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Genetic analysis and FISH mapping of the *Colourless non-ripening* locus of tomato

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Abstract *Cnr* (*Colourless non-ripening*) is a dominant pleiotropic ripening mutation of tomato (*Lycopersicon esculentum*) which has previously been mapped to the proximal region of tomato chromosome 2. We describe the fine mapping of the *Cnr* locus using both linkage analysis and fluorescence in situ hybridisation (FISH). Restriction fragment length polymorphism (RFLP)-, amplified restriction fragment polymorphism (AFLP)-, and cleaved amplified polymorphic sequence (CAPS)-based markers, linked to the *Cnr* locus were mapped onto the long arm of chromosome 2. Detailed linkage analysis indicated that the *Cnr* locus was likely to lie further away from the top of the long arm than previously thought. This was confirmed by FISH, which was applied to tomato pachytene chromosomes in order to gain an insight into the organisation of hetero- and euchromatin and its relationship to the physical and genetic distances in the *Cnr* region. Three molecular markers linked to *Cnr* were unambiguously located by FISH to the long arm of chromosome 2 using individual BAC probes containing these single-copy sequences. The physical order of the markers coincided with that established by genetic analysis. The two AFLP markers most-closely linked to the *Cnr* locus were located in the euchromatic region 2.7-cM apart. The physical distance between these markers was measured on the pachytene spreads and estimated to be approximately 900 kb, suggesting a bp:cM relationship in this region of chromosome 2 of about 330 kb/cM. This is less than half the average value of 750 kb/cM for the tomato genome. The relationship between genetic and physical distances on chromosome 2 is discussed.

Keywords Molecular markers · Heterochromatin · Physical distance · Map-based cloning · *Lycopersicon esculentum*

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Introduction

The *Cnr* (Colourless non-ripening) mutation in tomato (*Lycopersicon esculentum*) produces a severe non-ripening phenotype, where fruit develop a colourless and mealy pericarp (Thompson et al. 1999). The profound effects of the *Cnr* mutation on fruit ripening suggest that it may represent a gene involved in the regulation of this important developmental process, and consequently it is of sufficient importance to justify an attempt to clone the gene using a map-based approach. Cloning a gene by a genetic map-based approach can be greatly facilitated by high-density genetic maps, already available for a number of plant species including tomato (Tanksley et al. 1992; Haanstra et al. 1999), and a high frequency of recombination in the region of interest. Linkage maps, however, cannot provide definitive detail of the physical location of markers on the chromosomes, since map distances are not directly proportional to physical distances. In general the relative positions of loci on the physical map differ greatly from their corresponding sites on a genetic map (de Jong et al. 1999). This is caused by variation in recombination frequency in different regions of the genome, with crossing-over being almost absent in the heterochromatin, and more frequent in some areas of the euchromatin (Sherman and Stack 1995).

We have recently produced an integrated cytogenetic map of the short arm of *Arabidopsis* chromosome 4 which demonstrates that heterochromatic organisation of the chromosome is associated with low recombination activity (Fransz et al. 2000). In tomato and other species where a physical map is still lacking or incomplete, information about the location of a gene of interest can be

critical in cases where map-based cloning of that locus is planned. A gene in a heterochromatin-rich region may prove to be a much more difficult target to clone due to lack of recombination and the presence of blocks of highly repeated sequences. Furthermore, markers that are apparently genetically close to the gene, may be physically very far from it (Zhong et al. 1999).

Fluorescence in situ hybridisation (FISH) is a powerful tool for positioning unique or repetitive sequences on chromosomes. Single- and low-copy sequences can be localised on tomato synaptonemal complex spreads, which are highly extended and contain four copies of each locus (Peterson et al. 1999). FISH can therefore be undertaken as a prelude to map-based cloning, in order to determine if the gene of interest lies within heterochromatin or euchromatin and, prior to chromosome walking, to determine the physical distance between the closest markers flanking the gene. Information of this type may be crucial before embarking on positional cloning of a target gene merely on the basis of very tight linkage to molecular markers (Zhong et al. 1999).

We are currently using FISH to determine the physical distances between markers on chromosome 2 as part of a programme to clone the *Cnr* gene. Our initial genetic mapping experiments indicated that the gene residing at the *Cnr* locus was in the sub-telomeric region of the long arm of chromosome 2 within a 13-cM interval flanked by the RFLP loci TG31/CT251 and CT106A (Thompson et al. 1999). Gaps in the linkage map occur in a number of regions of the tomato molecular map and may indicate areas of low gene density or else are 'hot spots' for recombination (Tanksley et al. 1992). If a map-based approach to isolate the *Cnr* gene is to be considered, it is important to determine if the gene is in a heterochromatic region, and to identify its position relative to the centromere. Here we report on genetic mapping experiments to establish the order and relative distances of RFLP-, AFLP- and CAPS- based markers linked to the *Cnr* locus. Selected markers were then located on chromosome 2 by FISH, thus providing a comparison of the genetic and physical distances between the markers close to the *Cnr* locus.

