Delayed Ripening Tomato Plants Expressing the Enzyme 1-Aminocyclopropane-1-carboxylic Acid Deaminase. 1. Molecular Characterization, Enzyme Expression, and Fruit Ripening Traits

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Tomato plants that are delayed in fruit ripening have been developed by *Agrobacterium tumefaciens*-mediated transfer of a gene encoding 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) into the tomato genome. Two delayed ripening (DR) tomato lines are characterized in this paper. Line 8338, transformed by a double border plasmid vector, contains a single copy of the *accd* gene, and DNA outside the plasmid border sequences was not transferred to the plant genome. Line 5673, transformed by a single border plasmid vector, contains a single, complete copy of the *accd* gene plus tandem, incomplete copies of the gene. The mean expression levels of ACCd in fruit collected from four field trials were 39.4 and 20.6 μg/g of fresh weight for lines 8338 and 5673, respectively. Fruit ethylene synthesis was significantly reduced, and time for fruit to ripen was extended for both DR tomato lines relative to the parental control line. Introduction of the *accd* gene and the delayed ripening trait into appropriate tomato varieties will potentially allow production of tomato fruit with superior taste quality.

Keywords: *Delayed ripening; 1-aminocyclopropane-1-carboxylic acid deaminase; transgenic tomatoes; ethylene synthesis; ELISA; Southern blot*

INTRODUCTION

Consumption of fresh tomatoes in the United States is relatively high among vegetable crops (U.S. Department of Agriculture, 1992). However, commercial tomatoes are generally considered by the consumer as having poor taste quality (Stevens, 1986). The poor taste quality of fresh tomatoes can be attributed to a production system that is based on fruit harvest at the mature green stage of development. Mature green fruit have the firmness and market life attributes necessary for a national and international distribution system. However, fruit harvest typically consists of both mature green and immature green fruit (the two green fruit types are externally indistinguishable), and immature green fruit do not develop full flavor qualities when ripened to red ripe (Grierson and Kader, 1986). To avoid contamination with inferior immature green fruit, many growers harvest fruit that have developed some red color. Although these "vine-ripened" fruit develop good flavor quality, the fruit typically have a relatively short market life and do not maintain the physical characteristics necessary for national distribution. Tomato plants with delayed fruit ripening traits have been previously described (Tigchelaar et al., 1978; Hamilton et al., 1990; Oeller et al., 1991; Klee et al., 1991). Introduction of a delayed ripening trait into fresh market tomatoes will allow harvest of vine-ripened tomatoes with extended market life and supply of good flavor quality fruit to the consumer nationwide.

The biosynthetic pathway of the phytohormone ethylene, and the stimulatory effect of ethylene on tomato fruit ripening have been well characterized (Taiz and Zeiger, 1991; Grierson and Kader, 1986). The amino acid methionine is converted to ethylene in a series of reactions involving *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC). Tomato lines that are delayed in fruit ripening have been developed by stable insertion of a gene encoding enzyme ACC deaminase (ACCd) into the tomato chromosome (Klee et al., 1991). The enzyme catalyzes metabolism of ACC to ammonia and α-ketobutyrate (Honma and Shimomura, 1978). Removal of ACC in the transformed plants reduces ethylene synthesis and delays fruit ripening (Klee et al., 1991; Klee, 1993). Two delayed ripening (DR) tomato lines, designated 8338 and 5673, have been characterized in detail. Both lines contain an *accd* gene isolated from the soil bacterium *Pseudomonas chlororaphis*, strain 6G5, as described by Klee et al. (1991), and the neomycin phosphotransferase (*nptII*) selectable marker gene (Fraley et al., 1983). The delayed ripening and marker genes were introduced into tomato variety UC82B using an *Agrobacterium tumefaciens* transformation system (Klee and Rogers, 1989). Tomato lines 8338 and 5673 were produced with different transformation vectors. The vectors contained the same *accd* and *nptII* genes but differed with respect to the number of T-DNA borders (nucleotide sequences of the T-DNA necessary for the *Agrobacterium* Ti plasmid transformation system) and promoters for the *accd* gene.

In this paper, both DR tomato lines 8338 and 5673 are characterized with respect to (a) the inserted DNA present in the genome of each tomato line, (b) expression levels ACCd protein in tomato fruit, and (c) ethylene synthesis and the resulting delayed fruit ripening trait. An ELISA was developed and validated for quantitation of ACCd protein in tomato fruit. Protein expression, ethylene synthesis, and fruit ripening traits were measured in fruit of DR lines and parental control line,

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UC82B, harvested from field-grown plants at four locations in Florida. Results of this study were used to obtain the safety and regulatory approval of genetically modified tomato plants that express the ACCd protein.

MATERIALS AND METHODS

Production of Delayed Ripening Tomato Lines. Delayed ripening tomato lines 8338 and 5673 were produced by *A. tumefaciens*-mediated transformation of the commercial tomato variety UC82B. Lines 8338 and 5673 were produced with binary plasmid vectors pMON10117 and pMON10028, respectively (Figure 1). Both vectors contain two genes that are expressed in plants, the *accd* gene (isolated from *P. chloroaphis*, strain 6G5; Klee et al., 1991) and the selectable marker gene *nptII* (Fraley et al., 1983), which confers resistance to the aminoglycoside antibiotic kanamycin (KAN). The *accd* gene in vector pMON10117 is driven by a caulimovirus 35S promoter isolated from a modified figwort mosaic virus (Shepherd et al., 1987; Richins et al., 1987), with a 5′ nontranslated leader from a *Petunia hybrida* HSP70 gene (Winter et al., 1988). The *accd* gene in vector pMON10028 is driven by a 0.6 kb 35S cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985) with the duplicated enhancer region (Kay et al., 1987). In both vectors the *accd* gene is followed by a nontranslated region of the pea rbc-E9 gene (Coruzzi et al., 1984; Morelli et al., 1985), which directs polyadenylation of the mRNA. Both vectors contain the same constructs for the *nptII* gene. The CaMV 35S promoter region (Sanders et al., 1987; Gardner et al., 1981) drives expression of the *nptII* gene, which is followed by the nopaline synthase (NOS) 3′ region that directs polyadenylation of the mRNA (Fraley et al., 1983; Depicker et al., 1982). Both vectors contain origins of replication required for plasmid replication in *Agrobacterium* and *Escherichia coli* hosts and a bacterial selectable marker gene, aminoglycoside adenylyltransferase (*aad*), which confers spectinomycin/streptomycin (Spc/Str) resistance.

