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Characterization of a Tomato (*Lycopersicon esculentum* Mill.) Ripening-associated Membrane Protein (TRAMP) Gene Expression and Flavour Volatile Changes in TRAMP Transgenic Plants

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Abstract

The tomato ripening associated membrane protein (TRAMP) (Fray et al., 1994) is a member of the major intrinsic protein (MIP) family, defined as channels facilitating the passage of water and small solutes through membranes. During normal fruit ripening the TRAMP mRNA levels were increased whereas the expression levels of TRAMP in low ethylene ACO1-sense suppressed lines, *Nr* and *rin* fruits, were lower than at the breaker stage of wild type fruit. TRAMP mRNA is inhibited by $LaCl_3$, which is an inhibitor of Ca^{2+}-stimulated responses, treatment but drought condition did not affect TRAMP expression. The levels of TRAMP mRNA transcripts were substantially higher in the dark treated seedlings and fruits. These suggest that TRAMP function as a water channel may be doubted because of several reasons; no water content was changed during ripening in wild type, antisense and overexpression lines, TRAMP expression under light condition was lower than dark condition and TRAMP expression was not changed in drought condition. Co-suppression plant, 3S88 was one of sense suppression lines, which contain CaMV 35S promoter and sense pNY507 cDNA, produced small antisense RNA, approximately 21-25 nucleotides in length, mediated post-transcriptional gene silencing. Therefore, TRAMP expression was inhibited by small antisense and multiple copies might induce gene silencing without any production of double strand RNA. Total seven selected volatile productions, isobutylthiazole, 6-methyl-5-hepten-2-one, hexanal, hexenal methylbutanal, hexenol, and methylbutanol, were highly reduced in sense line whereas total volatile production was increased in TRAMP antisense line. These results suggested TRAMP might change volatile related compounds.

Key words: aquaporin, aroma, ethylene, major intrinsic protein (MIP), tomato ripening associated membrane protein (TRAMP)

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Introduction

The tomato ripening associated membrane protein originally identified from the partial cDNA clone Tom 75 and encoded by the full length clone PNY507 (TRAMP; GeneBank accession no. X73848) (Fray et al. 1994) is a member of the MIP family. It was found by further study that pTOM75 is a hybrid clone, containing a short antisense sequence homologous to another ripening- related clone E8 (Lincoln et al. 1987) at the 5' end. The full length cDNA clone, pNY507 (GeneBank accession no. X73848) (Fray et al. 1994) was isolated by screening a λ-ZAP cDNA library (Picton et al. 1993) with a pTOM75 cDNA fragment as a probe.

The tomato ripening associated membrane protein (TRAMP) (Fray et al. 1994) is a member of the MIP family. The MIP family is defined as a class of integral membrane proteins that function as channels facilitating the passage of water and small solutes through membranes. The MIP family was named after the first member to be identified and characterised was MIP of bovine eye lens fiber cells

(Gorin et al. 1984). The MIP family members generally have a similar molecular weight of 23-31 kDa. The function of some MIPs has been investigated and there are now hundreds of homologues known from different species, including aquaporins which facilitate the diffusion of water, glycerol, urea, and carbon dioxide (Borgnia et al. 1999; Maurel 1997; Prasad et al. 1998). The first demonstration of the function of a plant aquaporin was in 1993 when a tonoplast intrinsic protein, γ-TIP, which was isolated by homology cloning using a cDNA corresponding to α-TIP, a seed-specific tonoplast integral protein (Johnson et al. 1989) in Arabidopsis, was shown to exhibit water transport activity when expressed in *Xenopus* oocytes (Maurel et al. 1993). Some have been shown to transport other solutes in addition to water. The bacterial protein, GlpF, has been shown to facilitate 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). AQP1 of human erythrocytes has been reported to cause a 4-fold increase in both water and CO_2 permeability when inserted into proteoliposomes (Prasad et al. 1998). Furthermore, this increase in CO_2 and water permeability was inhibited by Mercuric chloride ($HgCl_2$), the water transport inhibitor, and mercaptoethanol reversed the inhibition. Thus, in vivo, AQP1 may facilitate the transport of CO_2 in addition to water, although evidence against physiologically significant CO_2 transport by AQP1 has also been reported (Yang et al. 2000). MIPs have been found in the plasma membrane (PIPs), in the tonoplast (TIPs) and in the peribacteroid membrane of root nodules (called Nodulin 26-like MIPs or NLMs). In *Arabidopsis*, 35 MIP family genes were identified and at least 54 family genes in *Brassica rapa* were identified (unpublished data). GFP-fused TRAMP was detected predominantly in the plasma membrane when stably expressed in transgenic tomato fruit and stem cells and in onion cells following microprojectile bombardment (Kim and Grierson 2005).

