

Molecular and Genetic Characterization of a Non-Climacteric Phenotype in Melon Reveals Two Loci Conferring Altered Ethylene Response in Fruit¹

Christophe Périn², MariCarmen Gomez-Jimenez², Lynda Hagen, Catherine Dogimont, Jean-Claude Pech, Alain Latché, Michel Pitrat*, and Jean-Marc Lelièvre

Institut National de la Recherche Agronomique, Station de Génétique et d'Amélioration des Fruits et Légumes, Domaine St. Maurice, Boîte Postale 94, 84143 Montfavet cedex, France (C.P., L.H., C.D., M.P.); and Unité Mixte de Recherche Institut National de la Recherche Agronomique 990, Ecole Nationale Supérieure Agronomique de Toulouse, Boîte Postale 107, 31326 Castanet-Tolosan cedex, France (M.G.-J., J.-C.P., A.L., J.-M.L.)

Fruit ripening and abscission are associated with an ethylene burst in several melon (*Cucumis melo*) genotypes. In cantaloupe as in other climacteric fruit, exogenous ethylene can prematurely induce abscission, ethylene production, and ripening. Melon genotypes without fruit abscission or without ethylene burst also exist and are, therefore, non-climacteric. In the nonabscising melon fruit PI 161375, exogenous ethylene failed to stimulate abscission, loss of firmness, ethylene production, and expression of all target genes tested. However, the PI 161375 etiolated seedlings displayed the usual ethylene-induced triple response. Genetic analysis on a population of recombinant cantaloupe Charentais × PI 161375 inbred lines in segregation for fruit abscission and ethylene production indicated that both characters are controlled by two independent loci, *abscission layer* (*Al*)-3 and *Al*-4. The non-climacteric phenotype in fruit tissues is attributable to ethylene insensitivity conferred by the recessive allelic forms from PI 161375. Five candidate genes (two *ACO*, two *ACS*, and *ERS*) that were localized on the melon genetic map did not exhibit colocalization with *Al*-3 or *Al*-4.

In general, fleshy fruits are divided in two large groups, climacteric and non-climacteric, based upon the presence or absence of an autocatalytic ethylene burst during ripening (McMurchie et al., 1972). Numerous results have demonstrated the key role of ethylene in the regulation of several ripening processes in climacteric fruit, including ethylene biosynthesis itself (Abeles et al., 1992; Lelièvre et al., 1997; Giovannoni, 2001). However, the regulatory mechanisms governing fruit ripening remain largely unknown.

The ripening of melon (*Cucumis melo*) fruit of several cultivated varieties and wild ecotypes is climacteric and often associated with fruit detachment (Abeles et al., 1992). Interestingly, there exist several melons that apparently do not abscise and display a long shelf life. It has been shown in some cases that these melon fruits emit little or no ethylene and

therefore behave like non-climacteric fruit (Shiomi et al., 1999). Although a number of pleiotropic tomato (*Lycopersicon esculentum*) mutants resulting in partial or total inhibition of ripening have been described (Thompson et al., 1999; Giovannoni, 2001), studies in different species may lead to identification of other genes or regulatory mechanisms. Cantaloupe is a cultivated species of African origin with a high phenotypic and molecular variation (Naudin, 1859; Stepansky et al., 1999). It is an annual diploid plant, and the size of its genome (450 Mb) is relatively small. The construction of genetic maps with high-density markers was recently achieved (Wang et al., 1997; Périn et al., 2000).

The ethylene biosynthetic pathway is well established (Yang and Hoffman, 1984). The regulation of the expression of the main ethylene biosynthetic genes, 1-aminocyclopropane-1-carboxylic acid (*ACC*) synthase (*ACS*) and *ACC* oxidase (*ACO*), has been described in many species and tissues (Zarembinski and Theologis, 1994; Giovannoni, 2001), including melon fruit (Yamamoto et al., 1995; Lasserre et al., 1996; Shiomi et al., 1999). This knowledge was used to alter ethylene production in climacteric melon types such as Védrantais (Charentais subgroup of *C. melo* subsp. *melo*, *cantalupensis*). Védrantais displays a high rate of ripening followed also by rapid senescence; both are inhibited in transgenic melon fruit, in which the capacity for ethylene synthesis is inhibited 99% by an anti-sense *ACO* gene (Ayub et al., 1996).

¹ This work was supported in part by the Institut National de la Recherche Agronomique Action Incitative Programmée Aptitude à l'utilisation des Grains et des Fruits contracts in both laboratories. C.P. was a recipient of a doctoral fellowship from the Institut National de la Recherche Agronomique and Région Provence-Alpes Cote-d'Azur (France). M.G.-J. was a recipient of a postdoctoral fellowship from the Spanish "Ministerio de Educacion y Cultura."

² These authors contributed equally to the paper.

* Corresponding author e-mail Michel.Pitrat@avignon.inra.fr; fax 33-4-32-72-27-02.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010613.

Studying transgenic melon led to the characterization of ripening events that are highly dependent upon ethylene action (Guis et al., 1997; Hadfield et al., 2000). Among these are fruit abscission, degreening of the rind, the duration of shelf-life, and chilling injury (Guis et al., 1997; Ben Amor et al., 1999). The stimulated expression of numerous genes observed during ripening is also strongly dependent on high ethylene levels (Guis et al., 1999; Shiomi et al., 1999; Hadfield et al., 2000).

The non-climacteric phenotype in melon fruit may be attributable to alteration of either upstream developmental processes or any element of the ethylene signal transduction pathway (Giovannoni, 2001). Spectacular progress has been made in the comprehension of the molecular mechanisms of ethylene perception (for review, see Bleecker and Kende, 2000; Stepanova and Ecker, 2000) essentially thanks to *Arabidopsis* mutants altered in the triple response. Exposure of etiolated seedlings to exogenous ethylene inhibits root and shoot elongation and induces radial swelling of the shoot as well as changes in its orientation to gravity; the whole response is referred to as the triple response (Bleecker and Kende, 2000). Moreover, root hair formation is also regulated by ethylene, at least in *Arabidopsis* (Kieber et al., 1993; Raz and Ecker, 1999). The tomato *Nr* mutant, originally identified as a pleiotropic fruit ripening mutant, turned out to display a similar phenotype of global ethylene insensitivity and was identified as an allelic mutation in an *ERS*-like ethylene receptor gene (Lanahan et al., 1994; Wilkinson et al., 1995). In *Arabidopsis* and other plants exists a small multigenic family of redundant ethylene receptors (Bleecker and Kende, 2000) expressed in many tissues. All ethylene receptors act as negative regulators of the transduction pathway as shown by elegant genetic analysis in *Arabidopsis* (Hua and Meyerowitz, 1998) and confirmed in transgenic tomato (Tieman et al., 2000). No receptor gene appears to be specifically expressed during tomato and melon fruit development, although the observed hormonal, temporal, and spatial regulation of the different genes probably play a role in modulating ethylene sensitivity (Wilkinson et al., 1995; Lashbrook et al., 1998; Sato-Nara et al., 1999; Tieman and Klee, 1999). Transcription factors *EIN3* and *EIN3*-like (*EIL*; Chao et al., 1997) have also been identified. These DNA-binding proteins positively regulate the expression of target genes during the primary response in *Arabidopsis* (Solano et al., 1998), but little is known about the regulation and role of these elements during fruit ripening.