The plasmid vectors pMON10117 and pMON10028 were assembled in *E. coli* K-12 cells, mated into a disarmed ABI *Agrobacterium* strain, and used to transform tomato line UC82B, as described (Klee et al., 1991). During the transformation process, part of the vector DNA (referred to as T-DNA) is stably transferred to the tomato genome. The T-DNA is delineated on the plasmid vector by border sequences (DNA sequences necessary for the *Agrobacterium* Ti plasmid transformation system). For pMON10117, a double border plasmid, the *accd* and *nptII* genes within the T-DNA delimited by the two borders are expected to transfer to the plant genome, whereas the origin of replication and *spc/str* gene that lie outside the border sequences are not expected to transfer. For pMON10028, a single border plasmid vector, the *accd, nptII,* and *spc/str* genes and the origin of replication are expected to transfer to the tomato genome, since T-DNA transfer typically initiates at the right border and continues to include the whole plasmid for this type of vector (Klee and Rogers, 1989).

One of the transformants obtained with vector pMON10117, homozygous line 8338, was crossed to commercial tomato line Florida-1-B for determination of DNA insert stability in the tomato genome. The F_1 progeny were subsequently backcrossed to three different lines, designated BHN-B, BHN-H, and BHN-N, through three generations to BC_3F_1 material.

Control Tomato Lines. The control line, UC82B, is the parental tomato variety from which transformed DR lines 8338 and 5673 were developed. Tomato lines BHN-B, BHN-H, and BHN-N are the parental controls for backcrosses with line 8338 (BC_3F_1 material). The control lines do not contain the genes encoding the ACCd and NPTII proteins.

Plant Seed. Plants of DR tomato lines 8338 and 5673 were grown from homozygous R_2 seed, selected from original R_0 transformants of each line. Seeds of parental control line UC82B were obtained from Ferry Morse Seed Co., Modesto, CA. Plants of backcross lines (8338 with BHN-B, BHN-H, and BHN-N) were grown from BC_3F_1 seed. Seeds of BHN-B, BHN-H, and BHN-N parental control lines were obtained from the BHN-Joint Venture commercial breeding program.

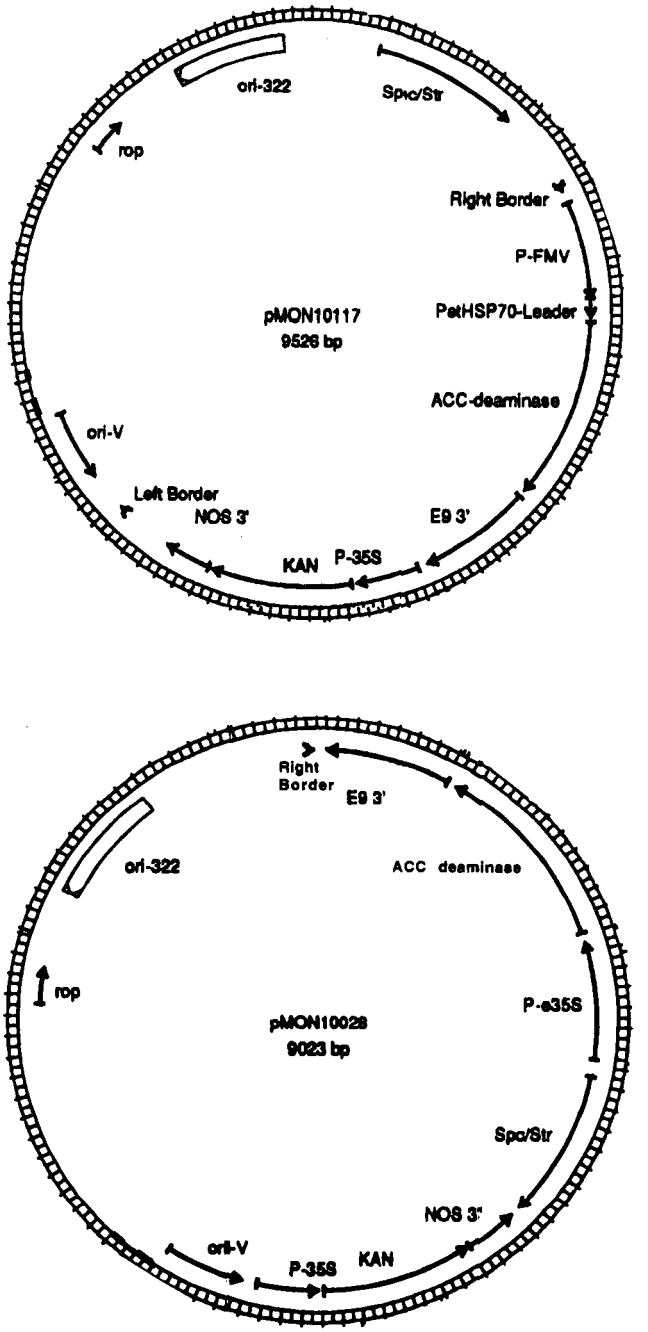

Figure 1. Map of plasmid vectors pMON10117 and pMON10028 used to produce DR tomato lines 8338 and 5673, respectively. Both vectors contain the *accd* gene, the *nptII* gene (the kanamycin resistance marker gene, KAN), and the *aad* gene that confers spectinomycin/streptomycin (Spc/Str) resistance. Promoters for the *accd* and *nptII* genes and DNA sequences following each of these genes are defined under Materials and Methods. The vectors also contain genetic elements that comprise the bacterial origin of replication. These elements include the vegetative origin of replication (ori-V), replication of the primer region (rop), and plasmid replication origin (ori-322).

Molecular Analyses. *(a) Plant Material.* Plants of each tomato line were grown in 3 in. pots in Metro-Mix 350 growth medium (Grace Sierra Horticultural Products Co., Milpitas, CA) under greenhouse conditions. At 19 days after planting, all leaves were excised from plants, pooled by line, and immediately frozen on dry ice. Samples were stored at −80 °C prior to analysis.