Ethylene plays an important role in regulating plant responses to environmental changes such as drought, wounding, chilling or waterlogging and also during normal developmental processes such as leaf senescence and, in climacteric fruit, ripening. Drought stress has been found to induce ethylene production in some plant species (El-Beltagy and Hall 1974; Apelbaum and Yang 1981; Kimmerer and Kozlowski 1982; Stumpff and Johnson 1987; Chen and Yu 1988). Fruit of the *rin* mutant turn yellow but fail to ripen normally, whereas *Nr* fruit turn pale orange but also do not ripen. The application of exogenous ethylene does not restore ripening to either *rin* or *Nr*. The enzyme ACC oxidase (ACO) is responsible for the final stage in the production of ethylene by higher plants. Two sense suppressed lines, T4B11 and V11B7, shared a greatly enhanced degree of silencing compared to controls carrying the ACO construct without the inverted repeat (Hamilton et al. 1998). Fruit ripening of these plants was delayed for 1-2 weeks and ethylene production was more than 90% lower than wild type during ripening (Alpuche-Solis 1999).

Recently, we (Chen et al. 2001) have shown by antisense and gene silencing that inhibition of TRAMP expression alters organic acids and sugar levels in tomato fruit. The aims of this work was to characterize TRAMP expression and co-suppressing plants by Southern and RNAs gel blot analysis and to measure the accumulation of the volatiles in wild type and transgenic plants containing antisense or sense pNY507 cDNA.

Materials and Methods

Plant materials

All experiments were performed using control and transgenic lines derived from a line of *Lycopersicon esculentum* Mill. cv. Ailsa Craig grown at Sutton Bonington, Leics., UK for over 20 years. T4B11 contains the CaMV 35S promoter, and the untranslated region and ACO1 coding sequence (Alpuche-Solis 1999). A stop codon was included at the thirty eighth codon near the 5' end of the ACO1 coding sequence. The V11B7 construct contains the CaMV 35S promoter, unntranslated region, ACO1 coding sequence and an inverted repeat (79 bp) of the ACO1 5' untranslated region upstream of the regular 5' untranslated region sequence. Plants containing these constructs shared a greatly enhanced degree of silencing compared to controls carrying the ACO constuct without the inverted repeat (Hamilton et al. 1998). In earlier experiments (Chen et al. 2001; Kim and Grierson 2005) TRAMP antisense, sense and cosuppression transgenic lines were made with the near-constitutive CaMV 35S promoter and antisense or sense pNY507 cDNA and the expression levels of these plants were studied.

Unless otherwise stated, wild type, *Nr*, *rin* and other transgenic plants were grown in 24 cm diameter pots in M2 compost (Levington Horticulture Ltd., Ipswich, Suffolk, UK) in growth chambers with a diurnal regime of 16 h continual light (250 mmol m^{-2} s^{-1} photosynthetic photon flux) at 23℃ followed by 8 h continual dark at 18℃. Plants were watered daily and fed with high nitrogen liquid fertilizer at regular intervals. Fruit development and ripening were divided into various stages for sampling and samples were taken at the

same time each day. Flowers were tagged at anthesis and fruit development recorded as days post-anthesis (dpa). Mature green fruit were defined as those fruit being at 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. Fruits 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.

Seeds were surface sterilised prior to germination by soaking for 10 min in 50% (v/v) ethanol, 20 min in a saturated solution of trisodium orthophosphate and rinsed briefly several times with sterile distilled water (SDW). This was then followed by soaking for 10 min in 50% domestic bleach solution (sodium hypochlorite/water 1:1 (v/v) and finally rinsed 3-4 times with SDW before sowing. All plant samples were frozen in liquid nitrogen and stored at-70℃ until required.

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 4M LiCl at -20℃ for 1 h. RNA was quantified by spectrophotometry and following formamide denaturation 10 μg samples and RNA size markers (GIBCO BRL Life Technologies Ltd., 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℃ in 5×SSPE, 1% (w/v) SDS, 5× Denhardt's solution and 150 μgml^{-1} sheared, denatured, salmon sperm DNA. The RNA was hybridised in the same buffer to probes generated from pNY507 or GFP cDNA sequences using the Megaprime system from Amersham International plc., Little Chalfont, Buckinghamshire, UK. After hybridization, membranes were washed in 0.5×SSPE, 0.1 % (w/v) SDS at 65℃ and were autoradiographed. Hybridisation signals were quantified using a phosphoimage scanner (AMBIS Radioanalytical Image System, AMBIS Inc., 3939 Ruffin Road, San Diego, CA 92123, USA).

Extraction and detection of sense (sRNAs) and antisense (asRNAs) RNAs

The RNAs were extracted and transferred to Hybond-Nx membrane (Amersham Pharmacia Biotech) as described previously (Hamilton and Baulcombe 1999; Han and Grierson 2002). The initial steps for extraction of small RNAs were the same as those described above for total RNA extraction. After the first ethanol precipitation, the pellet was re-dissolved in 2 ml water. High molecular weight nucleic acids were removed by precipitation in 10% PEG 8000/0.5 M NaCl and small RNAs were enriched using a Qiagen-tip 20 (Qiagen). Small RNAs were separated through 15% polyacrylamide/7 M urea gels, transferred onto Hybond NX filters by electrophoretic transfer at 250 mA for 30 min and cross-linked by UV using a Stratalinker®(Stratagene). Prehybridisation was performed in 40% formamide, 7% SDS, 0.3 M NaCl, 0.05 M Na_2HPO_4-NaH_2PO_4 (pH 7), 1×Denhardt's solution, 100 μg/ml sheared and denatured salmon sperm DNA for 30 min at 30℃. Hybridisation was in the same solution for 16 h at 30℃ and the filters were washed with 2× SSC/0.2% SDS at 50℃ for 3×10 min.