We initiated physiological and genetic analysis of the non-climacteric phenotype of a nonabscising, long-shelf-life melon fruit. These data provide a starting point to dissect the physiological and molecular differences between climacteric and non-climacteric melons. We report on the genetic control of the climacteric burst, ethylene synthesis, and associated

response. This genetic study was conducted on segregating populations between a non-climacteric melon (PI 161375) and a climacteric variety (Védrantais). To exploit natural variation, molecular genetic mapping offers powerful tools to define major genes and quantitative trait loci (QTL) involved in complex traits (Tanksley and McCouch, 1997). Immortalized lines such as recombinant inbred lines (RILs; Burr and Burr, 1991) are choice material because trial replications in different environments and for different characters can be achieved. Here, we show that variation in climacteric versus non-climacteric ripening is due to two loci.

RESULTS

The PI 161375 Fruit That Does Not Abscise Is Non-Climacteric

As observed previously (Guis et al., 1997), Védrantais melon fruit abscised and displayed a characteristic peak of ethylene production (Fig. 1A) and ACC content (Fig. 1C) 37 d after pollination (DAP). This was associated with a peak of ACS activity 36 DAP (Fig. 1B). In contrast, PI 161375 fruit did not abscise up to 60 to 80 DAP and did not display any peak of ethylene production, ACC content, or ACS activity (Fig. 1, A–C). The PI 161375 fruit remained firm (6 kg cm^{-2}) during the same period, whereas firmness had decreased down to 2 kg cm^{-2} in Védrantais at 42 DAP (Fig. 1D).

Ethylene production and all ethylene-dependent events can be prematurely induced in climacteric fruit by exogenous ethylene or its analog propylene

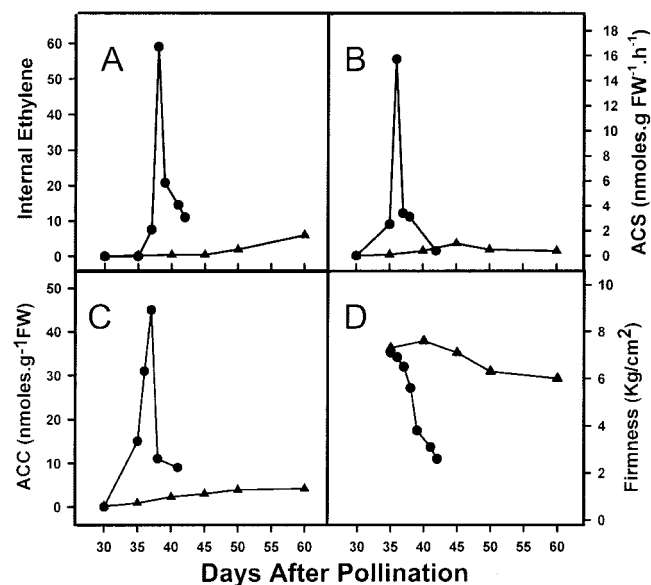


Figure 1. Comparison of internal ethylene content (A; in microliters per liter), ACS activity (B), ACC content (C), and flesh firmness (D) in melon fruit of climacteric (Védrantais, ●) and non-climacteric (PI 161375, ▲) genotypes.

(McMurchie et al., 1972). In Védrrantais melon, such treatment can trigger ethylene production and cell separation in the fruit abscission zone (Guis et al., 1997) but not in PI 161375 fruit (not shown). Figure 2A shows that propylene treatment did not induce endogenous ethylene production in PI 161375 fruit. Moreover, neither firmness nor rind color were significantly different between air- or propylene-treated fruit (Fig. 2, B and C). In contrast, a high level of ethylene was produced after wounding in PI 161375 fruit (data not shown) as in Védrrantais (Bouquin et al., 1997). Taken together, these results demonstrate that PI 161375 melon fruit can be considered non-climacteric, although they can synthesize high amounts of ethylene after wounding.

Comparison of the Expression of Genes between Védrrantais and PI 161375 Fruit

We examined the expression of ripening- or ethylene-related melon genes previously isolated, such as *ACO1*, *ERS1*, *PG1*, *PG2*, *RM4*, *RM5*, *RM7*, *RM8*, *RM11*, and *RM16*, or isolated during this study

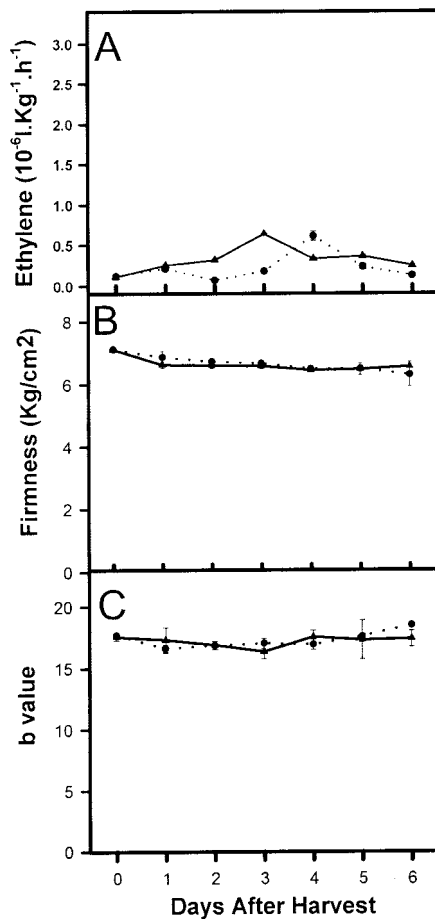


Figure 2. Effect of exogenous propylene on PI 161375 melon fruit harvested 45 DAP for 1 or 6 d on ethylene production (A), flesh firmness (B), and color of the peel (C). Dashed line, Fruit in air; solid line, fruit exposed to propylene.

