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Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression

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Abstract

A tomato phytoene synthase gene, *Psy1*, has recently been isolated as the clone GTOM5 [20] and shown by sequence identity to be the gene from which the major fruit-ripening cDNA clone TOM5 [19] was derived. Sequence analysis of transcripts from two allelic yellow-fruited tomato mutants, mapped to chromosome 3, has shown the lack of carotenoids in fruit of these mutants to be due to the production of aberrant TOM5 transcripts which are unlikely to encode a functional phytoene synthase enzyme. In one mutant (*yellow flesh*) the aberrant transcript contained a sequence that, by its strong hybridization to a wide size range of genomic fragments, appeared to be repeated many times within the genome. Southern and PCR analysis of the phytoene synthase genes in the mutant revealed restriction fragment length polymorphisms, suggesting that the production of altered mRNAs was associated with specific genomic rearrangements. Constitutive over-expression of a TOM5 cDNA clone in transgenic mutant plants restored synthesis of the carotenoid lycopene in ripening fruit and also led to unscheduled pigment production in other cell types. In some mutant plants transformed with the TOM5 cDNA construct, inhibition of carotenoid production in immature green fruit, leaves and flowers was observed, due to the phenomenon of co-suppression, indicating that different insertion events with the same gene construct can lead to overexpression or co-suppression in transgenic plants. Green organs of these plants were susceptible to photobleaching, due to the lack of carotenoids. These results suggest the existence of separate *Psy* genes for carotenoid synthesis in green organs.

Introduction

The history of tomato fruit colour mutants is a long one and appears to predate the European cultivation of tomato. As early as 1623, yellow, golden, red and white fruit varieties were known [9]. In 1700, seven forms of tomato were described, including a pale red one. By the begin-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X67143 (*r* mutant) and X67144 (r^y mutant).

ning of the 19th century four red varieties and two yellow varieties were grown in Europe, the white, golden and pale red varieties having been lost. Tomato mutants featured as early as 1906 for genetic studies when they were used for testing Mendel's rediscovered laws of inheritance [18]. It was at this time that the *yellow flesh* mutant locus was given the symbol *r* (mutant form of red), thereby deviating from the now standard nomenclature rules by being named after the wild-type rather than mutant phenotype.

The *r* phenotype is pale yellow fruit flesh with more intensely yellow-coloured fruit skin, and is also associated with a paler, lemon-yellow flower corolla. The ripe fruit contain virtually no carotenoids [12] and the yellow skin pigment is primarily due to the accumulation of the flavonoid chalconaringenin [11]. Given the long history of cultivation of yellow-fruited tomatoes, it seems probable that the original mutation that gave rise to *r* occurred in an ancestral polymorphic population in South America. A second mutant, r^y, with a similar phenotype to *r*, but with a normal coloured corolla, arose spontaneously [6]. The two mutants map to the same location on chromosome three and, as they do not complement each other, are probably mutations of the same gene. Both mutant alleles have been transferred to cv. Ailsa Craig by repeated backcrossing, as part of a programme to create near-isogenic lines only differing in the mutant genotype [6]. Plant carotenoids, together with phytol, plastoquinone, ubiquinone and other terpenoids, are biosynthesized via the isoprenoid pathway. The first step unique to carotenoid synthesis is the condensation of two molecules of the C_{20} compound geranylgeranyl diphosphate (GGPP) to give the first C_{40} compound, phytoene. In *Erwinia* and *Rhodobacter*, two enzymes are believed to be required for this step, the first making the intermediate prephytoene pyrophosphate and the second converting this to phytoene. An alternative interpretation, that there is only one enzyme that catalyses both steps in bacteria has been proposed recently by Bartley *et al.* [2]. A single bifunctional enzyme that catalyses both reactions has been purified from *Capsicum annum* [8]. This monomeric enzyme has a similar molecular weight to that predicted for the protein encoded by the tomato fruit cDNA TOM5, and it has been proposed that TOM5 encodes a similar bifunctional tomato fruit enzyme [5].

Evidence that TOM5 encodes phytoene synthase was obtained by transforming wild-type tomato plants with a construct designed to make antisense RNA to the first 794 bp of the TOM5 message. Some of these transgenic plants produced yellow fruit and flowers with a pale yellow corolla [5]. This altered phenotype was associated with a severe reduction in TOM5 homologous mRNA during ripening and a reduction in fruit carotenoid levels to between 3% and 5% of normal. However, leaf carotenoid levels were unaffected, suggesting that in this tissue any enzyme catalysing the same step must be encoded by a mRNA substantially different from the antisense sequence used. By the homology of the derived amino acid sequence to those of the *crtB* genes of *Erwinia herbicola* and *Rhodobacter capsulatus* [1], the protein encoded by TOM5 was proposed as being involved in phytoene synthesis [5]. We show in this paper that the *r* and r^y mutations, which map to chromosome 3, encode defective TOM5 sequences and that the *r* mutation can be complemented by over-expressing the wild-type sequence in transgenic mutant plants. In other transformants, TOM5-related genes were down-regulated by co-suppression, resulting in white flowers and photobleaching of green tissues.

Materials and methods

Construction of transgene and plant transformation

The construct pBD5ST was designed to over-express a functional TOM5 transgene. A 1455 bp *Ssp* I fragment that contained all of the translated region was ligated into the *Sma* I site of pT7T3α18 (Bethesda Research Laboratories). This fragment was then removed by cutting with enzymes that cut within the plasmid multiple cloning site [24]. First an *Eco* RI digest was performed, the ends were made blunt by incubation with Klenow en-

zyme and the fragment removed by a further digestion with *Bam* HI. The expression vector, pDH51 [17], was cut with *Sal* I, blunted with Klenow then digested with *Bam* HI. This allowed the TOM5 fragment to be directionally cloned into pDH51 between the CaMV 35S promoter and terminator. This expression cassette was then removed by an *Eco* RI digest and ligated into the *Eco* RI site of the binary transformation vector, BIN19 [3], to give pBDH5ST (Fig. 6). After being transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating [3], this construct was used for transforming cotyledons of the *r* mutant of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) [4]. Transgenic plants that rooted on kanamycin were transferred to compost and grown under glass with day and night temperatures of 22 °C and 18 °C respectively and 16 h daylight with supplementary lighting where required (tungsten bulbs, 59 μE m^{-2} s^{-1} at ground level).

