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Inhibiting expression of a tomato ripening-associated membrane protein increases organic acids and reduces sugar levels of fruit

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Abstract. Tomato (*Lycopersicon esculentum* Mill.) ripening-associated membrane protein (TRAMP) is a channel protein of the membrane intrinsic protein (MIP) class encoded by the cDNA clone pNY507 [R.G. Fray et al. (1994) Plant Mol Biol 24: 539–543]. It has been suggested that these proteins encode water channels or aquaporins. TRAMP mRNA accumulated in all tomato tissues tested and was elevated in fruit during post-anthesis development and again during ripening. Transgenic plants that constitutively expressed a TRAMP antisense RNA sequence were generated with a 94% reduction of endogenous TRAMP mRNA in fruit. They showed no obvious phenotype that could be associated with gross perturbation of water relations, but ripening fruit of these plants showed marked alterations in the normal pattern of accumulation of both organic acids and sugars. At the onset and during ripening, levels of the organic acids L-malate and citrate were significantly elevated while levels of D[+]-glucose and D[+]-fructose were reduced. Additional transgenic lines were generated with reduced TRAMP mRNA, and the phenotype of increased acids and reduced sugars during fruit maturation and ripening was shown to be reproducible and stably inherited. Fruit of plants that over-expressed TRAMP mRNA showed no significant alteration in the sugars or acids investigated. These results suggest a role for TRAMP in the movement of solutes between cell compartments.

Key words: Antisense gene – Aquaporin – *Lycopersicon* (aquaporin) – Organic acid – Sugar – Transgenic tomato

The EMBL data library accession number for the TRAMP cDNA nucleotide sequence in clone pNY507 (Fray et al. 1994) is X73848

Abbreviations: B = breaker; CaMV = cauliflower mosaic virus; dpa = day post anthesis; MG = mature green; MIP = membrane intrinsic protein; TRAMP = tomato ripening-associated membrane protein

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Introduction

The MIP family of proteins, named after the first member to be characterised, the major intrinsic protein (MIP) of bovine lens fibers (Gorin et al. 1984), is defined as a class of hydrophilic, integral membrane proteins that function as channels facilitating the passage of water and small solutes through membranes. Amino acid sequence comparisons suggest that all MIPs possess six membrane-spanning domains, exhibit a channel-like structure and have amino and carboxy termini that face the cytoplasm (Chrispeels and Maurel 1994). Ranging in size from 25 to 30 kDa, MIPs from species as diverse as bacteria, plants and mammals have highly conserved peptide sequences, especially throughout the membrane-spanning regions. The signature sequence NPAVT found in the amino-terminal halves of the proteins and partially repeated as NPA in the carboxy-terminal halves is absolutely conserved (Daniels et al. 1994).

Although the function of some MIPs has been investigated, there are now hundreds of homologues from different species and the true roles and membrane localisations of the majority await elucidation. Transient over-expression of the human erythrocyte channel-forming integral protein (CHIP28) (Denker et al. 1988) and a rat homologue in *Xenopus laevis* oocytes caused rapid influx of water and rupture of the egg cells following their transfer to hypotonic solutions (Preston et al. 1992; Fushimi et al. 1993). Similar experiments with *Arabidopsis thaliana* γ-TIP (Maurel et al. 1993), two other plant homologues (Chrispeels and Agre 1994) including the turgor-responsive protein RD28 (Yamaguchi-Shinozaka et al. 1992) and an *Escherichia coli* homologue (AqpZ) (Calamita et al. 1995) gave similar results. Thus, the growing trend is to suggest that the proteins encoded by many of these cDNAs are also "aquaporins", defined as channel proteins that permit only the selective membrane passage of water and not protons or other ions (Agre et al. 1993). However, not all MIPs can be strictly classified as aquaporins. Some have been shown to transport other solutes in addition to water. The bacterial protein, GlpF, facilitates the

transport of glycerol and other straight-chain polyols across the cytoplasmic membrane (Heller et al. 1980; Sweet et al. 1990) and the peribacteroid membrane protein, NOD26, has been suggested to be involved in malate transport (Ouyang et al. 1991). The bovine MIP from lens fibers formed ion channels when incorporated into planar lipid bilayers (Zampighi et al. 1985; Ehring et al. 1990), and a yeast MIP homologue restored growth on fermentable sugars of the yeast mutant *fdp1* and may be involved in sugar transport (Aelst et al. 1991). Studies on a *Nicotiana tabacum* aquaporin (NtAQP1) have shown it to be localised in the plasma membrane and to mediate glycerol uptake when expressed in *Xenopus laevis* oocytes (Biela et al. 1999). Recently, the use of *X. laevis* oocytes as a heterologous expression system for the functional study of membrane channels has been questioned (Barkla and Pantoja 1996). The potential difficulty of expressing inward rectifying MIPs from the membranes of subcellular organelles in *X. laevis* in the same orientation as they are found in vivo has been overlooked, since this may cause solute to move from the cell to the medium rather than the other way round.

The tomato ripening-associated membrane protein (TRAMP) (Fray et al. 1994) is a member of the MIP family. Virtually identical homologues to TRAMP exist in *Nicotiana* (Yamada et al. 1997), *Arabidopsis* (Shagan et al. 1993), *Brassica* (Ruiter et al. 1997), *Hordeum* (Hollenbach and Dietz 1995), *Pisum* (Guerrero et al. 1990) and in many other plant species, but until now, although suggestions of aquaporin identity have been made, no membrane transport function has been experimentally ascribed to most of these proteins. We have generated transgenic antisense and sense tomato plants bearing fruit in which TRAMP mRNA has been reduced or over-expressed in order to resolve some of the questions regarding the function of this protein. Measurements suggest that during fruit ripening this MIP is involved in regulating the balance between the levels of organic acids and sugars and may function in the movement of solutes, rather than just the movement of water, between the cytosol and the vacuole.

Materials and methods

Plant material

All experiments were performed using a near-isogenic line of diploid *Lycopersicon esculentum* Mill. cv. Ailsa Craig plants that has been grown at Sutton Bonington for over 20 years. Unless otherwise stated, plants were grown in 24 cm-diameter pots in M2 compost (Levington Horticulture, Ipswich, Suffolk, UK) in growth chambers with a diurnal regime of 16 h continual light (250 µmol m^{-2} s^{-1} photosynthetic photon flux) at 23 °C followed by 8 h continual dark at 18 °C. Plants were watered daily and fed with high-nitrogen liquid fertiliser at regular intervals. Flowers were tagged at anthesis and fruit development recorded as days post-anthesis (dpa). Mature green (MG) fruit were defined as 35 dpa and were characterised as being green and shiny with no obvious ripening-associated colour change. Breaker (B) fruit were defined as those fruit showing the first signs of ripening-associated colour change from green to yellow. Fruit of subsequent ripening stages were defined in days post-breaker so that B + 3 fruit were yellow/orange in colour while B + 7 fruit were a full red-ripe. All plant samples were taken at the same time each day, frozen in liquid nitrogen and stored at −70 °C until required.