(b) DNA Isolation and Restriction Enzyme Digestion. Isolation of DNA from leaf tissue and digestion of genomic DNA

by restriction enzymes were by standard laboratory methods, as described by Sambrook et al. (1989). Restriction enzymes used for characterization of the lines included *Spe*I, *Eco*NI, *Eco*RV, *Bam*HI, *Eco*RI, *Pst*I, *Bgl*II, and *Ssp*I. Enzymes *Spe*I, *Eco*NI, and *Eco*RV do not cleave within the transformation plasmids and were used to determine the number of T-DNA insert loci in the tomato genome. Other restriction enzymes listed above cleave within the plasmids (sites shown in Figures 3 and 4) and were used to characterize the T-DNA integrity and content.

(c) Hybridization Probes. Four hybridization DNA probes were derived by polymerase chain reaction (PCR) using plasmid pMON10117 as the template. The PCR probes were purified by agarose gel electrophoresis/DEAE membrane transfer and subsequently radiolabeled with ^{32}P by the random priming method, essentially as described by Sambrook et al. (1989). Specific probes were synthesized for the *accd, nptII,* and *spc/str* genes and for the bacterial plasmid origin of replication.

(d) Southern Blot Hybridization. Southern blot analyses of tomato DNA were essentially as described by Southern (1975) and Sambrook et al. (1989). Restricted DNA samples were resolved using 1% agarose gel electrophoresis. DNA fragments were transferred to a Zeta-probe nylon membrane, hybridized to ^{32}P labeled probes, and exposed to film to identify the hybridizing fragments. Molecular weight standards were included on each gel to estimate the approximate size of the hybridizing fragments.

Extraction of ACCd from Red Ripe Tomato Fruit. Red-ripe frozen fruit samples were homogenized in dry ice to coarse frozen tissue powders using a Robot-Coupe commercial food processor (Ridgeland, MS). The samples were further homogenized in liquid nitrogen to fine frozen tissue powders using a steel Waring blender. The frozen fruit powders were extracted in phosphate buffered saline plus Tween-20 (PBST; 0.14 M sodium chloride, 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride, and 0.05% Tween-20) at a tissue-to-buffer ratio of 1:40, using a Brinkman overhead Polytron (Kinematica AG, Switzerland). The extraction method was optimized for maximal extraction of ACCd from tomato fruit tissue. Extracts were centrifuged, and the supernatant assayed for ACCd concentration by ACCd ELISA.

ACCd ELISA. *(a) ACCd Protein Standard.* ACCd protein was purified in gram quantities from recombinant *E. coli* containing the *accd* gene, as described (Finn et al., 1995). The ACCd protein was more than 90% pure and was used for immunizations to generate anti-ACCd antibody and for standards in the ACCd ELISA.

(b) Antisera Production and Conjugation. Antibodies specific to ACCd were produced in two goats using an intramuscular immunization procedure. The animals were immunized at 3 week intervals with approximately 500 μg of purified ACCd per goat in Freund's complete adjuvant (two immunizations). Subsequent immunizations used approximately 250 μg of antigen per goat in Freund's incomplete adjuvant. Blood was drawn from the jugular vein 7–14 days after each immunization. Antibody titer was determined on the basis of ACCd-specific antibody binding to ACCd protein adsorbed to the wells of microtiter dishes. The IgG fraction of goat serum from the third bleed was affinity purified on protein G Sepharose (Pharmacia LKB, Uppsala, Sweden). Affinity-purified polyclonal goat anti-ACCd was conjugated to alkaline phosphatase using the method described by Engvall and Perlmann (1971).

(c) ELISA Procedure. The ACCd ELISA is a direct double-antibody sandwich assay specific for the ACCd protein. The ELISA procedure consisted of three general steps.

Step 1. Affinity-purified antibody was diluted in plate coating buffer (1.5 mM sodium carbonate, 3.5 mM sodium bicarbonate, and 15 mM sodium chloride) and 0.5 μg of antibody in 100 μL of buffer adsorbed to the wells of microtiter plates overnight at 4 °C.

Step 2. Standard ACCd and samples were appropriately diluted in PBST. Extracts of control line UC82B red ripe tomato fruit were added to all ACCd standards to correct for tissue matrix effects in the assay. Diluted standards and samples and alkaline phosphatase-conjugated antibody (0.5 μg) were added to the plates in a total volume of 100 μL and incubated overnight at 4 °C in a humid box.

Step 3. *p*-Nitrophenyl phosphate (pNPP) substrate (Kirkegaard and Perry Laboratories, Inc., MD) was added to the plates (200 μL per well) and allowed to react for approximately 1 h at room temperature. Absorbance was read at 405 nm (reference wavelength 655 nm) using a Bio-Rad (Richmond, CA) Model 3550 microplate processor.

Wells were washed three times between each of the above steps with buffer containing 0.1 M sodium chloride, 44 mM Tris, 10 mM sodium azide, and 0.05% Tween 20. Quantitation of ACCd concentration in samples was accomplished by extrapolation from a logistic curve fit of the ACCd standard curve.

ELISA Validation: Precision, Accuracy, and Sensitivity. Assay validation parameters were evaluated for measurement of ACCd expression levels in red ripe tomato fruit. Fruit of DR line 5673 and control line UC82B were collected from field sites described below (ACCd expression) for assay validation studies.

Precision. Interassay precision was estimated from the analysis of aliquots of red ripe fruit extracts prepared from a pool of delayed ripening tomato tissue. These data were obtained from a minimum of 20 separate assays conducted over an 8 month period.

Accuracy. (i) Spike and Recovery. Red ripe tomato tissue of control line UC82B was extracted in buffer spiked with known amounts of ACCd protein, and recovery of ACCd was estimated by ELISA. The ACCd protein was spiked at two different concentrations, which approximated to high and low values commonly observed in delayed ripening fruit tissue. The percent recovery of ACCd was estimated as the average for the two spike concentrations.

(ii) Extraction Efficiency. The efficiency of ACCd extraction from red ripe tomato fruit tissue was estimated by the sequential extraction method described by Rogan et al. (1992). Extraction efficiency was calculated as the percent of ACCd in the first extraction compared to the total amount of ACCd extracted from the tissue in sequential extractions.

Sensitivity. The sensitivity (lowest detectable dose) of the ELISA was defined as the amount of ACCd that could be measured by an absorbance reading of 3 standard deviations above the background absorbance. The background absorbance and associated standard deviation were estimated from the ELISA absorbance values for more than 20 independent analyses of control samples.