RNA isolation and northern analysis

RNA was extracted from tomato fruit pericarp and leaves as described for leaves by Smith *et al.* [25] except that contaminating carbohydrate and DNA were removed by differential precipitation of the RNA in 4 M lithium chloride at −20 °C for 1 h. RNA was quantified by spectrophotometry and 15 μg fractionated on 1.2% agarose after denaturing with glyoxal. RNA was blotted onto GeneScreen (NEN Research Products) membranes which were then prehybridized in 5 × SSPE [21], 1% SDS, 5 × Denhardt's solution and 100 μg/ml sheared denatured salmon sperm DNA. The RNA was hybridized in the same buffer, to probes made by nick-translating pTOM5 cDNA sequences. Membranes were washed in 0.2 × SSPE, 1% SDS at 65 °C and autoradiographed.

DNA extraction and Southern analysis

Tomato genomic DNA was extracted according to the method of Shure *et al.* [23], except that precipitation of DNA with 10% polyethylene glycol, 0.5 M sodium chloride replaced buoyant density ultracentrifugation. Genomic DNA was cut with the appropriate restriction enzymes and 10 μg of each sample were fractionated on a 1% agarose gel. This was then transferred to GeneScreen Plus membranes (NEN Research Products) according to the manufacturer's instructions. Hybridization and wash conditions were the same as those described above for northern analysis.

DNA sequencing

DNA sequence determination was carried out by the dideoxynucleotide chain termination method of Sanger *et al.* [22]. Sequence-specific synthetic primers were used where appropriate so that the novel sequences were sequenced on both strands and unambiguous sequence was obtained on at least one strand for the previously published TOM5 sequence.

Mutant analysis by PCR

A thermal cycler (TECHNE PHC-3) was set for the following program; one cycle of 95 °C for 5 min, 53 °C for 1 min, 72 °C for 2 min; thirty cycles of 94 °C for 1 min, 53 °C for 1 min, 73 °C for 2 min. This last cycle was repeated once with the final incubation at 73 °C extended to 15 min. 5 units of *Taq* DNA polymerase (Boehringer Mannheim) were used in each reaction. For the analysis of the *Psy1* gene 0.5 μg of genomic DNA were used. For the cloning of the mutant cDNAs, first-strand cDNA generated from 20 μg of total RNA were used in the amplification reactions.

Extraction and estimation of carotenoids and chlorophylls

Tissue samples of 1 g were ground with hexane-acetone (60:40 v/v) with an ice cold pestle and mortar. The supernatant was taken and the cell

debris repeatedly extracted with fresh solvent until colourless. The pooled supernatants were centrifuged at 3300 × *g* for 5 min, the solvent volume measured, made up to 20 ml and the absorption spectrum measured (400–700 nm). The carotenoids and chlorophylls present were estimated according to published extinction coefficients [7, 15].

Results and discussion

Two genes with sequence homology to the tomato ripening specific cDNA clone, TOM5, have previously been cloned from a wild-type Ailsa Craig background [20]. Both have been sequenced and one, GTOM5 [20], contains exon sequences identical to TOM5 and probably represents the gene (designated *Psy1* by Bartley *et al.* [2]) from which it was transcribed (Fig. 1). The other clone, clone F, showed only limited homology and appears to be derived from a second gene (*Psy2*). Due to its apparent non-expression it was proposed that it might be a pseudogene [20]. Loci with homology to TOM5 have previously been mapped to chromosomes 2 and 3 [14]. Comparison of the molecular and classical maps showed that the TOM5-related gene on chromosome three was close to the position of the *r* and r^y mutant alleles [14]. Therefore, the possible relationship between these mutations and TOM5-related genes was investigated.

Analysis of mutant TOM5 mRNAs

When the expression of TOM5 in the ripening fruit of the two mutants was studied by northern analysis it was found that the size of the TOM5 mRNA of the *r* mutant was approximately 850 nucleotides, 750 bases shorter than the wild-type message (Fig. 2). The corresponding mRNA produced in the r^y mutant also appeared to be slightly reduced in length, and was estimated as ca. 50 nucleotides shorter than in the wild type (Fig. 3). When a similar northern blot was probed with a TOM5 probe that lacked the first 500 nucleotides, no hybridization to the *r* fruit RNA was detectable (data not shown).

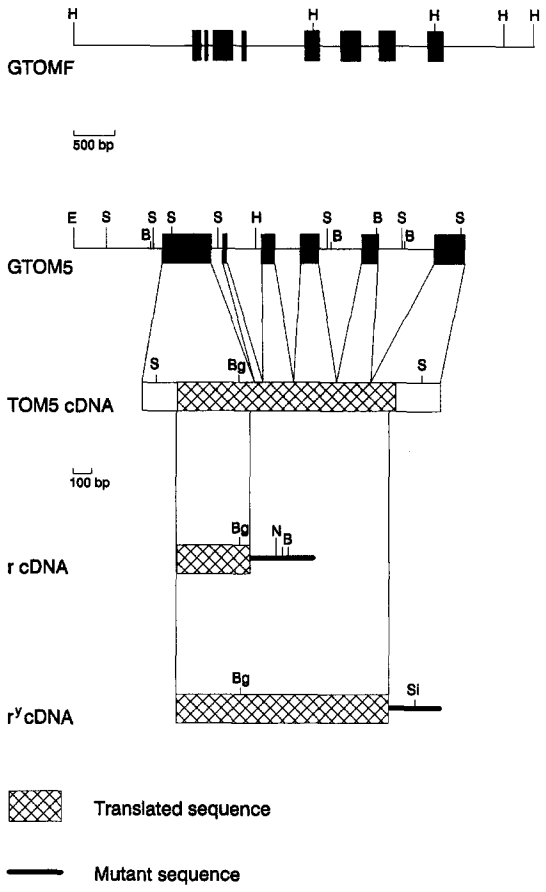

Fig. 1. Relationship between TOM5 cDNA, the cloned chromosomal sequences GTOM5 and clone F, and mutant cDNAs from the *r* and r^y mutants; B, *Ban* II; Bg, *Bgl* II: H, *Hind* III; N, *Nar* I; S, *Ssp* I; Si, *Sin* I. The gene corresponding to the GTOM5 sequence, which gives rise to the TOM5 cDNA, has been named *Psy1* [2].