(*EIL1* and *EIL2*). The expression level of *PG2*, *ERS1*, *EIL1*, *EIL2*, *RM7*, and *RM8* was similar in Védrrantais (just before the climacteric crisis) and PI 161375 (Fig. 3A). However, it is noticeable that the decrease in expression of *PG2*, *ERS1*, *EIL2*, *RM7*, and *RM8* observed in Védrrantais fruit after d 37 or 38, i.e. after the climacteric ethylene (Fig. 1A), did not occur in PI 161375 fruit (Fig. 3A). Two of these genes (*RM7* and *RM8*) were found to be expressed at lower levels in transgenic (antisense *ACO*) Védrrantais with 99% inhibition of ethylene production but were not up-regulated by exogenous ethylene (Hadfield et al., 2000). We checked their regulation by propylene in PI 161375 fruit harvested 45 DAP (Fig. 3B) and continuously exposed to air supplemented or not with propylene. The expression of none of these genes was different in air- or propylene-treated PI 161375 fruit.

A second group of genes were expressed at low levels in PI 161375 fruit (Fig. 4A). Most genes of this group had a maximum expression level at the climacteric crisis in Védrrantais fruit (Fig. 4A). This group includes the ethylene biosynthetic genes *ACO1* and *ACS1*. The latter was slightly detected in Védrrantais and not at all in PI 161375 fruit (data not shown). *RM2* and *PG3* transcripts were not detected in PI 161375. Again, regulation by ethylene of the expression of these genes was examined in PI 161375 fruit and no propylene-induced changes were observed (Fig. 4B). Propylene treatment performed on older PI 161375 fruit (50 DAP) yielded identical results (not shown). Wound-induced ethylene production, which is largely independent of ethylene action, correlated with strong stimulation of *ACO1* and *ACS1* expression in both PI 161375 and Védrrantais fruit (data not shown; Bouquin et al., 1997).

Seedling Tissues of the PI 161375 Display the Triple Response Induced by Ethylene

Both PI 161375 and Védrrantais seedlings displayed the triple response (Fig. 5). In both genotypes, ethylene inhibited root and shoot elongation and induced radial swelling. However, some differences were noted: (a) root elongation was partially inhibited by ethylene in PI 161375, and root hair formation was completely inhibited; and (b) the apical hook in ethylene treated Védrrantais seedlings did not display the exaggerated curvature that is seen in PI 161375 (Fig. 5). There was almost no hook curvature at any stage in Védrrantais seedlings grown in air (data not shown), contrary to what is observed in Arabidopsis plants.

Two Loci Control the Fruit Abscission Phenotype and Are Localized in Two Different Linkage Groups (LG)

F_1 (Védrrantais \times PI 161375) fruits displayed the Védrrantais phenotype, i.e. they abscised and dis-

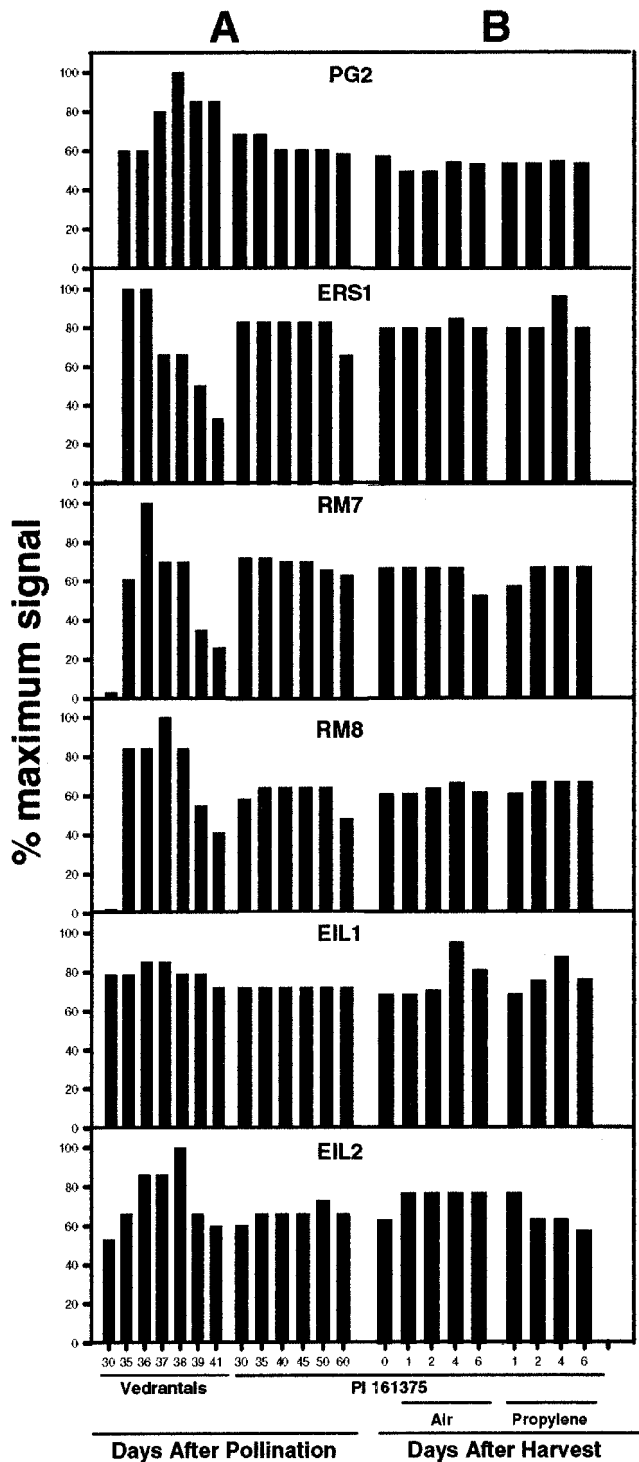


Figure 3. RNA analysis of genes whose expression in PI 161375 fruit is at the same level as in Védrantais fruit before the climacteric crisis. Phosphor imager data of RNA gel-blot analysis of *PG2*, *ERS1*, *RM7*, *RM8*, *EIL1*, and *EIL2* in developing Védrantais and PI 161375 melon fruit expressed for each individual blot in terms of percentage of the maximum signal detected. A, Comparison of gene expression between Védrantais and PI 161375 melon fruit during ripening on the vine. Tissues were from seven stages of fruit development (30, 35, 36, 37, 38, 39, and 41 DAP) for Védrantais climacteric line or from 6 stages (30, 35, 40, 45, 50, and 60 DAP) for the non-climacteric PI