Construction of transgenes and plant transformation

Three transgene constructs were produced. The first (p507BAS) was designed to constitutively express an antisense RNA for TRAMP under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter. The second (p507B2AS) was designed to express the same antisense RNA as the first, but under the control of the *2A11* (Van Haaren and Houck 1991) tomato fruit-specific promoter. The third (p507BS) was designed to constitutively over-express a functional TRAMP transgene. For these experiments, the *2A11* promoter was supplied by Sumant Chengappa of the Unilever Research Colworth Laboratory, UK as a −3967 to +27 polymerase chain reaction (PCR) fragment cloned into the *Eco*RI site of pBluescript II SK– (Stratagene, Cambridge Science Park, Cambridge, UK) and termed pBS2A11. Throughout, all basic methods were as described by Sambrook et al. (1989).

To construct p507BAS, a 760-bp *Sac*I / *Sph*I 5′ TRAMP cDNA fragment from the clone pNY507 (Fray et al. 1994) was first ligated so as to replace the CaMV 35S terminator in the similarly digested plasmid pDH51 (Pietrzak et al. 1986) to yield pDH507. The cDNA was then re-excised from pDH507 as a *Pst*I fragment and was positioned in the antisense orientation between the CaMV 35S promoter and terminator by ligation into *Pst*I-digested pDH51 to yield pDH507T. The antisense gene was excised from pDH507T with *Eco*RI and ligated into the transformation vector pBIN19 (Bevan 1984) to yield p507BAS.

To construct p507B2AS, a 4-kbp *Bam*HI/*Sal*I *2A11* promoter-containing fragment from pBS2A11 was inserted between the CaMV 35S promoter and the antisense cDNA in pDH507T to yield pDH5072AT. The *2A11*-driven antisense gene and CaMV 35S terminator cassette were excised from pDH5072AT with *Kpn*I and ligated into similarly digested pBIN19 to yield p507B2AS.

To construct p507BS, a 1107-bp *Bam*HI/*Xho*I cDNA fragment, including the full coding sequence and polyA + tail from pNY507, was first ligated in the sense orientation between the CaMV 35S promoter and terminator of *Bam*HI/*Sal*I-digested pDH51 to yield pDH507ST. The sense gene was then excised from pDH507ST with *Eco*RI and ligated into similarly digested pBIN19 to yield p507BS.

The correct orientations of ligated fragments used to produce the antisense and sense constructs were verified by restriction digest analysis and by sequencing. After transfer to *Agrobacterium tumefaciens* strain LBA4404 (Bevan 1984) by the freeze-thaw method of An et al. (1988), the constructs were used to transform tomato cotyledon explants as described by Bird et al. (1988). Transgenic plants that rooted on kanamycin were transferred to compost and grown as described above.

Extraction and analysis of RNA

RNA was extracted from tomato fruit pericarp and from other vegetative tissues as previously described (Smith et al. 1986) except that contaminating carbohydrates and DNA were removed by differential precipitation of the RNA from 4 M LiCl at −20 °C for 1 h. RNA was quantified by spectrophotometry and, following formamide denaturation, 10-µg samples and RNA size markers (GIBCO BRL Life Technologies, Inchinnan, Paisley, UK) were fractionated in 1% (w/v) agarose gels containing 3% (v/v) formaldehyde. RNA was capillary-blotted onto GeneScreen Plus (NEN Life Science Products, Hounslow, UK) membranes which were then prehybridised at 65 °C in 5 × SSPE [1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4], 1% (w/v) SDS, 5 × Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl pyrrolidone, 0.1% (w/v) bovine serum albumin]

and 150 µg ml^{-1} sheared, denatured, salmon sperm DNA. The RNA was hybridised in the same buffer to probes generated from pNY507 cDNA sequences using the Megaprime system from Amersham International, Little Chalfont, Bucks., UK. After hybridisation, membranes were washed in 0.5 × SSPE, 0.1% (w/v) SDS at 65 °C and were autoradiographed. Hybridisation signals were quantified using a phosphoimage scanner (AMBIS Radioanalytical Image System; AMBIS Inc., San Diego, Calif., USA).

Extraction and analysis of genomic DNA

Genomic DNA was extracted by grinding 5 g of young leaf tissue in 25 ml of ice-cold homogenisation buffer [25 mM Tris-HCl (pH 7.6), 20% (v/v) glycerol, 2.5% (w/v) Ficoll 400, 0.44 M sucrose, 10 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100]. The homogenate was filtered through muslin and nuclei pelleted by centrifugation (1,000 *g*, 4 °C, 15 min). The nuclei in the pellet were lysed at 70 °C in urea buffer [42% (w/v) urea, 25 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 50 mM EDTA, 1% (w/v) *N*-lauryl sarcosine] and the DNA allowed to dissolve. The solution was extracted twice with phenol/chloroform (1:1, v/v) and the DNA precipitated from the aqueous phase by the addition of an equal volume of ethanol. The DNA was washed successively with 50 mM potassium acetate in 70% (v/v) ethanol, 70% (v/v) ethanol and 95% (v/v) ethanol, allowed to partially air dry and was dissolved in sterile distilled water (SDW) containing 10 µg ml^{-1} DNase-free calf pancreatic RNase A (Boehringer Mannheim UK, Lewes, East Sussex, UK) and stored at 4 °C until required. Individual genomic DNA (30 µg) samples were completely digested with one or more of the following: *Eco*RI, *Bam*HI, *Xba*I, *Hin*dIII and *Bgl*II, separated in 0.8% (w/v) agarose gels and capillary blotted to GeneScreen Plus (NEN Life Science Products, Hounslow, UK) membranes. Membranes were prehybridised as for Northern analysis and the DNA hybridised to probes generated from either the cDNA sequences of pNY507 or from the DNA sequences of the neomycin phosphotransferase gene (*npt*II) (Pridmore 1987) located within the T-DNA borders of pBIN19. Membranes were washed at 65 °C in 1 × SSPE, 0.1% (w/v) SDS and were autoradiographed.

