) surface-spreading technique for displaying SCs from animal primary spermatocytes in two dimensions would seem to be ideal for locating RNs on SCs, there has been only one report in which this technique was used for observing RNs on the SCs of a plant (Gillies 1984). On the other hand, we have developed a new, generally applicable hypotonic bursting technique for spreading SCs from primary microsporocytes of vascular plants (Stack 1982). By hypotonic bursting, SCs are displayed essentially free of other protoplasm, and RNs are clearly visible

[Figs. 1–6; also see Albini and Jones (1984) for another spreading technique that demonstrated RNs in *Allium fistulosum*].

For a study of RNs and SCs, we selected tomato (*Lycopersicon esculentum*) primary microsporocytes because (Stack and Anderson 1986): (1) tomato is a true diploid with $2n=2x=24$ small chromosomes. (2) Meiosis in tomato is regular and conforms to the classic meiotic stages. (3) The cultivars of tomato differ little in their chromosome cytology or genetic map. (4) All tomato chromosomes have pericentric heterochromatin that offers the opportunity to study SCs and RNs in eu- and heterochromatin. (5) The relatively short SCs of tomato are largely immune to the stretching and fragmentation that are more common in SC spreads from species with longer SCs. (6) The genetic map of tomato is one of the best available for plants.

In our study of tomato, we have observed a pattern of RNs on SCs in zygonema and early pachynema that is different from the pattern reported so far in animals and fungi (von Wettstein et al. 1984). We interpret this pattern to be compatible with current molecular models for recombination and to give clues for answering several classic cytogenetic questions such as: how is at least one crossover per bivalent assured? How are crossovers localized? What is the basis for positive chiasma interference?

Materials and methods

Cherry tomatoes ($2n=24$, *Lycopersicon esculentum*) were grown to flowering year round in a controlled temperature greenhouse (21°–24° C).

The hypotonic bursting technique for spreading SCs from primary microsporocytes of tomato has been reported in detail elsewhere (Stack 1982). The following description only stresses those steps that are significantly different from the previously described technique.

The primary microsporocytes from one of the five anthers normally found in a bud were squeezed into a drop of 2% aceto-orcein on a slide and squashed to determine their stage of meiosis. If the stage was leptonema, zygonema, pachynema, or diffuse stage (early diplonema), the remaining anthers were assumed to be at the same stage (Stack and Anderson 1986) and placed in a depression slide containing 0.1 ml of a solution containing 0.7 M sorbitol or mannitol, 0.6 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 0.1 mM Pipes (acid), 0.3% potassium dextran sulfate, and 2% polyvinylpyrrolidone with the pH adjusted to 5.1 with 0.1 N KOH. The anthers were bisected transversely, and the primary microsporocytes were squeezed out. Then 0.25–0.5 mg β-glucuronidase was stirred in. The rods of cells were digested for 10–15 min, then drawn into a micropipet, and transferred to a plastic-coated glass microscope slide that had been glow discharged to make it hydrophilic. A siliconized cover glass was placed on the cell suspension and 3 µl of distilled water was drawn under it to burst the cells. The cover glass was removed by the dry ice method, and the slides were air dried before fixation in 4% formaldehyde, pH 8.5. The slides were then rinsed briefly in 0.4% Kodak Photoflo 200, air dried, and stained either with phosphotungstic acid (PTA), as described by Moses (1977), or with uranyl acetate and lead citrate (UP). For UP staining the slides were incubated 5 min in 1% uranyl acetate in water, rinsed in water, incubated 5 min in Reynolds' (1963) lead citrate, rinsed in water, and air dried. SCs were located by phase contrast microscopy, and 50 mesh copper grids were placed over the SCs. The plastic and grids were lifted by water, picked up on glassine paper, and air dried.