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, 10mM β-mercaptoethanol, 0.1% (v/v) Triton X-100). The homogenate was filtered through muslin and nuclei pelleted by centrifugation (1000×g, 4℃, 15 min). The nuclei in the pellet were lysed at 70℃ 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/CHCl3 (1:1) 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 K^+ acetate in 70% (v/v) ethanol, and 95% (v/v) ethanol, allowed to partially air dry and was dissolved in SDW containing 10 μgml^{-1} DNase-free calf pancreatic RNase A (Boehringer Mannheim UK Ltd., Lewes, East Sussex, UK) and was stored at 4℃ until required. Individual genomic DNA (30 μg) samples were completely digested *with BamHI* and were 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 (Fray et al. 1994) or from the DNA sequences of the neomycin phosphotransferase gene (*NPTII*) (Pridmore, 1987) located within the T-DNA borders of pBIN19. Membranes were washed at 65℃ in 1×SSPE, 0.1% (w/v) SDS and were autoradiographed.

Analysis of volatiles by APCI-MS (Atmospheric Pressure Chemical Ionisation-Mass Spectrometry)

The general set-up was as described in the introduction. A Platform Quadrupole mass spectrometer (Micromass, Altrincham, UK) operating in the API (Atmospheric Pressure Ionisation) positive ion mode was fitted with a custom-built air-sampling interface (Linforth and Taylor 2000; Boukobza and Taylor 2002). The method volatiles analysis by APCI-MS was described by Boukobza and Taylor (2002). Tomato seedlings were blended in a modified commercial food blender (total volume: 355 ml) with three Swagelok bulkhead fittings bolted into the blender lid. The headspace within the device was continually flushed with air at 170 ml/min to rapidly remove volatiles produced. As the total volume of the device is 355 ml, the air was changed every 2 min. A portion of the airflow was continuously sampled into the API-MS at 11.5 ml/min through a heated transfer line (0.53 mm inner diameter is fused silica tube held at 100℃). The headspace was initially monitored for about 30 sec to obtain a baseline measure of volatiles above the intact seedlings, then seedlings were blended (2-5 sec) and monitoring of the headspace above the macerate carried out for a further 1 min to follow the release of the nine selected volatile compounds (Boukobza and Taylor 2002). The selection of seven volatile compounds monitored (i.e. hexanal, hexenal, hexenol, 6-methyl-5-hepten-2-one, methylbutanal, methylbutanol, isobutylthiazole) was based on the different chemical origin and odour impact of these compounds (Buttery and Ling 1993). Four identical maceration devices were used to allow rapid sampling, which minimise the time from harvest to sampling. Each device was thoroughly cleaned with water and dried after use.

Results and Discussion

TRAMP expression in mutants and low ethylene plants

TRAMP is a hybrid clone, containing a short antisense sequence homologous to another ripening-related clone E8 (Lincoln et al. 1987) at the 5' end. Transcription of the E8 gene is regulated during fruit ripening both by ethylene, and by ethylene-independent ripening signals (Deikman et al. 1992), and E8 is also expressed at a high level in anthers (Kneissl and Deikman, 1996). Therefore, RNA gel blot analysis of gene expression was carried on wild type, *Nr, rin* and low ethylene T4B11 and V11B7 tomato plants.

RNA gel blot analysis was carried out on total RNA extracted from wild type, Nr and rin tomato fruits at various stages: MG, B, B+3, B+7, B+10 (Figure 1a) and from fruits from wild type and ACO1 co-suppression plants T4B11 and V11B7, using fruit at B and B+7 (Figure 1b). Equal amounts (40 μg) of total RNA from each fruit stage were probed for