Fruit L*-malate, citrate,* D*[+]-glucose and* D*[+]-fructose content*

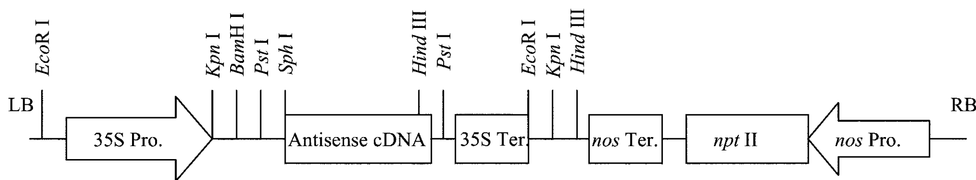
Fruit at the MG, B, B + 3 and B + 7 stages from wild-type and transgenic plants were analysed for their L-malate, citrate, D[+]-glucose and D[+]-fructose contents using standard enzymatic analytical kits (Boehringer Mannheim) for the detection of these compounds in foods. For each fruit stage at least five fruit from each of the plant types were analysed separately. Fruit pericarp was frozen in liquid nitrogen and ground to a fine powder. The powder (5 g) was added to 25 ml of boiling SDW and incubated at 100 °C for 10 min with vigorous shaking. After centrifugation (10,000 *g*, 25 °C, 10 min) and removal of the supernatant, the pellet was re-extracted with a further 15 ml of boiling SDW and centrifugation as before. The supernatants were combined, the pH adjusted to pH 8.0 with 0.1 M NaOH and the total volume was brought to 50 ml with SDW. Between 100 and 200 µl from each 50-ml sample was used for assay following the kit instructions and the formation of products derived enzymatically from the acids and sugars under test was measured spectrophotometrically. Duplicate measurements of each analysis were performed.

Results

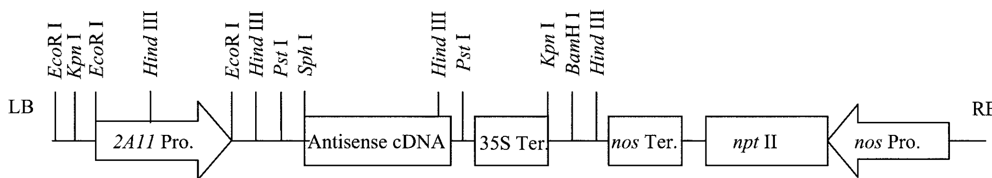
Construction of TRAMP antisense and sense genes and transformation of tomato

Two constructs containing TRAMP antisense genes and one sense gene construct were produced as described in the *Materials and methods* (Fig. 1). The first, an antisense construct p507BAS, contained a 760-bp antisense fragment from the 5′ end of the TRAMP cDNA and was designed for constitutive expression under the control of the CaMV 35S promoter. The second, a fruit-specific antisense construct p507B2AS, contained the same antisense fragment, but under the control of a 4-kb

(a). p507BAS



(b). p507B2AS



(c). p507BS

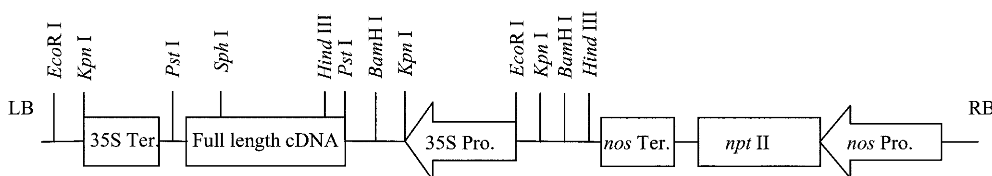


Fig. 1a–c. Sense and antisense TRAMP gene constructs used to transform tomato plants. The two antisense transgenes and one sense transgene were cloned into pBIN19 (Bevan 1984) to yield, respectively, p507BAS (**a**), p507B2AS (**b**) and p507BS (**c**). The construct p507BAS was designed to constitutively express a TRAMP antisense RNA while the construct p507B2AS was designed to express the same antisense RNA in a fruit-specific manner. The construct p507BS was designed to constitutively express a functional TRAMP transgene. *LB* and *RB* indicate the left and right borders, respectively, of the T-DNA region of pBIN19. The schematic is not drawn to scale and the design of the constructs is fully described under experimental procedures

fragment of the tomato fruit-specific *2A11* (Van Haaren and Houck 1991) promoter. The third, an over-expression construct p507BS, contained a 1,107-bp TRAMP cDNA fragment including the full coding sequence in the sense orientation, downstream of the CaMV 35S promoter. All three constructs, which included the CaMV 35S terminator downstream of the transcribed TRAMP sequences and a kanamycin-resistance selectable marker gene, were transferred to the tomato cultivar Ailsa Craig using *Agrobacterium* transformation. Three independent transformants containing construct p507BAS, 19 containing p507B2AS and 5 containing p507BS were selected by their ability to grow on kanamycin and were grown to maturity.

Inhibition and over-expression of TRAMP mRNA

Total RNA was extracted from the fruit, flowers, leaves, stems and roots of wild-type Ailsa Craig plants and was analysed for the expression of TRAMP mRNA by Northern analysis using probes generated from the cDNA insert of the clone pNY507. All the wild-type samples tested yielded a 1.1-kb hybridising band that differed in signal intensity depending on the stage of development of the fruit. TRAMP mRNA was present throughout development, but accumulated to its highest level at the breaker (B) +7 (7 d after the start of ripening) stage of ripening (Fig. 2). Early during development, up to 15 dpa the mRNA was at approximately 45% of the level found in B + 7 ripening fruit. Thereafter, the level declined and at the mature green (MG) stage was reduced to 17%. At the breaker stage of ripening the mRNA again began to show an increase in concentration, reached a maximum at B + 7 and then declined again. TRAMP mRNA was also detected in flowers, leaves, stems and roots (data not shown).

Total RNA was also analysed from the fruit of transgenic plants transformed with antisense (p507BAS), fruit-specific antisense (p507B2AS) and over-expression (p507BS) constructs in order to identify plants showing the greatest reduction (p507BAS and p507B2AS transformants) or increase (p507BS transformants) in the level of TRAMP mRNA. For plants transformed with the 35S antisense construct p507BAS, a line (3A29) was selected in which, at the MG stage before ripening and at the breaker, B + 3 and B + 7 stages during ripening the level of TRAMP mRNA was reduced to between 4% and 7% of the maximum expression levels found in wild-type controls (Fig. 3a). Of the 19 plants independently transformed with the fruit-specific antisense construct p507B2AS, the plant (2A90) showing the greatest reduction in endogenous TRAMP mRNA had from 12% to 25% of the levels in wild-type controls (Fig. 3b). Most of the other p507B2AS primary transformants showed lesser reduction of endogenous TRAMP mRNA in fruit. The 3S03 plants transformed with the over-expression construct p507BS (Fig. 3c) produced fruit at the MG stage with approximately 10-fold higher levels of TRAMP mRNA than found in equivalent fruit from wild-type controls.