For partial, three-dimensional reconstructions of primary microsporocyte nuclei, the stage of meiosis was determined by aceto-orcein squashing as described for hypotonic bursting above. If the primary microsporocytes of one anther in a bud were in zygonema through pachynema, the other anthers of the bud were placed in 0.1 M potassium phosphate buffer at pH 7.4 containing 2% formaldehyde and 2% glutaraldehyde. The anthers were bisected transversely, the rods of primary microsporocytes squeezed out, and the anther walls discarded. Fixation continued 2 h before rinsing in the same buffer without fixatives. The cells were then postfixed for 2 h in the same buffer containing 2% OsO_4 and subsequently dehydrated through an acetone series. The cells were embedded in Epon-Araldite (Mollenhauer 1964). Serial sections were cut on a diamond knife at a thickness of approximately 100 nm. Ribbons of serial sections were dried into the openings of Formvar-coated slot grids and post stained by UP as described for spread SCs.

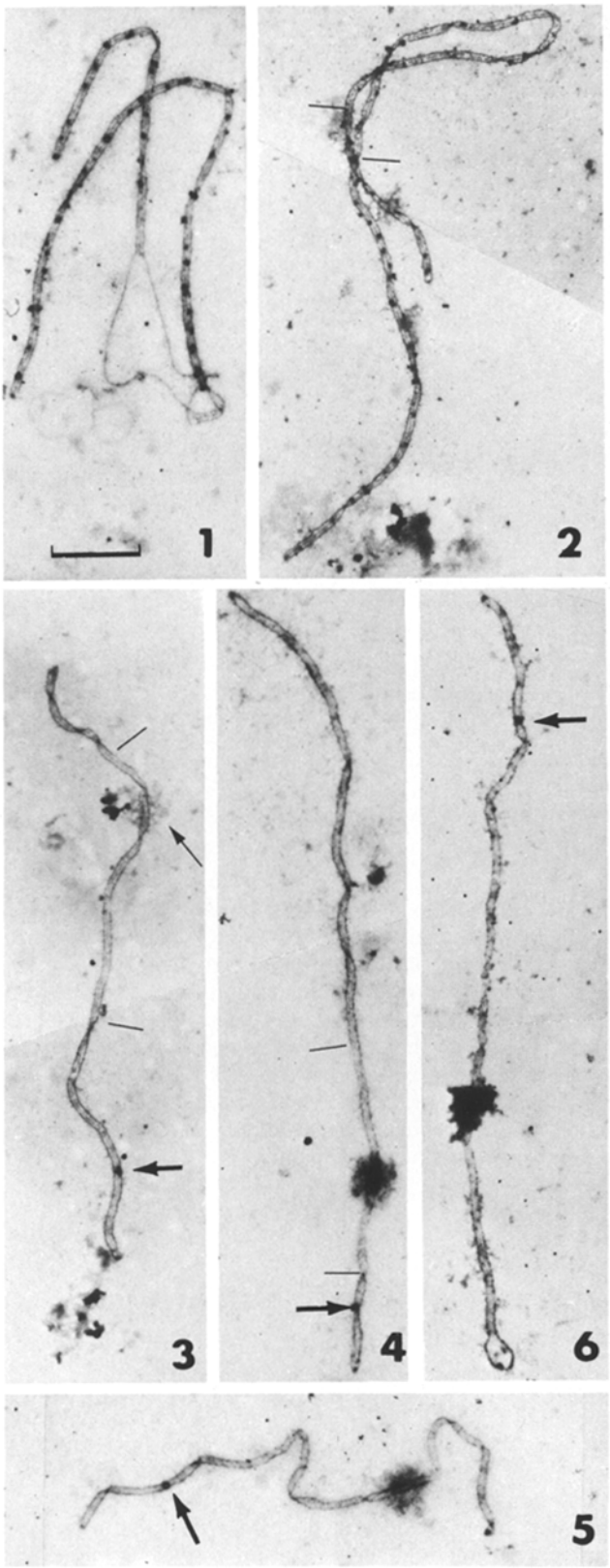
All preparations were examined and photographed in an AEI EM6B or an AEI 801 electron microscope at an accelerating voltage of 60 kV. Magnifications on negatives were determined from carbon gratings. Lengths of spread SCs were measured from prints using a Hewlett Packard 86B computer and 9111A graphics tablet. For three-dimensional reconstructions, serial sections of nuclei were photographed at 10,000× and printed at 20,000×. The outline of the nuclear envelope, the location of SCs and axial cores, the location of heterochromatin, and the location of RNs were marked on clear acetate sheets. By superimposing the outline of the nuclear envelope on adjacent acetate sheets that represented serial sections, portions of 75 nuclei were reconstructed. SC lengths were corrected for section thickness by applying the Pythagorean theorum (Gillies 1972). An average partial reconstruction involved 10–11 serial sections (about 1.05 µm) of a nucleus that averaged 5 µm in diameter.

