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The Tomato *Never-ripe* Locus Regulates Ethylene-Inducible Gene Expression and Is Linked to a Homolog of the *Arabidopsis ETR1* Gene[1]

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Fruit ripening represents a complex system of genetic and hormonal regulation of eukaryotic development unique to plants. We are using tomato ripening mutants as tools to elucidate genetic components of ripening regulation and have recently demonstrated that the *Never-ripe* (*Nr*) mutant is insensitive to the plant growth regulator ethylene (M.B. Lanahan, H.-C. Yen, J.J. Giovannoni, H.J. Klee [1994] Plant Cell 6: 521–530). We report here ethylene sensitivity over a range of concentrations in normal and *Nr* tomato seedlings and show that the *Nr* mutant retains residual sensitivity to as little as 1 part per million of ethylene. Analysis of ripening-related gene expression in normal and mutant ethylene-treated fruit demonstrates that *Nr* exerts its influence on development at least in part at the level of ethylene-inducible gene expression. We have additionally used cloned tomato and Arabidopsis sequences known to influence ethylene perception as restriction fragment length polymorphism probes, and have identified a tomato locus linked to *Nr* that hybridizes to the *Arabidopsis ETR1* gene at low stringency, suggesting the possibility that *Nr* may be homologous to *ETR1*.

Ripening of fleshy fruits represents a tightly synchronized system of development unique to plant species. Numerous biochemical and physiological processes are coordinated during ripening to effect changes in color, texture, flavor, and aroma, thereby making the fruit organ desirable to seed-dispersing organisms. Nuclear genes encoding enzymes contributing to many aspects of this process have been isolated and characterized in terms of expression patterns and function (for reviews, see Speirs and Brady, 1991; Gray et al., 1992; Giovannoni, 1993). In climacteric fruit such as tomato, banana, apple, peach, and many others, ripening is coordinated by the biosynthesis of the gaseous plant growth regulator ethylene (Abeles et al., 1992). Fruit-ripening-related ethylene biosynthesis is governed by the induction of genes encoding enzymes of the ethylene biosynthetic pathway that are themselves influenced by ethylene, although initiated at the onset of ripening by some as yet undefined developmental signal(s) (Theologis, 1992). Previously described mutations in tomato fruit ripening such as *rin*, *nor*, and *Nr* likely represent loci involved in the production and/or perception of primary cues initiating the ripening cascade (Tigchelaar et al., 1978).

The ethylene biosynthetic pathway has been studied extensively and elucidated by Yang and Hoffman (1984). Genes encoding specific enzymes of this pathway, including ACC synthase and ACC oxidase, have been cloned and utilized in antisense gene repression studies to confirm the critical role of ethylene in climacteric ripening (Oeller at al., 1991; Picton et al., 1993). In addition, the model plant species *Arabidopsis thaliana* has proven extremely valuable as a genetic tool for advancing knowledge concerning mechanisms by which ethylene exerts its influence in living organisms. In particular, aberration of the seedling triple response to ethylene has been used both as a screen for identification of mutants in ethylene perception and for the isolation of corresponding loci (reviewed by Kieber and Ecker, 1993).

Cloning of the *Arabidopsis CTR1* gene revealed sequence homology with members of the Raf family of protein kinases, suggesting a role for this gene product in ethylene signal transduction (Kieber et al., 1993). Bleeker et al. (1988) reported identification of the dominant *Etr1* ethylene-insensitive mutant of *Arabidopsis*, which is likely allelic with the *Ein1* mutant described by Guzmán and Ecker (1993). The corresponding *ETR1* gene has since been isolated by a chromosome walk and shown to have sequence homology with bacterial two-component regulators, suggesting that the *ETR1* gene product may be involved in ethylene sensing and signaling (Chang et al., 1993). Analysis of double

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Abbreviations: *ain1*, ACC-insensitive mutant of *Arabidopsis*; cM, centimorgan; *ctr1*, constitutive triple response mutant of Arabidopsis; *Ein1*, ethylene-insensitive mutant of *Arabidopsis*; *Etr1*, ethylene-response mutant of *Arabidopsis*; *nor*, nonripening mutant of tomato; *Nr*, Never-ripe mutant of tomato; PG, polygalacturonase; ppm, parts per million; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; *rin*, ripening-inhibitor mutant of tomato.

mutants supports the hypothesized functions of ETR1/EIN1 and CTR1 because the activity of ETR1/EIN1 occurs prior to that of CTR1 (Kieber and Ecker, 1993).

We have recently shown that tomato *Nr* seedlings are phenotypically similar to Arabidopsis seedlings harboring the *Etr1/Ein1* allele, suggesting that *Nr* may represent a tomato homolog of *ETR1* (Lanahan et al., 1994). Nevertheless, it is easy to construct sound models in which *Nr* could represent a homolog of any of a number of Arabidopsis ethylene-response mutants including *ETR1* and *CTR1*. Alternatively, *Nr* may represent an as-yet uncharacterized component of ethylene signal transduction. As a first step toward isolation and characterization of the tomato *Nr* locus, we have mapped *Nr* relative to RFLP markers on tomato chromosome 9. Genetic localization has permitted the testing of cloned sequences from both tomato and Arabidopsis for linkage to *Nr*, thus permitting rapid appraisal of whether particular genes represent potential homologs of this critical component of fruit ripening. The data presented here eliminate seven candidate loci as possible homologs of *Nr* and indicate that one of five loci hybridizing to ETR1 is in fact linked to *Nr*.

Ethylene dose-response analysis of nearly isogenic normal and mutant tomato seedlings suggests that *Nr* is either a leaky allele or a member of a redundant ethylene signal transduction system, because residual sensitivity to as little as 1 ppm of ethylene is seen via observation of the triple response. Analysis of ripening-related gene expression in both normal and mutant tomato fruit is also presented and demonstrates that *Nr* inhibits fruit ripening at least in part through down regulation of ethylene-inducible genes.

MATERIALS AND METHODS

Plant Materials

Seed for the normally ripening tomato (*Lycopersicon esculentum*) cv Ailsa Craig and lines nearly isogenic and homozygous for the *Nr* and *rin* mutations were obtained from the Glasshouse Crops Research Institute. Seed homozygous for the *nor* mutation and a corresponding normally ripening near isoline (cv MH1) was kindly provided by B. Fisher (University of California, Berkeley). Seed for normally ripening *Lycopersicon cheesmannii* (accession No. LA483) was kindly provided by C. Rick (University of California, Davis). The large F_2 population scored for fruit ripening phenotype was grown in the field at Cornell University in the summer of 1992; all other mature plants were grown under standard greenhouse conditions at either Cornell or Texas A&M. Normal "mature green" fruit were estimated to be 3 to 5 d from the "breaker" stage by tagging flowers at anthesis and measuring days to onset of red color development as a standard. Normal "breaker plus 7 d" fruit are red ripe fruit harvested 7 d after the first signs of color development. Fruit from all *L. esculentum* genotypes were tagged at anthesis, and mutant fruit were harvested at the same time as the corresponding normal fruit of the same age.