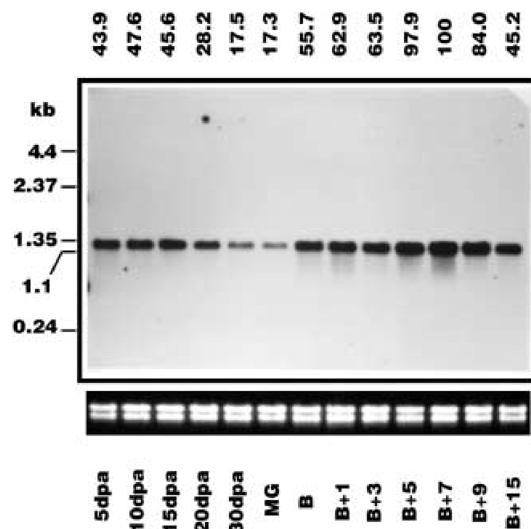


Fig. 2. Expression of TRAMP mRNA during tomato fruit development and ripening. RNA gel-blot analysis of TRAMP gene expression in wild-type tomato fruit was carried out using total RNA isolated from developing tomato fruit at various days post-anthesis (*dpa*), at mature green (*MG*) and breaker (*B*) (first visible colour change) stages of ripening and at various days post-breaker. Blotted RNA was hybridised with TRAMP cDNA from the clone pNY507 (Fray et al. 1994). Autoradiography exposure time was 48 h and the hybridisation signals were quantified using a phosphoimage scanner. For each sample, the percentage of the maximum expression level is indicated above each lane of the autoradiograph. Gel RNA loadings are represented below the autoradiograph by inclusion of a UV image of the ethidium bromide-stained rRNAs in each sample lane. The position attained by co-migrating RNA size markers is indicated as is the size of the single 1.1-kb hybridising band

As the fruit ripened this increase became less apparent, as the mRNA from the endogenous gene accumulated, so that at the B + 7 stage the fruit contained only 3-fold higher levels of the mRNA. Two other transformants (3S05 and 3S53) with the p507BS over-expression construct showed slightly lower mRNA levels than 3S03 at equivalent stages (data not shown).

Incorporation and inheritance of antisense and sense transgenes

Genomic Southern analysis was used to confirm the incorporation of TRAMP antisense and sense genes into transformants and to make an estimation of copy number and heritability. The DNA was digested with either *Xba*I, *Bgl*II, *Hin*dIII, *Bam*HI or *Eco*RI. Probing of Southern blots of wild-type DNA with TRAMP cDNA resulted in multiple hybridising bands and indicated that TRAMP is encoded by a small multigene family (data not shown). Digestion of genomic DNA extracted from the leaves of primary transformants (35S antisense: 3A29; sense over-expression: 3S03; and 2A11 antisense: 2A34 plus 2A90) with *Bam*HI or *Hin*dIII, which do not cut within the *npt*II gene, released a fragment containing a variable length of plant genomic DNA plus the *npt*II gene. For the line 3A29 (35S antisense), three hybridising bands were inherited

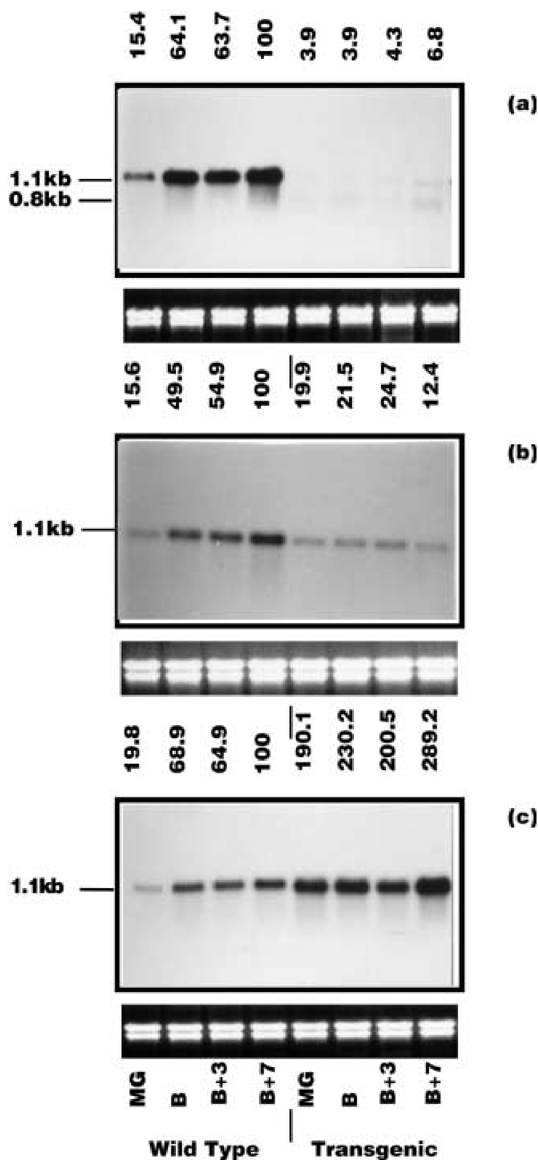


Fig. 3a–c. Comparitive levels of TRAMP mRNA in antisense and sense transgenic tomato plants. RNA gel blot analysis of TRAMP gene expression in fruit of wild-type control and p507BAS transformant plants (line 3A29) which constitutively expressed TRAMP antisense RNA under the control of the CaMV 35S promoter (**a**), wild-type and p507B2AS transformant plants (line 2A90) which expressed fruit specific TRAMP antisense RNA under the control of the *2A11* promoter (**b**), and wild-type and p507BS transformant plants (line 3S03) which constitutively expressed full-length sense TRAMP RNA under the control of the CaMV 35S promoter (**c**). Total RNA was extracted from mature green (*MG*) fruit and from fruit at the breaker (*B*), B + 3 and B + 7 stages of ripening. Blotted RNA was hybridised with TRAMP cDNA from the clone pNY507 (Fray et al. 1994). Autoradiography exposure times were 48 h (**a**), 16 h (**b**), 16 h (**c**), and the hybridisation signals were quantified using a phosphoimage scanner. For each sample, the percentage of the maximum expression level detected in wild-type plants is indicated above each lane of the autoradiographs. Gel RNA loadings are represented below each autoradiograph by the inclusion of a UV image of the ethidium bromide-stained rRNAs in each sample lane. The sizes of hybridising bands are indicated. For ripening fruit of 3A29 plants the antisense RNA of approximately 0.8 kbp is just visible (**a**)

together (data not shown). Similar genomic Southern analyses show that the over-expressing sense line 3S03 (p507BS) and the 2A11 antisense line 2A34 (p507B2AS) resulted from a single transgene insertion, whilst the antisense line 2A90 (p507B2AS) resulted from multiple transgene insertions (data not shown). F_1 plants derived from the antisense parental line 3A34 (3A34-4, 3A34-6, 3A34-9, 3A34-15 and 3A34-17, generated by selfing the primary transformant 3A34) yielded different multiple bands, indicating that multiple antisense inserts co-segregated to generate a range of antisense plants with different transgene insertions (Fig. 4a).