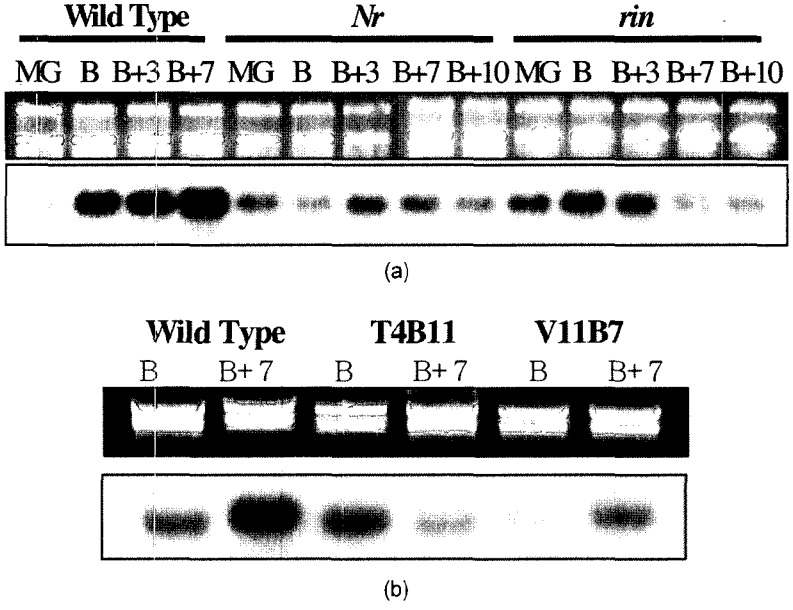

Figure 1. TRAMP mRNA expression in the fruit of wild type, *Nr, rin* and ACO1 co-suppression plants measured by RNA gel blot analysis. (a) Upper panel shows ethidium bromide stained total RNA extracted from wild type, *Nr, rin* fruits at various ripening stages and lower panel shows TRAMP mRNA transcript expression. (b) Upper panel shows ethidium bromide stained total RNA from fruit of wild type and ACO1 co-suppression (T4B11 and V11B7) plants and lower panel shows TRAMP mRNA transcript expression. Total RNA 40 μg, cDNA probe pNY507, exposure 24 h.

TRAMP transcripts with pNY507 cDNA (Figure 1a). TRAMP mRNA transcript levels increased through B+7 in wild type fruit whereas levels in *Nr* and *rin* fruit showed a lesser increase through B+3 and declined there after. mRNA transcript levels of TRAMP from low ethylene T4B11 and V11B7 fruit were significantly reduced during ripening, although the reduction was not consistent for the two stages tested of each line. Transcript levels in the co-suppressed lines varied, with T4B11 showing a decrease at B+7 from similar levels to wild type at breaker stage, whereas V11B7 showed lower levels at breaker stage increasing at B+7 (Figure 1b). Therefore, TRAMP expression levels were inhibited in low ethylene tomato fruits.

Effect of calcium, water stress and dark treatment on expression of TRAMP mRNA

Recently, it has been shown that calmodulin (CAM) interacts with the major intrinsic protein (MIP), and possibly regulates MIP channel permeability (Swamy-Mruthinti, 2001). Chen (Ph.D. thesis 1997) showed that levels of TRAMP mRNA expression were reduced by 6 days water deficit. Long term stress as the 6 day stress period might affect gene expressions of many other genes and could also nearly kill seedling plants but it is not clear that the expression is directly reduced by water deficit or not. In the predicted TRAMP protein sequence there is a putative protein kinase C phosphorylation site in the N-terminal region (TDK), where T may be the phosphorylation site. In TRAMP, casein kinase II phosphorylation sites are also present in the N-terminal (TDKD), and loop C (TKGD), and between loop D and the 5th membrane spanning domain (SATD), where S or T might be the phosphorylation site. Johansson et al. (1998) have proposed a model where, at high apoplastic water potential, a Ca^{2+} channel in the plasma membrane opens in response to a signal, leading to an influx of Ca^{2+} which activates a protein kinase phosphorylating aquaporins.

To study the early effects of Ca^{2+} on TRAMP expression, wild type tomato seedlings were grown on 0.471 g/l of Murashige and Skoog basal salt mixture with 30 g/l of sucrose and addition of 0.0 mM, 10 mM or 50 mM $CaCl_2$ (Figure 2a). In wild type, TRAMP mRNA transcript levels increased with the addition of 10 mM $CaCl_2$ but was reduced at 50 mM $CaCl_2$.

In order to confirm the effect of water stress and calcium inhibition on the expression of TRAMP in tomato, mRNA transcript levels were examined during simulated drought with/without $LaCl_3$, which is an effective inhibitor of membrane Ca^{2+} channels. Three week-old wild type tomato seedlings were soaked in SDW or in 10 mM $LaCl_3$ for 1h and then dried in the air for 1-3 h. TRAMP expression was unchanged or only slightly decreased in drought conditions for 3 h whereas mRNA levels were greatly reduced by $LaCl_3$ (Figure 2b). These results indicate that short drought conditions may not affect the expression of TRAMP directly. However, the expression might be reduced by water deficit in long term. Ca^{2+} concentration in cells may be important for the TRAMP membrane channel expression but not Cl^-.

