Analysis of Plant Genes Involved in Aromatic Volatile Production

by

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ABSTRACT

The cDNA *CM-AAT1* from melon was expressed as a fusion protein in yeast, *Saccharomyces cerevisiae*. The protein exhibited alcohol acyl-transferase (AAT) activity, producing ester compounds from a wide range of alcohols and acyl-CoAs. A second cDNA clone, *Le-AAT1* was identified by heterologous screening of a tomato fruit library with the melon *CM-AAT1* probe. The amino acid sequence of the encoded protein showed some similarities to many proteins using acyl-CoAs as substrates, including CM-AAT1. The *Le-AAT1* open reading frame (ORF) consists of 1329 nucleotides, encoding 442 amino acids, while the *CM-AAT1* ORF is 1431 nucleotides in length with a deduced sequence of 476 amino acids. Although the Le-AAT1 showed 43% identity to the CM-AAT1 at the amino acid level, the yeast-expressed protein demonstrated AAT activity. On the other hand, a second melon clone, *CM-AAT2*, encoding a 475 amino acid protein, which is 86% identical to the CM-AAT1 protein, did not show AAT activity. The CM-AAT1 fusion protein was active over pH 6.0 to pH 8.0 *in vitro* with activity being enhanced by Mg^{2+}, whereas the Le-AAT1 protein performed efficiently at pH between 6.0 and 9.0 with Na^{+}. The activity of the CM-AAT1 protein probably requires posttranslational modifications since the protein expressed in *Escherichia coli* was inactive.

Northern analysis of RNA from a range of tissues including developing fruit showed that the melon *CM-AAT1* and the tomato *Le-AAT1* are fruit ripening specific genes. The endogenous *CM-AAT1* mRNA was ethylene inducible and the expression dramatically reduced in transgenic low ethylene melon. The expression of *Le-AAT1* mRNA was also enhanced by exogenous ethylene, but the levels were still high in low ethylene tomatoes. The ADH2 protein, which can transform aldehydes to alcohols,

was found to be expressed in various organs of tomato and highly expressed at the late ripening stages. Exogenous ethylene could not induce high accumulation of the *ADH2* mRNA.

Tomato plants were transformed with gene constructs containing the *CM-AAT1* sense and partial *Le-AAT1* antisense cDNAs with either CaMV 35S promoter or tomato ACO1 promoter. The production of all selected volatile compounds greatly increased during tomato fruit ripening. Although ester volatiles were rarely generated at the start of fruit development, they were produced in significant amounts as ripening proceeded, but were still low, compared to other volatiles. There was, however, no statistical difference of the ester concentrations in fruit between control and transgenic tomatoes.

ABBREVIATIONS

α	alfa
[α-^{32}P] dCTP	deoxycytidine 5'-[α-^{32}P] triphosphates
A	adenine
AAT	alcohol acyl-transferase
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ADH	alcohol dehydrogenase
asRNA	antisense RNA
β	beta
B	breaker (the stage of tomato fruit development)
BAMT	*S*-adenosyl-*L*-methionine:benzoic acid carboxyl methyl-transferase
BCIP	5-bromo-4-chloro-3-indolye phosphate
BEAT	acetyl-CoA:benzylalcohol acetyl-transferase
BEBT	benzoyl-CoA: anthranilate-*N*-benzoyl-transferase
bp	bases pair(s)
BSA	bovine serum albumin
CaMV35S	cauliflower mosaic virus 35S promoter
cDNA	complementary deoxyribonucleic acid
CoA	Coenzyme A
cv.	cultivar(s)
°C	degree Celsius
daa	days after anthesis
DAT	acetyl-CoA:deacetylvindoline 4-*O*-acetyl-transferase
dATP	2'-deoxyadenosine 5'-triphosphates
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxyribonucleoside triphophates
dsRNA	double strand RNA
DTT	dithiothreitol

DW	distilled water
E	*trans* (configuration)
E. Coli	*Escherichia coli*
EDTA	diamioethanetetra-acetic acid, disodium salt
ed.	editor(s)
EFE	ethylene forming enzyme;
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tags
et al.	*et alia* (and other; Latin)
EtBr	ethidium bromide
γ	gramma
g	gram(s) or relative centrifugal force (RCF)
h	hour(s)
HCBT	hydroxy-cinnamoyl/benzoyl-CoA:anthranilate-*N*-hydroxycinnamoyl/benzoyl-transferase
HPL	hydroperoxide lyase
HPO	hydroperoxides
IMG	immature green (the stage of tomato fruit development)
Kb	kilobases
kDa	kilo daltons
Km	a kinetic parameter used to characterise an enzyme
L, l	litre(s)
LAH	lipolytic acyl hydrolase
LB medium	Luria and Bertani medium
LB	left T-DNA border
LOX	lipoxygenase
Ltd.	limited company
M	molar(s)
1-MCP	1-methyl-cyclopropane
MG	mature green (the stage of tomato fruit development)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)

mM	millimolar(s)
mRNA	messenger ribonucleic acid
MS medium	Murashige and Skoog basal medium
Mw	molecular weight
NBT	nitro blue tetrazolium chloride
ng	nanogram(s)
nm	nanometre(s)
NPTII	neomycin phosphotransferase
Nr	Never ripe
nt	nucleotide(s)
OD	optical density
PCR	polymerase chain reaction
PDS	phytoene desaturase
PEG	polyethylene glycol
PG	polygalacturonase
pH	negative logarithm of hydrogen ion concentration
PL	phospholipase
PME	pectin methylesterase (or pectin esterase)
PSY	phytoene synthase
pp	pages
ppb	part(s) per billion
ppm	part(s) per million
PTOX	plastid terminal oxidase
RB	right T-DNA border
rin	ripening inhibitor, tomato mutant
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	retention time
RT-PCR	reverse transcriptase – polymerase chain reaction
sec	second(s)
SDS	sodium dodecyl sulphate
SDW	sterile distilled water

SSC	sodium salt/citrate buffer
SSPE	sodium salt/phosphate/EDTA buffer
TBG	β-galactosidase
T-DNA	transfer DNA
TEMED	*NNN'N'* tetramethylethylene diamine
TrisHCl	Tris(hydroxymethyl) aminomethane hydrochloride
U	uracil
UTR	untranslated region
UV	ultraviolet
V	voltage (s)
v/v	volume per volume
var	variety
µg	microgram(s)
µl	microlitre(s)
µM	micromolar(s)
w/v	weight to volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indoly-β-*D*-galactopyranoside
Z	*cis* (configuration)
ζ	zeta

CHAPTER 1

GENERAL INTRODUCTION

1.1 Nuclear Genomes

In recent years, the amount of nucleic acid sequence data, made available for the public databases, has been immensely increased, mainly due to intensive efforts to sequence the genomes of *Homo sapiens*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and some other organisms. The sequencing of the *Saccharomyces cerevisiae* genome was completed in 1996 (Cherry *et al.*, 1997) and the completion of the *Caenorhabditis elegans* genome in 1998 (The *C. elegans* sequencing consortium, 1998). *Arabidopsis thaliana* is a flowering plant containing a small genome, the sequence of which was published in 2000 (The *Arabidopsis* genome initiative, 2000).

The *Arabidopsis* complete genome size is about 125 megabases, while the sequenced regions cover 115.4 megabases, containing 25,498 genes encoding proteins from 11,000 families (The *Arabidopsis* genome initiative, 2000). Although flowering plants have gradually evolved during the past hundred million years ago and might be expected to be very similar at the genetic level, developmental and metabolic diversity still exists (Somerville and Somerville, 1999). As a result of the availability of genetic information and mutants, *Arabidopsis thaliana* is used worldwide for the study of many aspects of plant biology. Tomato, with a genome of approximately 950 megabases (Arumuganathan and Earle, 1991), has not yet been sequenced but the *Arabidopsis* genome and nucleic acid sequences can be used for comparison with tomato ETS (expressed sequence tags) and the map positions of related genes are often correlated (Mysore *et al.*, 2001). Because it produces a climacteric fruit with a

short life cycle, tomato has also been a basic model for plant science research especially in fruit quality, ripening and senescence for more than 30 years (Chalmers and Rowan, 1971; Goodwin and Jamikorn, 1952; Hill *et al.*, 1970; Hobson, 1967; Rattanapanone *et al.*, 1977; Rattanapanone *et al.*, 1978). In respect of fruit ripening, melon is another climacteric fruit with a number of important quality traits, including the production of high scent during ripening. Recently, melon has been increasingly applied for research about fruit ripening sharing genetic information from *Arabidopsis* and tomato (Balagué *et al.*, 1993; Karvouni *et al.*, 1995; Sato-Nara *et al.*, 1999). The melon genome size is estimated to be about 454-502 megabases (Arumuganathan and Earle, 1991) and the construction of a genetic map is in progress (Wang *et al.*, 1997).

1.2 Tomato

1.2.1 Tomato History

Tomato (*Lycopersicon esculentum* Mill.) is a vegetable fruit in the *Solanaceae* family, which involves some economically famous genera, including *Capsicum*, *Lycopersicon*, *Nicotiana*, and *Solanum* (Langer and Hill, 1999). Soon after the discovery of America, the cultivated tomatoes, originating in the coastal strip of western south America (Andean region), were introduced into southern Europe (Davies and Hobson, 1981). Tomato was first known as *pomid'oro* and *mala aurea* (golden apple) and also as *poma amoris* (love apple). The name '*tomatl*', from the Nahua Tongue of Mexico, was the origin of the modern name 'tomato' (Goodenough, 1990). The first types cultivated in Europe bore large fruit instead of small fruit of all wild type species (Rick, 1978). *Lycopersicon esculentum* Mill. has been widely accepted as the scientific name for most commercial tomatoes, but there are, however, other alternatively names such as *Solanum lycopersicum* L., or *Lycopersicon lycopersicum*.

1.2.2 Tomato Biology

All species in the genus *Lycopersicon* have an identical genome formula, where 2n is 24 (Taylor, 1986). In the *Solanaceae*, the genus *Lycopersicon*, consisting of relatively modern-day species, is subdivided into two subgenera, the *Eulycopersicon* and the *Eriopersicon.* The characteristics of the subgenera are shown in Table 1.1. Although tomato is a perennial plant, almost all commercial varieties are cultivated as annuals. Tomatoes can grow up to 2 metres in height, but are unable to bear their own weight, and only the young plants are naturally erect, although bush varieties are grown commercially. Inflorescences containing 4 to 12 flowers are developed from their leaf axils, frequently after the plant has produced 9 leaves. Flowers are perfect flowers, which show a high degree of self-pollination (Rick, 1978).

In early stage of fruit development, seeds are engulfed and locule cavities are filled by growing placental cells. Tomato is separated into 2 groups: bilocular and multilocular fruit (Grierson and Kader, 1996; Ho and Hewitt, 1996). Fruit shapes are diverse, but in general are round having an average of three locules (Hobson and Grierson, 1993). Mature fruit, containing a large number of seeds, usually turn from green to red in pericarp when ripen due to accumulations of lycopene and β-carotene and degradation of chlorophyll (Grierson and Kader, 1996). However, there are a number of tomato ripening mutants that show abnormalities in fruit ripening (see section 1.6.3.4), for example, by remaining either green or yellow and delaying ripening processes such as flavour development and softening. Tomatoes are chilling sensitive below 11°C; suitable conditions for growing and producing good flavoured fruit are 12-16 hours of daylight, 16-25°C at day and 11-18°C at night with 70-90% humidity (Goodenough, 1990).

Table 1.1 Characteristics and origins of modern-day tomato from two subgenera of the genus *Lycopersicon* (from Goodenough, 1990; Hobson and Grierson, 1993; Taylor, 1986)

Subgenera	Species	Characteristics	Origins
Eulycopersicon - Glabrous, coloured-fruit with normally red or reddish-yellow colour	*L. esculentum* (normal species cultivated)	Automatic self-pollination	Mexico, central and southern America
	L. esculentum var. cerasiforme (cherry tomato)	Resistance to root rot and fungal disease	Central and southern America in Andean foothills
	L. pimpinellifolium (currant tomato)	Disease resistance and factors for improved composition and colour	Low altitudes in Ecuador and northern interandean Peru
	L. cheesmanii	Jointless fruit stalks, salt tolerance	Galapagos islands
Eriopersicon - Hairy, whitish-green-fruit with purplish stripes.	*L. peruvianum*	Pest and disease resistance, vitamin C content	High Peruvian Andes plus a few coastal races
	L. hirsutum	Pest and disease resistance, low temperature tolerance, source of improved fruit colour	Ecuador and Peru at high elevations
	L. chilense	Drought, cold, virus and disease resistance	Southern Peru and northern Chile
	L. chmielewski	Improved composition, total solids, and fruit colour	High altitudes in the Peruvian Andes
	L. parviflorum	Improved composition, total solids, and fruit colour	High altitudes in the Peruvian Andes

1.3 Melon

1.3.1 Melon History

Melon (*Cucumis melo* L.) together with cucumber and gherkin are cultivated species in the genus *Cucumis* of the family *Cucurbitaceae*. Most plants in the *Cucurbitaceae* are chilling and frost sensitive and are found in subtropical and tropical regions throughout the world. Melon and other species in the subgenus *Melo* originated in Africa and spread across the Middle East and Asia (Robinson and Decker-Walters, 1997) and are grown for their large fruits. Many common names have been used to describe the different varieties, including cantaloupe, muskmelon, nutmeg melon, winter melon, sweet melon, rock melon, snap melon and others.

1.3.2 Melon Biology

Like tomato, the chromosome number of melon (n) is equal to 12. Plants typically are trailing vines, having tendrils borne singly at the node to help support the stem. Most melon cultivars are andromonoecious, bearing male and female flowers on the same stem. Exogenous application of ethylene can induce a bisexual floral bud to develop into a pistillate flower in melon, cucumber and squash (Robinson and Decker-Walters, 1997). Melon shows a huge diversity of fruit types, varying in skin colour from green, white, yellow, orange to gray; in skin texture from smooth to netted skinned; in flesh colour from green, pink to orange; in weight from around 500 grams to a few kilograms (Seymour and McGlasson, 1993). Groups of melons and their characteristics are described in Table 1.2. Cantaloupe, showing a sigmoid pattern in the fruit growth, is well known for a netted melon with excellent flavour quality. In netted melons, the onset of ripening is accompanied by the development of an abscission

layer (McGlasson and Pratt, 1963). Therefore, at full slip, melon fruit can easily be pulled from the vine and can complete abscission from the stem within 3 days. Melons are of best quality when raised at 25-30°C, 16 h light and proper irrigation (Robinson and Decker-Walters, 1997).

Table 1.2 Characteristics of 6 groups of melons (modified from Robinson and Decker-Walters, 1997)

Group	Characteristics
Cantalupensis - Cantaloupe, muskmelon	Fruits are a medium-size with netted rind; flesh usually orange; flavour aromatic or musky. Fruit detach from peduncle at maturity.
Inodorus - Winter melon	Fruit are large with smooth or wrinkled, but not netted rind; flesh typically white or green and lacking a musky odour. Fruit do not detach from the peduncle when mature with longer keeping than those of the Cantalupensis group.
Flexuosus - Snake melon	Fruit are very long, slender and often ribbed.
Conomon - Pickling melon	Fruit are small with smooth rind; tender skin; white flesh; little sweetness or odour.
Dudaim - Pomegranate melon, Queen Anne's pocket melon	Fruit are small, round to oval in shape with white flesh and thin rind.
Momordica - Phoot, snap melon	Fruit are ovoid to cylindrical in shape with a smooth surface; white or pale orange flesh. They are low in sugar content, mealy and insipid. The fruit disintegrates when barely ripe.

1.4 Fruit Ripening

Fruit are physiologically divided into 2 groups according to their respiratory behaviour during ripening: climacteric fruit such as apple, melon, pear, banana, tomato, and non-climacteric fruit such as citrus and strawberry. In climacteric fruit, a rise in respiration is associated with the onset of ripening, while there is no respiratory rise in non-climacteric fruit (Wills *et al.*, 1989). During the onset of ripening only climacteric fruit produce a huge increase in ethylene, which is a critical factor, stimulating the respiratory rate and the ripening processes. Ethylene production at the onset of ripening is autocatalytic, and is enhanced by increasing the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC Oxidase (ACO or ethylene forming enzyme; EFE) activity (Kende, 1993). Ripening consists of many processes, including softening, colour change, generation of aroma volatiles and other biochemical changes affecting the quality, appeal, nutritional value and storage life of fleshy fruit. It is unclear what substitutes for ethylene in non-climacteric fruit.

Tomato has been a popular climacteric fruit for genetic studies for over 30 years and a great deal is known about the role of specific genes in ripening. Some genes are switched off during tomato ripening such as genes related to generation of chlorophyll and photosynthetic enzymes, whereas housekeeping genes remain expressed and new ripening genes are switched on such as those associated with ethylene production, cell wall degradation or colour development (Grierson, 1998).

Melon is a fragrant fruit, showing a climacteric pattern when ripening (Hadfield *et al.*, 1995). The ripening process occurs rapidly after harvest, causing a short shelf-life of less than 10 days at 21°C for full-slip fruit (Seymour and McGlasson, 1993). Melon shows an increase of ethylene production, related to ripening and sensitivity to

exogenous provided ethylene (Lui *et al.*, 1985). The characteristics of melon ripening are the softening of the mesocarp, in some varieties the colour changes both in peel and in flesh, and especially the development of aroma volatiles and sweet taste (Seymour and McGlasson, 1993).

1.5 Ripening Processes

1.5.1 Biochemical Changes

Dry matter content of ripe tomato fresh fruit is between 5 and 7.5%. Approximately 50% and 27% of the dry matter are in the form of reducing sugars and alcohol-insoluble solids, respectively, while the rest of constituents are amino acids, pigments, vitamins, polyphenols and minerals (Davies and Hobson, 1981). Glucose and fructose are the main sugars and found in about equal proportions in ripe tomatoes (Davies and Kempton, 1975; Md Islam *et al.*, 1996; Winsor, 1966). Ripe tomato accumulates a higher sugar content in the pericarp than in the locules (Winsor *et al.*, 1962). Sucrose, presented at less than 0.1% of fruit fresh weight, dramatically reduces from the mature to red stage of fruit development in contrast to glucose and fructose content (Davies and Kempton, 1975; Md Islam *et al.*, 1996). Sucrose synthase is highest in young fruit and then declines rapidly, whereas acid invertase sharply increases during fruit maturity (Md Islam *et al.*, 1996) and is responsible for converting sucrose to glucose and fructose. Starch, a storage form of important carbon, increases in level during early stages of fruit development until nearly the mature green stage and rapidly declines during ripening (Davies and Cocking, 1965; Yu *et al.*, 1967). Malic acid is found to be the predominant acid during early fruit growth but declines in abundance in ripe tomato while citric acid increases during the green-yellow stage of ripening (Carangal *et al.*, 1954). In 'Cal Ace' tomato, four amino acids, glutamine,

glutamic, γ-aminobutyric, and aspartic acid, make up about 80% of the total free amino acids in fruit (Kader *et al.*, 1978). The fruit harvested at the ripe stage contains more alanine and less glutamic acid than those picked at the green or breaker stage.

Melon fruit mostly accumulates carbohydrate in form of sucrose as the principal sugar, not starch (Bianco and Pratt, 1977). The galactosyl-sucrose oligosaccharides stachyose and raffinose are synthesised in melon leaves and translocated to sink tissues such as the fruit (Hubbard *et al.*, 1990; Mitchell *et al.*, 1992) and can be hydrolised to sucrose by the alkaline form I of α-galactosidase (Gao and Schaffer, 1999). Sugar content in fruit is an important aspect of fruit quality, because sugar content of melon does not increase after the fruit has been removed from the vine (Mutton *et al.*, 1981). Sucrose is initially detected in the mature stage and increases dramatically during ripening (Bianco and Pratt, 1977; Wang *et al.*, 1996). Sucrose synthase activity increases in the maturing sweet melon fruit, whereas soluble acid invertase activity massively decreases (Lingle and Dunlap, 1987; Schaffer *et al.*, 1987). Citric acid is a major organic acid in many cultivars of *Cucumis melo* (Leach *et al.*, 1989; Wang *et al.*, 1996), while succinic acid is found in immature fruit, but disappears during maturing stages. pH increases from 5.52 in 20 daa (days after anthesis) fruit to 6.48 in 48 daa fruit in Makdimon melon (Wang *et al.*, 1996).

1.5.2 Texture Change

The cell wall is an important component of plant cells, making them firm and providing shape and support to the plant. The primary cell wall generally consists of approximately 30% cellulose, 30% hemicellulose, 35% pectin and 5% protein in dicotyledonous plants (Fischer and Bennett, 1991). During ripening, many enzymes are apparently involved in partial degradation of the cell wall structure to undergo

fruit softening. Polygalacturonase (PG) is involved in softening in many fruit by cleaving the α-1,4 linkage of polygalacturonic acids in plant cell walls. A PG cDNA clone, *TOM6*, was the first ripening cDNA to be cloned (Grierson *et al.*, 1986b). PG activity in tomato is initially detected at the onset of ripening and the enzyme dramatically increases in activity during ripening (Tucker *et al.*, 1982). PG mRNA, protein, and enzyme activity sequentially peak at the turning, ripe and red ripe stages of pericarp ripening, respectively (Biggs and Handa, 1989). PG expression is specific in fruit ripening, which is in agreement with the lack of *TOM6*, encoding tomato PG, expression in unripe fruit, roots and leaves (Maunders *et al.*, 1987). Pectin methyl esterase (PME or Pectin esterase), another pectin hydrolysing enzyme, is the enzyme that demethoxylates pectin to a carboxylated pectin in the cell wall, seemingly to prepare it for degradation by PG. The mRNA levels of PME are highest in immature green fruit and then decline throughout maturation and ripening (Ray *et al.*, 1988). However, there are 3 isoforms, found in tomato fruit and *PME1* shows highest accumulation at the breaker stage (Hall *et al.*, 1994).

Xyloglucan endo-transglycosylase (XET), considerably referred to be in a xyloglucan endotransglucosylase/hydrolase (XTH) group (Rose *et al.*, 2002), catalyses the cleavage and transfer of one xyloglucan molecule to another and probably play an important component of cell wall metabolism, particularly in expanding tissue and ripening fruit. *tXET-B1* and *tXET-B2* were isolate from tomato fruit that the *tXET-B1* mRNA is highly expressed in pink fruit pericarp and in stem (Arrowsmith and de Silva, 1995). Expansins are also cell wall extension proteins in large multigene families (Shcherban *et al.*, 1995), found to be expressed in plant growing tissues, including hypocotyls (McQueen-Mason and Cosgrove, 1995). In tomato, expansin *LeExp1* mRNA is accumulated in high levels specifically during fruit ripening (Rose *et al.*,

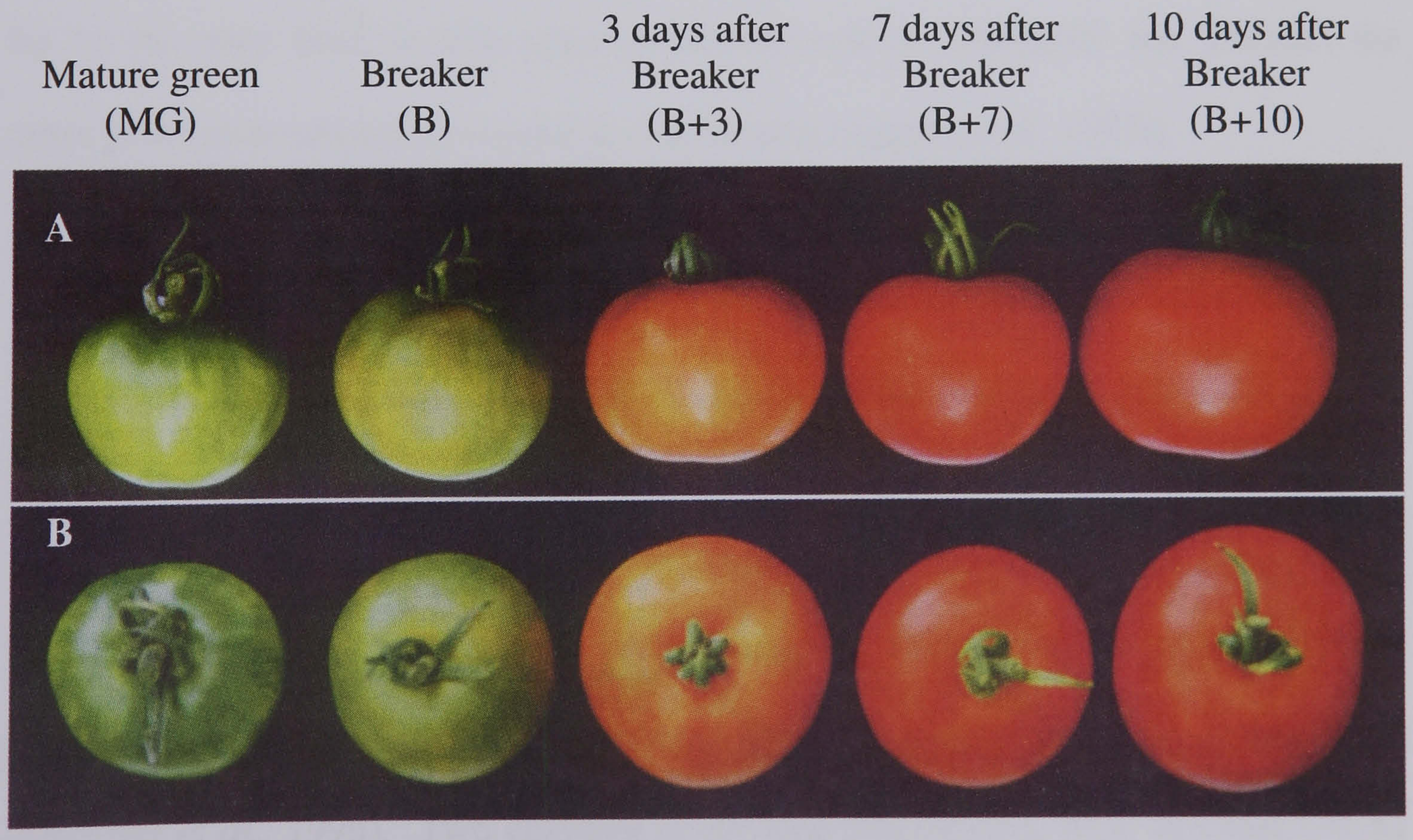
1997). Beta-galactosidases show exo-galactanase ability, which hydrolyses terminal nonreducing β-*D*-galactosyl residues from β-*D*-galactosides. Beta-galactosidase II (β-galactosidase/exogalactanase) presents during tomato fruit ripening and is capable of degrading tomato fruit galactan (Smith *et al.*, 1998). Seven clones of tomato β-galactosidase (TBG) were isolated and *TBG4* clone showed high expression in wild type fruit during ripening, but very low in the mutants, *rin*, *nor*, and *Nr* (Smith and Gross, 2000). Speirs *et al.* (2002) found that rate of an increase in ADH (alcohol dehydrogenase) in six tomato cultivars during ripening was strongly correlated with rate of fruit softening, although this relationship is probably indirect.

Firmness of melon fruit flesh gradually declines during fruit development and dramatically drops at fruit ripening (Aggelis *et al.*, 1997a). Changes in cell wall composition in muskmelon consist of an increase in soluble pectin, a decrease of pectin molecular size, loss of galactosyl residues and changes in molecular size of hemicelluloses (McCollum *et al.*, 1989). In many fruits, softening is associated with increased PG activity during ripening, but in melon, it has been reported that PG is not involved in the change to the structure of the cell wall (Lester and Dunlap, 1985; McCollum *et al.*, 1989; Ranwala *et al.*, 1992). However, Hadfield *et al.* (1998) reported that three PG clones, including an endo-PG clone, were highly expressed during ripening and this was related to decrease of molecular weight of pectin. The study of galactan hydrolase enzymes, such as β-galactosidases, has revealed an increase in activity during ripening (Huber, 1983; Ranwala *et al.*, 1992). The soluble isoform II of β-galactosidases starts accumulating around 34 days after anthesis when fruit ripening is initiated (Ranwala *et al.*, 1992). PME and cellulases were detected in melon fruit, but the activity of both enzymes remained constant during fruit development and decreased during fruit ripening and senescence (Lester and Dunlap, 1985).

1.5.3 Colour Change

During development, green tomato fruit accumulate chlorophyll in the thylakoid membranes of chloroplasts. During ripening, the chloroplasts change into chromoplasts, accumulating the carotenoids, lycopene and β-carotene pigments, and degrading chlorophyll and the thylakoid membranes (Bathgate *et al.*, 1985). Tomato fruit turn from green to red when ripening as shown in Figure 1.1. However, in the tomato mutant, green flesh (*gf*), since chlorophyll degradation is defective, detectable amounts of chlorophyll remain in the ripe fruit, causing a rusty red fruit colour (Cheung *et al.*, 1993). Tomato plastid terminal oxidase (PTOX), expected to play a role during early chloroplast development, participates in carotenoid desaturation in chromoplasts (Josse *et al.*, 2000). *TOM5* (phytoene synthase; the enzyme, which catalyses the production of phytoene for carotenoid synthesis) mRNA is hardly present

Figure 1.1 The tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) ripening stages showing colour change from the side-view (A) and top-view (B)



in immature fruit, but increases in ripe fruit and then drops during the over ripening stage (Slater *et al.*, 1985). Phytoene desaturase (PDS), another enzyme associated with carotenoid synthesis catalyses conversion of phytoene to ζ- carotene. Although PDS activity was higher in green than in orange fruit (Fraser *et al.*, 1994), the mRNA levels of PDS increased ten-fold in ripe tomato compared to in green fruit (Pecker *et al.*, 1992). The *Cnr* tomato mutant has a colourless fruit, which has low levels of total carotenoids, phytoene and lycopene, due to the virtual absence of phytoene synthesis in the ripe *Cnr* fruit (Fraser *et al.*, 2001).

Beta-carotene is the predominant pigment in many ripening melons (Curl, 1966). In orange-fleshed muskmelon, the carotenoid content of flesh increases around 10 days before the climacteric rise of respiration, while chlorophyll content declines sharply during the respiratory climacteric (Reid *et al.*, 1970). Increasing carotenoid content of flesh was accompanied by a great accumulation of phytoene synthase mRNA (*MEL5*) from the onset of the climacteric rise of Charentais cantaloupe (Karvouni *et al.*, 1995). In normal ripening varieties, the flesh turned from green to orange rapidly during ripening, but on the other hand, in slow ripening cultivars such as 'Marygold' and 'Delada', the green flesh colour seemed to increase during ripening (Aggelis *et al.*, 1997a).

1.5.4 Respiration and Ethylene Production

Tomato shows a respiratory pattern during ripening, as do all climacteric fruits. Respiration occurs at the preclimacteric stage but increases after the mature green stage and reaches a peak at the orange or fully red stage in both attached and detached fruit (Andrews, 1995). Ethylene production, like respiration, increases after the mature green stage and is at a maximum production at the orange or red stage (Martinez *et al.*, 1996). Two enzymes in ethylene biosynthesis, ACC synthase (ACS)

and ACC oxidase (ACO), are key factors in the control of fruit ripening and the regulated expression of members of multigene families encoding these enzymes has been studied in detail in tomato. Some factors such as auxin treatment and some stress conditions enhance ACS (Cervantes, 2002). Yang and Hoffman (1984) concluded that ACS is the rate-limiting step of ethylene biosynthesis, but regulation of ACO is now known to be important also. *TOM13*, encoding a 35 kDa polypeptide induced during ripening and after wounding (Smith *et al.*, 1986), was identified as encoding ACC oxidase (ACO1) (Hamilton *et al.*, 1991; Hamilton *et al.*, 1990). It is now widely believed that the ACS-ACO pathway is the main route for ethylene biosynthesis, although lipoxygenase activity may play under same circumstances by an additional sources of ethylene (Sheng and Wainwright, 2000).

Netted melons have a rapid climacteric respiration near the time that they reach maturity and also generate high ethylene amounts at harvest. In honeydew melon, however, ethylene synthesis may extend over several days or a climacteric rise may be absent or they may produce ethylene at 20 days postharvest (McGlasson and Pratt, 1963). Both respiratory and ethylene climacterics in harvested Charentais cantaloupe occur earlier than those in unharvested fruit, but the ethylene production of fruit attached to the plant is much higher than from harvested fruit (Hadfield *et al.*, 1995). ACO and ACS activities are low in green fruit, but dramatically increase at the same time as climacteric rise of ethylene generation (Martinez-Madrid *et al.*, 1999). Ethylene biosynthesis starts to increase in the placental tissues of fruit and then in the mesocarp tissues (Yamamoto *et al.*, 1995). The level of ACO mRNA (*MEL1*) is very low in unripe fruit, but a rise is first detected in the placental tissue at the preclimacteric stage and then the levels increase in the mesocarp tissue during the climacteric rise in the ethylene production. Three ACO clones were isolated (Lassaere *et al.*, 1996) that *CM-ACO1* corresponding to *MEL1* (Balague' *et al.*, 1993)

highly expressed in wounded leaves and ripe fruit while *CM-ACO2* and *CM-ACO3* are highly expressed in flowers and etiolated hypocotyls, respectively. Furthermore, levels of two ACS mRNAs, *ME-ACS1* and *ME-ACS2*, were detected in seeds and the placental tissue at the pre-climacteric phase, but only *ME-ACS1* was increasingly expressed in placental tissue, mesocarp and seeds during ripening (Yamamoto *et al.*, 1995).

1.5.5 Flavour Generation

Flavour is generally regarded as a combination between odour, taste and probably mouth feel together (Seidman, 1979). Volatile compounds, detected in the nose, are responsible for aroma, whereas nonvolatile compounds, presented in the mouth, perceived by the tongue and adjacent tissues, are responsible for taste (Taylor, 1996; Taylor and Linforth, 2000).

1.5.5.1 Taste

The characteristic of tomato taste is mainly determined by a balance between sugars, acids and their interaction in fruit (Stevens *et al.*, 1977a). Sweetness is due mainly to reducing sugars (glucose and fructose) and sucrose, whereas sourness is caused by the organic acid content, comprising mostly malic and citric acids. Both high sugars and acids are required for the best flavour and to be satisfactorily accepted by the consumer (Hobson, 1981). Differences in composition between locular and pericarp tissue can have dramatic effects on flavour. Cultivars with large locular portion, containing high concentration of acids and sugars, have been found to have good flavour quality (Stevens *et al.*, 1977b). Glucose and fructose are the main sugars found in ripe tomato (Winsor, 1966) and may constitute 50% of the soluble solids. Citric acid, a main organic acid in ripe tomato, increases during the maturation stage (Carangal *et al.*, 1954).

Melon mesocarp contains sugar pools such as glucose, fructose and sucrose throughout fruit development, but only sucrose is accumulated during the final stages of fruit ripening (Lingle and Dunlap, 1987). Sucrose accumulation is a major determinant of sweetness (Hubbard *et al.*, 1989) while citric acid is a major organic acid in many cultivars of melon, but is present in low amount and the content slightly reduces during ripening (Leach *et al.*, 1989).

1.5.5.2 Aroma

Odour is due to a mixture of volatile compounds and is probably the most complicated quality sensation. Ripe fruit contains a large number of volatiles due to breakdown products from macromolecules and/or generation of new volatiles including aldehydes, alcohols, ketones and esters.

1.5.5.2.1 Tomato Aroma

The main flavour compounds in tomato are aldehydes and ketones with a few alcohols. These are produced in intact plant tissues by intracellular biosynthetic pathways during the ripening period. The formation of carotenoid-derived, amino acids-derived, lipid-oxidised, and glycoside hydrolysis volatiles are the major routes involved in this process. Some secondary products are generated in small amounts in intact cells, but are formed at high rates during cell disruption by crushing, cutting and slicing (Goodenough, 1990). The lipid oxidation process has been proven to be the major biogenesis pathway for volatiles generation in tomato fruit after maceration (Boukobza *et al.*, 2001; Boukobza and Taylor, 2002; Leahy and Roderick, 1999). Major volatiles produced following maceration of fresh ripe fruit are shown in Table 1.3.

Table 1.3 Concentration and odour thresholds of major components in fresh ripe tomatoes using blending procedure (from Butter, 1993)

Compound	Concentration (ppb)[a]	Odour Threshold (ppb in H_2O)[b]	Log Odour Units[c]
Z-3-Hexenal	12,000	0.25	3.7
β-Ionone	4	0.007	2.8
Hexanal	3,100	4.5	2.8
β-Damascenone	1	0.002	2.7
1-Penten-3-one	520	1	2.7
3-Methylbutanal	27	0.2	2.1
E-2-Hexenal	270	17	1.2
2-Isobutylthiazole	36	3.5	1.0
1-Nitro-2-phenylethane	17	2	0.9
E-2-Heptanal	60	13	0.7
Phenylacetaldehyde	15	4	0.6
6-Methyl-5-hepten-2-one	130	50	0.4
Z-3-Hexenol	150	70	0.3
2-Phenylethanol	1,900	1,000	0.3
3-Methylbutanol	380	250	0.2
Methyl salicylate	48	40	0.08
Geranylacetone	57	60	-0.02
β-Cyclocitral	3	5	-0.2
1-Nitro-3-methylbutane	59	150	-0.4
Geranial	12	32	-0.4
Linalool	2	6	-0.5
1-Penten-3-ol	110	400	-0.6
E-2-Pentenal	140	1,500	-1.0
Neral	2	30	-1.2
Pentanol	120	4,000	-1.5
Pseudoionone	10	800	-1.9
Isobutyl cyanide	13	1,000	-1.9
Hexanol	7	500	-1.9
Epoxy-β-ionone	1	100	-2.0

[a]Parts (ml) of compound per 10^9 parts (ml) of fresh tomato

[b]Parts (ml) of compound per 10^9 parts (ml) of water

[c]Logarithm of odour unit value.

Odour unit is assigned based on dividing the concentration of a component (in ppb) by the components detection threshold level (in ppb).

The odour threshold is defined as the minimum physical intensity detected by sensory analysis.

Log odour units are defined as the relative odour contribution in fruit.

1.5.5.2.1.1 Amino Acid-Related Compounds

Amino acid metabolism, generating aliphatic and branched chain alcohols, acids, carbonyls and esters, contributes to fruit flavour (Wyllie *et al.*, 1996). These compounds are formed in the intact tomato during the ripening processes; there is very little difference in the formation of these compounds after maceration. The main formation of these amino acid-related compounds seems to occur between the breaker and ripe stages (Buttery *et al.*, 1987). Alanine, isoleucine, leucine, phenylalanine, and valine are main sources of the aroma compounds in fresh tomato (Table 1.4).

Table1.4 Volatiles in fresh tomato fruit related to amino acids (from Buttery and Ling, 1993)

Amino Acid	Volatile compound	Concentration (ppb of tomato)
Alanine	Acetaldehyde	800
Valine	1-Nitro-2-methylpropane	<5
Leucine	3-Methylbutanol	150-380
	3-Methylbutyric acid	200
	3-Methylbutanal	27-65
	3-Methylbutylnitrile	13-42
	1-Nitro-3-methylbutane	59-300
	2-Isobutylthiazole	36-110
Isoleucine	2-Methylbutanol	100
	2-Methylbutyric acid	5
Phenylalanine	Phenylacetaldehyde	15-18
	2-Phenylethanol	1000
	1-Nitro-2-phenylethane	17-54
	Phenylacetonitrile	3-8

1.5.5.2.1.2 Terpene-Related Compounds

Terpenes contain a number of isoprene units (C5) derived from 3-*S*-hydroxymethyl glutaryl-CoA. Terpene-related compounds are constituents of essential oils and photoalexins, mostly containing relatively few simple functional groups such as hydroxyls, carbonyls, and double bonds.

1.5.5.2.1.2.1 Terpenoid Volatiles

Many terpenoids are stored in plants as non-volatile glycosides. Terpenoid hydrocarbons generally occur in leaves and other parts of tomato plants, but not many in fruit. During *de novo* synthesis, geranyl pyrophosphate (C10) is an intermediate precursor to be converted to many monoterpene essential oils such as linalool, 8-hydroxylinalool (Lewinsohn *et al.*, 2001). The breakdown of lycopene in blended-fresh fruit releases C10 oxygenated terpenoids such as linalool, neral, α-pinene and geranial (Buttery and Ling, 1993). The major terpenoids found in blended leaves and fruit are listed in Table 1.5.

1.5.5.2.1.2.2 Carotenoid-Related Compounds

Carotenoids (C40) consist of 50-80% lycopene and 2-7% β-carotene in fully ripe tomato (Davies and Hobson, 1981). The oxidative decomposition of carotenoids leads to the formation of terpenes and terpene-like compounds (Buttery and Ling, 1993). 6-methyl-5-hepten-2-one and geranylacetone are the main volatile from lycopene degradation. The branched chain ketonic volatile compounds, formed from thermal breakdown of lycopene and production of C9 to C13 cyclic compounds from β-carotene are shown in Table 1.6

Table1.5 C10 and C15 terpenoid volatiles in blended tomato leaves and fruit (from Buttery and Ling, 1993)

Compound	Concentration, ppb (leaves)	Concentration, ppb (fruit)
α-Pinene	100	<1
(+)-2-Carene	1700	<1
Limonene	1000	<1
β-Phellandrene	8000	<1
Linalool	<10	2
Neral	<5	2
(-)-α-capaene	<5	12
Caryophyllene	350	<1
Humulene	250	<1
Caryophyllene epoxide	64	<1

Table1.6 Carotenoid-related fresh tomato volatiles (from Buttery and Ling, 1993)

Compound	Concentration, ppb (intact)	Concentration, ppb (macerated)
Open chain		
6-Methyl-5-hepten-2-one	100	210
6-Methyl-5-hepten-2-ol	8	8
Geranylacetone	20	330
Pseudoionone	11	6
Cyclic		
2, 2, 6-Trimethylcyclohexanone	<5	<5
β-Cyclocitral	3	5
β-Damascenone	<5	<5
β-Ionone	11	18
Epoxy-β-ionone	<5	<5

1.5.5.2.1.3 Lignin-Related Compounds

Formation of shikimic acid in plants is derived from the modification of phosphoenol pyruvate and *D*-erythose-4-phosphate. The shikimate is converted to chorismate, an intermediate in many aromatic compounds related to several lignin-related compounds such as benzaldehyde and cinnamaldehyde (Fisher and Scott, 1997). Other lignin-related compounds, eugenol, guaiacol (2-methyloxyphenol) and methyl salicylate, have been identified in tomato volatiles (Kazeniac and Hall, 1970) that ferulic acid and *p*-coumaric acid are main precursors of these compounds (Whitfield and Last, 1991).

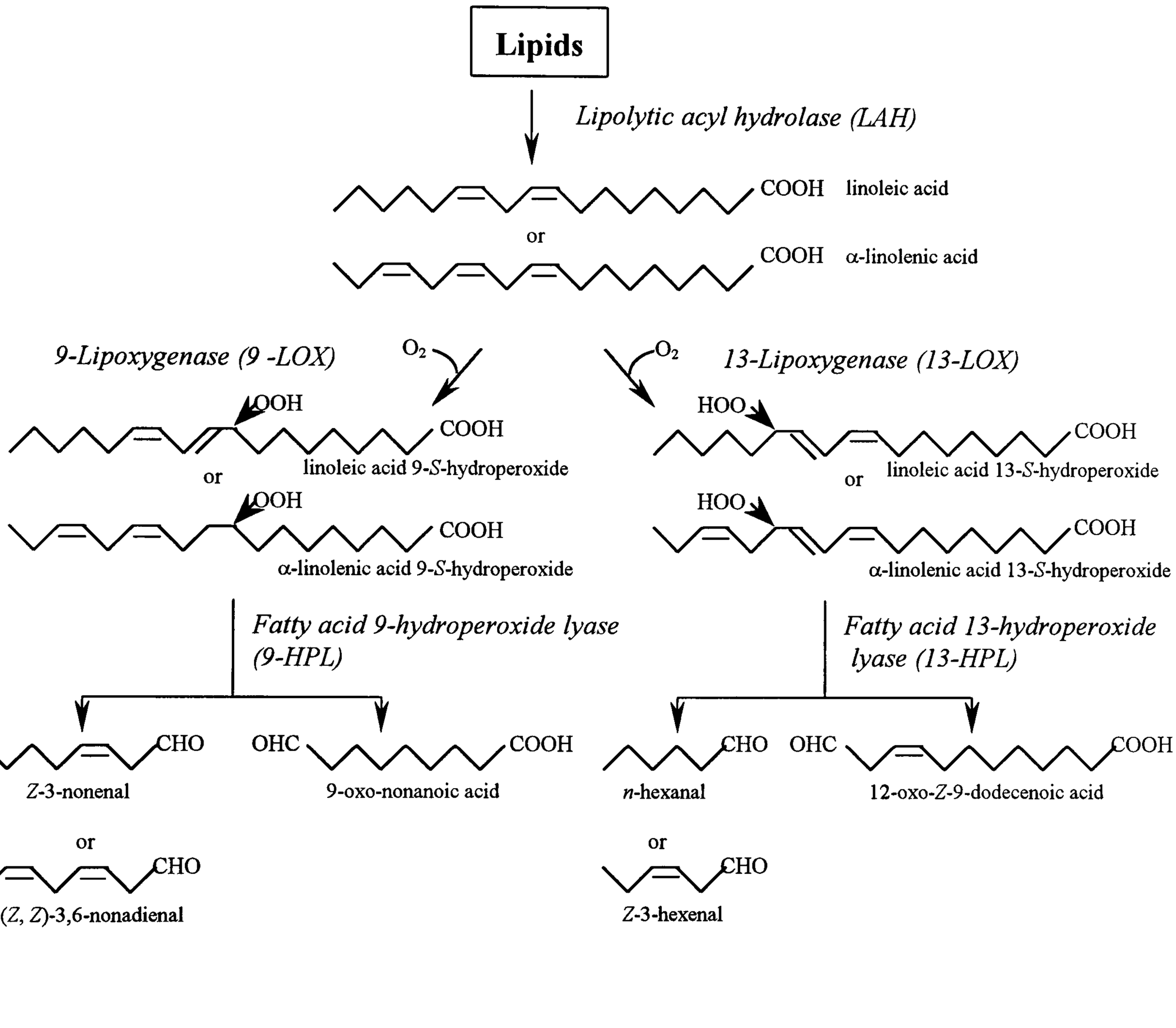
1.5.5.2.1.4 Lipid-Oxidised Compounds

Unsaturated fatty acid metabolism in plants reveals many prospects for plant development. Free forms of the fatty acids, which are released from phospholipids by either phospholipase A_1 (PLA_1), or phospholipase A_2 (PLA_2) (Laxalt and Munnik, 2002), or from triglycerides by lipases, can be involved in pathways of lipid oxidation, flavour generation, or plant defensive metabolism (Bleé, 1995; Feussner *et al.*, 1997).

Lipoxygenases (LOX) play a major role in the generation of volatile C6 aldehydes and alcohols in tomato through lipid oxidation pathways (Figure 1.2). Linoleic (C18:2) and linolenic (C18:3) acids are the main substrates of LOX. Biosynthesis of short-chain carbon volatiles occurs via the sequential action of enzymes starting from lipolytic acyl hydrolase (LAH) to release free fatty acids from lipids. The free fatty acids are oxidised to form fatty acid hydroperoxides (HPOs) by LOX. Cleavage of these HPOs to produce short chain aldehydes and oxo-acids is carried out by fatty

acid hydroperoxide lyase (HPL). The pathway of C6 aldehyde generation predominating in tomato is shown by the metabolism on the right-hand side in Figure 1.2 (Gray *et al.*, 1999).