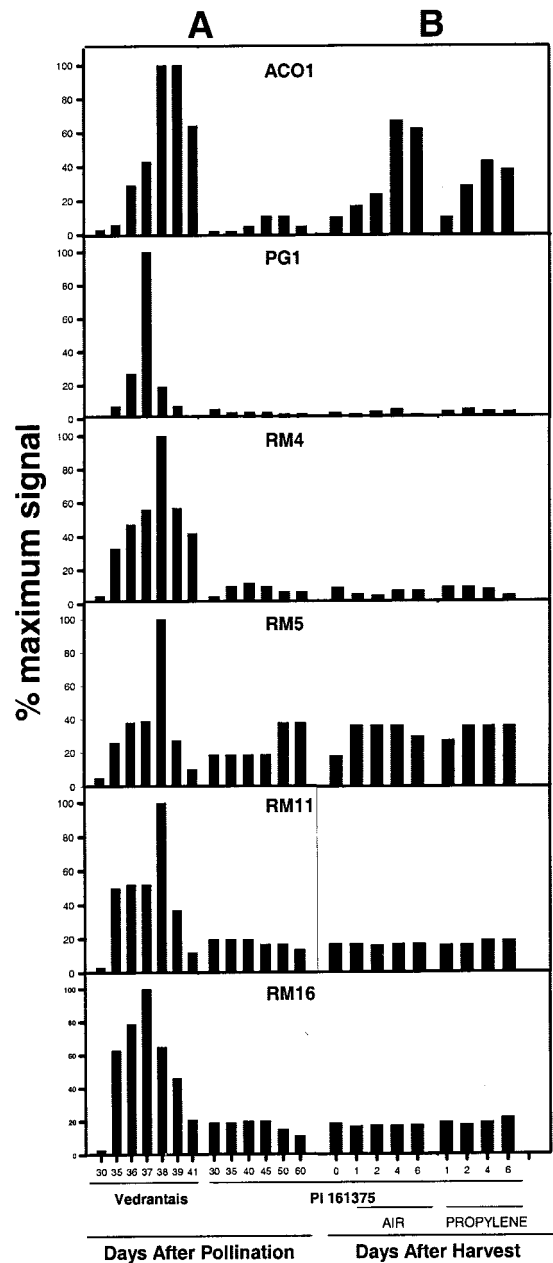
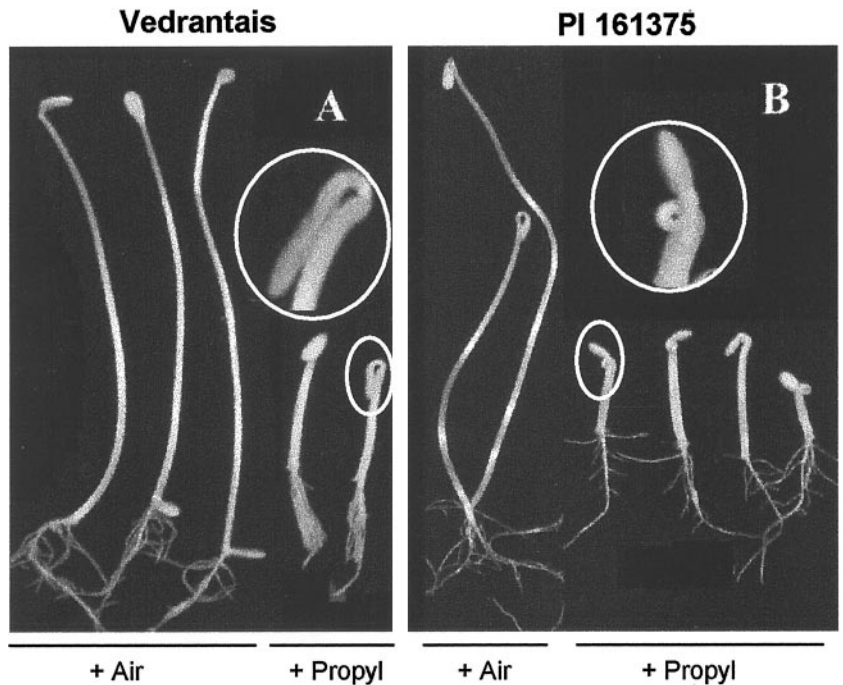


Figure 4. RNA analysis of genes whose expression in PI 161375 fruit is lower than in Védrantais fruit before the climacteric crisis. Legend is identical to Figure 3.

played a peak of ethylene during ripening (data not shown). This indicates that both phenotypes are dominant over the phenotypes of nonabscission and absence of peak of ethylene. On a population of 111 RILs derived from the Védrantais × PI 161375 cross, the observed distribution of the fruit abscission phenotype was consistent with a 3:1 distribution (Table

161375 line. B, Comparison of gene expression in harvested PI 161375 fruit (45 DAP) treated with air or 2,000 $\mu\text{L L}^{-1}$ of propylene for 1 to 6 d.

Figure 5. Comparison of ethylene-induced triple response between Védrantais (A) and PI 161375 (B) etiolated seedlings. An enlargement of the apical region in ethylene-treated seedlings is shown in the right corners of A and B.



I), which corresponded to the segregation of two independent genes coding for the same function. We propose the names *abscission layer-3* and *abscission layer-4* (*Al-3* and *Al-4*) for these genes.

A logarithm-of-odds (LOD) score mapping method developed to localize *Al-3* and *Al-4* on the melon genome (Périn, 2000) led to the identification of only two genomic regions with an LOD score above the threshold 2.0. *Al-3* and *Al-4* genes were mapped close to the amplified fragment-length polymorphism (AFLP) markers H33/M43_21 (LOD 3.79) on LGVIII and H36/M37_11a (LOD 3.09) on LGIX, respectively (Fig. 6).

Al-3 and *Al-4* Control the Presence of (Climacteric) Autocatalytic Ethylene Production

Sixty-six RILs were chosen at random, and the ethylene production during fruit ripening was measured. Fruit abscission was always found to be associated with the peak of ethylene in fruit (internal ethylene = $31 \pm 20 \mu\text{L L}^{-1}$), whereas the absence of abscission was always correlated with the lack of ethylene burst (mean value = $2 \pm 2 \mu\text{L L}^{-1}$). More-

over, in all nonabscising lines, propylene treatment failed to induce fruit abscission and ethylene production (data not shown).

QTLs Controlling the Level of Climacteric Ethylene Production Are Not Linked to *Al-3* and *Al-4*

We used ethylene measurements observed among 43 climacteric RILs during fruit ripening for quantitative trait analysis of the internal ethylene maximum in fruit. Four QTLs were detected at an LOD score threshold of 2.0 (Table II) and were localized on LGI, LGII, LGIII, and LGXI of the composite map (Fig. 6). Each of them explained the same phenotypic variance explained (PVE) of about $9 \mu\text{L L}^{-1}$ maximum of fruit internal ethylene. PI 161375 alleles *eth1.1* and *eth11.1* increased internal ethylene even if PI 161375 fruits did not produce ethylene during ripening.

The Difference for the Apical Hook Curvature Is under Monogenic Control

We further analyzed the genetic control of the apical hook curvature. The F_1 (Védrantais \times PI 161375)

Table I. Segregation of fruit abscission and hook curvature

Fruit abscission was evaluated only on the RIL Védrantais \times PI 161375 population, whereas hook curvature was evaluated on the RIL and BC1 (Védrantais \times PI 161375) \times PI 161375 populations.

