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Genetic mapping of ripening and ethylene-related loci in tomato

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Abstract The regulation of tomato fruit development and ripening is influenced by a large number of loci as demonstrated by the number of existing non-allelic fruit development mutations and a multitude of genes showing ripening-related expression patterns. Furthermore, analysis of transgenic and naturally occurring tomato mutants confirms the pivotal role of the gaseous hormone ethylene in the regulation of climacteric ripening. Here we report RFLP mapping of 32 independent tomato loci corresponding to genes known or hypothesized to influence fruit ripening and/or ethylene response. Mapped ethylene-response sequences fall into the categories of genes involved in either hormone biosynthesis or perception, while additional ripening-related genes include those involved in cell-wall metabolism and pigment biosynthesis. The placement of ripening and ethylene-response loci on the tomato RFLP map will facilitate both the identification and exclusion of candidate gene sequences corresponding to identified single gene and quantitative trait loci contributing to fruit development and ethylene response.

Key words RFLP mapping · Fruit ripening · Ethylene · Tomato

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Introduction

The development and ripening of flesh fruits represents the coordinated regulation of numerous biochemical pathways which in summation are manifested as the ripe phenotype. While specific ripening phenomena vary among fruiting species, ripening can be generally characterized as changes in pigmentation, texture, cell-wall ultrastructure, carbohydrate metabolism, accumulation of aromatic and flavor volatiles, and susceptibility to opportunistic pathogens. Many of these characteristics are inter-related. Physiologists have historically divided ripening into two general classifications, climacteric and non-climacteric, based on the presence or absence of dramatic ripening-related increases in respiration, respectively (Baile and Young 1981). In general, climacteric ripening is also associated with substantially increased ethylene biosynthesis and sensitivity. Ethylene produced during climacteric fruit ripening has a profound influence on the ripening process, as demonstrated by the inhibition of ripening in fruits blocked in ethylene biosynthesis or perception via chemical (Davies et al. 1988) or genetic means (Hamilton et al. 1990; Oeller et al. 1991). Numerous fruiting species of agricultural and scientific importance, including apples, pears, most stone fruits, tomatoes, bananas, melons, and many others, utilize the gaseous hormone ethylene as a coordinator of expression for genes contributing to the ripening process (Hobson and Grierson 1993). Additional aspects of ripening in both climacteric and non-climacteric fruit are controlled by developmental signals (Theologis et al. 1993).

The most extensively studied model system of fruit development and ripening to date is tomato. The physiology, molecular biology, and genetics of tomato ripening has been facilitated by the dramatic phenotype, and relatively uniform and rapid ripening, particularly of small-fruited cultivars. Continuous effort for many years and by numerous groups has resulted in the

isolation of a large number of ripening-related genes (many of which are regulated by ethylene) and genetic loci defined by mutation. Tomato gene isolation efforts have primarily targeted genes involved in cell-wall-metabolism (DellaPenna et al. 1986; Grierson et al. 1986; Harriman et al. 1991; Lashbrook et al. 1994) and ethylene biosynthesis or perception (Theologis 1992: Wilkinson et al. 1995) in addition to genes whose expression is regulated during ripening and/or by ethylene (reviewed in Gray et al. 1994). Analysis of transgenic tomato plants altered in ethylene biosynthesis (Hamilton et al. 1990; Oeller et al. 1991; Klee et al. 1991), cell-wall metabolism (Smith et al. 1988; Teiman and Handa; 1994), and carotenoid biosynthesis (Bird et al. 1991; Fray et al. 1995) has provided genetic verification of function for candidate genes in said biochemical pathways in addition to representing some of the first demonstrations of antisense and sense gene repression in plants.

While ethylene biosynthesis has been extensively characterized in tomato, analysis of ethylene signal transduction has been performed primarily in the model plant *Arabidopsis*. Numerous mutants altered in ethylene perception and signal transduction have been identified by screening for defects in the seedling "triple-response" to ethylene, and in many instances corresponding genes have been cloned (reviewed in Ecker 1995). For example, the *ETR1* gene encodes a "two-component" sensor-histidine kinase which has been shown to bind ethylene and which when mutated can confer dominant insensitivity to ethylene in *Arabidopsis*, tomato, and petunia (Chang et al. 1993; Wilkinson et al. 1995; 1997). Similar effects are observed in *Arabidopsis* when a related gene, *ERS*, is inflicted with a mutation analogous to that observed in the *Etr1-1* mutant (Hua et al. 1995). The inability of the mutant *ETR1-1* gene product to bind ethylene is correlated with ethylene insensitivity (Schaller and Bleeker 1995). These results in combination with analyses of epistasis among ethylene signal transduction mutants which place *ETR1* at the initial stage of the pathway (Ecker 1995) provide strong evidence that this gene encodes an ethylene receptor.

Genetic mapping of gene sequences related to a particular biological phenomenon or response represents a simple, yet powerful tool for gaining insight into specific gene function. This approach is particularly useful for species such as tomato in which a large number of single gene loci and quantitative trait loci (QTLs) related to fruit development, ripening, and quality have been mapped. For example, mapping of tomato loci homologous to the *Arabidopsis ETR1* gene revealed co-segregation of a related sequence with the *Never-ripe (Nr)* locus also characterized by insensitivity to ethylene (Lanahan et al. 1994; Yen et al. 1995). Subsequent isolation of the *Nr*-linked tomato *ETR1* locus led to verification that the *NR* gene in fact represents a tomato ethylene receptor most similar in structure to the *Arabidopsis ERS* gene (Wilkinson et al. 1995). Another example is mapping of the tomato TOM5 phytoene synthase gene to the same location as the *r* (*yellow-flesh*) locus on chromosome 3 (Kinzer et al. 1990).

Here we present RFLP mapping data for 47 tomato loci homologous to DNA sequences related to fruit ripening and/or ethylene biosynthesis or perception, including 32 sequences which had not been previously mapped. Mapped sequences can generally be classified in terms of (1) ethylene biosynthesis, (2) ethylene signal transduction, or (3) ripening- (and often ethylene)-induced. Our results suggest that both ripening and ethylene-related loci are dispersed throughout the tomato genome. Placement of these loci on the tomato DNA marker map facilitates both the identification and elimination of candidate genes for characterized QTL and single-gene mutant loci related to fruit development and ethylene hormone responses.