Figure 1.2 Short-chain aldehyde forming system in plants (redrawn from Matsui *et al.*, 2001)



The C6 aldehyde compounds, which are an important part of tomato flavour volatiles, are the predominant compounds identified in both the blended leaves and tomato fruit (Buttery, 1993). Although more than 400 substances have been found to contribute to

ripe tomato flavour, a combination of 10 major volatiles, consisting of *Z*-3-hexenal, *E*-2-hexenal, hexanal, 1-penten-3-one, 3-methyl butanal, *Z*-3-hexenol, 6-methyl-5-hepten-2-one, methyl salicylate, 2-Isobutylthiazole and β-ionone, was considered to be very similar to the aroma compounds of a sliced fresh ripe tomato (Buttery, 1993). The most outstanding aroma compound, indicated thorough panel test, is *Z*-3-hexenal (Buttery, 1993; Buttery *et al.*, 1987) and is also considered to be indicative of a tomato-like smell (Tandon *et al.*, 2000).

1.5.5.2.1.4.1 Lipoxygenase (LOX)

Purified linoleate oxygenase from tomato fruit optimally functions at pH 6.8 at 25°C in the absence of any detergent and the Km values for linoleic acid and linolenic acid are 1.42 and 2.60 mM, respectively (Regdel *et al.*, 1994). Riley *et al.*, (1996) reported that activities of the soluble and membrane-bound LOXs, extracted from ripening fruit, were higher at pH 6 than at pH 7 or 8 and the LOX activity was predominantly in a post-microsomal pellet. There have been at least five LOX genes isolated from tomato, namely *TomloxA*, *TomloxB (Ferrie et al., 1994)*, *TomloxC*, *TomloxD* (Heitz *et al.*, 1997) and *TomloxE* (Accession No AY008278). Nevertheless, only three LOX genes, *TomloxA*, *TomloxB*, and *TomloxC*, are expressed at significant levels in the fruit during ripening, but probably have different functions (Griffiths *et al.*, 1999). *TomloxD* is largely absent from fruit, but is found in leaves and the levels of expression are increased in wounded leaves (Heitz *et al.*, 1997). The gene product of *TomloxD* is expected to be involved in defence signaling in response to herbivore and pathogen attack by forming a component of the octadecanoid-signaling pathway.

1.5.5.2.1.4.2 Hydroperoxide Lyase (HPL)

Fatty acid hydroperoxide lyase (HPL) is an enzyme that cleaves a C-C bond near to a hydroperoxide group of polyunsaturated fatty acids to form short chain aldehydes and ω-oxoacids. Most HPLs exhibit some substrate specific, utilising preferentially 13-hydroperoxides of α-linolenate and linoleate, but some enzymes like cucumber and melon HPLs modify both 9-and 13-hydrperoxides (Grechkin, 2002). HPL, highly purified from green bell pepper fruits, appears to be a heme protein whose spectrophotometric properties greatly resemble a cytochrome P-450 (Matsui *et al.*, 1996; Psylinakis *et al.*, 2001; Shibata *et al.*, 1995). However, no heme group was demonstrated in HPL purified from tomato; only 13-hydroperoxides from linoleic and linolenic acids were cleaved by the enzyme having optimum pH at 6.5 (Suurmeijer *et al.*, 2000). This suggests that C-6 compounds are the main volatiles in tomato flavour. Tomato HPL showing high activity in microsomal membranes did not show any significant change in activity as the fruit ripened (Riley *et al.*, 1996).

1.5.5.2.1.5 Other Pathways

Alcohol dehydrogenase (ADH) has been shown to play an important role in the interconversion of aldehydes to alcohols in ripening tomato fruit (Bicsak *et al.*, 1982; Prestage *et al.*, 1999; Speirs *et al.*, 1998). The tomato-genomic Southern analysis by Van Der Straeten *et al.* (1991) suggested the presence of a multigene family for ADH and at least 3 *ADH* genes were subsequently isolated. *ADH1* is expressed only in pollen, seeds and young seedling, while *ADH2* accumulates in many parts of tomato including ripe fruit (Chen and Chase, 1993). The *ERT10* clone, 'short chain' ADH, (Accession No X72730) was also cloned from ripe tomato fruit (Picton *et al.*, 1993b). Genetic manipulation of *ADH2* levels in ripening tomato fruit has been shown to affect the balance of some flavour aldehydes and alcohols and fruit with increased

ADH2 levels had a more intense "ripe-fruit" flavour (Speirs *et al.*, 1998). Transgenic tomato fruit with enhanced *ADH2* activity had not shown significant difference in alcohol levels to controls, but down-regulation showed much higher aldehydes: alcohols ratio (Prestage *et al.*, 1999). High induction of ADH was observed under hypoxic conditions, both in tomato seedlings and roots. However, in tomato after the onset of ripening, the induction of ethanol production and ADH gene expression is not accompanied by induction of pyruvate decarboxylase (PDC) activity (Or *et al.*, 2000).

1.5.5.2.2 Melon Aroma

Melon produces a range of volatile compounds from various sources during ripening, including alcohols, aldehydes, ketones, esters, sulphur-containing compounds, and furans.

1.5.5.2.2.1 Amino Acid-Related Compounds

The pathway of the production of amino acid-related volatiles is generally the same as the tomato pathway described in section 1.4.5.2.1.1. Branched-chain compounds are typically generated from leucine and isoleucine (Bauchot *et al.*, 1998; Wyllie *et al.*, 1996).

1.5.5.2.2.2 C9 Saturated and Unsaturated Aliphatic Aldehydes and Alcohols

Many of the melon volatiles consist of C9-aldehyde, C9-alcohols and their esters. These C9-aldehydes and alcohols are known as off-flavour components of hydrogenated vegetable oils and are found in members of the family *Cucurbitaceae* (Engel *et al.*, 1990). Melon LOX plays a major role in the generation of C9 and C6 aldehydes and alcohols through the lipid oxidation pathways (Grechkin, 2002). The C9 aldehyde metabolism is shown in the left-hand side of the pathway in Figure 1.2. Some C9 volatile compounds are presented in Table 1.7

Table 1.7 Odour detection thresholds in water of some C9 compounds in melon (Modified from Engel *et al.*, 1990)

Compound	Odour threshold (ppb)
Nonanyl acetate	200
E-Non-3-enyl acetate	60
E, *Z*-Nona-3, 6-dienyl acetate	15
Z, *Z*-Nona-3, 6-dien-1-ol	10
E, *Z*-Nona-3, 6-dien-1-ol	3
Z-Non-6-enyl acetate	2
Z-Non-6-en-1-ol	1
Nonanal	1
E-Non-2-enal	0.5-1

HPL as purified from cucumber has a Mw about 5.5 kDa and optimum activity at pH 6 with both 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid substrates (Hornostaj and Robinson, 1999). The *C15* cucumber HPL clone, characterised as a P-450 protein, was also shown to act on both 9- and 13-hydroperoxides, but the *C17* HPL clone was revealed specifically to be only active against 13-hydroperoxides, when both clones were expressed in *E. coli* (Matsui *et al.*, 2000). *CYP74* melon HPL, encoding a protein of 481 amino acids, shows 35-50% homology at amino acid level with other members of the *CYP74* family. The melon HPL, expressed in *E. coli*, is also a cytochrome P-450 enzyme, showing the highest rate of reaction with 9-hydroperoxy-linolenic acid, followed by 9-hydroperoxy-linoleic acid and then the corresponding 13-hydroperoxides (Tijet *et al.*, 2001).

1.5.5.2.2.3 Ester Compounds

Volatile carboxylic esters contribute an important group of aroma substances in many fruit such as apple, strawberry, banana and melon (Harada *et al.*, 1985; Pérez *et al.*, 1993; Vanolia *et al.*, 1995; Wyllie *et al.*, 1996). The aroma profiles of many ripe melons are notable for the presence of a range of esters significantly contributing to the characteristic odour and quality perception of the fruit. Esters were detected in low amount at the initial stages of fruit development, but were produced in abundance during melon ripening and were related to the rates of respiration and ethylene production (Wang *et al.*, 1996). A significant proportion of esters containing a branched alkyl chain originate from leucine and isoleucine (Wyllie *et al.*, 1996).

1.5.5.2.2.3.1 Plant Alcohol Acyl Transferases (AAT)

Since aldehydes can be converted into alcohols in plants by enzymatic reduction, for example by alcohol dehydrogenase (ADH), esters are derived from alcohols and acyl-CoAs (Erickson, 1979). The last enzyme in the pathway producing esters are alcohol acyl-transferases (AAT) (Figure 1.3), which produce ester aroma in various parts of plants. The ester benzyl acetate, produced by the enzyme acetyl-CoA:benzylalcohol acetyl-transferase (BEAT), is a major floral scent of *Clarkia breweri* (Dudareva *et al.*, 1998). In snapdragon, the main scent compound is the volatile ester methyl benzoate, which is generated by *S*-adenosyl-*L*-methionine:benzoic acid carboxyl methyl-transferase (BAMT), the final enzyme of the pathway. Most of BAMT is in petals and the peak of the activity occurs during full bloom during daytime (Dudareva *et al.*, 2000). Benzoyl-CoA:anthranilate-*N*-benzoyl-transferase (BEBT) converts anthranilate and benzoyl-CoA to N-benzoyl anthranilate, which is the precursor of several kinds of dianthramides in carnation (Yang *et al.*, 1997). *In vitro* the enzyme showed limited

substrate specificity for anthranilate but accepted a variety of aromatic-CoAs. Harada *et al.* (1985) purified and investigated alcohol acetyl-transferase from banana fruit and found that the esterification ability in pulp slices appeared in the fruit at the yellow-green stage and increased, reaching a maximum at the full yellow stage. Pérez *et al.* (1993) studied AAT substrate specificity to various alcohols and acyl-CoAs, in strawberry and showed that hexyl alcohol and acetyl-CoA are the preferred substrates. Relative activities of AAT from some fruit are shown in Table 1.8

Table 1.8 Relative activities of alcohol acyl-transferase from fruit protoplasts towards a rang of alcohols (acyl donor acetyl CoA) (from Wyllie *et al.*, 1996)

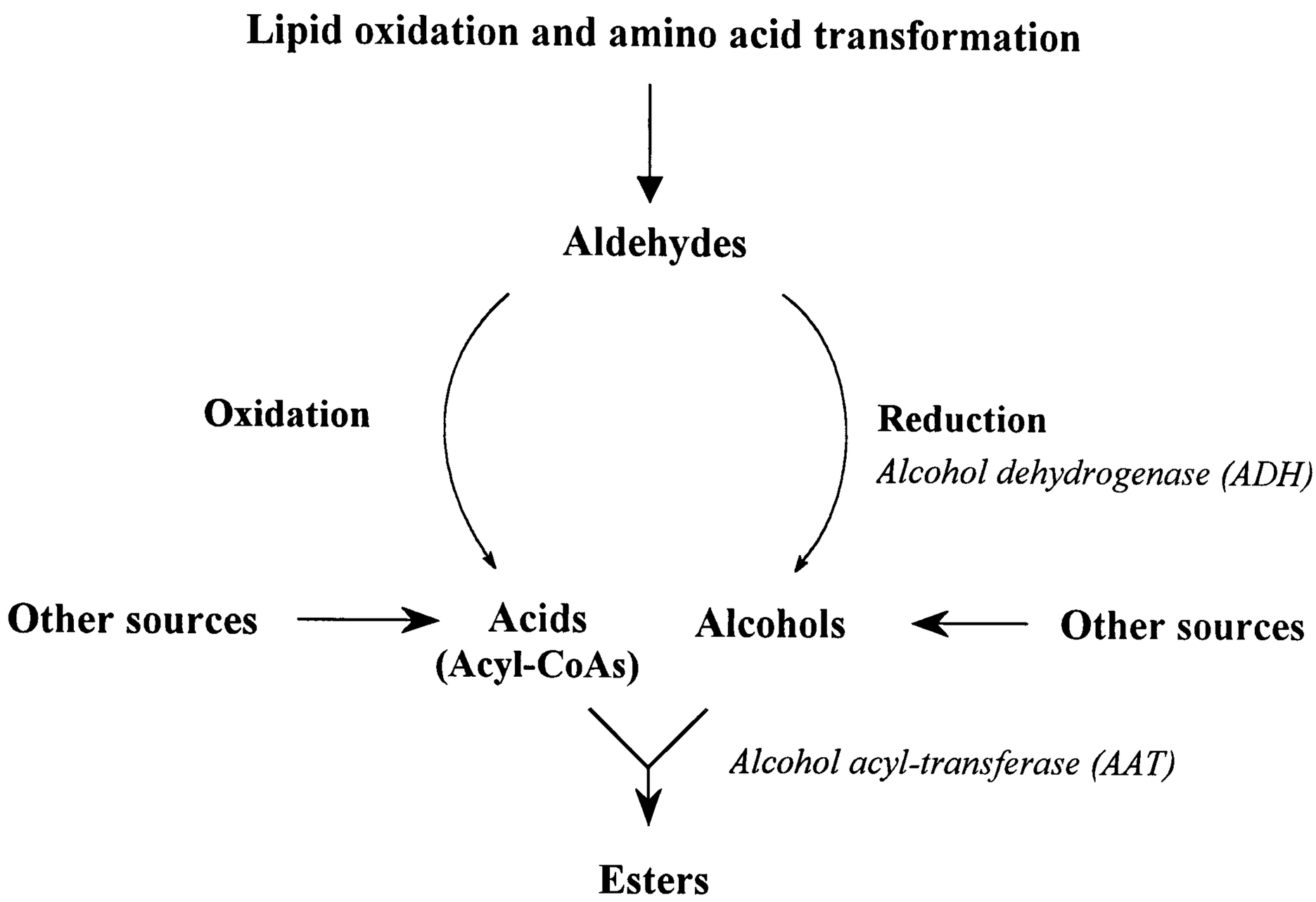
Alcohol substrate	Strawberry tissue	Strawberry enzyme	Banana tissue	Melon tissue
Methanol	0	nd	0	0
Ethanol	0	100	0	0
Propanol	8	5	0	7
Butanol	54	40	47	57
2-Methylpropanol	24	7	65	50
2-Methylbutanol*	100	84	93	88
3-Methylbutanol	95	75	100	100

nd : not determined

* : S enantiomer

There are several alternative pathways that can generate esters, such as β-oxidation shortening of fatty acids; reduction and oxidation of carbonyl groups; decarboxylation of keto-acids; degradation of unsaturated fatty acids (Berger *et al.*, 1992). A summary of routes producing carboxylic esters is shown in Figure 1.3.

Figure 1.3 Summary of pathway leading to the formation of volatile carboxylic esters in plants



1.5.5.2.2.3.2 Melon AAT

In melon, purification of AAT protein has been reported and its activity demonstrated during ripening (Shalit *et al.*, 2001; Ueda *et al.*, 1997; Wyllie *et al.*, 1996), but AAT related genes have not been isolated. Aggelis *et al.* (1997b) found 2 novel ripening-related cDNA clones, *MEL2* and *MEL7*, from screening the melon-ripening library. *MEL2* was found to be expressed specifically during fruit ripening, while *MEL7*, although it increased greatly during ripening, was expressed in many melon organs including roots and stems. Southern analysis indicated that there is more than one gene corresponding to *MEL2*. It was proposed that they could play an important role in fruit and ripening, because their expression is specific, but the function has not been identified (Aggelis *et al.*, 1997a).

A study of floral aroma in *Clarkia breweri* by Durareva *et al.* (1998) revealed the first evidence to support the possibility that the *MEL2* may function as a BEAT. One region of BEAT, containing 35 amino acid residues (amino acid position between 135 and 163), shows 40-50% identity to a part of the *MEL2* predicted protein. Moreover these 35 residues have a significant similarity to several proteins known to use an acyl-CoA substrate. For example, the region is 56% and 42% identical to the corresponding region in the CER2 protein of *Arabidopsis* (Negruk *et al.*, 1996) and in HCBT (hydroxy-cinnamoyl/benzoyl-CoA:anthranilate-*N*-hydroxycinnamoyl/benzoyl-transferase) from carnation (Yang *et al.*, 1997), respectively. Furthermore, from genetic database alignment, *MEL2* shares some similarities to many genes in the acyl-transferase family at both the nucleotide and nucleic acid levels. **'*MEL2*' was subsequently renamed '*CM-AAT1*'** (Yahyaoui *et al.*, 2002).

Melon, a fragrant fruit, accumulates large amounts of sugar and produces abundant aromatic volatiles (Ayub *et al.*, 1996; Horvat and Senter, 1987). The volatiles are rarely produced at the initial stage of fruit development, but they are generated in abundance during ripening stages (Wang *et al.*, 1996). Many papers have reported that the major volatiles producing during melon ripening are ester compounds (Homatidou *et al.*, 1992; Horvat and Senter, 1987; Schieberle *et al.*, 1990; Wang *et al.*, 1996; Wyllie and Leach, 1990; Wyllie and Leach, 1992; Yabumoto and Jenning, 1977). The enzymes of importance in biochemical pathways for the formation of esters in ripening melons are α-aminotransferase, ADH, and AAT (Wyllie *et al.*, 1996). Ueda *et al.* (1997), for example, studied an AAT from melon involved in the esterification of alcohol with acetyl-CoA. Levels of acetate ester, ethyl acetate and alcohol remain low at maturation but they increase rapidly in mid and fully ripened

fruit (Harvat and Senter, 1987; Wang *et al.*, 1996). This evidence suggests that *CM-AAT1* (*MEL2*) may be involved in aromatic production in ripening melon and the encoded protein may be an esterase enzyme using acyl-CoAs as substrates.

1.6 Genetic Modification of Fruit

1.6.1 *Agrobacterium*-Mediated Gene Transformation

Agrobacterium, a soil bacterium, is the causative agent of crown gall disease in many dicotyledonous plants. The pathogenic mechanism of *Agrobacterium* on host plants is based on the ability to transfer a particular DNA segment, the T-DNA of the *Ti*-plasmid (from *Agrobacterium tumefaciens*) or the *Ri*-plasmid (from *A. rhizogenese*), to the plant genomes during the cellular interaction between the two organisms (Haung *et al.*, 1993). The *Ti*-plasmid contains a 35 Kb virulence region including several virulence (*vir*) genes, whose products are directly involved in T-DNA processing and transfer (Zupan and Zambryski, 1995). The expression of genes in the *vir* region is induced by phenolic compounds from cells or wounded tissues of the host plants. The products of the *vir* region generate the transfer intermediate (T-complex) and mediate T-DNA transport into the plant cells (Zupan *et al.*, 2000). T-DNA, therefore, can be engineered to contain interesting genes and/or a selectable marker, which is required to be inserted into a nuclear genome of the host plant. The *Agrobacterium*-mediated transformation method is activated by a set of *vir* gene with optimal expression occurring at acidic pH and in the presence of phenolic inducers released by wounded plant cells (Hansen and Wright, 1999). A binary artificial chromosome vector has been developed that is capable of transferring up to 150 Kb of foreign DNA into a plant genome (Hamilton *et al.*, 1996).

Gene transformation into tomato by *Agrobacterium tumefaciens*, which greatly contributes to rapid molecular studies, and transgenic tomato plants can be regenerated relatively easily, is successfully achieved by tissue culture techniques (Bird *et al.*, 1988; Koornneef *et al.*, 1986; McCormick *et al.*, 1986). Several gene promoters have been widely applied in transgenic tomatoes to study ripening and senescence, for example, CaMV 35S (a constitutive promoter, (Benfey *et al.*, 1990)), 2A11 (a tomato fruit-specific promoter, (van Haaren and Houck, 1993)), PG (a tomato fruit-ripening-specific promoter, (Nicholass *et al.*, 1995a)), ACO1 (a tomato fruit ripening and wounding specific promoter, (Blume *et al.*, 1997)). A reliable system for the *Agrobacterium*-mediated transformation in melon was recently reported in 2000 (Guis *et al.*, 2000), although the *ACO1* antisense plant had been achieved previously in melon (Ayub *et al.*, 1996).

1.6.2 Control of Gene Expression

Gene expression mechanisms include transcription and processing of mRNA, which is influenced by chromatin architecture and availability of transcription factors, RNA stability, and translational mechanisms. The 'central dogma' of molecular biology states that DNA makes RNA and the RNA makes protein (Thieffry and Sarkar, 1998). Each step of the pathway of gene expression is complex and, furthermore, may be involved in regulation. The importance of gene regulation in eukaryotes is that the expression of many genes is controlled at more than one step (Latchman, 2002). The regulation frequently occurs at either the level of transcription or translation.

1.6.2.1 Gene Over-Expression

Manipulation of organisms can be used to study gene function as well as to produce low cost production factories by over-expressing the required genes. Transgenes or foreign genes can be introduced to produce required proteins in *E. coli* or eukaryotes such as yeast, insects, plants, or mammals. Some aspects in gene expression have to be concernedly considered for making proper sense gene constructs in order to produce the appropriate proteins. The most importance is to obtain the correct DNA-reading frame containing in the gene cassette, because the proper protein will be transcribed and translated from the correct ORF (Brown, 1986). In addition, there are sections of RNA before and after its start and stop sequences that are not translated. These regions, known as the 5'UTR and 3'UTR, have been studied in term of controls of gene expression. In many eukaryotes, several structural features of 5'UTRs have been shown to be important for the mRNA translational efficiency (Kozak, 1987; Kozak, 1991; Pain, 1996). On the other hand, stability of mRNA transcripts is regulated by 3'UTR secondary structure (Kozak 1990). Some 3' UTRs can be interacted with one or more protein factors and allow the mRNAs remain stable during active translation (Ji *et al.*, 2003).

1.6.2.2 Gene Silencing

Gene silencing is an important strategy in understanding and analysing gene function. Two effective strategies for down-regulating the expression of target endogenous genes are antisense and sense gene expression.

1.6.2.2.1 Antisense Gene Silencing

Down regulation of endogenous genes in plant has been observed over past 15 years. Inverted-sense nucleotides have proven to be a specific method for the down-

regulation of a number of cellular targets. The first evidence in the transgenic tomato, containing an inverted partial PG cDNA under the CaMV 35S promoter, showed the inhibition of endogenous PG, varied between different transformants (Smith *et al.*, 1988). The antisense genes were stably inherited and PG activity was found to be inhibited by 99% or more in plants homozygous for the antisense construct with other ripening aspects unaffected (Smith *et al.*, 1990b). The antisense techniques have been used worldwide for study of gene identification. For example, the antisense *TOM13* tomato inhibited ethylene synthesis in ripening fruit and wounded leaves that was correlated with a decrease in ACO activity (Hamilton *et al.*, 1990). That indicates that *TOM13* encodes ACO during fruit ripening and in wounded leaves.

The efficiency of antisense gene silencing depends on the formation of *in vivo* transcribed antisense RNAs, which are supposed to interact with their target mRNAs, resulting in suppressed gene expression (Kuipers *et al.*, 1997). This complementary strand of nucleotides binding to the mRNA to prevent production of the protein is antisense nucleotides. In side the transgenic cells, antisense nucleotides are targeted to interact with complementary regions of a specific mRNA (RNA:RNA) or are formed triple-helix structure with genomic DNA (RNA:DNA)(Toulmé *et al.*, 1997). Cleavage of target antisense RNA based on hammerhead (hairpin)-ribozyme structure has been also studied (Sun *et al.*, 2000; Tabler and Sczakiel, 1997).

1.6.2.2.2 Co-Suppression Gene Silencing

Transformation with homologous sense genes can frequently effect a reduction of endogenous gene expression in plants. Sense gene silencing has been initially reported from 1990 (Napoli *et al.*, 1990; Smith *et al.*, 1990a), although the mechanisms had been unknown at the time.

The repeated two additional upstream inverted copies of 5' untranslated region of *ACO1* enhanced the probability of gene silencing of the endogenous gene in tomato due to post-transcriptional suppression (Hamilton *et al.*, 1998), which was found to correlate with the production of small antisense RNAs (asRNAs), preferentially generated from the 3' region of the transgene (Han and Grierson, 2002a). Small antisense RNAs and sense RNAs were present in transgenic plants showing transgene-induced sense gene silencing and these small RNAs were homologous to their target RNAs (Hamilton and Baulcombe, 1999). Furthermore, approximately 23 nucleotides of asRNAs were shown to be produced from the transgene in silencing of the fruit ripening-specific endogenous PG (Han and Grierson, 2002b). The silencing mechanism is proposed to involve the production of antisense RNA from the 3' region of the transgene by an RNA-dependent RNA polymerase (RdRP) (Baulcombe, 1999; Han and Grierson, 2002b). The double strand RNAs (sense and antisense RNAs formed) could be cleaved into 21-25 nt RNAs by a dsRNA-specific RNase called 'Dicer' (Baulcombe, 2002; Han and Grierson, 2002b; Hannon, 2002). Annealing of the cleaved asRNAs to an RNase would target it to the endogenous RNA and result in endonucleotytic cleavage, causing gene silencing (Han and Grierson, 2002b).

1.6.3 Manipulation of Ripening-Related Genes

1.6.3.1 Cell Wall Altering

The techniques of gene silencing have been successfully used to inhibit the function of ripening-related genes. The first gene manipulated in transgenic tomato fruit was PG, which had been believed to be the key enzyme in tomato fruit softening. In wild type tomato, PG mRNA increases around 100-fold during ripening (DellaPenna *et al.*, 1986), while levels of PG mRNA in *rin* mutant is less than 1% comparing to normal (DellaPenna *et al.*, 1989). The accumulation of PG mRNA from antisense PG fruit

was reduced to approximately 1% of the normal (Sheehy *et al.*, 1988; Sheng and Wainwright, 2000; Smith *et al.*, 1988). However, although PG activity was dramatically reduced and pectin degradation was inhibited, the fruit softening during ripening was not different to the normal (Smith *et al.*, 1988). Furthermore, PG gene was manipulated to be over-expressed in *rin* mutant and polyuronide structure was degraded, but the transgenic fruit still did not restored to normal softening (Giovannoni *et al.*, 1989). Subsequently, it was shown that the texture of low PG fruit was in fact altered (Grierson and Schuch, 1993). This indicates that PG is not the major enzyme of tomato fruit softening. PME functions to de-esterify methyl-esterified polyuronides in the cell wall. Although large pectin fragments extracted from cell wall were increased, the fruit softening of PME antisense fruit was not different to the normal (Tieman *et al.*, 1992). However, transgenic tomato, introduced with an antisense *Rab11* GTPase gene, showed a decrease in PG and PME levels, resulting in reduction of the fruit softening (Lu *et al.*, 2001). This indicates that *Rab11* GTPase may play a role in transport or secretion of cell wall modifying enzymes. *TBG4*, encoding a β-galactosidase/*exo*-galactanase, is another gene involved in fruit softening and antisense transgenic fruit of the gene have been shown to be 40% firmer than controls (Smith *et al.*, 2002). In tomato, expansin *Exp1* is highly expressed during fruit ripening (Rose *et al.*, 1997). *Exp1*-suppressed fruit were firmer than control throughout ripening, while over-expressed fruit were much softer than controls even at the mature green stage (Brummell *et al.*, 1999).

1.6.3.2 Colour Development

The *TOM5* expression is involved in carotenoid biosynthesis during tomato ripening. Levels of *TOM5* mRNA, encoding phytoene synthase (PSY), were markedly reduced in *TOM5*-antisense tomato, resulting in yellow fruit and pale-yellow flowers (Bird *et*

al., 1991). The antisense fruit were similar in phenotypes to the *yellow flesh* (*r*) mutant. Over-expression of *TOM5* in transgenic *yellow flesh* tomato restored the synthesis of lycopene, confirming the mutant lacked a functional PSY (Fray and Grierson, 1993). Since lycopene and β-carotene have been reported to be such an important part of a healthy diet, there have been efforts of improving carotenoid content in tomato. For instance, bacterial *Psy* was recently over-expressed in tomato under an PG promoter causing increase of 1.8 fold in lycopene and 2.2 fold in β-carotene (Fraser *et al.*, 2002). The transgenic tomatoes containing a bacterial carotenoid gene (*crtI*), encoding the enzyme phytoene desaturase, did not elevate total carotenoid levels but the β-carotene content increased about threefold of the total carotenoid content. Endogenous carotenoid genes were concurrently up-regulated, except for *Psy*, which was repressed (Romer *et al.*, 2000).

1.6.3.3 Volatile Production

The C6 aldehydes are an important part of tomato flavour volatiles identified in both blended leaves and tomato fruit (Buttery, 1993). LOX is the main enzyme in the lipid oxidation pathway, but silencing of *TomloxA* and *TomloxB* by antisense genes failed to reduce aldehyde volatiles in ripe fruit (Griffiths *et al.*, 1999). *TomloxC*, however, may play a role in lipid oxidation in fruit during ripening, since inhibiting its expression has been shown to greatly reduce fruit volatile concentration (Dr Guoping Chen, this laboratory, personal communication). Tomato plants transgenic for a heterologous *Clarkia breweri S*-linalool synthase (LIS) gene, under the control of the tomato late-ripening-specific E8 promoter, synthesise and accumulate *S*-linalool and 8-hydroxylinalool in ripening fruit (Lewinsohn *et al.*, 2001). Speirs *et al.*, (1998) reported over-expression of *ADH2* in tomato resulted in increased hexanol and *Z*-3-

hexenol levels during ripening. However, in further experiment using a shear maceration procedure, *ADH2* down-regulated fruit showed significant reduction of alcohols especially *Z*-3-hexenol, but up-regulation showed no difference in alcohol levels to controls (Prestage *et al.*, 1999).

1.6.3.4 Reduction of Ethylene

The reduction of ethylene production causes a dramatic ripening delay of many aspects of the fruit ripening process. There are many ripening mutants in tomato, shown in Table 1.9, some of which have altered ethylene synthesis or response. Green, breaker, orange, or red tomato fruit, treated with 1-MCP (1-methyl-cyclopropane, an ethylene-action inhibitor), show great reductions of the mRNA accumulation of *ACO1*, *PSY1*, and *EXP1* (Hoeberichts *et al.*, 2002). Transforming tomato with an antisense ACC oxidase gene (*ACO1, TOM13*) effectively reduced ethylene both in ripening fruit and wounded leaf Hamilton *et al.*, 1990; Picton *et al.*, 1993a). The percentage of reduction depends on the number of antisense genes introduced into the plant, and perhaps the position of the antisense gene. The levels of mRNA homologous to *TOM5* were significantly lower in ACO antisense tomato and exogenous ethylene treatment at least partially restored expression of this gene compared to that in wild type (Picton *et al.*, 1993a). Antisense ACS tomato showed 99% inhibition of ethylene production and never fully ripened without exogenous ethylene application. It was reported that PG mRNA was still accumulating in spite of the low levels of ethylene production in ACS antisense fruit (Oeller *et al.*, 1991). This suggests that PG is an ethylene-independent gene. However, Theologis *et al.* (1993) reported that ethylene may play a translational or post-translational role in PG expression. Subsequently, however, a role for ethylene in controlling expression of PG has been established (Nicholass *et al.*, 1995b; Sitrit and Bennett, 1998).

Table 1.9 Tomato ethylene production mutants (Modified from Biggs and Handa, 1989; Grierson and Kader, 1996)

Name	Chromosome on which the gene is located	Fruit phenotype
Ripening inhibitor (*rin*)	5	Fruit do not ripen even when treated with exogenous ethylene. Pericarp turns yellow in colour and softens very slowly. There is no rise of respiration or ethylene. The fruit have very low levels of PG activity and poor tomato flavour. The mutation is now known to be in a transcription factor required for ripening gene expression.
Never ripe (*Nr*)	9	Ripe fruit turn on orange colour and soften slowly. Ethylene, lycopene and PG production is much reduced from normal tomato. The mutation is in a fruit-expression ethylene receptor gene.
Non ripening (*nor*)	10	Similar to *rin* but the final ripe fruit colour is pale orange. Ethylene production is very low. PG activity is less than 1% of the wild type fruit. The mutation is now known to be in a transcription factor.
Alcobaca (*alc*)	10	Ripe fruit attached to the vine are pale red. The flavour is almost normal but the storability is increased due to a slow softening rate. Fruit picked mature green show reduced ethylene production and respiration and only ripen to a yellow colour.
Longkeeper	10	Fruit ripen to a golden-orange-red colour. There are reductions of PG activity, softening and carotenoid synthesis.

In a transgenic tomato line over expressing ACC deaminase, the bacterial enzyme using ACC as a substrate, although ethylene synthesis was partially inhibited, the levels of ACO and PG activity indicated no significant differences from controls while transgenic fruit are significantly firmer than controls. This indicates that other enzymes may have a significant role in fruit softening (Klee, 1993; Klee *et al.*, 1991). In other experiments, ethylene production was inhibited by expressing S-adenosylmethionine (SAM) hydrolase that catalyzes the conversion of SAM, the metabolic precursor of ACC, to methylthioadenosine and homoserine (Good *et al.*, 1994).

In Charentais melon fruit transformed with an *ACO1* antisense gene, a sharp reduction in ethylene production and fruit ripening occurred both on and off the vine (Ayub *et al.*, 1996). Chlorophyll loss was strongly inhibited in the rind but the accumulation of carotenoids in the flesh continued. As antisense fruit can be kept on the vine longer, they accumulate higher amounts of soluble sugars, thus reaching better sensory quality. Flores *et al.* (2001) reported some physiological characterisation of *ACO1* suppressed cantaloupe lines: degreening of the rind and cell separation in the peduncular abscission zone were totally dependent on ethylene, but softening and membrane deterioration displayed some ethylene-independent components. However, the total volatiles of the *ACO1* antisense variety were 60-85% lower than those of the non-transformed variety (Bauchot *et al.*, 1998). The production of aliphatic esters such as butyl and hexyl acetates was found in trace levels in *ACO1* antisense fruit and, furthermore, were highly reduced in the discs from wild-type and transgenic fruits treated with 1-methylcyclopropane, the ethylene antagonist (Flores *et al.*, 2002). Expression of the *MEL2* (*CM-AAT1*) mRNA, which has some homology to genes involved in flavour production, was induced 27-fold after exogenous ethylene treatment to unripe melon fruit (Aggelis *et al.*, 1997b). These results suggest that

ethylene may be involved in expression of genes important in production of aroma volatiles.

1.7 Genetic Screening

1.7.1 Differential Screening

Differential screening is an important tool to isolate new or unique genes from cDNA libraries. The differential expression screen can involve cDNA libraries from different sources, different treatments, different times, organs, tissues or varieties. For example, five unique clones, *pERT1*, *10*, *13*, *14* and *15*, were isolated using differential screening of fruit ripening cDNA libraries from wild type tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) and *rin* (ripening inhibitor) mutant (Picton *et al.*, 1993b) and many ripening-related clones, expressed in ripe but not unripe fruit, were reported (Grierson *et al.*, 1986a).

Aggelis *et al.* (1997b) isolated 2 novel ripening-related cDNA clones, *MEL2* (*CM-AAT1*) and *MEL7*, using differential screening on melon cDNA libraries. The *MEL2* (*CM-AAT1*) cDNA clone was of unknown function and found to be expressed specifically during fruit ripening while *MEL7*, which was expressed in many melon organs including roots and stems, shows a significant similarity with a latex protein from opium-poppy (*MLP22*) (Aggelis *et al.*, 1997b; Hadfield *et al.*, 2000) and other major latex proteins (Strömvik *et al.*, 1999). Hadfield *et al.* (2000) used differential screening to isolate 16 melon ripening-regulated cDNAs from Charentais melon with half of them showing no significant sequence homology to any known protein or nucleotide sequence in the database. Hong *et al.* (2002) recently found 2 melon fruit-specific cDNA clones, *RFS3* and *RFS8*, showing no significant homology with known protein sequences.

1.7.2 Homologous Screening

Homologous Screening is based on homology between a probe (normally a nucleic acid sequence) and one or some clones represented in a DNA library. An ethylene receptor, *ETR1*, isolated from *Arabidopsis thaliana* (Chang *et al.*, 1993), was used from homologous screening for tomato ethylene receptors such as the *Nr* gene (Payton *et al.*, 1996; Wilkinson *et al.*, 1995; Yen *et al.*, 1995), *LeETR1* and *LeETR2* cDNAs (Lashbrook *et al.*, 1998).

Detailed molecular genetic research on melon ripening has started relatively recently by using genome database information from tomato. *MEL1* (*CM-ACO1*), encoding a melon ACO, was isolated from a melon fruit cDNA library using homologous screening with *pTOM13*, the tomato ACO fragment, as a heterologous probe (Balagué *et al.*, 1993). *CM-ACO2* and *CM-ACO3*, melon ACO gene family members were isolated from screening of a melon genomic library with the *MEL1* probe (Lassaere *et al.*, 1996). Karvouni *et al.* (1995) also isolated *MEL5* encoding a melon phytoene syntase using *pTOM5*, the tomato phytoene synthase, as a probe. Two muskmelon ethylene receptor clones, *CM-ERT1* and *CM-ERS1*, were isolated using RT-PCR using *Arabidopsis* and tomato DNA sequences as database for making primers (Sato-Nara *et al.*, 1999) and there are many other sequences examples of this approach.

1.8 Recombinant Gene Expression

The expression of cloned eukaryotic genes in microorganisms allows the isolation of large quantities of protein products that are present in only trace amounts from natural sources (Taun, 1997). The production of recombinant gene products is a very successful technique of modern molecular biology, and can enable many different proteins to be characterised at the molecular level.

1.8.1 Proteins Expressed in *E. coli*

The extensive genetic and biochemical studies of *E. coli* laid the foundation for modern recombinant DNA technology. *E. coli* has been widely used as a host for gene expression because of its simplicity, the availability of strong regulatable promoters and high levels of protein production. Many experiments analysing enzymes related to plant aroma volatile generation have used *E. coli*, for example, hydroxycinnamoyl/benzoyl-CoA: anthranilate-*N*-hydroxycinnamoyl/benzoyl-transferase from carnation (Yang *et al.*, 1997), BEAT from *Clarkia breweri* (Dudareva *et al.*, 1998), acetyl-CoA:deacetylvindoline-4-*O*-acetyl-transferase (DAT) from *Catharanthus roseus* (St-Pierre *et al.*, 1998), BAMT from Snapdragon flower (Dudareva *et al.*, 2000), benzoyl-CoA:benzyl alcohol benzoyl-transferase (BEBT) from *Clarkia breweri* (D'Auria *et al.*, 2002), orcinol-*O*-methyl-transferases (OOMT) from rose petals (Lavid *et al.*, 2002), and a plant 4-coumarate:coenzyme A ligase involved in aromatic ester metabolism (Beuerle and Pichersky, 2002). Anthocyanin 5-aromatic acyl transferase, the aromatic acylation enzyme involved in the colour and stability of anthocyanins from *Gentiana triflora* was also studied in *E. coli* (Fujiwara *et al.*, 1998)

1.8.2 Proteins Expressed in Yeast

Many eukaryotic gene products do not fold properly and are not biological active when expressed in *E. coli*. Proteins expressed in *E. coli* may lack proper biological function and antigenicity because of the absence of eukaryotic posttranslational modifications. Yeast are single-celled eukaryotic microorganisms, which quickly grow by dividing into two daughter cells every 2 h so that thousands of clonal yeast colonies can be cultured on petri-dished in 2 days (Walker, 1998). Therefore, protein expression in yeast is a good alternative to study the function of eukaryotic genes. Modern recombinant DNA technology is very beneficial for biotechnology research;

for example, shuttle vectors allows plasmid to be manipulated by conventional recombinant DNA methods, propagated in bacteria like any other plasmid, and then returned to yeast for study (Singh and Heinemann, 1997; Walker, 1998). The shuttle vectors must contain selectable markers and origins of DNA replication that work in each organism.

pRC13, the correct coding sequence of ACO corresponding to *pTOM13*, was heterologously expressed in *Saccharomyces cerevisiae*, and the cells produced a protein showing similar characteristics to the ACO activity found in plant (Hamilton *et al.*, 1991). The *Arabidopsis ETR1* gene, one of the ethylene receptors, encodes a *His*-kinase of the two component class prevalent in bacteria (Chang *et al.*, 1993), where signal transduction systems response to a wide variety of environmental stimuli (Parkinson, 1993). The *ETR1* expressed in yeast was shown to have a saturable binding site for ethylene, but yeast lacking *ETR1* and having an *ETR1* mutant (*etr1-1*) showed no detectable ethylene binding (Schaller and Bleecker, 1995). Subsequently, *Arabidopsis ERS1* was biochemically characterised by heterologous expression in yeast showing an ethylene-binding protein (Hall *et al.*, 2000). Degan *et al.* (2001) reported at the N-terminal domain from *RDPG1*, the endo-PG involved in pod dehiscence in oilseed rape was not required for protein folding or dimerisation, when expressed in *Pichia pastoris*. The function of anthocyanin 5-aromatic acyl transferase, a protein involved in the synthesis of flavonoid colours in *Gentiana triflora*, was studied using yeast, *Saccharomyces cerevisiae* (Fujiwara *et al.*, 1998). These studies demonstrate the wide range of phenomena that can be studied by heterologous protein expression in yeast.

1.9 Thesis Objectives

From the previous research and a consideration of the literature, it is timely to consider the generation of aroma during fruit ripening from the point of view of the enzymes controlling the process and their related genes. Tomato and melon are among the model plants used for fruit ripening research. *CM-AAT1* (*MEL2*), a novel unidentified ripening specific gene, is expected to play an important role in ester generation during melon fruit ripening, based on its similarity to other enzymes. Therefore, the aims of the project are:

1. To obtain more *CM-AAT1* homologues from tomato and avocado cDNA libraries by screening the libraries with the *CM-AAT1* probe. Computer programs will be used for sequence alignment and molecular analysis.

2. To investigate expression of *CM-AAT1*, and its homologues, and some hypothetical related-flavour genes in wild type and low ethylene production varieties in tomato and melon.

3. To identify the functions of the *CM-AAT1* gene and its homologues using recombinant proteins expressed in yeast.

4. To produce transgenic tomatoes by over-expression and down-regulation of *CM-AAT1* genes or homologues to scrutinize their functions *in vivo*, and measure flavour profiles in transgenic and control plants.

CHAPTER 2

MATERIALS AND METHODS

2.1 Non-Biological Materials

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK), Fisher Scientific Ltd. (Loughborough, UK), Gibco BRL Life Technologies (Paisley, UK), or BDH Chemicals Ltd. (Dorset, UK), unless otherwise stated. Microbiological media were supplied by Oxoid Ltd. (Basingstoke, UK). All pure authentic standards of volatile compounds were supplied from Sigma-Aldrich Co. Ltd. (Dorset, UK).

2.1.2 Blotting Membrane Filters

GeneScreen Plus nylon membrane NEN#NEF-986 (Dupont, MA, USA) was used for northern or Southern blotting.

A 137 mm Hybond-N^+ charged nylon membrane disk filter supplied by Amersham Biosciences Ltd. (Bucks, UK) was used for blotting of cDNA library and colony blot analysis.

Reinforced cellulose nitrate membrane supplied by Schleicher and Schuell GmbH (Einbeck, Germany) was used for western blotting.

2.1.3 Radiochemicals

Radiolabelled chemicals were supplied by Amersham Biosciences Ltd., Bucks, UK:

Deoxy-cytidine 5'-[α-^{32}P] triphosphates ([^{32}P]dCTP) Redivue

(Specific activity: ~110TBq/mmol; ~3000 Ci/mmol Concentration: 10 mCi/ml)

2.2 Biological Materials

2.2.1 Microorganism Strains

2.2.1.1 Bacterial Strains

Escherichia coli	**Genotype:**
XL1 Blue	MRF' Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F' *pro*AB *lab*I^q ZΔM15 Tn10 (Tet^r)] (Stratagene Ltd., Cambridge, UK)
DH5α	*sup*E44, Δ*lac*U169(ϕ80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi-1*, *rel*A1 (Hanahan, 1983)
TOP10F'	F' {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ*80lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G (Invitrogen Ltd., Paisley, UK)

2.2.1.2 Yeast Strain

Saccharomyces cerevisiae	**Genotype:**
INVSc1	MAT α/a, his3-Δ1/ his3-Δ1, leu2/ leu2, trp1-289/ trp1-289, ura3-52/ura3-52 (Invitrogen Ltd., Paisley, UK)

2.2.1.3 *Agrobacterium* Strain

Agrobacterium tumefaciens **Genotype:**

LBA4404	Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404) (Hoekema *et al.*, 1983).