Results and discussion

The primary microsporocytes of all five anthers in a tomato bud are closely synchronized from leptonema through midpachynema (Stack and Anderson 1986). By squashing the contents of one anther for examination in the light microscope, the meiotic stage of the primary microsporocytes in the other anthers can be determined accurately. Using this technique as an initial guide, it was observed that during zygonema and early pachynema, axial cores and SCs in spread preparations do not display visible kinetochores (Figs. 1–3). In contrast, kinetochores are visible in late pachynema (Figs. 4, 5) and in early diffuse stage (early diplonema), when synapsis is beginning to break down (Fig. 6). Once these correlations had been established, the stage could be determined reliably by observing spread SCs alone.

Pattern of RNs on spread SCs

From mid through late pachynema there are usually one to three ellipsoidal particles that are approximately 100 nm in their longest dimension lying on the central element of



Figs. 1–6. Synaptonemal complexes (SCs) from hypotonically burst primary microsporocytes of tomato from late zygonema through early diplonema. Dark staining telomeres that superficially resemble recombination nodules (RNs) are visible at the ends of all the SCs. The bar represents 2 μm. **1** Late zygonema SC. There are numerous RNs (about 36) on the completed SC except near the unsynapsed segment that corresponds to late synapsing pericentric heterochromatin. **2** Early pachynema SC indicated by the absence of a kinetochore and complete synapsis. There are numerous RNs except in the presumptive location of pericentric heterochromatin (between the two *bars*). Although it is not clear in this case, the SC in pericentric heterochromatin is often lighter staining and the lateral elements thinner than in euchromatin (see Figs. 3, 4). **3** Early pachynema SC with a single RN (*large arrow*) in euchromatin. The SC in heterochromatin lies between the *bars*. A forming kinetochore is visible on the heterochromatic segment (*small arrow*). **4** Late pachynema SC with a single RN visible in side view on a twist of the SC (*arrow*). Heterochromatin lies between the *bars* with a prominent kinetochore visible on the SC in heterochromatin. **5** Similar to **4**, but the single RN in euchromatin is seen in frontal view (*arrow*). **6** Early diplonema SC with a single RN visible distally in euchromatin (*arrow*). A prominent kinetochore is also visible closer to the other end of the SC where desynapsis has just begun. Here the ends of the lateral elements are still held together at the telomere.

the SC in euchromatin (1.7 ± 0.89 particles per SC, $n = 139$ SCs; Figs. 4, 5). This represents 0.085 particles per μm of SC (Table 2). Judging from their size, shape, and location, these particles must be RNs. This frequency is similar to the frequency of RNs from mid- to late pachytene nuclei of animals and fungi (von Wettstein et al. 1984). The frequency of RNs per individual SC in tomato indicates that a complete set of 12 SCs at mid- to late pachynema has an average of 20.5 RNs (Table 1). This estimate is similar to the average of 21.3 crossover events per diploid set of chromosomes derived from the genetic linkage map for tomato (Rick 1982). Also like chiasmata in tomato (Brown 1949), RNs tend to be located distally (Figs. 4, 5).