Treatments of Seedlings and Fruit

Ethylene sensitivity was scored as the seedling triple response, either directly in F_2 seedlings or indirectly in F_3 progeny, via germination in water-agar supplemented with 20 μM ACC (Sigma; A-0430) as described by Lanahan et al. (1994). Direct scoring of F_2s was confirmed by analysis of F_3 seedlings.

Tomato seedlings used in dose-response experiments were harvested from mature fruit, surface sterilized with 2.5% sodium hypochlorite for 10 min, and rinsed with deionized water. Seeds were germinated for 9 d in the dark in 500-mL sealed glass canning jars containing 50 mL of solidified water-agar (1.5%). A 1-cm-diameter septum was introduced into the lid of each jar and sealed with silicon glue for introduction of ethylene gas. Gas samples (1 mL) were taken from each jar every 24 h and analyzed for ethylene with a Hewlett-Packard 5890 series II gas chromatograph. Additional ethylene was added if leakage resulted in more than a 5% reduction in the original ethylene concentration. Ethylene concentration never varied more than 10% in any jar. Control seedlings were germinated in identical jars without the addition of exogenous ethylene.

Ethylene-treated mature green ("mature green plus ethylene") fruit were harvested and placed in open 500-mL glass jars for 6 h. Jars were then sealed and 20 ppm of ethylene were added via the septum. Seeds were extracted and pericarp tissue was frozen in liquid nitrogen after 8 h of ethylene exposure. All nonethylene-treated fruit were treated identically except for the addition of ethylene to the jar.

DNA Analysis

Procedures for both genomic DNA extraction from expanding tomato leaves and DNA gel-blot hybridization were performed as described previously (Tanksley et al., 1992). Alkaline DNA gel blotting was as described by the supplier of the nylon membrane used (Hybond-N^+, Amersham). High-stringency hybridizations were at 65°C in 5× SSC, 0.5% (w/v) SDS, 50 mM Na-P (pH 7.5), and 5× Denhardt's solution and followed by 20-min 65°C washes in first 2× SSC, 0.1% (w/v) SDS and then 1× SSC, 0.05% (w/v) SDS. Low-stringency hybridizations were as for high, except they were performed at 37°C and followed by two 20-min washes in 2× SSC, 0.1% (w/v) SDS also at 37°C. Identification of RFLPs was as described by Tanksley et al. (1992), except that the following additional restriction endonucleases were used to survey for differences between the *L. esculentum* (*Nr/Nr*) and *L. cheesmannii* (*nr/nr*) alleles of RFLP loci: *Alu*I, *Ava*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*II, *Bst*II, *Cla*I, *Hinc*II, *Hind*III, *Hin*fI, *Hpa*I, *Kpn*I, *Mbo*I, *Nco*I, *Nde*I, *Pst*I, *Pvu*I, *Pvu*II, *Rsa*I, *Taq*I, *Sal*I, *Sau*3A, *Sca*I, *Sma*I, *Sph*I, *Sst*I, *Xba*I, and *Xho*I. Restriction enzymes yielding RFLPs were used to digest genomic DNA extracted from individual members of segregating populations for linkage analysis.

Genetic Mapping

F_2 seed segregating for the *Nr* and RFLP loci were generated from the cross *L. esculentum* (*Nr/Nr*) × *L. cheesmannii*

(*nr/nr*). Genetic linkage analysis was performed using MapMaker software (Lander et al., 1987). All RFLP markers used had been localized previously to tomato chromosome 9 (Tanksley et al., 1992). The "group," "compare," and "ripple" commands were utilized, respectively, to verify the order of markers in our cross. Additional RFLP and RAPD markers were added to the map using the "try" and "ripple" commands, respectively. Centimorgan distances were calculated using the Kosambi (1944) function.

RNA Analysis

Total fruit RNA was isolated from fresh-frozen tomato pericarp tissue as described by DellaPenna et al. (1986). RNA gel blotting and hybridization were as described by the manufacturer of the nylon membrane used (Hybond N; Amersham).

Construction of Nearly Isogenic DNA Pools and RAPD Analysis

Two DNA pools ("mutant" and "normal") nearly isogenic for the *Nr* locus region of chromosome 9 were constructed from segregating F_2 progeny as described by Giovannoni et al. (1991) except that the genotype at the target locus itself (Michelmore et al., 1991), in addition to flanking RFLP loci, was considered for inclusion in a particular pool. Genomic DNA (5 μg) from each of six *Nr/Nr* F_2 individuals was combined to generate the mutant DNA pool, whereas DNA from six *nr/nr* F_2 individuals was used to generate the normal DNA pool. Heterozygosity at flanking loci (TG291 and TG551) for at least one member of each pool was utilized to narrow the target region. Pool members were derived from the same F_2 population described above and analyzed for *Nr* and RFLP segregation. Three hundred random 10-base primers (Operon Technologies, Alameda, CA) were individually tested in amplification of 10 to 20 ng of each pooled DNA template under the conditions described by Giovannoni et al. (1991). Linked RAPD PCR products were cloned into pGEM-T (Promega, Madison, WI).

RESULTS

Ethylene Dose-Response Analysis of Normal and *Nr* Seedlings

We have previously demonstrated that the *Nr* mutant exhibits insensitivity to ethylene via the triple response and that the severity of this response is dependent on *Nr* gene dosage, suggesting incomplete dominance (Lanahan et al., 1994). To (a) test whether the homozygous *Nr* mutant completely blocks ethylene perception or retains residual ethylene responsiveness and (b) quantitate saturation of any residual ethylene sensitivity, dose-response analysis was performed on normal and *Nr/Nr* seedlings germinated in concentrations of ethylene ranging from 0 to 1000 ppm.

Twenty seedlings of each genotype (*Nr/Nr* and *nr/nr*) were dark grown for 9 d in 500-mL jars containing 0, 1, 3, 20, 100, 250, or 1000 ppm of exogenous ethylene. Seedlings were then scored for hypocotyl and root elongation. Treatment of normal (*nr/nr*) seedlings in 0 ppm of ethylene and all treatments of *Nr/Nr* seedlings demonstrated considerable variation in hypocotyl elongation and were, therefore, performed in triplicate. Figure 1a shows variable hypocotyl elongation of nonethylene-treated *Nr/Nr* and *nr/nr* seedlings. Saturation of the ethylene response in *nr/nr* seedlings, as measured by inhibition of hypocotyl elongation, occurs between 1 and 3 ppm of exogenous ethylene. Interestingly, *Nr/Nr* hypocotyls demonstrate responsiveness to ethylene even at 1 ppm, although this response remains greatly attenuated even in concentrations of ethylene as high as 1000 ppm. The attenuated triple response of *Nr/Nr* seedlings saturates between 20 and 100 ppm of ethylene (Fig. 1a; variation in percentage of individuals greater than 6 cm is not significant among the 100, 250, and 1000 ppm of ethylene treatments: $\chi^2 = 0.177$, $P > 0.9$; whereas the percentage of individuals greater than 6 cm from the 20 ppm treatment can be considered statistically the same as those from the 100, 250, and 1000 ppm treatments only at $P > 0.25$: $\chi^2 = 3.04$). Together, these results suggest that the *Nr* mutant retains partial ethylene sensitivity and is partially dominant in its inhibition of ethylene perception related to hypocotyl elongation of tomato seedlings. Inhibition of root elongation does not saturate until approximately 20 ppm of ethylene in *nr/nr* seedlings and demonstrates nearly complete insensitivity to ethylene in *Nr/Nr* (Fig. 1a). Examples of seedlings from each treatment are shown in Figure 1b.