2.2.2 Plasmids

pGEM®T Easy was supplied by Promega UK (Southampton, UK), as shown in Figure 2.1.

pYES2.1/V5-His-TOPO® TA was supplied by Invitrogen Ltd (Paisley, UK), as shown in Figure 2.2.

pDH51 (Pietrzak *et al.*, 1986) as shown in Figure 2.3.

pBin19 (Accession No U09365) (Bevan, 1984; Frisch *et al.*, 1995) as shown in Figure 2.4. There are more extra fragments containing in the vector, published by Fray *et al.* (1994)

Figure 2.1 Map of plasmid pGEM®T Easy copied from the Promega website (www.promega.com; web figure number: 1473va)

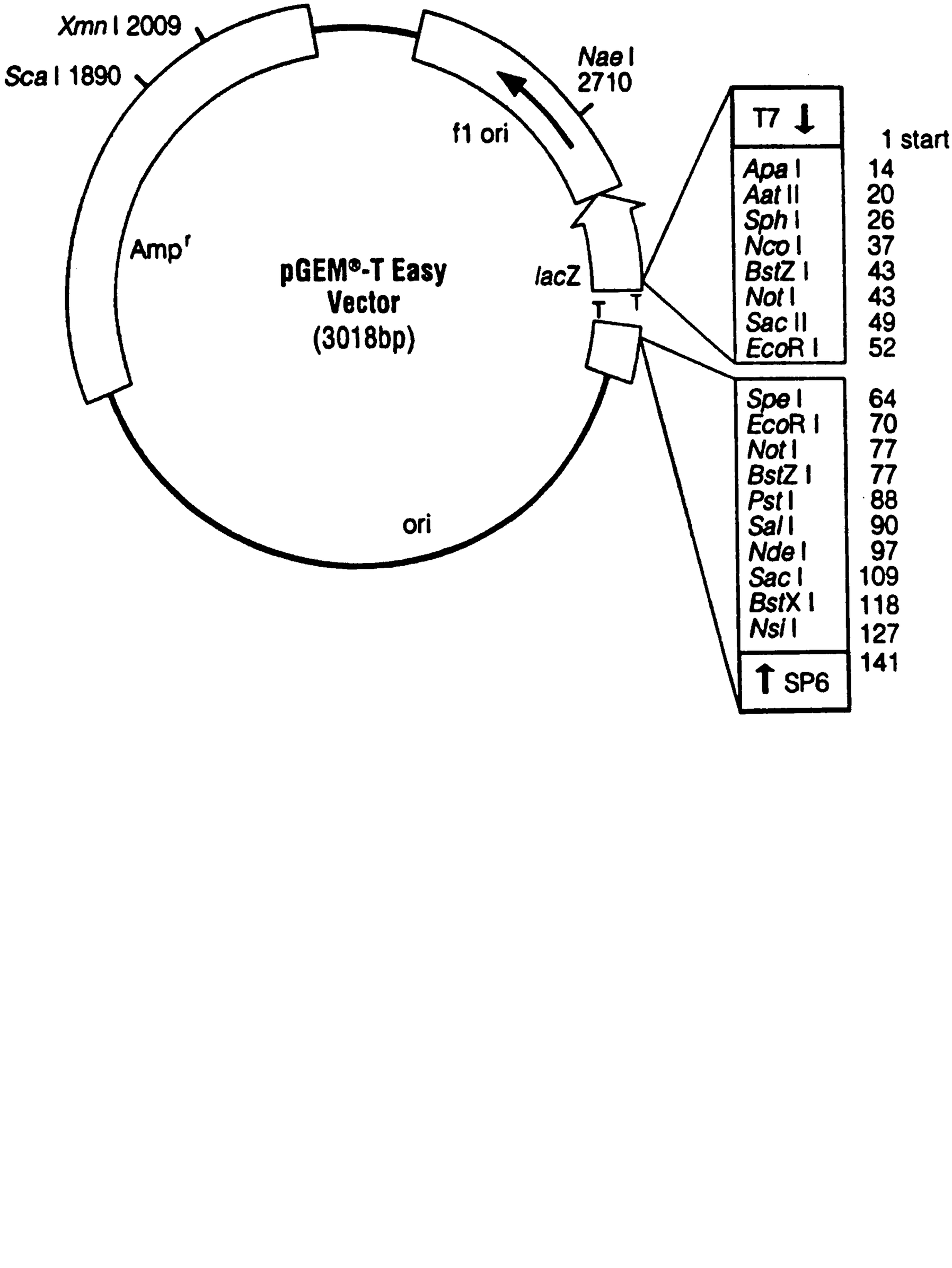
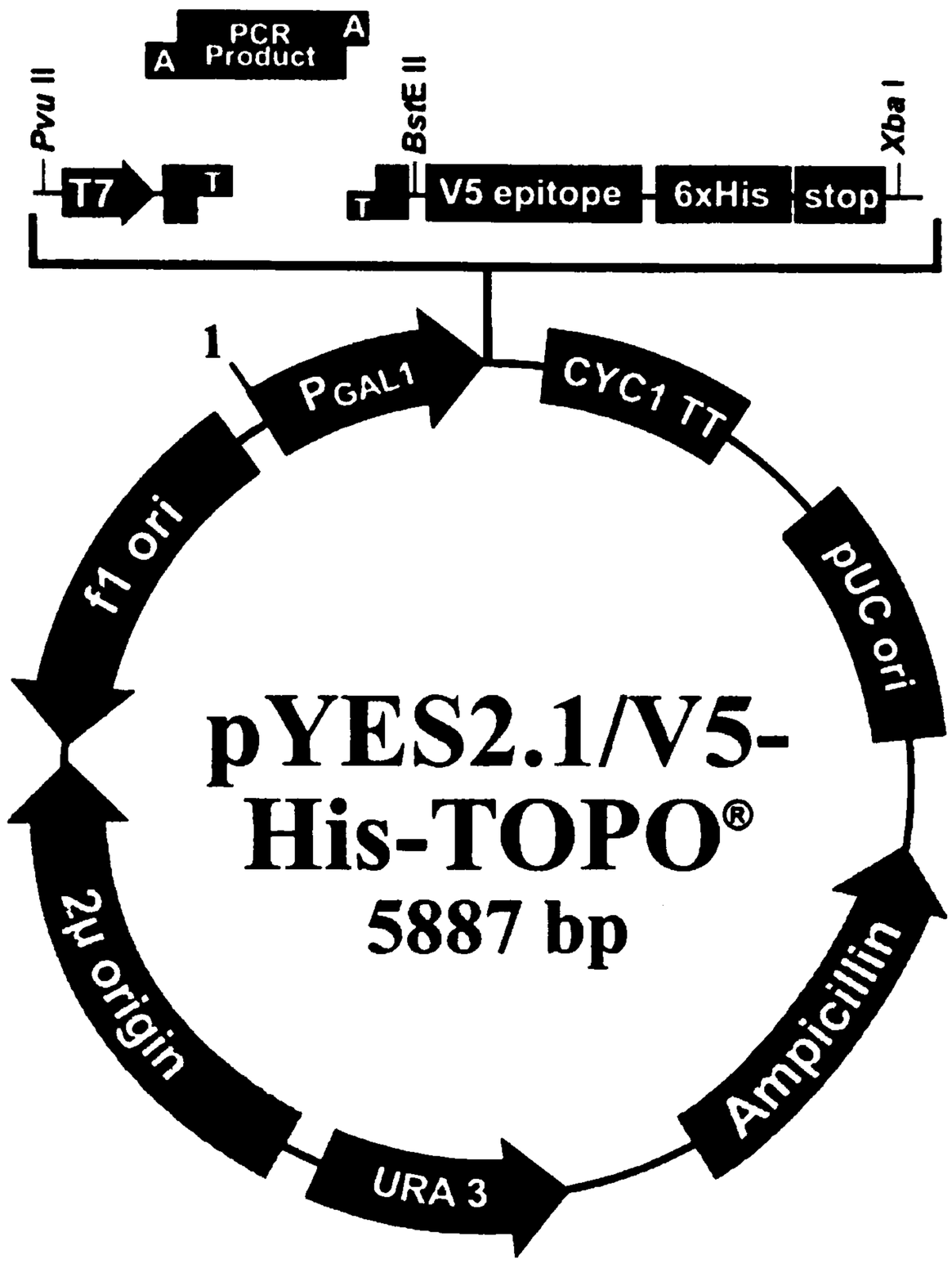


Figure 2.2 Map of plasmid pYES2.1/V5-His-TOPO® TA copied from the Invitrogen instruction manual (Catalogue No: K4150-01)



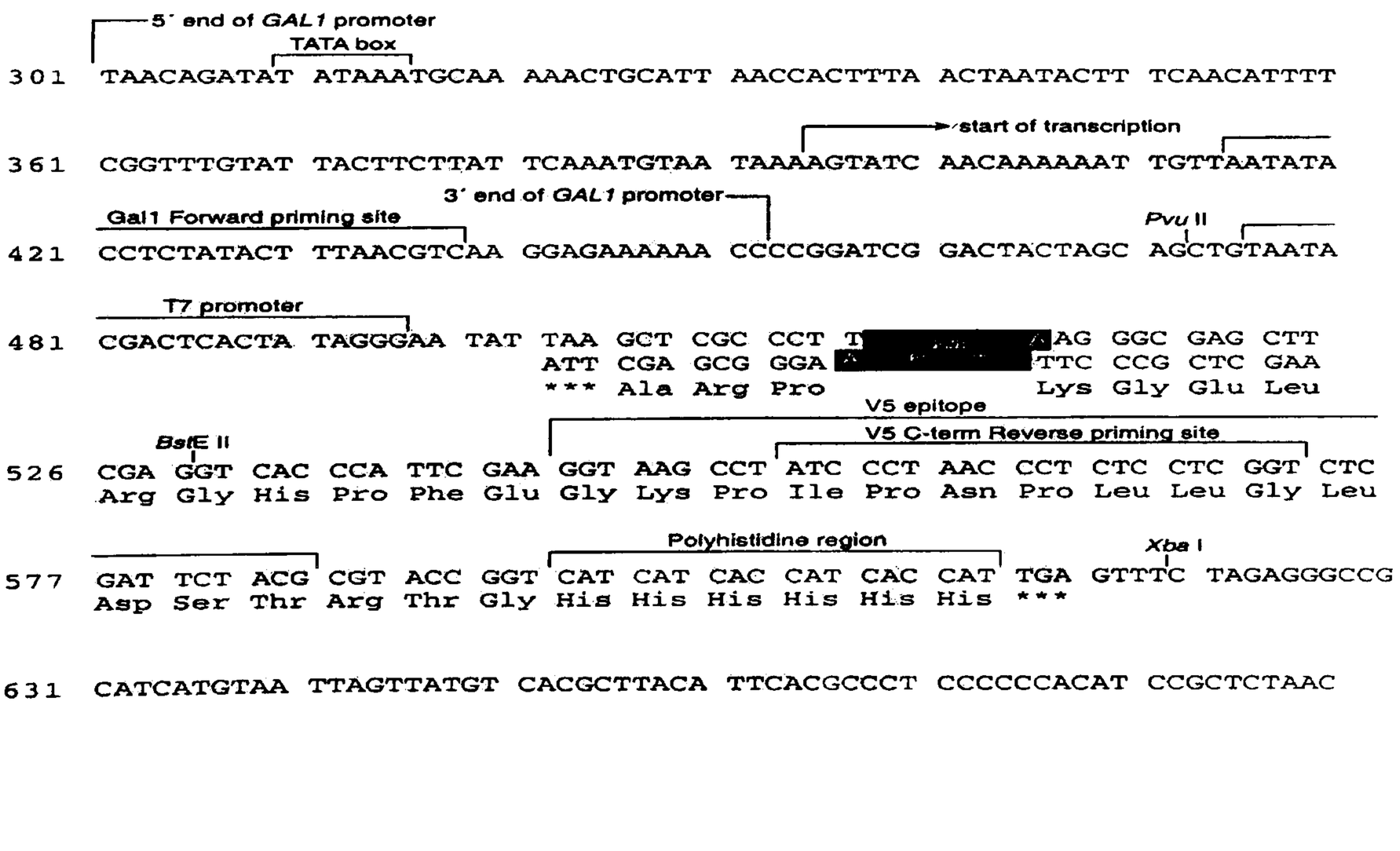


Figure 2.3 Map of plasmid pDH51 copied from the Plant Molecular Biology: Lab Fax (Guerineau and Mullineaux, 1993)

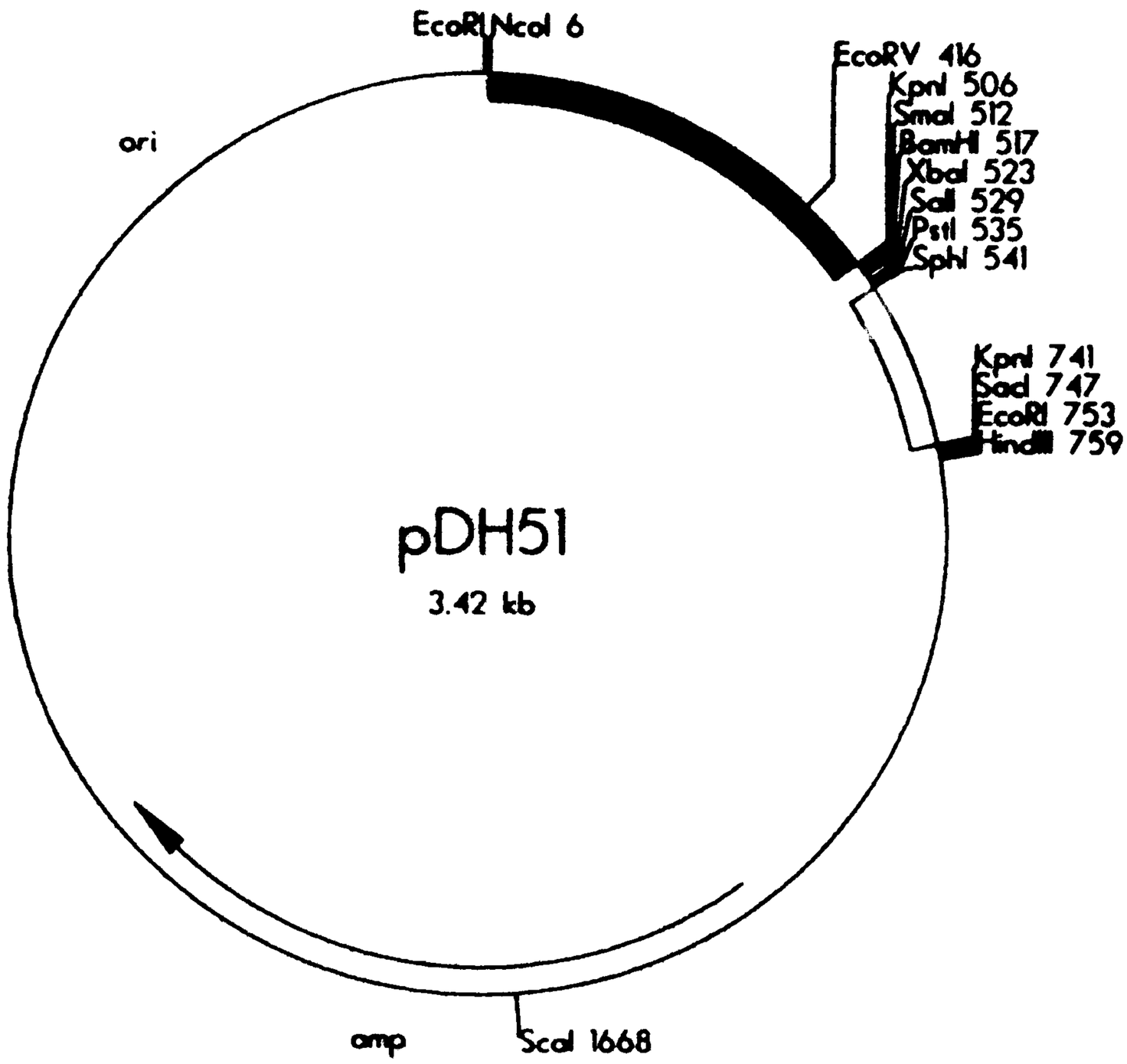
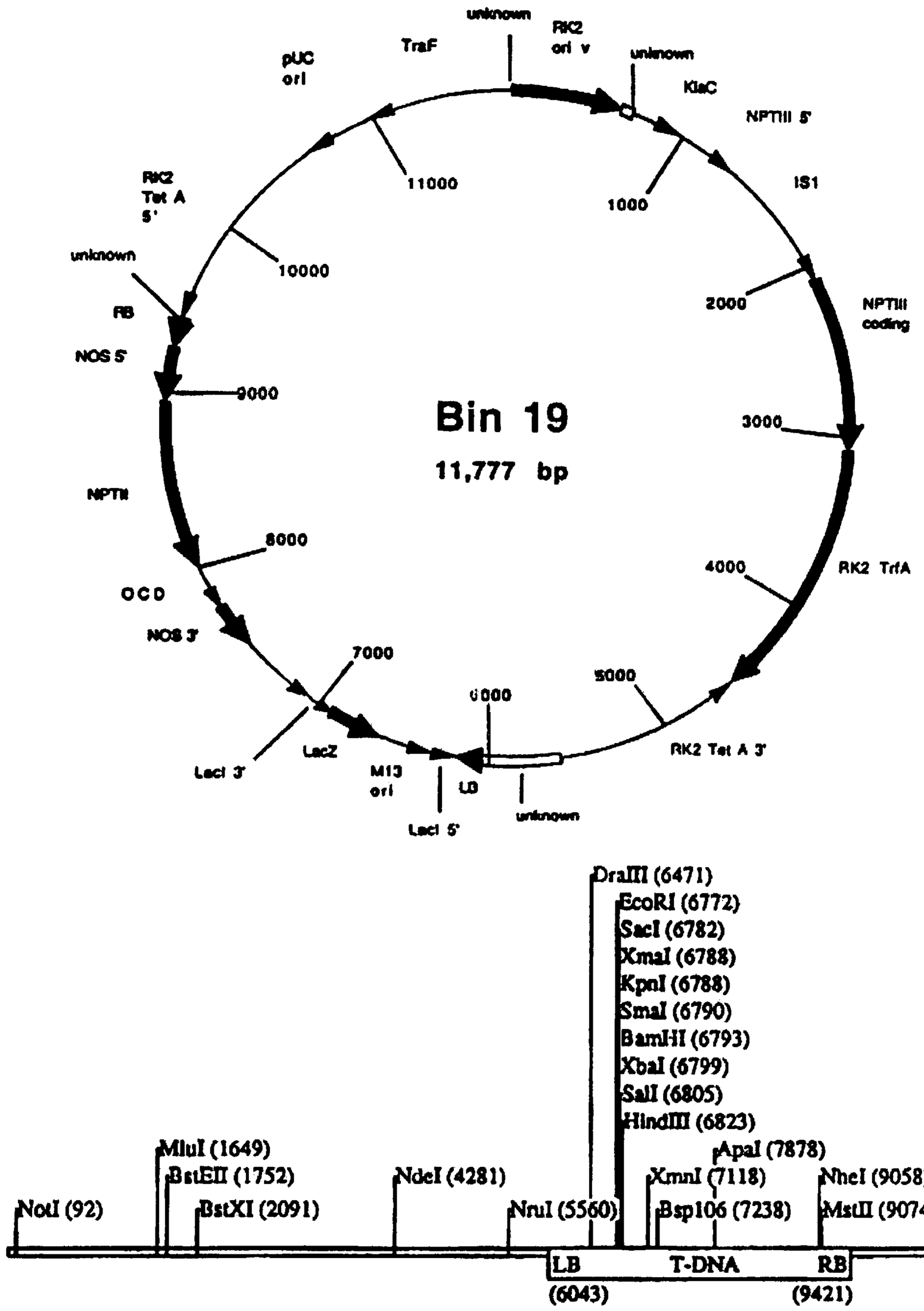


Figure 2.4 Map of plasmid pBin19 copied from the published paper by Frisch *et al.*, (1995)



Note: Three extra fragments were discovered by sequencing to contain between LB and RB regions of the vector (Fray *et al.*, 1994).

2.2.3 Oligonucleotide Primers

All DNA primers were synthesised by MWG Biotech (Ebersberg, Germany). The underlined sequences of Mel2F2K and Tomm7FK indicate start codons.

Mel2F: 5'-GGAAAGGCGTTGGTGTTCTAC-3'

Mel2R: 5'-GAGGCATGAACATTTGCCC-3'

Mel2F2K: 5'-GCCATGGGCTCTGGATCCGGTG-3')

Mel2R3: 5'-TTTCGAAGCAGATTGAATAGTTTGC-3'

Mel2BR: 5'-TAAAGCAAGATAGAATGGTTTGCAT-3'

Mel2FSma: 5'-CCCGGGATGGGCTCTGGATCCGGTGAT-3'

Mel2RXba: 5'-GCTCTAGAAACTCAATGGTGATGGTGATG-3'

Tomm7FK: 5'-GATATGGCAAATATTCTACCAAT-3'

Tomm7R: 5'-TGACTCTGAAAGTC ATGTTGTAG-3'

Tomm74R1: 5'-GGCCAATGGAGGTAAACTTATAGC-3'

ATomm7FSal: 5'-CGTCGACGGAAATGTCGTACCATTGCTC-3'

Atomm7RBam: 5'-CGGATCCCAGAAACACCAAAACTAGTG-3'

BamHI-XbaITCF: 5'-AGCCGGATCCTCTAGATAGTTTGATAGTGAAAGATA-3'

Mel2-BamHIR: 5'-CACCGGATCCAGAGCCCATATGTGATATACAACAAA-3'

2.2.4 Plant Materials

Wild type tomato (*Lycopersicon esculentum* Mill cv. Ailsa Craig) seeds were obtained from homozygous lines, grown at Sutton Bonington since 1979. Seeds of the homozygous Never ripe (*Nr*) mutant were near isogenic lines of the cultivar Ailsa Craig. Seed stocks were supplied by the BBSRC plant gene regulation laboratory, the University of Nottingham.

Melon seeds (*Cucumis melo* L. cv. Charentais), both wild type and *ACO1* antisense, were provided by F. El Yahyaoui and J-C. Pech, ENSAT, Toulouse, France.

2.2.5 cDNA Libraries

A tomato cDNA library (Biggs and Handa, 1989) was obtained from Prof Handa, Department of Horticulture, Purdue University, USA. The tomato cDNA library, which was prepared from the turning stage of 'Rutgers' tomato pericarp, was constructed from oligo-dT primed poly (A)+ mRNA in λgt11 phage.

A avocado (*Persea americana* Mill. cv. Hass) fruit cDNA library (Dopico *et al.*, 1993) was made from a mixture of equal amounts of poly(A)+ mRNA isolated from fruit after 25 and 53 days of storage at 7°C using a λZAP-phage.

2.2.6 Antibodies

Primary antibody

Anti-V5 antibody (Invitrogen Ltd., Paisley, UK) was used at a 1:2000 dilution

Secondary antibody

Anti-Mouse IgG (whole molecule), alkaline phosphatase conjugation, was obtained from Sigma-Aldrich Co. Ltd., Dorset, UK.

2.2.7 Enzymes and Other Biological Materials

Most restriction enzymes were purchased from MBI Fermentas GMBH (Heidelberg, Germany) and Promega UK (Southampton, UK). Other enzymes and biological reagent were supplied by New England Biolabs Ltd. (Herts, UK), Bioline Ltd. (London, UK), Gibco BRL Life Technologies (Paisley, UK), Roche Diagnostics Corp. (Basel, Switzerland), or Promega UK (Southampton, UK), unless stated otherwise.

2.3 Plant Growth and Maintenance

2.3.1 Tomato

Transgenic seeds were surface sterilised prior to germination by soaking in 50% sodium hypochloride for 10 min and rinsed 3-4 times with sterile distilled water (SDW) before sowing. Wild type and *Nr* seeds were sown on F2S compost (Levington Horticulture Ltd., Ipswich, UK) while transgenic seeds were sown on MSR3 agar (see section 2.4.2.2) plus 50 μg/ml kanamycin sulphate. Seeds were germinated in a growth room with supplementary light to provide 16 h day length at 23°C and 8 h night length at 18°C. Seeds sown on MSR3 were germinated in a tissue culture room at 24°C.

After 2 to 3 weeks, seedlings were individually separated and transferred to 7-cm pots containing Levington F2 compost and left to grow in a propagation room. The plants were transferred into larger pots as necessary. After the tomato plants were big enough, indicated by the appearance of the first flower truss, the plants were transferred into 25-cm pots containing Levington M2 compost mixed with volcanic rock (Willium Sinclair Horticulture Ltd., Lincoln, UK) to improve aeration and drainage. Plants were supported with jute string and a balanced 1:1:1 NPK Sangral fertilizer (Henry Alty Ltd., Preston, UK) was fed daily. All side-shoots and senescent leaves were regularly removed from the plants.

2.3.2 Melon

Seeds were surface sterilised in 50% sodium hypochloride for 20 min and rinsed 3-4 times with SDW and sown on MSR3 agar media (50 μg/ml kanamycin sulphate was added for transgenic seeds). Seeds were germinated at 25°C in a growth room until

root tips were approximately 1-2 cm long. Seedlings were then transferred to 12 cm pots containing Levington M1 compost and placed in an ACGM1 glasshouse under 16 h of light. Once 6-7 true leaves had appeared, plants were transferred into 25 cm pots filled with Levington M3 compost. Plants were supported by jute string and a balanced 1:1:1 NPK Sangral fertilizer was fed daily. Freshly full-bloomed female flowers with swollen ovaries were hand-pollinated with fresh male pollen, and covered with a plastic bag to maintain high humidity and to protect from other pollen contamination. Hand-pollinated flowers were tagged to identify stages of fruit development. Two fruit per plant were developed and harvested at 20, 25, 30, 35, 40, 45, 50, 55 days after anthesis (daa). The ripening stage of fruit was measured by detecting internal ethylene production immediately after harvesting. The fruit or other organs were then cut in small pieces, frozen immediately in liquid N_2 and stored at -80◦C.

2.4 Growth Media

2.4.1 Microorganism Growth Media

2.4.1.1 LB Medium

LB medium contained 0.1% (W/V) Bacto-tryptone, 0.05% (W/V) Bacto-yeast extract, and 0.1% (W/V) sodium chloride (NaCl). The medium was adjusted to pH 7.0 with NaOH and then sterilised by autoclaving.

2.4.1.2 NZY Medium

Five g of NaCl, 2 g of $MgSO_4.7H_2O$, 5 g of Bacto-yeast extract, and 10 g of casein hydrolysate were added to 800 ml of distilled water (DW). The medium was adjusted to pH 7.5 using NaOH, made up to 1 litre with SDW, and sterilised by autoclaving.

2.4.1.3 Top Agarose Overlay

Seven g of agarose were added to a 1 litre of NZY medium, and the medium was autoclaved.

2.4.1.4 CM Medium

Amino Acid Drop-out Mix Stock (for 20 litres)

The stock of amino acid mixture contained 1.25 g of L-adenine, 0.6 g of L-argenine, 3.0 g of L-aspartic acid, 3.0 g of L-glutamic acid , 0.6 g of L-isoleucine, 0.9 g of L-lysine, 0.6 g of L-methionine, 1.5 g of L-phenylalanine, 11.0 g of L-serine, 6.0 g of L-threonine, 0.9 g of L-tyrosine, 4.5 g of L-valine, 1.8 g of L-leucine, 0.6 g of L-histidine, 1.2 g of L-tryptophane.

Two g of amino acid drop-out mix and 6.7 g of yeast nitrogen base (without amino acids) were added to 800 ml of DW. The mixture was stirred for around 1-2 h to dissolve amino acids and adjusted to pH 5.8. The medium was autoclaved for 15 min in a bench top autoclave. Two hundred ml of 10% raffinose were added to the medium when cooled to approximately 65°C.

2.4.1.5 YEPD Medium

A litre of YEPD medium contained 10 g of Bacto-yeast extract, 20 g of Bacto-peptone, and 20 g of glucose. For plating, 20 g of Bacto-agar was added to the medium and then autoclaved for 15 min in a bench top autoclave.

2.4.1.6 APM Medium

The mixture of 5 g of yeast extract, 0.5 g of casamino acids, 8 g of mannitol, 2 g of ammonium sulfate, and 5 g of NaCl, was dissolved in 800 ml of DW. The medium was adjusted to pH 6.6, made up to 1 litre with SDW, and then autoclaved.

2.4.2 Plant Transformation and Tissue Culture Media

2.4.2.1 MS Medium

Thirty g of sucrose and 4.4 g of MS salts were dissolved in 800 ml of DW. The medium was adjusted to pH 5.9, filled to 1 litre with SDW, and then autoclaved.

2.4.2.2 MSR3 Medium

Thirty g of sucrose, 4.4 g of MS salts, and 1 ml of R3 vitamins (0.1% thiamine, 0.05% nicotinic acid, 0.05% pyridoxine) were dissolved in 800 ml of DW. The medium was adjusted to pH 5.9, filled to 1 litre with SDW, and then autoclaved.

2.4.2.3 MS 2-4D Medium

A litre of MS 2-4D medium consisted of 4.4 g of MS salts, 30 g of sucrose, 1 ml of R3 vitamins, 0.1 mg of Kinetin, 0.2 mg of 2-4D, and 0.2 g of potassium phosphate. The medium was adjusted to pH 5.7 and sterilised by autoclaving.

2.4.2.4 3C5ZR Medium

After autoclaving, MSR3 medium was cooled down to 65◦C and then 1.75 mg of Zeatin and 0.78 mg of IAA were added.

2.5 General Molecular Biology Methods

2.5.1 Polymerase Chain Reaction (PCR) Amplification

2.5.1.1 PCR Amplification by Taq DNA Polymerase

PCR Reaction

A PCR reaction consisted of 1-10 μl (1-10 ng) of DNA template, 5 μl of 10X NH4 buffer, 1.5 μl of 50 mM $MgCl_2$ solution, 1 μl of 10 mM dNTPs (Bioline Ltd., London, UK), 1 μl of 100 nM forward primer and reversed primer and 1 μl of Taq DNA polymerase. The volume was adjusted to 50 μl with SDW.

PCR Program

The RoboCycler® Gradient 40 PCR machine (Stratagene Ltd., Cambridge, UK) was set to the following program:

95°C for 3 min (hot start)

95°C for 1 min

50-58°C for 1 min (according to primer melting temperature) — 35 cycles

72°C for 1-2 min

72°C for 5 min

2.5.1.2 PCR Amplification by Proofreading DNA Polymerase

A reaction in 50 ml was consisted of 1-10 μl (1-10 ng) of DNA template, 5 μl of 10X Deep Vent® DNA polymerase buffer, 1 μl of 100 mM $MgSO_4$ solution, 2 μl of 10 mM dNTPs, 2 μl of 100 nM forward primer and reverse primer, and 1 μl of Deep Vent® DNA polymerase. The volume was adjusted to 50 μl with SDW. The PCR program was run as following the section 2.5.1.1 with a 4 min elongation time.

2.5.1.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Synthesis of First Strand cDNA

cDNA was synthesised from total RNA (section 2.5.12). Reaction components were 2 µl (approximately 2 µg) of total RNA, 2 µl of 100 ng/µl oligo d (T) and 7 µl of SDW. The reaction was heated at 65°C for 1 min, cooled to room temperature for 2 min, and incubated on ice for 5 min. Four µl of 5X reverse transcriptase buffer, 3 µl of 10 mM dNTPs, and 2 µl of reverse transcriptase (MMLV) (Stratagene Ltd., Cambridge, UK) were added into the reaction. The mixture was incubated at 37°C for 1 h and then heated at 65°C for 10 min.

Synthesis of Second Strand DNA and PCR Amplification

The second strand DNA and amplification of the sequence were done using either Taq or proofreading DNA polymerase.

3 µl of first strand cDNA

5 µl of 10X DNA polymerase buffer

1 µl of 100 mM $MgSO_4$ solution

2 µl of 10 mM dNTPs

2 µl of 100 nM forward primer

2 µl of 100 nM reverse primer

1 µl of Taq or Deep Vent DNA polymerase

The mixture was adjusted to 50 µl with SDW and then subjected to PCR (section 2.5.1.1)

2.5.2 Gel Electrophoresis

2.5.2.1 DNA Gel Electrophoresis

DNA was subjected to gel electrophoresis under standard conditions using 1X TAE buffer (50x stock solution: 2 M Tris base, 5% glacial acetic acid, 50 mM EDTA pH 8.0.) Loading buffer (50% Glycerol, 0.2% Bromophenol blue, 5 mM Sodium phosphate buffer pH 6.8) was added to each sample before electrophoresis (Mini Sub™ DNA Cell, Bio-Rad Laboratories Ltd., CA, USA). DNA size were calculated using lambda DNA marker #SM0191 (digested with *EcoR*I/*Hind*III, MBI Fermentas GMBH, Heidelberg, Germany) and gel concentrations used were 0.8 - 1.0% and gels were subjected to 80-100 volts (Bio-RAD Model 200/2.0 Power Supply) for 1 h. One μg of ethidium bromide (EtBr) per 10 ml of agarose gel was added to allow the visualisation of DNA fragments under UV light.

2.5.2.2 Formaldehyde Denaturing Gel Electrophoresis of Total RNA

3.5 g of agarose were added to 225 ml of 20 mM Sodium phosphate buffer pH 6.8, microwave-melted, and cooled to 65°C. Twenty five ml of 37% formaldehyde pH 7.0 were then mixed well with the solution, and poured into a gel tray.

Total RNA (5-40 μg) was diluted to a total volume of 12.5 μl with SDW and mixed with 12.5 μl of 2x EtBr buffer (500 μl of 37% deionised formaldehyde, 165 μl of 37% formaldehyde pH 7.0, 20 μl of 0.5 M EDTA, 40 μl of 1M sodium phosphate buffer pH 6.8, 2 μl of 10 mg/ml EtBr, 273 μl of SDW). Samples were then incubated at 65°C for 15 min and put on ice for 5 min. Five μl of loading buffer were added to the samples, mixed and spun down. After chilling on ice for 5 min, the samples were loaded onto the agarose-formaldehyde gel in a northern-gel electrophoresis apparatus

GNA-200 (Amersham Pharmacia Biotech Ltd., Bucks, UK) using 10 mM sodium phosphate pH 6.8 with 3% (v/v) formaldehyde pH 7.0 as running buffer. The gel was run at a constant 100 volts (Bio-RAD Model 200/2.0 Power Supply) for 3-4 h with continuous circulation of running buffer (Amersham Pharmacia Biotech Pl peristaltic pump). After electrophoresis the RNA was visualised on an UV transilluminator and photographed to aid future size estimations.

2.5.3 Recovery of DNA from Agarose Gels

After fragmentation by gel electrophoresis, the target DNA fragment was excised from agarose gel under UV light with a clean scalpel and put into an Eppendorf tube. The QIAquick gel extraction kit (Qiagen Ltd., West Sussex, UK) was used to extract the DNA from the gel according to the manufacture instructions.

2.5.4 Addition of 3'A Overhang to PCR Products

Addition of an adenosine at the 3' ends of PCR products was carried out at 72°C for 20 min. A 20 µl reaction was consisted of 5 µl of purified PCR product, 1 µl of 0.2 mM dATP, 2 µl of Taq polymerase buffer, 1 µl of 50 mM $MgCl_2$, 1 µl of Taq DNA polymerase, and 10 µl of SDW.

2.5.5 Cleavage of DNA with Restriction Endonucleases

2.5.5.1 Verification of Insert by Enzymatic Digestion

Plasmid DNA from mini-preps was digested by appropriate restriction enzymes. Digests were carried out in a total volume of 20 µl (10 µl of plasmid DNA solution, 2 µl of 10x appropriate buffer, 1 µl of restriction enzyme, 7 µl of SDW). The reaction was incubated at 37°C for 1 h then subjected to gel electrophoresis.

2.5.5.2 Digestion of Genomic DNA

Ten to 30 μg of genomic DNA were used in the digestion reaction, containing 7 μl of 10X corresponding buffer, and 5-8 μl of 10 Units/μl restriction enzyme. The mixture volume was adjusted to 70 μl with SDW and then incubated overnight at 37°C. Five μl of the overnight digested DNA was fractionated by mini-gel electrophoresis to check complete digestion.

2.5.6 Introduction of DNA Fragments to Vectors

A 10 μl reaction volume contained 1 μl of vector DNA, 1-5 μl of DNA insert, 1X ligation buffer and 1 unit of T4 DNA ligase. The ratio of insert to vector was between 3:1 and 5:1. The ligation reaction was incubated overnight at 4°C and then used for bacterial transformation.

2.5.7 Preparation of Competent Cells

2.5.7.1 DH5α Competent Cells

ψ Broth: 2% (W/V) Bacto-tryptone, 0.5% (W/V) Bacto-yeast extract, 0.4% (W/V) $MgSO_4$, 10 mM KCl. pH of the medium was adjusted to 7.6 with KOH

TFBI: 100 mM Rubidium chloride (RbCl), 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% glycerol. The solution was adjusted to pH 5.8 with 0.2 N acetic acid and then sterilised by filtration.

TFBII: 10 mM MOPS (3-N-morpholino propanesulfonic acid) pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol. The solution was sterilised by filtration.

An LB agar plate was streaked from a glycerol stock culture of host strain *E. coli* and incubated overnight. A 5 ml ψ broth culture was inoculated with a single colony of

E. coli strain DH5α, and shaken overnight at 37°C. The starter culture was then transferred to 500 ml ψ broth culture and shaken at 37°C for about 2-3 h. When the optical density at 550 reached 0.48, the culture was chilled on ice for 15 min before harvesting by centrifugation at 1000g for 5 min at 4°C in a pre-chilled rotor. The pellet was gently resuspended in 130 ml of ice-cold TFBI and kept on ice for 15 min. Cells were pelleted at 1000g for 5 min at 4°C and resuspended gently in 20 ml of ice-cold TFBII. The cell suspension was dispensed into pre-chilled tubes in aliquots of 100 µl. The aliquots were frozen in liquid N_2 and were stored at -80°C.

2.5.7.2 XL1 Blue Competent Cells for Library Screening

A 10 ml liquid NZY broth, containing 100 µl of 2% maltose and 100 µl of 1 M $MgSO_4$, was inoculated with a single colony of *E. coli* strain XL1 Blue and incubated overnight at 37°C with shaking. Bacterial cells were collected by centrifugation at 3000g for 5 min at 4°C. The cells were resuspended in 3 ml of ice-cold 10 mM $MgSO_4$.

2.5.7.3 *Agrobacterium* Competent Cells

An APM agar plate plus 500 µg/ml streptomycin was streaked from a glycerol stock culture of *Agrobacterium* strain LBA4404, containing Ti helper plasmid, and incubated at 29°C for 3 days. A single colony was used to inoculate in 5 ml APM liquid media containing streptomycin, which was incubated while shaking in dark conditions at 29°C (shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA) overnight. 2 ml of the overnight culture were added into 50 ml of fresh APM media containing streptomycin. The culture was shaken in the dark at 29°C until the culture OD (A_{600}) was between 0.5 and 1.0. The culture was chilled on ice for 15 min, centrifuged at 2000g for 5 min at 4°C, and the cell pellet was resuspended in 1 ml of

ice-cold 20 mM $CaCl_2$. The cells were snap frozen in liquid N_2 and stored -80°C until needed.

2.5.8 Bacterial Transformation with Plasmid DNA

Competent cell suspension was thawed at room temperature, put on ice for 5 min, and then mixed gently with 5 to 10 µl of ligation mixture. The tubes were incubated on ice for 20 min, heat shocked at 40-42°C for 60-90 sec and then immediately put on ice for 2 min. Seven hundred µl of LB medium were added and the cells were incubated at 37°C for 1 h with shaking. The cells were then plated onto appropriately selective medium plates and incubated at 37°C overnight.

2.5.9 Amplification of Recombinant Vectors in *E. coli*

2.5.9.1 Plasmid DNA Mini-Preps: STET Preps or Boiling Method

A single transformed bacterial colony from selective media was inoculated into 5 ml liquid LB culture with appropriate antibiotic. The culture was incubated at 37°C overnight with shaking and then transferred to a 1.5 ml Eppendorf tube. The bacterial cells were collected by centrifuging the culture for 20 sec at 12000g. The bacterial pellet was resuspended in 200 µl of STET (8% sucrose, 5% Triton X-100, 50 mM EDTA pH 8.0, 50 mM TrisHCl pH 8.0) and mixed briefly, avoiding formation of bubbles. Twenty µl of 10 mg/ml lysozyme were added to the tubes, quickly mixed by vortexing, and boiled for 1 min. The suspension was centrifuged for 10 min at 12000g and the pellet (denatured proteins and chromosomal DNA) was discarded. An equal volume of phenol/chloroform (49% phenol, 49% chloroform, 0.1% 8-hydroxyquinoline sulphate, 1.9% isoamyl alcohol) was added and mixed by vortexing. The samples were then centrifuged at 12000g for 5 min and protein-free

supernatant was transferred to a fresh tube. This step was then repeated and the DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate (NaOAc) pH 5.2 and 0.7 volumes of isopropanol. Plasmid DNA was collected by centrifugation at 12000g for 10 min and the DNA pellet was washed with 1 ml of 70% ethanol, allowed to dry, and resuspended in 20-30 µl of SDW.

2.5.9.2 Plasmid DNA Mini-Preps: Alkaline Lysis Method

Bacterial cells from an overnight culture were harvested in an Eppendorf tube (1.5 ml) by centrifugation at 12000g for 1 min. The cell pellet was resuspended in 100 µl of ice-cold solution 1 (25 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1% Glucose), and left for 5 min at room temperature. Two hundred µl of solution 2 ((make fresh) 200 mM NaOH and 1% SDS) were added to the samples, mixed by inversion, and incubated for 5 min on ice. One hundred and fifty µl of solution 3 (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of SDW) were added, and the samples were again mixed by inversion before being incubated on ice for 5 min. After centrifugation at 12000g for 5 min, 0.5 µl of 100 µg/µl DNase-free RNase was added to the supernatant before incubating at room temperature for 5 min. The mixture was extracted once with phenol/chloroform and once with chloroform. Two and a half volumes of ice-cold absolute ethanol was added into the aqueous phase and the mixture was put on dry ice for 5 min and then centrifuged at 12000g for 5 min. The DNA pellet was rinsed with ice-cold 70% ethanol, dried under vacuum, and resuspended in 30 µl of SDW.

2.5.9.3 Large-Scale Plasmid DNA Preps

A 100 ml liquid LB culture with 50 μg/ml appropriate antibiotic was inoculated with a selected single colony and left to grow overnight at 37°C with shaking. The bacterial cells were pelleted by centrifuging the culture at 4000g for 10 min at 4°C. The bacteria were resuspended in 5 ml of solution 1, and then thoroughly mixed by inverting gently with 10 ml of solution 2. After leaving for 5 min at room temperature, 7.5 ml of solution 3 was added, mixed gently, and then placed on ice for 20 min. Cell debris and chromosomal DNA were removed by spinning at 16000g for 30 min at 4°C. The supernatant was filtered through muslin (or glass wool) into a new sterile centrifuge tube. Two volumes of absolute ethanol were added to the solution, which was then placed on ice for 10 min and centrifuged at 9000g for 10 min at 4°C. The pellet was resuspended in 2 ml of SDW and mixed well with 2 ml of an ice-cold solution of 5 M lithium chloride ($LiCl_2$). After incubation at -20°C for 30 min, the samples were centrifuged at 12000g for 10 min at 4°C and the supernatant was transferred to a new tube. An equal volume of isopropanol was added, and the precipitated plasmid DNA was recovered by centrifugation at 12000g for 10 min at room temperature. The pellet was washed with 70% ethanol and left to dry. Five hundred μl of SDW, containing, 5 μl of 10 μg/μl RNase, DNase-free, was added to dissolve the pellet and the tubes were incubated at 37°C for 30 min. The solution was extracted once with phenol/chloroform, and once with chloroform. The DNA, suspended in the aqueous phase, was precipitated by the addition of 0.1 volumes of 3 M NaOAc pH 5.2 and 2 volumes of absolute ethanol, and then left at -20°C for 20 min. The plasmids were pelleted by centrifugation at 12000g for 10 min, washed with 1 ml 70% ethanol, air-dried, and resuspended in SDW (30-50 μl).

2.5.9.4 Mini- or Mega-Scale Plasmid Preps by Kit

For high quality of plasmid DNA, recombinant vector was amplified and purified by QIAprep Spin or Qiagen Plasmid Mega Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions.

2.5.10 Preparation of Glycerol Stocks

Bacterial glycerol stocks were prepared by inoculating 2 ml of media culture (containing the appropriate antibiotic) with a single bacterial colony and incubated at 37◦C, with shaking, until the OD_{600} reached 0.5-0.7. A 0.85 ml culture was transferred to a sterile cryovial and mixed with 0.15 ml of sterile glycerol. The culture was frozen under liquid N_2 and stored at –80◦C.

2.5.11 DNA Sequencing

DNA sequencing was carried out in house using a CEQ 2000XL DNA Analysis System (Beck-Man-Coulter, Inc., CA, USA) at the Sutton Bonington sequencing facility, in the Plant Sciences building, the University of Nottingham.

2.5.12 RNA Extraction

Ten g of frozen tissue were ground to fine powder using a chilled mortar and pestle with liquid N_2. Ten ml of homogenising buffer (6% 4-amino-salicylic acid, 1% 1, 5-naphthalenedisulfonic acid disodium salt, 5% phenol reagent, 50 mM TrisHCl pH 8.5) was added and ground until the mixture became liquid, then an equal volume of phenol chloroform was added. The mixture was transferred to a 50 ml tube, shaken well and centrifuged at 1000g for 15 min. The aqueous phase was transferred to a fresh Eppendorf tube. The phenol/chloroform extraction was repeated until the

aqueous phase was clear. 0.1 volumes of 3 M NaOAc pH 6.0 and 2.5 volumes of cold ethanol were added, mixed well and left at –20°C for an hour. The samples were then centrifuged at 12000g for 30 min at 4°C. The pellet was dissolved in 750 µl of SDW and an equal volume of 8 M $LiCl_2$ was added. The RNA was then precipitated at -20°C for an hour. The solution was centrifuged at 12000g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and $LiCl_2$ precipitation was repeated twice as above. The aqueous phase was collected, mixed well with 0.1 volumes of 3 M NaOAc pH 6.0 and 2.5 volumes of cold ethanol, and then precipitated at -20°C for an hour. The nucleic acid pellet was collected by centrifugation at 13000g for 10 min, washed with 200 µl 70% ethanol, air-dried, and resuspended in 180 µl of SDW. 20 µl of 10X DNase buffer and 2 µl of RQ1 DNase were added, and incubated at 37°C for 30 min. The solution was extracted by an equal volume of phenol chloroform, and then chloroform. The aqueous phase was collected and mixed well with 0.1 volumes of 3 M NaOAc pH 6.0 and 2.5 volumes of cold ethanol, and left at -20°C for an hour. The RNA pellet was collected at maximum speed in a microcentrifuge for 10 min, washed with 200 µl of 70% ethanol and air-dried. The RNA was resuspended in 50 µl of SDW and then stored at -80°C.

2.5.13 Genomic DNA Extraction

Three to 5 g of young tomato leaves were finely powdered by grinding in a mortar and pestle with liquid N_2. Ten ml of urea extraction buffer (42% (W/V) urea, 0.3 M NaCl, 50 mM TrisHCl pH 8.0, 20 mM EDTA, 1% *N*-Lauryl sarcosine) were added to the powder, mixed by grinding for 2 min further, and then the mixture was transferred into a 50 ml tube. Ten ml of phenol/chloroform were added and the mixture was shaken for 10 min, and then centrifuged at 12000g for 15 min. The supernatant was

transferred to a new tube and gently mixed with an equal volume of isopropanol. The samples were kept at -20°C for 30 min and centrifuged at 12000g for 15 min. The DNA pellet was washed with 70% ethanol, dried out, and resuspended in 700 µl of SDW containing RNase-DNase free. After 15 min incubation at 37°C, the samples were extracted 2 or 3 times of phenol/chloroform extraction. 0.1 volumes of 3 M NaOAc pH 5.2 and 2 volumes of ethanol were mixed well with the aqueous phase and the mixture was kept at -20°C for 30 min. The pellet was collected by micro-centrifugation at high speed for 15 min, washed with 70% ethanol, resuspended with 30-50 µl of SDW. The DNA suspension was stored at -80°C.