Organic acid and hexose levels in ripening fruit of wild-type and transgenic plants

L-Malate, citrate, D[+]-glucose and D[+]-fructose levels were determined for two stages of fruit, B and B + 7, taken from wild-type control plants, transgenic plants from lines 3A29 (35S antisense), 3S03 (35S sense over-expression) and 2A90 (2A11 antisense) (Table 1). Leaf size, plant height, and fruit size and growth rate were not found to be different in transgenic and control plants. The dry weight as a percentage of the fresh weight of all the fruit was always between 6 and 7%. For fruit of the wild-type controls the normal pattern of ripening-associated changes in the levels of L-malate, citrate, D[+]-glucose and D[+]-fructose were observed. At the breaker stage of ripening L-malate and citrate levels were approximately equimolar as were D[+]-glucose and D[+]-fructose. By the B + 7 stage L-malate and citrate levels had declined by approximately 60 and 20% respectively while the levels of both D[+]-glucose and D[+]-fructose had increased by approximately 20%. For fruit of the antisense line 3A29, in which the endogenous TRAMP mRNA was reduced to only 6% of wild-type levels (Fig. 3a), substantial increases in the levels of L-malate and citrate and decreases in the level of D[+]-glucose and D[+]-fructose were observed at both the breaker and B + 7 stages of ripening when compared to wild-type control fruit (Table 1). Fruit of the sense over-expressing line 3S03 had L-malate and citrate levels that were similar to those of the control plants at the breaker and B + 7 stages and, although there were small differences in sugar levels, these were not significantly different from the controls (Table 1).

The significance and reproducibility of these altered levels of organic acids and sugars were tested further in new transgenic plants. Six additional F_1 plants were identified with reduced TRAMP mRNA in fruit, and designated 2A34-7, 3A34-4, 3A34-6, 3A34-9, 3A34-15 and 3A34-17. Line 2A34-7 contained a 2A11-promoter driving the TRAMP antisense gene (construct p507B2AS) and the remaining plants were from a segregating F_1 population, described above, derived from a selfed primary transformant (3A34) with multiple 35S promoter antisense inserts (construct p507BAS) characterised in Fig. 4a. Quantitative measurements of TRAMP mRNA levels showed major

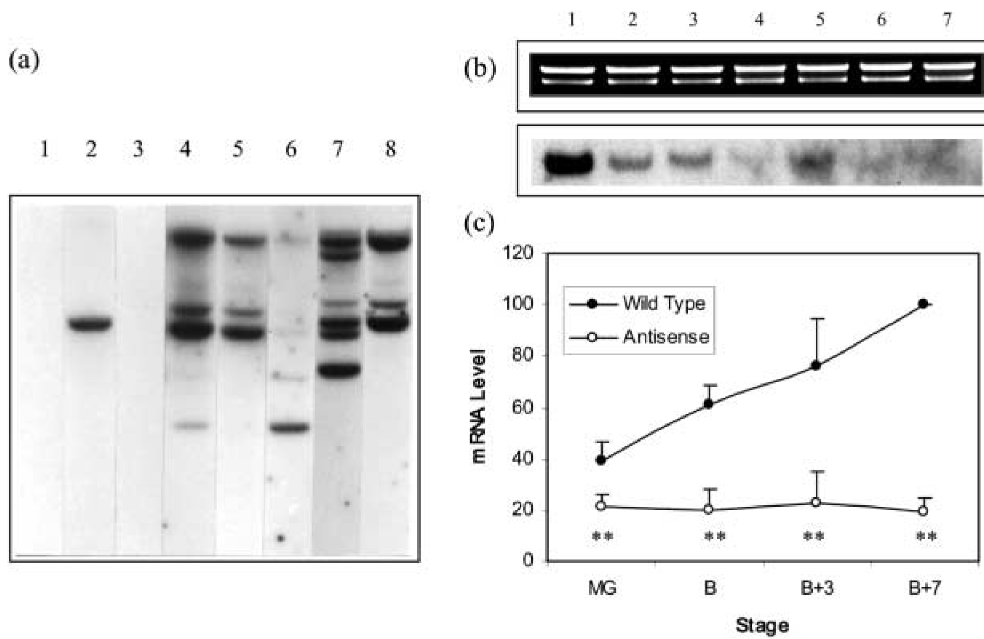


Fig. 4a–c. Inheritance of the transgene and reduction in TRAMP mRNA in fruit of antisense tomato plants at different stages of development. **a** Detection of the transgene in antisense plants. Leaf genomic DNA was extracted from wild-type control plant (*lanes 1, 3*), the F_1 progeny of 2A34 that were homozygous for the p507B2AS transgene (*lane 2*), and the F_1 progeny of 3A34 (p507BAS transgene): 3A34-4 (*lane 4*), 3A34-6 (*lane 5*), 3A34-9 (*lane 6*), 3A34-15 (*lane 7*), and 3A34-17 (*lane 8*). The genomic DNA was digested with *Hin*dIII (*lanes 1, 2*) and *Bam*HI (*lanes 3–8*). Blotted DNA was hybridised to the kanamycin resistance (*npt*II) gene. **b** RNA gel blot analysis of TRAMP gene expression in breaker +7 fruit of wild-type control plants (*lane 1*), and F_1 antisense plants: *lane 2*, 2A34; *lane 3*, 3A34-4; *lane 4*, 3A34-6; *lane 5*, 3A34-9; *lane 6*, 3A34-15; *lane 7*, 3A34-17. The upper panel shows the ethidium bromide-stained gel. Blotted RNA was hybridised with TRAMP cDNA from the clone pNY507 (lower panel). Autoradiography exposure time was 48 h and the hybridisation signals were quantified using a phosphoimage scanner. **c** RNA was extracted from fruit of each plant shown in **b** at four stages of ripening. RNA gels were fractioned, hybridised with TRAMP cDNA as described and the average TRAMP mRNA levels determined for each. For each sample, the percentage of the maximum expression level detected in wild-type plants is shown for each of the four stages of ripening investigated. Standard deviations of the means of duplicate assays on at least five separate (wild type) and six (TRAMP antisense) fruit for each sample type are indicated by the error bars on each point. Significantly different results are marked at $P < 0.01$(**)