In order to determine the nature of the mutant messages, cDNA was generated from total RNA extracted from the mutant fruit using as a primer a 25-nucleotide poly T primer attached to 20 bp of 3′ sequence (boxed in Fig. 3) containing several restriction enzyme sites. After removal of RNA and primer, the TOM5 messages were amplified by PCR using an oligonucleotide corresponding to the 20 bp sequence as the 3′ primer and a similar lengthed oligonucleotide complementary to the beginning of the translated sequence as the 5′ primer (boxed in Fig. 3), with a *Bam* HI site added at its 5′ end. The resulting PCR products were cloned via an *Eco* RI-*Bam* HI

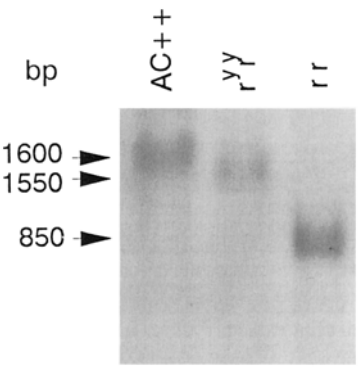

Fig. 2. Northern blot of fruit RNA from Ailsa Craig wild type and the r^y and *r* mutants. The approximate sizes of the mRNAs, estimated by fractionating size markers in the same gel, are indicated on the left.

digest into the plasmid pT7T3α18. Transformants were screened for those that released an insert hybridizing to TOM5 when cut with these enzymes. In this way cDNAs corresponding to the TOM5 mRNAs from the normal translation start site to the poly(A) tail were cloned from the two mutants. Insert sizes were 650 bp and 1350 bp for the *r* and r^y mutants, respectively, 200 nucleotides shorter than the sizes determined by northern hybridization due to the absence of the untranslated 5′ sequences (Figs. 1 and 3). Upon sequencing, the cloned fragment of the *r* cDNA was found to be identical to the TOM5 sequence for the first 319 nucleotides of translated sequence. Thereafter, homology broke down completely for a further 328 nucleotides until the poly(A) tail was reached. An in-frame stop codon exists within this 328 base sequence and a protein of 17.9 kDa would be predicted. Of its 164 amino acids only the first 107 would be shared with the 413 amino acids encoded by the wild type TOM5 message. The r^y cDNA was found to be mutated at its 3′ end, lacking the last 237 bases of pTOM5 homologous sequence and containing 185 nucleotides of an unrelated sequence in its place (Fig. 3). This would result in a protein in which the terminal 25 amino acids of the normal protein were replaced by an unrelated sequence of 23 amino acids. It is interesting that the mRNA sequence of the *r* allele is identical to that of the wild type for the first 319 nucleotides until the boundary with the novel sequence, particularly in view of its potentially ancient origin.

Computer searches revealed no significant homologies between the novel mutant sequences and any published sequence. However, when Southern blots of both *yellow flesh* and wild-type *Dra* I-cut genomic DNA were probed with the 328 bp of the cloned *r* mutant sequence (contained on a *Bgl* II-*Eco* RI cDNA fragment), an intensely hybridizing ladder of bands was observed (Fig. 4). Thus it would appear to represent a highly repeated sequence, such as a fragment of a transposable element.

Analysis of Psy1 *genes from mutant and wild-type plants*

In order to establish that the novel 328 bp sequence of the cloned *r* TOM5 cDNA was derived from transcription of a mutated *Psy1* gene, Southern analysis was performed. Knowledge of the cloned GTOM5 (*Psy1*) and *r* cDNA sequences allowed predictions to be made for expected DNA fragment sizes following digestion with various restriction enzymes (Fig. 1). The *r* sequence contains *Ban* II and *Nar* I sites within the 328 bp 3′ region, but it does not contain any *Ssp* I sites, whereas the wild type GTOM5 (*Psy1*) sequence has no *Nar* I site. Thus, if the cloned *r* sequence is derived directly from a mutated *Psy1* gene, an *Eco* RI-*Nar* I digest of genomic DNA, probed with an *Ssp* I-*Bgl* II fragment from the 5′ end of the TOM5 cDNA, would be expected to produce a band of 1730 bp in the case of the *r* mutant, and a band greater than 4800 bp in that of wild type (see Fig. 1). Similarly, bands of 2143 bp in wild type and 800 bp in *r* would be predicted for an *Eco* RI-*Ban* II digest, whereas an *Ssp* I digest should generate a band of 573 bp in wild type and a size greater than this in *r*. All of these predicted bands were observed (Fig. 5a). In addition, an oligonucleotide with the sequence GGCATTCAACATCCGGCT, complementary to the 3′ sequence that spans the boundary of the TOM5 and mutant sequence in the *r* cDNA (shown boxed in Fig. 3) was used successfully to amplify