Materials and methods

Plant material

Inbred cultivated tomato (*Lycopersicon esculentum*, cv 'Ailsa Craig') and the wild relative *L. pennellii* (accession TA56) were grown under standard greenhouse conditions from seed as needed. TA56 seed was kindly provided by the Tomato Genetics Stock Center at the University of Calfornia, Davis. Thirty-two individuals from the F_2 mapping population described by Tanksley et al. (1992) were generously provided by Steve Tanksley (Cornell University) and propagated as cuttings. Expanding leaves from all plants were harvested and frozen in liquid nitrogen on a bi-monthly basis.

Isolation and hybridization of plant nucleic acids

Procedures for both genomic DNA extraction from expanding tomato leaves and DNA gel-blot hybridization were performed as described previously (Fulton et al. 1995; 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 for 36 h at 65°C in 5 × SSC, 0.5% (w/v) SDS, 50 m*M* Na-P (pH 7.5), and 5 × Denhardt's solution, and were followed by 20-min 65°C washes first in 2 × SSC, 0.1% (w/v) SDS, then in 1 × SSC, 0.05% (w/v) SDS, and finally in 0.5 × SSC, 0.05% (w/v) SDS. Identification of restriction fragment length polymorphisms (RFLPs) was as described by Tanksley et al. (1992), including additional restriction endonucleases as in Yen et al. (1995). Restriction enzymes yielding RFLPs were used to digest genomic DNA extracted from individual members of segregating populations for subsequent linkage analysis.

DNA marker probes

All probes used for linkage analysis were cDNAs previously cloned into various vectors containing M13 forward/reverse and/or T3/T7 primers. Chemistry for polymerase chain reaction (PCR) amplification of RFLP probe templates was as described by the *Taq* polymerase supplier (Promega) in a 100-µl total reaction volume and performed in a Perkin Elmer 4800 DNA thermocycler. Thermocycler

conditions were, 28 cycles of 1 min. 95°C; 1 min, 55°C; 2 min, 72°C, followed by 1 cycle of 1 min, 95°C; 1 min, 55°C; 10 min, 72°C and terminating in a 4°C hold. PCR products were quantitated on agarose gels with known amounts of lambda DNA (BRL) as standards and purified from unincorporated nucleotides by passage through a sephadex G-50 (Sigma) spin column constructed from a 1-ml syringe. Radiolabeling was performed using random hexamers as described previously (Tanksleyet al. 1992).

Genetic linkage analysis

Autoradiograms resulting from hybridization of radiolabeled cDNA probes for F_2 genomic DNAs were scored for segregation of RFLP alleles by at least two individuals. Conflicting scores were resolved by re-examining the autorads or scored as "no data" if unclear. Genetic linkage analysis was performed using *MapMaker* software (Lander et al. 1987) and the RFLP marker segregation database used to construct the tomato RFLP map in the larger population of which our materials were a subset (Tanksley et al. 1992). RFLPs corresponding to cDNAs of interest here were added to the map using the "try" and "ripple" commands, respectively. Only those markers which mapped with a LOD score of 3.0 or higher were included in the map. CentiMorgan distances were calculated using the Kosambi (1944) function.

Results and discussion

Placement of loci corresponding to gene sequences of known or hypothesized function was carried out using a subset of the F_2 DNA marker mapping population described by Tanksley (1992) and maintained by vegetative propagation. Over 1200 DNA, isozyme, and morphological markers have been mapped in this population, and the resulting database of segregation data was used for placement of the loci mapped in this report.

Sources of ripening and ethylene-related cDNAs

Mapped cDNAs were provided from several laboratories and fall into three general categories: (1) ripening-induced (most of which are also ethylene-induced); (2) ethylene biosynthesis; and (3) ethylene signal transduction. These categorical distinctions are not completely descriptive as some sequences fall into multiple categories. Examples include ACC oxidase (TOM13) and TETR-3 (*NR*), which are both induced during ripening and involved in ethylene biosynthesis and ethylene perception, respectively (Hamilton et al. 1990; Wilkinson et al. 1995). A tomato pedicel abscission zone polygalacturonase (*TAPG1*; Kalaitzis et al. 1997) which responds to ethylene but is apparently not induced in ripening fruit tissues was also mapped during this study.

Table 1 lists the specific loci whose map position is reported here. Most of these loci correspond to cDNAs which were isolated via differential screening for genes induced during ripening. Specifically, DDTFR (Differential Display Tomato Fruit Ripening) clones were isolated by differential displays of mature green versus early breaker-stage tomato fruit and confirmed for expression pattern by RNA gel-blot analysis (P. Kannan and J. Giovannoni, unpublished). E and J cDNAs were isolated from differential screens of mature green versus ethylene-treated mature green tomato fruit (Lincoln et al. 1987), while ERT cDNAs resulted from differential screens of normal breaker-stage fruit versus equivalent-age fruit homozygous for the *ripening-inhibitor* (*rin*) mutation which greatly impairs the ripening process (Tigchellar et al. 1978; Picton et al. 1993). TOM cDNAs were originally isolated via differential screening of mature green versus ripe fruit mRNAs (Slater et al. 1985) with many of the corresponding genes subsequently tested for function via targeted gene repression or over-expression in transgenic tomato plants (reviewed in Gray et al. 1992, 1994).

The remainder of the tomato loci reported here correspond to genes isolated either on the basis of homology to previously isolated genes or biochemical activity. Putative ethylene signal transduction loci designated *Le-ETR*, *TCTR*, and *TEIN3* were isolated based on homology to the *ETR1* (Chang et al. 1993), *CTR1* (Keiber et al. 1993), and *EIN3* (Chao et al. 1997) genes of *Arabidopsis*, respectively. A number of loci corresponding to *ETR* and *CTR* sequences were originally mapped by Yen et al. (1995) using *Arabidopsis ETR1* and *CTR1* genes as probes, while positions of gene-specific probes derived from their tomato counterparts are reported here. Tomato ACC synthase (ACS) genes were also isolated based on homology to previously isolated *ACS* genes (Theologis 1992 and references therein). The tomato polygalacturonase (*PGAL*) and beta-subunit (*B-PG*) genes were isolated using immunological probes specific to the corresponding protein products (DellaPenna et al. 1986; Zheng et al. 1994). *PGAL* was simultaneously identified as TOM6 (Grierson et al. 1986).