Materials and methods

Plant material

The *Cnr* mutation was detected in an F_1 hybrid tomato cultivar Liberto (Thompson et al. 1999) and a homozygous mutant line was subsequently produced after selfing for four generations. A cross was made between this *L. esculentum Cnr* line and *Lycopersicon cheesmanii* (LA483). The resulting F_1 was selfed and an initial mapping population of more than 300 F_2 progeny was generated.

Isolation of genomic DNA

Genomic DNA was isolated from young leaves using the Phytopure kit (Anachem, Bedford,UK) following the manufacturer's instructions. C_0t-1 DNA, which was used for blocking repetitive sequences in the FISH experiments, was generated from tomato genomic DNA following the protocol of Zwick et al. (1997).

AFLP and RFLP markers and probe labelling

For RFLP analyses, typically 10 µg of genomic DNA *from L. cheesmanii* and *L. esculentum* were digested with the restriction enzymes, *Bam*HI, *Bgl*II, *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*cII, *Hin*dIII and *Pst*I (Life Technologies, Paisley, UK), fractionated on 0.8% (w/v) agarose gels in 1×TAE buffer and transferred to a Hybond N+ membrane (Amersham, Bucks, UK). Plasmids containing the tomato RFLP markers CT251, TG276, CT205, CT277 and CT244 (Tanksley et al. 1992) were obtained from S.D. Tanksley (Cornell University). The 721AL marker was obtained from J.D.G. Jones (Sainsbury Laboratory, UK). DNA probes were radiolabelled with [^{32}P]dCTP using the Hyper Prime Labelling Kit (Bioline, London, UK). DNA blots were hybridised with labelled probes using standard techniques (Ausubel et al. 1990). AFLP analyses were performed using a kit obtained from Life Technologies, except for the ^{33}P (Amersham). Manufacturers' instructions were followed with minor modifications. PCR products were separated on 5% polyacrylamide gels (19:1 acrylamide. N,N´methlyene-bis-acrylamide, 8.0 M urea, 1×TBE buffer). The gels were run for 3 h, vacuum dried and exposed to X-Ray films (Kodak). PCR-based markers (CAPS) were designed for the CT205, TG276, CT277 and CT244 loci on chromosome 2. RFLP, AFLP and CAPS markers were mapped relative to the *Cnr* mutant locus on 255 F_2 plants using the Kosambi mapping function (Kosambi 1944) and the JoinMap programme (Stam and Van Ooijen 1995).

Tomato BACs containing the CT251, 721AL, E6M7 and E24M1 sequences were isolated by PCR screening of a pooled tomato BAC library obtained from J.Verbalov and J.J.Giovannoni (Texas A&M University) with sequence-specific primers. BAC DNA for use as probes in subsequent FISH experiments was isolated using an alkaline lysate protocol described by Woo et al. (1994). The molecular size of the BAC clones containing the probes 721AL and E24M1 was determined by PFGE according to standard protocols and was around 100 kb in both cases. The presence of AFLP markers E6M7 and E24M1 on the BACs was confirmed by sequencing of the PCR products from the BACs and mapping of BAC ends or internal sequences. For identification of the 45S rDNA locus, the pTa71 clone from *Triticum aestivum* L. (Gerlach and Bedbrock 1979) containing a 9-kb *Eco*R1 fragment consisting of the conserved 18S-5.8S-25S rDNA genes and the non-transcribed sequences was used. Probes for FISH were labelled with either dUTP-digoxygenin or dUTP-biotin using a Nick Translation Kit (Roche, East Sussex, UK) according to the manufacturer's instructions. The 45S rDNA probe was labelled directly with dUTP spectrum green (Amersham).

Fixation

Flower buds were taken from tomato plants (*L. esculentum* cv Ailsa Craig) and carefully dissected under a stereo-microscope. Single anthers from individual buds in the 0.3–0.5 mm size range were removed and the meiotic stages of single anthers were checked by lacto-proprionic orcein squashing. The remaining anthers were fixed in 3:1 ethanol:glacial acetic acid.