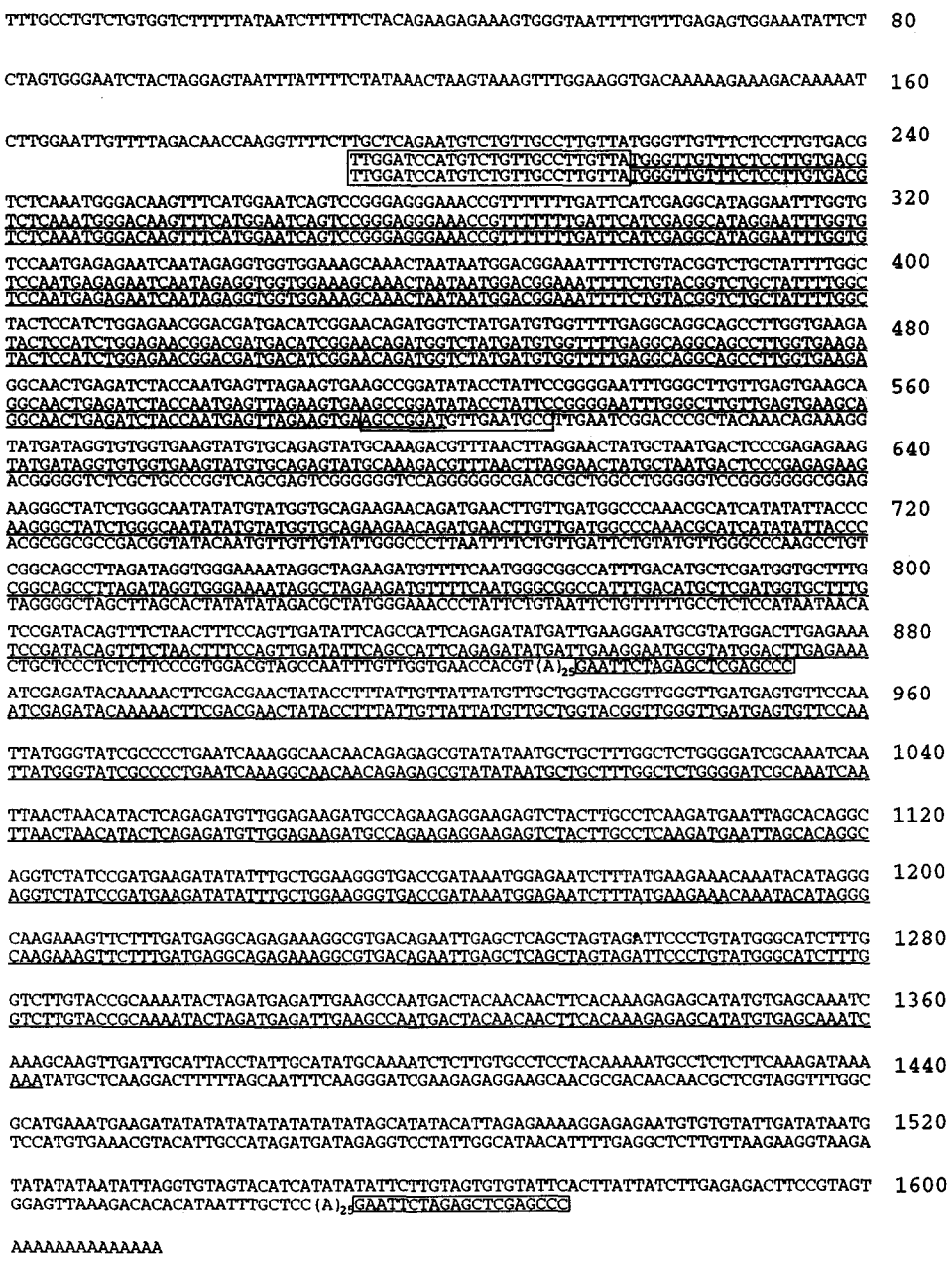

Fig. 3. Sequence of TOM5 cDNA with the cloned sequences of r^y and *r* beneath. Regions of the mutant sequences that are homologous to the wild-type message are underlined. The oligonucleotide sequences used for PCR are shown boxed.

a fragment of 340 bp from *r* but not wild-type DNA when used in a PCR reaction in conjunction with the 5′ primer used in the cDNA cloning described above (Fig. 5b). Taken together these results suggest that the novel 328 bp of the *r* cDNA clone are directly derived from a mutated *Psy* I gene and are not the result of post-transcriptional RNA processing. In separate experiments, bands of 2910 and 1500 bp were observed both for wild type and *r* when a *Hin*d III digest was hybridized with a full-length TOM5 cDNA probe in 5× SSPE 50% formamide at

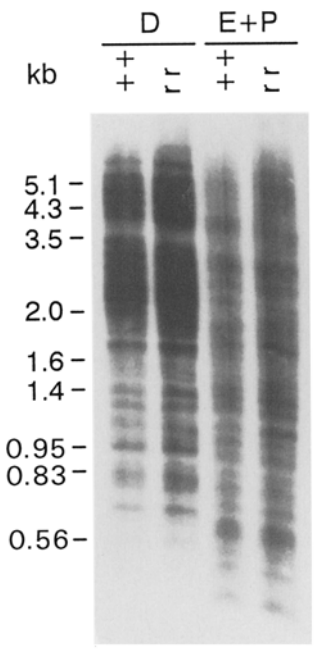

Fig. 4. Southern blot of wild type (+ +) and *yellow flesh* (*rr*) genomic DNA probed with the 328 bp of the novel mutant sequence. DNA was digested with: D, *Dra* I, E, *Eco* RI; P, *Pst* I.

42 °C and washed at the lower stringency of 1 × SSPE (data not shown). These bands are the sizes predicted for the clone F (*Psy2*) gene based on the cloned sequence (Fig. 1). When autoradiography of the probed membrane shown in Fig. 5a was carried out for a longer period, a number of additional bands were observed both in the mutant and wild type lanes. These bands were of a size that could not be explained by partial digestion of the DNA or by hybridization to fragments derived from the known clone F (*Psy2*) sequence, thus they may indicate the presence of a further TOM5-related gene or genes. To test for the presence of the TOM5 sequence that is absent in the cloned cDNA of the *r* mutant, an *Eco* RI-*Sin* I DNA digest was probed with a labelled *Ssp* I-*Bgl* II fragment from the 3′ end of the TOM5 cDNA (Fig. 5). The presence of a hybridizing band in *r* demonstrates the presence in the genome of at least some of this sequence, thus the mutation is probably due to an insertion, inversion or translocation rather than a simple deletion. In addition to wild type and *r*, *Eco* RI-*Sin* I-cut genomic DNA from the r^y mutant and a non-allelic mutant, *white flower* (*wf*), was also probed with the same TOM5 cDNA fragment. The r^y TOM5 cDNA clone contains a *Sin* I site in the terminal 185 bp of sequence, the cloned wild type *Psy1* sequence contains no such site. Thus if the novel 185 bp of the r^y cDNA are derived directly from a mutated *Psy1* gene, a hybridizing fragment of 4700 bp would be predicted, which is the approximate size of the observed fragment (Fig. 5b).