Nr-Associated Ethylene Insensitivity Occurs at the Level of Gene Expression

The effects of the mutant *Nr* allele on ethylene-mediated development and response phenomena of tomato, including the seedling triple response, fruit ripening, pedicel abscission, epinasty, and petal senescence, have been characterized previously (Rick and Butler, 1956; Hobson 1967; Tucker et al., 1984; Lanahan et al., 1994). Although reduced expression of several ripening-related genes has been observed in the presence of the *Nr* mutation (DellaPenna et al., 1989; Knapp et al., 1989), regulation of gene expression by ethylene has not been analyzed in *Nr* tomato plants. Four ripening-related cDNAs (PG1.9, E4, E8, and J49) previously isolated from tomato (DellaPenna et al., 1986; Lincoln et al., 1987) were used as probes to measure ethylene responsiveness at the level of nuclear gene expression in *Nr/Nr* fruit.

Total RNA was extracted from normal tomato fruit at the mature green, breaker, and red ripe stages of development and from fruit of identical age harvested from nearly isogenic *Nr/Nr*, *rin/rin*, and *nor/nor* mutants. In addition, total RNA was extracted from mature green fruit of all four genotypes following treatment with 20 ppm of ethylene for 8 h.

PG1.9 mRNA was detected by RNA gel-blot analysis in normal ripening fruit and to a lesser extent in red ripe age *Nr* fruit, as reported previously by DellaPenna et al. (1989). No PG1.9 signal was detected in ethylene-treated fruit from any of the four genotypes tested (Fig. 2), confirming the previous observation that PG mRNA does not accumulate rapidly in response to exogenous ethylene (Lincoln et al.,

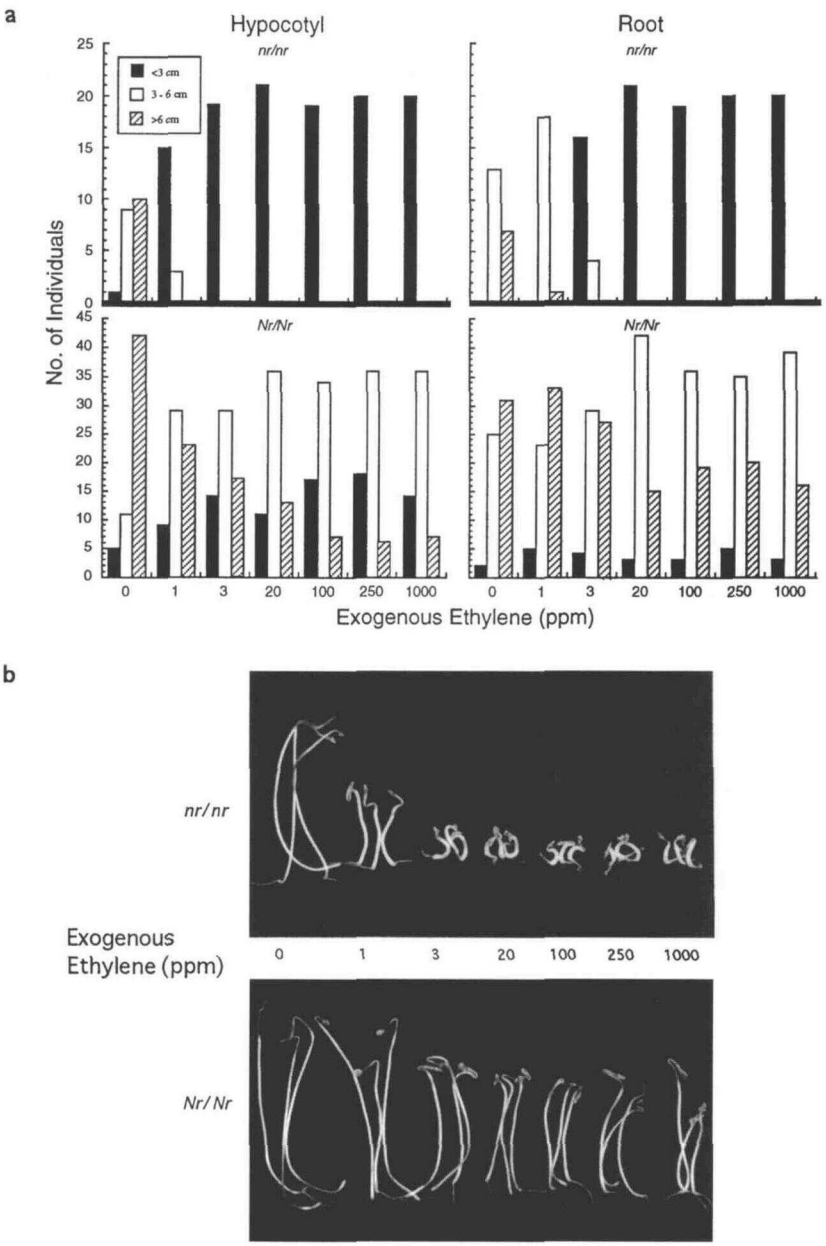

Figure 1. Ethylene dose-response analysis of tomato seedlings. Tomato seedlings of cv Ailsa Craig and a line nearly isogenic for the *Nr* mutation were germinated in the indicated concentrations of exogenous ethylene. After 9 d, hypocotyl and root length measurements were taken. Because of the uniformity of ethylene response in the normal near isoline, only 20 individuals were scored as opposed to 60 *Nr/Nr* seedlings per ethylene treatment. a, Hypocotyl and root length distributions; b, examples of individual seedlings from each treatment following removal of roots.

1987). As anticipated, all three cDNA probes corresponding to genes known to respond rapidly to ethylene (E4, E8, J49; Lincoln and Fischer, 1988a) detected homologous mRNA in normal ethylene-treated mature green fruit. In addition, significant E4 mRNA accumulation occurred in ethylene-treated mature green fruit harboring either the *rin* or *nor* mutation, whereas only ethylene-treated *rin* fruit accumulated detectable E8 mRNA. The E8 message observed in red ripe age *rin* fruit is likely due to the nonethylene-mediated component of E8 gene expression previously shown to be active in *rin* mutant fruit (Lincoln and Fischer, 1988b; Giovannoni et al., 1989).