Preparation of material for microscopy

The spreading method of Zhong et al. (1996) was modified as follows. Anthers were washed 3× for 5 min in citrate buffer (10 mM, pH 4.5) and digested in an enzyme mixture containing 0.3% (w/v) cellulase, 0.3% (w/v) pectinase and 0.3% (w/v) cytohelicase (Sigma, UK) in 10 mM citrate buffer pH 4.5 for 2 h inside a moist chamber at 37°C. The enzyme solution was replaced with cold distilled water. A single anther with a small amount (10 µl) of water was placed on a glass slide and quickly broken up with a fine

glass needle; 20 µl of 60% acetic acid was added and the slide was placed on a hot block (45°C) for 30 s. The slide was placed on the bench and the material re-fixed by adding 100 µl of cold ethanol:acetic acid fix (3:1 v/v), initially in a circle around the acetic acid drop, until the fixative and acetic acid were throughly mixed. The slide was tilted, flushed with fresh fixative and air-dried. For in situ hydridisation freshly made slides (up to 1 week old) were found to give superior results.

In situ hydridisation

FISH was carried out according to the method of Franz et al. (1996) and Armstrong et al. (1998) with the following additions: before the paraformaldehyde step slides were incubated in 0.01 g of pepsin in 100 ml of 0.01 M HCl for 2 min at room temperature and immediately rinsed in distilled water. Between 50 and 100 ng of probe and (where appropriate) 1 µg of C_0t-1 DNA were used for each slide and made to 20 µl in a hybridisation mixture containing 50% de-ionised formamide, 2×SSC and 10% dextran sulphate. This was applied to the slides which were then sealed under a 22×22-mm coverslip using a vulcanising solution. Slides were placed on a hot block at 75°C for 4 min, which simultaneously denatured the target DNA and probe DNA. Hybidisation was carried out overnight at 37°C in a humid chamber. Post-hybridisation washes were performed as described by Fransz et al. (1996). Biotin-labelled probes were detected by FITC-antiavidin or Texas Red-antiavidin. Signal amplification was obtained by using biotin-conjugated goat antibody according to the manufacturer's instructions (Vector, UK). Detection of digoxygenin labelled probes was performed by the use of FITC- or rhodamine-antidigoxygenin antibody, according to the manufacturer's instructions (Roche). Slides were counterstained with DAPI (1 µg/ml) in Vectasheild antifade mounting medium (Vector). For photomicroscopy, slides were examined on a Nikon Eclipse T300 microscope. Capture and analysis of images was achieved using an image analysis system (Vysis, UK). Up to 30 good quality pachytene cells probed with either single or dual probe combinations were selected for the measurements. The captured pachytene images were measured directly on the screen.

Results and discussion

Identification of markers linked to *Cnr*

A bulked segregant approach (Michelmore et al. 1991) was undertaken to identify markers linked to the *Cnr* locus. From the segregating F_2 population, ten wild-type plants were selected at random and their pre-amplified DNAs were pooled (wild-type pool). Similarly, another pool was made from ten mutant-type plants (mutant pool). Screening wild-type and mutant pools with 430 *Eco*R1×*Mse*1 primer combinations identified four co-dominant and nine dominant AFLP linked markers. One of the markers (E6M7) which co-segregated with the mutant phenotype within these pools was cloned and used as an RFLP probe. Initial mapping experiments with this marker placed *Cnr* at the top of the long arm of chromosome 2, between the RFLP markers CT251 and CT106A (Thompson et al. 1999). E6M7 and a dominant marker (E24M1), which was in coupling-phase with the wild-type allele, were investigated further. CAPS markers were generated from BACs containing E6M7 and E24M1, and were mapped relative to the *Cnr* locus. Using 255 F_2 plants linkage between E6M7, E24M1 and *Cnr* was confirmed and their positions determined relative to published markers on the upper portion of the long arm of chromosome 2 (see Tanksley et al. 1992; Thomas et al. 1994). CT251, 721AL, TG276 and TG205 were all shown to be located above E6M7, E24M1 and the *Cnr* locus (Fig. 1), while markers CT277 and TG244 were located below the target locus. The LOD scores for markers mapped in relation to *Cnr* were 34 (E6M7), 41 (E24M1) and 19 (CT277). These mapping data place the *Cnr* gene a few cM from CT277 (Fig. 1a). This location is below that reported previously (Thompson et al. 1999). However, the current map is based on 255 F_2 plants, whereas the previous estimate relied on scores from less than 50 individuals.