Transformation of yellow flesh *plants with a functional TOM5 cDNA*

A chimaeric TOM5 sense gene was constructed (Fig. 6) by cloning a 1455 bp *Ssp* I fragment of the cDNA containing the full-coding sequence (Fig. 1) into the plant expression cassette of pDH51. The chimaeric gene was removed by digestion with *Eco* RI and inserted into the *Eco* RI site of the binary transformation vector BIN19 [13] to give the construct pBDH5ST. This plasmid was transferred to *Agrobacterium tumefaciens* LBA4404 by triparental mating and used to transform tomato cotyledon explants of the *r* mutant. Plants were selected for their ability to grow on kanamycin. An early indication that the transgene was producing a functional protein was the generation of pink/red-brown callus in some of the transgenic lines (Fig. 7a). This novel colour was not seen with callus generated using unrelated constructs and was presumably due to the accumulation of pigmented carotenoids such as lycopene. Upon differentiation, the pink colouration was not observed in any subsequent vegetative tissue, with the exception of the abscission zones of flowers and fruit (Fig. 7b).

Transgenic plants were transferred to the glasshouse and grown to maturity. Southern analysis was performed on genomic DNA extracted from leaves of several of these plants. The presence of an intact transgene was confirmed in each case by the presence of a hybridizing band of 2250 bp when the transgenic plant DNA was digested with

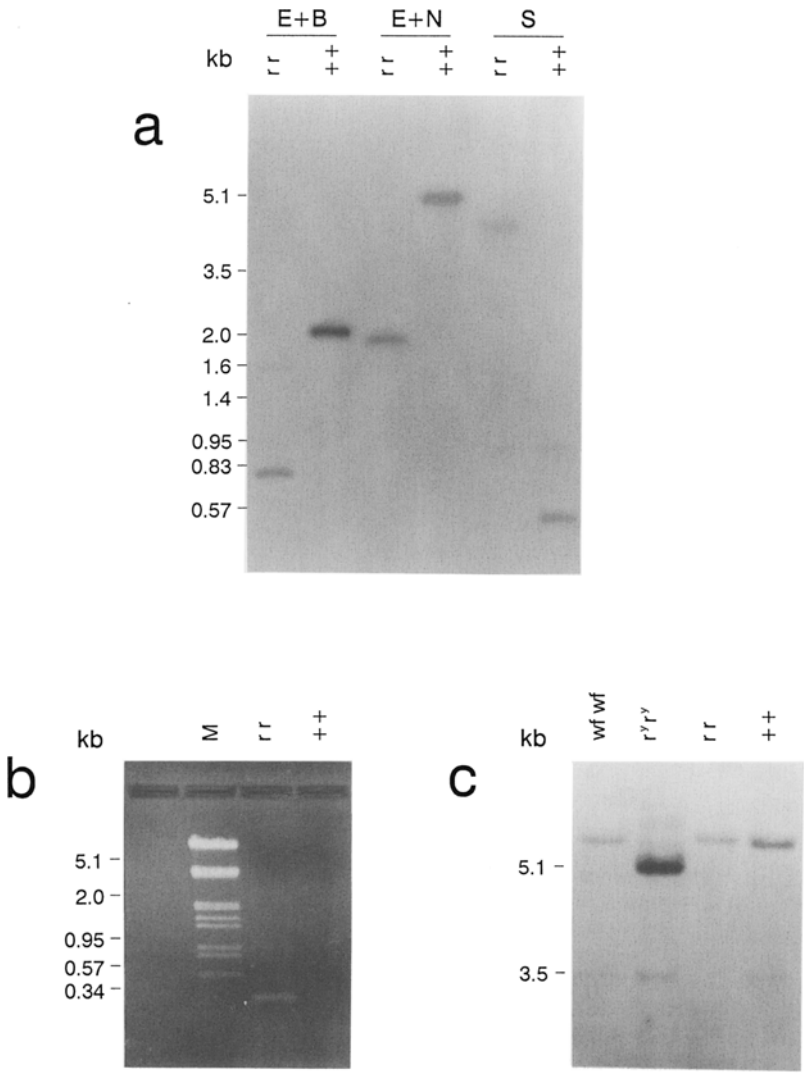

Fig. 5. Characterisation of the mutant phytoene synthase gene. a. Southern blot of *r* mutant and wild-type genomic DNA probed with the 5′ *Ssp* I-*Bgl* II fragment of TOM5 cDNA. Restriction enzyme abbreviations are the same as in Fig. 1. The major hybridizing bands (left to right) correspond to the predicted sizes of 800, 2143, 1730, <4800, >573 and 573 bp. b. Ethidium-stained gel of the products of a PCR carried out using a 3′ oligonucleotide specific to the *r* mutant and the 5′ oligonucleotide shown in Fig. 2. A band of 340 bp is present in the mutant but not wild-type lanes. *Eco* RI-*Hin*d III-cut lambda phage DNA (lane M) was run as size markers. c. Southern blot of *wf*, r^y, *r* and wild type digested with *Eco* RI and *Sin* I and probed with the 3′ *Ssp* I-*Bgl* II fragment of TOM5. Examination of the ethidium stained gel prior to DNA transfer indicated that the amount of r^y DNA loaded was ca. 1.5 that of the other samples.

Eco RI and probed with TOM5 cDNA (Fig. 6 and Fig. 8). A range of phenotypes were observed in the ripe fruit of these plants, from a yellow colour indistinguishable from the *r* parent (seven plants), through varying shades or orange to red (six plants) (Fig. 7c). Red transgenic fruit, but not the untransformed yellow controls, were shown to contain lycopene (Table 1). Fruit mRNA was analysed for three of the plants that produced orange or red tomatoes. As well as the endogenous truncated *r* TOM5 message, there was also a larger transcript derived from the transgene. This larger transcript was not apparent in a yellow-fruited transformant (Fig. 9).

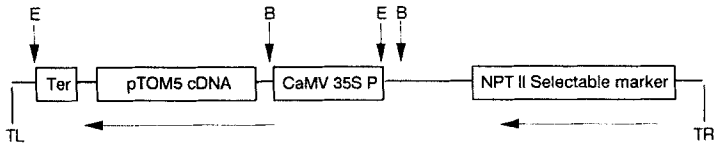

Fig. 6. Structure of the T-DNA containing the wild-type TOM5 cDNA sequence under the control of the CaMV 35S promoter. E, *Eco* RI; B, *Bam* HI restriction enzyme sites.

The successful complementation of the *r* mutant by a functional TOM5 transgene implies that the rearranged *Psy1* gene is the only lesion in the mutant responsible for conferring the yellow phenotype.