Character	Population	Observed Nos.	Theoretical Segregation	χ^2 Value (Probability)	Gene Symbol
Fruit abscission	RIL	76:35	3:1	2.5 (11.2%)	<i>Al-3</i> , <i>Al-4</i>
Hook curvature	RIL	35:30	1:1	0.38 (53%)	<i>ech</i>
Hook curvature	BC1	102:100	1:1	0.02 (89%)	<i>ech</i>

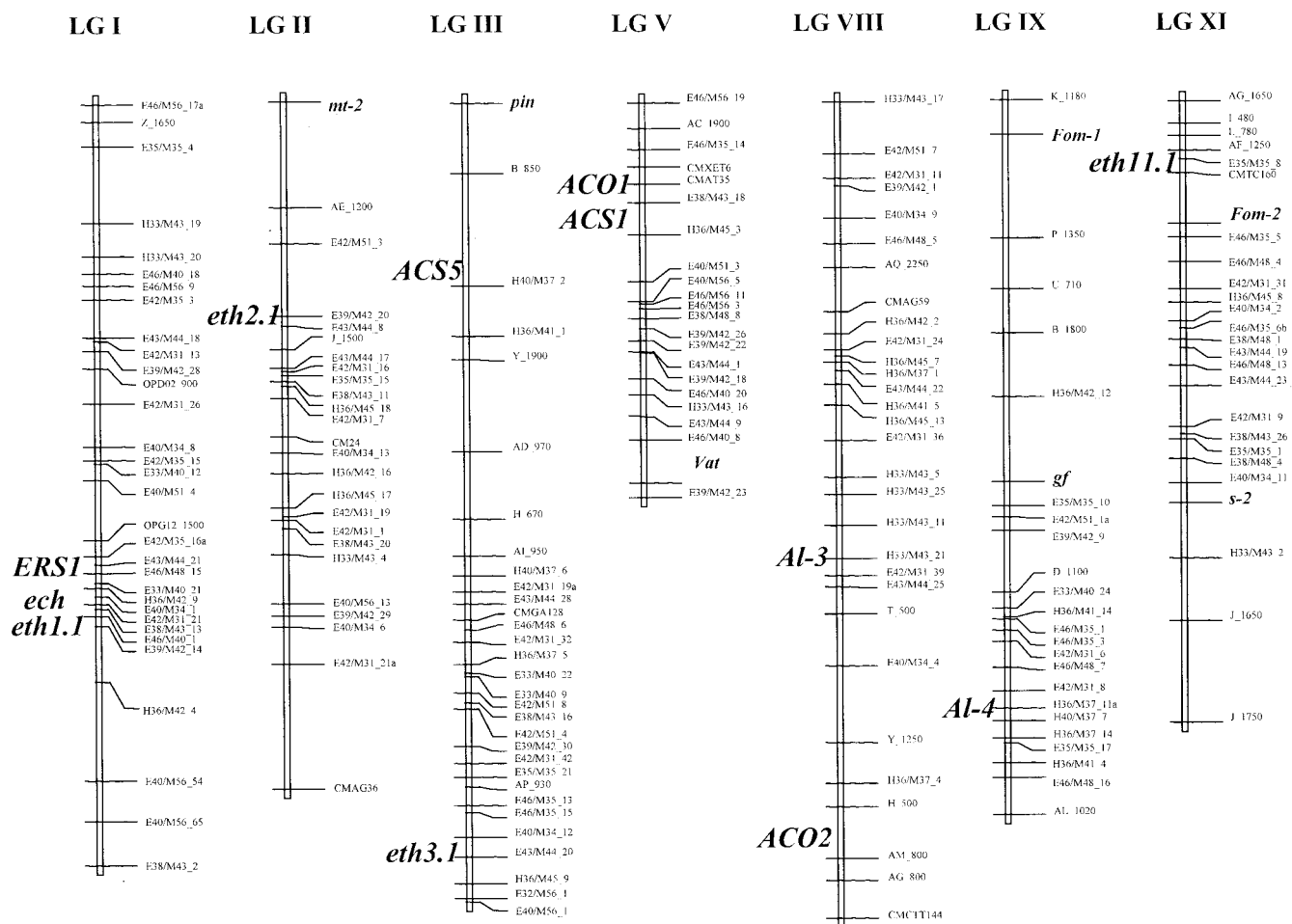


Figure 6. Mapping of the genes *ech*, *Al-3*, and *Al-4*; the QTLs *eth1.1*, *eth2.1*, *eth3.1*, and *eth11.1*; and some candidate genes (in bold on the left side of each LG) on the composite map of melon. All of them were mapped on a population of RILs generated between Védrañtais, a climacteric line, and PI 161375, a non-climacteric line, with the exception of the *ERS1* locus, which was mapped on a RIL population derived between Védrañtais and PI 414723.

seedlings displayed the Védrañtais phenotype, i.e. absence of hook curvature in air-grown seedlings and simple curvature in ethylene-treated seedlings. We examined the distribution of the hook phenotype under ethylene on the RIL and back-cross (BC_1 ; Védrañtais \times PI 161375) \times PI 161375 populations and found that it was fully compatible with a monogenic control (Table I). On the BC_1 population, one-half of

the population tested displayed the exaggerated hook curvature, whereas the other half displayed the phenotype of the Védrañtais parent. We propose the name *exaggerated curvature of the hook* (*ech*) for this locus, which was localized on the LG I on the melon reference map (Périn et al., 2000) close to the E40/M34_1 AFLP marker. The same genomic region comprised the QTL *eth1.1*.

Table II. QTLs detected for fruit ethylene production in the RIL Védrañtais \times PI 161375 population

QTLs were named with trait abbreviations and the linkage group number (LG). The second number was used to distinguish two QTLs detected in the same linkage group. QTL position was given as the most significantly associated marker by QTLcartographer. QTLs were detected by interval mapping (IM) and/or CIM; CIM data (i.e. QTL position, LOD score, and PVE) are indicated.

QTL	LG	IM	CIM	Position	LOD	PVE	Phenotypic Effect
<i>eth1.1</i>	I		x	E39/M42.14	3.1	34.2	-9.8 $\mu\text{L L}^{-1}$
<i>eth2.1</i>	II		x	E39/M42.20	2.7	26.3	8.4
<i>eth3.1</i>	III		x	E43/M44.20	2.9	30.1	8.8
<i>eth11.1</i>	XI	x	x	E35/M35.8	3.0	28.9	-8.6

An Ethylene Receptor Gene *ERS1* Was Tightly Linked to *eth1.1* and *ech*

We tried to map several melon sequences that are homologs to Arabidopsis genes encoding elements of the ethylene signal transduction pathway, such as *ETR1* or *ERS1* (Bleecker et al., 1998; Hua and Meyerowitz, 1998), *CTR1*, related to MAP kinase genes (Kieber et al., 1993), and *EIN3* (Chao et al., 1997). Unfortunately, the available polymorphism in the two populations allowed mapping of only melon *ERS1* to LGI at a single locus near the AFLP marker E42/M35_16a. This region also contained *ech* and *eth1.1*. Among ethylene biosynthetic genes, *ACS5* was mapped to LGIII, *ACO1* and *ACS1* to LGV, and *ACO2* to LGVIII (Fig. 6). None of these genes colocalizes with *Al-3* or *Al-4*.