2.5.14 Measurement of Nucleic Acids Concentration

DNA or RNA concentration was quantified by measuring the absorbance at A_{260} and A_{320} using a PU8720 UV/VIS scanning spectrophotometer (Thermo Spectronic, Madison, WI, USA). The concentration of DNA and RNA was calculated by the following formula:

$$\text{DNA concentration } (\mu g/\mu l) = (OD_{260}\text{-}OD_{320}) \times 50\ \mu g/\mu l$$

$$\text{RNA concentration } (\mu g/\mu l) = (OD_{260}\text{-}OD_{320}) \times 40\ \mu g/\mu l$$

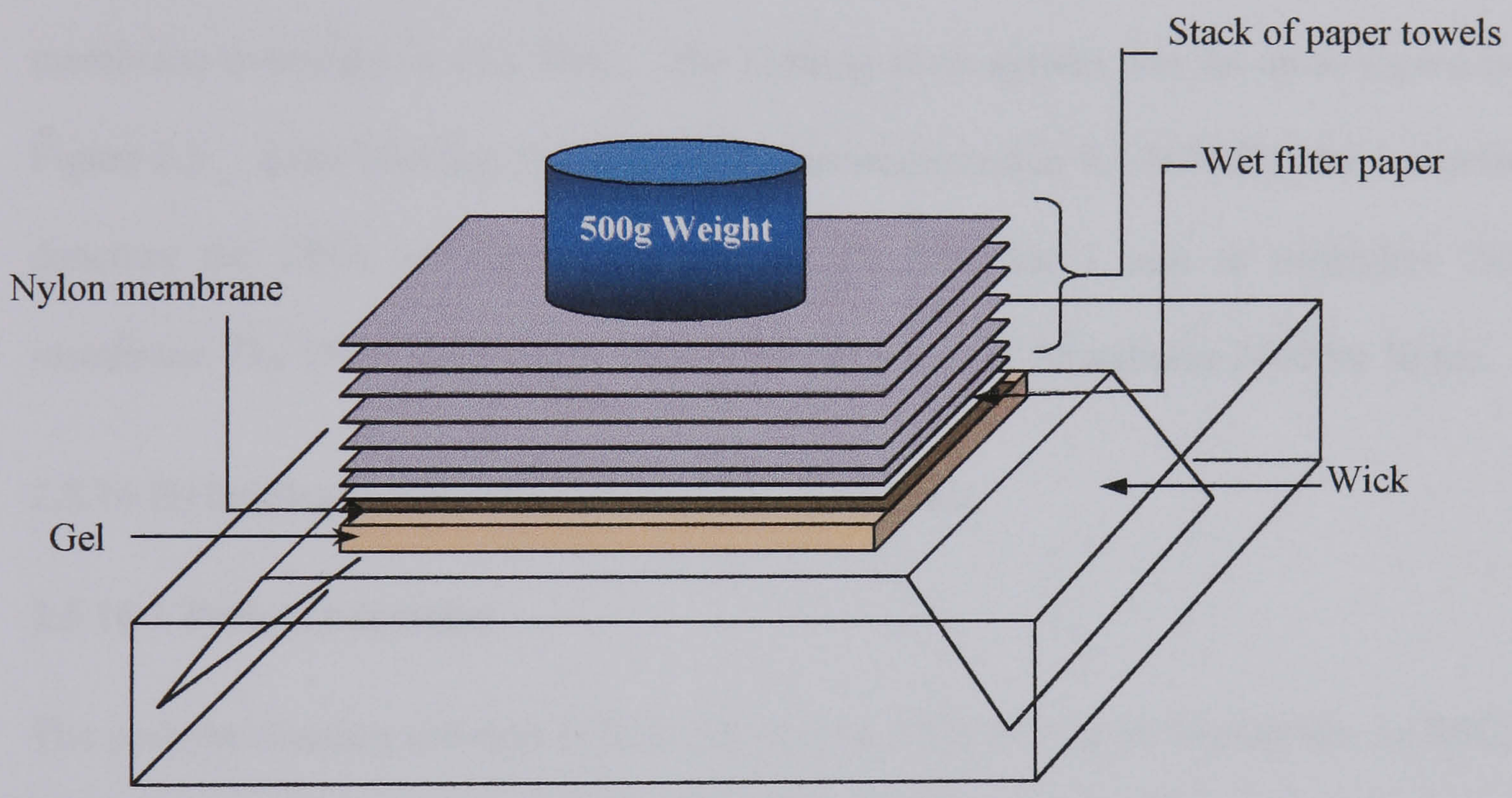
2.5.15 Polynucleotides Blotting

2.5.15.1 Northern Blotting

RNA was fractionated by gel electrophoresis as described in section 2.5.2.2. The northern gel was washed with SDW for 3 X 5 min and RNA blotting was carried out by the capillary protocol as shown in Figure 2.5. Twenty five mM sodium phosphate pH 6.8 and GeneScreen Plus membrane were used as the transfer buffer and the hybridisation filter, respectively. After overnight (16 h) blotting at room temperature,

the positions of the RNA fragments were visualised by UV and their position and location of the wells were marked on the membrane with a pencil. The membrane was briefly rinsed in 25 mM phosphate buffer to remove any residual agarose gel and placed RNA-side up on Whatman paper to air-dry. RNA was bound to the membrane by a 30 sec exposure to UV (UV Stratalinker 2400, Stratagene Ltd., Cambridge, UK). Dried membranes were sealed in plastic bags and stored at -20°C until needed.

Figure 2.5 Organization of capillary-polynucleotide blotting



2.5.15.2 Southern Blotting for Mini-Gel

After mini-gel electrophoresis, DNA pieces or PCR products fractionated on the gel were blotted to a GeneScreen Plus nylon membrane using a hybrid vacuum blotter (Bio-Rad Laboratories Ltd., CA, USA) according to the manufacturer's protocol. During blotting, the blotting stack was soaked with denaturing solution (0.6 M NaCl, 0.4 N NaOH). Blotting was carried out for 1 h under vacuum and the membrane was then neutralized in 2X SSC for 5 min, dried in air and incubated at 80°C for 2 h.

2.5.15.3 Southern Blotting

Digested genomic DNA was fractionated by gel electrophoresis as described in section 2.5.2.1. The fractionated DNA was depurinated by agitating the gel in 0.25 N HCl for 15 min and the gel then was rinsed 3 times in SDW. The gel was then shaken gently in denaturing solution (0.4 N NaOH, 0.6 M NaCl) for 30 min and then in neutralising solution (1.5 M NaCl, 0.5 M TrisHCl pH 7.5) for 30 min. The gel was soaked in 20X SSC (3 M NaCl, 0.3 M sodium citrate) for 15 min. The DNA in the gel was transferred by blotting onto a GeneScreen Plus positively charged nylon membrane overnight in 10X SSC. The blotting stack system was set up as shown in Figure 2.5. After blotting, the membrane was immersed in 0.4 N NaOH for 1 min to denature the DNA and then immersed in 2X SSC for 1 min to neutralize the membrane. The DNA was fixed to the membrane by the UV Stratalinker 2400 for 50 sec.

2.5.16 Hybridisation and Detection of Nucleic Acids

2.5.16.1 Prehybridisation

The prehybridisation solution (1% filtrated SDS, 50% deionised formamide, 5x SSC, 50 m sodium phosphate buffer pH 6.8, 0.1% sodium pyrophosphate, 10% dextrose sulphate) was mixed together and pre-heated at the hybridisation temperature for 1 h. The nylon membrane either from a plaque lift, a northern or Southern blot was rewashed with 2X SSC and put into a hybridisation bottle. One hundred and fifty μl of 10 mg/ml boiled salmon sperm (ss) DNA was then added to the hybridisation solution and then poured into the hybridisation bottle. The membrane filter was prehybridised in a hybridisation oven (Hybaid Ltd., Ashford Middx, UK) at 42◦C (northern analysis), 60-62◦C (library screening), or 65◦C (genomic Southern analysis) for at least 2 h.

2.5.16.2 Hybridisation

DNA probes were labelled with [^{32}P]dCTP using a Rediprime™ II Kit (Amersham Pharmacia Biotech Ltd., Bucks, UK), according to the manufacturer's instructions. The radioactive probe solution was then added to the prehybridisation bottle containing the membrane and then incubated overnight at the temperature used for prehybridisation.

2.5.16.3 Membrane Washing

After hybridisation, the radioactive liquid in the bottle was poured off and non-specifically bound probe was removed by washing solution 1 (2X SSC, 1% SDS) for 20 min at room temperature, solution 2 (0.5X SSC, 1% SDS) for 30 min at the hybridisation temperature, and solution 3 (0.1X SSC, 1% SDS) for 15-30 min at the hybridisation temperature.

2.5.16.4 Detection of Hybridisation Signals Using Autoradiography

Autoradiography was used to detect the signals generated by membrane bound ^{32}P-labelled probes after hybridisation. The membranes were sealed in plastic bags and then exposed to Kodak X-OMAT AR film (Kodak Ltd., Hempstead, UK) inside a film cassette and incubated at -80∘C for 2 to 120 h. After the appropriate exposure time, the film was developed under a red safe light by agitating in Kodak developing solution (1:4 dilution) for 5 min, rinsed in water, and fixed in Kodak fixing solution (1:9 dilution) for 5 min. The film was dried and the positions of the wells were aligned on the dried autoradiogram.

2.6 General Biochemical Methods

2.6.1 Western Blot Analysis

2.6.1.1 Protein Fractionation by SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Protein suspension was mixed with an equal volume of SDS-PAGE sample buffer (50 mM TrisHCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and placed in boiling water for 5 min. Twenty µl of each lysate were loaded onto an SDS-PAGE with 10 or 12% separating gel (10 or 12% acrylamide mix, 375 mM TrisHCl pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, 0.004% TEMED, adjusted with SDW). The gel was set in the BIO-RAD apparatus with Tris-glycine buffer (25 mM Trisbase, 250 mM glycine, 0.1% SDS). Proteins were electrophoresed at 120 volts for 90 min.

2.6.1.2 Western Blotting

The gel, a piece of nitrocellulose membrane, and 6 pieces of Whatman paper (slightly bigger than the gel) were soaked in transfer buffer (125 mM TrisBase, 1 M glycine, 20% methanol) for 5 min. The gel and the membrane were placed between 3 sheets of upper and lower paper layers, moistened with buffer on a 'Trans-Blot SD' blotter (Bio-Rad Laboratories Ltd., CA, USA). The proteins were transferred from the gel to the membrane by blotting at 10 volts for 30 min.

2.6.1.3 Immunodetection of Fusion Proteins

After blotting, the membrane was incubated in 20 ml of TBS buffer (150 mM of NaCl and 10 mM TrisHCl pH 8.0) with 5% dried milk at room temperature while gently shaking for 15 min. The membrane was then gently agitated overnight at 4°C in 10 ml of TBS buffer containing 2 µl of 1.0 mg/ml Anti-V5 primary antibody. The

membrane was washed 3 times with 10 ml of TBS buffer for 5 min each (for the first wash, 0.05% Tween20 was added to the buffer). Immunodetection was carried out using alkaline-phosphate conjugation by gently shaking the membrane in 10 ml of TBS buffer containing 2 µl of secondary antibody, Anti-Mouse IgG (whole molecule) for 1 h. The membrane was washed 3 times with TBS buffer for 5 min each and shaken in 20 ml of carbonate buffer (5mM $MgCl_2$ and 100 mM Tris adjusted to pH 9.5) with 66 µl of nitro blue tetrazolium (NBT) and 33 µl of 5-bromo-4-chloro-3-indolyl-phospahte (BCPIP) for 10-15 min. When bands were strong enough, the membrane was washed with H_2O and dried in air between white roll papers.

2.6.2 Ethylene Measurement

Melon or tomato fruit were placed in a sealed glass jar for 2 h at 25°C, and 1 µl of headspace air in the jar was measured. Ethylene concentration in the gas sample was measured by a Gas Chromatograph (GC 610 Series, ATI Unicam, Cambridge, UK) linked to a PC with UNICAM 4880 chromatography data handling software (ATI Unicam, Cambridge, UK). The GC was combined with a flame-ionisation detector (FID) with the operating conditions of 25 psi of air and 10 psi of N_2, 18 psi of H_2 carrier gas. Column specifications were 150 mm length, 6 mm outer dimension, and 4 mm inner dimension with support of alumina F_1 mesh range 80 to 100. The analysing temperatures were set to 110°C for the oven/column, 108°C at the injector, and 160°C for the detector.

2.7 Exogenous Ethylene Treatment

Melon fruit at 30 daa or tomato fruit at the mature green (MG) stage were kept in a glass jar containing 10 ppm ethylene for 16 h or 20 ppm for 24 h. The mesocarp of the fruit was then cut into small pieces and frozen immediately in liquid N_2.

2.8 Methods for Screening of Tomato and Avocado Fruit cDNA Libraries

2.8.1 Plating of Phage Lysate

One hundred µl of *E. coli* XLi Blue competent cells in 10 mM $MgSO_4$ were mixed with 1 µl of tomato cDNA tomato ripening library or avocado cDNA fruit library and incubated at 37°C for 20 min. Each suspension was added into 3 ml of top agarose (pre-warmed at 60°C), mixed well, and poured onto NZY agar plates. After cooling to room temperature for 10 min, the plates were incubated inverted at 37°C overnight. Cell lysis was visualized as plaques, appearing as transparent spots, on a lawn of the bacteria of the top agarose media.

2.8.2 Plaque Lift

After incubation overnight, plates were chilled at 4°C for at least 1 h to allow the top agarose to harden. A 137 mm Hybond-N^+ charged nylon membrane disk filter was placed onto the top agarose for 1 min in order to blot DNA phages to the membrane. The membranes and plate were marked with a pencil or needle containing waterproof black drawing ink to allow membrane orientation. After 1 min, the filter was peeled off and treated with DNA denaturing solution (0.6 M NaCl and 0.4 N NaOH) for 7 min, washed twice with neutralising solution (1.5 M NaCl and 0.5 M TrisHCl pH 7.5)

for 3 min, and then washed with 2X SSC to remove the trace of the top agarose. The upside-down filters were air dried for 1 h and then incubated at 80°C for 2 h.

2.8.3 Hybridisation of Plaque Lift Membranes

A 1103 nucleotide fragment was amplified by PCR, using pCM-AAT1 (pMEL2) as a template and Mel2F and Mel2R as primers. The plaque lift membrane was hybridised with the probe at 60°C as described in section 2.5.16.

2.8.4 Identification of Positive Plaques

Plaques corresponding to positive signals on the autoradiograph film were cored out from the phage plate culture media using Pasteur pipettes. The cored plaques were transferred to Eppendorf tubes containing 500 µl of SM buffer (100 mM NaCl, 10 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin solution) and 10 µl of chloroform. The tubes were then kept at 4°C for overnight to allow phage to diffuse into the buffer. The solution was used for second round screening by repeating section 2.8.1 to 2.8.4.

In second round screening, selected plaques were cored out from the media and transferred to Eppendorf tubes containing 100 µl of SM buffer to dissolve the medium. The DNA inserts were then amplified by PCR.

2.8.5 Isolation of Selected cDNA Clones

2.8.5.1 PCR Amplification of Selected cDNA Clones

T7 and T3 DNA sequences, integrated on the phagemid between DNA inserts, were used as primers and 10 µl of cDNA library lysate (in SM buffer) from the second round screening was used as DNA template for the PCR reaction using Tag DNA polymerase.

2.8.5.2 Cloning of DNA Fragments in Bacteria

The fragment was cloned into the pGEM®T Easy vector, according to the manufacturer's instructions. The ligation was carried out at 4°C overnight and introduced to DH5α competent cells. The cells were plated onto medium plates containing 50 μg/ml ampicillin and 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-*D*-galactoside) and incubated at 37°C overnight.

2.8.5.3 Selection of Positive Colonies

White colonies growing were selected and the bacteria grown up for mini preps. The presence of the inserts was confirmed by enzymatic digestion using *EcoR*I, followed by gel electrophoresis, and the plasmids were sequenced (section 2.5.11) using T7 and SP6 primers.

2.8.5.4 Obtaining Additional Sequence from *TOMM7* by PCR

The addition sequence at the 5' end of *TOMM7* (the sequenced tomato clone from the second round screening), was obtained by PCR. The T7 primer was used as a forward primer and Tomm74R1 was used as a reverse primer, which corresponds to the sequence inside *TOMM7* clone. Ten μl of cDNA library lysate were used as a DNA template. The sequence was cloned and sequenced.

2.9 Recombinant Protein Methods

2.9.1 Making Constructs of Fusion Proteins

As part of the *MEL2* project collaboration with F. El Yahyaoui and J-C. Pech, ENSAT, Toulouse, France, we received 2 melon ORF clones, which are pMEL2 (subsequently named *CM-AAT1*; Accession No CAA94432, (Yahyaoui *et al.*, 2002))

and pMEL2' (subsequently renamed *CM-AAT2*; Accession No AF468022, (Yahyaoui *et al.*, 2002)). The tomato EST sequence TC98820 (TIGR, http://www.tigr.org/tdb/tgi/) was used to design primers for cloning the ORF sequence (designated as *LeAAT1*) from total tomato fruit RNA.

2.9.1.1 Cloning of ORF Fragments into Yeast Expression Vector

The clones were isolated, cloned and then expressed in yeast using the pYES2.1 TOPO TA Cloning Kit. The expression vector carries the promoter and enhancer sequences from the *GAL1* gene for regulated expression (Figure 2.2).

2.9.1.1.1 Amplification of *CM-AAT1* and *CM-AAT2* ORFs

The ORF inserts in pCM-AAT1 and pCMAAT2 were amplified by PCR using Deep Vent® DNA polymerase (section 2.5.1.2). To optimise expression, an extra residue, G, was added at position -3 before the ATG initiation codon of the Mel2F2K forward primer (section 2.2.3) for cloning both *CM-AAT1* and *CM-AAT2*. This enhances the translation initiation in many mRNA sequences (Kozak, 1987; Kozak, 1990). The reverse primers, Mel2R3 for *CM-AAT1* and Mel2BR for *CM-AAT2*, were designed to remove the native stop codon (TAA) to allow cloning in frame with the V5 epitope and polyhistidine tag (C-terminus) for expression in yeast.

2.9.1.1.2 Amplification of *Le-AAT1* ORF by RT-PCR

Total RNA from ripe tomato was used for RT-PCR with Deep Vent® DNA polymerase as described in section 2.5.1.3. The PCR reaction was consisted of 3 µl of the first strand cDNAs, Tomm7FK primer and Tomm7R.

2.9.1.1.3 Insertion of DNA Fragments into the Yeast Expression Vector

The PCR fragments were recovered from agarose gels after gel electrophoresis. Before ligation, the *CM-AAT1*, *CM-AAT2* and *Le-AAT1* inserts were post-amplified by adding 3' A-overhang, as described in section 2.5.4. The DNA inserts were then introduced into pYES2.1/V5-His-TOPO® vector. The reaction consisted of 0.5 to 2 μl (10-30 ng) of fresh PCR product, 1 μl (10 ng) of the vector and was adjusted to the final volume of 5 μl with SDW. The reaction was mixed gently and incubated for 5 min at room temperature (around 25°C). One μl of the 6X TOPO Cloning stop solution was mixed into the reaction and the mixture was then put on ice.

2.9.1.2 Bacterial Transformation and Verification of Insert

Recombinant plasmids from the ligation were introduced into *E. coli* competent cells, strain TOP10F'. The transformants were plated onto medium containing 50 ng/ml ampicillin and the plates were incubated at 37°C overnight.

The size and orientation of the inserts in the plasmids, amplified by mini-preps, was checked by enzymatic digestion. The *CM-AAT1* and *CM-AAT2* inserts were digested by *Pvu*II (5' end on the vector) and *Nco*I (3' end on the clones), resulting in an 1.37 Kb product size. The *Le-AAT1* insert was digested by *Hind*III (5' end on the clone) and *Xba*I (3' end on the vector), releasing an 1.15 Kb fragment. The reactions were carried out according to section 2.5.5.1. The sequence of each clone was confirmed by sequencing using the GAL1 and V5 C-TERM primers.

2.9.2 Expression of the Clones in Yeast

The sequenced recombinant vectors named as pYES-CMAAT1, pYES-CMAAT2 and pYES-LeAAT1 were used for yeast transformation.

2.9.2.1 Small-Scale Yeast Transformation

A single colony of yeast (*Saccharomyces cerevisiae*), strain INVSc1, was inoculated in 10 ml of YEPD medium, and the culture was shaken overnight at 30°C. The starter culture was diluted to an OD_{600} of 0.4 in 50 ml of YEPD medium and grown for 2-4 h. The yeast cells were collected by centrifugation at 1000g at 4°C for 5 min and resuspended in 40 ml of 1X TE (10 mM TrisHCl pH 7.5, 1 mM EDTA). The cells were again centrifuged at 1000g for 5 min and resuspended in 2 ml of solution I (1X LiAc, 0.5X TE). After incubation at room temperature for 10 min, 1 μg of recombinant vector and 100 μg of denatured sheared salmon sperm DNA were added in 100 μl of the yeast suspension. To the yeast/DNA mix was added 700 μl of solution II (1X LiAc, 40% PEG-3350, and 1X TE), which was mixed well, and then incubated at 30°C for 30 min. Eighty eight μl of dimethyl sulfoxate (DMSO) was added and the cells were heat shocked at 42°C for 7 min. The cells were collected by centrifuging for 10 sec in a microcentrifuge at maximum speed and the supernatant was removed. The yeast cells were then washed in 1 ml of 1X TE and re-pelleted, and resuspended in 100 μl of 1X TE before being plated on solid CM media without uracil (CM-uracil), containing 2% raffinose. Transformed yeast colonies took approximately 5-7 days to grow.

2.9.2.2 Fusion Protein Expression Induced by Galactose

The GAL1 promoter is galactose inducible. The expression of *CM-AAT1*, *CM-AAT2* and *Le-AAT1* fusion proteins in yeast was induced by incubation in medium containing galactose. A single transformed yeast colony was inoculated in 15 ml liquid CM-uracil medium plus 2% raffinose, and incubated overnight at 30°C while

shaking. Cells of the overnight culture were pelleted by centrifugation at 1000g for 5 min at 4°C, resuspended in 1 ml of CM-uracil medium containing 2% galactose (induction medium) and inoculated into 50 ml of induction medium to give an OD_{600} of 0.4 and the culture was incubated at 30°C with shaking. Five ml aliquot of cells was harvested at 0, 4, 8, 12, 16 and 24 h from the culture flask. The collected aliquot was centrifuged at 1000g for 5 min at 4°C and the pellet resuspended in 500 µl of SDW. The suspension was repelleted and the cell pellet was stored at -80°C until required for SDS PAGE analysis.

2.9.2.3 Protein Extraction for SDS PAGE Analysis

The frozen cell pellet was resuspended in 500 µl of breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM EDTA, 5% glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF)) and repelleted by centrifuging at 1000g for 5 min at 4°C. The cells were resuspended in 250 µl of breaking buffer and an equal volume of acid-washed glass beads (0.4-0.6 mm size) was added. The mixture was vigorously vortexed for 30 sec, followed by 30 sec on ice. This was repeated 4 times for a total of 4 min to lyse the cells. Cell debris was collected by centrifugation at maximum speed by a microcentrifuge for 10 min. The supernatant was removed promptly.

2.10 Methods for Alcohol Acyl-Transferase (AAT) Activity Assays

2.10.1 Preparation of Fusion Proteins

A colony of transformed yeast containing pYES-CMAAT1, pYES-CMAAT2, or pYES-LeAAT1 was inoculated in 5 ml of CM-uracil culture, containing 2% raffinose, and left to grow overnight at 30°C. The cells were collected by centrifugation at 1500g for 5 min at 4°C, and resuspended in 1 ml of induction medium. The cell

suspension was added to 50 ml of fresh induction media and the culture was shaken overnight at 30°C. The cells were collected by centrifugation at 1500g for 10 min at 4°C, resuspended in 2 ml of 50 mM TrisHCl pH 7.5, and frozen with liquid N_2. The frozen suspension was mechanically ground to a fine powder and stored at -80°C until enzyme activity assays.

2.10.2 Quantitative Measurement of Proteins

The frozen powder was thawed, vortexed for 1 min, and centrifuged at 12000g for 15 min at 4°C. The supernatant was transferred to a new Eppendorf tube and kept on ice. The total proteins from the extraction were quantified according to the Bradford method (Bradford, 1976).

2.10.3 pH Optimisation of *Le-AAT1*

The enzyme activities of the fusion proteins were tested to find out the optimum pH *in vitro*. AAT activity was tested in 500 µl total volume containing 40mM hexanol, 250 µM acetyl-CoA, 20 mM $MgCl_2$, 166 µg protein extract. The reaction was adjusted to 500 µl with 50 mM TrisHCl pH 5.5 to 9.0, containing 1 mM dithiothreitol (DTT) and then incubated at 30°C for 20 min.

2.10.4 Effect of Ionic Strength and Nature of Ions on *Le-AAT1*

Various concentrations of $MgCl_2$, NaCl, or KCl were applied to the reaction mixture to measure specificity of the recombinant proteins. The reaction contained 40 mM hexanol, 250 µM acetyl-CoA, 166 µg protein extract, 0.01 to 1 M $MgCl_2$, NaCl, or KCl, and was adjusted to 500 µl with buffer A (50 mM TrisHcl pH 7.5, 1 mM DTT). The mixture was incubated at 30°C for 20 min.

2.10.5 Substrate Specificity

Alcohols and acyl-CoAs were used as substrates to test the AAT activity of the fusion proteins. The reaction conditions were 166 µg protein extract, 40 mM alcohols (R-OH), 250 µM acyl-CoAs, 20 mM $MgCl_2$ (for *CM-AAT1* and *CM-AAT2* proteins) or 20 mM NaCl (for *Le-AAT1* protein), and the total volume was adjusted to 500 µl with buffer A. The mixture was then incubated at 30°C for 20 min.

2.10.6 Kinetic Properties of *Le-AAT1*

The affinity of the *Le-AAT1* activity for butanol, hexanol, acetyl-CoA, and propionyl-CoA was tested. The reaction contained 166 µg protein extract, 5 - 40 mM butanol or hexanol, 50 to 250 µM acetyl-CoA or propionyl-CoA, 20 mM NaCl, and was adjusted to 500 µl with buffer A. The mixture was then incubated at 30°C for 20 min

2.10.7 Extraction of Esters Formed

After 20 min incubation at 30°C, the esters formed were extracted by adding 250 µl of pentane, containing 5 µl/L α-pinene as internal standard, to the enzymatic reaction. The mixture was vigorously vortexed for 1 min and 1 µl of the pentanic phase (upper phase) was used for GC analysis.

2.10.8 Ester Analysis

2.10.8.1 Gas Chromatography/Mass Spec (GC/MS) Operation

A Fison 8000 series gas chromatograph (GC) (Fisons Scienctific, Manchester, UK) with a 60 m x 0.250 mm DB-5 column (J&W Scientific, Folsom, CA, USA) and a MD-800 mass spectrometer (MS) (Fisons Scienctific, Manchester, UK) was used. Helium was used as carrier gas run at 4 psi. The injector temperature was set at

250∘C while the column temperatures were programmed to change gradually, starting at 40∘C for 1 min, increasing at 8-12∘C/min and holding at 220∘C for 1 min. The mass spectra in *El+* ionisation mode at 350eV were recorded in a full or selective scan mode with solvent delay time of 5 min. The GC/MS was linked to a desktop computer with the Mass lab program ver. 1.4 (Finnigan Corp., San Jose, CA, USA).

2.10.8.2 Ester Identification and Quantification

Compounds were identified by comparing their mass spectra against the National Institute of Standards and Technology (NIST) mass spectral reference collections. The Lab–Base software ver. 2.22 (LAB-BASE system) was used for data acquisition analysis. Ester identity was verified and quantified by comparing the retention time and peak area with corresponding pure authentic standards of each ester when available.

2.11 Methods for Generating of Transgenic Tomatoes

Antisense and sense constructs were made and used to study aromatic compound manipulation. Two sense constructs used the CaMV35S promoter with *CM-AAT1* ORF and *CM-AAT1* ORF fused with a heterologous 212 bp 5'UTR from *Le-AAT1*. This construct was also made with the ACO1 promoter. A 411 bp antisense *Le-AAT1* fragment near the 3'end, driven by the CaMV35S promoter, was also made.

2.11.1 Making Gene Expression Cassettes

2.11.1.1 Introduction of Inserts into Cloning Vectors

2.11.1.1.1 Open Reading Frame of *CM-AAT1*

An ORF sequence of *CM-AAT1* (*MEL2*) cDNA was cloned from pYES-CMAAT1 (section 2.9.2) using proofreading PCR with Mel2FSma and Mel2RXba primers and

introduced into pGEMT Easy vector. The recombinant vector was designated as pGEM-CMAAT1.

2.11.1.1.2 *CM-AAT1* Heterologously Fused with the 5'UTR from *Le-AAT1*

A 211 bp 5' UTR fragment of *Le-AAT1* (TC98820, TIGR) was cloned using proofreading RT-PCR with BamHI-XbaITCF .and Mel2-BamHIR primers and introduced into the pYES-CMAAT1 vector using the *BamH*I restriction site. The recombinant vector was named pYES-UCMAAT1.

2.11.1.1.3 Antisense *Le-AAT1* fragment

A partial 411 bp sequence near the 3' end of *Le-AAT1* was cloned in the antisense orientation using RT-PCR with ATomm7FSal and ATomm7RBam primers and introduced into pGEMT Easy vector. The recombinant vector was designated as pGEM-ASLeAAT1.

2.11.1.2 Making CaMV 35S Promoter Constructs

2.11.1.2.1 Digestion of Inserts

The inserts in pGEM-CMAAT1, pYES-UCMAAT1, pGEM-ASLeAAT1 were digested with the relevant restriction enzymes (shown with the dashed arrows in Figure 2.6, Figure 2.7, and Figure 2.8, respectively). The digested reactions were extracted once with phenol/chloroform (1:1), and once with chloroform. The DNA were precipitated by two volumes of alcohol, collected by centrifugation, washed and resuspended in SDW to 50 ng/μl.

Figure 2.6 Construction of pBIN35S-CMAAT1

Dashed arrows indicate restriction sites used.

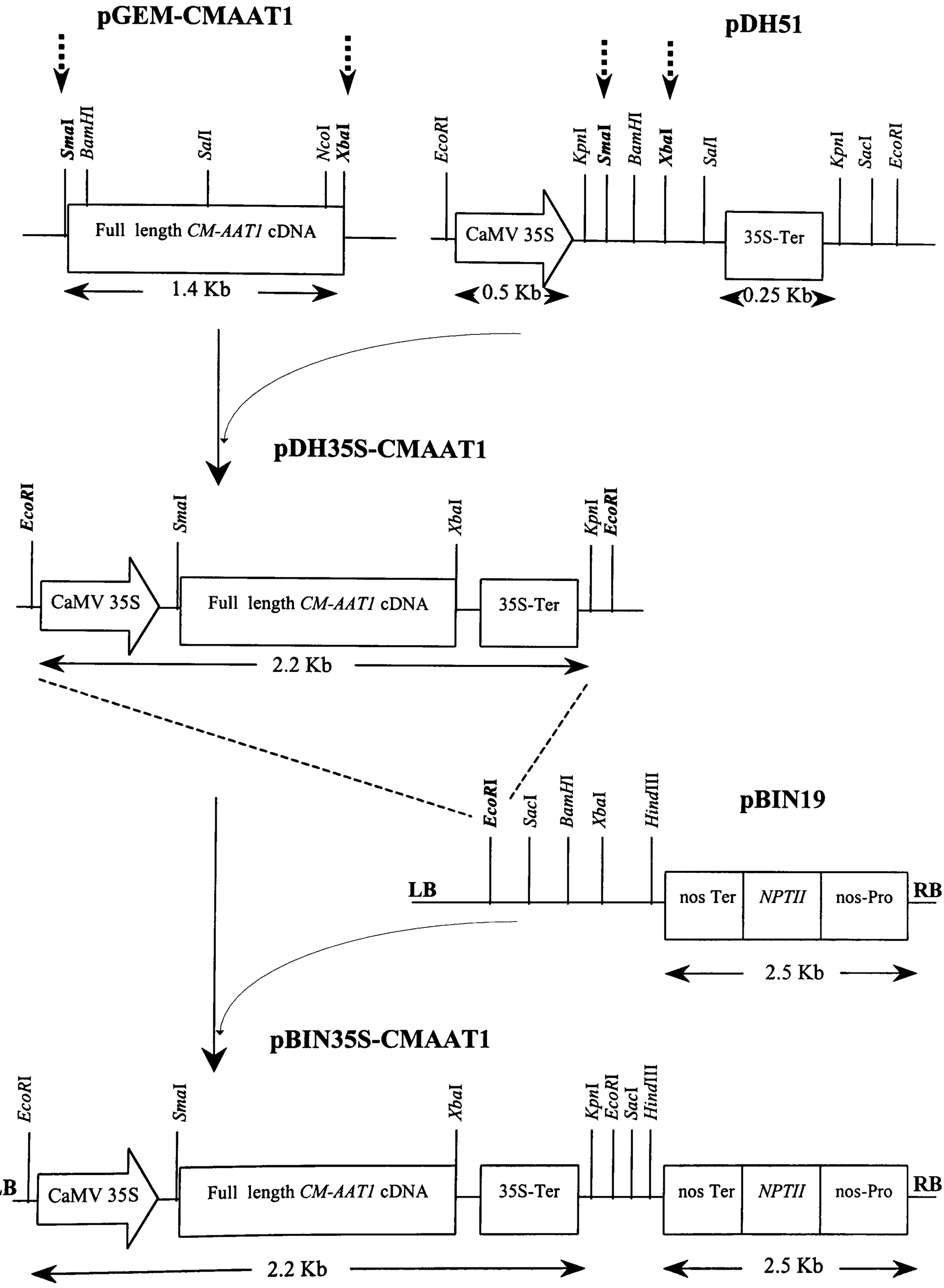


Figure 2.7 Construction of pBIN35S-UCMAAT1

Dashed arrows indicate restriction sites used.

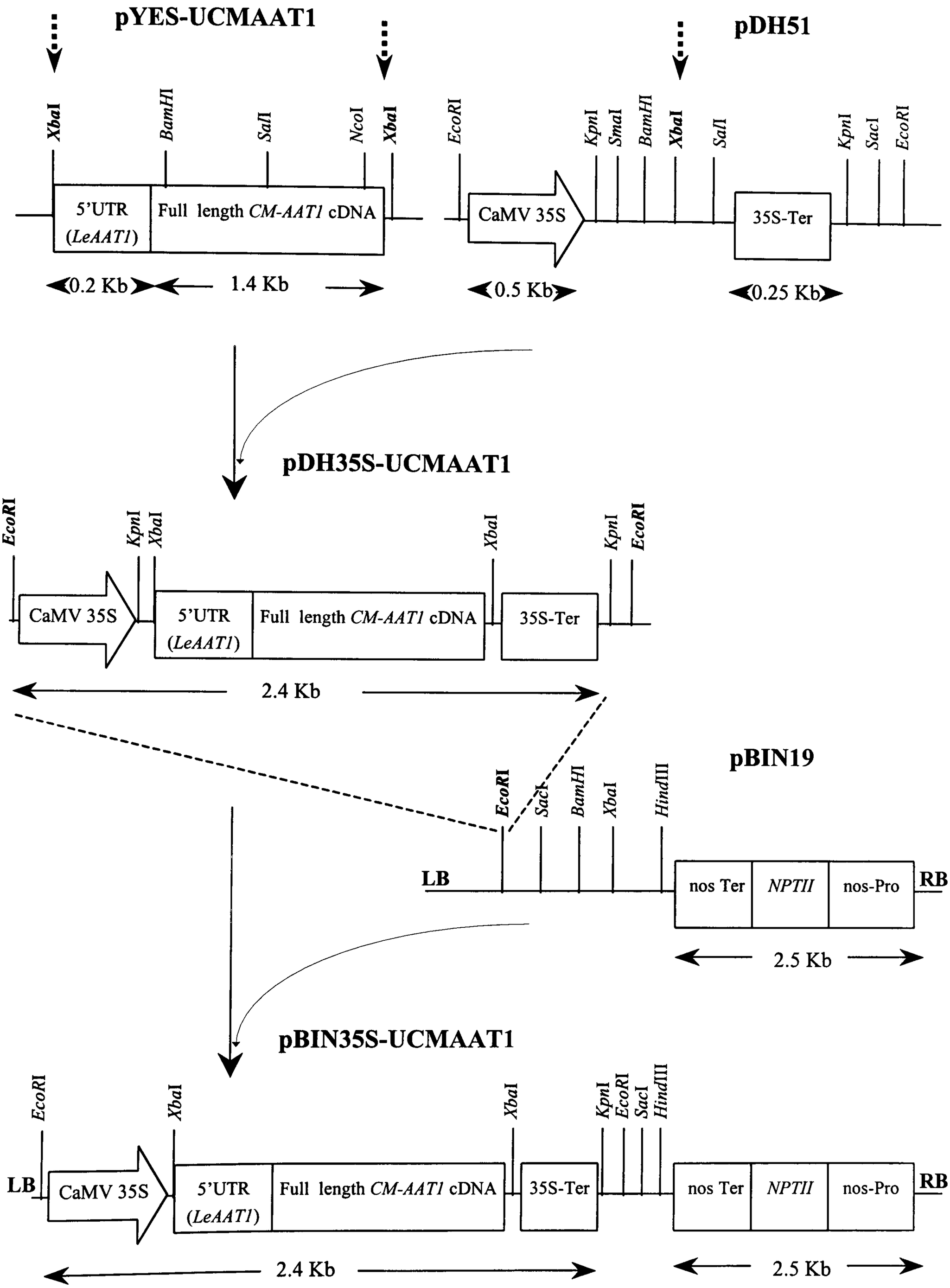
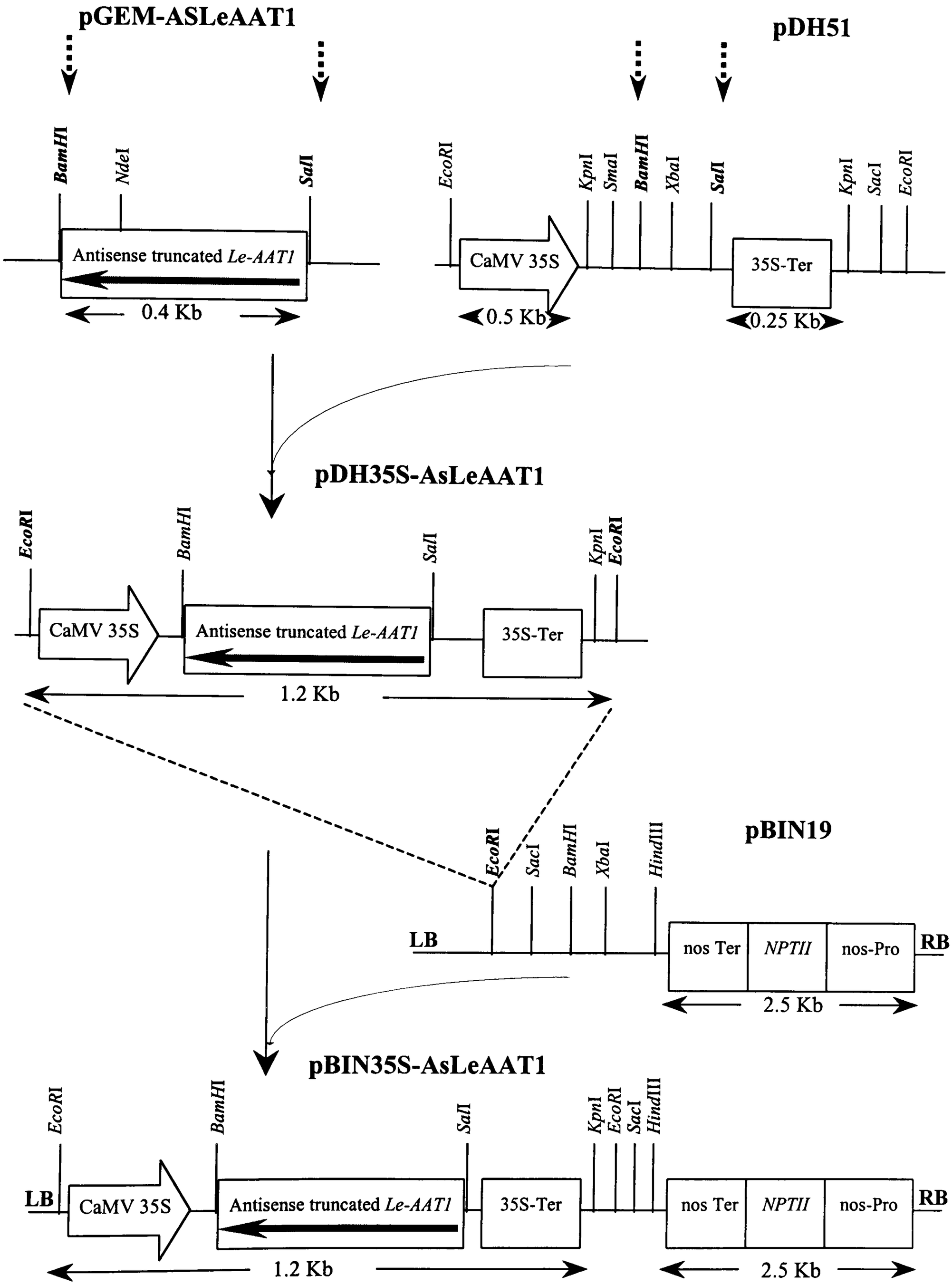


Figure 2.8 Construction of pBIN35S-ASLeAAT1

Dashed arrows indicate restriction sites used.



2.11.1.2.2 Digestion and Dephosphorylation of Host Vector

pDH51 (Pietrzak *et al.*, 1986), containing the CaMV35S promoter, the 35S terminator and an ampicillin resistance gene, was cleaved with the restriction enzymes according to section 2.11.1.2.1. In case of single restriction enzyme digestion, the 5' phosphate of both protruding ends of the digested vector were removed using calf intestinal alkaline phosphatase (CIP) to prevent the possibility of self-ligation. To the digested-vector reaction was added 0.01 unit CIP/ρmoles DNA, which was then incubated at 37°C for 1 h. The reaction was extracted with phenol/chloroform (1:1), and chloroform. The pellet was collected by ethanol precipitation, washed with 75% (v/v) ethanol, dried and dissolved in 50 ng/µl SDW.

2.11.1.2.3 Bacterial Transformation and Colony Blot

The DNA inserts were introduced into the linearised pDH51 vector DNA using a ligation kit (Stratagene Ltd., Cambridge, UK). The ligation mix was then used to transform DH5α competent cells. The transformants were identified using colony blot analysis. Colonies on the overnight selective plates were transferred with a sterile toothpick to 2 media plates containing ampicillin and incubated at 37°C overnight. A master plate was kept at 4°C for further analysis, and the other one was used for colony blot analysis. A 137 mm nylon membrane disk filter was placed on the plate for 1 min. The orientations of the plate were marked on the membrane using a pencil or needle containing waterproof black drawing ink. The filter was peeled off and placed upside-down on Whatman paper soaked with DNA denaturing solution (1.5 M NaCl and 0.5 N NaOH) for 7 min, and then placed on paper soaked with neutralizing solution (1.5 M NaCl and 0.5 M TrisHCl pH 7.5) for 2x3 min each. The

membrane was washed with 2X SSC to remove traces of the medium. The upside-down filters were air-dried and the DNA was fixed on the membrane by crosslinking (UV Stratalinker 2400). Colonies containing the correct insert were identified using a corresponding radio probe.

For one site ligation, the plasmid was digested by restriction enzymes, one on the vector arm and the other one on the insert. The inserts were confirmed by sequencing.

2.11.1.2.4 Introduction the CaMV35S Promoter Cassettes into pBIN 19

The recombinant pDH51 vectors were digested with *EcoR*I to release the CaMV35S promoter cassettes. pBin19 (Bevan, 1984; Frisch *et al.*, 1995), a binary plasmid DNA containing a kanamycin resistance gene, was digested with *EcoR*I and dephosphorylated. The CaMV 35S promoter cassettes were then ligated into pBin19 and the ligation reaction was transformed into DH5α competent cells.

2.11.1.2.5 Screening for Insertion into pBIN19

Insertion of the cassette from recombinant pDH51 to pBIN19 was firstly screened by inactivation of plasmid-borne antibiotic resistance genes. Colonies, surviving and growing on both ampicillin and kanamycin selective media, were likely to contain the pDH51-pBIN19 combined vector. Therefore colonies, which did not grow in the presence of ampicillin, but grew on kanamycin, were selected for enzymatic digestion. The recombinant vectors were named pBIN35S-CMAAT1, pBIN35S-UCMAAT1, and pBIN35S-AsLeAAT1.

2.11.1.3 Making ACO1 Promoter Constructs

2.11.1.3.1 Transferring ACO1 Promoter to pBIN 19

pBluescript, containing the ACO1 promoter (Blume *et al.*, 1997), and pBIN 19 were digested with *EcoR*I and *Sac*I (Figure 2.9). The ACO1 promoter was ligated into the linearised pBIN 19 and the recombinant vector was introduced into DH5α competent cells with kanamycin selection. Positive colonies were screened following the method in section 2.11.1.2.5.

2.11.1.3.2 Transformation of *UCM-AAT1* into pBINACOP

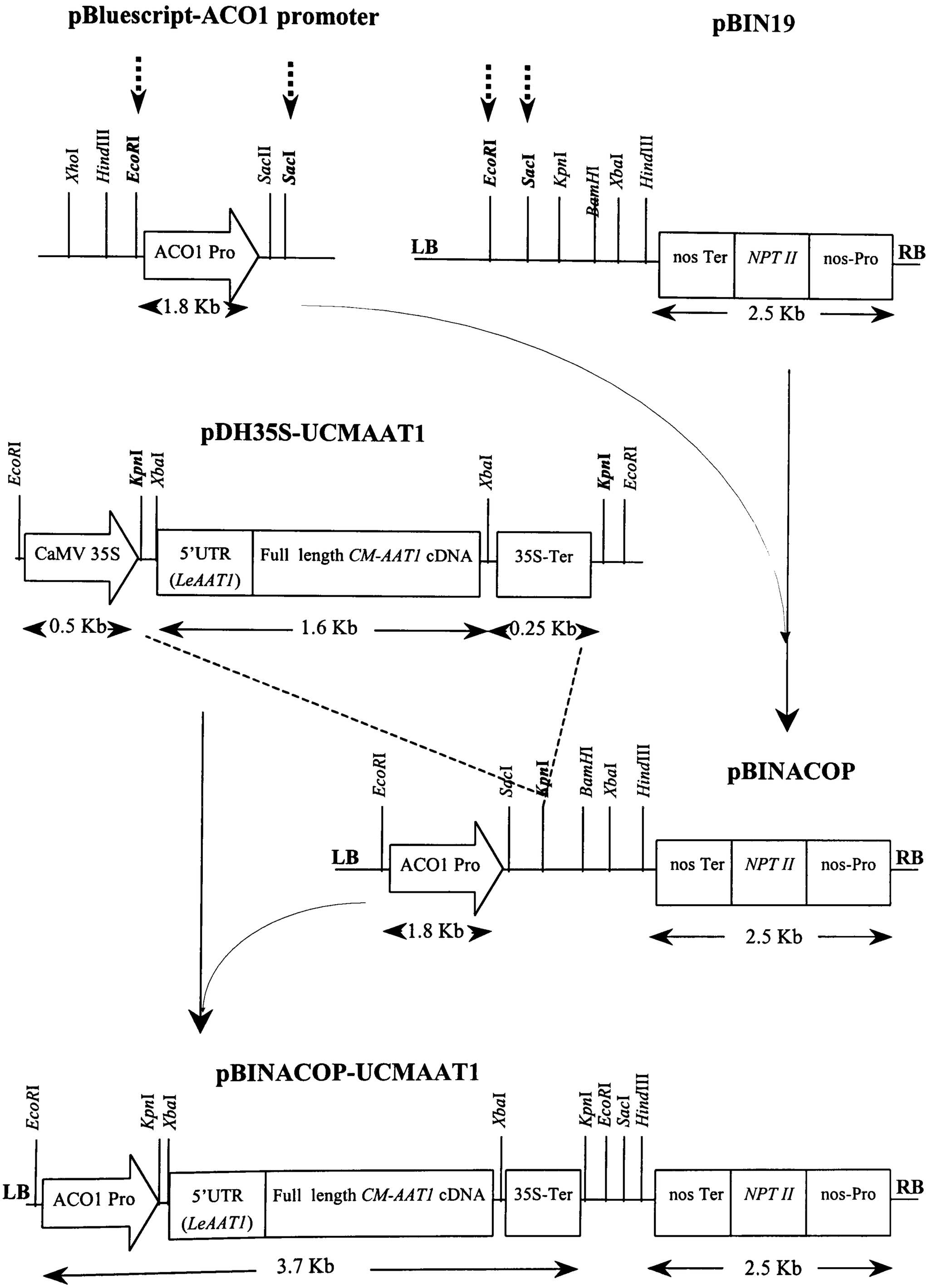
The *CM-AAT1* fragment, fused with tomato 5'UTR and 35S terminator, was released from pDH35S-UCMAAT1 (Figure 2.7) by *Kpn*I digestion. pBINACOP was digested using *Kpn*I and then dephosphorylated. The *UCM-AAT1* fragment was introduced into the linearised pBINACOP. The ligation mix was transformed into DH5α competent cells and transformants were selected using inactivation of plasmid-born antibiotic resistance genes. The recombinant vector was named pBINACOP-UCMAAT1 (Figure 2.9).