The most significant observation reported here is that the E4 and E8 probes showed no detectable signal upon hybridization with RNA extracted from ethylene-treated mature green *Nr* fruit, suggesting that the *Nr* mutation manifests itself, at least in part, at the level of nuclear gene expression (Fig. 2). In addition, Figure 2 demonstrates that J49 is expressed to a significant level in mature green ethylene-treated fruit from all four genotypes, including *Nr*. This result most likely represents an additional manifestation of the residual ethylene responsiveness observed in *Nr* seedlings. The previous observation that J49 mRNA accumulates to its highest levels in the presence of the relatively low ethylene concentrations sufficient to elicit the residual seedling response shown in Figure 1 (1 ppm; Lincoln and Fischer, 1988a) supports this hypothesis.

Localization of the *Nr* Locus on the Tomato RFLP Map

To localize the *Nr* locus on the tomato genetic map, an interspecific cross between a cultivated tomato variety (*L.*

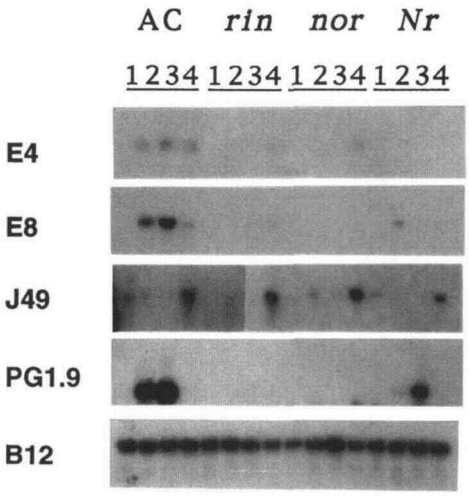

Figure 2. RNA gel-blot analysis of fruit ripening-related genes. Total RNA was isolated from mature green (lane 1), breaker (lane 2), red ripe (lane 3), and mature green plus ethylene-treated (lane 4) fruit. Shown are RNAs extracted from normal (AC) and equivalent-age mutant fruit (*rin, nor, Nr*). Fifteen micrograms of total RNA was loaded per lane. cDNA probes were generously provided by B. Fischer. B12, a constitutively expressed gene of unknown function isolated from a breaker stage tomato fruit cDNA library (D. Ruezinsky and J. Giovannoni, unpublished data), was hybridized as an RNA-loading control.

esculentum; cv Ailsa Craig) homozygous for the *Nr* mutation and a wild relative (*L. cheesmannii*; LA483) bearing normally ripening fruit (*nr/nr*) was performed to yield an F_2 population segregating for both RFLPs and the *Nr* locus. *L. cheesmannii* was selected as the normal parent because (a) considerable RFLP polymorphism has been demonstrated between this species and *L. esculentum* (Miller and Tanksley, 1990) and (b) F_2 progeny resulting from this cross have a high degree of fertility necessary for production of fruit and F_3 seed used to score the *Nr* phenotype. Unfortunately, *L. cheesmannii* also harbors the dominant β allele on chromosome 6 (Rick, 1980) resulting in orange-colored, ripe fruit due to preferential β-carotene accumulation. Because of the visual similarity between fruit harboring either the *Nr* or β alleles, only red-fruited individuals resultant of this cross (*nr/nr, b/b*) can be accurately scored at either locus. Thus, only one in 16 F_2 progeny was expected to be useful in RFLP mapping the *Nr* locus.

Previous linkage analysis using morphological markers suggested localization of the *Nr* locus to tomato chromosome 9 (Rick, 1980). Thus, the resulting F_2 population segregating for *Nr* was subjected to RFLP analysis using previously isolated chromosome 9 DNA markers polymorphic between the parents of this cross (Tanksley et al., 1992). Approximately 450 fruiting F_2 progeny from the *L. esculentum* × *L. cheesmannii* cross were field grown to maturity to obtain a subpopulation of 29 red-fruited individuals used for preliminary localization of the *Nr* locus toward the center of the chromosome 9 RFLP map (Fig. 3).

The recent observation of *Nr*-associated insensitivity to ethylene (Lanahan et al., 1994) facilitated the construction of a higher resolution mapping population in which all seed-setting individuals could be accurately genotyped at the *Nr* locus. Eighteen F_2 progeny of the *L. esculentum* × *L. cheesmannii* cross were germinated in the dark on water-agar plus 20 μM ACC for 9 d, scored for the *Nr* phenotype, and transplanted to potting media. The genotype at the *Nr* locus was confirmed via identical analysis of F_3 seed. Twenty-four additional F_2 progeny were grown without ACC selection and genotyped by analysis of ACC sensitivity in F_3 seed.

The resulting population of 42 F_2 individuals was scored for phenotypic and chromosome 9 RFLP segregation. The segregation data was analyzed with MapMaker software (Lander et al., 1987) to place the *Nr* locus in the 21.2-cM interval flanked by TG291 and TG551 toward the center of the chromosome 9 map (Fig. 3). It is interesting to note that TG291 and TG551 border an interval of approximately half this genetic distance (9.4 cM) in an F_2 population derived from a cross between *L. esculentum* × *Lycopersicon pennellii*, which was not segregating for the *Nr* locus (Tanksley et al., 1992). The tightest RFLP marker linkage observed to *Nr* was with TG551 at 7.8 cM. Additional DNA markers shown to reside within or adjacent to the interval harboring *Nr* (for example, TG390) were not mapped because of lack of a clear polymorphism between the population parents among the 34 restriction enzymes tested. Finally, several RFLP markers from tomato chromosomes 5 and 10 were scored in the *Nr* F_2 population and shown to have no significant linkage to *Nr* or any of the chromosome 9 RFLP loci shown in Figure 3 (data not shown).

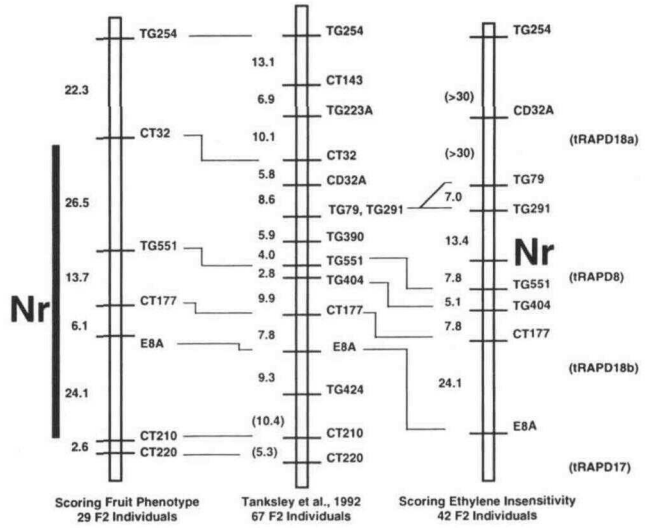

Figure 3. Placement of the *Nr* locus on the tomato RFLP map. An F_2 population segregating for *Nr* and RFLP loci was generated from the cross *L. esculentum* (*Nr/Nr*) cv Ailsa Craig × *L. cheesmannii* (*nr/nr*). A subpopulation of 29 individuals was scored on the basis of fruit-ripening phenotype and an additional subpopulation of 42 individuals was scored on the basis of seedling ethylene sensitivity as described in "Materials and Methods." Linkage analysis was performed with MapMaker software (Lander et al., 1987).