DISCUSSION

The Non-Climacteric Phenotype of PI 161375 Is Associated with an Alteration of Ethylene Perception, Limited to Fruit Tissue

Non-climacteric fruit are defined by the absence of ethylene burst, which may correspond to the inability to synthesize ethylene autocatalytically either because synthesis, as in anti-sense tomato (Oeller et al., 1991; Picton, 1993) and melon (Ayub et al., 1996) fruit, or perception, as in spontaneous *Nr* tomato mutant (Wilkinson et al., 1995), is blocked. As an alternative, the non-climacteric phenotype may be due to the alteration of early steps of the ripening process as in *rin*, *nor*, and *Cnr* tomato mutants (Giovannoni, 2001).

In melon fruit, ethylene-dependent events during fruit ripening have been defined, in particular using antisense ACC oxidase transgenic lines (Ayub et al., 1996; Guis et al., 1999; Hadfield et al., 2000). Events that are dependent on high levels of ethylene production have been defined, whereas others are either ethylene independent or extremely sensitive to low levels of ethylene (Hadfield et al., 2000). All events of the first category are absent in PI 161375 fruit. The non-climacteric phenotype of PI 161375 was confirmed by treating fruit with exogenous ethylene or propylene; fruit firmness, rind color, ethylene synthesis, ACC content, and expression of known targeted genes were not stimulated. Hence, ethylene-regulated genes (*RM5*, *RM11*, *PG1*, *PG3*, and *ACO1*) were all expressed at low levels or undetected and were never induced by exogenous ethylene/propylene.

This makes PI 161375 different from the non-climacteric Earl's Favourite melon in which ethylene treatment increases *ACO1* gene expression (Shiomi et al., 1999). Both *ACO1* and *ACS1* gene expression were strongly stimulated in all genotypes by wounding (Shiomi et al., 1999; this report). In the case of *ACO1*, promoter analysis led to the identification of two clearly separated cis-regulatory regions, one neces-

sary for wounding response and the other for ethylene response (Bouquin et al., 1997). Thus, wound-induced signaling proceeds independently of ethylene-induced signaling. The fruit-specific inhibition of ethylene perception in PI 161375 is mediated by a different mechanism than that present in *Nr* tomato because the dominant mutation in the *NR* (*ERS1*) ethylene receptor ethylene response blocks ethylene response in all *Nr* tissues (Lanahan et al., 1994; Wilkinson et al., 1995; Yen et al., 1995). Besides the *Nr* tomato mutant already mentioned, other known non-climacteric mutant fruit are different. For instance, the *rin* tomato mutant fruit retains the capacity to display ethylene-stimulated *ACO* gene expression (Knapp et al., 1989; Shiomi et al., 1999) among other responses. In this report, we have shown that in the non-climacteric *chinensis* melon, ethylene perception is highly—if not completely—inhibited.

Al-3 and *Al-4* Proteins Are Most Likely Fruit-Specific Elements Necessary for Climacteric Fruit Phenotype and Complete Ethylene Response

The redundant genes *Al-3* and *Al-4* identified by genetic analysis are controlling ethylene-dependent abscission and ethylene production in fruit. Two unlinked genes named *Al-1* and *Al-2* were reported previously to control fruit abscission in another population of melon (Takada et al., 1975). We have not performed an allelism test, and the map location of *Al-1* and *Al-2* and their involvement in fruit ethylene production and sensitivity remain unknown.

Two other features observed in PI 161375 and the RILs provide additional clues for *Al-3* and *Al-4*. First, the PI 161375 allelic forms are recessive, and one Védraçais allele at any of these loci confers normal ethylene perception. The *Al-3* or *Al-4* loci had no major effect on the level of ethylene production during fruit ripening. The high level of variation found among the set of climacteric RI lines is under a distinct genetic control with four different QTLs modulating fruit ethylene production. Second, both recessive alleles confer ethylene insensitivity in fruit tissues only. A partial ethylene insensitivity in PI 161375 roots was observed in abscising (one Védraçais allele at any locus) and nonabscising (*al-3/al-3* and *al-4/al-4*) RILs (data not shown).

These results are in favor of the hypothesis that ethylene perception is faulty in PI 161375 and in non-climacteric RIL fruits but could be also attributable to the absence of some ethylene-independent developmental regulation in PI 161375 fruit (McCourt, 1999). Thus, distinction between climacteric/non-climacteric and ripening/non-ripening fruit is not significant to characterize a ripening mutant in melon as for other climacteric species. For instance, the tomato ripening mutants *rin* and *nor* are non-climacteric but are part of a developmental control upstream ethylene signaling (Giovannoni, 2001).

Cosegregation of QTL *eth1.1* with *ech* Raises the Possibility That Regulatory Networks for Ethylene Biosynthesis or Response Are Operating in Multiple Tissues

The presence of the ethylene burst in melon fruit is controlled by two independent loci. However, at least four modifier loci (QTL) localized on other genomic regions are controlling its maximum intensity. None of the ethylene biosynthetic melon genes studied (*ACO1*, *ACO2*, *ACS1*, and *ACS5*) were linked to any QTL. On the other hand, the locus controlling the curvature of the hook was localized close to the QTL *eth1.1* and to the gene encoding a member of the ethylene receptor family *ERS1*. This colocalization is still approximate and does not exclude the examination of other candidate genes for *eth1.1* or *ech* (Raz and Ecker, 1999; Bleecker and Kende, 2000). Nevertheless, the hypothesis that *ERS1*, *eth1.1*, and/or *ech* are the same gene fits well with what we know of ethylene receptors if one assumes that PI 161375 *ERS1* is a loss-of-function allele. One way to test the role of *ERS1* in the melon hook formation and fruit ethylene production could be to compare the effect of the dominant allele (Védrantais in the hook, PI 161375 in fruit) in transgenic plants.