In zygonema, although interstitial (away from the ends of chromosomes) initiation of synapsis can occur, distal (toward the ends of a chromosome) initiation is much more common (Stack and Anderson 1986). Since all of the chromosomes have pericentric heterochromatin, this means that most if not all initiation of synapsis occurs in euchromatin. During early and mid-zygonema, numerous elipsoidal to spherical RNs associate with the central element almost anwhere that the SC has formed (Fig. 1). Observed differences in size of individual RNs during zygonema and early pachynema may indicate different steps in their assembly. Pericentric heterochromatin synapses late in zygonema, and relatively few RNs associate with the SC in these segments of the chromosomes (Stack 1984; Stack and Anderson 1986; Figs. 2, 3). Possible reasons for the differential associations of RNs with SCs in eu- and heterochromatin have been discussed recently (Stack 1984). In tomato the frequency of RNs during zygonema is 1.41 per μm of SC (Table 2). If this frequency were maintained into late pachynema, when the combined length of a complete set of 12 SCs is 240 ± 35.9 μm ($n = 25$) (Table 1), there would be 358 RNs per set or an average of 28.1 RNs per synapsed bivalent. Instead, there is a 16.6-fold reduction in the frequency of RNs per unit length of SC by late pachynema. The reduction seems to occur in early pachynema since SCs at this stage have frequencies of RNs between those observed in zygonema and late pachynema (Figs. 2, 3 and 7).

Patterns of RNs on sectioned SCs

Most previous studies of the frequency of RNs on SCs have utilized three-dimensional reconstructions of serially sectioned nuclei. For comparison, we prepared a series of

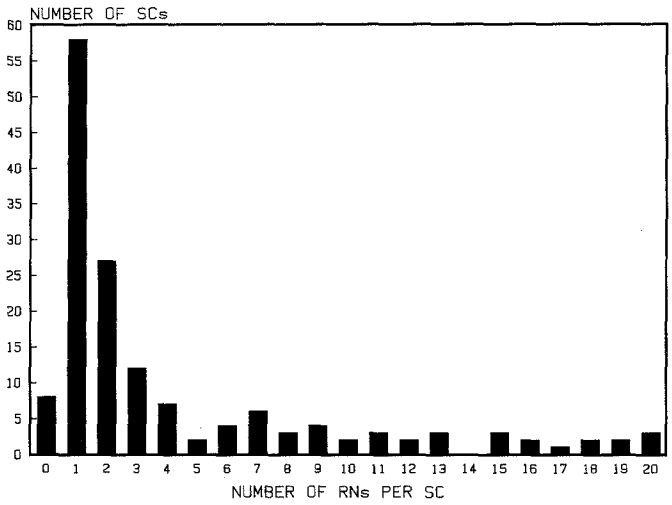


Fig. 7. Bar graph of the number of recombination nodules (RNs) per synaptonemal complex (SC) in early pachynema. SCs were selected at random from those scattered on slides. The SCs seem to fall either into a relatively distinct group containing SCs with four or fewer RNs or a less distinct group containing SCs with five or more RNs. The data have been interpreted to indicate a precipitous loss of RNs during late zygonema or early pachynema to leave only a few RNs per SC. We suspect the zero RN category is an artifact due to some mechanical loss of RNs during hypotonic bursting (Stack and Anderson 1986, with permission of the American Journal of Botany)