Estimation of copy number of ripening- and ethylene-related genes

Representation in the genome of each sequence mapped was estimated using at least one of the following methods. First, all sequences were hybridized to tomato genomic DNA digested with a minimum of four different restriction enzymes to determine the minimum number of hybridizing bands which could be detected for each probe sequence. Tomato genomic DNA from cultivated tomato (*L. esculentum*), in addition to that from the wild relatives *L. pennellii* and *L. cheesmannii*, were digested with each restriction enzyme and analyzed side-by-side for RFLPs (Fig. 1a). *L. esculentum* and *L. pennellii* represent the parents of the cross used to generate the F_2 mapping population employed for linkage analysis, while *L. cheesmannii* has

Table 1 Summary of mapped ripening- and ethylene-related cDNA clones from tomato. All loci listed including those which had been mapped previously were mapped again in this study

Clone	Copy no.	Chromosome[a]	Function (homology)	Reference
ACS	>5	1*, 5*, 5, 8, 8*	ACC synthase	Rottmann et al. 1991 Theologis et al. unpublished
B-PG	1	5	Beta-subunit polygalacturonase	Watson et al. 1994
DDTFR1	1	9	None	Kannan and Giovannoni, unpublished
DDTFR5	1	10	None	Kannan and Giovannoni, unpublished
DDTFR8	1	4	(Progesterone receptor)	Kannan and Giovannoni, unpublished
DDTFR10	1–2	7	(Translation elongation factor 1b)	Kannan and Giovannoni, unpublished
DDTFR10 A	2–3	5	(EREBP)	Kannan and Giovannoni, unpublished
DDTFR13	1	11	None	Kannan and Giovannoni, unpublished
DDTFR18	1	4	None	Kannan and Giovannoni, unpublished
E4	1	3*	(*Drosophilia* ecdysone responsive gene)	Cordes et al. 1989
E8	2–3	3*, 9*	(Ethylene perception)	Penarrubia et al. 1992
E17	1	8	(Proteinase inhibitor)	Margossian et al. 1987
ERT1	1–2	10	(UDP-glucosyl transferase)	Picton et al. 1993
ERT10	1	10	(Dehydrogenases)	Picton et al. 1993
ERT13	1	11	(TUB8, stolin-tip induced)	Picton et al. 1993
ERT14	1	7	None	Picton et al. 1993
ERT15	1–2	10	None	Picton et al. 1993
ERT16	2	4	(ABA stress-related protein)	Picton et al. 1993
ERTD1	3	3	NA	Picton, Whotton and Grierson, unpublished
ERTS2	1–2	2	NA	Picton, Whotton and Grierson, unpublished
J49/DDTFR4	2	2, 8	(NP24 protein)	Lincoln et al. 1987 Kannan and Giovannoni, unpublished
Le-ETR	5	6, 7‡, 9‡, 11‡, 12‡	Ethylene receptor-kinase	Wilkinsen et al. 1995 Lashbrook et al. 1998
PHYS/TOM5	3	2^, 3^,7	Phytoene synthase	Ray et al. 1987 Bird et al. 1991
PGAL	1	10*	Fruit polygalacturonase	DellaPenna et al. 1986 Grierson et al. 1986 Smith et al. 1988
SENU5	1–2	1	Senescence upregulated	John et al. 1997
TAPG1	2–3	12	(Abscission polygalacturonase)	Kalaitzis et al. 1997
TCTR	2	1, 2‡	(CTR1 kinase; *A thaliana*)	(1) Lee and Giovannoni, unpublished (2) Lin et al. 1998
TEIN3	3	1, 1, 6	(*A. thaliana* nuclear-localized ethylene response regulator)	Chao et al. 1997 Tieman and Klee, unpublished
TOM13	3	7^	ACC Oxidase	Holdsworth et al. 1987 Hamilton et al. 1990
TOM66	1	6	(Heat-shock protein)	Fray et al. 1990
TOM75	1–3	1	(Membrane solute transporter)	Fray et al. 1990
TOM92	2–4	8	(Histidine decarboxylase)	Picton et al. 1993b

[a] Loci previously mapped by Kinzer et al. (1990) (^), Tanksley et al. (1992) (*), and Yen et al. (1995) (‡) are indicated
Sequences for which no putative function was identified via a GENBANK search are designated NA (not available) under function

been used to create a number of mapping populations segregating for specific fruit-ripening loci (Giovannoni et al. 1995, *rin*, *nor*; Yen et al. 1995, *Nr*, 1997, *hp-1*). Analysis of all three genotypes permits not only the estimation of genome copy number, but also the simultaneous identification of RFLPs that are useful in determining chromosome position (*L. esculentum* × *L. pennellii*) and the possible relationship to a number of known ripening mutants should a clone map in the vicinity (*L. esculentum* × *L. cheesmannii*).

Second, all probes which showed RFLPs between *L. esculentum* and *L. pennellii* were hybridized to genomic DNAs extracted from individuals comprising the F_2 mapping population (Fig. 1b). Segregation analysis indicated that some sequences of interest such as DDTFR13 and ACS8 were clearly single-copy (Figs. 1b and 2a, respectively), while others such as TOM5 (phytoene synthase) were represented multiple times in the tomato genome (Fig. 2b). Placement of more than one locus at LOD > 3 was also considered to be a strong indication of multiple loci. In some instances hybridization to mapping population genomic DNAs digested with alternative enzymes was used to localize additional loci. For example, TOM5 A (PSY) and TOM5B were mapped to chromosomes 3 and 2, respectively (Fig. 2b, 3), using F_2 genomic DNAs digested