reductions at all stages of fruit development and ripening tested (Fig. 4b,c). For each plant, sugars and acids were measured in fruit sampled at the MG, B, B +3 and B +7 stages of ripening. The results (Fig. 5) confirmed the increase in organic acids observed in the initial analysis of antisense TRAMP fruit (Table 1). The approximately 50% increase in organic acid level at the B + 3 and B + 7 stages was significant at $P < 0.01$ (Fig. 5). A corresponding decrease in sugar levels of approximately 30% was significant at $P < 0.05$ for the B and B + 7 stages (the same stages that were investigated for the original transgenic fruit, Table 1), but the concentration of sugars was not significantly different at B + 3 (Fig. 5).

Table 1. Changes in sugars and acids during ripening of wild-type and transgenic TRAMP fruit. Fruits were taken from wild-type control plants, the 35S antisense (p507BAS) transformant 3A29, the 2A11 antisense (p507B2AS) transformant 2A90 and the 35S over-expressing (p507BS) transformant 3S03. Data are the means ± SD of duplicate assays on five independent fruit for each sample type. Significant differences are indicated for $P < 0.05$(*) and $P < 0.01$(**)

		Acids (μmol g^{-1}FW)		Sugars (μmol g^{-1}FW)	
		Citrate	Malate	Glucose	Fructose
Breaker	Wild type	19.72 ± 2.20	18.90 ± 2.30	70.78 ± 7.68	68.71 ± 6.87
	3A29	28.50 ± 0.79**	28.15 ± 4.11**	50.11 ± 3.63**	51.75 ± 4.56**
	2A90	24.80 ± 1.69*	23.08 ± 1.21*	50.75 ± 8.96*	55.20 ± 9.05
	3S03	19.72 ± 2.34	19.74 ± 2.65	62.11 ± 10.57	63.43 ± 10.13
B+7	Wild type	15.93 ± 2.26	7.20 ± 1.02	84.03 ± 5.05	83.93 ± 4.28
	3A29	31.85 ± 2.05**	15.01 ± 2.57**	48.41 ± 0.69**	51.67 ± 2.20**
	2A90	21.83 ± 1.04*	7.72 ± 1.39	71.56 ± 2.34**	72.39 ± 1.70**
	3S03	17.86 ± 0.99	7.37 ± 1.93	76.27 ± 2.90	77.71 ± 1.87

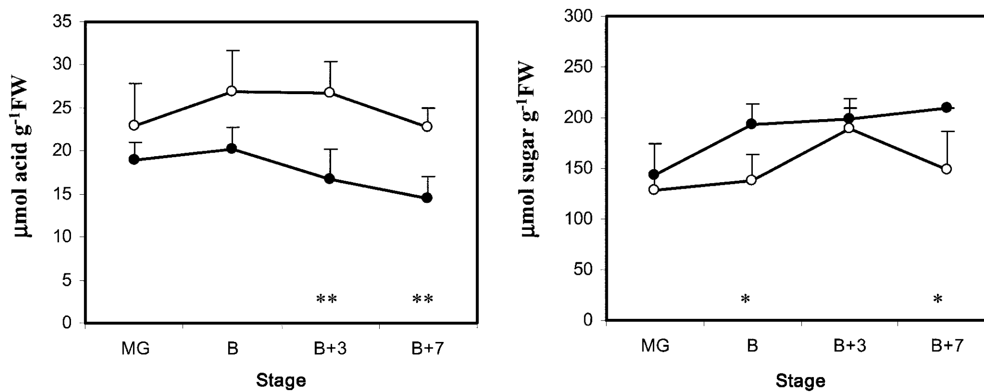


Fig. 5. Organic acid and sugar compositions of fruit from wild-type and TRAMP antisense tomato plants. Plants deficient in TRAMP mRNA shown in Fig. 4 were grown in a growth room. The citric acid, L-malic acid, D[+]-glucose and D[+]-fructose levels were measured for MG, breaker, B + 3 and B + 7 fruit from wild-type control (●) and TRAMP antisense (○) plants produced by selfing the primary transformants 2A34 and 3A34 for the p507B2AS- and p507BAS-derived transgenes. Standard deviations of the means of duplicate assays on at least 3 separate (wild type) and 12 (TRAMP antisense) fruit for each sample type are indicated by the error bars on each point. Significantly different results are marked at $P < 0.05$(*) and $P < 0.01$(**)

Discussion

Expression of TRAMP

The TRAMP mRNA was detected in roots, stems, leaves and flowers (data not shown). High expression of TRAMP mRNA in fruit was associated with two phases of development: growth and assimilate accumulation, and ripening (Fig. 2). At the MG stage, when growth has virtually ceased, the fruit are in a quiescent stage prior to ripening. It is notable that TRAMP mRNA levels were at their lowest at this stage. The dramatic increase in TRAMP mRNA during ripening is associated with major changes in fruit composition, with very little change in bulk water movement. These observations are consistent with TRAMP being involved in assimilate accumulation or distribution and inconsistent with a role in bulk water movement.

Altered levels of TRAMP mRNA using antisense and sense transgenes

Transgenic plants were successfully produced in which TRAMP mRNA was greatly reduced using antisense or increased by sense over-expression. As with other experiments involving over-expression or inhibition of genes in tomato, different transformants with a range of expression levels were obtained with the same gene construct (Smith et al. 1988). While the use of the CaMV 35S promoter to drive antisense expression was generally efficient at effecting a major reduction to 4% in the level of the endogenous TRAMP mRNA, the fruit-specific *2A11* promoter was less effective (Fig. 3). The reason for this is unknown, but may relate to the stability of the endogenous TRAMP message or the relatively low activity of the *2A11* promoter early during fruit development (Pear et al. 1989; Van Haaren and Houck 1991). Thus, the most effective *2A11* antisense plants described here only had a reduction in the endogenous TRAMP mRNA to between 12% and 25% of that found in wild-type ripening fruit (Fig. 3b). In *A. thaliana*, Kaldenhoff et al. (1998) showed that inhibition of a plasma-membrane aquaporin (PIP1b) by an antisense gene driven by the 35S promoter only reduced mRNA levels to 20–60% of normal.