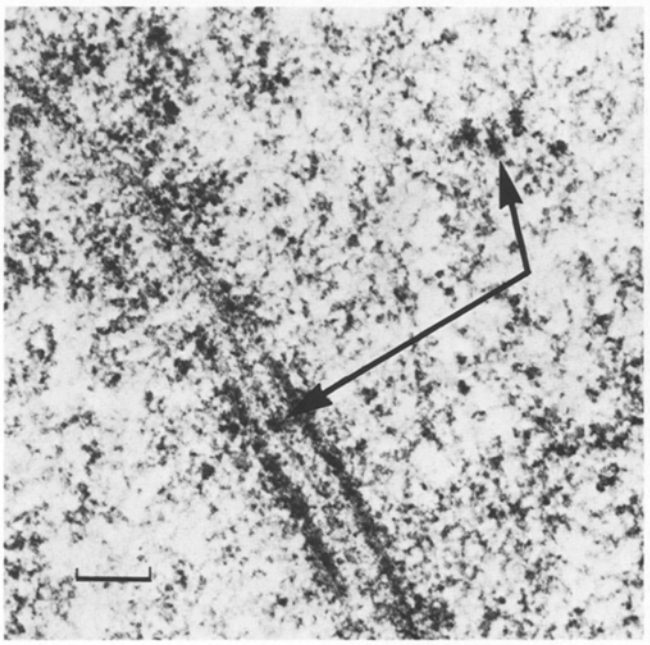


Fig. 8. Section of a primary microsporocyte in early pachynema in which recombination nodules (RNs) are visible on the central element of synaptonemal complexes (SCs) in both transverse (*short arrow*) and frontal sections (*long arrow*). Bar represents 0.2 μm

partial reconstructions of serially sectioned nuclei from primary microsporocytes of tomato in zygonema, early pachynema, and late pachynema (Fig. 8; Table 2). At all three stages, RNs are approximately twice as frequent on SCs in spread preparations as compared to three dimensional

Table 1. Average lengths of complete sets of 12 synaptonemal complexes in early, mid-, and late pachynema from tomato primary microsporocytes

Early pachynema	mid-pachynema	late pachynema
252.2±36.3 μm ($n=26$)	255.8±31.9 μm ($n=25$)	240.2±35.9 μm ($n=25$)

Table 2. Frequency of recombination nodules (RNs) on synaptonemal complexes (SCs) in tomato primary microsporocytes

Stage	Hypotonic bursting[a]	Three-dimensional reconstructions
Zygonema	1.41 RN/μm of SC (335 μm of SC analyzed from 23 bivalents[a])	0.74 RN/μm of SC (217 μm of SC analyzed from 21 different nuclei)
Early pachynema	0.35 RN/μm of SC (33 bivalents analyzed)	0.18 RN/μm of SC (584 μm of SC analyzed from 27 different nuclei)
Late pachynema	0.085 RN/μm of SC (139 bivalents analyzed)	0.05 RN/μm of SC (972 μm of SC analyzed from 27 different nuclei)

[a] SCs were selected at random from those scattered over slides. Few are likely to have come from the same nucleus

reconstructions. We suspect this difference is due to a greater difficulty of identifying RNs in sectioned material, and we consistently missed approximately the same proportion of RNs in sectioned nuclei from all three stages. More important than the absolute frequency of RNs in sectioned material is the fact that the relative frequency of RNs from zygonema through pachynema is nearly the same as in hypotonically burst nuclei, i.e., there is roughly a 15-fold reduction in the frequency of RNs per unit length of SC from zygonema to late pachynema.

An hypothesis

Taking into account the frequency of RNs in zygonema, if each RN is capable of producing a recombination intermediate with a 50:50 chance of forming a crossover, the question is not how at least one crossover per bivalent is guaranteed, but why are there no more crossovers, e.g., an average of 14 per bivalent based on the extrapolated value of 28 RNs per bivalent in completely synapsed chromosomes? The answer may lie in the temporal pattern of synapsis and positive chiasma interference. To explain, we assume that RNs cannot mediate recombination until they attach to a completed segment of SC. The SC forms progressively during zygonema. During early and mid-zygonema, almost any segment of completed SC is occupied by one or more RNs. Crossing over must require a certain amount of time to be completed and because of this, the first RNs to attach will have a head start on recombination compared to RNs that attach later. Since distal initiation of synapsis in euchromatin is much more common in tomato primary microsporocytes than is proximal initiation

in heterochromatin, euchromatin obtains RNs before heterochromatin. Aside from the fact that heterochromatin receives few RNs when it does synapse (Stack 1984), one might assume crossing over would be achieved in euchromatin before heterochromatin. Considering the proposed steps in molecular models for forming a crossover (Holliday 1977; Radding 1978), it is reasonable to assume that establishing a crossover intermediate would take much longer than completing reciprocal exchange or resolving a crossover intermediate after it had formed. Positive crossover interference (the tendency for one crossover to inhibit the formation of another crosssover nearby) may be directly related to the loss of RNs during early pachynema. One might hypothesize that the first successful reciprocal recombination event causes a "signal" to be transmitted in either direction down the SC. This signal attenuates with distance so it causes the release only of nearby RNs and inhibits only nearby crossing over. The signal may not be able to cross an unsynapsed region. Presumably the released RNs have not yet established crossover intermediates and are removed without lasting effect on the DNA. Recombination intermediates that are resolved without crossing over may be involved in gene conversion but not in chiasma interference. This mechanism would essentially guarantee a small number of crossovers with only rare failures, e.g., an average failure rate in tomato of $(0.5)^{28}$ per SC based on an extrapolated frequency of RNs in late pachynema of 28 per SC.