ACCd Expression. *(a) Plant Material.* Seeds of each tomato line were germinated in minicell trays, transplants grown under greenhouse conditions at BHN-Joint Venture, Bonita Springs, FL, for approximately 6–7 weeks, and tomato seedlings transplanted to four separate field sites in Florida. Field site locations were as follows: field site 1, Gulfcoast farm 2, Greenway Rd N., Collier County, FL; field site 2, Gulfcoast farm 7, 15000 E. Tamiami Trail, Naples, FL; field site 3, BHN-Joint Venture, 16750 Bonita Beach Rd., Bonita Springs, FL; and field site 4, Gulfcoast farm 11, Immokalee Road N., Collier County, FL. Plant beds at all field sites were spaced as 6 ft center-to-center rows. Fertilizer was applied in the beds according to soil test recommendations, and beds were fumigated (67% methyl bromide, 33% chloropicrin) at 2.5 lb/100 ft linear row. Seven or more days after fumigant application, plants were transplanted into the beds through the polyethylene mulch and were spaced approximately 20 in. apart.

The plot design was completely random at field sites 1 and 2 and was a randomized complete block at field sites 3 and 4. There were four replicates of each tomato line at each of the field sites. Normal Florida fresh market tomato production practices were used for plant culture at each field test site. These practices included resetting weak transplants, plant staking, tying, seep irrigation, and use of registered pesticides within the labeled application rates for control of weeds, insects, and diseases.

Approximately 30 fruit at the orange stage of ripening (30–60% orange or red color) were harvested from each plot. The

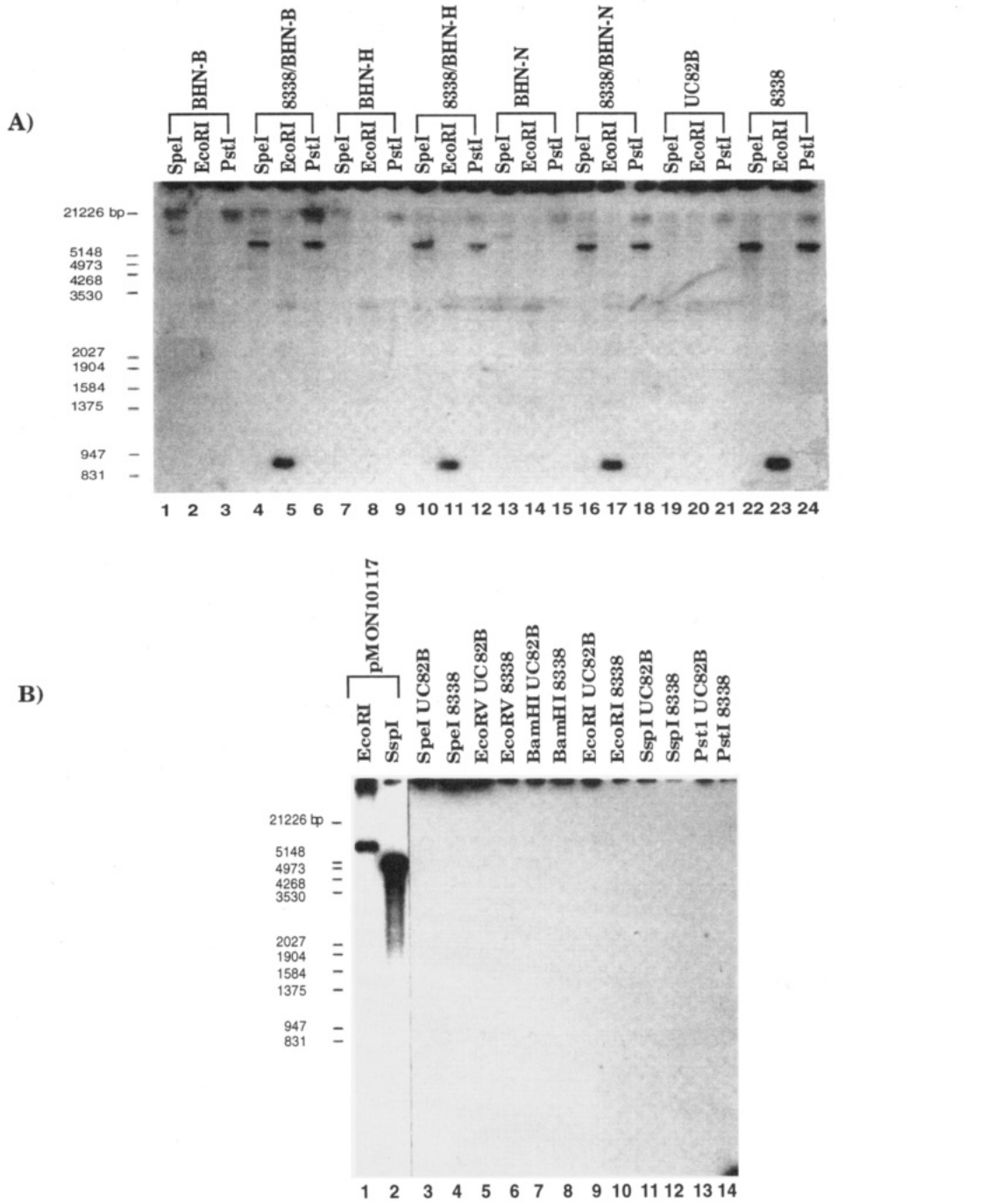

Figure 2. Southern blot analysis of genomic DNA of DR tomato line 8338. (A) Blot of lines 8338, parental control UC82B, backcross lines (8338 with BHN-B, BHN-H, and BHN-N), and corresponding nontransgenic controls. DNA was digested with *Spe*I, *Eco*RI, and *Pst*I and probed for *accd*. (B) Blot of lines 8338 and UC82B and plasmid vector pMON10117. DNA was digested with enzymes that included *Spe*I, *Eco*RV, *Bam*HI, *Eco*RI, *Ssp*I, and *Pst*I and probed for *spc/str*. The blot is a composite of two exposures. Lanes 1 and 2 were exposed for 30 min,; lanes 3–14 were exposed for 20 h. DNA standards are shown to the left of each blot.

fruit were washed in a 100 ppm chlorine solution, rinsed thoroughly in water, and dried at ambient temperature. The fruit were packaged separately by plot and shipped by overnight carrier to Monsanto Co., St. Louis, MO. The fruit were ripened in a controlled environment chamber (relative humidity 80%, no exogenous ethylene applied). When most fruit reached the red ripe stage of ripening (greater than 90% red color), fruit that showed signs of disease or that were at earlier/later stages of ripening were discarded, and red ripe fruit were frozen in dry ice and stored at −80 °C prior to analysis.