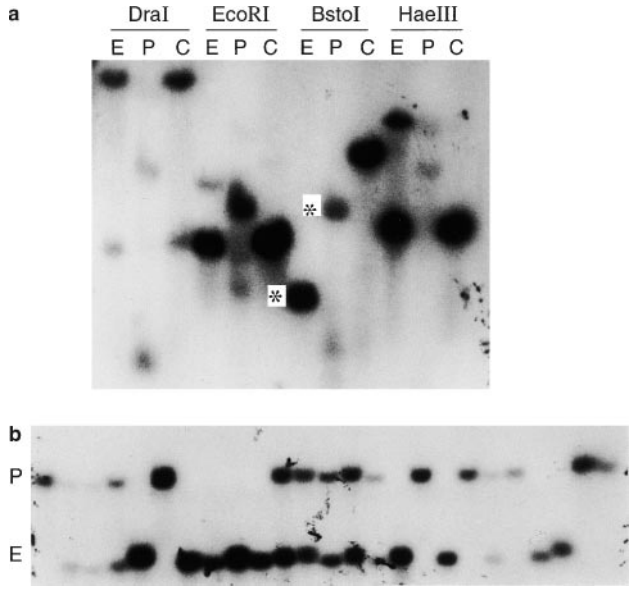


Fig. 1a, b RFLP identification and mapping of ripening and ethylene-related sequences. DNA makers were initially surveyed for RFLPs by hybridizing [^{32}P]-labeled marker sequences to DNA gel-blots containing genomic DNA extracted from the parents of RFLP mapping populations and digested with several different restriction enzymes. Following identification of an RFLP(s) the same probe was utilized on a subset of the F_2 mapping population described by Tanksley et al. (1992) to determine chromosomal assignment and position. **a** Autoradiogram resulting from hybridization of [^{32}P]-labeled DDTFR13 to a DNA gel-blot containing *L. esculentum* (E), *L. pennellii* (P), and *L. cheesmannii* (C) genomic DNA digested with either *Dra*I, *Eco*RI, *Bsto*I, or *Hea*III. *Bsto*I RFLPs between the *E* and *P* parents of the mapping population are designated by the *asterisks*. **b** Autoradiogram resulting from DDTFR13 hybridization to the Tanksley et al. (1992) F_2 mapping population subset following digestion with *Bsto*I. *P* and *E* designate the *L. pennellii* and *L. esculentum* alleles of DDTFR13, respectively

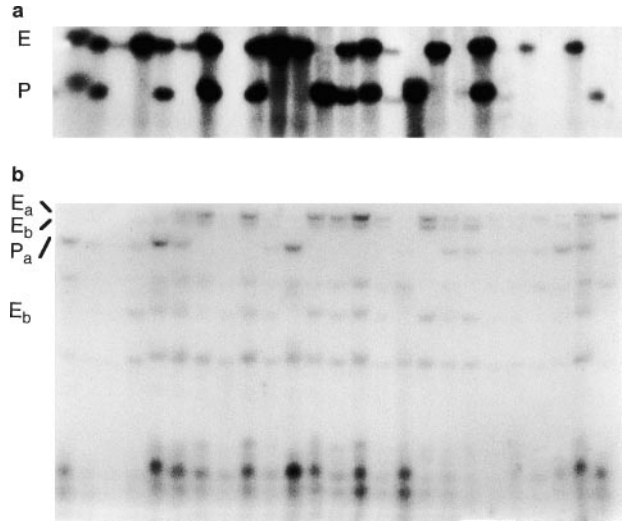


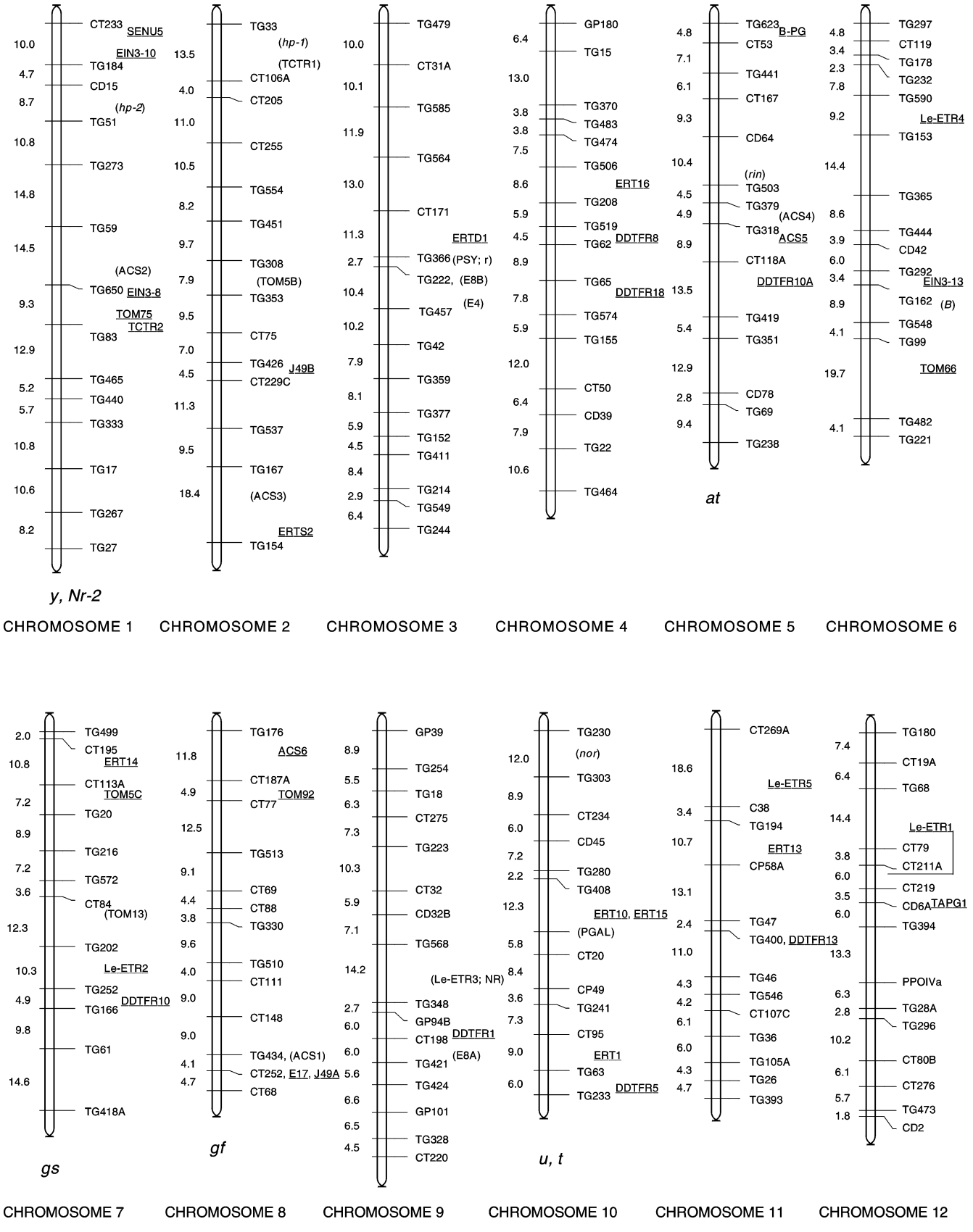
Fig. 2a, b RFLP segregation of single and multiple-copy loci. **a** RFLP segregation of a single-copy sequence derived from the ACC Synthase 8 (ACS8) gene. Genomic DNA extracted from a subset of the Tanksley et al. 1992) RFLP mapping population was digested with *Hae*III. *E* and *P* designate the *L. esculentum* and *L. pennellii* alleles of ACS8, respectively. **b** RFLP segregation of the multi-copy tomato phytoene synthase (pTOM5) cDNA. Genomic DNA extracted from a subset of the Tanksley et al. (1992) RFLP mapping population was digested with *Bsto*I. E_a and P_a correspond to the pTOM5 locus on chromosome 3 (PSY), while E_b and P_b correspond to the chromosome 2 locus (TOM5B). Note that additional non-segregating bands could represent additional restriction fragments located at the two mapped loci or could represent additional independent loci. Using *Dra*I we positioned an additional TOM5-related locus (TOM5C) on chromosome 7 (data not shown)