Classic observations of an increase in recombination in one segment of a chromosome if recombination is inhibited in another segment of the same chromosome have been explained on the basis of a reduction in chiasma interference (Rhoades 1968). We note that even short synapsed segments of chromosomes have enough RNs to be likely to undergo exchange if they are not inhibited by positive chiasma interference from adjacent segments.

As suggested by others (Darlington 1937; John and Lewis 1965), localization of exchange would be dependent on the pattern of synapsis. Thus, while there may be other explanations for the lack of crossing over in heterochromatin (Stack 1984), RNs attach first in euchromatin, and this is where practically all crossing over and chiasma formation occurs (Rick 1971). Variability of the exact site of exchange depends on variability in the pattern of synaptic initiation, the exact sites on SCs where RNs are located, and the probability that any particular RN will be involved in crossing over after forming a crossover intermediate.

Pattern of RNs on SCs in other organisms

Hobolth (1981) made complete three-dimensional reconstructions of a late zygotene nucleus and an early pachytene nucleus of hexaploid bread wheat (*Triticum aestivum*, $2n=42$) in which he observed 97 and 88 RNs, respectively. Abirached-Darmency et al. (1983) reconstructed one early and three mid- to late pachytene nuclei of rye (*Secale cereale*, $2n=14$) and counted 48 and an average of 24 RNs per nucleus, respectively. Kehlhoffner and Dietrich (1983) reconstructed one pachytene nucleus of *Paeonia tenuifolia* ($2n=10$) and counted six RNs. Assuming these are accurate counts of the number of RNs per nucleus, there is an average of one to eight RNs per SC, similar to the numbers of RNs per SC in early pachytene nuclei of tomato (Table 2). In our unpublished investigations of *Solanum tuberosum* (a tetraploid dicotyledonous plant), and *Allium cepa* and *Tradescantia edwardsiana* (diploid monocotyledonous plants), we have found high frequencies of RNs per unit length of SC in zygonema and comparatively low frequencies of RNs per unit length of SC in pachynema. This suggests a common pattern of RN distribution for all angiospermous plants. In addition, we have found the tomato pattern of RN distribution in *Psilotum nudum*, a primitive, seedless vascular plant (unpublished), suggesting that this pattern of RN distribution may be common to vascular plants in general.

Animals and fungi similarly exhibit a decrease in the number of RNs from late zygonema through pachynema (Carpenter 1979a; Holm and Rasmussen 1980). However, the decrease in RNs is usually 1.5- to 2-fold and involves many fewer RNs (von Wettstein et al. 1984). This difference may simply be related to the rate of synapsis and the number of RNs available at any one time. Generally all steps of meiosis are shorter in plants compared to animals that have even roughly similar genome sizes (Bennett 1977). For instance, zygonema lasts 3 h in snapdragon (Ernst 1938) and 8 h in rye (Riley and Bennett 1971) compared to about 1 day in man (Clermont 1963; Heller and Clermont 1963) and 5 days in a newt (Callan 1968). In tomato, leptonema through zygonema lasts approximately 18 h, and pachynema lasts approximately 6 h (Stack and Anderson 1986). With fast synapsis and many RNs available, possibly tomato and vascular plants in general rapidly achieve a level of crossing over sufficient to assure at least one chiasma per bivalent. Animals require longer times for synapsis, and with relatively few RNs they may only slowly achieve a crossover in each bivalent by using and reusing either RNs or components of RNs.

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