FISH

Tomato chromosome 2 is submetacentric with a secondary constriction in the short arm. Ribosomal sequences have been located previously both by RFLP mapping and FISH (Tanksley et al. 1992; Xu and Earle 1994, 1996; Peterson et al. 1999) to the secondary constriction and provide a convenient marker for this chromosome. A DAPI-negative staining region delineates the centromere, whereas proximally on the long arm there is a densely stained region of heterochromatin and an extended euchromatin tail (see Fig. 1b). Preliminary experiments with tomato BAC clones containing the markers CT251 and 721AL gave a substantial amount of non-specific labelling. This indicated that the BAC probes for these markers contained dispersed repetitive DNA elements. The addition of C_0t-1 DNA can effectively block such repeats in a number of species, including tomato (Zwick et al. 1997). Using C_0t-1 DNA, CT251 could be localised close to the centromere of chromosome 2 (data not shown). However, the C_0t-1 DNA block was only partly effective in this case. The reason could reside in the particular abundance of repetitive DNA likely to be associated with the location of the CT251 BAC clone (Peterson et al. 1998). On the other hand, blocking of repetitive elements was highly successful for the BAC probe containing 721AL, even though this marker was situated in the pericentromeric heterochromatin on the long arm of chromosome 2 (Fig. 1b). This 721AL marker was previously mapped to the proximal region of chromosome 2 (Thomas et al. 1994). Our physical mapping experiments confirm the original location of 721AL close to CT251 and indicate that it is located within the pericentric heterochromatin (Fig. 1b). Markers E6M7 and E24M1, which are linked to the *Cnr* gene (Fig. 1a), were positioned relatively close to each other in the middle of the euchromatic region of the long arm (Fig. 1c, d). These experiments demonstrated that *Cnr* is likely to be situated in a euchromatic region containing few repetitive sequences. Indeed, no C_0*t*-1 DNA was required for hybridisations with E6M7, although it gave some improvements to the resolution of E24M1, by reducing a limited amount of non-specific labelling observed with this BAC probe.