2.11.2 Transformation of Recombinant pBIN19 into *Agrobacterium tumefaciens*

One μg of binary plasmid DNA was added to 100 μl of *Agrobacterium* competent cell LBA4404 suspension (section 2.5.7.3), mixed gently, and frozen in liquid N_2 for 3 min. The frozen mixture was thawed by incubation in a water-bath at 37°C for 5 min. 1 ml of APM medium was added and the cells were incubated at 29°C with shaking at 200 rpm for 2-4 h in the dark. The cell pellet was collected by microcentrifugation for 30 sec and resuspended in 100 μl of APM media. The cells were spread out on an APM

Figure 2.9 Construction of pBINACOP-UCMAAT1

Dashed arrows indicate restriction sites used.



agar plates containing 500 µg/ml streptomycin and 50 µg/ml kanamycin and the plates were incubated in the dark at 29°C in an inverted position until colonies appeared. Southern analysis of recombinant plasmids was done to check the insert cassettes.

2.11.3 Tomato Plant Transformation and Regeneration

2.11.3.1 Preparation of Transformation Explants

Wild type tomato seeds were surface sterilised by soaking for 1 min in 100% ethanol, and then soaked in 10% bleach solution for 10 min. The seeds were thoroughly rinsed using 1 litre of SDW and evenly sown onto MSR3 medium at a high density of about 200 seeds per pot (9 cm diameter). The seeds were left to germinate in a tissue culture growth room under conditions of 16 h daylight at 26°C followed by 8 h dark at 18°C.

Ten-12 day old cotyledons were harvested to be used as explants. The explants were dipped in MS2-4D media for 1 h, dried on autoclaved Whatman No1 filter paper, and then placed on 3CSZR plates. The plates were sealed with parafilm, and incubated in the tissue culture room under low light conditions by covering the plates with 2 layers of muslin. The explants were ready to use for transformation after 24 h pre-incubation.

2.11.3.2 Preparation of Transformed *Agrobacterium tumefaciens*

Fifty µl of transformed *A. tumefaciens* glycerol stock were inoculated into 10 ml of LB culture with antibiotics (500 µg/ml streptomycin and 50 µg/ml kanamycin) and incubated in the dark at 29°C with shaking at 200 rpm overnight. The cells were subcultured by transferring 200 µl of the cell culture into 10 ml of fresh LB media

containing the antibiotics and the culture shaken overnight (200 rpm) at 29°C in the dark.

2.11.3.3 *Agrobacterium*-Mediated Gene Transformation

The overnight transformed *A. tumefaciens* cell culture, was grown to A_{600} of 1.8 –2.0, and the cells were collected by centrifugation at 2000g for 10 min. The cell pellet was resuspended in 10 ml of LB media and collected as previously. The cells were evenly resuspended in 10 ml of MS media. The pre-incubated cotyledon explants were transformed by placing into the *A. tumefaciens* cell suspension and agitated gently for 15 min. Excess bacteria were absorbed by placing the explants on sterile Whatman No1 filter paper and the explants were returned to their plates. The plates were sealed with parafilm and incubated in the tissue culture room for 2 days at low light conditions.

2.11.3.4 Selection of Transgenic Calli

After 2 days, the cotyledon explants were transferred onto selective 3C5ZR plates containing antibiotics, 75 μg/ml kanamycin sulphate and 400 μg/ml augmentin (Smithkline Beecham Pharmaceuticals, Cambridge, UK), and incubated as above. The explants were transferred to fresh selective media plates every 3 weeks until calli and shoots developed. Individual shoots from calli were cut and placed in MSR3 pots containing 50 μg/ml kanamycin sulphate and 400 μg/ml augmentin to induce growth of roots. The subcultured pots were incubated in the tissue culture room until roots formed. Selected rooting shoots were taken and grown in compost at glasshouse room as described in section 2.3.1.

2.12 Analysis of Flavour Volatiles in Tomatoes

2.12.1 Sample Preparation

2.12.1.1 Volatile Compounds from Blended Tomato Fruit

A tomato fruit (25-30 g) from each fruit ripening stage was properly blended in a blender (Phillip HR-2914) for 1 min. The homogenate was transferred into a 250 ml Duran bottle, sealed, and incubated at room temperature for 20 min.

2.12.1.2 Volatile Compounds from Blended Tomato Fruit with Added Substrates

Twenty-five g of B+7 tomato fruit were blended with 10 ml of 1 M phosphate buffer pH 7.0 in the blender for 1 min. The homogenate was transferred into a 250 ml Duran bottle, with 2 mM 3-methyl butanol, hexanol, *Z*-3-hexenol, and phenyl ethanol. Acetyl-CoA was then added into the homogenate to final concentration of 5 μM and the mixture was incubated at 30°C for 60 min.

2.12.1.3 Volatile Compounds from Ground Tomato Leaves with Added Substrates

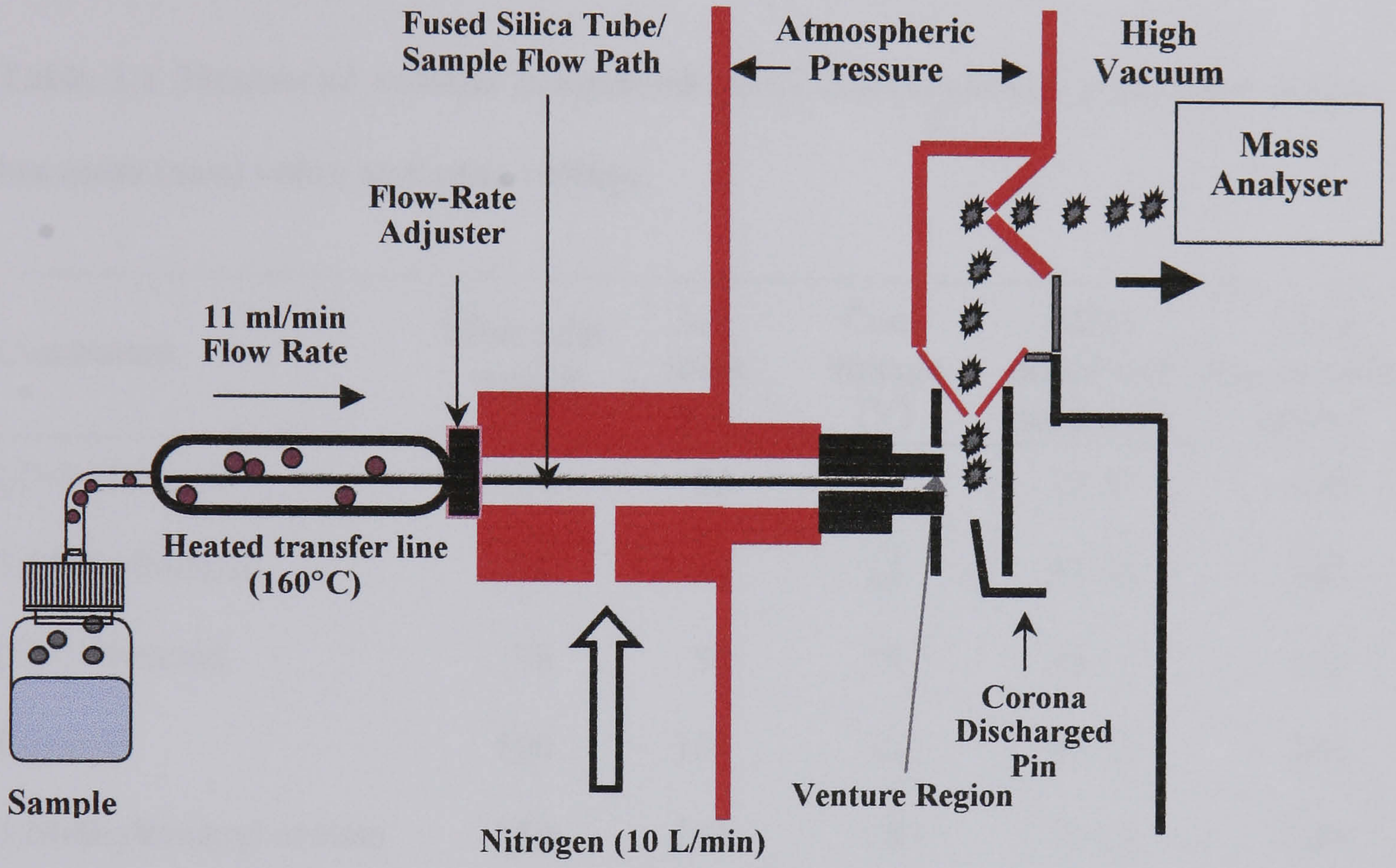
Five g of tomato leaves were ground to a fine powder in liquid N_2. The powder was put into a 100 ml Duran bottle with 5 ml of 0.1 M phosphate buffer pH 7.0. 3-Methyl butanol, hexanol, *Z*-3-hexenol, phenyl ethanol and acetyl-CoA were then added into the homogenate to a final concentration of 2 mM for alcohols and 5 μM for acetyl-CoA. The mixture was incubated at 30°C for 60 min.

2.12.2 Atmospheric Chemical Ionisation (APCI) Operation

A Microcromass Platform II quadrupole mass analyser based detector (Micromass, Manchester, UK.) operating in an APCI positive ion mode for gas phase was fitted with a specifically designed air-sampling interface (Linforth and Taylor, 1998; Taylor

et al., 2000). The system was composed of a heat transfer line, an ionisation chamber and a mass spectrometer. The APCI operation diagram is shown in Figure 2.10

Figure 2.10 Schematic diagram of the APCI system



Gas was continuously pulled into the APCI-MS source by a high flow rate of N_2 (10 L/min). The sample was analysed by APCI at a flow rate of 11.0 ml/min through a heat transfer line (a deactivated fused silica tube) held at 160°C to prevent water and volatile condensation. Volatile compounds were ionised by a 4 kV corona discharged pin at cone voltage ranging between 12 and 37 volts in an ionisation chamber before they were extracted orthogonally into the quadrupole mass analyser. The mass spectrometer was operated in a selected ion-recording mode. The APCI operating method was followed to Boukobza *et al.* (2001). Headspace analysis was performed for 2 min after the baseline was steady line.

2.12.3 Aroma Volatile Specification

Eight different volatile compounds with different chemical and biochemical origins and contributions to tomato volatiles were monitored. The characteristic, ion mass (m/z) values and cone voltages used for the analysis of each of the compounds of interest are given in Table 2.1.

Table 2.1 Monitored volatile compounds with corresponding molecular weight, ion mass (m/z) value and cone voltage

Compound	Molecular weight	Ion mass m/z	Cone voltage (V)	Mass added into stock (µl)	Final concentration (ppbv)
(*Z*)-3-Hexenol	100	83	18	18.47	100
3-Methylbutanal	86	87	18	38.86	200
(*E*)-2-Hexenal	98	99	18	22.11	100
Hexanal	100	101	12	44.98	200
3-Methylbutanyl acetate	130	131	18	29.19	100
(*Z*)-3-Hexenyl acetate	142	143	18	31.87	100
Hexyl acetate	144	145	18	32.16	100
Phenylethyl acetate	164	165	18	73.50	200

Data supplied from the 'Compounds Ionisation, Specifications, Calibration' laboratory book by R. S. T. Linforth, M. S. Brauss, and A. J. Taylor

2.12.4 Data Processing

Data from APCI-MS analysis were collected using the Masslynx software (Masslynx 3.2, Micromass Ltd., UK). The intensity of each ion was analysed using the CDC-2000 (Cut, Delete and Calibration 2000) software. The ion current intensities were converted to headspace concentration (mg/m^3 or ppbv), which was subjected to a 5-point

smoothing algorithm as well as background signal subtraction. Results were expensed as mg per m^3.

2.13 Sequence Analysis and Database Searching

Alignment and comparison of DNA sequences in genetic database was carried out using the **National Center for Biotechnology Information** (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/), and **The Institute for Genetic Research** (TIGR) website (http://www.tigr.org/tdb/tgi/).

Protein prediction, identification, and characterization were carried out at the **ExPASy Molecular Biology Server** (http://ca.expasy.org/tools/dna.html).

Multiple sequence alignment was carried out using the **Baylor College of Medicine HGSC** (BCM) Search Launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

Box shade comparison of multiple sequence alignments was carried out using the **Boxshade 3.21** program (http://www.ch.embnet.org/software/BOX_form.html).

Tree dendograms were constructed using **Clustal-X 1.83** (ftp://ftp-igbmc.u-strasbg.fr/pub/) and **Treeview32** (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.14 Statistical Analysis

The data were subjected to statistical analysis using the SAS system ver. 8.02 TS level 02M0 (SAS Institute Inc., Cary, NC, USA). Completely randomised design (CRD) was used for analysing variances whereas means of treatments were compared at P=0.05 by the Duncan's multiple range test (DMRT) comparison.

CHAPTER 3

RESULTS

3.1 Screening of a Tomato Fruit cDNA Library

Approximately 200,000 phages from the tomato fruit cDNA library were screened using an 1.1 Kb *CM-AAT1* (*MEL2*) probe. After second round screening, 10 selected phages were amplified by PCR and separated by gel electrophoresis (Figure 3.1A). The Southern blotted membrane of the gel was heterologously hybridised with the melon *CM-AAT1* probe (Figure 3.1B, 3.1C). The results showed that only one fragment hybridised strongly to the probe (Figure 3.1B, Lane 7). However, seven inserts, including 6 weakly hybridising bands, were isolated, cloned, and sequenced. The cDNA clone, hybridising most strongly to the *CM-AAT1* probe, was designated as *TOMM7* (Figure 3.1A, Lane7), whereas the rest were named *TOMM1U*, *TOMM1L* (Figure 2.1A, Lane 1), *TOMM4* (Figure 3.1A, Lane 4), *TOMM5* (Figure 3.1A, Lane 5), *TOMM9* (Figure 3.1A, Lane 9) and *TOMM10* (Figure 3.1A, Lane 10). A summary of the characteristics of the clones is presented in Table 3.1

3.1.1 *TOMM1U*

TOMM1U, corresponding to the upper of the bands in Figure 3.1A Lane 1, is an 866 nucleotide sequence including a poly A tail (Figure 3.2). From Southern analysis, *TOMM1U* showed very weak hybridization with the *CM-AAT1* probe (Figure 3.1B, 3.1C). It was subsequently confirmed by DNA sequence alignment that *TOMM1U* had no similarity with *CM-AAT1*. *TOMM1U* was 98% identical to TC99331, the tomato sequence from TIGR. As a result of NCBI BlastX alignment (DNA translated

Figure 3.1 Gel electrophoresis of the PCR amplification of selected tomato clones from second round screening (A) and Southern analysis with the 1.1 Kb *CM-AAT1* probe (B, C)

A) Amplified cDNA inserts from selected 10 phage from 2nd round tomato screening, separated by gel electrophoresis and stained with ethidium bromide.

B) Southern analysis of the PCR products from Figure 3.1 A with 3 h exposure.

C) Southern analysis of the PCR products from Figure 3.1 A with 14 h exposure.

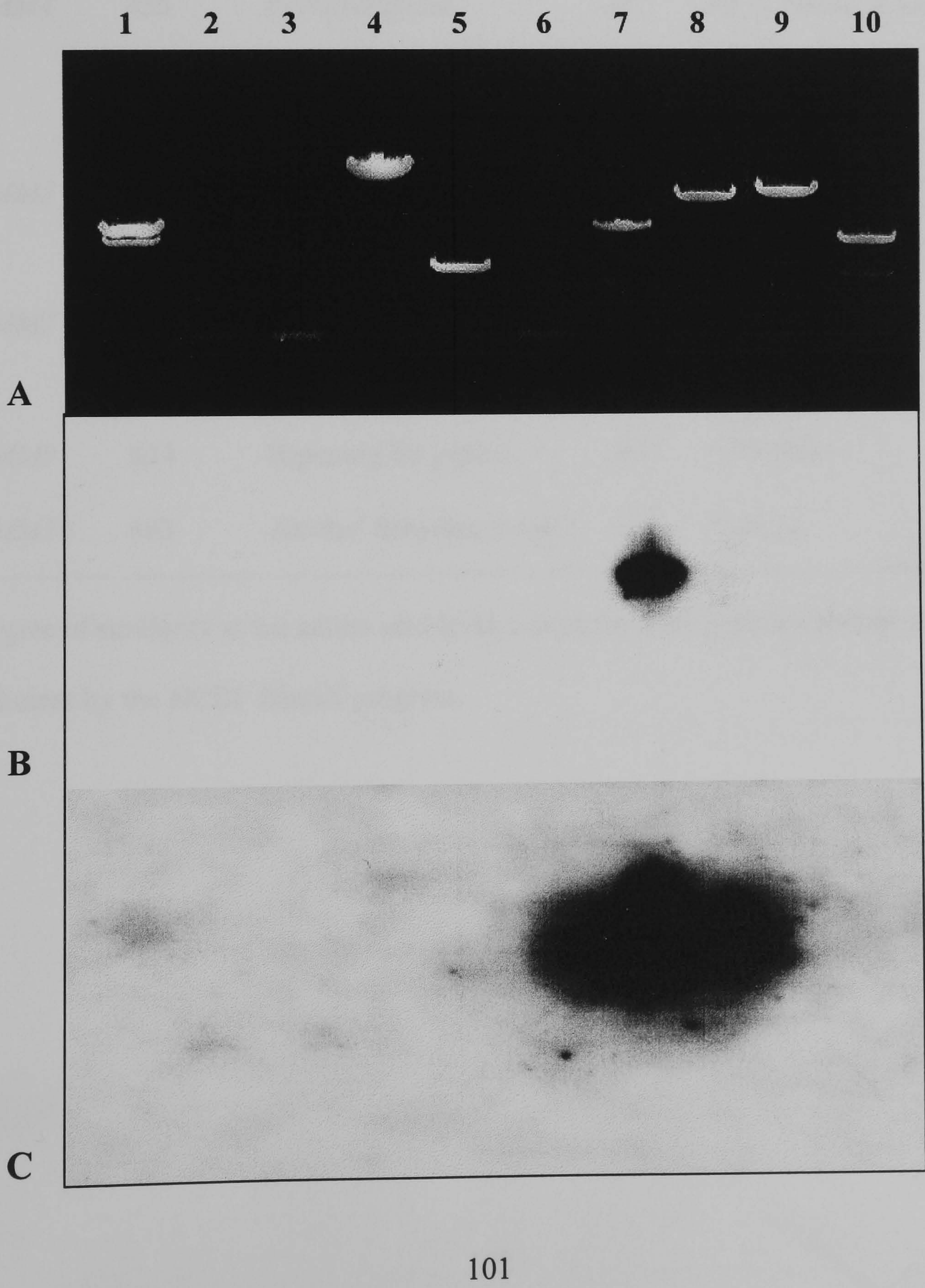


Table 3.1 Tomato cDNA clones initially identified by screening with the heterologous *CM-AAT1* probe.

cDNA clone	Sequenced size (bp)	Homology	P (N)[a]	Related sequence Accession No
TOMM1U	866	Serine/threonine protein phosphatase PP2A	$8e^{-74}$	CAB46506, AAD22116, NP_567066, BAA92699
TOMM1L	824	Pepper *Sn-1*, a vacuolar membrane protein	$3e^{-35}$	S65081
TOMM4	435	Pyrophosphate-fructose-6-phosphate-1-phosphotransferase	$2e^{-65}$	NP_179834, AAK98672
TOMM5	430	Actin protein	$8e^{-38}$	CAA39280, NP_187818, AAC31886, AAO38821
TOMM7	1328	Various acyl-transferases	$2e^{-95}$	AAN85436, AAN09798 AAN09796, CAA64636
TOMM9	634	Ripening E8 protein	$2e^{-39}$	AF004914
TOMM10	483	Alcohol dehydrogenase 2	$6e^{-41}$	P28032

[a] Degree of similarity at the amino acid level, calculated from genetic-database sequence alignment by the NCBI BlastX program.

Figure 3.2 Partial DNA sequence of the *TOMM1U* cDNA clone (866 bp) corresponding to the upper of two DNA bands from Lane 1 of Figure 3.1 A

The underlined sequences indicate the putative polyadenylated region.

```
  1  GCACGAGCTC GTGCCGTTGA AACTCTTGAT GATATACGCA ATTTTGACCG
 51  GGTCCAAGAA GTTCCACATG AGGGGGCCAT GTGTGATCTT TTATGGTCTG
101  ATCCTGATGA TCGTTGTGGT TGGGGCATCT CTCCAAGGGG TGCTGGATAT
151  ACATTTGGCC AGGATATCTC TGAGCAATTT AACCACACCA ACAACTTGAA
201  TCTAATTGCT AGAGCACACC AGCTGGTTAT GGAGGGATTC AATTGGGCCC
251  ATGATCAAAA GGTGGTTACC ATCTTTAGTG CACCTAATTA TTGTTACCGC
301  TGTGGTAATA TGGCTTCCAC CTTGGAAGTG GATGATGCCA AGGACCGTAC
351  ATTCATTCAG TTTGAACCAG CTCCCAGGAG AGGGGAGCCA GATGTAACCC
401  GAAGAACACC CGATTACTTC CTATAATGCA ATTGGGCAAA TCTATTGATT
451  TCTGTGCAGA AGGTCCTCCT AACTGCTTCA TATGTTGTGA GCCTCTTTCG
501  AGCTTATAGT TCTTTCTGCT ACAATGGTGT GAGCGGATGA TATGGAATGA
551  AGCATGTAAA TCAGTCATGT GATACTTTTT CTTTCCATTG TTGAAACCTC
601  ATTAAGATGC TGTGAAGAGA TGGTGAAACC AGTGACAGTT AGGGTGCATG
651  ATTCATTTTT CCGCGAACAT GTGTCAAATC TTTTTTTCCC TTAGATCCTA
701  TTCTTTTTTT TTTCTTCTGT TAGTGGGAGA AAGGACAATG ATCTGCTTCC
751  TCGATATGTT TTGTCCCTTT ACAAGGACAA TCACTATCGT CTAAGCTCCA
801  TAGAGGTAAA CGATAATCAT TTTTACACCT TTTAATGAGT GTTCGTGTAA
851  AAAAAAAAAA AAAAAA
```

protein sequence aligned to the protein database), the clone showed 94% homology to the serine/threonine protein phosphatase PP2A catalytic subunit (EC 3.1.3.16, Accession No CAB46506) from *Nicotiana tabacum*, and was also similar to many plant PP2A phosphatases, such as in *Oryza sativa* cv. Indica (Accession No AAD22116), *Arabidopsis thaliana* (Accession No NP_567066), *Vicia faba* (Accession No BAA92699), *Catharanthus roseus* (Accession No CAA07471.1) and *Medicago sativa* (Accession No CAA49849).

3.1.2 *TOMM1L*

TOMM1L, corresponding to the lower band of Lane 1 in Figure 3.1A, consisted of 824 nucleotides including a poly-A tail (Figure 3.3). There was no similarity with the *CM-AAT1* probe from sequence alignment. *TOMM1L* was 97% identical to TC105267, the tomato sequence from TIGR, whereas, at the amino acid level, it revealed 65% identity to the wound-induced protein pepper *Sn-1* (Accession No S65081), encoding a vacuolar membrane protein in *Capsium annum*, Yolo Wonder. Furthermore it was 39% similar to the Csf-2 protein from *Cucumis sativus* (Accession No BAA83470), a gene expressed during fruit growth in cucumber, and 36% to a major latex-like protein in *Prunus persica* (Accession No AF239177).

3.1.3 *TOMM4*

TOMM4 (Figure 3.1A, Lane 4) was sequenced and found to consist of 435 bp (Figure 3.4) showing 99% homology to the tomato TIGR sequence, TC104488. There was no homology with the probe sequence at the DNA sequence level. Alignment of the deduced amino acid sequence using the NCBI BlastX program showed that *TOMM4* was 80% identical to putative pyrophosphate-fructose-6-phosphate-1-phospho transferases

Figure 3.3 Partial DNA sequence of the *TOMM1L* cDNA clone (824 bp) corresponding to the lower of the two DNA bands from Lane 1 of Figure 3.1 A

The underlined sequences indicate the putative polyadenylated region. N is defined as an unclear nucleotide presented in sequencing.

```
  1  TCCCCNNGGG GNNNNCCNNN NNNNNGGGGT NAACCCTAAT TAAGGGGAAC
 51  AAAAGGTGGG GGGTTCCCCC CCGGGGGGCG GCCNGGTTTA GAATTAGTGG
101  AATCCCCCCG GTGCAGGGAA TTCGGCACGG AGCGGCAAGG AGAAAATATG
151  GGTGTGAAAG GCAAATTAAT TGGCTTTAGT GGAGGTAAAG TGTGGAGGAC
201  ACCCGATTCT TGATTTTTTT CACATCCATA CCCATCATAT ACCTAATATA
251  AGTCCTAATA TTATCAACCA TTTGGAGATA CACGCAGGTG AAGCTGTAAA
301  AGTTGGTTCG ATCGTTAGCT GCAATTATAA CGAAGCTGGA CAAAAGAAGT
351  CTGCTAAGCA AGTAATTGAA GCCATCGATC TTGACAAGAA ATCAATCACT
401  TGGAAAGTGA TTGAAGGAGA TGTGTTAGAG TCGTATAGTT CCTTTACTGG
451  TATCCTATCT TGTGAACACG AGTGGACAAC ATGGACAATT GAATACGAAA
501  AGAAAACTGA AGATATTCCA TAGCCTCTCA TTCAATTGGT CTTCTCCTTG
551  ACTTAACCAA GGATATAGAG CGTCACCTTT TCAAGAAATA AAGGATATAC
601  GAAAAGAAAT AAACGATATA TATCAATGTG TGTTTGTGTT TGTGTTTGTG
651  TGATGATAAT TTGCGATGTT GGCTAGCTCT AGCTAGCGTG TGGAATAAAG
701  AATATTGTGT GAAGAGTATA TTATAATATG TCTATCTCTT TCTACGAATG
751  TTATGTATTT TCAATGGTCA ATATATGTAA AATATCATGC ATTTTGTTAT
801  AATAAAAAAA AAAAAAAAAA AAAA
```

Figure 3.4 Partial DNA sequence of the *TOMM4* cDNA clone (435 bp) corresponding to the DNA band from Lane 4 of Figure 3.1 A

```
  1  GCACGAGCGG CACGAGCTAA CAACATCGAT TTCAACGATC CGGATTGGAA
 51  AGTGAAGTAT GAGAGAGAAT TCGAAGCTCG TTTCAATATT CCTCACATTA
101  CTGATCTATT TCCCTACGCC GTTTCGTATC CTTCCACTTT CTGTCTCAAA
151  ATGAGAACTC CAGTCACTGA GGATTTTGCA CAAGGATATC CTTCAGATGA
201  AGAATGGCAT GGATACATCA ATAACAATGA CAGAGTACTT CTTAAAACTA
251  TTAATTATTC CTCTCCGACT TCTGCTGGTG CTGAGTGCAT TGATCCCGAT
301  TGTACGTGGG TAGAGCAATG GGTTCATCGT GCTGGTCCTC GAGAGAAAAT
351  ATATTTCAAA CCAGAGGAGG TGAAAGCAGC AATCATAACC TGTGGTGGAC
401  TATGCCCAGG TCTTAATGAC GTTATTCGAC AGATA
```

from *Arabidopsis thaliana* (Accession No NP_179834) and *Oryza sativa* cv. Japonica (Accession No AAK98672).

3.1.4 *TOMM5*

The *TOMM5* (Figure 3.1A, Lane 5) clone was a 430 nucleotide sequence including a poly-A signal (Figure 3.5) showing no similarity to the *CM-AAT1* probe. The *TOMM5* fragment was identical to TC98620, the tomato TIGR sequence, which is described as a tomato actin gene. The translated sequence was also revealed to have a highly significant homology to numerous actin proteins such as potato actin (*Solanum tuberosum*, Accession No CAA39280), *Arabidopsis* actin (Accession No NP_187818), upland cotton actin (*Gossypium hirsutum*, Accession No AAC31886) and Japonica rice (*Oryza sativa*, Accession No AAO38821).

3.1.5 *TOMM7*

TOMM7 (Figure 3.1A, Lane 7) was the only clone, exhibiting high homology from the Southern hybridisation with the heterologous *CM-AAT1* probe (Figure 3.1B, 3.1C). However *TOMM7* as first isolated and sequenced was found to consist of only 819 nucleotides with a poly-A tail, corresponding to positions 510 to 1328 of the sequences in Figure 3.6. It was deduced to lack the 5' end of the sequence and a DNA primer was used to obtain the extension. The 509 nucleotide addition at the 5' end was subsequently derived from PCR amplification using the reverse primer inside the *TOMM7* clone, T7 and T3 inside the Phage arms as forward primers, and the tomato cDNA library as a template (see section 2.10.3.2). The complete sequence of the *TOMM7* clone from the cDNA library was finally established as 1328 nucleotides in length with the predicted ORF between positions 111 and 1169 (Figure 3.6)

Figure 3.5 Partial DNA sequence of the *TOMM5* cDNA clone (430 bp) corresponding to the DNA band from Lane 5 of Figure 3.1 A

The underlined sequences indicate the putative polyadenylated region.

```
  1  TCAGTGGTGG TACTACCATG TTCCCAGGTA TTGCTGATAG AATGAGCAAA
 51  GAAATTACTG CATTGGCTCC TAGCAGCATG AAGATTAAGG TGGTCGCTCC
101  ACCAGAGAGG AAATACAGTG TCTGGATTGG AGGCTCTATC TTGGCTTCCC
151  TCAGCACCTT CCAGCAGATG TGGATTGCAA AGGCAGAGTA TGACGAATCT
201  GGTCCCTCTA TTGTCCACAG GAAGTGCTTC TAATTTTTCC AAGATTGACA
251  ATGTTGGTGA AAGGAAAAGA CTTCTTATTT CCTACTGGAC CAGAGATGCA
301  ATTGTAGTGT TATATTCTGG CTTTATTTTC TGTATTTTTG TTCTCATGTT
351  GGATTGATGA TATTGAGAGG GCAAAGGAGT TAATTGTTGG GTTATGTTAA
401  TTCTTTTATT TCTAAAAAAA AAAAAAAAAA
```

Figure 3.6 Partial DNA sequence of the *TOMM7* cDNA clone (1328 bp). The sequence from 509 to 1328 (bold fonts) **corresponds to the DNA band from Lane 7 of Figure 3.1 A**

The underlined sequences indicate the putative polyadenylated region. *** is defined as a predicted initiation codon while **ooo** is predicted as a stop codon.

```
  1  TCAATGAAGG TAAAGATCTA GCAAAAATTA TCAAAGATGG ATTATCTAAA
 51  ACACTTGTGT TTTACTATCC ATTAGCTGGT AGACTCATTA AGGGCCTAA
                ***
101  TAAAAAGCTT ATGGTAAATT GCAATGGTGA AGGAGTCTTG TTTATCGAAG
151  GTGATGCTAA TATAGAGCTT GAAAAATTAG GTGAATCTAT TAAGCCACCA
201  TGTCCATACT TGGATTTACT ACTTCATAAT GTTCATGGTT CTGATGGAAT
251  TATTGGTTCT CCTCTTTTGT TAATTCAGGT GACTCGTTTT ACTTGTGGTG
301  GATTTGCTGT TGGATTTAGA TTTAATCACA CAATGATGGA TGCTTATGGC
351  TTCAAAATGT TTCTAAATGC GTTAAGTGAA TTAATTCAAG GAGCTTCAAC
401  ACCTTCTATA TTGCCTGTAT GGGAAAGACA TCTCCCAAGT GCTAGATCAT
451  CACCAAGTAT TACATGTATT CATCATGAGT TTGATGAGGA AATTGAATCA
501  AAAATTGCGT GGGAATCTAT GGAAGATAAG TTGATACAAC AATCATTTTT
551  CTTTGGAAAT GAGGAGATGG AAGTCATTAA AAATCAAGTT CCTCCAAATT
601  ATGAATGTAC AAAATTCGAG TTATTAATGG CATTTTTATG GAAATGTCGT
651  ACCATTGCTC TTAATTTGCA CTCTGATGAA ATTGTTCGTT TGACATACGT
701  TATTAATATA CGTGGAAAAA AGTCACTCAA CATTGAATTA CCAATTGGTT
751  ATTATGGGAA TGCGTTTATT ACTCCAGTTG TTGTATCAAA AGCAGGTTTG
801  TTATGTTCAA ATCCAGTGAC ATATGCAGTT GAATTGATCA AGAAAGTTAA
851  AGATCATATA AATGAAGAAT ACATCAAATC ATTGATAGAT TTAATGGTTA
901  CTAAAGGGAG ACCAGAGTTA ACAAAATCTT GGAATTTTTT GGTCTCAGAT
951  AATAGATATA TTGGATTTGA TGAATTTGAT TTTGGATGGG GAAACCCCAT
1001 TTTTGGAGGG ATCTTAAAGG CTATATCTTT CACTAGTTTT GGTGTTTCTG
1051 TTAAAAATGA CAAAGGAGAA AAAGGTGTTT TGATAGCTAT AAGTTTACCT
1101 CCATTGGCCA TGAAAAAACT TCAAGATATC TACAACATGA CTTTCAGAGT
                ooo
1151 CATAATTTCA AATATATAAG GCTTTTTCCT ATTTGAAAGT ACTTGTATTT
1201 TAGTATTTTG TTTCAATTTA TGTGACAGTG TTTGAAAAAA TAAACGACTT
1251 GTTTGTATCA ATAATGGTGA CTTGGTTTTA TGTATAAATC AACTGAATTC
1301 CATTTACAAT TTAAAAAAAA AAAAAAAA
```

TOMM7 was highly identical to TC98820, the tomato TIGR sequence, and *pTOM36* (Accession No A16155), previously cloned in this laboratory (Davies and Grierson, 1989). The TC98820 sequence is combined from the EST (Expressed Sequence Tag) sequences, derived only from ripe tomato tissues (information from TIGR website). TC98820 is 1,918 bp, predicted to encode 442 aa with the ORF from positions 473 to 1801 while *TOM36* is 1,080 bp with a predicted protein of 273 aa from positions 90 to 905 (Figure 3.7). There are some differences in the DNA sequences near the 3' end of *TOM36* compared to TC98820 and *TOMM7* (Figure 3.7). At the amino acid level, the TOMM7-predicted protein was identical to the TC98820-predicted protein, but 90-amino acid shorter, with only one position difference at the position 109 of TOMM7 (Figure 3.8). The TOM36-encoded protein was also highly similar to the TOMM7 and TC98820 proteins, but there are a number of sequence differences near the C-terminal end of the *TOM36* protein sequences from positions 242 to 271 (Figure 3.8).

Regarding the NCBI BlastX alignment, the TC98220-predicted protein showed some similarities to various plant acyl-transferases. It showed 80% identity to the acyl-transferase 2 of *Capsicum chinense* (Accession No AAN85436), a placental specific acyl-transferase gene from habanero chile. The deduced protein was furthermore 65% similar to the benzoyl coenzyme A: benzyl alcohol benzoyl-transferases (BEBT) from *Nicotiana tabacum* (Accession No AAN09798) and from *Clarkia breweri* (Accession No AAN09796), the hsr201 protein, hypersensitivity-related gene from *Nicotiana tabacum* (Accession No CAA64636).

From sequence analysis, the TC98820 predicted protein was revealed to have high homology, including some conserved regions, with AATs, namely melon AAT (CMAAT1), apple AAT (MaAAT), rice BEBT (OrBEBT), *Clarkia* BEAT, and strawberry AAT (Figure 3.9).

Figure 3.7 Multiple DNA sequence alignment of *TOMM7* with *TOM36* and TC98820

TOM36 (1080 bp) the tomato clone from our Laboratory, Accession No A16155

TOMM7 (1328 bp) the clone from our tomato library screening showing homology to *CM-AAT1*

TC98820 (1918 bp) the clone from tomato TIGR database

xxx - predicted start codon of *TOM36* clone

+++ - predicted start codon of *TOMM7* clone

*** - predicted start codon of TC98820 clone

@@@ - predicted stop codon (TAA) of *TOM36* clone

ooo - predicted stop codon (TAA) of TC98820 and *TOMM7* clones

```
TC98820     1 AAACCATCTAGGGATTCGTACTAAATTTCAGGTGCCCTAAACGGCTCGTTTAGGGTTTCG
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820    61 TGAAGGGTCCCTGGCCTGTTAACTCACTCTAGTCTCTCTCGAAGTCCTAGGTGTTACAAT
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   121 GACATAGCATGAAACTTTAAAATTTAGCAAATGTTGAGCAAAAAAAAGAGTATTGAAGCA
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   181 AAACTTTAAAATTTTCAACCTATAAGATGAGAAATCTAAGCCTATAAAGAATAAAGCTAA
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   241 AATGGTCAATTCATGAATGCATAGTTTGATAGTGAAAGATATATATATATAAGTTAATTT
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   301 TTAATTTTTTGTATCTCCCATCTTTTAATACCTCATAAAATCAACATCAATAAAAATTTG
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   361 TAAAAAATTTGAAGGTTGATATTTATTTGATACATTGGCCCTCTATAAATCTAATTCACA
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

                                                                      ***
TC98820   421 ATACAAAATTGTATTACTCTCATCATACACTACTCTTTGTTGTATATCACATATGGCAAA
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------
```

Figure 3.7 Multiple DNA sequence alignment of *TOMM7* with *TOM36* and TC98820 (Continued)

```
TC98820   481 TATTCTACCAATTTCAATAAATTACCACAAGCCAAAACTAGTAGTTCCATCAAGTGTAAC
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   541 ATCTCATGAGACAAAACGTCTTTCTGAAATAGATGATCAAGGGTTTATTCGACTCCAAAT
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98820   601 CCCCATACTAATGTTTTACAAATACAATTCTTCAATGAAAGGTAAAGATCTAGCAAAAAT
TOMM7       1 ------------------------------TCAATGAA-GGTAAAGATCTAGCAAAAAT
TOM36       1 ------------------------------------------------------------

TC98820   661 TATCAAAGATGGATTATCTAAAACACTTGTGTTTTACTATCCATTAGCTGGTAGACTCAT
TOMM7      29 TATCAAAGATGGATTATCTAAAACACTTGTGTTTTACTATCCATTAGCTGGTAGACTCAT
TOM36       1 ------------------------------------------------------------

                                      +++
TC98820   721 TGAAGGGCCTAATAAAAAGCTTATGGTAAATTGCAATGGTGAAGGAGTCTTGTTTATCGA
TOMM7      89 TAAAGGGCCTAATAAAAAGCTTATGGTAAATTGCAATGGTGAAGGAGTCTTGTTTATCGA
TOM36       1 ----------------------ATGGTAAATTGCAATGGTGAAGGAGTCTTGTTTATCGA

                                                                   XXX
TC98820   781 AGGTGATGCTAATATAGAGCTTGAAAAATTAGGTGAATCTATTAAGCCACCATGTCCATA
TOMM7     149 AGGTGATGCTAATATAGAGCTTGAAAAATTAGGTGAATCTATTAAGCCACCATGTCCATA
TOM36      39 AGGTGATGCTAATATAGAGCTTGAAAAATTAGGTGAATCTATTAAGCCACCATGTC-ATA

TC98820   841 CTTGGATTTACTACTTCATAATGTTCATGGTTCTGATGGAATTATTGGTTCTCCTCTTTT
TOMM7     209 CTTGGATTTACTACTTCATAATGTTCATGGTTCTGATGGAATTATTGGTTCTCCTCTTTT
TOM36      98 CTTGGATTTACTACTTCATAATGTTCATGGTTCTGATGGAATTATTGGTTCTCCTCTTTT

TC98820   901 GTTAATTCAGGTGACTCGTTTTACTTGTGGTGGATTTGCTGTTGGATTTAGATTTAATCA
TOMM7     269 GTTAATTCAGGTGACTCGTTTTACTTGTGGTGGATTTGCTGTTGGATTTAGATTTAATCA
TOM36     158 GTTAATTCAGGTGACTCGTTTTACTTGTGGTGGATTTGCTGTTGGATTTAGATTTAATCA

TC98820   961 CACAATGATGGATGCTTATGGCTTCAAAATGTTTCTAAATGCGTTAAGTGAATTAATTCA
TOMM7     329 CACAATGATGGATGCTTATGGCTTCAAAATGTTTCTAAATGCGTTAAGTGAATTAATTCA
TOM36     218 CACAATGATGGATGCTTATGGCTTCAAAATGTTTCTAAATGCGTTAAGTGAATTAATTCA

TC98820  1021 AGGAGCTTCAACACCTTCTATATTGCCTGTATGGGAAAGACATCTCCTAAGTGCTAGATC
TOMM7     389 AGGAGCTTCAACACCTTCTATATTGCCTGTATGGGAAAGACATCTCCCAAGTGCTAGATC
TOM36     278 AGGAGCTTCAACACCTTCTATATTGCCTGTATGGGAAAGACATCTCCTAAGTGCTAGATC

TC98820  1081 ATCACCAAGTATTACATGTATTCATCATGAGTTTGATGAGGAAATTGAATCAAAAATTGC
TOMM7     449 ATCACCAAGTATTACATGTATTCATCATGAGTTTGATGAGGAAATTGAATCAAAAATTGC
TOM36     338 ATCACCAAGTATTACATGTATTCATCATGAGTTTGATGAGGAAATTGAATCAAAAATTGC

TC98820  1141 GTGGGAATCTATGGAAGATAAGTTGATACAACAATCATTTTTCTTTGGAAATGAGGAGAT
TOMM7     509 GTGGGAATCTATGGAAGATAAGTTGATACAACAATCATTTTTCTTTGGAAATGAGGAGAT
TOM36     398 GTGGGAATCTATGGAAGATAAGTTGATACAACAATCATTTTTCTTTGGAAATGAGGAGAT

TC98820  1201 GGAAGTCATTAAAAATCAAGTTCCTCCAAATTATGAATGTACAAAATTCGAGTTATTAAT
TOMM7     569 GGAAGTCATTAAAAATCAAGTTCCTCCAAATTATGAATGTACAAAATTCGAGTTATTAAT
TOM36     458 GGAAGTCATTAAAAATCAAGTTCCTCCAAATTATGAATGTACAAAATTCGAGTTATTAAT

TC98820  1261 GGCATTTTTATGGAAATGTCGTACCATTGCTCTTAATTTGCACTCTGATGAAATTGTTCG
TOMM7     629 GGCATTTTTATGGAAATGTCGTACCATTGCTCTTAATTTGCACTCTGATGAAATTGTTCG
TOM36     518 GGCATTTTTATGGAAATGTCGTACCATTGCTCTTAATTTGCACTCTGATGAAATTGTTCG
```

Figure 3.7 Multiple DNA sequence alignment of *TOMM7* with *TOM36* and TC98820 (Continued)

```
TC98820 1321 TTTGACATACGTTATTAATATACGTGGAAAAAAGTCACTCAACATTGAATTACCAATTGG
TOMM7    689 TTTGACATACGTTATTAATATACGTGGAAAAAAGTCACTCAACATTGAATTACCAATTGG
TOM36    578 TTTGACATACGTTATTAATATACGTGGAAAAAAGTCACTCAACATTGAATTACCAATTGG

TC98820 1381 TTATTATGGGAATGCGTTTATTACTCCAGTTGTTGTATCAAAAGCAGGTTTGTTATGTTC
TOMM7    749 TTATTATGGGAATGCGTTTATTACTCCAGTTGTTGTATCAAAAGCAGGTTTGTTATGTTC
TOM36    638 TTATTATGGGAATGCGTTTATTACTCCAGTTGTTGTATCAAAAGCAGGTTTGTTATGTTC

TC98820 1441 AAATCCAGTGACATATGCAGTTGAATTGATCAAGAAAGTTAAAGATCATATAAATGAAGA
TOMM7    809 AAATCCAGTGACATATGCAGTTGAATTGATCAAGAAAGTTAAAGATCATATAAATGAAGA
TOM36    698 AAATCCAGTGACATATGCAGTTGAATTGATCAAGAAAGTTAAAGATCATATAAATGAAGA

TC98820 1501 ATACATCAAATCATTGATAGATTTAATGGTTACTAAAGGGAGACCAGAGTTAACAAAATC
TOMM7    869 ATACATCAAATCATTGATAGATTTAATGGTTACTAAAGGGAGACCAGAGTTAACAAAATC
TOM36    758 ATACATCAAATCATTGATAGATTTAATGGTTACTAAAGGGAGACCAGAGTTAACAAA-TC

TC98820 1561 TTGGAATTTTTTGGTCTCAGATAATAGATATATTGGATTTGATGAATTTGATTTTGGATG
TOMM7    929 TTGGAATTTTTTGGTCTCAGATAATAGATATATTGGATTTGATGAATTTGATTTTGGATG
TOM36    817 TTGGAATTTTTTGGTCTCAGATAATAGATATATTGGATTTGATGAATTTGATTTTGGATG
                                                                @@@
TC98820 1621 GGGAAACCCCATTTTTGGAGGGATCTTAAAGGCTATATCTTTCACTAGTTTTGGTGTTTC
TOMM7    989 GGGAAACCCCATTTTTGGAGGGATCTTAAAGGCTATATCTTTCACTAGTTTTGGTGTTTC
TOM36    877 GGGAAACCCCATTTTTGGAGGGATCTTAAAGGCTATATCTTTCACTAGTTTTGGTGTTTC

TC98820 1681 TGTTAAAAATGACAAAGGAGAAAAAGGTGTTTTGATAGCTATAAGTTTACCTCCATTGGC
TOMM7   1049 TGTTAAAAATGACAAAGGAGAAAAAGGTGTTTTGATAGCTATAAGTTTACCTCCATTGGC
TOM36    937 TGTTAAAAATGACAAAGGAGAAAAAGGTGTTTTGATAGCTATAAGTTTACCTCCATTGGC
                                                                  OO
TC98820 1741 CATGAAAAAACTTCAAGATATCTACAACATGACTTTCAGAGTCATAATTTCAAATATATA
TOMM7   1109 CATGAAAAAACTTCAAGATATCTACAACATGACTTTCAGAGTCATAATTTCAAATATATA
TOM36    997 CATGAAAAAACTTCAAGATATCTACAACATGACTTTCAGAGTCATAATTTCAAATATATA
               O
TC98820 1801 AGGCTTTTTCCTATTTGAAAGTACTTGTATTTTAGTATTTTGTTTCAATTTATGTGACAG
TOMM7   1169 AGGCTTTTTCCTATTTGAAAGTACTTGTATTTTAGTATTTTGTTTCAATTTATGTGACAG
TOM36   1057 -GGCTTTT--CTATTGAAAAAAAAAAA----------------------------------

TC98820 1861 TGTTTGAAAAAATAAACGACTTGTTTGTATCAATAATGGTGACTTGGTTTTATGTATA--
TOMM7   1229 TGTTTGAAAAAATAAACGACTTGTTTGTATCAATAATGGTGACTTGGTTTTATGTATAAA
TOM36        ------------------------------------------------------

TC98820      ----------------------------------------
TOMM7   1289 TCAACTGAATTCCATTTACAATTTAAAAAAAAAAAAAAAA
TOM36        ----------------------------------------
```

Figure 3.8 Multiple amino acid sequence alignment of *TOMM7* with TC98820 and *TOM36*

TC98820 is predicted to encode a 442 aa protein.