TRAMP expression in wild type 3 weeks old seedling grown on MSR3 (8 g/l bacteriological agar (Oxoid), 4.4 g/l Murashige and Skoog salts, 30 g/l sucrose, 1 ml/l R3

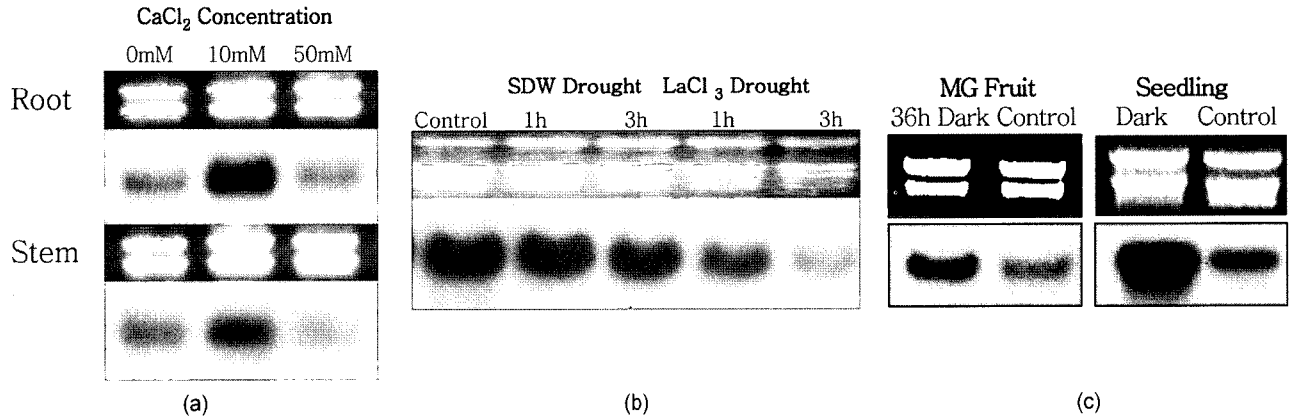

Figure 2. RNA gel blot analysis of the effect of calcium, calcium inhibition, water stress and dark on TRAMP mRNA expression in wild type tomato seedlings. (a) Roots and stems of one month old wild type seedlings treated with increasing concentrations of $CaCl_2$ in 471 mg/l of Murashige and Skoog medium with 3% sucrose additions. (b) 3 week-old wild type shoots soaked in SDW or 10mM $LaCl_3$ for 1h and dried for 1 h or 3 h. (c) MG fruit under normal growth room conditions or in the dark for 36 h and 3 week-old wild type seedlings grown on MSR3 in dark or normal growth conditions (8 h dark, 18℃ and 16 h light, 24℃). 25 μg (a) or 40 μg (b) of total RNA; cDNA probe pNY507; autorad exposure 24 h. UV images of ethidium bromide stained total RNA are shown in the panel above each autoradiograph.

vitamin; pH 5.9) medium in dark or normal growth condition (8 h dark, 18℃ or 16 h light, 24℃) was investigated by RNA gel blot analysis (Figure 2c). The levels of TRAMP mRNA transcripts were substantially higher in the dark seedlings. The higher levels of TRAMP expression in the dark were also observed when harvested MG fruits were incubated in the dark or normal growth condition (8 h dark, 18℃ or 16 h light, 22℃) for 36 h (Figure 2c). Tomato fruits have low transpiration rates and solute and water influx is dominated by phloem transport. At high light intensities, the uptake rates of water are increased and have been shown to decline at low light intensities (Adams 1986). In this study it was found that mRNA expression of TRAMP in dark conditions was much higher than in the light under normal growth condition. These suggests that TRAMP function as a water channel may be doubted because of several reasons; no water content changed during ripening, in light condition TRAMP expression was lower and drought condition was not changed TRAMP expression.

Gene silencing

In many cases, rather than achieving overexpression, the transgene and homologous host gene are coordinately suppressed in the plants, a phenomenon called "co-suppression" or "sense suppression" (Napoli et al. 1990; Smith et al. 1990). A transgenic line, which was used in experiments, carries a TRAMP gene in the sense orientation and showed co-suppression (gene silencing) of the TRAMP mRNA approximately 90% lower than wild type. Flowering was normal, leaves larger and darker, but fruit setting was extremely low because of abnormal pollen. Root growth was also poor and slower than controls.

Similar examples of gene silencing in tomato have been shown to be due to post transcriptional gene silencing (Hamilton et al. 1998). Post transcriptional gene silencing (PTGS) involves targeted degradation of sequencing-specific and homologous RNAs in the cytoplasm so that the control mechanism can act against transgenes, endogenous genes, and viruses (reviewed in Depicker and Van Montagu 1997; Stam et al. 1997; van den Boogaart et al. 1998). One possible explanation for gene silencing in tomato is that antisense RNAs may induce a double-stranded RNA-dependent degradation system. When radioactively labelled double-stranded RNA is added to an extract of *Drosophila* embryos (Tuschl et al. 1999), the RNA is degraded into oligonucleotides 21 to 23 nucleotides long. Hamilton and Baulcombe (1999) also suggested that small antisense RNA (asRNA), approximately 25 nucleotides in length, was likely to be synthesized from an RNA template and may represent the specificity determinant of PTGS.