Altered organic acids and sugars in transgenic fruit

One of the most important factors contributing to the taste of ripened fruit is the balance between the levels of organic acids and sugars (Oleski et al. 1987a) with high acid and sugars being favoured (Hobson and Grierson 1993). In tomato fruit the major organic acids are L-malate and citrate (Davies and Hobson 1981). In developing green fruit these acids are present at near equimolar levels (Goodenough et al. 1985). However, as fruit reaches maturity and begins to ripen, increased catabolic activity of the malic enzyme, which decarboxylates L-malate to pyruvate, and the continued activity of malate dehydrogenase and citrate synthase result in a decline in L-malate levels and the preferential accumulation of citrate (Goodenough et al. 1985). Different reports indicate slight increases (Davies and Hobson 1981), slight decreases (Carangal et al. 1954) or homeostasis (Davies 1966) of citrate levels as tomato fruit ripen, possibly reflecting varietal differences. As ripening commences, plastid-stored starch is converted to sugars such as D[+]-glucose and D[+]-fructose, which accumulate to levels above those required for respiration (Tucker 1993). Thus, in comparison to mature green fruit, ripened tomatoes normally have reduced levels of L-malate, similar or elevated citrate and increased levels of sugars. Much of the excess cytoplasmic sugar and citrate in ripening tomatoes is transported into and stored in the vacuole (Oleski et al. 1987a, b). Tomato plants in which TRAMP mRNA was greatly reduced by antisense showed major differences with regard to the normal pattern of accumulation of these acids and sugars in

ripening fruit, with organic acids increased and hexoses decreased (Table 1, Fig. 5). Over-expression of TRAMP mRNA, on the other hand, appeared to have little effect on the acid and sugar balance of ripening fruit. It is possible that the normal level of TRAMP mRNA is not rate limiting. Alternatively, although increased levels of the mRNA are readily apparent in over-expressing fruit (Fig. 3), this may not be efficiently translated. Furthermore, the excess protein may not all be correctly localised or functional in the cells. Function of MIPs is regulated by phosphorylation (Ouyang et al. 1991; Maurel et al. 1995; Daniels et al. 1996) and such modification may determine the actual level of TRAMP activity.

The question arises as to whether TRAMP functions in the distribution of acids and sugars within cells, or their translocation from one part of the plant to the other. The finding that similar trends (increased organic acids and reduced sugars) were obtained with antisense inhibition throughout the plant (35S antisense) and specifically in fruit (2A11 antisense) indicates that these results are not likely to be due to altered translocation of acids or sugars from one part of the plant to the other but occur as a result of reduced TRAMP protein in cells of tomato fruit.

Role of TRAMP

Some close MIP homologues of TRAMP have been classified as water channels or aquaporins (Cairney et al. 1995; Campos et al. 1997; Yamada et al. 1995, 1997). At least 23 MIPs have been identified in *Arabidopsis thaliana* and many of these have been suggested to be aquaporins (Weig et al. 1997). In our experiments, reducing TRAMP mRNA had no profound effect on the growth of the transgenic plants or fresh weight or dry weight of fruit. The plants of the 35S antisense line (3A29) rooted and grew at normal rates, showed no obvious signs of abnormal wilting, flowered normally and produced fruit which developed and ripened over a normal time span. The leaves and vine-ripened fruit showed normal dry weight to fresh weight ratios and seed development in the fruit appeared to be normal. Although not described here in detail, protoplasts isolated from immature (10 dpa) fruit of wild-type control and transgenic 3A29 antisense plants were found to burst at equivalent rates when transferred to hypotonic solutions. Thus, the balance of evidence indicates that TRAMP directly influences the relative levels of organic acids and sugars rather than movement of water. These findings contrast with those of Kaldenhoff et al. (1998) who showed inhibiting aquaporin PIP1b with an antisense gene decreased the cellular osmotic water permeability coefficient approximately three times and led to a 5-fold increase in root abundance in seedlings. In order to explain our observations in transgenic plants with reduced TRAMP mRNA, we propose that TRAMP is actually involved in the passage of solutes such as organic acids and/or sugars between the vacuole and the cytosol, although we do rule out a possible role in the movement of water also. Such a suggestion would be consistent with the recent work of Gerbeau et al. (1999), who concluded that a tobacco tonoplast intrinsic protein (TIP) was permeable to solutes such as urea and glycerol. According to our model, the TRAMP protein would be located in the tonoplast membrane, and experiments are under way to test this proposal. Inhibiting solute movement in antisense fruit could explain the reduction in sugars and give rise to enhanced production or retention of organic acids. Clarification of the role of TRAMP may result from an examination of the subcellular distribution and permeability of acids and sugars in wild-type and TRAMP antisense plants.

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References

Aelst LV, Hohmann S, Zimmermann F, Jans AWH, Thevelein JM (1991) A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisiae* mutant on fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling. EMBO J 10: 2095–2104

Agre P, Sasaki S, Chrispeels MJ (1993) Aquaporins – a family of water channel proteins. Am J Physiol 265: F461

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. In: Gelvin S, Schilperoot RA, Verma DPS (eds) Plant molecular biology manual. Kluwer, Dordrecht, pp A3/1–19

Barkla BJ, Pantoja O (1996) Physiology of ion transport across the tonoplast of higher plants. Annu Rev Plant Physiol Plant Mol Biol 47: 159–184

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 24: 8711–8721

Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R (1999) The *Nicotiana tabacum* plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. Plant J 18: 565–570

Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MW, Bird AS, Hughes S, Morris PC, Grierson D, Schuch W (1988) The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. Plant Mol Biol 11: 651–662

Cairney J, Newton RJ, Funkhouser EA, Chang S, Hayes D (1995) Nucleotide sequence of a cDNA for a water channel protein (aquaporin) homolog from *Atriplex canescens* (Pursh.) Nutt. Plant Physiol 108: 1291–1292

Calamita G, Bishai WR, Preston GM, Guggino WB, Agre P (1995) Molecular cloning and characterization of *AqpZ*, a water channel from *Escherichia coli*. J Biol Chem 270: 29063–29066

Campos F, Solorzano RM, Garciarrubio A, Covarrubias AA (1997) A *Phaseolus vulgaris* cDNA encoding a putative aquaporin. Plant Physiol 115: 313

Carangal ARJ, Alban EK, Varner JE, Burrell RC (1954) The influence of mineral nutrition on the organic acids of the tomato, *Lycopersicon esculentum*. Plant Physiol 29: 355–360