TOMM7 is predicted to encode a 352 aa protein

TOM36 is predicted to encode a 273 aa protein.

Black and gray boxes indicate identical and similar amino acid residues, respectively.

```
TC98220     1 MANILPISINYHKPKLVVPSSVTSHETKRLSEIDDQGFIRLQIPILMFYKYNSSMKGKDL
TOMM7       1 ------------------------------------------------------------
TOM36       1 ------------------------------------------------------------

TC98220    61 AKIIKDGLSKTLVFYYPLAGRLIEGPNKKLMVNCNGEGVLFIEGDANIELEKLGESIKPP
TOMM7       1 ----------------------------MVNCNGEGVLFIEGDANIELEKLGESIKPP
TOM36       1 ------------------------------------------------------------

TC98220   121 CPYLDLLLHNVHGSDGIIGSPLLLIQVTRFTCGGFAVGFRFNHTMMDAYGFKMFLNALSE
TOMM7      31 CPYLDLLLHNVHGSDGIIGSPLLLIQVTRFTCGGFAVGFRFNHTMMDAYGFKMFLNALSE
TOM36       1 MSYLDLLLHNVHGSDGIIGSPLLLIQVTRFTCGGFAVGFRFNHTMMDAYGFKMFLNALSE

TC98220   181 LIQGASTPSILPVWERHLLSARSSPSITCIHHEFDEEIESKIAWESMEDKLIQQSFFFGN
TOMM7      91 LIQGASTPSILPVWERHLPSARSSPSITCIHHEFDEEIESKIAWESMEDKLIQQSFFFGN
TOM36      61 LIQGASTPSILPVWERHLLSARSSPSITCIHHEFDEEIESKIAWESMEDKLIQQSFFFGN

TC98220   241 EEMEVIKNQVPPNYECTKFELLMAFLWKCRTIALNLHSDEIVRLTYVINIRGKKSLNIEL
TOMM7     151 EEMEVIKNQVPPNYECTKFELLMAFLWKCRTIALNLHSDEIVRLTYVINIRGKKSLNIEL
TOM36     121 EEMEVIKNQVPPNYECTKFELLMAFLWKCRTIALNLHSDEIVRLTYVINIRGKKSLNIEL

TC98220   301 PIGYYGNAFITPVVVSKAGLLCSNPVTYAVELIKKVKDHINEEYIKSLIDLMVTKGRPEL
TOMM7     211 PIGYYGNAFITPVVVSKAGLLCSNPVTYAVELIKKVKDHINEEYIKSLIDLMVTKGRPEL
TOM36     181 PIGYYGNAFITPVVVSKAGLLCSNPVTYAVELIKKVKDHINEEYIKSLIDLMVTKGRPEL

TC98220   361 TKSWNFLVSDNRYIGFDEFDFGWGNPIFGGILKAISFTSFGVSVKNDKGEKGVLIAISLP
TOMM7     271 TKSWNFLVSDNRYIGFDEFDFGWGNPIFGGILKAISFTSFGVSVKNDKGEKGVLIAISLP
TOM36     241 TNLGIFWSQIIDILDLMNLILDGETPFLEGS----------------------------

TC98220   421 PLAMKKLQDIYNMTFRVIISNI
TOMM7     331 PLAMKKLQDIYNMTFRVIISNI
TOM36         ----------------------
```

```
TC98220     1 ------------------MANILPISINYHKPKLVVPSSVTS-HETKRLSEIDDQGFIRL
CMAAT1      1 MGSGSGDDDDKLALMETMQTIDFSFHVRKCQPELIAPANPTP-YEFKQLSDVDDQQSLRL
MaAAT       1 ------------------MSFSVLQVKRLQPELITPAKSTP-QETKFLSDIDDQESLRV
OrBEBT      1 --------------------MALSFAVRRRATELVAPAAPTP-RETKRLSDVDDPESLRW
BEAT        1 ------------------------MNVTMHSKKLLKPSIPTPNHLQKLNLSLLDQIQIPF
SAAT        1 --------------------MEKIEVSINSKHTIKPSTSST-PLQPYKLTLLDQLTPPA

TC98220    42 QIPILMFYKYN-SSMKGKDLAKIIKDGLSKTLVFYYPLAGRLIEGPNKKLMVNCNGEGVL
CMAAT1     60 QLPFVNIYPHN-PSLEGRDPVKVIKEAIGKALVFYYPLAGRLREGPGRKLFVECTGEGIL
MaAAT      41 QIPIIMCYKDNPSLNKNRNPVKAIREALSRALVYYYPLAGRLREGPNRKLVVDCNGEGIL
OrBEBT     40 QVPVVFVYRPS---AAAADPVDTIRRALAAALVPYYPFAGRLREVEGRKLVVDCTGEGVM
BEAT       37 YVGLIFHYETLS--DNSDITLSKLESSLSETLTLYYHVAGRYN---GTDCVIECNDQGIG
SAAT       39 YVPIVFFYPITDHDFNLPQTLADLRQALSETLTLYYPLSGRVKN---N-LYIDDFEEGVP

TC98220   101 FIEGDANIELEKLGESIKPPCP-YLDLLLHNVHGSDGIIGSPLLLIQVTRFTCGGFAVGF
CMAAT1    119 FIEADADVSLEEFWDTLPYSLSSMQNNIIHNALNSDEVLNSPLLLIQVTRLKCGGFIFGL
MaAAT     101 FVEASADVTLEQLGDKILPPCP-LLEEFLYNFPGSDGIIDCPLLLIQVTCLTCGGFILAL
BEBT       97 FVEADADVRVVELEAAGLRAPFPCMDQLLFDVDGSAAVLGTPLLLIQVTRLLCGGFVLGI
BEAT       92 YVETAFDVELHQFLLGEESNNL---DLLVGLSGFLSETETPPLAAIQLNMFKCGGLVIGA
SAAT       95 YLEARVNCDMTDFLRLRKIECLNEFVPIKPFSMEAISDERYPLLGVQVNVFDSG-IAIGV

                *****                                         *****
TC98220   160 RFNHTMMDAYGFKMFLNALSELIQGASTPSILPVWERHLLSARSSPSITCIHHEFDEEIE
CMAAT1    179 CFNHTMADGFGIVQFMKATAEIARGAFAPSILPVWQRALLTARDPPRITFRHYEYDQVVD
MaAAT     160 RLNHTMCDAAGLLLFLTAIAEMARGAHAPSILPVWERELLFARDPPRITCARHEYEDVIG
OrBEBT    157 RLNHAMCDASGIVQFMDAVADLARGAREPAVSPAWSRELLDARKPPKLAFHLREYNDFAA
BEAT      149 QFNHIIGDMFTMSTFMNSWAKACRVGIKEVAHPTFGLAPLMPSAKVLNIPPPPSFEGVKF
SAAT      154 SVSHKLIDGGTADCFLKSWGAVFRGCRENIIHPSLSEAALLFP--PRDDLPEKYVDQMEA

TC98220   220 S--KIAWESMEDKLIQQSFFFGNEEMEVIKNQVPPN--YECTKFELLMAFLWKCRTIA-L
CMAAT1    239 MK---SGLIPVNSKIDQLFFFSQLQISTLRQTLPAH-LHDCPSFEVLTAYVWRLRTIA-L
MaAAT     220 HSDGSYASSNQSNMVQRSFYFGAKEMRVLRKQIPPHLISTCSTFDLITACLWKCRTLA-L
OrBEBT    217 APPAAPSVGALGDMVMRTFSFSPGDVAALKGALPPHLRGRATSFDVLASFVWRARARA-L
BEAT      209 VSKRFVFNENAITRLRKEATEEDGDGDDDQKKKRPSRVDLVTAFLSKSLIEMDCAKKE-Q
SAAT      212 LWFAGKKVATRRFVFGVKAISSIQDEAKSESVPKPSRVHAVTGFLWKHLIAASRALTSGT

TC98220   275 NLHSDEIVRLTYVINIRGKKSLNIELPIGYYGNAFITPVVVSKAGLLCSN-PVTYAVELI
CMAAT1    294 QFKPEEEVRFLCVMNLRS---K-IDIPLGYYGNAVVVPAVITTAAKLCGN-PLGYAVDLI
MaAAT     279 NINPKEAVRVSCIVNARG-KHNNVRLPLGYYGNAFAFPAAISKAEPLCKN-PLGYALELV
OrBEBT    276 ETPAGEDARLAIIVSFRN--NGELRLPRGYYGNVCVPVTVAMPAEALRRRGSLADVVEQV
BEAT      268 TKSRPSLMVHMMNLRKRTKLALENDVSGNFFIVVNAESKITVAPKITDLTESLGSACGEI
SAAT      272 TSTRLSIAAQAVNLRTRMNMETVLDNATGNLFWWAQAILELSHTTPEISDLKLCDLVNLL

                                                            ++++
TC98220   334 KKVKDHINEEYIKSLIDLMVTKGRPELTKSWNFLVS----------DNRYIGFDEFDFGW
CMAAT1    349 RKAKAKATMEYIKSTVDLMVIKGRPYFTVVGSFMMS----------DLTRIGVENVDFGW
MaAAT     337 KKAKATMNEEYLRSVADLLVLRGRPQYSSTGSYLIVS---------DNTRVGFGDVNFGW
OrBEBT    334 REAKKTVNAEYVRSVADTLVMRGRPAIDTANLLLLS----------DVRLAGFHRVDFGW
BEAT      328 ISEVAKVDDAEVVSSMVLNSVREFYYEWGKGEKNVFLY-------TSWCRFPLYEVDFGW
SAAT      332 NGSVKQCNGDYFETFKGKEGYGRMCEYLDFQRTMSSMEPAPDIYLFSSWTNFFNPLDFGW

                +
TC98220   384 GNPIFGGILKAIS-----FTSFGVSVKNDKGEKGVLIAISLPPLAMKKLQDIYNMTFRVI
CMAAT1    399 GKAIFGGPTTTGARITRGLVSFCVPFMNRNGEKGTALSLCLPPPAMERFRANVHASLQVK
MaAAT     388 GQPVFAGPVKALD-----LISFYVQHKNN-TEDGILVPMCLPSSAMERFQQELERITQEP
OrBEBT    384 GEPVYGGPSHAWYG----VSYLIAVKNGA-GEDGVAVPVVLPAAAMERFTSEIERLRKGQ
BEAT      381 GIPSLVDTTAVPFG----LIVLMDEAPAG---DGIAVRACLSEHDMIQFQQHHQLLSYVS
SAAT      392 GRTSWIGVAGKIES---ASCKFIILVPTQCG-SGIEAWVNLEEEKMAMLEQDPHFLALAS

TC98220   439 ISNI--------------
CMAAT1    459 QVVDAVDSHMQTIQSASK
MaAAT     442 KEDTCNNLRSTSQ-----
OrBEBT    439 QRGHFRVQTTSRI-----
BEAT      434 ------------------
SAAT      448 PKTLI-------------
```

The TC98820 protein contained two highly conserved consensus motifs, 'H-x-x-x-D' (positions 163 to 167 and 211-215) and 'DFGWG' (positions 380 to 384), found in many plant *O*-acyl-transferases (St-Pierre *et al.*, 1998). As a result of the mRNA size (see section 3.3.1.2) and sequence homology, **TC98820** was subsequently chosen, cloned, and renamed ***Le-AAT1*** for further study of AAT activity.

3.1.6 *TOMM9*

TOMM9 (Figure 3.1A, Lane 9) was sequenced and shown to consist of 634 bp including a poly-A region (Figure 3.10), unveiling no similarity with the *CM-AAT1* probe. At the DNA level, it was 98% identical to tomato TIGR TC98707, which was isolated as a fruit EST expressed during ripening. The TC98707 sequence is identical to the tomato ripening cDNA E8 (Accession No AF004914). The *TOMM9*-predicted protein was 69% similar to the tomato ACC oxidase (Accession No CAA31789), 58% to the melon E8-like gene (*CM-E8*, Accession No BAB68392), 61% to a putative oxidoreductase of *Arabidopsis thaliana* (Accession No AAN13044), and 60% to a putative *Arabidopsis* ACC oxidase (Accession NP_171933).

3.1.7 *TOMM10*

The *TOMM10* clone (Figure 3.1A, Lane 10) was sequenced and shown to be 483 bp (Figure 3.11), with no similarity to the *CM-AAT1* probe when aligned. The *TOMM10* DNA sequence was 99% identical to TC98767, the TIGR tomato sequence, which is highly homologous to the tomato alcohol dehydrogenase 2 (EC 1.1.1.1, Accession No P28032). From the NCBI BloatX alignment, the predicted *TOMM10* protein was 93% identical to the potato alcohol dehydrogenase 2 (Accession No P14674) and an alcohol dehydrogenase 3 (Accession No AAA33808), and was 86% and 81% identical to alcohol dehydrogenases from lettuce (Accession No BAA07911) and strawberry (Accession No CAA33613), respectively.

Figure 3.10 Partial DNA sequences of the *TOMM9* cDNA clone (634 bp) corresponding to the DNA band from Lane 9 of Figure 3.1 A

The underlined sequences indicate the putative polyadenylated region. N is defined as an unclear nucleotide presented in sequencing.

```
  1  NNNGCTCACA GATAATCGNG ACTCNAGTTC TTCNNANNAT CNATGTNTGA
 51  TNTCCTCNTA TACGTGGTGC TCTNGTCGTC NATNNNGAGA NTTTCTGCAG
101  CTCATGTCNA ATGANCANGT ACATGAGTGT TGAGCACAGA GCNATTGCNA
151  AGAAAGATGG ATCNAGNATG TCAGTTGCCT GCTTCTTTGG TGAAAATCCA
201  TTGCNATCTT CAAAGCTGTA TGAACCAATC ACTGAATTGT TATCAGAAGA
251  TAATCCTCCA AAATATCACA CAACCACAGT GATTGACTAC AAAAATTATG
301  TCCTTAATAA AGGCCTAGAT GGAACTTCTG CGCTGTTGAG TTACAAGATC
351  TAATATAATA ATGTACCAGT AAGTGGTGTT ACACCTCTTC ATCAAAATAA
401  GGAGTGTTCT TATCTTTTTT TCAACGTATT TATCAAGGAT AAGTTAGTGA
451  AAATACATCT TAATATCTAA GAAGTAGTTT TCTTAAAGGG TATTGCCCAA
501  AATAAAAACA TCACTTGTTT GGAAATGAAA GAAGTATCTA TAATTTAGTT
551  CGTTGAGTGT GAGTGTAACT ACTACTGCTA TTGAATAACG AAAACTAGTT
601  TGTGCATTTC ATACTTAAAA AAAAAAAAAA AAAA
```

Figure 3.11 Partial DNA sequences of the *TOMM10* cDNA clone (486 bp) corresponding to the DNA band from Lane 10 of Figure 3.1 A

N is defined as an unclear nucleotide presented in sequencing.

```
  1  GCACGAGAAA TCACTGAAAT TCTGTAAATA TACTTGAGAT AAAACAGAGA

 51  GAATTTGCAT TTTTTACAAG AATCGAAAGA GATATGGCAA AGCACAACAT

101  CTAAGCAAAA GACAAAAGAA GAGACATGGA TGGAATGTTA CAACTCAACC

151  AAACACGAAA GGGAAAATAA GACAGTGACA ATTTATTGGT CTTTTTTCTC

201  AAACAGTAGA AGCTCCTTTT CTAGGACAGA AAGTTTAGTC CGCCATGGTG

251  ATGATGCAAC GAAGGCCTTC TCCCTTCAGC ATTAAATCGA AAGCCTTATT

301  GATTTCAGCA AATGGAAGTG TATGAGTGAT GAATTTCTCC AATTCAAGTT

351  CTTTGTTCAT GTATTTCTCA ACAACACAAG GAATATCCGA ACGAGGTTTG

401  TAGTTTCCAA AGAAGGTTCC TTTGAGAGTC CGTTCATTCA AAAAGTTCAG

451  ANGATGTGTC NNGAACACAG CTTCTTTATG GGG
```

3.2 Screening of an Avocado Fruit cDNA Library

In the first attention, apart from screening of the tomato library we would like to screen on a mango fruit cDNA library for CM-AAT1 homologues, but unfortunately the library was lost from the laboratory. While an avocado fruit library is available, we therefore tried to screen on this library instead. Twelve selected clones from screening of the avocado library were amplified by PCR and were fractionated by gel electrophoresis (Figure 3.12A). There was no strong hybridisation with the *CM-AAT1* probe following Southern analysis (Figure 3.12 B, C). However, three putative clones were isolated and sequenced. *AVOM1* was designated corresponding to the band in Lane 1 of Figure 3.12A, whereas *AVOM2* and *AVOM8* were the bands in Lane 2 and Lane 8, respectively. None of the sequenced clones had any similarity with the *CM-AAT1* sequence when aligned. The summary of the features of these avocado clones is shown in Table 3.2.

Table 3.2 Avocado cDNA clones screened by heterologous *CM-AAT1* probe

cDNA clone	Sequenced size (bp)	Homology	P (N)[a]	Related sequence Accession No
AVOM1	615	None		
AVOM2	651	*Arabidopsis* cyclin 2b protein	$5.7e^{-06}$	Z31401
AVOM8	348	None		

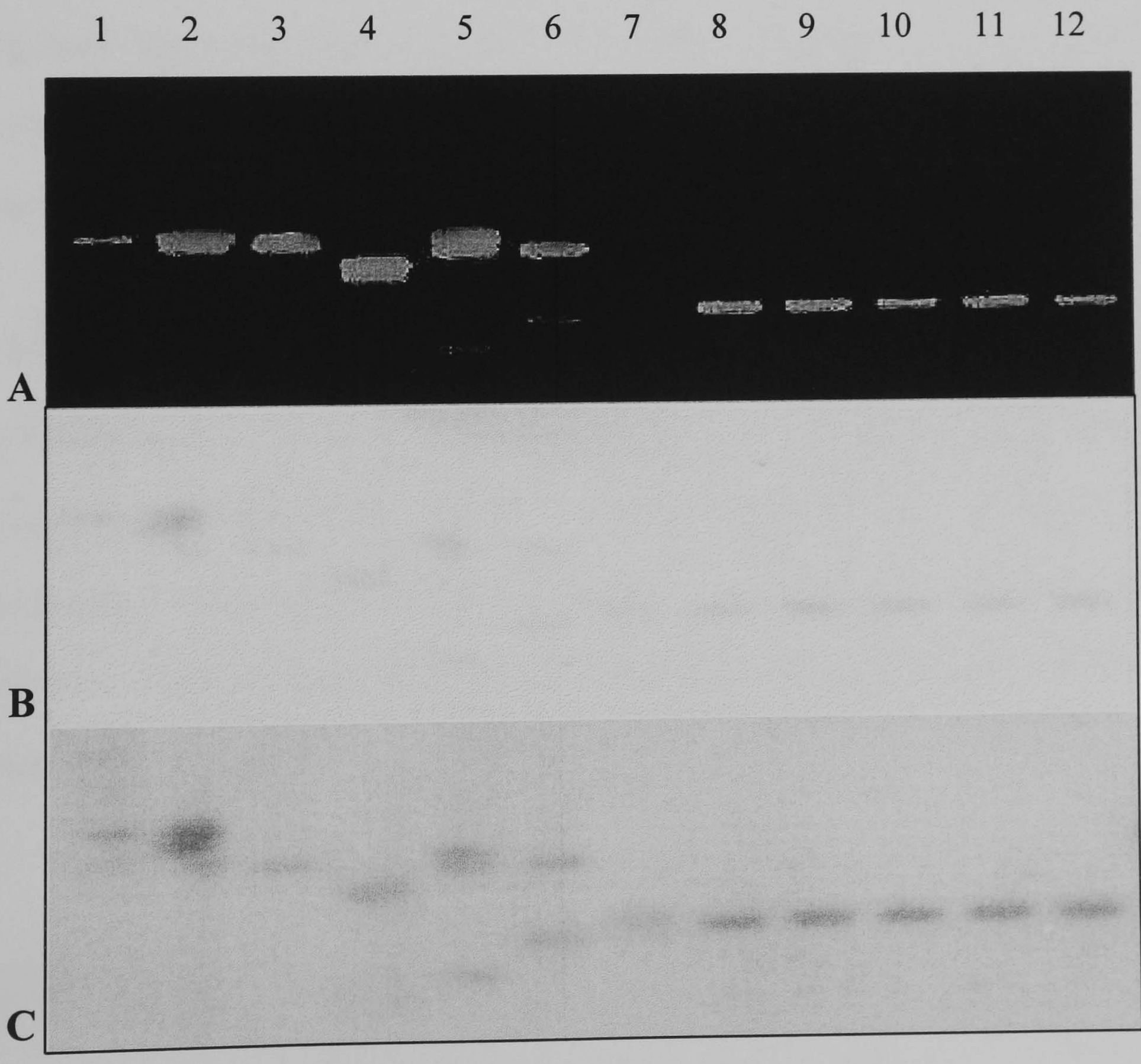
[a] Degree of similarity at the amino acid level, calculated from genetic-database sequence alignment by the NCBI BlastX program.

Figure 3.12 Gel electrophoresis of the PCR amplification products of selected avocado clones from second round screening (A) and Southern analysis with the 1.1 Kb *CM-AAT1* probe (B, C)

A) Amplified cDNA inserts from 12 selected phage from 2nd round avocado library screening with the *CM-AAT1* probe, separated by gel electrophoresis and stained with ethidium bromide.

B) Southern analysis of the PCR products from Figure 3.12 A, using the CM-AAT1 probe with 5 h exposure.

C) Southern analysis of the PCR products from Figure 3.12 A, using the *CM-AAT1* probe with 14 h exposure.



3.2.1 *AVOM1*

AVOM1 (Figure 3.12A, Lane 1) was a 615 bp sequence (Figure 3.13). *AVOM1* is 51% identical in DNA sequence to TC98998, the tomato TIGR sequence, which is similar to the P-rich protein EIG-I30 in *Nicotiana tabacum* (Accession No BAB16428). From NCBI BlastX alignment, *AVOM1* showed low homology to an unknown protein from *Arabidopsis thaliana* (Accession No AAM67318) and to an unnamed protein product in *Mus musculus* (Accession No BAC30584.1).

3.2.2 *AVOM2*

AVOM2 (Figure 3.12A, Lane 2) was sequenced and shown to consist of 651 nucleotides including a poly-A tail (Figure 3.14). The *AVOM2* DNA sequence was 51% identical to TC107525, the tomato TIGR sequence. From NCBI BlastX alignment, the predicted protein was shown to have 73% identity to the *Arabidopsis* cyclin 2b protein (Accession No Z31401), expressed during different intervals of the cell cycle.

3.2.3 *AVOM8*

AVOM8 (Figure 3.12A, Lane 8) was shown to be 348 nucleotides long, containing a poly-A region (Figure 3.15). The DNA sequence was 61% identical to TC110889, the tomato TIGR sequence, and 57 % identical to TC159088, the *Arabidopsis* TIGR sequence, which is similar to the GARP-like putative transcription factor *KANADI3* (Accession No AAL05438).

Figure 3.13 Partial DNA sequences of the *AVOM1* cDNA clone (615 bp) corresponding to the DNA band from Lane 1 of Figure 3.12 A

N is defined as an unclear nucleotide presented in sequencing.

```
  1  TCGGCACGAG AATCCCTTTA TTTCCCTCAG TTATCTCTTT ACACTGTAAA
 51  GCCCTTAATC ACCGCAACAC ATGGATGGCC TGGATAACTT GATCTTTCAG
101  TGAAGAAAAG AGGAAATTCA AGCATAAAAA TGTAGAAAAG AGAACATCCA
151  AAACCATTCC CTGTTATTCT CTCATTGTCT ATAGAGTATA AAATTCAAAG
201  CAGATGATAA AGATCCAAAA CACCCTAAAA ACATACAAAA AGGTAGAGAT
251  TTACATAAAT CAATCTAGAA CAAAGGTAAC CAAAGACACA AAGAAACACA
301  CTCAAATCAC ACCCTAAAAT ACCAAACACA CGCACACACA AAAAAGAGCA
351  GATTTACATG ATGTATGCAG CCGACGAGCA CGTTAGCCAG ACGGGGAGGC
401  GGGGGCTTAA CGTGAGCCGA TCATCAACCC CTTATTTTCT NGACCTNNTA
451  CCAGCCCTTC TNNCATGAAT CTCCCACAAC TNNCATNCCT TTCTNGAAGA
501  AATCTNTCAG GCCCTTTGCC CNTATCNTNA NCTGANGCTC AANNCANCCA
551  TTNCTCTCTC TCTCTCTCTC TACTAAACCA NCTAGNNTTC TTCTTCTTCN
601  TCNTCNAGGN TANAC
```

Figure 3.14 Partial DNA sequences of the *AVOM2* cDNA clone (651 bp) corresponding to the DNA band from Lane 2 of Figure 3.12 A

The underlined sequences indicate the putative polyadenylated region. N is defined as an unclear nucleotide presented in sequencing.

```
  1  GGGNACNAAN GCTGAGCTNC NCNGCGGNGG CGNCCGCTCT AGAACTAGTG
 51  ATCCNCNGGC CTGCAGAATT CGGCACGAGC ATNGCTCGGA GCTGAAAAGC
101  TACGNGCAGA GTTTGCTGCT CCTGATGGTA GGCCATGGGG CCCAGGTGGA
151  GCTTATGGAA TGAAACTTGG GGTACCAGAT GGTGCTTTCC CTCCTTATGG
201  GGATGGCTTT GGGCTTCACT CGGGTGCTGG TGACAAGGGT CATCTGTTTG
251  GTGCGGGTTC TGGTTCGTGG GGAGCATTTG ATAAGTCTCG CTTTGGACGA
301  CGCTAAATTT CATTTGGTTT TCATGTGGAA TGGGGAGATC ATGCCAGTTA
351  TTTAGGCCTC TGTTGCTTGA AAGGGTTAGA ATTGTGAGAA CTTTAATGGG
401  TTTGACAGAT CAGTAATCCT TTTACTGCAT TGCTATTAGT CGTGCTAGTA
451  TTCTGCCAGA CTTGTTATGT TACTATTGCA TTTGATGGAT GAAGACATAA
501  TGTCTATTTT ATCATTTTTG AATCTACATC CAAAGTTACT AATTTTCACA
551  TTTTGGCTTA GATTGTATCC AAGGGGATAC CGTTTAGTTC ATAGTGTGTT
601  ATGAAGTTAA TAGATTTACA ACATTATAGC TAAAAAAAAA AAAAAAAAAA
651  A
```

Figure 3.15 Partial DNA sequences of the *AVOM8* cDNA clone (348 bp) corresponding to the DNA band in Lane 8 of Figure 3.12 A

The underlined sequences indicate the putative polyadenylated region.

```
  1   AGGAATAAGT ACCAGAAAAG AAGATGTTCA TTTTGAGAAC AGAGTAATAG

 51   TAGTAGTAGT TTATAGGTAA GCTAGTTTGA GTTTCCTTCC TGTCTTTTGG

101   GGGTGTTTGT GCTTGGACTT TGACACTTTG TTATCGGTTT CTTGTTCTCT

151   TTCTTTGCTT TCTTGGGAAT AAAATCCTGT TTAGTGAAAA AAGAAAATGT

201   GTTTAAAAAG GAGATTTTGG TGTCGATGTC CAATGTGAGA TTGGACACGC

251   CCATGTTTCA TCTCGAAGTT TGACAAGCAT CCTTGTATGT TGGTAACTGA

301   ATAAATAAAA TGAGGACGAA ACCAATCATA AAAAAAAAAA AAAAAAAA
```

3.3 Gene Expression

3.3.1 mRNA Accumulation in Different Organs

3.3.1.1 *CM-AAT1* mRNA Expression

The evolution of ethylene is a good indicator for ripening stage of fruit. Ethylene production of wild type melon fruit showed a sharp increase from 35 to 45 daa (Figure 3.16). The wild type melon initiated physiological ripening at 35 days after anthesis (daa), when ethylene production was measured at 0.92 nl/h/g fresh weight and increased to 11.26 and 20.1 nl/h/g fresh weight at 40, and 45 daa, respectively. On the other hand, *ACO1* antisense fruit produced ethylene at 0.67 nl/h/g fresh weight at 45 daa. There was no ethylene detected from the green unripe fruits before 30 daa.

CM-AAT1 mRNA began to be accumulated in the 35 daa fruit and was highly expressed at 45 daa in wild type fruit, while *ACO1* antisense melon exhibited significant inhibition of the *CM-AAT1* mRNA accumulation, which was still present only at a low level in the 45 daa fruit (Figure 3.17). The *CM-AAT1* expression was stimulated in 30 daa fruit by exogenous ethylene application in both wild type and the low ethylene fruit. There were differences in phenotype between wild type fruit, which were very soft in flesh texture and had a rich aromatic scent after 45 daa, compared to the low ethylene variety, which was still firm and with much less aroma at 50 daa.

Figure 3. 16 Ethylene production during melon fruit ripening

The ethylene produced from detached melon fruits was measured at different ripening stages. Averages ± SD of 3 replicates are given.

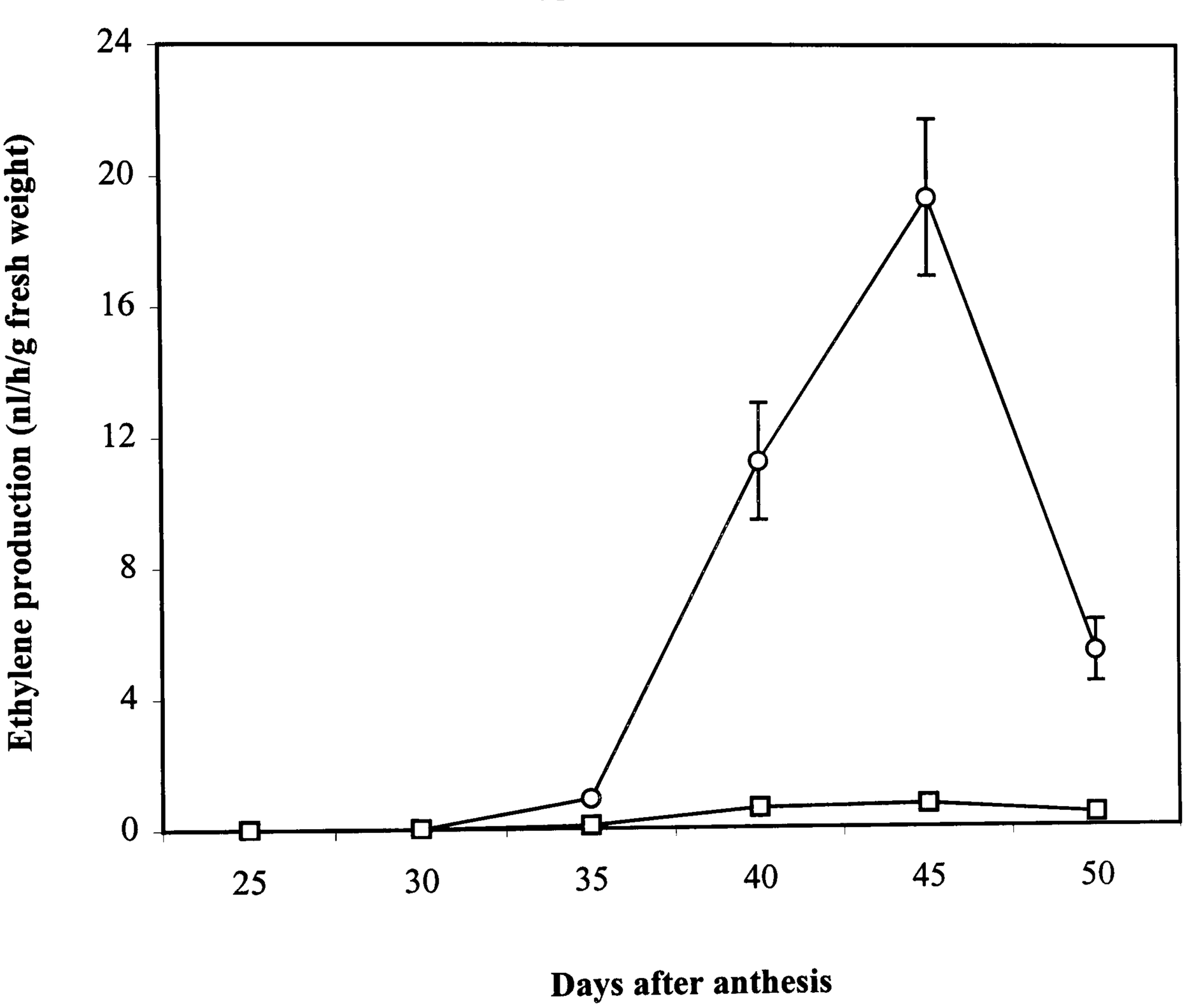
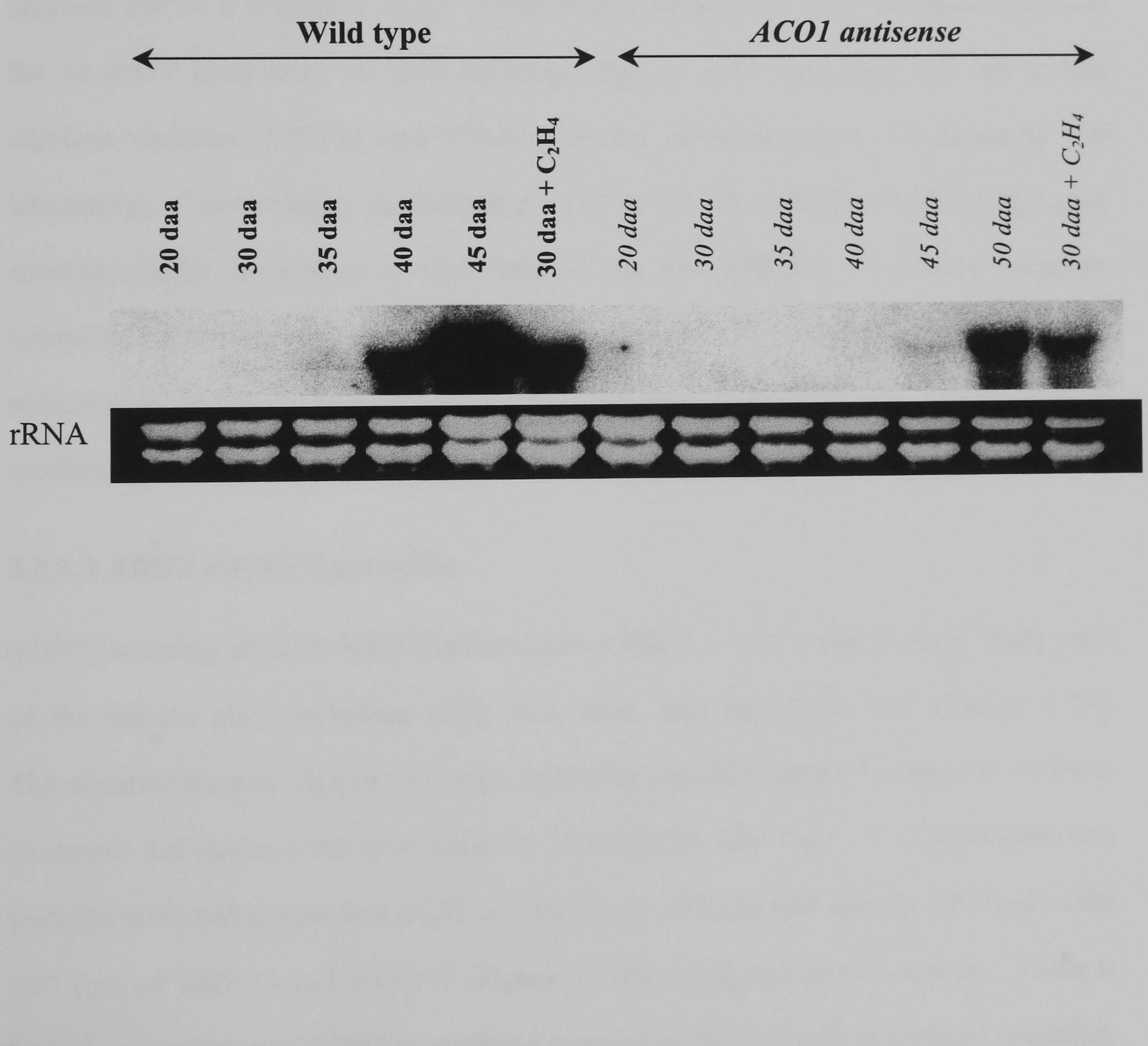


Figure 3. 17 Accumulation of *CM-AAT1* mRNA in melon during fruit development

Northern blot analysis of total RNA (25 μg) from different stages of melon fruit development of wild type (bold fonts) and *ACO1* antisense (italic fonts) fruit, including exogenous ethylene (20 ppm for 24 h) treatments, probed with *CM-AAT1*. The photograph below the northern blot film shows the corresponding rRNA in the gel, stained with ethidium bromide.



3.3.1.2 *Le-AAT1* mRNA Expression

Le-AAT1 (previously named TC98820, TIGR) shows a fruit ripening specific pattern of gene expression (Figure 3.18A). The *Le-AAT1* mRNA size from northern analysis is 1.6 Kb in length (Figure 3.18B) consistent with that of *TOM36* (Davies and Grierson, 1989). Accumulation of *Le-AAT1* started at the breaker stage and increased significantly during tomato ripening (Figure 3.18B) consistent with the information from TIGR that the TC98820 sequence was received only from the ETS derived only from ripe fruit tissues (section 3.1.5). The *Le-AAT1* expression in the MG fruit, treated with 10 ppm exogenous ethylene for 16 h, was detected at low level (Figure 13.18B), but the level of the expression was increased when treated with 20 ppm ethylene for 24 h (Figure 3.18A). There was a weak band, slightly expressing over the *Le-AAT1* band after the B+3 ripening stage in wild type fruit, but not in low ethylene varieties, T4B+11 and V11B+7 (*ACO1* co-suppression lines made by this laboratory). Interestingly, in contrast to *CM-AAT1*, the endogenous *Le-AAT1* gene showed similar expression in ripe fruit of the low ethylene production varieties, compared to the controls (Figure 3.19B). However, the expression showed slight reduction in *Nr* mutant fruit (Figure 3.19A), and there was no mRNA accumulation in seedlings growing in light and dark conditions (Figure 3.19B).

3.3.1.3 *ADH2* mRNA Expression

ADH2, encoding alcohol: NAD^+ oxidoreductase (EC 1.1.1.1), is expressed in many parts of the tomato plant including seed, root, stem, and especially fruit (Figure 3.20). The accumulation is high in fully ripe fruit after the B+5 stage. Exogenous ethylene treatment did increase the expression of *ADH2* in the MG fruit. The expression was partially inhibited in ripe fruit of *Nr* mutant (Figure 3.21A) and slightly inhibited in the B+7 fruit of T4B+11 and V11B+7 (Figure 3.21B), compared to the controls. There is higher accumulation of *ADH2* in seedlings growing in the dark than in the light condition.

Figure 3.18 Accumulation of *Le-AAT1* mRNA in tomato organs

Northern blot analysis of total RNA (25 μg) probed with *Le-AAT1* A) RNA from fruit at different ripening stages, including an exogenous ethylene (20 ppm for 24 h) treatment, plus other organs B) different ripening stages of tomato fruit, including an exogenous ethylene (10 ppm for 16 h) treatment. The photographs below the northern blot film show the corresponding rRNA in gel stained with ethidium bromide.

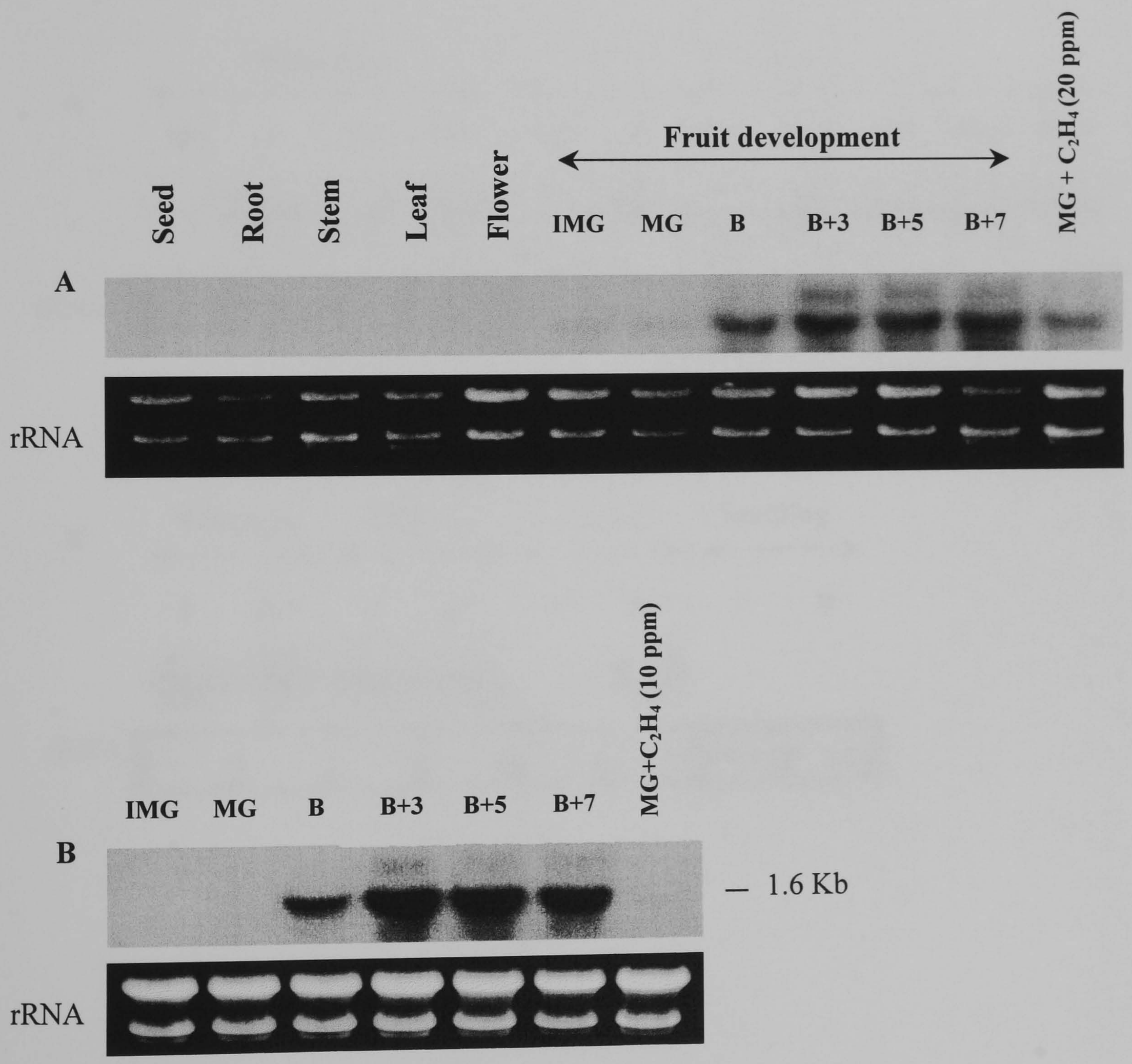


Figure 3.19 Accumulation of *Le-AAT1* mRNA in seedlings and in low ethylene producing transgenics during fruit ripening

Northern blot analysis of total RNA (40 μg) from different ripening stages of tomato fruit of wild type and *Nr* varieties (A), and low ethylene production lines, T4B+11 and V11B+7 (*ACO1* co-suppression lines), including RNA from light-dark-growing seedlings (B), probed with *Le-AAT1*. The photographs below the northern blot film show the corresponding rRNA in the gel, stained with ethidium bromide.

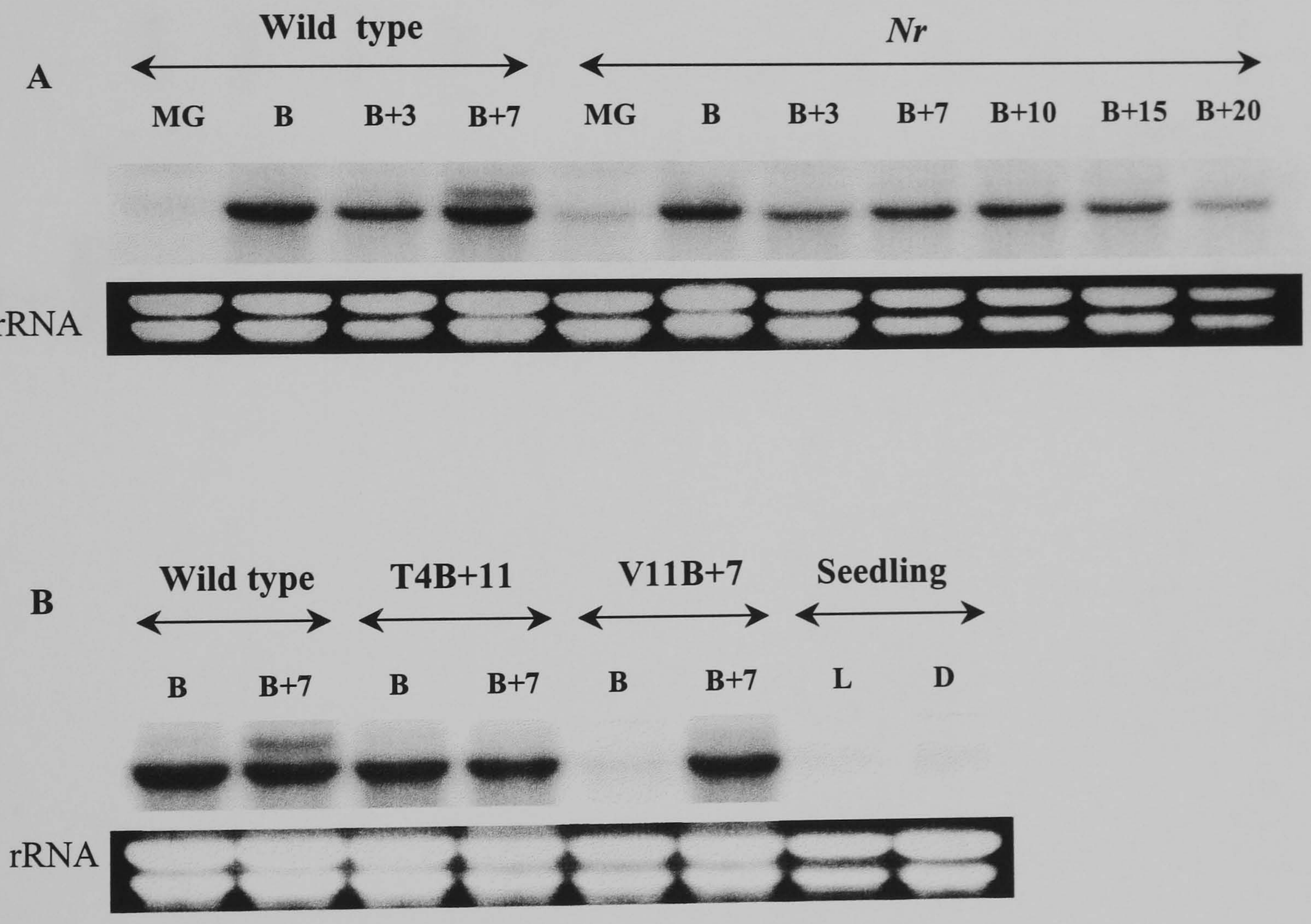


Figure 3. 20 Accumulation of *ADH2* mRNA in tomato organs

Northern blot analysis of total RNA (25 μg) from different ripening stages of tomato fruit, including an exogenous (20 ppm for 24 h) ethylene treatment, and various organs, probed with the *TOMM10* clone. The photograph below the northern blot shows the corresponding rRNA in the gel stained, with ethidium bromide.