The 3S88 contains a full length pNY507 cDNA including the poly (A) tail from the plasmid pNY507 (Fray et al. 1994) in the sense orientation under the control of the CaMV 35S promoter (Chen et al. 2001). RNA gel blot analysis of gene expression was carried on wild type tomato and transgenic

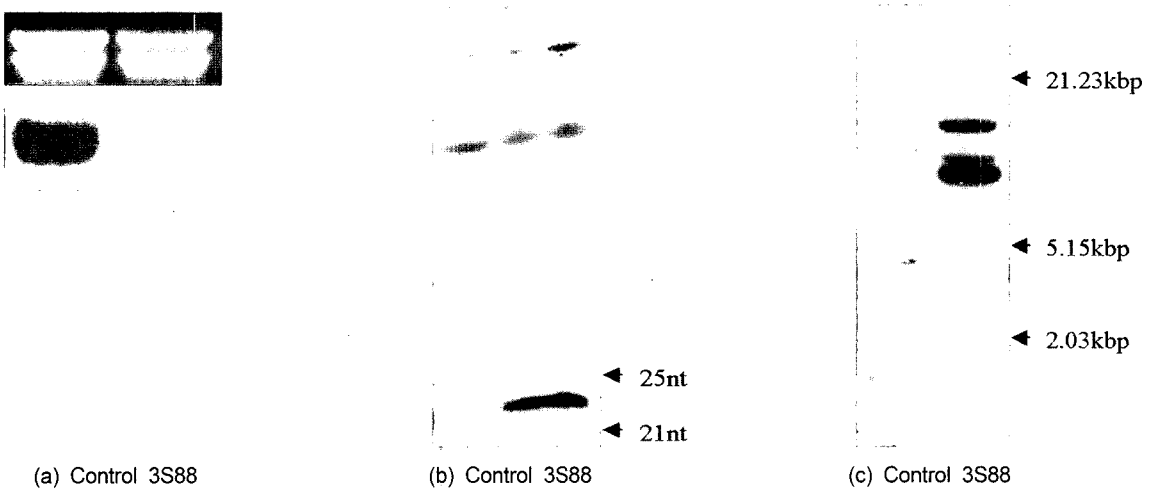

Figure 3. asRNA derived from transgenic plants showing co-suppression of TRAMP expression. (a) RNA gel blot analysis of TRAMP gene expression in tomato wild type and 3S88 line tomato fruits (B+7). 25 μg of total RNA; cDNA probe pNY507; autorad exposure 24 h. UV images of ethidium bromide stained total RNA are shown in the panel above a autoradiograph. (b) 3S88 line plants, carrying a sense TRAMP transgene and control, were analyzed for the presence of small RNAs implicated in co-suppression. Antisense-specific riboprobes used to detect small TRAMP asRNAs were derived from PCR product of pNY507, to whose 5' end T3 promoter incorporated. (c) Southern analysis of 3S88. 40 μg of genomic DNA extracted from young leaves of transgenic (3S88) and wild type, *BamHI* digestion, cDNA probe *NPTII*, exposure 24 h. The positions attained by co-migrating DNA size markers are indicated on the autoradiogram.

plant. The levels of TRAMP mRNA transcripts were substantially higher in wild type tomato fruit at B+7 but the expression in co-suppression line was highly inhibited (Figure 3a). Co-suppression line plants were analysed to confirm small RNA-mediated co-suppression. The antisense-specific riboprobe used to detect small TRAMP asRNAs was derived from a PCR product of pNY507 with T3 promoter incorporated at the 5' end. Two co-suppression plants showed small asRNA, approximately 21-25 nucleotides in length, by the antisense-specific riboprobe but control did not (Figure 3b). The results suggest that small asRNA may be involved in co-suppression of the TRAMP mRNA in sense gene silenced (co-suppressing) plants. Genomic DNA samples of co-suppression and wild-type plants were digested with *BamHI* and analysed by Southern gel blot (Figure 3c). Three bands in the co-suppression plants were identified by the *NPTII* probe, showing presence of the sense transgene whereas none was identified in wild type controls. Intensity of the weakest band was a half of upper band and intensity of lower band showed more stronger intensity than upper band in Southern result. Therefore, transgene copy number might be approximately 6-7 copies (1 from middle, 2 from upper, 3-4 from lower) and this result was same as gene silencing induction by high copy number of transgene (Kim and Grierson 2005). Further experiments results also showed same results (Data was not shown) that high copy number of transgene produced small antisense RNA, approximately 21-25 nucleotides in length, mediated post-transcriptional gene silencing without any production of double strand RNA.