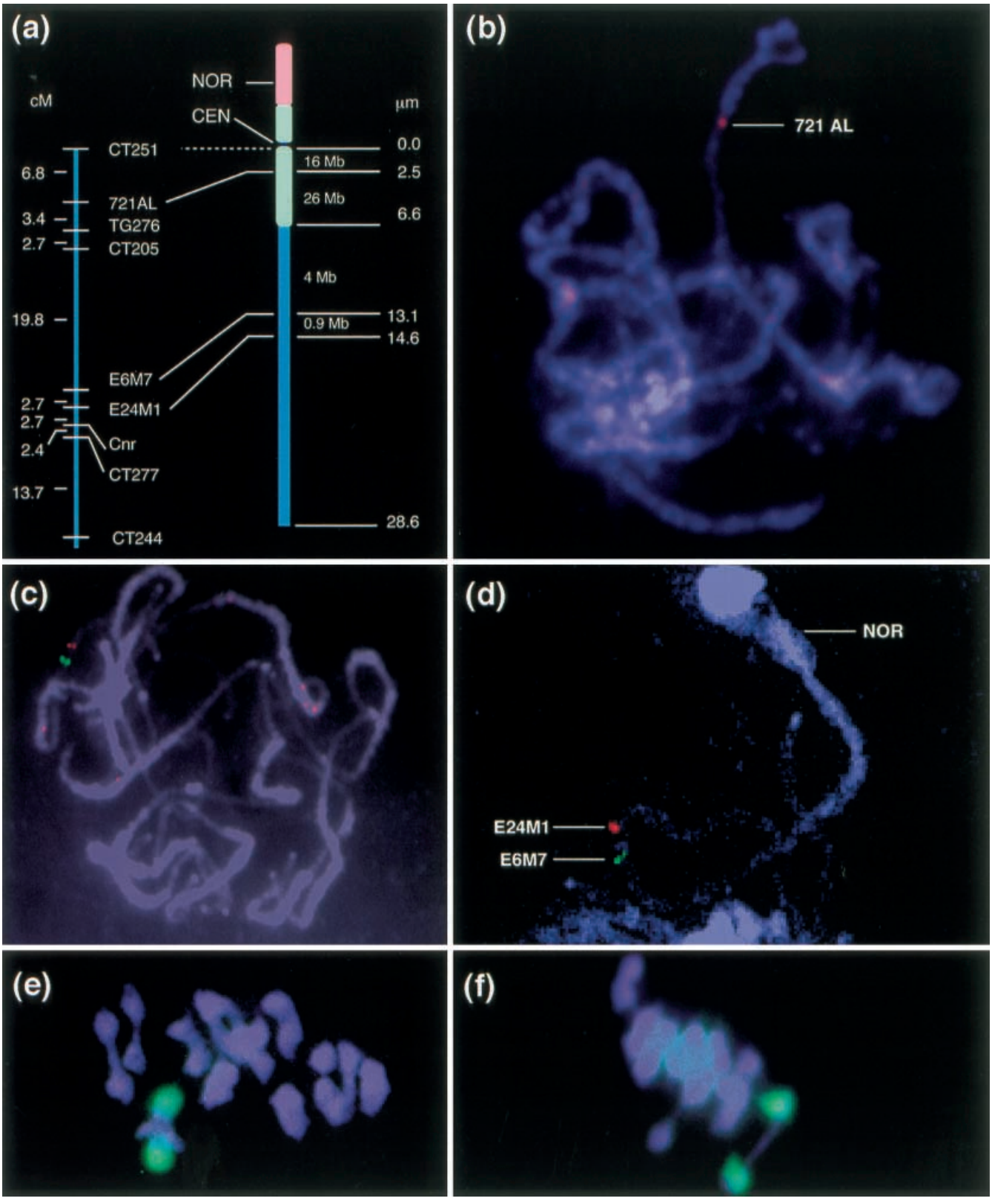

Fig. 1 **a** Genetic map of the tomato chromosome 2 region containing the *Cnr* locus and linked markers together with an ideogram of the pachytene chromosome 2 showing lengths and DNA content of heterochromatic (*green*) and euchromatic (*blue*) regions. These data are based on physical distances determined on the basis of FISH experiments. Detail of the short-arm, except for the NOR location, is not shown and is not drawn to scale. **b** Pachytene showing a single locus for the 721AL probe detected by rhodamine. Note the locus is in a heterochromatic region. **c, d** Pachytene showing positions of markers E6M7 and E24M1 detected by FITC (*green*) and rhodamine (*red*), respectively, within the euchromatin. **d** Similar to **c** but an enlarged view from another chromosome spread. **e, f** Meiotic MI showing a single locus for 45S rDNA detected by FITC. **e** Interstitial chiasma, **f** distal chiasma

The genetic distance separating E6M7 and E24M1 was 2.7 cM. The physical distance between the locations of the E6M7 and E24M1 markers was measured to be 1.5±0.09 µm (Fig. 1a). Based on previous calculations for DNA compactness of 6.3 Mb/µm in the heterochromatin and of 0.6 Mb/µm in the euchromatin (Zhong et al. 1999), we estimated a distance between E6M7 and E24M1 of about 0.9 Mb (Fig. 1a) and thus a bp:cM relationship in this region of 330 kb/cM. This is less than half the average value of 750 kb/cM estimated for the whole tomato genome (Tanksley et al. 1992), but similar to the relationship reported for the region spanning the *rin* locus on tomato chromosome 5 (Giovannoni et al. 1995). In contrast, the genetic distance between 721AL and E6M7 was 25.9 cM, and the presence of heterochromatin in this region gave an average bp:cM relationship of around 1.1 Mb/cM (Fig. 1). This difference presumably reflects highly reduced meiotic recombination within the pericentromeric heterochromatin. Similar FISH data were obtained using the E6M7 and E24M1 probes, irrespective of whether pachytene spreads were from *L. esculentum* or from the F_1 originating from the cross between *L. esculentum* and *L. cheesmanii* (data not shown).

The organisation of chromosome 2 is likely to have a significant effect on recombination. Much of the short arm is occupied by the highly repetitive rDNA locus and pericentromeric heterochromatin. In 50 MI cells, we found that the chromosome 2 bivalent always had a single chiasma, which in 24 (48%) cases was interstitial and in 25 (50%) were distal (Fig. 1e, f). Only one cell (2%) appeared to have a chiasma in the short arm. A study of the location of recombination nodules in tomato by Sherman and Stack (1995) suggested that very limited recombination occurs within the heterochromatin. The same authors found no evidence of recombination in the short arm of chromosome 2. Our data suggest that the relatively large interval between the markers CT251 and CT106A in the proximal portion of the long arm of chromosome 2 (Tanksley et al. 1992) represents a region which contains a significant amount of heterochromatin. The *Cnr* locus appears to lie outside this region, within the more-distal euchromatin, and is closely linked to the CT277 marker.

We have used FISH as a complementary approach to genetic analysis to obtain information about the physical location of markers associated with the tomato *Cnr* locus on chromosome 2. These experiments have provided important information about the relationship between genetic and physical distances on the long arm of chromosome 2. The indication that the *Cnr* gene is in a region of euchromatin within an area of relatively high recombination makes it a realistic target for cloning using a map-based strategy. We are currently generating further recombinants and anchored BACs in this region for this purpose. Once isolated, the *Cnr* gene will serve as an important tool for elucidating the developmental regulation of ripening in fleshy fruits.

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