(b) ACCd Expression. The fruit were processed to frozen tissue powders and ACCd extracted as described. The amount of ACCd protein in extracts was determined by the validated ACCd ELISA.

Fruit Ripening Traits. Fruit ripening traits (ethylene synthesis and time-to-red) were measured on fruit harvested from plants grown at field site 3, as described above (ACCd Expression). All fruit were washed in 100 ppm chlorine, rinsed in water, and dried prior to measurement of ripening traits.

(a) Ethylene Synthesis. Fruit were harvested at either mature green, breaker (0–10% orange color), or orange (30–60% orange color) stages of ripening. Ten fruit of each line and ripening stage were selected on the basis of size uniformity, and ethylene synthesis was measured by a modification of the method described by Ward et al. (1978). Whole fruit were sealed in half-pint Mason jars and incubated at room temperature, and gas samples were withdrawn after approximately 1–3 h. Ethylene in the gas samples was quantified by gas chromatography (Varian 3400 gas chromatograph)

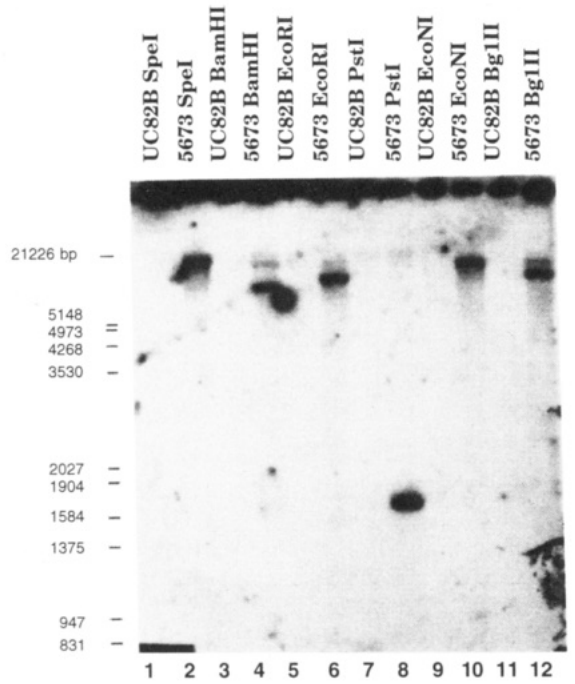

Figure 3. Southern blot analysis of genomic DNA of DR tomato line 5673. Genomic DNA of line 5673 and control line UC82B was digested with enzymes that included *Spe*I, *Bam*HI, *Eco*RI, *Pst*I, *Eco*NI, and *Bgl*II. The digested DNA was probed for *spc/str*. DNA standards are shown to the left of each blot. The circular band in lane 5 is a contaminating, nonspecific hybridization on the blot.

equipped with an alumina column and a flame ionization detector.

(b) Time-to-Red. Twenty fruit of each line and ripening stage (mature green, breaker, and orange) were placed in a controlled environment chamber (approximately 22 °C and 50% relative humidity). The time (in days) for each fruit to reach red ripe was measured, and the data presented as average time-to-red for each stage of ripening of a line. Differences in average time-to-red between DR lines and the control were tested by a two-sample Student's *t*-test, at the 5% level of significance.

RESULTS

Molecular Characterization. Genomic DNA of DR tomato lines 8338 and 5673 and control line UC82B was analyzed by Southern blot. Hybridization probes for the *accd*, *nptII*, and *spc/str* genes and bacterial origin of replication were used in these analyses. Hybridizing fragments that appear in the DR tomato lanes but do not appear in the control tomato establish the presence of insert DNA in the DR tomato line that is homologous to the probe fragment. Examples of Southern blots are shown in Figures 2 and 3; not all Southern blots for these studies are presented. In these analyses, differences between expected and observed fragment sizes were within the variability associated with fragment size determinations using the Southern blot method. Also, there was no specific hybridization of the *accd, nptII, spc/str,* and origin of replication probes with restriction digest fragments of control tomato line UC82B (examples in Figures 2 and 3). Results of molecular analyses of DR tomato lines 8338 and 5673 are summarized in schematic diagrams shown in Figure 4.

(a) Number of Insert Loci. Plant DNA was digested with restriction enzymes that do not cleave within the original transformation plasmid (*Spe*I and *Eco*RV for line 8338, *Spe*I and *Eco*NI for line 5673). By use of these enzymes, genomic fragments were produced that contained the entire inserted DNA. Therefore, each fragment that hybridized with the probes, and was not present in the UC82B control, represents a unique insert locus. Single unique genomic fragments from *Spe*I and *Eco*RV digests were observed for line 8338 with either the *accd* or *nptII* probes (an example is shown in Figure 2A). Since the unique fragments hybridized with both probes, the T-DNA in line 8338 must be present at a single locus in the plant genome. Line 8338 DNA fragments produced by *Spe*I and *Eco*RV digests did not hybridize with either the *spc/str* or origin of replication probes (see Figure 2B for an example), showing the absence of the *spc/str* gene and origin of replication in line 8338. Single unique genomic fragments from *Spe*I and *Eco*NI digests of line 5673 were observed with each of the four probes, *accd, nptII, spc/str,* and origin of replication (an example is shown in Figure 3), showing a single T-DNA insert in the genome of line 5673. Since line 5673 genomic fragments hybridized with the *spc/str* and origin of replication probes (see Figure 3 for an example), these DNA sequences are present in this DR tomato line.

(b) Insert Composition and Structure. (i) Line 8338. Genomic DNA from DR tomato line 8338 was digested with *Bam*HI, *Eco*RI, *Ssp*I, and *Pst*I restriction enzymes that cleave within the original transformation plasmid, pMON10117, to elucidate the structure of the insert DNA. The sizes of the DNA fragments that hybridized with the *accd* and *nptII* probes were of the predicted size (see Figure 2A for example), based on known restriction sites within the plasmid (Figure 4A). These data show that the *accd* and *nptII* genes are present as single copies at a single locus in the genome of line 8338. There were no DNA fragments that hybridized with either the *spc/str* or origin of replication probes (see Figure 2B for example), confirming the absence of these DNA sequences in the genome of line 8338. A schematic diagram of the T-DNA insert in the genome of line 8338 summarizes these results (Figure 4A), which confirm the expected functioning of the two T-DNA border sequences. Only the *accd* and *nptII* genes within the T-DNA were transferred from vector pMON10117 to a single locus in the tomato genome. Also, the structural integrity of the T-DNA was maintained during transfer.