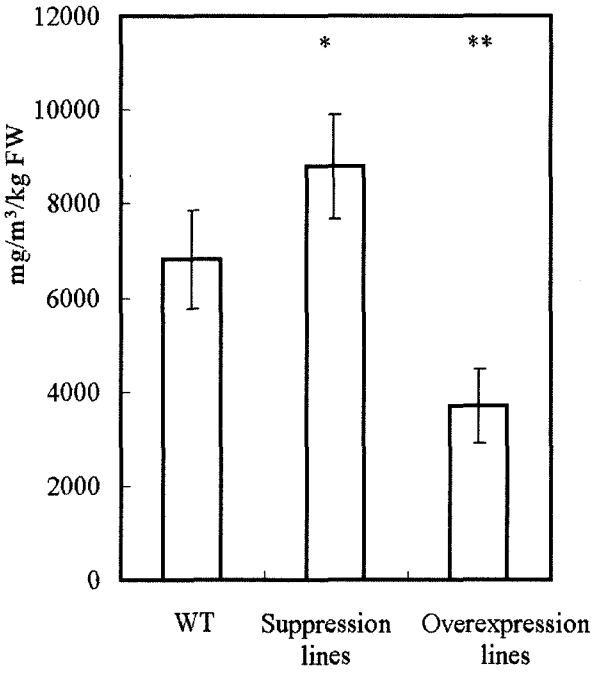

Figure 4. Total amounts of seven different compounds generated by wild type (wt), TRAMP antisense(3A29), TRAMP sense (3S03) tomato seedlings. Seven different compounds-isobutylthiazole, 6-methyl-5- hepten-2-one, hexanal, hexenal methylbutanal, hexenol, and methylbutanol -were measured from 4 weeks old seedlings. Data shows the average maximum signal intensity of four replicates, representing the maximum concentration, over a 1 min incubation period after maceration. Concentrations of the samples were expressed as mg volatile compound/ Fresh weight kg $\times m^3$ air. Error bars are $\pm$S.D. Superscripts indicate levels of significance (paired t-test), *P<0.05 and **P<0.01

Flavour and aroma compounds in fresh tomato seedling.

The full flavour and aroma of tomato fruit is a reflection of the many volatiles present. Over 400 compounds have been identified, including phenols, hydrocarbons, ethers, aldehydes, alcohols, esters, ketones, lactones, sulphur compounds, amines and a variety of heterocyclic molecules that are thought to influence fruit flavour and aroma (Hobson and Grierson, 1993). The volatiles of tomato leaves have been investigated previously (Andersson et al. 1980; Urbasch 1981), however, the compounds responsible for the characteristic tomato leaf aroma was not identified in these previous studies until further investigation of tomato leaf volatiles was carried out by Buttery et al (1987).

The homozygous wild type tomato seeds (*Lycopersicon esculentum* Mill. Cv. Ailsa Craig), TRAMP antisense and sense line seeds (Chen et al 2001) were sterilized and then sown on MSR3 agar medium. Volatile generation by 4 weeks old tomato seedlings (TRAMP antisense and sense lines) were monitored in order to determine whether there were differences compared with wild type. Tomato seedlings were blended in a modified commercial food blender with three Swagelok bulkhead fittings bolted into the blender lid. The headspace within the device was continually flushed with air (170 ml/min) so that volatiles formed were removed rapidly (total volume of device 355 ml; one change of air every 2 min).

Total seven selected volatiles, isobutylthiazole, 6-methyl-5-hepten-2-one, hexanal, hexenal methylbutanol, hexenol, and methylbutanol, were produced less in sense line than wild type and antisense line (Figure 4). Total volatile production in sense line was 3697.5 mg/m^3/kg FW while total volatile productions in wild type and antisense lines were 6812.5 mg/m^3/kg FW and 8794.5 mg/m^3/kg FW respectively. Total volatile production in sense line was 3115 mg/m^3/kg FW lower than wild type and also 5097 mg/m^3/kg FW lower than antisense line. Total volatile production in antisense line was 1982 mg/m^3/kg FW higher than wild type and also 5097 mg/m^3/kg FW higher than antisense line. Therefore, total volatile production was increased in TRAMP

antisense line whereas the production was decreased in TRAMP sense line.

The main precursors of endogenous volatile compounds are carbohydrates, lipids and proteins. The two major routes involved are from the carotenoid (isoprenoid) and amino acid pathways. A number of volatiles are derived from amino acids and the principal substrates are leucine, isoleucine, valine, alanine and phenylalanine (Buttery and Ling 1993). In TRAMP antisense, decarboxylation might be induced as results as an increase of amino acids (Chen et al. 2001) and volatiles but volatile productions were decreased in TRAMP sense line. Therefore, TRAMP might *inhibit the volatile productions.*

References

Adams P (1986) Mineral nutrition. In: Atherton JG, Rudich J (eds) The Tomato Crop. Chapman and Hall Ltd., pp 281-334

Alpuche-Solis AG (1999) Analysis of transgenic tomato plants with ACC oxidase suppressed by sense constructs. PhD thesis

Andersson BA, Holman RT, Lundgren L, Stenhagen G (1980) Capillary gas chromatography of leaf volatiles. J Agric Food Chem 28: 985

Apelbaum A, Yang SF (1981) Biosynthesis of stress ethylene induced by water deficit. Plant Physiol 68: 594-596

Borgnia M, Nielsen S, Engel A, Agre P (1999) Cellular and molecular biology of the aquaporin water channels. Annu Rev Biochem 68: 425-458

Boukobza F, Taylor AJ (2002) Effect of postharvest treatment on flavour volatiles of tomatoes. Postharvest Biol Technol 25: 321-331

Buttery RG, Ling LC (1993) *Volatile components of tomato fruit* and plant parts. In: Bioactive Volatile Compounds from Plants, Symposium series 525, American Chemical Society, Washington DC, pp 23-34