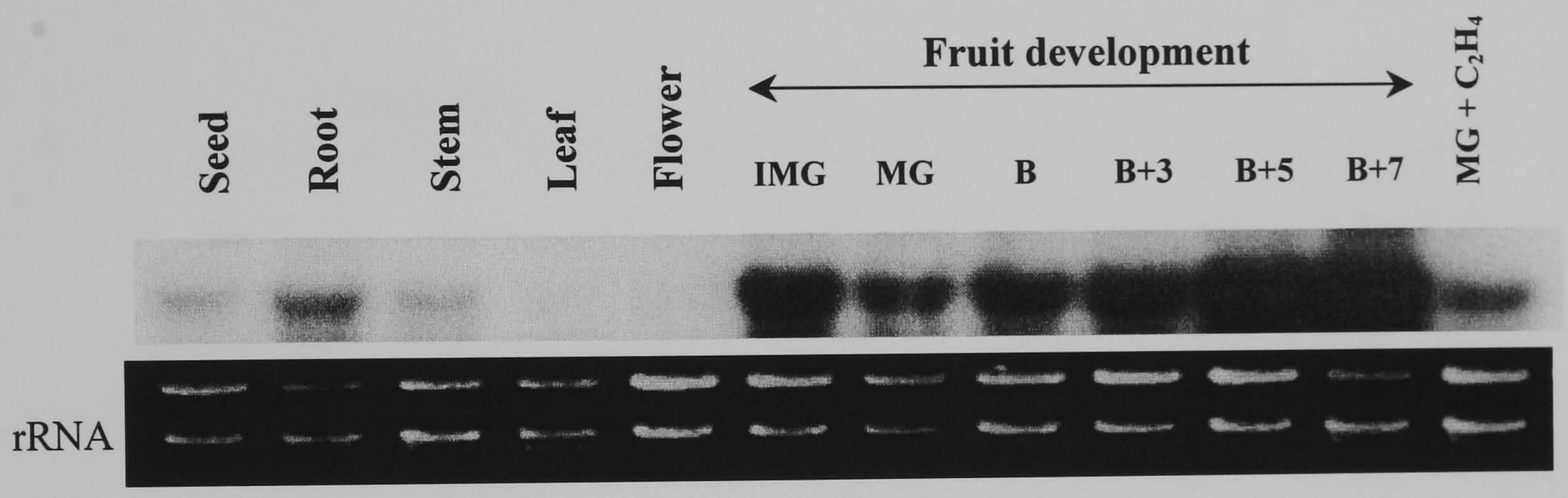
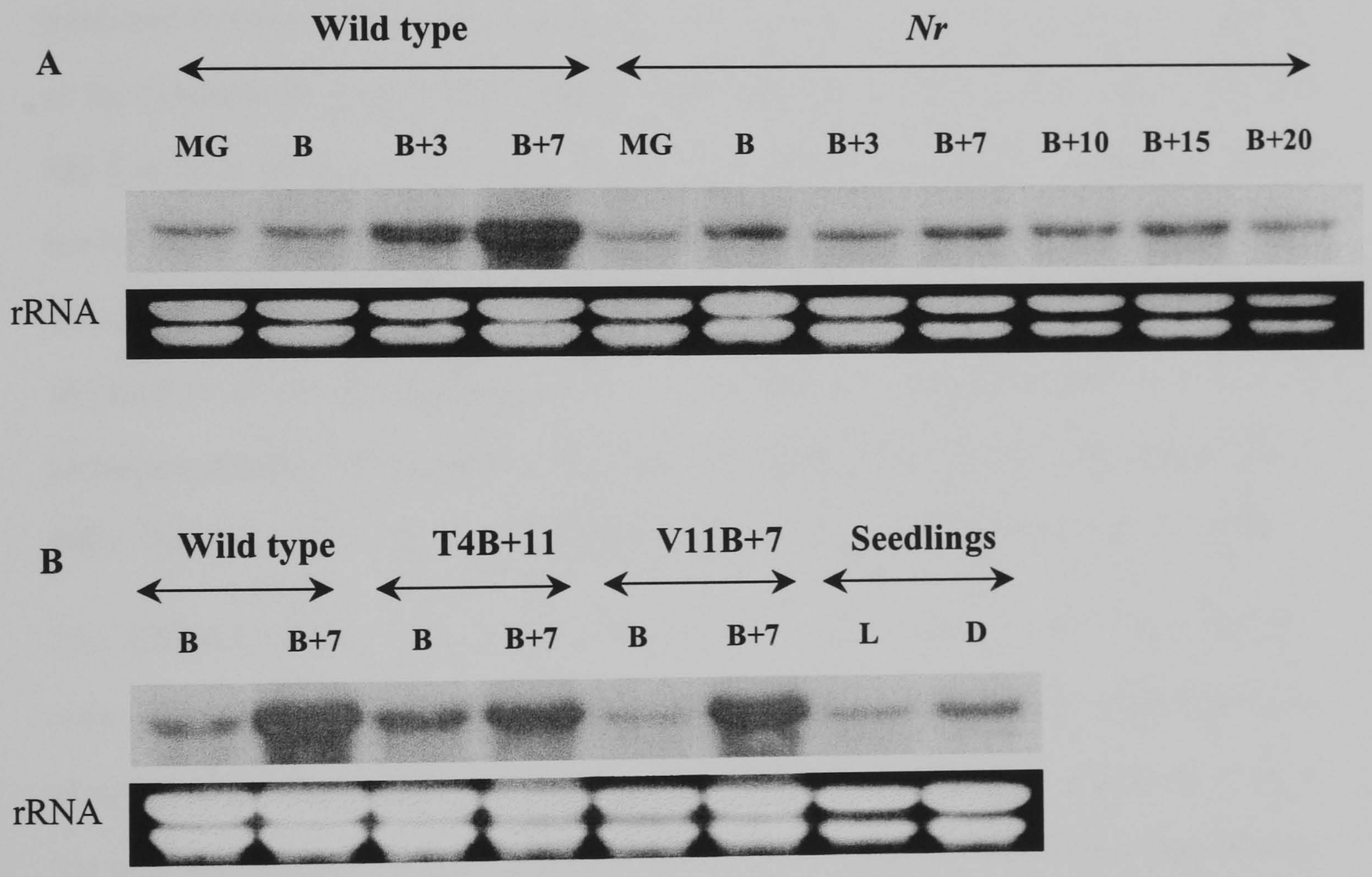


Figure 3.21 Accumulation of *ADH2* mRNA in tomato during fruit ripening and in seedlings

Northern blot analysis of total RNA (40 μg) from different ripening stages of tomato fruit of wild type and *Nr* varieties (A), and low ethylene production varieties, T4B+11 and V11B+7, including RNA from light-dark-growing seedlings (B), probed with the *TOMM10* clone. The photographs below the northern blot show the corresponding rRNA in the gel, stained with ethidium bromide.



3.3.2 Analysis of Proteins Encoded by AAT Clones

3.3.2.1 *CM-AAT1* and *CM-AAT2* cDNA Clones

A novel truncated *CM-AAT1* (*MEL2*) clone was first isolated from ripe melon by our group, Aggelis *et al.* (1997b). The full length clone of *CM-AAT1* and its homologue *CM-AAT2* were subsequently cloned by the J.-C. Pech group, ENSAT, Tolouse, and sent to us for protein expression study in yeast.

pYES-CMAAT1 and pYES-CMAAT2 were sequenced to confirm the presence of the inserts. The *CM-AAT1* open-reading frame (ORF) contains 1431 nucleotides, predicted to encode 476 amino acids. 21 additional amino acid residues were encoded at the N-terminus, compared to pMEL2 (Aggelis *et al.*, 1997b). The *CM-AAT2* ORF has 3 nucleotides less than *CM-AAT1* at the end of the C-terminus, resulting in a 1428 nucleotide length sequence encoding a deduced 475 amino acid protein (Figure 3.22). The *CM-AAT2* DNA sequence is 92% identical to that of the *CM-AAT1* clone, and 86% identical at the protein level (Figure 3.23). Both predicted proteins have two conserved consensus motifs, 'H-x-x-x-D' (positions 181 to 186) and 'DFGWG' (positions 396 to 400), found in many *O*-acyl-transferases (Aharoni *et al.*, 2000; St Pierre *et al.*, 1998).

The CM-AAT1 and CM-AAT2 predicted proteins contain 54.18% and 55.37%, respectively, of hydrophobic amino acids, such as alanine, leucine, valine, proline, phenylalanine, tryptophane, and methionine (Table 3.3), and were predicted to be in the cytosolic phase (the PSORT prediction program from ExPASy Molecular Biology Server). The CM-AAT1 protein was estimated to have a molecular weight (Mw) of 53 kDa with an isolectric point (pI) of 7.10, while the CM-AAT2 protein, an Mw of 53.1 kDa, and pI of 7.60. There is a *N*-link site of glycosylation at positions 181 to 184 of CM-AAT1, but none for CM-AAT2 (Table 3.4). The positions of phophorylation and amidation sites of both proteins are almost the same, but CM-AAT2 contains less *N*-myrislation sites, which are acylated by the covalent addition of myristate (a C14-saturated fatty acid) to the *N*-terminal residue.

Figure 3.22 DNA sequence alignment between *CM-AAT1* and *CM-AAT2*

Black and gray boxes indicate identical and similar amino acid residues, respectively.

```
CM-AAT1      1 ATGGGCTCTGGATCCGGTGATGACGATGACAAGCTCGCCCTTATGGAAACAATGCAAACC
CM-AAT2      1 ATGGGCTCTGGATCCGGTGATGACGATGACAAGCTCGCCCTTATGGAAACAATGCAAACC

CM-AAT1     61 ATCGACTTCTCTTTTCACGTACGAAAATGCCAACCAGAATTGATTGCACCAGCAAATCCT
CM-AAT2     61 ATCGACTTTTCTTTTCAAGTACGAAAATGCCAACCAGAATTGATTGCTCCAGCAAATCCT

CM-AAT1    121 ACACCCTATGAATTTAAACAACTTTCTGATGTGGATGATCAACAAAGCTTAAGGCTTCAA
CM-AAT2    121 ACACCGTATGAATTTAAACAACTTTCTGATGTGGATGATCAACAAAGCTTAAGGTTTCAA

CM-AAT1    181 TTGCCATTCGTAAATATCTATCCCCATAATCCAAGTTTGGAGGGAAGAGATCCAGTGAAG
CM-AAT2    181 TTGCCATTGGTAAATATCTATCACCATAATCCAAGTTTGGAGGGACGAGATCCAGTGAAG

CM-AAT1    241 GTAATAAAGGAAGCAATTGGAAAGGCGTTGGTGTTCTACTATCCTTTAGCAGGAAGATTG
CM-AAT2    241 GTAATAAAGGAAGCAATCGCAAAGGCATTGGTGTTTTACTATCCTTTAGCAGGAAGATTG

CM-AAT1    301 AGAGAAGGCCCAGGTAGAAAGCTTTTTGTTGAATGTACAGGTGAAGGAATCTTGTTTATT
CM-AAT2    301 AGAGAAGGGCCAGGAAGAAAGCTTTTTGTAGAATGTACAGGTGAAGGAATCTTGTTCATT

CM-AAT1    361 GAAGCGGATGCAGATGTGAGCTTAGAAGAATTTTGGGATACTCTTCCATATTCACTTTCA
CM-AAT2    361 GAAGCGGATGCAGATGTGAGCTTAGAGCAATTTAGGGATACTCTTCCATATTCACTTTCA

CM-AAT1    421 AGCATGCAGAACAATATTATACATAACGCTTTAAATTCTGATGAAGTCCTCAATTCTCCA
CM-AAT2    421 AGCATGGAGAACAATATTATACATAACTCTTTAAATTCTGATGGAGTCCTTAATTCTCCA

CM-AAT1    481 TTATTGCTCATTCAGGTGACACGACTCAAGTGTGGAGGTTTCATTTTTGGTCTTTGTTTC
CM-AAT2    481 TTATTGCTCATTCAGGTGACACGACTCAAGTGTGGAGGTTTCATTTTTGGTATTCATTTC

CM-AAT1    541 AATCATACTATGGCAGATGGTTTTGGTATTGTCCAATTCATGAAGGCTACAGCGGAGATA
CM-AAT2    541 GATCATACTATGGCAGATGGTTTTGGCATTGCCCAATTCATGAAGGCTATAGCAGAGATA

CM-AAT1    601 GCTCGTGGAGCTTTTGCTCCATCTATTTTACCAGTATGGCAAAGAGCTCTCTTAACCGCA
CM-AAT2    601 GCTCGTGGAGCTTTTGCTCCATCTATTTTACCAGTATGGCAAAGAGCTCTCTTAACCGCA

CM-AAT1    661 AGAGACCCTCCCAGAATCACTTTTCGCCACTATGAATACGACCAAGTAGTCGACATGAAG
CM-AAT2    661 AGAGACCCTCCCAGAATCACTGTTCGCCACTATGAATACGACCAAGTAGTCGACACAAAG

CM-AAT1    721 AGCGGCCTCATTCCAGTCAATAGCAAGATCGATCAATTATTCTTCTTTAGCCAACTTCAA
CM-AAT2    721 AGCACCCTCATTCCAGCCAATAACATGATCGATCGATTATTCTTCTTTACCCAACGTCAA

CM-AAT1    781 ATCTCCACCCTTCGCCAAACTTTGCCAGCCCACCTTCACGATTGCCCTTCCTTCGAGGTC
CM-AAT2    781 ATCTCTACTCTTCGCCAAACTTTGCCTGCCCATCTTCACGATTGCTCCTCCTTCGAGGTC

CM-AAT1    841 CTCACTGCCTATGTTTGGCGCCTCCGTACCATAGCCCTTCAATTTAAGCCAGAGGAGGAA
CM-AAT2    841 CTCGCTGCCTATGTTTGGCGCCTCCGTACCATAGCCTTTCAACTCAAGCCAGAGGAGGAA
```

Figure 3.22 DNA sequence alignment between *CM-AAT1* and *CM-AAT2*

Black and gray boxes indicate identical and similar amino acid residues, respectively.

```
CM-AAT1      1 ATGGGCTCTGGATCCGGTGATGACGATGACAAGCTCGCCCTTATGGAAACAATGCAAACC
CM-AAT2      1 ATGGGCTCTGGATCCGGTGATGACGATGACAAGCTCGCCCTTATGGAAACAATGCAAACC

CM-AAT1     61 ATCGACTTCTCTTTTCACGTACGAAAATGCCAACCAGAATTGATTGCACCAGCAAATCCT
CM-AAT2     61 ATCGACTTTTCTTTTCAAGTACGAAAATGCCAACCAGAATTGATTGCTCCAGCAAATCCT

CM-AAT1    121 ACACCCTATGAATTTAAACAACTTTCTGATGTGGATGATCAACAAAGCTTAAGGCTTCAA
CM-AAT2    121 ACACCGTATGAATTTAAACAACTTTCTGATGTGGATGATCAACAAAGCTTAAGGTTTCAA

CM-AAT1    181 TTGCCATTCGTAAATATCTATCCCCATAATCCAAGTTTGGAGGGAAGAGATCCAGTGAAG
CM-AAT2    181 TTGCCATTGGTAAATATCTATCACCATAATCCAAGTTTGGAGGGACGAGATCCAGTGAAG

CM-AAT1    241 GTAATAAAGGAAGCAATTGGAAAGGCGTTGGTGTTCTACTATCCTTTAGCAGGAAGATTG
CM-AAT2    241 GTAATAAAGGAAGCAATCGCAAAGGCATTGGTGTTTTACTATCCTTTAGCAGGAAGATTG

CM-AAT1    301 AGAGAAGGCCCAGGTAGAAAGCTTTTTGTTGAATGTACAGGTGAAGGAATCTTGTTTATT
CM-AAT2    301 AGAGAAGGGCCAGGAAGAAAGCTTTTTGTAGAATGTACAGGTGAAGGAATCTTGTTCATT

CM-AAT1    361 GAAGCGGATGCAGATGTGAGCTTAGAAGAATTTTGGGATACTCTTCCATATTCACTTTCA
CM-AAT2    361 GAAGCGGATGCAGATGTGAGCTTAGAGCAATTTAGGGATACTCTTCCATATTCACTTTCA

CM-AAT1    421 AGCATGCAGAACAATATTATACATAACGCTTTAAATTCTGATGAAGTCCTCAATTCTCCA
CM-AAT2    421 AGCATGGAGAACAATATTATACATAACTCTTTAAATTCTGATGGAGTCCTTAATTCTCCA

CM-AAT1    481 TTATTGCTCATTCAGGTGACACGACTCAAGTGTGGAGGTTTCATTTTTGGTCTTTGTTTC
CM-AAT2    481 TTATTGCTCATTCAGGTGACACGACTCAAGTGTGGAGGTTTCATTTTTGGTATTCATTTC

CM-AAT1    541 AATCATACTATGGCAGATGGTTTTGGTATTGTCCAATTCATGAAGGCTACAGCGGAGATA
CM-AAT2    541 GATCATACTATGGCAGATGGTTTTGGCATTGCCCAATTCATGAAGGCTATAGCAGAGATA

CM-AAT1    601 GCTCGTGGAGCTTTTGCTCCATCTATTTTACCAGTATGGCAAAGAGCTCTCTTAACCGCA
CM-AAT2    601 GCTCGTGGAGCTTTTGCTCCATCTATTTTACCAGTATGGCAAAGAGCTCTCTTAACCGCA

CM-AAT1    661 AGAGACCCTCCCAGAATCACTTTCGCCACTATGAATACGACCAAGTAGTCGACATGAAG
CM-AAT2    661 AGAGACCCTCCCAGAATCACTGTTCGCCACTATGAATACGACCAAGTAGTCGACACAAAG

CM-AAT1    721 AGCGGCCTCATTCCAGTCAATAGCAAGATCGATCAATTATTCTTCTTTAGCCAACTTCAA
CM-AAT2    721 AGCACCCTCATTCCAGCCAATAACATGATCGATCGATTATTCTTCTTTACCCAACGTCAA

CM-AAT1    781 ATCTCCACCCTTCGCCAAACTTTGCCAGCCCACCTTCACGATTGCCCTTCCTTCGAGGTC
CM-AAT2    781 ATCTCTACTCTTCGCCAAACTTTGCCTGCCCATCTTCACGATTGCTCCTCCTTCGAGGTC

CM-AAT1    841 CTCACTGCCTATGTTTGGCGCCTCCGTACCATAGCCCTTCAATTTAAGCCAGAGGAGGAA
CM-AAT2    841 CTCGCTGCCTATGTTTGGCGCCTCCGTACCATAGCCTTTCAACTCAAGCCAGAGGAGGAA
```

Figure 3.22 DNA sequence alignment between *CM-AAT1* and *CM-AAT2* (continued)

```
CM-AAT1   901 GTGCGGTTTCTTTGCGTAATGAATCTACGCTCGAAGATCGACATACCATTAGGGTATTAT
CM-AAT2   901 GTGCGGTTTCTTTGCGTCGTGAATCTACGCTCGAAGATCGACATACCATTAGGGTTTTAT

CM-AAT1   961 GGTAATGCGGTAGTTGTTCCTGCAGTAATCACCACCGCTGCGAAGCTTTGTGGGAACCCA
CM-AAT2   961 GGTAATGCAATAGTTTTCCCTGCAGTAATCACCACCGTTGCGAAGCTTTGTGGGAACCCA

CM-AAT1  1021 CTTGGTTATGCTGTAGACTTGATTAGGAAGGCCAAGGCTAAGGCAACGATGGAGTACATA
CM-AAT2  1021 CTTGGTTATGCTGTAGACTTGATTAGGAAGGCTAAGGCTAAGGCCACGAAGGAGTACATA

CM-AAT1  1081 AAGTCTACGGTGGATCTTATGGTGATTAAAGGACGACCCTATTTCACTGTAGTTGGATCA
CM-AAT2  1081 AAGTCTATGGTGGATTTTATGGTGATTAAAGGACGACCCCGTTTCACTGAAATTGGACCA

CM-AAT1  1141 TTTATGATGTCAGACCTAACGAGAATTGGGGTTGAAAACGTGGACTTTGGATGGGGAAAG
CM-AAT2  1141 TTTATGATGTCAGACATAACGAGGATTGGGTTCGAAAATGTGGACTTTGGATGGGGAAAG

CM-AAT1  1201 GCCATTTTTGGAGGACCTACAACCACAGGGGCCAGAATTACACGAGGTTTGGTAAGCTTT
CM-AAT2  1201 GCCATTTTTGGAGGACCTATAATTGGAGGGTGTGGAATTATCCGAGGTATGATAAGCTAT

CM-AAT1  1261 TGTGTACCTTTCATGAATAGAAATGGAGAAAAGGGAACTGCGTTAAGTCTATGCTTGCCT
CM-AAT2  1261 TCTATAGCTTTCATGAATAGAAATGGAGAAAAGGGAATTGTGGTACCTCTATGCTTGCCA

CM-AAT1  1321 CCTCCAGCCATGGAAAGATTTAGGGCAAATGTTCATGCCTCGTTGCAAGTGAAACAAGTG
CM-AAT2  1321 CCTCCTGCCATGGAAAGATTTAGGGCAAATGTTCATGCCTCATTGCAAGTGATACAAGTT

CM-AAT1  1381 GTTGATGCAGTTGATAGCCATATGCAAACTATTCAATCTGCTTCGAAATAA
CM-AAT2  1381 CTTGATAAAGTTGATAGAGATATGCAAACCATTCTATCTGCTTT--A-TAA
```

Figure 3.23 Amino acid sequence alignment between CM-AAT1 and CM-AAT2

Black and gray boxes indicate identical and similar amino acid residues, respectively. ***** is defined as the 'H-x-x-x-D' conserved region, found in the acyl transferase family. +++++ is defined as the 'DFGWG' conserved region, found in many *O*-acyl-transferase enzymes.

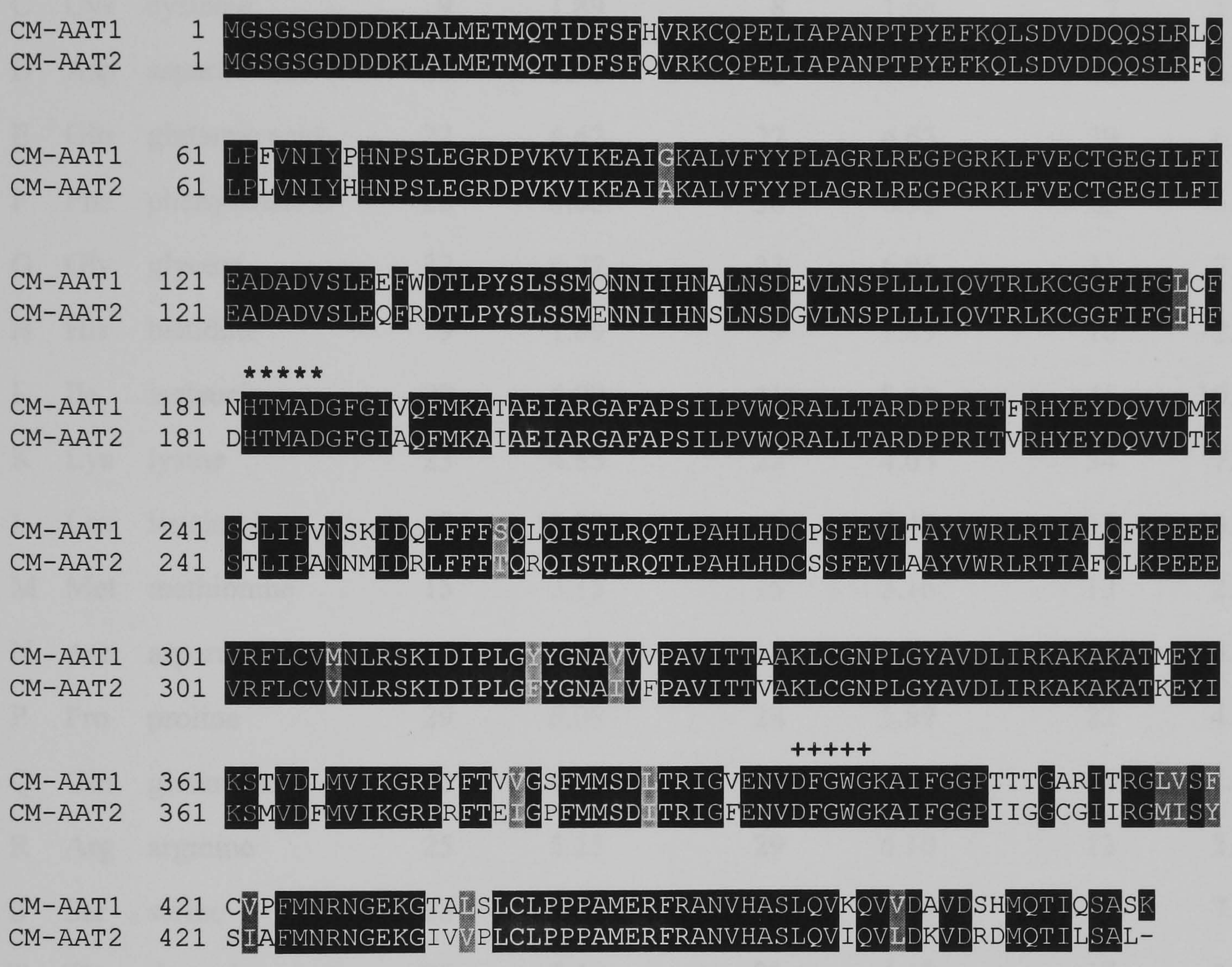


Table 3.3 Amino acid compositions of CM-AAT1 (476 aa), CM-AAT2 (475 aa), and Le-AAT1 (442 aa) predicted polypeptides (ProtParam program from ExPASy Molecular Biology Server)

			CM-AAT1		CM-AAT2		Le-AAT1	
Amino acid			**n**	**n%**	**n**	**n%**	**n**	**n%**
A	Ala	alanine	36	7.56	36	7.58	18	4.07
C	Cys	cysteine	9	1.89	8	1.68	7	1.58
D	Asp	aspartic acid	26	5.46	28	5.89	18	4.07
E	Glu	glutamic acid	22	4.62	22	4.63	29	6.56
F	Phe	phenylalanine	28	5.88	30	6.32	25	5.66
G	Gly	glycine	32	6.72	33	6.95	31	7.01
H	His	histidine	9	1.89	9	1.89	10	2.26
I	Ile	isoleucine	29	6.09	41	8.63	45	10.18
K	Lys	lysine	23	4.83	22	4.63	34	7.69
L	Leu	leucine	48	10.08	45	9.47	48	10.86
M	Met	methionine	15	3.15	15	3.16	13	2.94
N	Asn	asparagine	17	3.57	17	3.58	25	5.66
P	Pro	proline	29	6.09	28	5.89	22	4.98
Q	Gln	glutamine	20	4.20	19	4.00	8	1.81
R	Arg	arginine	25	5.25	29	6.10	13	2.94
S	Ser	serine	28	5.88	25	5.26	33	7.47
T	Thr	threonine	26	5.46	21	4.42	17	3.85
V	Val	valine	37	7.77	32	6.73	26	5.88
W	Trp	tryptophan	4	0.84	3	0.63	5	1.13
Y	Tyr	tyrosine	13	2.73	12	2.53	15	3.39
Z	---	other	0	0.00	0	0.00	0	0.00
Molecular weight (Daltons)			52972.4		53128.8		49835.2	
Theoretical pI			7.10		7.60		7.13	

3.3.2.2 *Le-AAT1* Clone

The *Le-AAT1* ORF was cloned from total mRNA of 'Ailsa Craig' ripe fruit and sequenced and shown to be a 1326 bp sequence, encoding 442 aa (Figure 3.24). It is 100% identical to the TC98220 (TIGR) ORF in Figure 3.7. The deduced protein contains both conserved consensus regions, 'H-x-x-x-D' (positions 163 to 167) and 'DFGWG' (positions 380 to 384), like CM-AAT1 and many plant *O*-acyl transferases (Figure 3.9).

The Le-AAT1 protein was calculated to have an Mw of 49835.2 Daltons and pI of 7.13, showing a high percentage of hydrophobic amino acids (Leu 10.9%, Ile 10.2%, Val 5.9%) (Table 3.3), and predicted to be localised in the cytoplasm (the PSORT prediction program from ExPASy Molecular Biology Server). Three sites of *N*-glycosylation were predicted at positions 52-55, 162-165, 432-435 of Le-AAT1 (Table 3.4).

3.3.2.3 Fusion Protein Expression from Yeast

The CM-AAT1 protein, fused with a V5 epitope and polyhistidine tag (5 kDa), was expressed in yeast, and immunologically detected after 8 h induction in media containing galactose and was present in large amounts after 16 h incubation (Figure 3.25A). The molecular weights of the fusion proteins expressed in yeast were approximately 64 kDa for CM-AAT1, CM-AAT2, and 60 kDa for Le-AAT1 (Figure 3.25B), which are bigger than estimated (section 3.3.2.1, 3.3.2.2). There is no linkage by disulfide bond (-*S*-*S*-) between protein subunits from those proteins because the same results were obtained when treated with dithiothreitol (DTT).

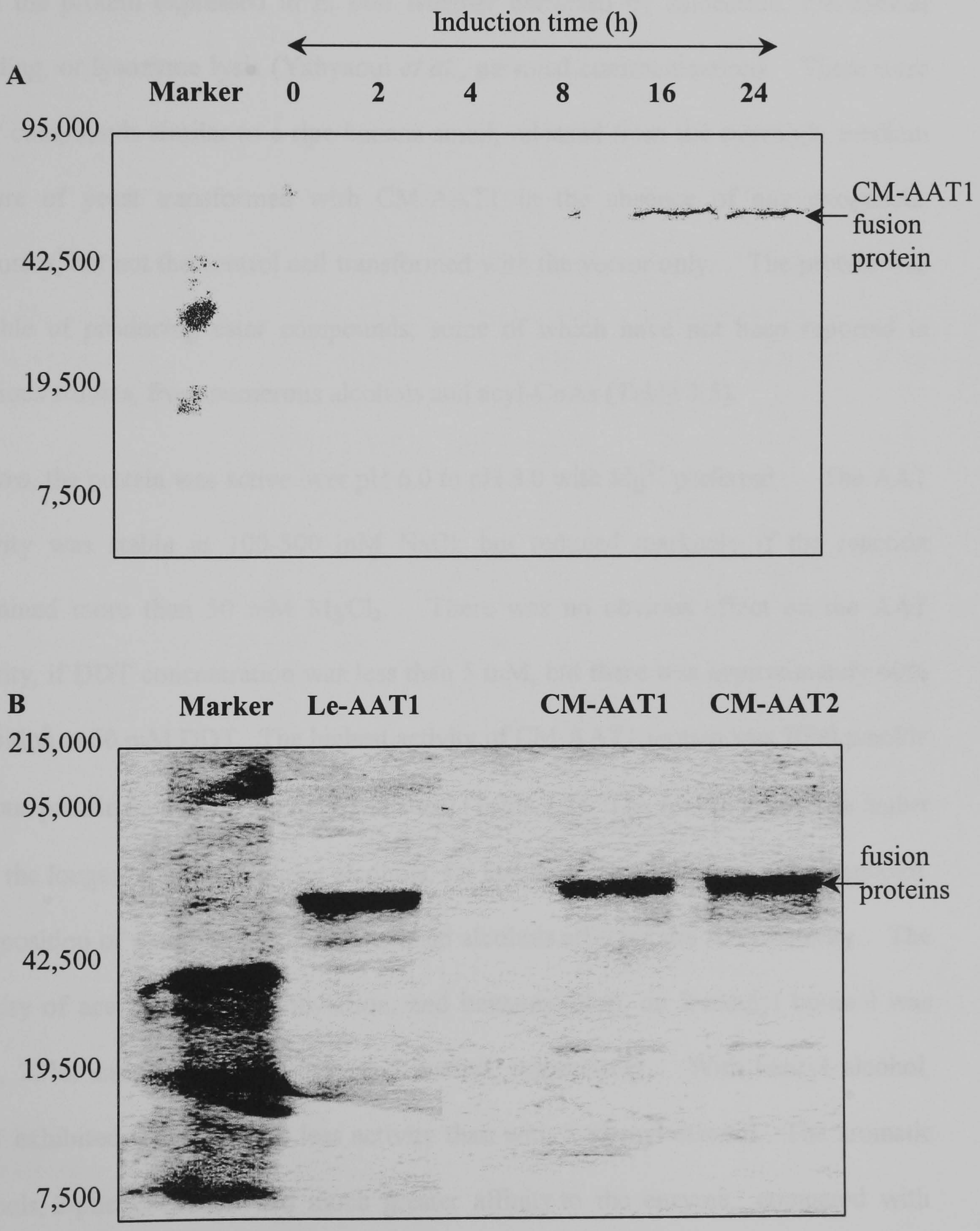
Figure 3.24 Nucleotide sequence of *Le-AAT1* open reading frame (1326 bp) and deduced amino acid sequence (422 aa)

The bold residues show the 'H-x-x-x-D' conserved region, found in the acyl transferase family. The underlined sequence is the 'DFGWG' conserved region, found in many *O*-acyl-transferase enzymes.

```
   1  ATGGCAAATA TTCTACCAAT TCAATAAAT TACCACAAGC CAAAACTAGT AGTTCCATCA
   1  M  A  N  I  L  P  I  S  I  N  Y  H  K  P  K  L  V  V  P  S
  61  AGTGTAACAT CTCATGAGAC AAAACGTCTT TCTGAAATAG ATGATCAAGG GTTTATTCGA
  21  S  V  T  S  H  E  T  K  R  L  S  E  I  D  D  Q  G  F  I  R
 121  CTCCAAATCC CCATACTAAT GTTTTACAAA TACAATTCTT CAATGAAAGG TAAAGATCTA
  41  L  Q  I  P  I  L  M  F  Y  K  Y  N  S  S  M  K  G  K  D  L
 181  GCAAAAATTA TCAAAGATGG ATTATCTAAA ACACTTGTGT TTTACTATCC ATTAGCTGGT
  61  A  K  I  I  K  D  G  L  S  K  T  L  V  F  Y  Y  P  L  A  G
 241  AGACTCATTG AAGGGCCTAA TAAAAAGCTT ATGGTAAATT GCAATGGTGA AGGAGTCTTG
  81  R  L  I  E  G  P  N  K  K  L  M  V  N  C  N  G  E  G  V  L
 301  TTTATCGAAG GTGATGCTAA TATAGAGCTT GAAAAATTAG GTGAATCTAT TAAGCCACCA
 101  F  I  E  G  D  A  N  I  E  L  E  K  L  G  E  S  I  K  P  P
 361  TGTCCATACT TGGATTTACT ACTTCATAAT GTTCATGGTT CTGATGGAAT TATTGGTTCT
 121  C  P  Y  L  D  L  L  L  H  N  V  H  G  S  D  G  I  I  G  S
 421  CCTCTTTTGT TAATTCAGGT GACTCGTTTT ACTTGTGGTG GATTTGCTGT TGGATTTAGA
 141  P  L  L  L  I  Q  V  T  R  F  T  C  G  G  F  A  V  G  F  R
 481  TTTAATCACA CAATGATGGA TGCTTATGGC TTCAAAATGT TTCTAAATGC GTTAAGTGAA
 161  F  N  H  T  M  M  D  A  Y  G  F  K  M  F  L  N  A  L  S  E
 541  TTAATTCAAG GAGCTTCAAC ACCTTCTATA TTGCCTGTAT GGGAAAGACA TCTCCTAAGT
 181  L  I  Q  G  A  S  T  P  S  I  L  P  V  W  E  R  H  L  L  S
 601  GCTAGATCAT CACCAAGTAT TACATGTATT CATCATGAGT TTGATGAGGA AATTGAATCA
 201  A  R  S  S  P  S  I  T  C  I  H  H  E  F  D  E  E  I  E  S
 661  AAAATTGCGT GGGAATCTAT GGAAGATAAG TTGATACAAC AATCATTTTT CTTTGGAAAT
 221  K  I  A  W  E  S  M  E  D  K  L  I  Q  Q  S  F  F  F  G  N
 721  GAGGAGATGG AAGTCATTAA AAATCAAGTT CCTCCAAATT ATGAATGTAC AAAATTCGAG
 241  E  E  M  E  V  I  K  N  Q  V  P  P  N  Y  E  C  T  K  F  E
 781  TTATTAATGG CATTTTTATG GAAATGTCGT ACCATTGCTC TTAATTTGCA CTCTGATGAA
 261  L  L  M  A  F  L  W  K  C  R  T  I  A  L  N  L  H  S  D  E
 841  ATTGTTCGTT TGACATACGT TATTAATATA CGTGGAAAAA AGTCACTCAA CATTGAATTA
 281  I  V  R  L  T  Y  V  I  N  I  R  G  K  K  S  L  N  I  E  L
 901  CCAATTGGTT ATTATGGGAA TGCGTTTATT ACTCCAGTTG TTGTATCAAA AGCAGGTTTG
 301  P  I  G  Y  Y  G  N  A  F  I  T  P  V  V  V  S  K  A  G  L
 961  TTATGTTCAA ATCCAGTGAC ATATGCAGTT GAATTGATCA AGAAAGTTAA AGATCATATA
 321  L  C  S  N  P  V  T  Y  A  V  E  L  I  K  K  V  K  D  H  I
1021  AATGAAGAAT ACATCAAATC ATTGATAGAT TTAATGGTTA CTAAAGGGAG ACCAGAGTTA
 341  N  E  E  Y  I  K  S  L  I  D  L  M  V  T  K  G  R  P  E  L
1081  ACAAAATCTT GGAATTTTTT GGTCTCAGAT AATAGATATA TTGGATTTGA TGAATTTGAT
 361  T  K  S  W  N  F  L  V  S  D  N  R  Y  I  G  F  D  E  F  D
1141  TTTGGATGGG GAAACCCCAT TTTTGGAGGG ATCTTAAAGG CTATATCTTT CACTAGTTTT
 381  F  G  W  G  N  P  I  F  G  G  I  L  K  A  I  S  F  T  S  F
1201  GGTGTTTCTG TTAAAAATGA CAAAGGAGAA AAAGGTGTTT TGATAGCTAT AAGTTTACCT
 401  G  V  S  V  K  N  D  K  G  E  K  G  V  L  I  A  I  S  L  P
1261  CCATTGGCCA TGAAAAAACT TCAAGATATC TACAACATGA CTTTCAGAGT CATAATTTCA
 421  P  L  A  M  K  K  L  Q  D  I  Y  N  M  T  F  R  V  I  I  S
1321  AATATA
 441  N  I
```

Figure 3.25 Western blot analysis of fusion proteins expressed in yeast

Western analysis (10% SDS gel) of CM-AAT1 fusion protein expressed in yeast after induction by galactose at 0, 2, 4, 8, 16, and 24 h (A), and Le-AAT1, CM-AAT1, CMAAT2 fusion proteins expressed after 16 h galactose induction (B). Anti-V5 antibody was specifically interacted with the polyhistidines tage of the fusion proteins and anti-mouse IgG, alkaline phosphatase conjugation, was then applied as the secondary antibody.



3.3.3 Enzyme Activity Assays

3.3.3.1 CM-AAT1

Most of the analysis of CM-AAT1 fusion protein was carried out in conjunction with colleagues at ENSAT, Toulouse (Yahyaoui *et al.* 2002). The CM-AAT1 fusion protein, expressed in yeast, showed AAT activity, but the activity was undetectable from the protein expressed in *E. coli* whether extracted by sonication, mechanical grinding, or lysozyme lysis (Yahyaoui *et al.*, personal communication). There were ester compounds similar to a ripe banana smell, released from the overnight medium culture of yeast transformed with CM-AAT1 in the absence of any exogenous precursor, but not the control cell transformed with the vector only. The protein was capable of producing ester compounds, some of which have not been reported in previous studies, from numerous alcohols and acyl-CoAs (Table 3.5).

In vitro, the protein was active over pH 6.0 to pH 8.0 with Mg^{2+} preferred. The AAT activity was stable at 100-500 mM NaCl, but reduced markedly if the reaction contained more than 50 mM $MgCl_2$. There was no obvious effect on the AAT activity, if DDT concentration was less than 5 mM, but there was approximately 60% inhibition at 50 mM DDT. The highest activity of CM-AAT1 protein was 2000 pmol/h/ μg total protein hexanoylation of *E*-2-hexenol (Table 3.5). The AAT activity was higher with the longer chain of aliphatic alcohols, for instance, butanol < hexanol < heptanol. The position of a methyl group on branched alcohols affected the AAT activity. The activity of acetylation, propionylation, and hexanoylation, on 2-methyl butanol was 23%, 22%, and 30% higher than on 3-methyl, respectively. With benzyl alcohol, AAT exhibited at least 2-fold less activity than with 2-phenyl ethanol. The aromatic alcohols, 2-phenyl ethanol had much greater affinity to the enzyme, compared with

Table 3.5 Substrate specificity of the recombinant *CM-AAT1* enzyme towards different types of alcohols and acyl-CoAs (Yahyaoui *et al.*, 2002).

Activity was measured in yeast extracts, expressed in pmol/h/µg protein as the mean ± SD of three replicates. TR: present at trace amounts; ND: non detectable; NT: not tested; +: reported in the literature; NR: not reported in the literature (Homatidou *et al.*, 1992; Wyllie and Leach, 1990).

Alcohols	Acetyl-CoA	Esters reported in melon	Propionyl-CoA	Esters reported in melon	Hexanoyl-CoA	Esters reported in melon
Ethanol	TR	+	ND	+	TR	+
Butanol	383 ± 12	+	535 ± 6	+	500 ± 19	+
Hexanol	1263 ± 35	+	1386 ± 21	NR	1883 ± 270	NR
Heptanol	1310 ± 135	+	ND	NR	NT	NR
Nonanol	ND	+	ND	NR	NT	NR
2-Methyl butanol	916 ± 35	+	1015 ± 17	NR	1434 ± 21	+
3-Methyl butanol	796 ± 4	+	875 ± 69	NR	1000 ± 36	NR
3-Methyl-2-buten-1-ol	610 ± 70	+	NT	NR	NT	NR
Linalool	ND	NR	NT	NR	NT	NR
Z-2-Hexenol	1000 ± 3	NR	670 ± 10	NR	814 ± 32	NR
E-2-Hexenol	1400 ± 15	NR	1285 ± 73	NR	2050 ± 75	NR
Z-3-Hexenol	1270 ± 33	+	ND	NR	1960 ± 33	NR
E-3-Hexenol	850 ± 19	+	ND	NR	1393 ± 123	NR
Benzyl alcohol	555 ± 48	+	1032 ± 47	+	935 ± 85	NR
1-Phenyl ethanol	322 ± 8	+	865 ± 23	NR	390 ± 49	NR
2-Phenyl ethanol	1323 ± 160	+	1760 ± 100	+	1915 ± 42	NR

Table 3.6 Kinetic properties of recombinant *CM-AAT1* (Yahyaoui *et al.*, 2002).

Co-substrate S1 (variable concentration)	Co-substrate S1 (saturating concentration)	Apparent Km (S1)	Vmax (pmol/h/µg protein)
1-Butanol	Acetyl-CoA	8.0 mM	400
1-Hexanol	Acetyl-CoA	1.4 mM	1200
Acetyl-CoA	1-Butanol	100 µM	350
Acetyl-CoA	1-Hexanol	85 µM	1100
Hexanoyl-CoA	1-Butanol	90 µM	350

1-phenyl ethanol. Alteration of the carbon double bond in the alcohols affected the CM-AAT1 activity. From *Z*-2 to *E*-2-hexenol, the activity was higher, but, in contrast, *E*-3 hexenol was a better substrate than *Z*-2-hexenol. Furthermore, when the position was changed from Z-2 to Z-3-hexenol, the activity was higher, but in contrast to the *trans*-forms.

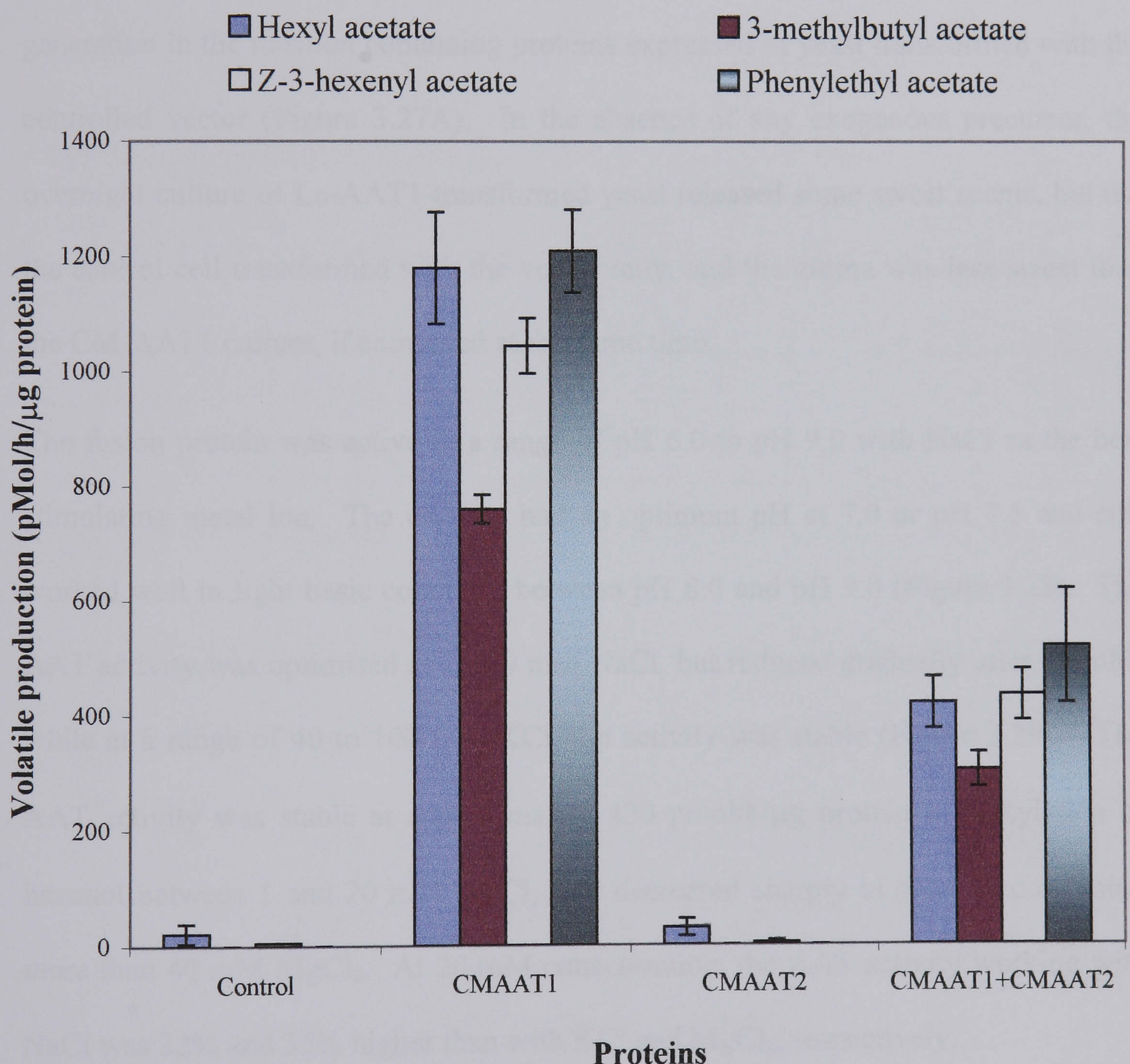
Table 3.6 shows some kinetic properties of the recombinant CM-AAT1 protein. Kinetic studies using a fixed concentration of acetyl-CoA (250 μM) showed that the Km value for butanol (8.0 mM) was much higher than the one for hexanol (1.4 mM). On the other hand, when concentrations of butanol and hexanol were fixed at 40 mM, the apparent Km for acetyl-CoA were 100 and 85 μM, respectively. The apparent Km for hexanoyl-CoA (90 μM) and for acetyl-CoA (100 μM) were similar under 40 mM-fixed butanol.