with *Bsto*I, while the TOM5 C locus was mapped to chromosome 7 using *Dra*I (data not shown).

Finally, for some sequences such as *ACS* and *ACO* (TOM13) more unique gene sequences corresponding to each gene family have been isolated and sequenced than could be positioned on the genetic map. For example, at least three unique tomato *ACO* genes have been isolated (Barry et al. 1996) while LOD > 3 segregation data could only be generated for the chromosome 7 locus. In instances such as this the number of known family members is indicated in Table 1 along with chromosomes containing any mapped loci whose specific position is shown in Fig. 3.

Placement of ripening- and ethylene-related loci on the tomato RFLP map

Localization of 47 ripening- and/or ethylene-related loci relative to DNA marker loci comprising the tomato RFLP map constructed by Tanksley et al. (1992) is shown in Fig. 3. Thirty-two loci represent gene sequence positions which have not been reported previously, while the remaining 15 represent either a confirmation of previous mapping results (Kinzer et al. 1990; Tanksley et al. 1992) or a mapping of gene-specific tomato probes corresponding to loci that had been previously positioned based on hybridization to a single tomato or heterologous sequence. In the latter case 5 tomato loci which hybridized to an *Arabidopsis ETR1* cDNA, and 1 locus hybridizing to an *Arabidopsis CTR1* cDNA had been identified previously in tomato (Yen et al. 1995). Five corresponding tomato ETR1-like genes (Le-ETR1, 2, 3, 4, 5) have been isolated (Lashbrook et al. 1998), and four have been positioned at loci mapped originally with the *Arabidopsis* gene. One of these loci, *Le-ETR3*, represents the tomato *NEVER-RIPE* gene known via mutation to regulate ethylene perception most likely via ethylene receptor function (Wilkinson et al. 1995). The additional locus, *Le-ETR4*, maps to chromosome 6 and was not detected using *ETR1* (Fig. 3). A tomato *CTR1*-like cDNA (TCTR1; Kannan and Giovannoni, unpublished) was mapped to tomato chromosome 2 at the identical location indicated by the *Arabidopsis CTR1* gene,


CT233 SENU5
10.0
EIN3-10
13.5
TG184
4.7
CD15
4.0
8.7
(hp-2)
CT205
TG51
11.0
10.8
CT255
TG273
10.5
14.8
TG554
8.2
TG59
TG451
14.5
9.7
(ACS2)
TG308
7.9
TG650 EIN3-8
(TOM5B)
9.3
TG353
TOM75
9.5
TCTR2
CT75
TG83
7.0
12.9
TG426 J49B
4.5
TG465
CT229C
5.2
11.3
TG440
5.7
TG537
TG333
9.5
10.8
TG167
TG17
18.4
10.6
(ACS3)
TG267
ERTS2
8.2
TG154
TG27
y, Nr-2
CHROMOSOME 1
TG33
(hp-1)
(TCTR1)
CT106A
CHROMOSOME 2
TG479
10.0
CT31A
10.1
TG585
11.9
TG564
13.0
CT171
11.3
ERTD1
2.7
TG366 (PSY; r)
TG222, (E8B)
10.4
(E4)
TG457
10.2
TG42
7.9
TG359
8.1
TG377
5.9
TG152
4.5
TG411
8.4
TG214
2.9
TG549
6.4
TG244
CHROMOSOME 3
GP180
6.4
TG15
13.0
TG370
3.8
TG483
3.8
TG474
7.5
TG506
8.6
ERT16
TG208
5.9
TG519
4.5
TG62 DDTFR8
8.9
TG65
DDTFR18
7.8
TG574
5.9
TG155
12.0
CT50
6.4
CD39
7.9
TG22
10.6
TG464
CHROMOSOME 4
TG623 B-PG
4.8
CT53
7.1
TG441
6.1
CT167
9.3
CD64
10.4
(rin)
TG503
4.5
TG379
4.9
(ACS4)
TG318 ACS5
8.9
CT118A
DDTFR10A
13.5
TG419
5.4
TG351
12.9
CD78
2.8
TG69
9.4
TG238
at
CHROMOSOME 5
TG297
4.8
CT119
3.4
TG178
2.3
TG232
7.8
TG590
9.2
Le-ETR4
TG153
14.4
TG365
8.6
TG444
3.9
CD42
6.0
TG292
3.4
EIN3-13
TG162 (B)
8.9
TG548
4.1
TG99
19.7
TOM66
TG482
4.1
TG221
CHROMOSOME 6
TG499
2.0
CT195
ERT14
10.8
CT113A
TOM5C
7.2
TG20
8.9
TG216
7.2
TG572
3.6
CT84
(TOM13)
12.3
TG202
Le-ETR2
10.3
TG252
4.9
DDTFR10
TG166
9.8
TG61
14.6
TG418A
gs
CHROMOSOME 7
TG176
ACS6
11.8
CT187A
4.9
TOM92
CT77
12.5
TG513
9.1
CT69
4.4
CT88
3.8
TG330
9.6
TG510
4.0
CT111
9.0
CT148
9.0
TG434, (ACS1)
4.1
CT252, E17, J49A
4.7
CT68
gf
CHROMOSOME 8
GP39
8.9
TG254
5.5
TG18
6.3
CT275
7.3
TG223
10.3
CT32
5.9
CD32B
7.1
TG568
14.2
(Le-ETR3; NR)
TG348
2.7
GP94B
6.0
CT198 DDTFR1
6.0
(E8A)
TG421
5.6
TG424
6.6
GP101
6.5
TG328
4.5
CT220
CHROMOSOME 9
TG230
12.0
(nor)
TG303
8.9
CT234
6.0
CD45
7.2
TG280
2.2
TG408
12.3
ERT10, ERT15
(PGAL)
5.8
CT20
8.4
CP49
3.6
TG241
7.3
CT95
9.0
ERT1
TG63
6.0
TG233 DDTFR5
u, t
CHROMOSOME 10
CT269A
18.6
Le-ETR5
C38
3.4
TG194
10.7
ERT13
CP58A
13.1
TG47
2.4
TG400, DDTFR13
11.0
TG46
4.3
TG546
4.2
CT107C
6.1
TG36
6.0
TG105A
4.3
TG26
4.7
TG393
CHROMOSOME 11
TG180
7.4
CT19A
6.4
TG68
14.4
Le-ETR1
CT79
3.8
CT211A
6.0
CT219
3.5
CD6A TAPG1
6.0
TG394
13.3
PPOIVa
6.3
TG28A
2.8
TG296
10.2
CT80B
6.1
CT276
5.7
TG473
1.8
CD2
CHROMOSOME 12