Chrispeels MJ, Agre P (1994) Aquaporins: water channel proteins of plant and animal cells. Trends Biol Sci 19: 421–425

Chrispeels MJ, Maurel C (1994) Aquaporins: the molecular basis of facilitated water movement through living plant cell. Plant Physiol 105: 9–13

Daniels MJ, Mirkov TE, Chrispeels MJ (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol 106: 1325–1333

Daniels MJ, Chaumont F, Mirkov TE, Chrispeels MJ (1996) Characterization of a new vacuolar membrane aquaporin sensitive to mercury at a unique site. Plant Cell 8: 587–599

Davies JN (1966) Changes in the non-volatile organic acids of tomato fruit during ripening. J Sci Food Agric 17: 396–400

Davies JN, Hobson GE (1981) The constituents of tomato fruit – the influence of environment, nutrition and genotype. CRC Crit Rev Food Sci Nutr 15: 205–280

Denker BM, Smith BL, Kuhajda FP, Agre P (1988) Identification, purification, and partial characterization of a novel M_r 28,000 integral membrane protein from erythrocytes and renal tubules. J Biol Chem 236: 15634–15642

Ehring GR, Zampighi GA, Horwitz J, Bok D, Hall JE (1990) Properties of channels reconstituted from the major intrinsic protein of lens fiber membranes. J Gen Physiol 96: 631–664

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

Fushimi K, Uchida S, Hara Y, Hirata Y, Marumo F, Sasaki S (1993) Cloning and expression of apical membrane water channel of rat kidney collecting tubule. Nature 361: 549–552

Gerbeau P, Guclu J, Ripoche P, Maurel C (1999) Aquaporin Nt-TIPα can account for the high permeability of tobacco cell vacuolar membrane to small neutral solutes. Plant J 18: 577–587

Goodenough PW, Prosser IM, Young K (1985) NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. Phytochemistry 24: 1157–1162

Gorin MB, Yancey SB, Cline J, Revel J, Horwitz J (1984) The major intrinsic protein (MIP) of the bovine lens fiber membrane: characterization and structure based on cDNA cloning. Cell 39: 49–59

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15: 11–26

Heller KB, Lin ECC, Wilson TH (1980) Substrate specificity and transport properties of the glycerol facilitator of *Escherichia coli*. J Bacteriol 144: 274–278

Hobson G, Grierson D (1993) Tomato. In: Seymour GB, Taylor JE, Tucker GA (eds) Biochemistry of fruit ripening. Chapman & Hall, London, pp 405–442

Hollenbach B, Dietz K (1995) Molecular cloning of emip, a member of the major intrinsic protein (MIP) gene family, preferentially expressed in epidermal cells of barley leaves. Bot Acta 108: 425–431

Kaldenhoff R, Grote K, Zhu J, Zimmermann U (1998) Significance of plasmalemma aquaporins for water-transport in *Arabidopsis thaliana*. Plant J 14: 121–128

Maurel C, Reizer J, Schroeder JI, Chrispeels MJ (1993) The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus* oocytes. EMBO J 12: 2241–2247

Maurel C, Kado RT, Guern J, Chrispeels MJ (1995) Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. EMBO J 14: 3028–3035

Oleski N, Mahdavi P, Peiser G, Bennett AB (1987a) Transport properties of the tomato fruit tonoplast: I. identification and characterization of an anion-sensitive H^+-ATPase. Plant Physiol 84: 993–996

Oleski N, Mahdavi P, Bennett AB (1987b) Transport properties of the tomato fruit tonoplast: II. Citrate transport. Plant Physiol 84: 997–1000

Ouyang LJ, Whelan J, Weaver CD, Roberts DM, Day DA (1991) Protein phosphorylation stimulates the rate of malate uptake across the peribacteroid membrane of soybean nodules. FEBS Lett 293: 188–190

Pear JR, Ridge N, Rasmussen R, Rose RE, Houck CM (1989) Isolation and characterization of a fruit-specific cDNA and the corresponding genomic clone from tomato. Plant Mol Biol 13: 639–651

Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res 14: 5857–5862

Preston GM, Carrol TP, Guggino WB, Agre P (1992) Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. Science 256: 385–387

Pridmore RD (1987) New and versatile cloning vectors with kanamycin-resistance marker. Gene 56: 309–312

Ruiter RK, van Eldik GJ, van Herpen MM, Schrauwen JA, Wullems GJ (1997) Expression in anthers of two genes encoding *Brassica oleracea* transmembrane channel proteins. Plant Mol Biol 34: 163–168

Sambrook J, Fritsch EF, Maniatis T (1989) In: Nolan C (ed) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Shagan T, Meraro D, Bar-Zvi D (1993) Nucleotide sequence of an *Arabidopsis thaliana* turgor-responsive TMP-B cDNA clone encoding transmembrane protein with a major intrinsic protein motif. Plant Physiol 102: 689–690

Smith CJS, Slater A, Grierson D (1986) Rapid appearance of an mRNA correlated with ethylene synthesis encoding a protein of molecular weight 35,000. Planta 168: 94–100

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

Sweet G, Gandor C, Voegele R, Wittenkindt N, Beuerle J, Truniger V, Lin ECC, Boos W (1990) Glycerol facilitator of *Escherichia coli*: cloning of *glpF* and identification of the *glpF* product. J Bacteriol 172: 424–430

Tucker GA (1993) Introduction: respiration and energy. In: Seymour GB, Taylor JE, Tucker GA (eds) Biochemistry of fruit ripening. Chapman & Hall, London, pp 3–9

Van Haaren MJJ, Houck CM (1991) Strong negative and positive regulation elements contribute to the high-level fruit-specific expression of the tomato *2A11* gene. Plant Mol Biol 17: 615–630

Van Haaren MJJ, Houck CM (1993) A functional map of the fruit-specific promoter of the tomato *2A11* gene. Plant Mol Biol 21: 625–640

Weig A, Deswarte C, Chrispeels MJ (1997) The major intrinsic protein family of *Arabidopsis* has 23 members that form three distinct group with functional aquaporins in each group. Plant Physiol 114: 1347–1357

Yamada S, Katsuhara M, Kelly WB, Michalowski CB, Bohnert HJ (1995) A family of transcripts encoding water channel proteins: tissue-specific expression in the common ice plant. Plant Cell 7: 1129–1142

Yamada S, Komori T, Imaseki H (1997) cDNA cloning of aquaporins from *Nicotiana excelsior*. Plant Physiol 114: 747

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 33: 217–224

Zampighi GA, Hall JE, Kreman M (1985) Purified lens junctional protein forms channels in planar lipid films. Proc Natl Acad Sci USA 82: 8468–8472