(ii) Line 5673. In a similar analysis, genomic DNA from DR tomato line 5673 was digested with *Bam*HI, *Eco*RI, *Bgl*II, and *Pst*I restriction enzymes that cleave within the transformation plasmid, pMON10028. DNA fragments of predicted size were detected by Southern blot when probed with *nptII*, *spc/str*, and the origin of replication (see Figure 3 for an example), based on known restriction sites within the plasmid (Figure 4B). When DNA fragments were probed with *accd,* additional fragments to those expected hybridized with the *accd* probe. The results suggest that the complete vector pMON10028 (T-DNA), containing the *accd*, *nptII*, and *spc/str*, genes and the bacterial origin of replication, and most probably two additional tandem, incomplete copies of the *accd* gene, transferred to a single locus in the genome of line 5673. Since the tandem, incomplete copies of the *accd* gene were not functional and line 5673 is not of commercial significance, the structure of these tandem copies was not further resolved. These results are summarized in a schematic diagram of the T-DNA insert in line 5673 (Figure 4B).

(c) Insert Stability. As part of a tomato breeding program, line 8338 was backcrossed to three different

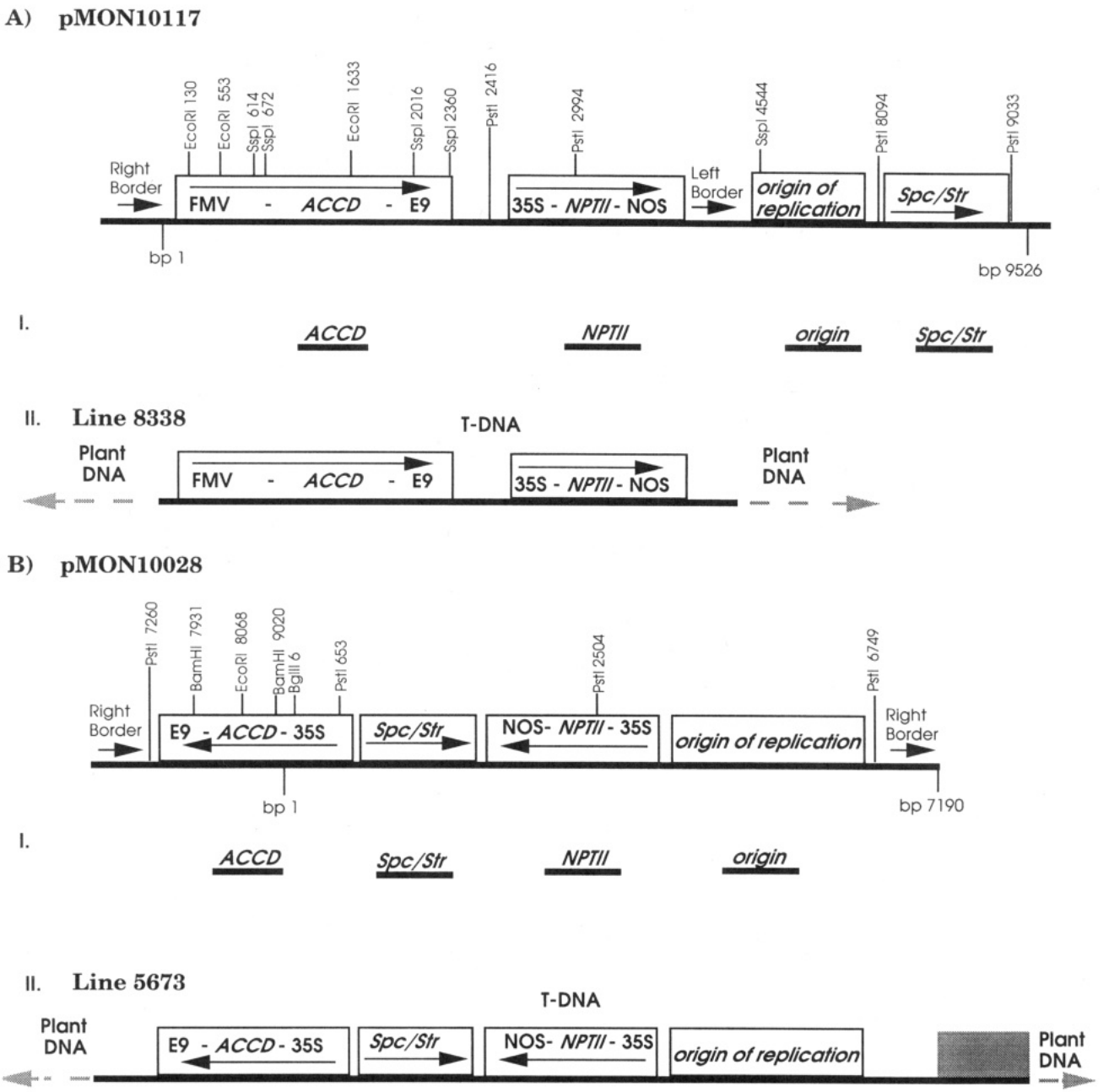

Figure 4. Schematic diagrams of plasmid vectors (A) pMON10117 used to produce DR tomato line 8338 and (B) pMON10028 used to produce DR tomato line 5673. The diagrams show restriction enzyme sites within each plasmid. Shown below each diagram are (I) the probe fragments used for Southern blot analysis of each DR tomato line and (II) the deduced structure of T-DNA inserted into the genome of each plant line. Line 8338 contains a single copy of the *accd* and *nptII* genes. Line 5673 contains a single complete copy of pMON10028, plus tandem, incomplete copies of the *accd* gene, shown as the shaded rectangle.

tomato lines (BHN-B, BHN-H, and BHN-N) through four generations. To determine the stability of the DNA insert in the genome of line 8338, genomic DNA of the backcross lines was analyzed by Southern blot, using the same restriction enzymes and hybridization probes as described for line 8338 analysis. An example of Southern blot analysis of these lines using the *accd* hybridization probe is shown in Figure 2A. Patterns of hybridization for the backcrossed lines were identical to those observed for the parental 8338 line. These results verify the stable integration of the inserted DNA in line 8338 through four backcross generations with nontransgenic tomato varieties. Stability of the DNA insert in line 5673 was not determined because this line is not of commercial significance.