Isolation of Additional DNA Markers Linked to the *Nr* Locus

Additional DNA markers linked to the *Nr* locus were recovered by RAPD analysis (Martin et al., 1991; Michelmore et al., 1991) of DNA pools nearly isogenic for the *Nr* region of chromosome 9 as described previously for the *nor* locus region of chromosome 10 (Giovannoni et al., 1991). F_2 seed from the same cross used in *Nr* RFLP mapping was germinated in the dark for 9 d on water-agar plus 20 μM ACC. Six homozygous *Nr/Nr* and six homozygous *nr/nr* individuals were identified based on insensitivity and sensitivity to ethylene (based on severity of the seedling triple response), respectively. Genomic DNA from the six *Nr/Nr* and six *nr/nr* individuals was then extracted and combined to form mutant and normal pools, respectively, to serve as templates for RAPD amplification of potential *Nr*-linked sequences.

Three hundred random 10-base oligonucleotide primers (Operon Technologies) were used to amplify the normal and mutant pools. Reactions utilizing the same primer but different pooled DNAs as template were analyzed side-by-side for polymorphisms among amplification products (Fig. 4a). Primers yielding reproducible differences between the normal and mutant DNA pools were then tested on DNA from a subset of four individuals making up each pool (Fig. 4b). Reproducible amplification polymorphisms were considered to be potentially linked to *Nr* if there were two scoreable recombinations or less between a given RAPD locus and *Nr* in this population of eight individuals. Based on these criteria, seven RAPD polymorphisms were mapped in the population of 42 F_2s as dominant amplification products, with four demonstrating linkage to *Nr* on chromosome 9 (Fig. 3). tRAPD8 maps to the interval defined by *Nr* and TG551 and, therefore, represents the most tightly linked DNA marker to *Nr* known. A summary of the RAPD screen is presented in Table I.

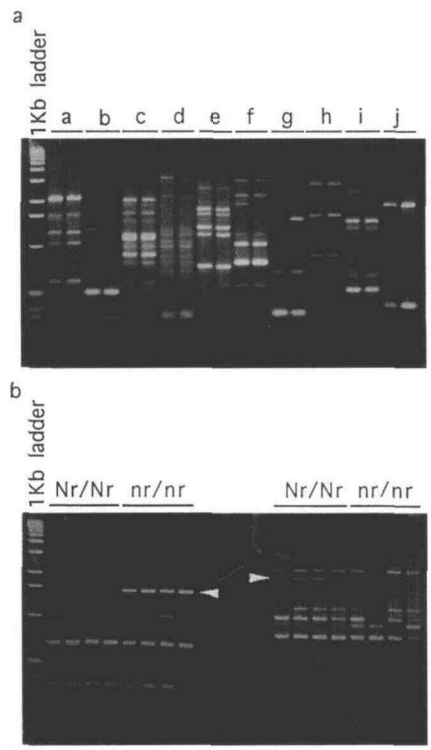

Figure 4. Identification of additional *Nr*-linked DNA markers. Two nearly isogenic DNA pools were constructed with DNA extracted from *nr/nr* or *Nr/Nr* F_2 individuals as determined by seedling ethylene sensitivity. The F_2 progeny were derived from the cross *L. esculentum* (*Nr/Nr*) cv Ailsa Craig × *L. cheesmannii* (*nr/nr*). a, Examples of RAPD PCR of the *Nr/Nr* (left) and *nr/nr* (right) DNA pools with 10 different random 10-base oligonucleotide primers (lanes a–j). b, RAPD primers that yielded reproducible polymorphisms were subsequently tested on four *Nr/Nr* and four *nr/nr* F_2 individuals to confirm linkage to the *Nr* locus. Examples of amplification products linked to the *Nr* locus and specific to the *nr/nr* (left) and *Nr/Nr* (right) parent are indicated by arrowheads.

RFLP Mapping of Cloned Tomato and *Arabidopsis* Ethylene-Response Genes Suggests the Possibility That *Nr* Is a Homolog of *Etr1*

Once the *Nr* locus was placed on the tomato RFLP map, it became possible to address the question of whether *Nr* represented a homolog to any previously isolated genes shown to be involved in the regulation of ethylene perception and/or fruit ripening. Two *Arabidopsis* ethylene-response genes (*ETR1*, Chang et al., 1993; *CTR1*, Kieber et al., 1993) have recently been cloned and characterized. In addition, a previously described ethylene/fruit-ripening-responsive gene from tomato (*E8*, Lincoln et al., 1987) was demonstrated via antisense gene repression to have a potential role in regulation of ethylene biosynthesis and perception (Penarrubia et al., 1992). cDNA clones corresponding to each of these three genes were acquired and utilized as hybridization probes for RFLP mapping of their respective loci in a subset of the *L. esculentum* × *L. pennellii* F_2 population described by Tanksley et al. (1992). More than 1000 RFLP loci have been mapped in this population, facilitating rapid and accurate localization of additional DNA markers to this map. Figure 5 displays the approximate positions of the tomato *Nr* and *E8* loci in addition to segregating RFLP loci observed via hybridization to the Arabidopsis *ETR1* and *CTR1* cDNAs. The *E8* and *CTR1* loci were mapped at high stringency, whereas *ETR1* could only be detected at low stringency (see "Materials and Methods").

The *E8A* locus showed stronger homology to the *E8* cDNA probe than did the *E8B* locus (based on relative hybridization signal intensity, data not shown), suggesting that *E8A* harbors the specific DNA sequence shown via antisense gene repression to be capable of influencing fruit-ripening-related ethylene biosynthesis and perception (Penarrubia et al., 1992).

Mutant Arabidopsis CTR1 alleles have been shown to confer a constitutive triple response in germinating seedlings in the absence of either exogenous ethylene or ACC (Guzmán and Ecker, 1993). Because *CTR1* and *Nr* both influence ethylene signal transduction in *Arabidopsis* and tomato, respectively, we mapped the *Arabidopsis* CTR1 cDNA in tomato to test for linkage with *Nr*. DNA gel-blot

Table I. *Summary of RAPD screen for Nr-linked markers*

Item	No.
Individual F_2 plants making up the normal (*nr/nr*) pool	6
Individual F_2 plants making up the mutant (*Nr/Nr*) pool	6
No. of random 10-mers tested	300
Loci (bands) observed	2091
Polymorphic PCR products reproduced 2 or more times	31
Polymorphic PCR products specific to the normal pool	17
Polymorphic PCR products specific to the mutant pool	14
Loci putatively linked to the *Nr* locus based on 8 F_2 plants	7
Loci linked to the *Nr* locus (chromosome 9) based on 42 F_2 plants	4

hybridization of *CTR1* to *Eco*RV-digested *L. esculentum* and *L. pennellii* genomic DNA revealed a single hybridizing fragment in the tomato genome at high stringency (data not shown) that mapped to a single locus on chromosome 2 designated *tCTR1* (Fig. 5). This result demonstrates that a tomato sequence with high homology to *CTR1* exists and yet does not reside at the *Nr* locus and that an additional component of ethylene signal transduction in tomato is likely to reside on chromosome 2.