As well as developing an orange or red colour upon ripening, a pink colouration was seen in many of the fruit at an early immature stage at which they would normally be green (Fig. 7d). This pink colour correlates with the presence of lycopene in this tissue (Table 1). The accumulation of pigment in the callus, abscission zone and immature fruit, but not in any other vegetative tissue is curious. It could be explained if the pro-

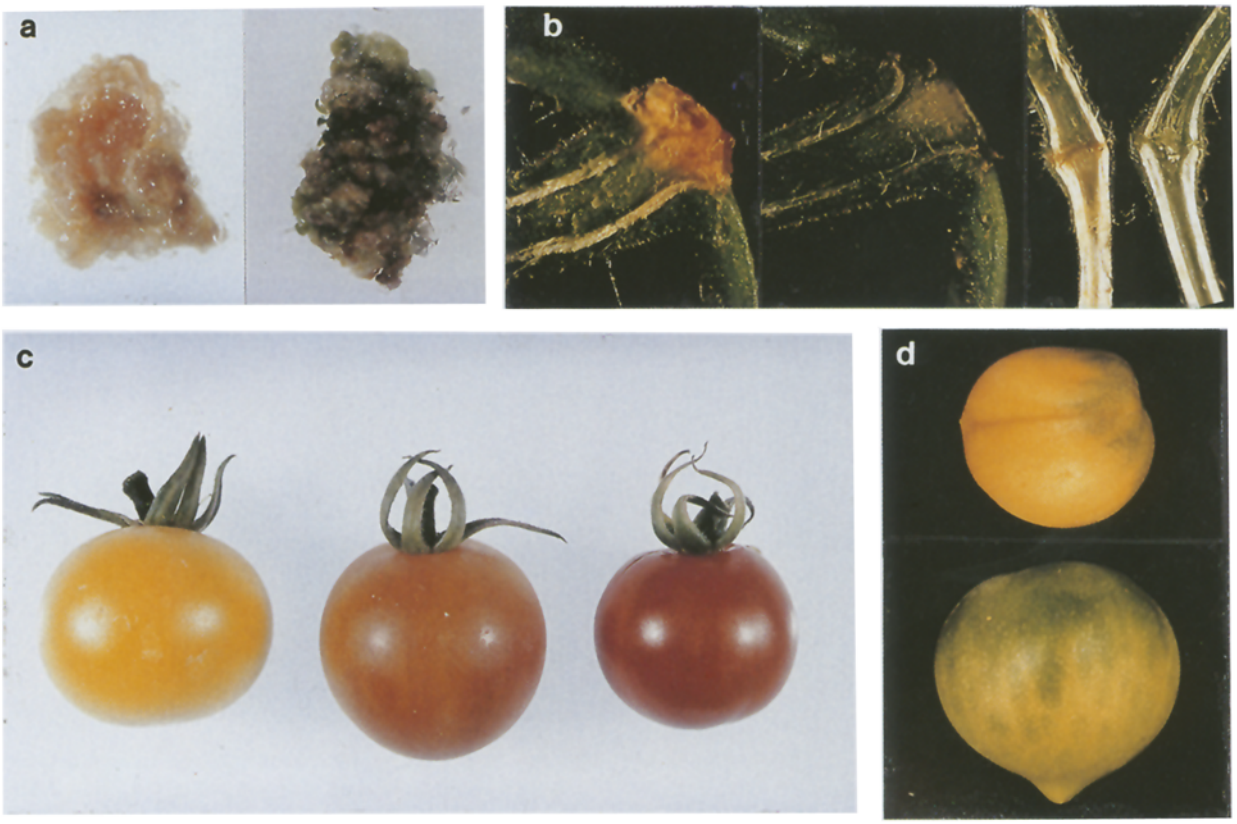

Fig. 7. Over-expression of phytoene synthase in the *r* mutant. a. Pink (pBDH5ST) and green (normal) transgenic callus. b. Red abscission zone of knuckle and pedicel in plant constitutively expressing TOM5 (pBDH5ST14) compared with abscission zones from a wild-type plant. c. Transgenic fruit from three *yellow flesh* lines transformed with pBDH5ST, no complementation (left, pBDH5ST13), partial (centre, pBDH5ST9) or full (right, pBDH5ST14) complementation of the mutant phenotype was observed in different transformants. d. Transgenic over-expresser and wild-type fruit at the 'immature green' stage showing premature accumulation of lycopene in fruit of the transgenic (upper) fruit plant (pBDH5ST14).

Table 1. Carotenoid and chlorophyll content of various tissues of transformed and untransformed tomato plants.

Plant	Tissue	Carotenoids (μg/g)		Chlorophylls (μg/g)
		total	lycopene	
Ailsa Craig	Leaf	321	0	2781
Wild type	Unripe fruit	28	0	84
	Ripe fruit	65	42	0
rr	Leaf	302	0	2611
	Unripe fruit	22	0	71
	Ripe fruit	7	0	0
pBDH5ST21	Leaf	321	0	2638
(*rr* over-expresser)	Unripe fruit	40	15	41
	Ripe fruit	52	22	0
pBDH5ST14	Ripe fruit	81	43	0
(*rr* over-expresser)				
pBDH5ST10	Leaf	130	0	556
(*rr* co-suppressor)	Unripe fruit	4	0	12.2
	Ripe fruit	1.6	0	0

Measurements were made on ripe fruit three days after the onset of ripening.

duction of phytoene from the transgene in these tissues resulted in the accumulation of lycopene at a greater rate than it can be converted to subsequent metabolites, such as β-carotene. Another possibility is that these cells normally contain enzymes required for synthesizing lycopene, but lack phytoene synthase.

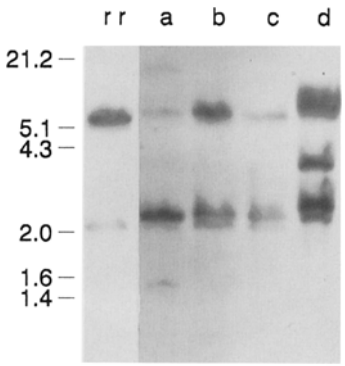

Fig. 8. Southern blot of genomic DNA from transgenic plants cut with *Eco* RI and probed with TOM5. The presence of a band of 2250 bp indicates the presence of an intact transgene. a, pBDH5ST10 (co-suppressor); b, pBDH5ST14; c, pBDH5ST9; d, pBDH5ST21 (over-expressers). As well as the band of 2250 bp, pBDH5ST10 and pBDH5ST21 each contain novel bands of an unpredicted size, implying that an incompletely transferred T-DNA is present in addition to the full length copy. The sizes of DNA fragments run in the same gel are shown on the left in kb.