ACCd ELISA Validation. ELISA validation parameters (precision, accuracy, and sensitivity) were determined for measurement of ACCd levels in red ripe tomato fruit. The interassay precision of the ACCd ELISA, measured as percent coefficient of variation (% CV), was 19.6%. Mean percent recovery (accuracy) of ACCd spiked into red ripe tomato fruit prior to extraction was 112.6%. There was no apparent loss of ACCd protein during extraction and assay. A single extraction of delayed ripening red ripe tomato fruit released 94.8% of the total ACCd protein present in the tissue (extraction efficiency). The sensitivity of the assay or lowest detectable level was 0.15 ng of ACCd per microtiter well. Under typical sample loading amounts per microtiter well, this is equivalent to a detection limit of approximately 1 ppm ACCd in fresh red ripe tomato fruit. The ACCd protein used as a standard in the ELISA (purified from recombinant *E. coli*) was immunologically and conformationally equivalent to the plant-expressed

Table 1. Expression of ACCd in Red Ripe Tomato Fruit of DR Tomato Lines 5673 and 8338 and Control Line UC82B[a]

field site[b]		mean[c] and SE[d] values (μg of ACCd/g of tissue fresh wt)		
		5673	8338	UC82B
1	mean	36.1	37.8	ND[e]
	SE	1.02	0.30	
2	mean	17.0	47.1	ND
	SE	0.71	1.08	
3	mean	14.5	47.4	ND
	SE	0.53	0.39	
4	mean	14.7	25.3	ND
	SE	0.32	0.57	
	mean across sites	20.6	39.4	ND
	SE across sites	0.51	0.45	

[a] Fruit were collected from four different field site locations. ACCd expression was determined by ELISA. Duplicate extracts of fruit samples from each of the four plots at each field site were analyzed by ELISA. [b] The location of each field site is described under Materials and Methods. [c] Means are the averages for each line across four plots. [d] SE, standard error. [e] ND, nondetectable (<1 μg ACCd/g of tissue fresh weight).

ACCd (Finn et al., 1995), which justifies use of the *E. coli*-produced enzyme as a standard for quantitation of ACCd expression levels in the tomato fruit.

ACCd Expression. The ACCd expression levels in red ripe tomato fruit of lines 8338 and 5673 are shown in Table 1. Protein expression was determined by ELISA, and results are presented as a mean of expression values across plots at each of the four field sites for the two DR tomato lines. The mean expression of ACCd across four field sites was 39.4 and 20.6 μg/g of fresh weight for lines 8338 and 5673, respectively. The range of expression across field sites was 25.3−47.4 μg/g of fresh weight for line 8338 and 14.5−36.1 μg/g of fresh weight for line 5673. The ACCd protein was not detected in red ripe tomato fruit of control line UC82B. The ACCd protein is a minor component of red ripe fruit of DR tomato lines, constituting approximately 0.39 and 0.21% of total fruit protein of lines 8338 and 5673, respectively.

Ethylene Synthesis. Ethylene synthesis of tomato fruit of DR lines 8338 and 5673 and control line UC82B was measured at the mature green, breaker, and orange stages of ripening. Individual measurements were performed on approximately 10 fruit of each line at each ripening stage. Average ethylene synthesis values are shown in Figure 5. Fruit ethylene synthesis increased in all tomato lines as fruit ripened from the mature green to breaker and orange stages. The rate of ethylene synthesis in the DR tomato fruit was reduced relative to the synthesis rate for control line fruit at each ripening stage. Percent reduction ranged from 97 (mature green) to 77% (orange stage) for line 8338 and from 90 (mature green) to 51% (orange stage) for line 5673. Levels of ACCd expression were correlated with decrease of fruit ethylene synthesis for both DR tomato lines (Table 1; Figure 5).

Time-to-Red. Time-to-red was measured from the mature green, breaker, and orange stages of tomato fruit ripening for lines 8338, 5673, and UC82B. The results are presented in Figure 6. Time-to-red from each ripening stage of line 8338 fruit was significantly extended relative to corresponding values for the control. Time-to-red from the breaker stage for DR line

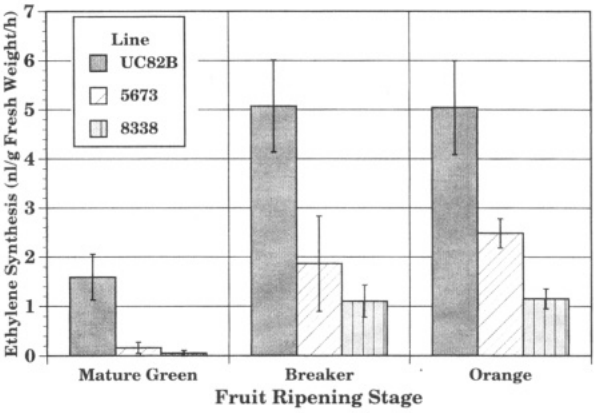

Figure 5. Ethylene synthesis by tomato fruit at different ripening stages for DR lines 8338 and 5673 and control line UC82B. Vertical lines around the bars represent ± SE.

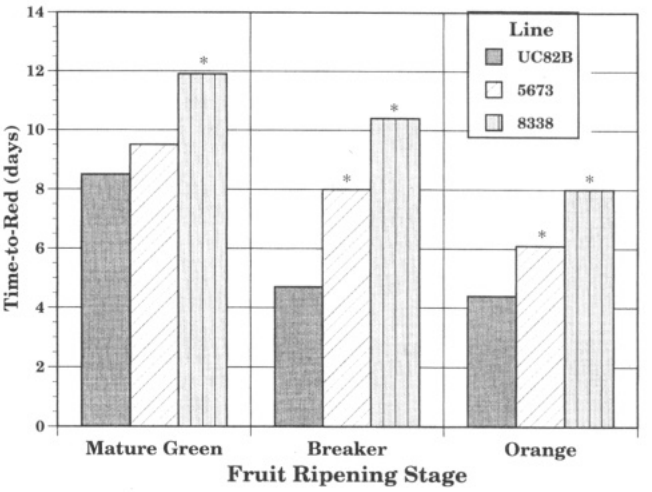

Figure 6. Time-to-red for tomato fruit at different ripening stages for DR lines 8338 and 5673 and control line UC82B. Asterisk shows statistically different from the UC82B control at the 5% level.