while a second tomato *CTR1*-like gene (*TCTR2*; Hackett, Payton, and Grierson, unpublished), more divergent from *CTR1* than *TCTR1*, was localized to chromosome 1 (Fig. 3).

Genes involved in ripening and/or ethylene response are not generally clustered

Mapped genes can be grouped on a number of bases, including the basis for isolation (eg. differential screen: TOM, DDTFR), specific function (eg. *ACS* genes), or general function (eg. ethylene signal transduction genes: *Le-ETR*, *TCTR*, *EIN*). However, there does not appear to be any substantial clustering of ripening or ethylene-response loci as has been observed, for example, for loci regulating self-incompatibility (Boyes and Nasrallah 1993) or disease resistance (Witsenboer et al. 1995) in plants or the major histocompatibility loci of animals (Graser et al. 1998). This may be due in part to the fact that ripening represents the convergence of numerous distinct biochemical pathways (and thus very different gene sequences) that have been selected for coordinate regulation at the terminal stages of fruit development to yield the "ripe" phenotype. Examples of a functional clustering of loci as those mentioned above tend to represent biological systems where divergence and selection of related gene sequences is required to maintain or maximize viability and thus are likely to represent a very different type of biological mechanism than the control of fruit development or ethylene responses.

Candidate gene function based on genetic linkage

One of the major benefits from determining chromosomal positions of ripening/ethylene-response genes is the ability to hypothesize corresponding gene function based on linkage with characterized ripening mutants. As more isolated genes and mutant loci are mapped relative to a common set of DNA marker loci the probability for identifying genes corresponding to functionally characterized loci will increase, thus circumventing the need for intensive gene isolation and functional characterization approaches (such as map-based cloning and antisense), in at least some instances.

A number of ripening-related and ethylene-response mutants have been identified and mapped relative to morphological markers in tomato (Rick et al. 1980 and references therein). In addition, a number of these loci have been mapped relative to RFLP markers, including those which influence most aspects of fruit development (*rin*, *nor*, Giovannoni et al. 1995), ethylene response (*Nr*, Yen et al. 1995), pigment accumulation (*r*, Kinser et al. 1990; *B*, Tanksley et al. 1992), and fruit development responses related to light quality and intensity (*hp-1*, Yen et al. 1992; *hp-2*, van Tiunen et al. 1997). A number of additional loci including *Nr-2*, *y*, *at*, *gs*, *gf*, *u*, and *t* remain to be localized to specific chromosomal regions relative to DNA marker loci.

Positioning of fruit ripening-related loci on the genetic map has and will continue to facilitate the determination of specific gene function. For example, co-segregation of the *Nr* locus with a RFLP locus homologous to the *Arabidopsis ETR1* gene provided the first molecular evidence that *Nr* represents an ethylene receptor mutation (Yen et al. 1995), as was later confirmed by cloning and analysis of the corresponding *Le-TETR3*/*NR* gene (Wilkinson et al. 1995). Here we show that two putative ethylene-response loci, *TEIN3-8* and *TCTR2*, map to chromosome 1 (Fig. 3) which also harbors the dominant *Nr-2* locus whose fruit are phenotypically similar to those from plants containing the *Nr* ethylene-receptor mutation (Kerr 1982). Mapping of *Nr-2* relative to RFLP loci will help to support or dismiss the possibility that *Nr-2* corresponds to a mutation in either of these genes.

Map position also allows for the exclusion of certain candidate genes for known mutations. The *rin* and *nor* mutants, for example, have been hypothesized to represent components of ethylene signal transduction or perception systems based on observations that fruit of plants homozygous for either mutation fail to ripen in response to exogenous ethylene. We have mapped 12 loci corresponding to genes of hypothesized or known roles in ethylene perception and/or signaling (5 Le-*ETR*, 2 *TCTR*, 2 *E8* and 3 *TEIN3* loci), none of which show linkage to *rin* or *nor*, thus eliminating all 12 as candidate *RIN* or *NOR* genes (Fig. 3).