Ethylene insensitivity in germinating *Nr/Nr* and *Nr/nr* seedlings (Fig. 1; Lanahan et al., 1994) is strikingly similar to that observed in *Arabidopsis* seedlings homozygous or heterozygous, respectively, for mutant alleles of *ETR1* (Bleeker et al., 1988). The *Arabidopsis ETR1* cDNA hybridizes at low stringency to several tomato genomic restriction fragments in *L. esculentum*, *L. cheesmannii*, and *L. pennellii* with all restriction enzymes tested (minimum of five enzymes tested per genotype), suggesting that a small family of related sequences exists in tomato (data not shown). RFLP mapping at low stringency in the same population used for *CTR1* linkage analysis, reveals four distinct tomato loci homologous to *ETR1* on chromosomes 7, 9, 11, and 12 (LOD > 3) and designated *tETR1f*, *tETR1n*, *tETR1x*, and *tETR1a*, respectively (Fig. 5). Hybridization fragments suggesting a possible fifth locus homologous to *ETR1* also segregated in this population and demonstrated linkage to chromosome 10 but could not be accurately localized within this linkage group.

As indicated in Figure 5, *tETR1n* maps to the region of chromosome 9 harboring the *Nr* locus, suggesting that *Nr* may represent a tomato homolog to *ETR1*. Alternatively, *tETR1n* may represent a separate but linked locus to *Nr*. Because *Nr* and *tETR1n* were mapped relative to identical DNA markers but in separate populations, we cannot conclusively confirm or eliminate either hypothesis at this time. Unfortunately, there is considerably less polymorphism between *L. esculentum* and *L. cheesmannii* (the parents of the population segregating for *Nr*) than between *L. esculentum* and *L. pennellii* (the parents of the population in which *ETR1* homologs were mapped). Thirty-four restriction enzymes were tested for RFLP polymorphisms, which could be utilized to score *tETRn* in the *Nr* mapping population, and no mappable polymorphisms were observed (data not shown). Nevertheless, the genetic linkage analysis summarized in Figure 5 demonstrates conclusively that seven of eight potential candidates for the *Nr* gene can be excluded as *Nr* homologs. These include genes residing at the *E8A*, *E8B*, *tCTR1*, *tETR1a*, *tETR1f*, *tETR1w*, and *tETR1x* loci. Current efforts are directed toward the isolation of the different *tETR1* loci (especially *tETR1n*) for use in both development of gene-specific probes that can be more effectively mapped in the population segregating for *Nr* and generation of transgenic plants overexpressing and inhibited in expression of *tETR1* gene family members.

DISCUSSION

Numerous aspects of plant growth and development, including fruit ripening, senescence, abscission, seed germination, and floral induction, have been implicated as being responsive to the plant growth regulator ethylene (Abeles et al., 1992). We are using the ethylene-insensitive *Nr* mutant of tomato to assist in elucidating the role of ethylene in fruit ripening and plant hormone signal transduction. We have previously shown that *Nr* influences normal ethylene responses in tomato, including the seedling triple response, epinasty, pedicel abscission, petal senescence, and fruit ripening (Lanahan et al., 1994). Here we demonstrate that the *Nr* mutant (a) retains residual ethyl-

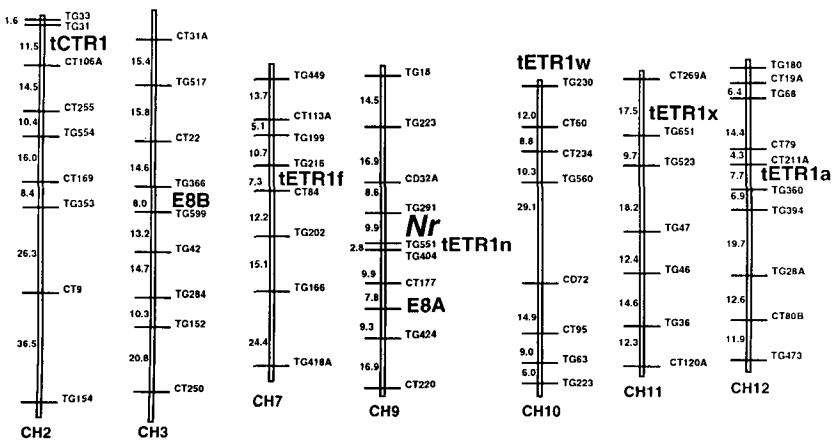

Figure 5. RFLP linkage mapping of ethylene perception loci. The tomato E8 and *Arabidopsis* ETR1 and CTR1 cDNA clones were used as RFLP probes to estimate gene family size and localization of specific loci. Tomato loci hybridizing to Arabidopsis sequences are designated with a "t." Placement of *tETR1w* at the top of chromosome 10 is meant to represent linkage to this chromosome, although specific localization within it could not be determined. All probes were mapped in a 50-member subset of an F_2 population previously utilized to generate a high-resolution RFLP map of tomato (Tanksley et al., 1992).

ene responsiveness, (b) exerts its effects at least in part through the regulation of ethylene-inducible gene expression, (c) maps genetically to a single locus on tomato chromosome 9, and (d) is linked to a locus that hybridizes to the *Arabidopsis* ethylene-response gene *ETR1* (Chang et al., 1993). This last result suggests the tantalizing possibility that *Nr* may be a functional homolog of *ETR1* in tomato and demonstrates the potential for molecular genetic tools in crossing species boundaries to develop hypothesis pertaining to gene function.

The *Nr* Mutant Retains Residual Ethylene Responsiveness

When attempting to interpret gene function based on mutant phenotype, it is important to remember that the affected characteristic(s) may be influenced by a leaky allele and/or epistatic effects. For example, two mutant alleles of the Arabidopsis *ain1* locus, *ain1–1* and *ain1–2*, exhibit relatively greater and lesser insensitivity to exogenous ethylene, respectively (Van Der Straeten et al., 1993), suggesting that the *ain1–2* allele is leaky compared to *ain1–1* and is capable of partial ethylene responsiveness. Based on the observations of residual *Nr* ethylene sensitivity in seedlings (Fig. 1) and at the level of ethylene-inducible J49 gene expression (Fig. 2), it is apparent that tomato plants harboring the mutant *Nr* allele are also capable of partial ethylene responsiveness. Unlike the Arabidopsis *etr-1/etr-1* mutant that displays virtually complete insensitivity to ethylene at concentrations as high as 100 ppm (Bleeker et al., 1988), the tomato *Nr/Nr* mutant, nearly isogenic in the cv Ailsa Craig, exhibits demonstrable sensitivity to ethylene in the presence of as little as 1 ppm of ethylene (Fig. 1).