8338 was 10.4 days compared to 4.7 days for the control, an increase of 5.7 days or approximately 2.2-fold. Time-to-red from the breaker and orange ripening stages of line 5673 was significantly extended over values for the control, but the difference in time-to-red from the mature green stage between line 5673 and the control was not statistically significant. Comparison of time-to-red values of lines 8338 and 5673 shows that rates of ethylene synthesis (Figure 5) and ripening time (Figure 6) are inversely related for these two lines, as expected, on the basis of the role of ethylene in fruit ripening (Taiz and Zeiger, 1991).

CONCLUSIONS

We report the characterization of two DR tomato lines expressing the ACCd protein. The two lines, designated 8338 and 5673, were developed by *A. tumefaciens*-mediated transfer of the *accd* gene into the tomato genome. Southern blot analysis showed a single T-DNA insert in both DR tomato lines, but insert composition and integrity differed between the lines. Line 8338 contained a single copy of the *accd* and *nptII* genes. The *spc/str* gene and bacterial origin of replication were not present in this line. This result confirmed the expected functioning of the two border sequences of transformation plasmid pMON10117. In addition, the genetic insert remained stably integrated in the plant genome

through four successive backcross generations of line 8338 to nontransgenic tomato lines, as determined by Southern blot. As further support for a single, stable DNA insertion, the *accd* gene in line 8338 behaves as a single dominant gene inherited in Mendelian fashion in crosses to other tomato lines (B. Sammons, Monsanto Co., unpublished results). Line 5673 contained a single copy of the *accd*, *nptII*, and *spc/str* genes and the bacterial origin of replication, plus tandem incomplete copies of the *accd* gene, at the same single locus in the genome. This result suggests that T-DNA transfer to the genome of tomato line 5673 initiated at the right border of plasmid pMON10028 and continued to include the whole transformation plasmid and additional incomplete copies of the *accd* gene. Inheritance of the *accd* gene in crosses between line 5673 and other tomato varieties has not been determined because this line is not of commercial significance. However, Mendelian inheritance is expected on the basis of a single T-DNA insert in the genome of this line.

The *accd* and *nptII* genes present in tomato lines 8338 and 5673 are driven by plant-specific promoters, with the corresponding ACCd and NPTII proteins being expressed in both lines. The *spc/str* gene in line 5673 is not expected to express because it is driven by its own bacterial promoter (Fling et al., 1985). ELISAs have been developed and validated to measure levels of ACCd and NPTII proteins in DR tomato fruit tissue. We describe the ACCd ELISA; the NPTII ELISA has been described in a previous paper (Fuchs et al., 1993). Establishing the precision, accuracy, and sensitivity of the ACCd ELISA has led to the conclusion that the ELISA is a valid assay for measurement of ACCd protein levels in red ripe tomato fruit. The mean expression of ACCd protein in fruit collected from four field trials was 39.4 μg/g of fruit fresh weight in line 8338 and 20.6 μg/g of fruit fresh weight in line 5673. Differences in ACCd expression levels between the two lines may reflect different promoters and enhancer sequences for the *accd* gene in the two lines. Alternatively, the expression difference may be associated with different positions of the *accd* gene in the genome of the two tomato lines or a combination of both factors. The ACCd protein is a minor component of DR tomato fruit (accounting for 0.004 and 0.002% of fruit fresh weight for lines 8338 and 5673, respectively). However, expression levels were sufficient to decrease ethylene synthesis by a maximum of 97 and 90% in lines 8338 and 5673, respectively, relative to the synthesis rate of the control line. The expression of ACCd and the decrease in ethylene synthesis were greater in tomato fruit of line 8338 than in fruit of line 5673.

Tomato fruit ripening involves a series of coordinated biochemical reactions that result in fruit compositional changes and in fruit flavor development (Grierson and Kader, 1986). The compositional changes include chlorophyll degradation and lycopene synthesis, observed as a change in fruit color from green to red. Therefore, time-to-red is a simple measure of the rate of fruit ripening. Results of the current study show that time-to-red for fruit of lines 8338 and 5673 is extended relative to that of the control line. Furthermore, the length of the extension is correlated with the degree of inhibition of ethylene synthesis in these lines and the level of expression of ACCd proteins. In addition, shelf life (time from red ripe to over-ripe) of tomato fruit expressing ACCd is extended relative to that of control fruit (Klee et al., 1991; Klee, 1993). Several benefits to commercial tomato producers, shippers, and retailers may be realized by introduction of the *accd* gene and associated delayed fruit ripening traits into fresh market tomato lines. Growers will be able to harvest fruit at the breaker ripening stage (first break of color) and eliminate the inferior immature green fruit from the harvest. Packers, shippers, and retailers will reduce fruit loss due to over-ripe fruit, thereby increasing the yield of marketable fruit. Packers and shippers will be able to expand the geographical distribution of tomato fruit. These benefits will result in supply of better flavor quality fruit to the consumer nationwide.

The ACCd protein produced in DR tomato fruit does not pose any human food safety concerns. We have shown that the ACCd protein is rapidly degraded in simulated mammalian digestive fluids (the half-life of ACCd in simulated gastric fluid is less than 15 s), which suggests that the protein should not pose any allergenic concerns (Reed et al., 1995). There were no adverse effects in mice administered ACCd protein by oral gavage at a dose that represents greater than a 5000-fold safety margin relative to the highest potential human consumption of ACCd protein. Therefore, the ACCd protein is not toxic, as expected, since ACCd protein is rapidly digested in simulated digestive fluids. Finally, consumption of red ripe tomatoes accounts for approximately 0.7% of the adult human recommended dietary allowance (RDA) for protein (National Research Council, 1989). Since ACCd protein is a small fraction of total tomato fruit protein (approximately 0.4 and 0.2% for lines 8338 and 5673, respectively), consumption of ACCd protein in DR tomatoes accounts for an extremely small fraction of the human RDA for total protein. Therefore, the ACCd protein in DR tomato fruit presents no risks for human consumption.

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