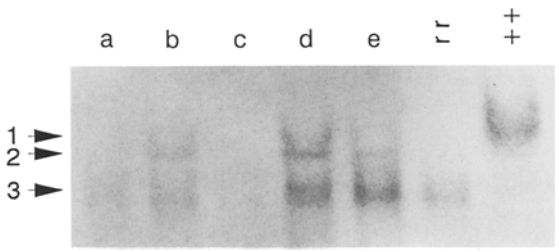

Fig. 9. Northern analysis of fruit RNA from transgenic plants of the *r* genotype, transformed with pBDH5ST. Lanes b, d and e contain RNA from the over-expressers pBDH5ST9, 14 and 21 respectively, lane c contains RNA from the co-suppressed pBDH5ST10 plant and lane a contains that from pBHH5ST13, a yellow-fruited transgenic in which the mutation was not complemented. The arrows labelled 1, 2 and 3 indicate the position of the wild-type (+ +), transgenic and mutant (*rr*) messages respectively.

Co-suppression of phytoene synthase genes in transgenic plants

Of seven transgenic plants containing the TOM5 expression cassette, in which the ripe fruit were still yellow, two produced flowers that were much paler than the *r* parent, being almost white in colour (Fig. 10a). This white flower phenotype parallels the observations made when wild-type plants were transformed with an *antisense* TOM5 gene to produce yellow fruit and pale flowers [5]. Of these two plants, one (pBDH5ST10) produced green fruit and foliage that were highly sensitive to photobleaching (Fig. 10b, c, d). Indeed, at the stage normally described as 'mature green', these fruit were virtually white, although they went on to accumulate a yellow pigment, presumably the flavonoid chalconaringenin, upon ripening (Fig. 10c). Leaf sectors masked from exposure to light by a wrap of aluminium foil remained green, indicating that photobleaching was the primary cause of chlorophyll loss (data not shown). This photobleaching is correlated with a reduction in carotenoids in foliage and unripe fruit (Table 1). A transcript from the TOM5 transgene could not be detected in ripe fruit RNA of this plant and the endogenous *r* message was also much reduced (Fig. 9). These findings are consistent with phytoene synthesis being blocked by co-suppression, the term given to the discovery that transferring

Fig. 10. Alterations in leaf, flower and fruit colour caused by co-suppression of phytoene synthase genes. a. Top row from left to right; flowers of wild-type, *yellow flesh* and *white flower* plants. Below; flowers from transgenic *yellow flesh* plants, line pBDH5ST9 (over-expresser) and line pBDH5ST10 (co-suppressor). Variation in petal number from 5 to 7 is normal in this tomato variety. b. Unripe and ripe fruit of pBDH5ST10: unripe fruit photobleach to a white colour, ripe fruit are yellow due to the accumulation of flavonoids. c. 'Mature green' fruit of pBDH5ST10 (left) and *yellow flesh* plants (right). d. Photobleached leaves of pBDH5ST10, unbleached green sectors are also apparent.

a gene or gene fragment in the sense orientation back into a plant can result in the inhibition of expression of both the transgene and the endogenous gene, from which it was derived. The phenomenon is manifest by reduced levels of mRNA from both the endogenous gene (and closely related sequences) and from the introduced transgene as was found in this case (Fig. 9, lane c). This indicates that the *r* mutant contains a phytoene synthase gene that is functional in leaves, but which can be co-suppressed by a transgene constructed with the TOM5 fruit sequence. Many cases of co-suppression have now been reported and a number of models have been proposed to explain the mechanism [27, 16, 26, 13, 10]. The photobleaching observed here showed pale leaf tissue which was interspersed with unbleached green sectors (Fig. 10d). Pigmented and unpigmented sectoring was also a characteristic of petunia flowers in which anthocyanin biosynthesis was inhibited by co-suppression when either a chalcone synthase or a dihydroflavonol-4-reductase sense transgene was used [27, 16].

It is interesting that none of the earlier antisense experiments of Bird *et al.* [5] produced plants that showed reduced levels of carotenoids in leaf or unripe fruit tissue. Transgenic material in which the antisense construct massively inhibited the production of leaf carotenoids might have been unable to survive at an early stage of tissue culture. However, Bartley *et al.* [2] have recently observed that the 794 bp antisense sequence used in these experiments only covers a segment of the 5′-untranslated region, including the transit peptide and a portion of what is likely to be the mature protein. Thus it is possible that the co-suppression described here, using a full-length TOM5 cDNA, relies on homology between TOM5 and a related gene expressed in the leaves, involving a region nearer the 3′ end of the TOM5 cDNA. This region may show greater sequence conservation than the 5′ region due to functional constraints. Evidence for the functional importance, and therefore probable sequence conservation, of the C-terminus of phytoene synthase, comes from the demonstration that the yellow-fruit r^y mutant sequence only lacks the 25 amino acids from the C-terminus (see above).

The fact that the *r* mutant produces normal levels of carotenoids in its leaves and immature fruit (Table 1) suggests that in these tissues synthesis of phytoene is catalyzed by an enzyme other than that encoded by *Psy1*, which appears to be nonfunctional in these plants. It is probably the mRNA from this unknown gene that is being targeted and reduced in the pBDH5ST10 plant that shows co-suppression and photobleaching (Fig. 10).