This result also suggests that the partial ripening observed in mature *Nr* fruit may be due to partial ethylene responsiveness. This hypothesis is consistent with the observations of more complete ripening inhibition of normal mature green tomato fruit treated with inhibitors of ethylene biosynthesis or action (Yang, 1985) and in fruit derived from tomato plants expressing antisense ACC synthase or ACC oxidase constructs (Oeller et al., 1991; Picton et al., 1993). If *Nr* does represent a tomato equivalent of *ETR1*, it is possible that *Nr* is a leaky allele. It is also possible that the ethylene-response mechanism, of which *Nr* is a part, is influenced by epistatic interactions that have not been observed in Arabidopsis. For example, if *Nr* is a functional homolog of *ETR1*, it may be possible that a related gene at one or a combination of the *tETR1a*, *tETR1f*, *tETR1w*, and/or *tETR1x* loci (Fig. 5) may provide partial compensation for the *Nr* mutation, resulting in the observed residual sensitivity to ethylene. Our previous observation that the *Nr* mutation results in a more severe ethylene-insensitive phenotype in cv Pierson than cv Ailsa Craig suggests at least some interaction between *Nr* and additional loci (Lanahan et al., 1994).

Nr Regulates Ethylene-Inducible Gene Expression

Because the *Nr* mutation blocks normal ethylene responses, including the seedling triple response and fruit ripening, it was reasonable to test whether *Nr* influenced the regulation of ethylene-inducible genes. Although expression of a number of ripening-related genes has been analyzed in normal and mutant tomato fruit, the previously observed perturbations in mRNA accumulation patterns in *Nr*, relative to normally ripening fruit (DellaPenna et al., 1989; Knapp et al., 1989), have not been correlated with or attributed to alteration of ethylene perception in the *Nr* mutant. Consequently, mRNA accumulation patterns for four tomato-ripening-related genes (PG, E4, E8, and J49) were characterized in ethylene-treated normal and mutant fruit, including *Nr*.

PG mRNA did not accumulate in any of the 8-h (20 ppm) ethylene-treated tissues, consistent with previous observations in normal and *rin* fruit, suggesting little if any direct role for ethylene in the regulation of this cell wall hydrolase (Lincoln et al., 1987; DellaPenna et al., 1989). Maunders et al. (1987) demonstrated that PG mRNA accumulates in response to longer exposure (24 h) to exogenous ethylene, possibly suggesting a less direct role of ethylene in modulation of PG gene expression. However, examination of PG mRNA accumulation in transgenic tomato fruit harboring an antisense ACC synthase gene resulting in less than 1% normal ethylene biosynthesis during ripening demonstrated that PG mRNA accumulation can be separated from the presence of the relatively high levels of ethylene characteristic of ripening tomato fruit (Oeller et al., 1991). The lack of PG mRNA accumulation in both normal and mutant ethylene-treated fruit, in combination with the significant accumulation of PG mRNA in ripening *Nr* fruit (Fig. 2), is consistent with a model ascribing a minimal role for ethylene in the regulation of this gene.

E4 mRNA accumulated in ethylene-treated normal, *rin*, and *nor* mature green fruit but not in *Nr* fruit of equivalent maturity and treatment. E8 mRNA accumulated in normal and *rin* fruit but not those harboring either the *nor* or *Nr* mutation, suggesting that the *nor* gene product may also play some role in ethylene signal transduction for at least a subset of ethylene-responsive genes. The residual ethylene-inducible J49 mRNA accumulation in *Nr* fruit (Fig. 2) may represent a partial ethylene response similar to that observed in germinating *Nr* seedlings (Fig. 1). It is important to note that a previous study of J49 mRNA accumulation in response to various concentrations of ethylene demonstrated maximal accumulation in the presence of 1 ppm of ethylene, a concentration similar to the maximum perceived by *Nr* mutant seedlings (Fig. 1), whereas E4 and E8 both demonstrated maximal accumulation at 23 ppm (Lincoln and Fischer, 1988a), consistent with their respective lack of expression in ethylene-treated *Nr* fruit.

Analysis of mRNA accumulation profiles in normal and mutant developing and ethylene-treated fruit, in conjunction with the observations of ethylene insensitivity in fruit of all three mutants with respect to overall ripening, suggests the model depicted in Figure 6. In this model the normal *Nr* gene product is necessary for proper regulation of most or all ethylene-regulated genes. Some genes, such as E4, are largely dependent on ethylene perception and signal transduction for expression, whereas others, such as

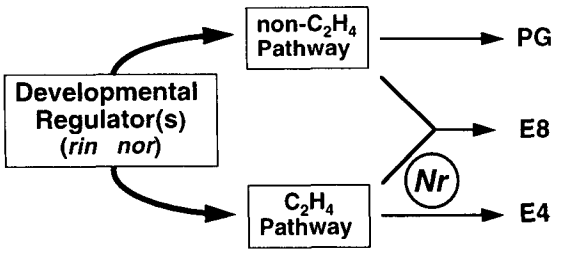

Figure 6. Model for genetic control of tomato fruit ripening. Three classes of ripening genes corresponding to those that are not ethylene regulated, partially ethylene regulated, and solely ethylene regulated are represented by PG, E8, and E4, respectively. *Nr* influences expression of those genes that are regulated by ethylene. *rin* and *nor* exert broader regulation over ripening influencing both ethylene-regulated and nonethylene-regulated genes.

E8, have both developmental and ethylene-mediated components and are, therefore, influenced only in part by the action of ethylene and proper *nr* function. The observations of ethylene-induced E8 mRNA accumulation in the *rin* but not the *nor* mutant suggest that *nor* may play a limited and/or less direct role in ethylene perception as well. Although both *rin* and *nor* seedlings demonstrate normal ethylene responsiveness (Lanahan et al., 1994), both mutations result in fruit that are insensitive to ethylene at the level of overall ripening (Tigchelaar et al., 1978). This fact likely represents developmental regulation of competence to respond to ethylene, which is dependent on normal *rin* and *nor* gene products during fruit development. *Nr*, meanwhile, is a necessary component of ethylene signal transduction, which when blocked impairs most observable ethylene responses, including regulation of ethylene-mediated gene expression. In this regard, the *Nr* mutant may serve as a useful tool for distinguishing ethylene-regulated from nonethylene-regulated ripening genes.

The Triple Response to Ethylene Is a Useful Marker for Mapping *Nr*

Rick (1980) performed linkage analysis with numerous morphological markers to construct a genetic map of tomato encompassing all 12 chromosomes. In his survey, *Nr* was placed tentatively on chromosome 9; however, specific placement relative to other chromosome 9 loci could not be established. The ability to accurately score the *Nr* phenotype can be greatly enhanced by analysis of *Nr*-associated ethylene insensitivity in germinating seedlings (a co-dominant marker; Fig. 1) as opposed to assessment of impaired fruit ripening (a dominant marker; Fig. 3), making phenotypic analysis of *Nr* simpler and more accurate.