A major limitation in identifying candidate genes at present is the significant number of mutations which remain to be positioned relative to DNA marker loci. Consequently, though positioning relative to other morphological loci on known chromosomes makes it possible to eliminate numerous candidate genes based on linkage to differing chromosomes, identification of tightly linked candidate genes is viable only for those mutant loci which have been mapped relative to DNA marker loci. Figure 3 shows the positions of 7 ripening-related morphological loci and 7 additional loci which remain to be mapped relative to DNA markers yet have been assigned to specific chromosomes based on

Fig. 3 Position of mapped fruit-ripening and ethylene-related loci on the tomato RFLP map. All 47 loci listed in Table 1 are positioned relative to DNA marker loci described by Tanksley et al. (1992). Positions of *underlined* DNA marker loci have not been reported previously, while those in *parenthesis* have been confirmed for previously reported map positions. Loci corresponding to tomato fruit-ripening and ethylene-related mutants are based on previously reported positions. Mutant loci listed at the *bottom* of linkage groups have been assigned to said group based on linkage to morphological markers (Rick 1980) but have not been positioned relative to DNA marker loci

linkage to other morphological markers (Rick 1980). Additional ripening-related mutants not reported and characterized to varying degrees here have also been identified by our group and others.

Summary

Forty-seven tomato loci homologous to 32 different ripening-related or ethylene response-related DNA sequences were ordered on the existing tomato RFLP map of Tanksley et al. (1992). Mapped loci corresponding to DNA sequences implied or directly shown to be related to ethylene biosynthesis and signaling, cell wall metabolism, carotenoid biosynthesis, and various stress-related responses were found on all 12 tomato chromosomes and did not demonstrate any significant clustering. Seven of the mapped loci are known to be ripening-related only by virtue of their expression pattern as no significant DNA or amino acid sequence homologies were identified.

The positioning of genes related to fruit development and ethylene response in tomato will facilitate the identification of candidate genes as more QTL and single gene mutant loci are localized relative to DNA marker loci. Of equal importance is the fact that the positioning of ripening-related and ethylene response-related loci to specific chromosomal regions also permits the exclusion of most genes as candidates for any particular locus of interest which has been accurately mapped to a specific chromosome using morphological markers.

Plant genome analysis and technology is rapidly approaching the juncture where the efficiency of isolating, characterizing, and mapping gene sequences related to a developmental stage or response of interest can be merged with the accurate and efficient mapping of QTL and single-gene mutant loci to predict candidate genes sequences which can be specifically targeted for confirmation via transgenic or genetic means. The data shown herein for example points to two tomato *EIN3*-like loci and one *CTR1*-like locus on chromosome 1 as candidates for sequences harbored at the *Nr-2* locus, and further dismiss 11 additional ethylene signal transduction-related sequences as candidate genes. Also, as shown previously in the case of the *NR* ethylene receptor and the *PYS* carotenoid biosynthesis gene, knowledge of candidate gene map position relative to loci of known QTLs or mutations can both accelerate gene identification and facilitate the characterization of gene function.

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References

Baile J, Young R (1981) Respiration and ripening in fruits-retrospect and prospect. In: Friend J, Rhodes M (eds) Recent advances in the biochemistry of fruits and vegetables. Academic Press, New York, pp. 1–39

Barry C, Blume B, Bouzayen M, Cooper W, Hamilton A, Grierson D (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant Journal 9: 525–535

Bird C, Ray J, Fletcher J, Boniwell J, Bird A, Teulieres C, Blain I, Bramley P, Schuch W (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. Bio/Technology 9: 635–639

Boyes D, Nasrallah J (1993) Physical linkage of the SLG and SRK genes at the self-incompatibility locus of *Brassica oleracea*. Mol Gen Genetics 236: 369–373

Chang C, Kwok S, Bleecker A, Meyerowitz E (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science 262: 539–545

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker J (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89: 1133–1144

Cordes S, Deikman J, Margossian L, Fischer R (1989) Interaction of a developmentally regulated DNA-binding factor with sites flanking two different fruit-ripening genes from tomato. The Plant Cell 1: 1025–1034

Davies K, Hobson G, Grierson D (1988) Silver ions inhibit the ethylene-stimulated production of ripening-related mRNAs in tomato. Plant Cell Environ 11: 729–738

DellaPenna D, Alexander D, Bennett, A (1986) Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening. Proc Natl Acad Sci USA 83: 6420–6424

Ecker J (1995) The ethylene signal transduction pathway in plants. Science 268: 667–675

Fray R, Lycett G, Grierson D (1990) Nucleotide sequence of a heat-shock and ripening-related cDNA from tomato. Nucl Acids Res 18: 7148

Fray R, Wallace A, Grierson D, Lycett G (1994) Nucleotide sequence of a ripening and water stress-related cDNA from tomato with homology to the MIP class of membrane channel proteins. Plant Molec Biol 24: 539–543

Fray R, Wallace A, Fraser P, Valero D, Hedden P, Bramley P, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J 8: 693–701

Fulton T, Julapark C, Tanksley S (1995) Miniprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13: 207–209

Giovannoni J, Noensie E, Ruezinsky D, Lu X, Tracy S, Ganal M, Martin G, Pillen K, Tanksley S (1995) Molecular genetic analysis of the *ripening-inhibitor* and *non-ripening* loci of tomato: a first step in genetic map-based cloning of fruit ripening genes. Mol Gen Genet 248: 195–206

Graser R, Vincek V, Takami K, Klein J (1998) Analysis of zebrafish Mhc using BAC clones. Immunogenetics 47: 318–325

Gray J, Picton S, Shabbeer J, Schuch W, Grierson D (1992) Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Mol Biol 19: 6553–6558

Gray J, Picton S, Giovannoni J, Grierson D (1994) The use of transgenic and naturally occurring mutants to understand and manipulate tomato fruit ripening. Plant Cell Environ 17: 557–571

Grierson D, Tucker G, Keen J, Ray J, Bird C, Schuch W (1986) Sequencing and identification of a cDNA clone for tomato polygalacturonase. Nucleic Acids Res 14: 8595–8603

Hamilton A, Lycett G, Grierson, D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature. 346: 284–287

Harriman R, Tieman D, Handa A (1991) Molecular cloning of tomato pectin methylesterase gene and its expression in Rutgers, ripening inhibitor, nonripening and Never ripe tomato fruits. Plant Physiol 97: 80–87

Hobson G, Grierson D (1993) Tomato. In: Seymour B, Taylor E, Tucker A (eds) Biochemistry of fruit ripening. Chapman and Hall, London pp 405–442

Holdsworth M, Bird C, Ray J, Schuch W, Grierson D (1987) Structure and expression of an ethylene-related mRNA from tomato. Nucl Acids Res 15: 731–739