Buttery RG, Ling LC, Light DM (1987) Tomato leaf volatile *aroma components.* J *Agric Food Chem* 35: 1039-1042

Chen G (1997) Molecular analysis of the pNY507 and ETR14 ripening-related mRNAs in transgenic tomatoes. PhD. Thesis

Chen G, Wilson ID, Kim SH, Grierson D (2001) Inhibiting expression of a tomato ripening-associated membrane protein *increases organic acids and reduces sugar levels of* fruit. Planta 212: 799-807

Chen YM, Yu SW (1988) Effects of different types of water stress on ethylene production, contents of ACC, and MACC in wheat plants. Acta Phytophysiol Sin 14: 281-288

Deikman J, Kline R, Fischer RL (1992) Organization of ripening and ethylene regulatory regions in a fruit-specific promoter from tomato (Lycopersicon esculentum). Plant Physiol 100: 2013-2017

Depicker A, Van Montagu M (1997) Post-transcriptional gene silencing in plants. Curr Opin Cell Biol 9: 372-382

El-Beltagy AS, Hall MA (1974) Effect of water stress upon endogenous ethylene levels in Vicia faba. New Phytol 73: 47-60

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

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

Hamilton AJ, *Baulcombe* DC (1999) A *novel species of small* antisense RNA in post-transcriptional gene silencing. Science 286: 950-952

Hamilton AJ, Brown S, Yuanhai H, Ishizuka M, Lowe A, Alpuche-Solis AG, Grierson D (1998) A transgene with *repeated DNA causes high frequency, post-transcriptional* suppression of ACC-oxidase gene expression in tomato. Plant J 6: 737-746

Han Y, Grierson D (2002) Relationship between small antisense RNAs and aberrant RNAs associated with sense transgene *mediated gene silencing in tomato.* Plant J 29: 509-519

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 and Hall, Lond, pp 1-51

Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10: 451-459

Johnson KD, Herman EM, Chrispeels MJ (1989) An abundant highly conserved tonoplast protein in seeds. Plant Physiol 91: 1006-1013

Kim SH, Grierson D (2005) Subcellular localisation and silencing of ripening-associated membrane protein (TRAMP) in tomato *Lycopersicon esculentum Mill.* Plant Science In press

Kimmerer TW, Kozlowski TT (1982) Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. Plant Physiol 69: 840-847

Kneissl ML, Deikman J (1996) The tomato E8 gene influences ethylene biosynthesis in fruit but not in flowers. Plant Physiol 112: 537-547

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc Natl Acad Sci USA 84: 2793-2797

Linforth RST, Taylor AJ (2000) Manual for gas phase APCI software. Samworth flavour laboratory, Division of food sciences, University of Nottingham

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 (1997) Aquaporins and water permeability of plant membranes. Annu Rev Plant Physiol Plant Mol Biol 48: 399-430

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *petunia* results in reversible co-suppression of homologous genes in trans. Plant Cell 2: 279-289

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

Picton S, Gray J, Barton S, Bakar UA, Lowe A, Grierson, D (1993) cDNA cloning and characterisation of novel ripening-related mRNAs with altered Pattern of accumulation in the *ripening inhibitor* (*rin*) tomato ripening mutant. Plant Mol Biol 23: 193-207

Prasad GV, Coury LA, Finn F, Zeidel ML (1998) Reconstituted aquaporin 1 water channels transport CO_2 across membranes. J Biol Chem 273: 33123-33126

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

Smith CJS, Slater A, Grierson D (1986) Rapid appearance of an mRNA correlated with ethylene synthesis encoding a protein of molecular weight 35000. Planta 168: 94-100

Smith CJS, Watson CF, Edwards K, Schuch W, Grierson D (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol Gen Genet 224: 477-481

Stam M, Mol JNM, Kooter JM (1997) The silence of genes in transgenic plants. Ann Bot 79: 3-12

Stumpff NJ, Johnson JD (1987) Ethylene production by loblolly pine seedlings associated with water stress. Plant Physiol 69: 167-172

Swamy-Mruthinti S (2001) Glycation decreases calmodulin binding to lens transmembrane protein, MIP. Biochimica et Biophysica Acta 1536 : 64-72

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 indentification of the glpF product. J Bacteriol 172: 424-430

Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA (1999) Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev 13: 3191-3197

Urbasch I (1981) Antimykotisch wirksame, Fluchtige stoffwechsel produkte aus dem kraut von tamatenpflanzen. Naturwis Senschaften 68: 204

van den Boogaart T, Lomonossoff GP, Davies JW (1998) Can we explain RNA-mediated virus resistance by homology-dependent gene silencing? Mol Plant-Microbe Interac 11: 717-723

Yang B, Fukuda N, van Hoek AN, Matthay MA, Ma T, Verkman AS (2000). Carbon dioxide permeability of aquaporin-1 measured in erythrocytes and lung of aquaporin-1 null mice and in reconstituted proteoliposomes. J Biol Chem 275: 2686-2692