Using the triple response seedling assay to determine genotype at the *Nr* locus and RFLP markers previously shown to span chromosome 9 (Tanksley et al., 1992), we have confirmed the presence of *Nr* within this linkage group. Specifically, the *Nr* locus is flanked by the tomato RFLP markers TG551 and TG291 (Fig. 3). In addition, DNA pools nearly isogenic for the *Nr* region of the tomato genome were constructed and utilized to identify four additional DNA (RAPD) markers that map to chromosome 9 in both the population segregating for *Nr* (Fig. 3) and that used previously to construct the tomato RFLP map (data not shown), providing additional support for localization of *Nr* on chromosome 9.

Use of Nearly Isogenic DNA Pools to Identify Additional *Nr*-Linked Markers

Although a high-density RFLP map of tomato has been developed (Tanksley et al., 1992), difficulty often arises in attempting to identify tightly linked DNA markers that are also polymorphic in the population segregating for the target locus. DNA markers derived from comparative RAPD amplification of nearly isogenic DNA pools represent an avenue by which additional linked markers can be identified. Consequently, our efforts in localizing the *Nr* locus on the tomato RFLP map were complimented by identification of additional tightly linked DNA markers, which could be used as (a) alternative reference points to aid interpretation of comparative linkage analysis between the *Nr* locus and previously cloned ethylene-response genes, which could not be mapped in the same population as *Nr*, and (b) starting points in a chromosome walk to isolate the normal *nr* gene in the event that genetic evidence indicated that *Nr* was not homologous to a previously characterized ethylene-response component. The identification of chromosome 9-linked RAPD markers resulting from nearly isogenic DNA pools selected for the *Nr* locus provided confirming evidence for placement of *Nr* on this chromosome. In addition, RAPD analysis of DNA pools nearly isogenic for the *Nr* region of chromosome 9 yielded tRAPD8, the most tightly linked DNA marker to *Nr* (Fig. 3).

Variation in Genetic Distance among Different Mapping Populations

Several possibilities exist for explaining the deviation in linkage distances observed between markers in the populations shown in Figure 3, including (a) relatively few progeny scored, (b) misscoring of RFLP and mutant genotypes/phenotypes, and (c) real variation in recombination rates among different parent pairs. The most likely cause for the difference is the relatively small size of all three populations, especially those used for mapping *Nr* by fruit or seedling phenotype (29 and 42 individuals, respectively). Although the subpopulation of the *L. esculentum* (*Nr/Nr*) × *L. cheesmannii* (*nr/nr*) cross scored for ethylene sensitivity yielded linkage distances on average greater than those observed in the *L. esculentum* × *L. pennellii* population described by Tanksley et al. (1992), the subpopulation scored for ripening phenotype did not (Fig. 3).

Misscoring mutant and/or RFLP phenotype could also contribute to the variation in linkage distances between loci shown in Figure 3. To control for RFLP-scoring error, DNA was extracted a second time from each individual of the 42-member F_2 population scored on the basis of ethylene sensitivity and analyzed again via gel-blot hybridization with chromosome 9 RFLP markers. All markers tested were scored identically both times. In addition, removal of

the *Nr* locus from the data set used to construct the RFLP map shown in Figure 3 (scoring ethylene sensitivity) results in only a 3.7-cM reduction in linkage distance between the RFLP markers (TG291 and TG551) flanking *Nr*. This reduction in linkage distance from 21.2 to 17.5 cM between TG291 and TG551 following the removal of *Nr* suggests little if any misscoring of the seedling ethylene-response phenotype.

Nr Is Linked to a Locus Sharing DNA Sequence Homology with the *Arabidopsis ETR1* Gene

A variety of mutants and several genes directly involved in or related to ethylene biosynthesis and/or signal transduction have been isolated in recent years (Kieber and Ecker, 1993). We have recently demonstrated that the *Nr* mutant represents a lesion in a gene necessary for ethylene responsiveness in tomato (Lanahan et al., 1994). Placement of the *Nr* locus on the tomato RFLP map has allowed us to address whether any of the cloned ethylene-response genes potentially represents an *Nr* homolog and, additionally, which locus (if a member of a multigene family) should be targeted for isolation and complementation testing in transgenic tomato plants. Although several ethylene-response genes, including *ETR1* and *CTR1*, have been isolated from the model plant species *Arabidopsis*, which does not produce fleshy fruits like those found in tomato, utilization of cloned *Arabidopsis* sequences as RFLP probes has permitted the mapping of related sequences in tomato (Fig. 5).

The impaired ethylene response observed in either the *Arabidopsis Etr1* (*Ein1*), *ctr1* mutants, or tomato E8 antisense mutants is consistent with a model in which *Nr* is considered a homolog of any of the three. For example, *ctr1* mutants represent genetic lesions in the C-terminal catalytic domain of a putative Raf protein kinase involved in the negative regulation of ethylene signal transduction and, therefore, result in continual stimulation of the ethylene-response pathway (Kieber et al., 1993). Mutation in the N-terminal domain of CTR1 may be expected to result in ethylene insensitivity because of constitutive activation of repressor function (Stanton et al., 1989; Heidecker et al., 1990; Kieber et al., 1993), which would be consistent with the phenotype of the tomato *Nr* mutant.

To test whether *CTR1* or any of the other available ethylene-response genes were related to *Nr*, cloned sequences were utilized as molecular probes in tomato RFLP-mapping populations. Figure 5 demonstrates the chromosomal locations of eight putative ethylene-response loci in the tomato genome based on RFLP mapping of the tomato *E8* and *Arabidopsis ETR1* and *CTR1* genes. The RFLP linkage analysis presented here demonstrates that *Nr* is not a homolog of seven of the eight loci mapped (*tCTR1*, *tETR1a*, *tETR1f*, *tETR1w*, *tETR1x*, *E8A*, and *E8B*) and suggests that *tETR1n* may be a candidate for the *Nr* locus. Unfortunately, genetic testing of this hypothesis is complicated by our inability to map *tETR1n* in the population segregating for *Nr*. Nevertheless, the only conclusive test for allelism will be accomplished via isolation of *tETR1n* from the *Nr* mutant, followed by insertion into the normal tomato genome and analysis of ethylene sensitivity and fruit ripening in transgenic plants.

The phenotypic similarity between seedlings of the tomato *Nr* and *Arabidopsis* Etr1 (*Ein1*) mutants is striking and suggests the possibility that the *Nr* locus harbors a tomato homolog of *ETR1*. RFLP linkage analysis implicates one of five *ETR1* homologs, *tETR1n*, as the only candidate locus and eliminates four others, demonstrating the utility of using DNA probes across species to (a) generate hypotheses whose testing may simplify the complexities associated with isolation of a gene corresponding to a particular mutant locus and (b) ascertain the in vivo role of specific gene family members. The alternative to such a short-cut in gene isolation, although often necessary because of the lack of cloned candidate genes from model species, would likely be the arduous task of genetic map-based cloning. Following isolation of tomato *tETR1* clones from normal and *Nr* genomic libraries, development of gene-specific probes should facilitate the rapid identification, via RFLP mapping, of *tETR1n* clones that can be tested for both variation in DNA sequence between normal and *Nr*-derived alleles and physiological function in transgenic plants.

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