Hua J, Chang C, Sun Q, Meyerowiitz E (1995) Ethylene insensitivity conferred by *Arabidopsis* ERS gene. Science 269: 1712–1714

John I, Hackett R, Cooper W, Drake R, Farrell A, Grierson D (1997) Cloning and characterization of tomato leaf senescence-related cDNAs. Plant Mol Biol 33: 641–651

Kalaitzis P, Solomos T, Tucker M (1997) Three different polygalacturonases are expressed in tomato leaf and flower abscission, each with a different temporal expression pattern. Plant Physiol 113: 1303–1308

Kerr E (1982) Never ripe-2 (NR2) a slow ripening mutant resembling *Nr* and *Gr*. Rep Tomato Genet Coop 32: 33

Kieber J, Rothenberg M, Roman G, Feldman K, Ecker J (1993) *CTR1*, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell 72: 427–441

Kinzer S, Schwager S, Mutschler M (1990) Mapping of ripening-related or -specific cDNA clones of tomato. Theor Appl Genet 79: 489–496

Klee H, Hayford M, Kretzmer K, Barry G, Kishore G (1991) Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell 3: 1187–1193

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12: 172–175

Lanahan M, Yen H, Giovannoni J, Klee H (1994) The *Never Ripe* mutation blocks ethylene perception in tomato. Plant Cell 6: 521–530

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Lashbrook C, Gonzalez-Bosch C, Bennett A (1994) Two divergent endo-B-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers. Plant Cell 6: 1485–1493

Lashbrook C, Tieman D, Klee H (1998) Differential regulation of the tomato *ETR* gene family throughout plant development. Plant Journal 15: 243–252

Lincoln J, Cordes S, Read E, Fischer R (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA 84: 2793–2297

Margossian L, Federman A, Giovannoni J, Fischer R (1988) Ethylene-regulated expression of a tomato fruit ripening gene encoding a proteinase inhibitor 1 with a glutamic residue at the reactive site. PNAS USA 85: 8012–8016

Oeller P, Wong L, Taylor L, Pike D, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense 1-aminocyclopropane-1-carboxylate synthase. Science 254: 427–439

Penarrubia D, Aguilar M, Margossian L, Fischer R (1992) An antisense gene stimulates ethylene hormone production during tomato fruit ripening. The Plant Cell 4: 681–687

Picton S, Gray J, Barton S, AbuBaker U, Lowe A, Grierson D (1993) cDNA cloning and characterization of novel ripening-related mRNAs with altered patterns of accumulation in the ripening inhibitor (rin) tomato ripening mutant. Plant Mol Biol 23: 193–207

Ray J, Bird C, Maunders M, Grierson D, Schuch W (1987) Sequence of pTOM5, a ripening-related cDNA from tomato. Nucl Acids Res 15: 10587

Rick C (1980) Tomato linkage survey. Rep Tomato Genet Coop 30: 2–17

Rottman W, Peter G, Oeller P, Keller J, Shen N, Nagy B, Taylor L, Campbell A, Theologis A (1991) 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. Journal of Molec Biol 22: 937–961

Schaller G, Bleeker A (1995) Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR*1 gene. Science 270: 1809–1811

Slater A, Maunders M, Edwards K, Schuch W, Grierson D (1985) Isolation and characterization of cDNA clones for tomato polygalacturonase and other ripening related proteins. Plant Mol Biol 5: 137–147

Smith C, Watson C, Ray J, Bird C, Morris P, Schuch W, Grierson D (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature 334: 724–726

Tanksley S, Ganal M, Prince J, de Vicente M, Bonierbale M, Broun P, Fulton T, Giovannoni J, Grandillo S, Martin G, Messeguer R, Miller J, Miller L, Paterson A, Pineda O, Röder M, Wing R, Wu W, Young N (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132: 1141–1160

Theologis A (1992) One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell 70: 181–184

Theologis A, Oeller P, Wong L, Rothmann W, Gantz D (1993) Use of a tomato mutant constructed with reverse genetics to study fruit ripening, a complex developmental process. Dev Genetics 14: 282–259

Tieman D, Handa A (1994) Reduction in pectin methylesterase activity modifies tissue integrity and action levels in ripening tomato (*Lycopersicon esculentum* Mill.) fruits. Plant Physiology 106: 429–436

Tigchelaar E, McGlasson W, Buescher R (1978) Genetic regulation of tomato fruit ripening. HortScience 13: 508–513

van Tuinen A, Cordonnier-Pratt MM, Pratt LH, Verkerk R, Zabel P, Koornneef (1997) The mapping of phytochrome genes and photomorphogenic mutants of tomato. Theor Appl Genet 94: 115–122

Watson CF, Zheng L, Dellapenna D (1994) Reduction of tomato polygalacturonase beta subunit expression affects pectin solubilization and degradation during fruit ripening. Plant Cell 6: 1623–1634

Wilkinson J, Lanahan M, Yen H, Giovannoni J, Klee, H (1997) An ethylene-inducuble component of signal transduction encoded by *Never-ripe*. Science. 270: 1807–1809

Wilkinson J, Lanahan M, Clark D, Bleeker A, Chang C, Meyerowitz E, Klee H (1997) A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nat Biotechnol 15: 444–447

Witsenboer H, Kesseli R, Fortin M, Stanghellini M, Michelmore R (1995) Sources and genetic structure of a cluster of genes for resistance to three pathogens in lettuce. Theor Appl Genet 91: 178–188

Yen H, Lee S, Tanksley S, Lanahan M, Klee H, Giovannoni J (1995) The tomato *Never-ripe* locus regulates ethylene-inducible gene expression and is linked to a homologue of the *Arabidopsis ETR1* gene. Plant Physiol 107: 1343–1353

Yen H, Shelton A, Howard L, Vrebalov J, Giovannoni J (1997) The tomato *high pigment* (*hp*) locus maps to chromosome 2 and influences plastome copy number and fruit quality. Theor Apl Genet 95: 1069–1079

Zheng L, Watson CF, DellaPenna D (1994) Differential expression of the two subunits of tomato polygalacturonase isoenzyme 1 in wild-type and rin tomato fruit. Plant Physiol 105: 1189–1195