MATERIALS AND METHODS

Plant Material

All physiological studies were performed on 144 RILs resulting from the cross between melon (*Cucumis melo*) types Védrantais and PI 161375 (Périn, 2000). A second population of 64 RILs issued from the cross between Védrantais and PI 414723 was used to map some candidate genes. The three parents belong to very different subspecies and cultigroup (Pitrat et al., 2000): Védrantais (released by Vilmorin S.A.) is a commercial variety of the Charentais type (*C. melo* subsp. *melo*, *cantalupensis* group); PI 161375 (*C. melo* subsp. *agrestis*, *chinensis* group) and PI 414723 (*C. melo* subsp. *agrestis*, *momordica* group) corresponded originally to two cultivars collected in Korea and India, respectively, and that have been maintained by self-pollination. To check the monogenic inheritance for hook phenotype in etiolated seedlings, we also used 200 individuals of the BC₁ population (Védrantais × PI 161375) × PI 161375.

Genetic Mapping for Monogenic Traits and Candidate Genes

A saturated map with molecular markers was developed by merging the two maps obtained on the two RIL populations (Védrantais × PI 161375) and (Védrantais × PI 414723; Périn, 2000).

Genetic linkage analysis was performed using Mapmaker software 3.0 (Lander et al., 1987). Monogenic traits were mapped through the Mapmaker commands "build" and "try" using the framework map. Distances (in centimorgans) were calculated with the Kosambi (1944) func-

tion. Candidate genes were mapped by RFLP or PCR on the two populations used for composite map construction.

Mapping of *Al-3* and *Al-4*

We derived a maximum likelihood equation for linkage between duplicated genes and molecular markers on a RIL population according to Fisher procedure. A maximum likelihood ratio was evaluated for each marker of the framework map published under the hypothesis of linkage versus independence (Périn, 2000). A threshold value of 2.0 was chosen to declare linkage of an *abscission layer* (*Al-3* or *Al-4*) gene and a molecular marker. The highest values were used to localize *Al-3* and *Al-4* on the composite map.

QTL Detection

QTL search for ethylene production by the fruits was performed using three methods: single-factor analysis of variance, simple interval mapping (Lander et al., 1987) and composite interval mapping (CIM) with QTL Cartographer software (Zeng, 1994). A threshold value of 2.0 was used for QTL detection. Only CIM data were used to estimate the genetic effect of the QTLs detected and to localize them on the composite map.

Ethylene and Propylene Treatments

Seeds were surface-sterilized in 30% (v/v) ethanol for 2 min then in 5% (w/v) hypochlorite solution for 5 to 8 min, rinsed abundantly in sterile water, and placed on half-concentrated Murashige and Skoog macronutrients medium supplemented with 0.8% (w/v) agar in boxes (Magenta, Chicago). Growth was performed in small compartments placed in darkness with an air flux containing 10 $\mu\text{L L}^{-1}$ ethylene or not. After 5 to 10 d at 28°C, seedlings were observed. Fruits were treated at room temperature under a similar gas flow, except that ethylene was eventually replaced by 1,000 $\mu\text{L L}^{-1}$ propylene. After harvest at 40, 50, and 60 DAP, fruits were placed in jars with air or air plus ethylene (propylene) flow. Firmness, ethylene production, and flesh and peel color were measured as previously described (Guis et al., 1997).

Molecular Techniques

DNA (10 μg) was digested with *EcoRI*, *EcoRV*, and *HindIII* enzymes and blotted. Southern and northern hybridization procedures were performed using standard protocols (Sambrook et al., 1989) and ³²P-labeled probes. Total RNA was extracted from fruit essentially as described by Ayub et al. (1996) and separated by electrophoresis on formaldehyde agarose gels (Sambrook et al., 1989). RNA blots were probed with either the entire sequence of melon *ACO1* (Lasserre et al., 1996), *ERS1* (Sato-Nara et al., 1999), *PG1*, *PG2*, *PG3* (Hadfield et al., 1998), *RM2*, *RM4*, *RM5*, *RM7*, *RM8*, *RM11*, *RM16* (Hadfield et al., 2000), *ACS5* (J.-M. Lelièvre, unpublished data), or partial sequence for *ACS1* (Yamamoto et al., 1995). Total fruit cDNA was used for a

PCR-based amplification of partial sequences of *CTR1*-like and *EIN3*-like cDNAs using degenerate primers coding for conserved regions of the proteins. The *CTR1*-like and the *EIN3*-like sequences have been deposited under the GenBank code numbers AF387794 and AF387795, respectively. This *EIN3*-like sequence detected three transcripts in fruits of both genotypes (data not shown) with two expression patterns that were named *EIL1* and *EIL2*.

Quantitative dosage of radioactive signals hybridizing with RNA was achieved with an Ambis 100. Signals were normalized according to the signal obtained with the DNA probe from the 18S rRNA gene of squash (*Cucurbita pepo*; Torres-Ruiz and Hemleben, 1994).

ACKNOWLEDGMENTS

We are grateful to Dr. Zheng-guo Li (Toulouse, France) for providing us the melon *EIN3* sequence and to Pr. Alan B. Bennett (Davis, CA) for the generous gift of the genes for polygalacturonases and of the RM series. The technical assistance of Didier Besombes and Nathalie Giovinazzo in growing and breeding plants is acknowledged.

Received July 10, 2001; returned for revision November 1, 2001; accepted February 4, 2002.