Expression of Psy *genes in flowers*

The fact that the flowers of both the TOM5 antisense plants [5] and those showing co-suppression in this study were paler than wild type (Fig. 10) indicates that a TOM5-related gene or genes are also normally expressed in flower tissue. Interestingly, the *r* mutant shows a pale corolla [6], suggesting that the gene expressed in fruit is also expressed in flowers. However, the corolla of both pBDH5ST10, and also the other transformant showing co-suppression, had flowers that were much paler than the corolla of *r*, being almost white (Fig. 10a). This suggests that a further phytoene synthase gene in addition to *Psy1* is involved in flower colour. A transcript the same size as that of the mutated TOM5 message of *r* fruit was detected in RNA from *r* flowers,

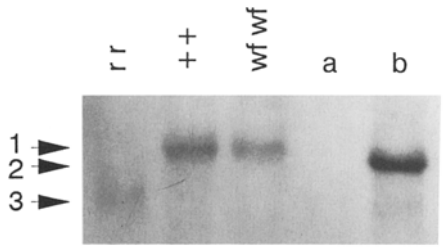

Fig. 11. Northern analysis of phytoene synthase transcripts in flowers from normal, mutant and transgenic plants. Flower RNA from *yellow flesh* (*rr*), wild type (++) and *white flower* (*wfwf*) was compared with the transgenic pBDH5ST10 (co-suppressor) in lane a and the over-expresser pBDH5ST9 in lane b. The arrows labelled 1, 2 and 3 indicate the position of the wild-type, transgenic and mutant messages respectively.

whilst a normal-sized message was seen in that of wild-type flowers (Fig. 11). It is unlikely that the *r* gene product would be even partially functional in the flower chromoplasts since it is inactive in fruit, and it therefore seems probable that a second, related gene is also expressed in this tissue and is responsible for the low level of phytoene produced and the pale colour of the flowers of *r* (Fig. 10a). So far we have been unable to detect such a mRNA by northern blot analysis.

Conclusions

These results establish that the *r* and r^y mutations, located on chromosome three, are caused by lesions in the phytoene synthase gene (*Psy1*) expressed in ripening tomato fruit. They also indicate that related gene(s) are expressed in green fruit and leaves, and in flowers. The successful over-expression of the TOM5 phytoene synthase to produce lycopene indicates that this sequence could be used as a reporter gene, at least in some tissues, and could also be used to modify pigment content of flowers and fruits. The demonstration that the same gene construct can give rise to over-expression or co-suppression in different transgenic plants is presumably related to the difference in insertion site of the transgene. Further analysis of the chromosomal context and position of the transgene relative to endogenous genes may provide an explanation of the phenomenon of co-suppression.

References

1. Armstrong GA, Alberti M, Hearst JE: Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes. Proc Natl Acad Sci USA 87: 9975–9979 (1990).
2. Bartley GE, Viitanen PV, Bacot KO, Scolnik PA: A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. J Biol Chem 267; 5036–5039 (1992).
3. Bevan M: Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12: 8711–8721 (1984).
4. Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MW, Bird AS, Hughes S, Morris PC, Grierson D, Schuch W: The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. Plant Mol Biol 11: 651–662 (1988).
5. Bird CR, Ray JA, Fletcher JD, Boniwell JM, Bird AS, Blain I, Bramley PM, Schuch W: Using antisense RNA to study gene function: Inhibition of carotenoid biosynthesis in transgenic tomatoes. Bio/technology 9: 635–639 (1991).
6. Darby LA, Ritchie DB, Taylor IB: Isogenic lines of tomato 'Ailsa Craig'. In: Davies JN, Hesling JJ (eds) The Glasshouse Crops Research Institute 1977 Annual Report, pp. 168–184. GCRI, Littlehampton (1978).
7. Davies BH: Carotenoids. In: Goodwin TW (ed) Chemistry and Biochemistry of Plant Pigments, 2nd ed., pp. 38–165, Academic Press, London (1976).
8. Dogbo O, Laferriere A, D'harlingue A, Camara B: Carotenoid biosynthesis: Isolation and characterization of a bifunctional enzyme catalyzing the synthesis of phytoene. Proc. Natl. Acad. Sci. USA. 85: 7054–7058 (1988).
9. Fleming HK, Myers CE: Tomato inheritance, with special reference to skin and flesh colour in the orange variety. Proc Am Soc Hort Sci 35: 609–624 (1937).
10. Grierson D, Fray RG, Hamilton AJ, Smith CJS, Watson CF: Does co-suppression of sense genes in transgenic plants involve antisense RNA? Trends Biotechnol 9: 122–123 (1991).
11. Hunt GM, Baker EA: Phenolic constituents of tomato fruit cuticles. Phytochemistry 19: 1415–1419 (1980).
12. Jenkins JA, Mackinney G: Carotenoids of the apricot tomato and its hybrids with yellow and tangerine. Genetics 40: 715–720 (1955).
13. Jorgensen R: Altered gene expression in plants due to trans interactions between homologous genes. Trends Biotechnol 8: 340–344.
14. Kinzer SM, Schwager SJ, Mutschler MA: Mapping of ripening-related or -specific cDNA clones of tomato (*Lycopersicon esculentum*). Theor. Appl. Genet. 79: 489–496 (1990).
15. MacKinney G: Absorbtion of light by chlorophyll solutions. J. Biol Chem 140: 315–323 (1941).
16. Napoli C, Lemieux C, Jorgensen R: Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2: 279–289 (1990).
17. Pietrzak M, Shillito RD, Hohn T, Potrykus I: Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucl Acids Res 14: 5857–5868 (1986).
18. Price HL, Drinkard AW Jr: Inheritance in tomato hybrids. Virg Agric Exp Stn Bull 177: 17–53 (1908).
19. Ray J, Bird CR, Maunders M, Grierson D, Schuch W: Sequence of pTOM5, a ripening related cDNA from tomato. Nucl Acids Res 15: 10587 (1987).
20. Ray J, Moureau P, Bird C, Bird A, Grierson D, Maunders M, Truesdale M, Bramley P, Schuch W: Cloning and characterisation of a gene involved in phytoene synthesis from tomato. Plant Mol Biol 19: 401–404 (1992).

21. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
22. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
23. Shure M, Wessler S, Fedoroff N: Molecular identification and isolation of the waxy locus in maize. Cell 35: 225–233 (1983).
24. Slater A, Maunders MJ, Edwards K, Schuch W, Grierson D: Isolation and characterisation of cDNA clones for polygalacturonase and other ripening-related proteins. Plant Mol Biol 5: 137–147 (1985).
25. Smith CJS, Slater A, Grierson D: Rapid appearance of an mRNA correlated with ethylene synthesis encoding a protein of molecular weight 35000. Planta 168: 94–100 (1986).
26. Smith CJS, Watson CF, Bird CR, Ray J, Schuch W, Grierson D: Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol Gen Genet 224: 477–481 (1990).
27. van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR: Flavonoid genes in petunia: addition of a limited number of copies may lead to a suppression of gene expression. Plant Cell 2: 291–299 (1990).