LITERATURE CITED

- Abeles FB, Morgan PW, Saltveit MEJ** (1992) Ethylene in Plant Biology. Academic Press, San Diego
- Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latché A, Bouzayen M, Pech JC** (1996) Expression of an antisense ACC oxidase gene inhibits ripening in cantaloupe melons fruits. *Nat Biotechnol* **14**: 862–866
- Ben Amor M, Flores B, Latché A, Bouzayen M, Pech JC, Romojaró F** (1999) Inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevents chilling injury in Charentais cantaloupe melons. *Plant Cell Environ* **22**: 1579–1586
- Bleecker AB, Estelle MA, Somerville C, Kende H** (1998) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* **241**: 1086–1089
- Bleecker AB, Kende H** (2000) Ethylene: a gaseous signal molecule in plants. *Annu Rev Cell Dev Biol* **16**: 1–18
- Bouquin T, Lasserre E, Pradier J, Pech JC, Balagué C** (1997) Wound and ethylene induction of the ACC oxidase melon gene CM AC01 occurs via two direct and independent transduction pathways. *Plant Mol Biol* **35**: 1029–1035
- Burr B, Burr FA** (1991) Recombinants inbreds for molecular mapping in maize: theoretical and practical considerations. *Trends Genet* **7**: 55–60
- Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR** (1997) Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* **89**: 1133–1144
- Giovinazzo J** (2001) Molecular biology of fruit maturation and ripening. *Annu Rev Plant Physiol Plant Mol Biol* **52**: 725–749
- Guis M, Botondi R, Ben Amor M, Ayub R, Bouzayen M, Pech JC, Latché A** (1997) Ripening-associated biochemical traits of cantaloupe Charentais melons expressing an antisense ACC oxidase transgene. *J Am Soc Hortic Sci* **122**: 748–751
- Guis M, Latché A, Bouzayen M, Pech JC** (1999) Understanding the role of ethylene in fruit softening using antisense ACC oxidase melons. In AK Kanellis, C Chang, H Klee, AB Bleecker, JC Pech, D Grierson, eds, *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 395–396
- Hadfield KA, Dang T, Guis M, Pech JC, Bouzayen M, Bennett AB** (2000) Characterization of ripening-regulated cDNAs and their expression in ethylene-suppressed Charentais melon fruit. *Plant Physiol* **122**: 977–983
- Hadfield KA, Rose JKC, Yaver DS, Berka RM, Bennett AB** (1998) Polygalacturonase gene expression in ripe melon fruit supports a role for polygalacturonase in ripening-associated pectin disassembly. *Plant Physiol* **117**: 363–373
- Hua J, Meyerowitz EM** (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**: 261–271
- Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR** (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
- Knapp J, Moueau P, Schuch W, Grierson D** (1989) Organization and expression of polygalacturonase and other ripening related genes in Ailsa Craig “never ripe” and “ripening inhibitor” tomato mutants. *Plant Mol Biol* **12**: 105–116
- Kosambi DD** (1944) The estimation of map distance from recombination values. *Ann Eugenism* **12**: 172–175
- Lanahan MB, Yen HC, Giovannoni J, Klee HJ** (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 CC, Tieman DM, Klee HJ** (1998) Differential regulation of the tomato ETR gene family throughout plant development. *Plant J* **15**: 243–252
- Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balagué C** (1996) Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Mol Gen Genet* **251**: 81–90
- Lelièvre JM, Latché A, Jones B, Bouzayen M, Pech JC** (1997) Ethylene and fruit ripening. *Physiol Plant* **101**: 727–739
- McCourt P** (1999) Genetic analysis of hormone signaling. *Annu Rev Plant Physiol Plant Mol Biol* **50**: 219–243
- McMurchie EJ, McGlasson WB, Eaks IL** (1972) Treatment of fruit with propylene gives information about the biogenesis of ethylene. *Nature* **237**: 235–236

- Naudin C** (1859) Essais d'une monographie des espèces et des variétés du genre *Cucumis*. Annales des Sciences Natureles **11**: 5–87
- Oeller PW, Lu MW, Taylor LP, Pike DA, Theologis A** (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254**: 437–439
- Périn C** (2000) Construction d'une carte génétique de référence du melon (*Cucumis melo* L.) et étude du contrôle génétique du développement et de la maturation du fruit. PhD thesis. Université de la Méditerranée, Marseille, France
- Périn C, Hagen L, Dogimont C, de Conto V, Lecomte L, Pitrat M** (2000) Construction of a reference genetic map of melon. In N Katzir, H Paris, eds, Cucurbitaceae 2000, Seventh EUCARPIA Meeting on Cucurbit Genetics and Breeding. Ma'ale Hahamisha, Israel. Acta Hort **510**: 367–374
- Picton SB** (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J **3**: 469–481
- Pitrat M, Hanelt P, Hammer K** (2000) Some comments on infraspecific classification of cultivars of melon. In N Katzir, H Paris, eds, Cucurbitaceae 2000, Seventh EUCARPIA Meeting on Cucurbit Genetics and Breeding. Ma'ale Hahamisha, Israel. Acta Hort **510**: 29–36
- Raz V, Ecker JR** (1999) Regulation of differential growth in the apical hook of *Arabidopsis*. Development **126**: 3661–3668
- Sambrook J, Fritsch EF, Maniatis T** (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Sato-Nara K, Yuhashi KI, Higashi K, Hosoya K, Kubota M, Ezura H** (1999) Stage- and tissue-specific expression of ethylene receptor homolog genes during fruit development in muskmelon. Plant Physiol **119**: 321–329
- Shiomi S, Yamamoto M, Nakamura R, Inaba A** (1999) Expression of ACC synthase and ACC oxidase genes in melons harvested at different stage of maturity. J Jpn Soc Hortic Sci **68**: 10–17
- Solano R, Stepanova A, Chao Q, Ecker JR** (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12**: 3703–3714
- Stepanova A, Ecker JR** (2000) Ethylene signaling: from mutants to molecules. Curr Opin Plant Biol **3**: 353–360
- Stepansky A, Kovalski I, Perl-Treves R** (1999) Intraspecific classification of melons (*Cucumis melo* L.) in view of their phenotypic and molecular variation. Plant Syst Evol **217**: 313–332
- Takada K, Kanazawa K, Takatuka K** (1975) Studies on the breeding of melon for resistance to powdery mildew. II. Inheritance of resistance to powdery mildew and correlation of resistance to other characters. Bull Veg Ornamental Crops Res Stn **A2**: 11–31
- Tanksley S, McCouch SR** (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. Science **277**: 1063–1066
- Thompson AJ, Tor M, Barry CS, Vrebalov J, Orfila C, Jarvis MC, Giovannoni JJ, Grierson D, Seymour GB** (1999) Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. Plant Physiol **120**: 383–390
- Tieman DM, Klee HJ** (1999) Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol **120**: 165–172
- Tieman DM, Taylor LP, Ciardi JA, Klee HJ** (2000) The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc Natl Acad Sci USA **97**: 5663–5668
- Torres-Ruiz RA, Hemleben V** (1994) Pattern and degree of methylation in ribosomal RNA genes of *Cucurbita pepo* L. Plant Mol Biol **26**: 1167–1179
- Wang YH, Thomas CE, Dean RA** (1997) A genetic map of melon (*Cucumis melo* L.) based on amplified fragment length polymorphism (AFLP) markers. Theor Appl Genet **95**: 791–797
- Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni J, Klee HJ** (1995) An ethylene-inducible component of signal transduction encoded by Never-ripe. Science **270**: 1807–1809
- Yamamoto M, Miki T, Ishiki Y, Fujinami K, Yanagisawa Y, Nakagawa H, Ogura N, Hirabayashi T, Sato T** (1995) The synthesis of ethylene in melon fruit during the early stage of ripening. Plant Cell Physiol **36**: 591–596
- Yang SF, Hoffman NE** (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol **35**: 155–189
- Yen HC, Lee S, Tanksley S, Lanahan MB, Klee HJ, Giovannoni J** (1995) The tomato Never ripe locus regulates ethylene inducible gene expression and is linked to a homolog of the Arabidopsis ETR1 gene. Plant Physiol **107**: 1343–1353
- Zarembinski TI, Theologis A** (1994) Ethylene biosynthesis and action: a case of conservation. Plant Mol Biol **26**: 1579–1597
- Zeng ZB** (1994) Precision mapping of quantitative trait loci. Genetics **136**: 1457–1468