3.3.3.2 CM-AAT2

Although CM-AAT2 is 86% identical to CM-AAT1at the amino acid level (Figure 3.22), CM-AAT2 fusion protein, expressed either in *E. coli* or in *Saccharomyces cerevisiae*, did not show AAT activity (Figure3.26; Yahyaoui et al., 2002). The experimental combinations of half CM-AAT1 (83 μg) and half CM-AAT2 (83 μg) fusion proteins in the reactions did not show a positive interaction of the AAT activity (Figure 3.26). The volatile compound productions from the reaction of the combination were less than 50% total activity from the CM-AAT1 protein.

Figure 3.26 Volatile production generated from fusion proteins

Hexanol, 3-mthylbutanol, *Z*-3-hexenol and phenyl ethanol were acetylated by CM-AAT1, CM-AAT2, and the combination of CM-AAT1 + CM-AAT2 fusion proteins. Data are means ± SD of 3 replicates.



Proteins

3.3.3.3 Le-AAT1

Le-AAT1 (the tomato ORF sequence of TC98820, TIGR) was expressed as a fusion protein in yeast, *Saccharomyces cerevisiae*, and also exhibited AAT activity. In Figure 3.27, for example, the Le-AAT1 protein modified butanol with acetyl CoA to butanyl acetate (Figure 3.27B, at RT 7.97), or with propionyl CoA to butanyl propionate (Figure 3.27C, at RT 10.33), respectively, but there was no ester generation in the reaction containing proteins expressed in yeast transformed with the controlled vector (Figure 3.27A). In the absence of any exogenous precursor, the overnight culture of Le-AAT1-transformed yeast released some sweet scents, but not the control cell transformed with the vector only, and the aroma was less sweet than the CM-AAT1 culture, if compared at the same time.

The fusion protein was active in a range of pH 6.0 to pH 9.0 with NaCl as the best stimulating metal ion. The enzyme had an optimum pH at 7.0 or pH 7.5 and still worked well in light basic condition between pH 8.0 and pH 9.0 (Figure 3.28). The AAT activity was optimised at 10-20 mM NaCl, but reduced gradually after 40 mM, while at a range of 40 to 100 mM KCl, the activity was stable (Figure 3.29). The AAT activity was stable at approximately 430 pmol/h/μg protein of acetylation of hexanol between 1 and 20 mM $MgCl_2$, but decreased sharply in solution containing more than 40 mM $MgCl_2$. At 20 mM concentration, the AAT activity working with NaCl was 22% and 35% higher than with KCl and $MgCl_2$, respectively.

Although ripe tomato comprises very low ester compounds, the Le-AAT1 fusion protein exhibited the ability to modify various alcohols and some acyl-CoAs (Table 3.7). The AAT activity of the Le-AAT1 fusion protein was effective at acetylation and propionylation, but not hexaonylation. In general, the activities of Le-AAT1

Figure 3.27 Volatile compounds extracted from enzyme assay reactions containing proteins expressed in transformed yeast with the vector only (A), and with the vector harboring the *Le-AAT1* gene (B, C, D) by GC/MS.

The reaction contained 40 mM butanol with 250 μM acetyl-CoA (A and B), or with 250 μM propionyl-CoA (C), or with 250 μM hexanoyl-CoA (D). IS is defined as the internal standard peak of 5 μl/L pentene.

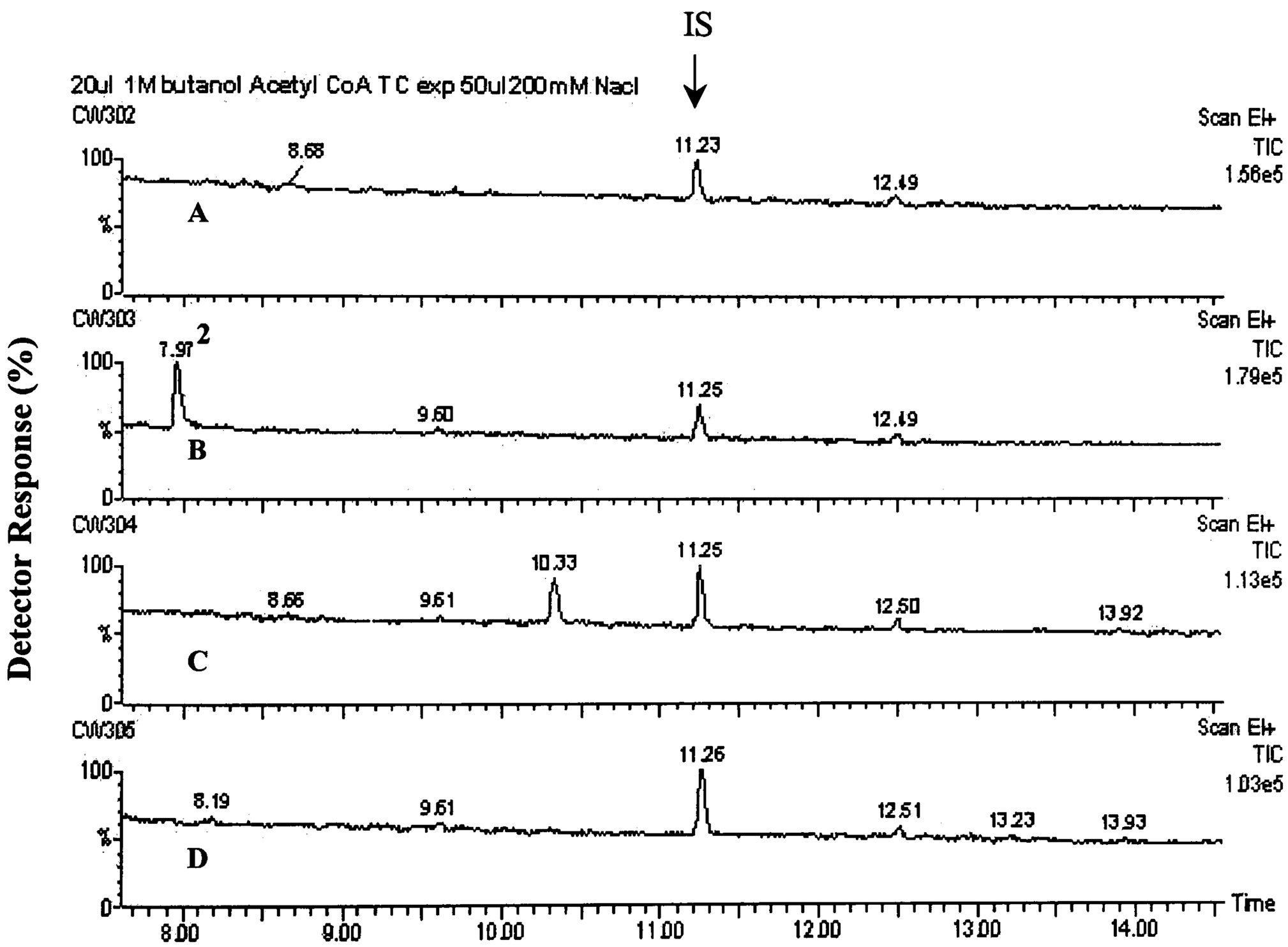


Figure 3.28 Le-AAT1 activities in buffer of different pH

Activities of Le-AAT1 protein on hexanol and acetyl-CoA were measured in buffer adjusted to between pH 5.5 and pH 9.0. Data are means ± SD of 3 replicates.

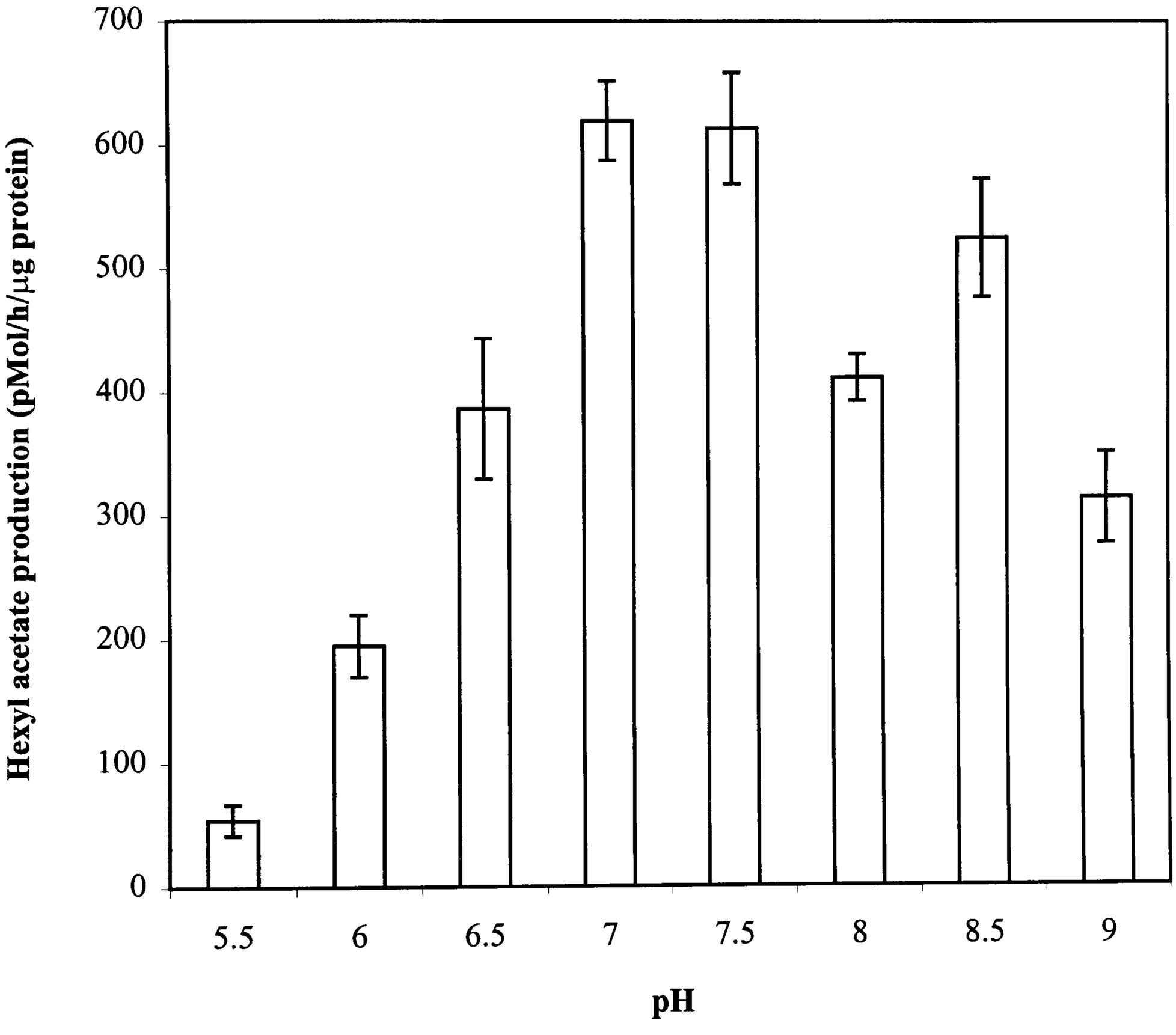


Figure 3.29 Le-AAT1 activities in buffer with different metal ions.

Activities of Le-AAT1 protein on hexanol and acetyl-CoA were measured in buffer with different concentration of $MgCl_2$ (O), KCl (□), or NaCl (Δ). Data are means ± SD of 3 replicates.

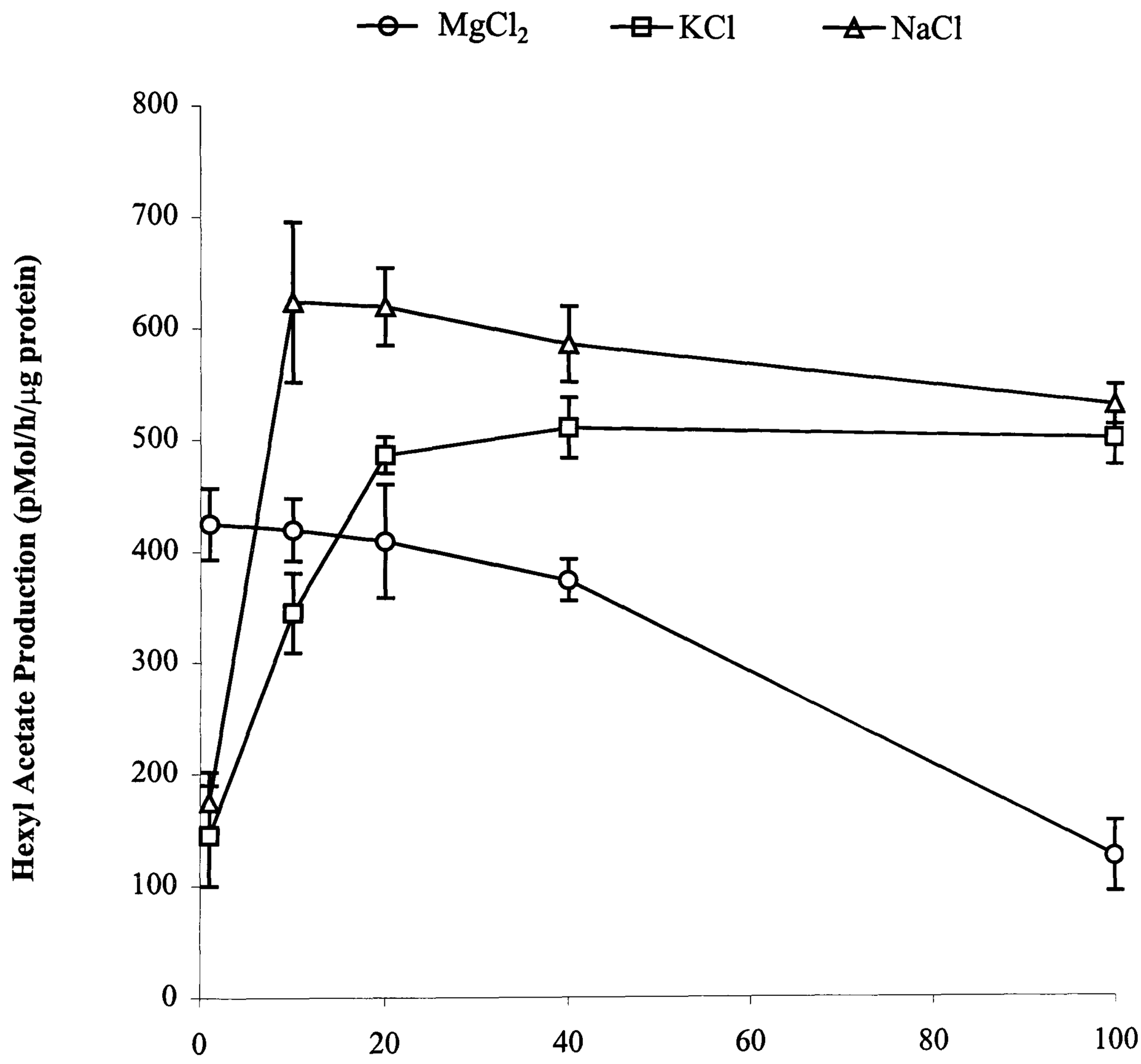


Table 3.7 Substrate specificity of the recombinant *Le-AAT1* enzyme towards different types of alcohols and acyl CoAs

Activity was measured in yeast extracts, expressed in pmol/h/μg protein as the mean ± SD of three replicates. TR: present at trace amounts; ND: non detectable; NT: not tested; +: reported in the literature; NR: not reported in the literature (Buttery and Ling, 1993; Buttery *et al.*, 1971).

Alcohols	Acetyl-CoA	Esters reported in tomato	Propionyl-CoA	Esters reported in tomato	Hexanoyl-CoA	Esters reported in tomato
Ethanol	TR	+	ND	NR	ND	NR
Butanol	419 ± 63	+	284 ± 45	NR	TR	NR
Hexanol	614 ± 43	NR	344 ± 84	NR	98 ± 7	NR
Heptanol	102 ±16	NR	TR	NR	NT	NR
Nonanol	169 ±14	NR	TR	NR	NT	NR
2-Methyl butanol	705 ±37	NR	980 ± 115	NR	112 ± 9	NR
3-Methyl butanol	415 ±30	+	348 ± 48	NR	96 ± 8	NR
3-Methyl-2-buten-1-ol	334 ±16	NR	TR	NR	TR	NR
Linalool	ND	NR	NT	NR	NT	NR
E-2-Hexenol	545 ± 74	NR	263 ±14	NR	TR	NR
Z-3-Hexenol	511 ± 43	+	630 ± 48	NR	102 ± 19	NR
E-3-Hexenol	472 ± 15	NR	526 ± 31	NR	86 ± 17	NR
Benzyl alcohol	349 ± 24	NR	323 ± 60	NR	NT	NR
1-Phenyl ethanol	126 ± 11	NR	TR	NR	ND	NR
2-Phenyl ethanol	287 ± 29	NR	269 ± 13	NR	NT	NR

Table 3.8 Kinetic properties of recombinant *Le-AAT1*

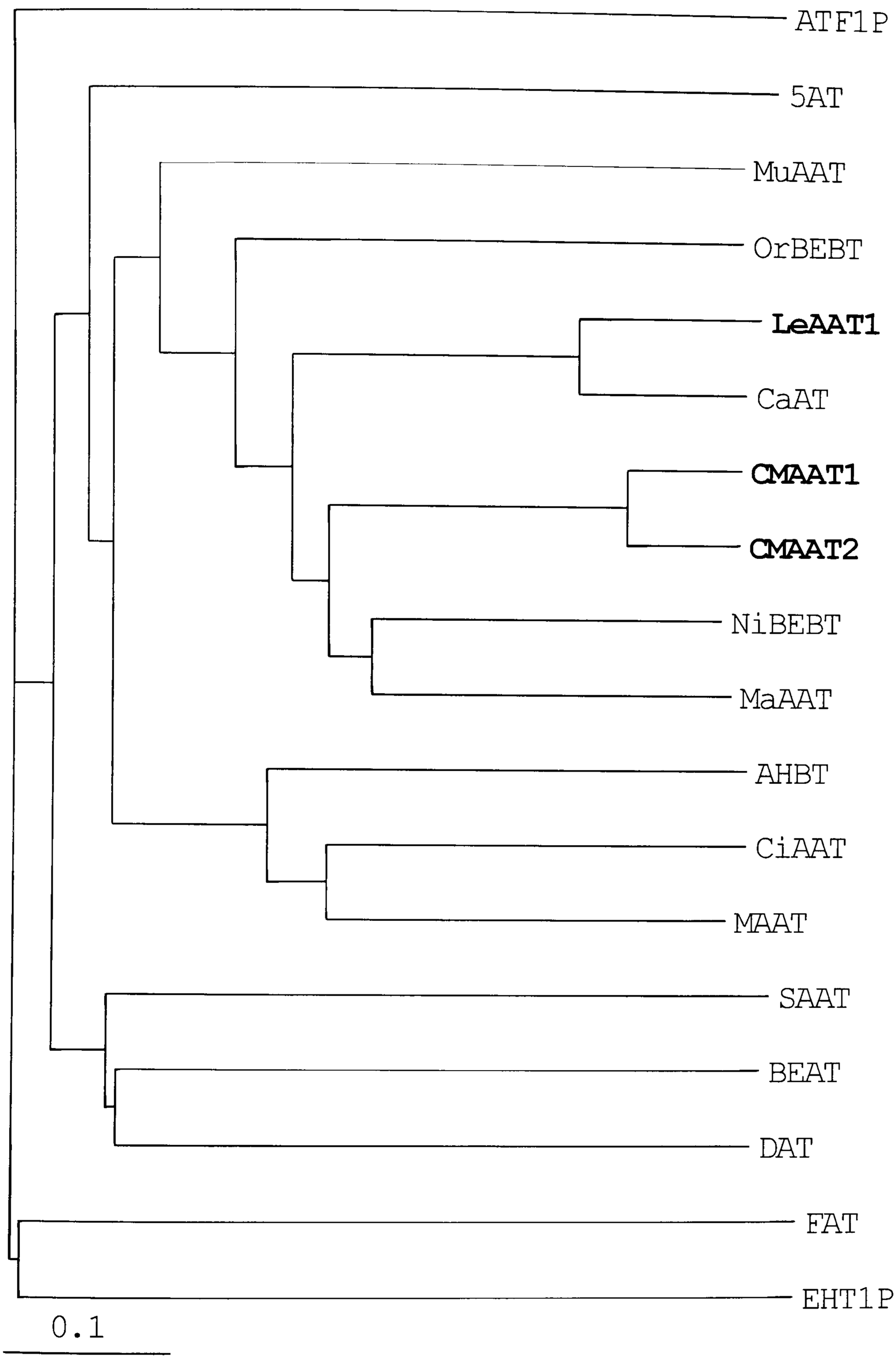
Co-substrate S1 (variable concentration)	Co-substrate S1 (saturating concentration)	Apparent Km (S1)	Vmax (pmol/h/μg protein)
1-Butanol	Acetyl-CoA	10.0 mM	400
1-Hexanol	Acetyl-CoA	6.5 mM	600
Acetyl-CoA	1-Butanol	90 μM	350
Acetyl-CoA	1-Hexanol	85 μM	550
Propionyl-CoA	1-Butanol	90 μM	300

were lower than those of CM-AAT1. Ethyl-donor was a preferred precursor for Le-AAT1. Ethanol was acetylated in trace amounts with no propionated and hexanoylated reactions. The activity was 46% higher, if hexanol was used as precursor instead of butanol, but the activity dropped sharply with longer carbon chain alcohols such as heptanol and nonanol (Table 3.7). From hexanol to *Z*-3-hexenol, the Le-AAT1 activity dropped by 15%, while the activity of *Z*-3-hexenol was similar to *E*-3 hexenol. The highest activity of Le-AAT1 was 1000 pmol/h/μg total protein propionylation of 2-methyl butanol. The methyl position showed some effects on the Le-AAT1 activity. The activity of the enzyme reduced dramatically from using 2-methyl to 3-methyl butanol both in acetylation and propionylation. In the aromatic alcohol group, unlike CM-AAT1, benzyl alcohol was acetylated to 350 pmol/h/μg protein that is 170% higher than acetylation of 2-phenyl ethanol of which exhibited 2-fold activity over 1-phenyl ethanol.

Kinetic studies using a fixed concentration of acetyl-CoA (250 μM) showed that the Km value for butanol (10.0 mM) was much higher than the one for hexanol (6.5 mM) (Table 3.8). When concentrations of butanol and hexanol were fixed at 40 mM, the apparent Km for acetyl-CoA were similar at 90 and 85 μM, respectively. The apparent Km for propionyl-CoA under 40 mM-fixed butanol was 90 μM and 85 μM for acetyl-CoA.

3.3.4 Phylogenetic Relationship of Some Acyl-Transferases

From amino acid sequence analysis of 18 proteins known to use acyl CoAs as substrates, they are separated into 3 main groups (Figure 3.30). One is the yeast ATF1P, an alcohol *O*-acetyl-transferase 1 (AATASE 1) of *Saccharomyces cerevisiae* (Accession No NP_015022) this showed no homology with the CM-AAT1 and Le-AAT1. Another one comprises the FAT, a long-chain-alcohol *O*-fatty-acyl-transferase family (wax synthase) (Accession No NP_199955) of *Arabidopsis thaliana*, and the yeast Eht1, an alcohol acyl-transferase (lipid metabolism) (Accession No NP_009736) of *Saccharomyces cerevisiae*. The other group is a big group of *O*-acyl-transferases, containing 2 subgroups. The SAAT (Accession No AAG13130) from strawberry fruit, the BEAT (acetyl-CoA:benzylalcohol acetyl-transferase, Accession No AF043464) from flower of *Clarkia breweri*, and the DAT (deacetylvindoline 4-*O*-acetyl-transferase, Accession No AAC99311) of *Catharanthus roseus*, are in the same group. The 5-AT (Anthocyanin 5-aromatic acyl transferase, Accession No BAA74428), producing coloured flavoniod compounds in *Gentiana triflora* (Fujiwara *et al.*, 1998), is among 9 AATs, producing aromatic volatiles. The melon AATs are closely related to the NiBEBT (52% identity) (benzoyl-Co A:benzyl alcohol benzoyl-ransferase, Accession No AAN09798) of *Nicotiana tabacum* and apple AAT (47% identity) (MaAAT, Accession No CAC09064) and then to the tomato Le-AAT1 (43% identity). The Le-AAT1 has highest homology with CaAT (80% identity) (Accession No AAN85436), the acyl-transferase 2 from *Capsicum chinense*, which is a placental specific acyl-transferase gene.


ATF1P
5AT
MuAAT
OrBEBT
LeAAT1
CaAT
CMAAT1
CMAAT2
NiBEBT
MaAAT
AHBT
CiAAT
MAAT
SAAT
BEAT
DAT
FAT
EHT1P
0.1

3.4 Analysis of Aromatic Volatiles from Transgenic Tomatoes

3.4.1 Selection of Transgenic Tomatoes

Four transgenic constructs were introduced to tomato, 3 constructs with the *CM-AAT1* cDNA as a sense and a construct with the *Le-AAT1* cDNA in the antisense orientation. According to the literature about mRNA translational efficiency in eukaryotes (Kosak, 1987; Kozak, 1991; Pain, 1996), 0.2 Kb 5'UTR of the endogenous *Le-AAT1* was considered to be fused at the 5' end of the *CM-AAT1* ORF. The fusion sequences were constructed under a 35S promoter (named 35S-UCMAAT1; Figure 2.7) and under a fruit-specific ACO1 promoter (named ACOP-UCMAAT1; Figure 2.9). Thus only *CM-AAT1* ORF was made under a 35S promoter (named 35S-CMAAT1, Figure 2.6) as an additional control for sense transgenics. The antisense construct was designed from a 0.5 Kb region near the 3' end of the *Le-AAT1* sequence, controlled by the 35S promoter (35S-ASLeAAT1; Figure 2.8). The transformants were screened according to their mRNA expression either in young leaves for those under a CaMV35S promoter, or in 1 h-wounded young leave for the ACOP-UCMAAT1 construct.

In 35S-CMAAT1 transformants, 6 lines showed strong expression of the transgene, *CM-AAT1*, and line 2 and line 4 (Figure 3.31A, encircled) were selected to grow in a glasshouse. Line 3 and line 7 from 35S-UCMAAT1 transformants (Figure 3.31B, encircled) and line 7 and 15 from ACOP-UCMAAT1 transformants (Figure 3.31C, encircled) were chosen to grow further in the glasshouse. The untransformanted Ailsa Craig, control, did not exhibit accumulation of the foreign *CM-AAT1* gene. The antisense lines 1, 2, 5, and 7 showed accumulation of 0.5 Kb *Le-AAT1* antisense (Figure 3.32, encircled); only line 1 and 2 were selected to grow for flavour analysis.

Figure 3.31 Selection of *CM-AAT1* over-expression in transgenic tomatoes.

Northern blot analysis of total RNA (5 μg), from tomato leaves carrying constructs 35S-CMAAT1 (A), 35S-UCMAAT1 (B) and from wounded leaves in ACOP-UCMAAT1 (C), probed with the *CMAAT1* probe. The numbers refer to the transgenic lines selected for further analysis. WT is defined as the control wild type. The numbers with cycle show the transgenic lines selected to be grown for volatile analysis. The photographs below the northern blot show the corresponding rRNA in the gel, stained with ethidium bromide.

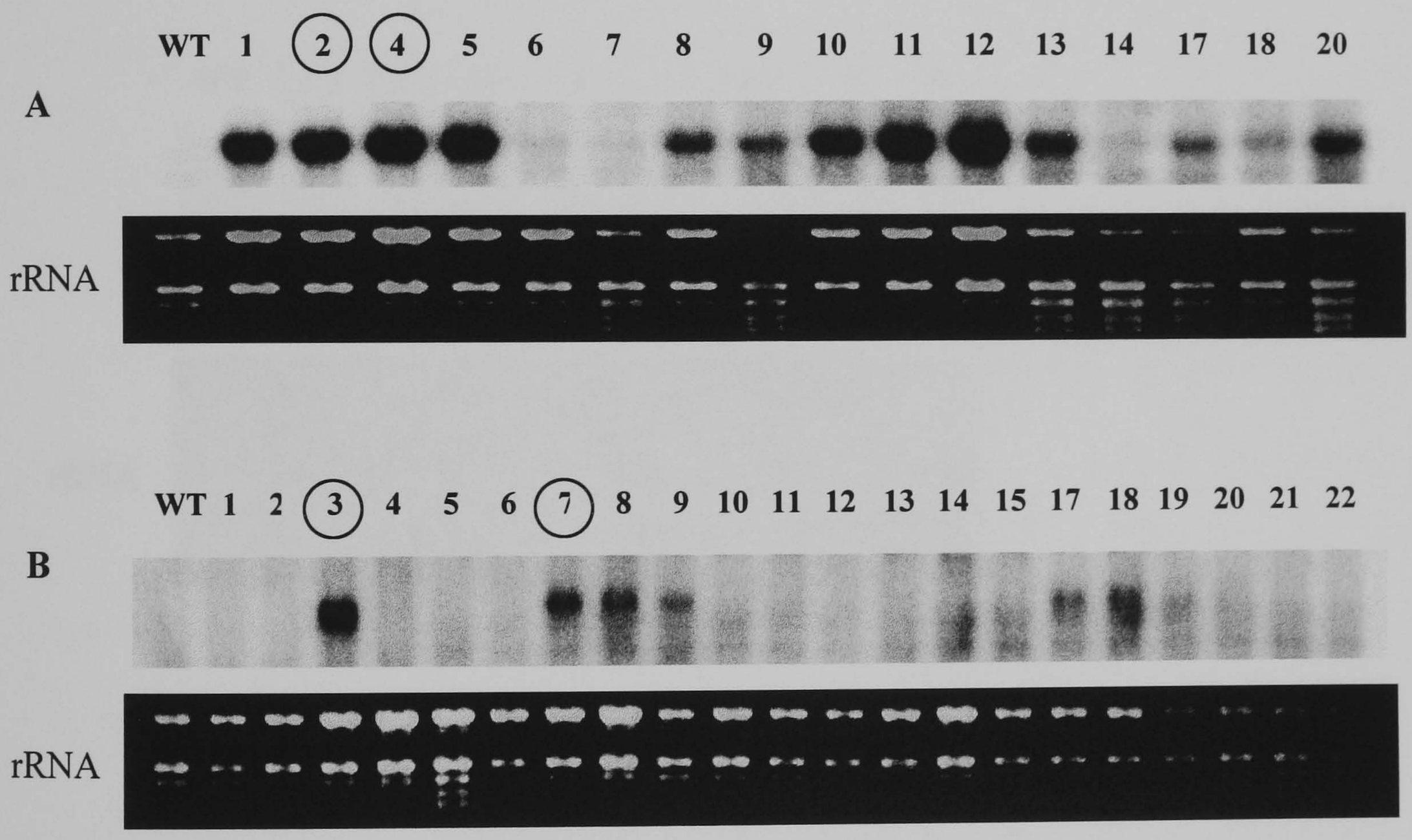
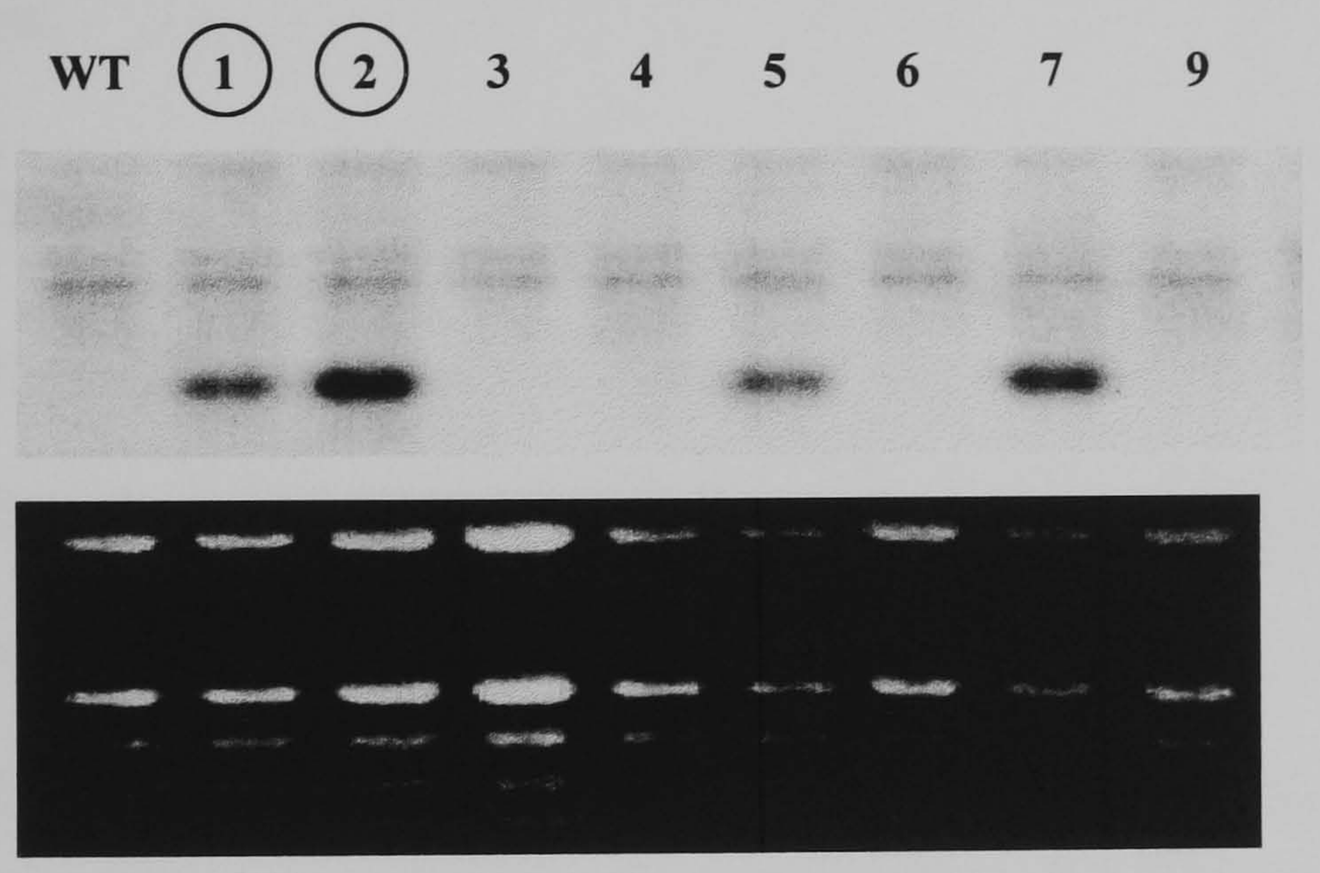


Figure 3.32 Selection of *Le-AAT1* antisense expression in transgenic tomato.

Northern blot analysis of total RNA (5 μg), from tomato leaves of 35S-ASLeAAT1 lines, was probed with the *Le-AAT1* antisense probe. The numbers refer to the transgenic lines selected for further analysis. WT is defined as the control wild type. The numbers with cycle show the transgenic lines selected to be grown for volatile analysis. The photograph below the northern blot shows the corresponding rRNA in the gel stained with ethidium bromide.



3.4.2 Aromatic Volatile Analysis

3.4.2.1 Phenotypes of Transgenic Tomato

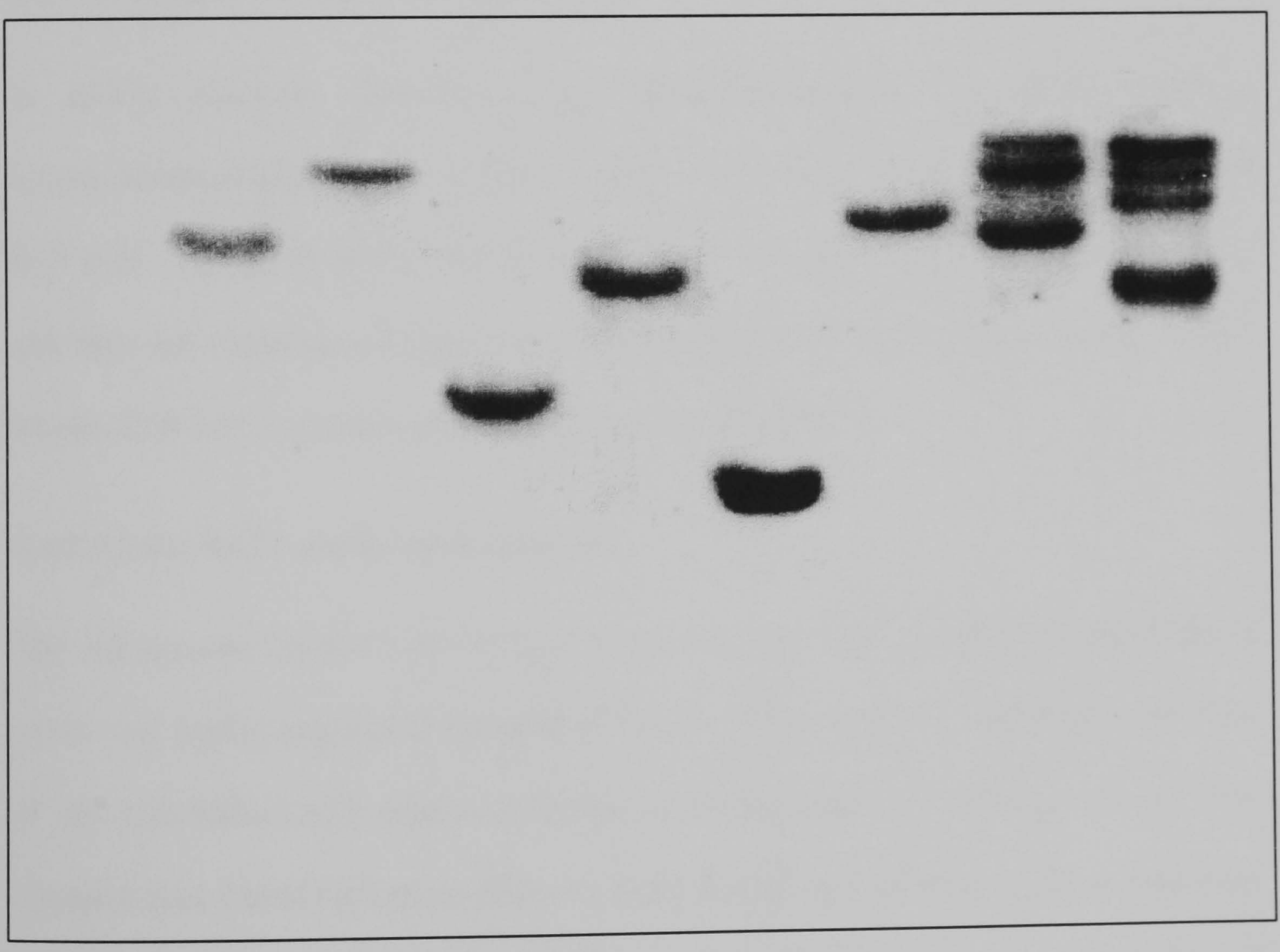
None of the *CM-AAT1* sense and *Le-AAT1* antisense transgenic tomato lines showed differences in growth or plant phenotypes such as stem, leaf, flower and fruit, compared to the control. Fruit ripened normally in all aspects such as colour, texture, or volatiles. There was apparently no difference in volatile scents, released from blended fruit between all transgenic lines and the controls, when making preliminary observations by nose. However the human panel test for fruit scent released was not carried out.

3.4.2.2 Genomic Southern Analysis

The *NPTII* sequence (Figure 2.4), which is fused with the constructs of the transgenes, was used as the probe for detecting the number of transgene copies incorporated into the genomic DNA of the transgenic plants. Six lines under a 35S promoter, namely 35S-CMAAT1 (2), 35S-CMAAT1 (4), 35S-UCMAAT1 (3), 35S-UCMAAT1 (7), 35S-ASLeAAT1 (1), and 35S-ASLeAAT1 (2), were identified as hemizygous lines, which have only one transgene, integrated into the genomic DNA (Figure 3.33). However, 35S-ASLeAAT1 (1) showed a smeared band, which might contains more than one fragment of slightly different lengths. ACOP-UCMAAT1 (7) contains 3 copies of the *CMAAT1* genes, whereas ACOP-UCMAAT1 (15) contains 4 copies of the gene in the genomic DNA. The *NPTII* fragment was not present in the wild type genomic DNA.

Figure 3.33 Genomic Southern of wild type and transgenic tomatoes.

Tomato genomic DNA (35 μg) from wild type (WT) and transgenic lines were digested with *EcoR*I, separated on gel electrophoresis and probed with the *NPTII* sequence.



3.4.3 mRNA Accumulation during Fruit Ripening

3.4.2.3.1 *CM-AAT1* Expression

The expression of *CM-AAT1* under a 35S promoter was found in every stage of fruit development, but the 35S-CMAAT1 (2) was highly expressed at the B+7 and the B+14 fruit, while 35S-CMAAT1 (4) highly expressed at the MG fruit, reduced at the breaker and B+4, and increased again at the B+7 and B+14 fruit (Figure 3.34). In 35S-UCMAAT1 fruit, line 3 exhibited accumulation of *CM-AAT1* at the MG, the B+7 and the B+14 fruit and the expression dropped at the B+4 stage (Figure 3.35). 35S-UCMAAT1 (7) showed high expression at the MG and the B+7 fruit, whereas at the other stages, the expression was lower.

In ACO1 promoter construct lines, ACOP-UCMAAT1 (7) showed highest accumulation of *CM-AAT1* at the breaker fruit and some accumulation at the B+4 and B+7 fruit. ACOP-UCMAAT1 (15) showed high accumulation at the B and B+4 fruit and then the expression dropped at the B+7 and B+14 stages (Figure 3.36). There was no *CM-AAT1* accumulation in any stage of the controls.

3.4.2.3.2 *Le-AAT1* Antisense Expression

The endogenous *Le-AAT1*, showing AAT activity when the protein was expressed in yeast, was highly expressed during fruit ripening (Figure 3.18). Although both lines of the 35S-ASLeAAT1 construct showed high expression of antisense during fruit ripening, line 1 exhibited more effective reduction of accumulation of the endogenous *Le-AAT1* than line 2 (Figure 3.37). The expression of the endogenous *Le-AAT1* was reduced sharply at the MG and the breaker fruit, but slightly increased at the B+4, B+7 and B+14 fruit, although it remained at a low level compared to the control.

Figure 3.34 *CM-AAT1* accumulations in transgenic tomato fruit during ripening

Northern blot analysis of total RNA (8 μg), from different stages of fruit ripening in wild type and the 35S-CMAAT1 Line 2 and Line 4, probed with the *CMAAT1* probe. The photograph below the northern blot shows the corresponding rRNA in the gel stained with ethidium bromide.

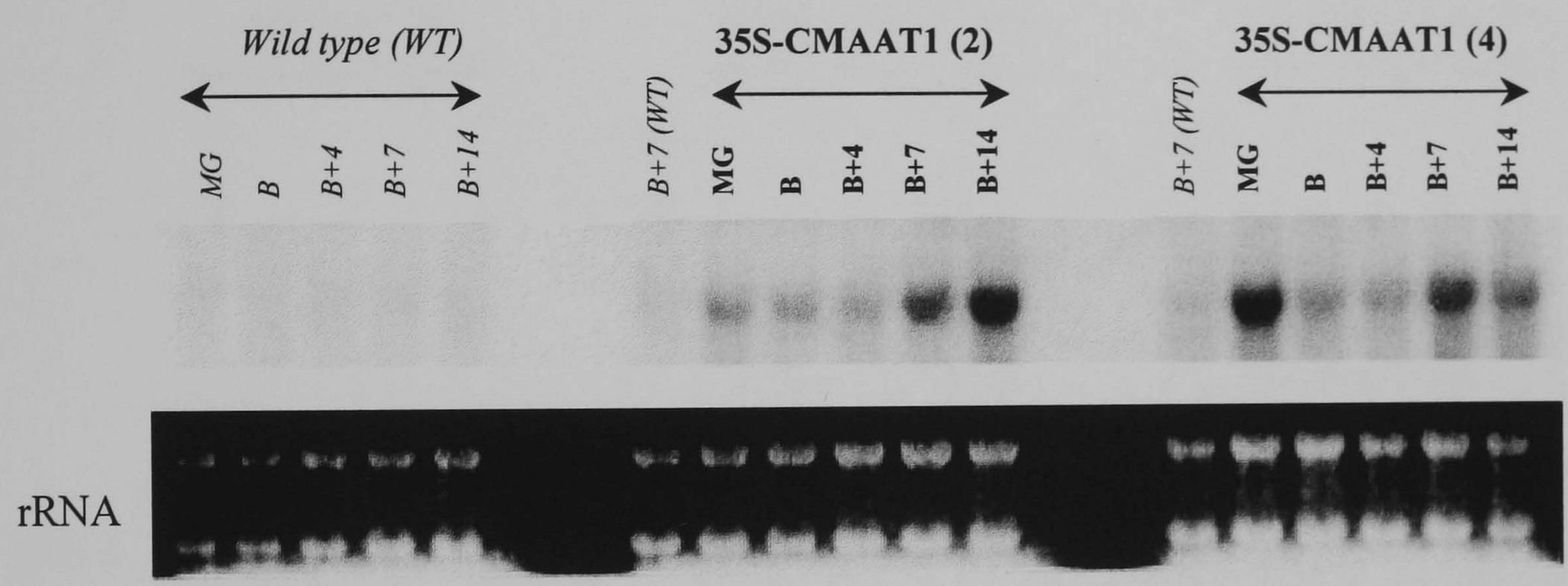


Figure 3.35 *CM-AAT1* accumulations in transgenic tomato fruit during ripening

Northern blot analysis of total RNA (8 μg), from different stages of fruit ripening in wild type and the 35S-UCMAAT1 Line 3 and Line 7, probed with the *CMAAT1* probe. The photograph below the northern blot shows the corresponding rRNA in the gel stained with ethidium bromide.

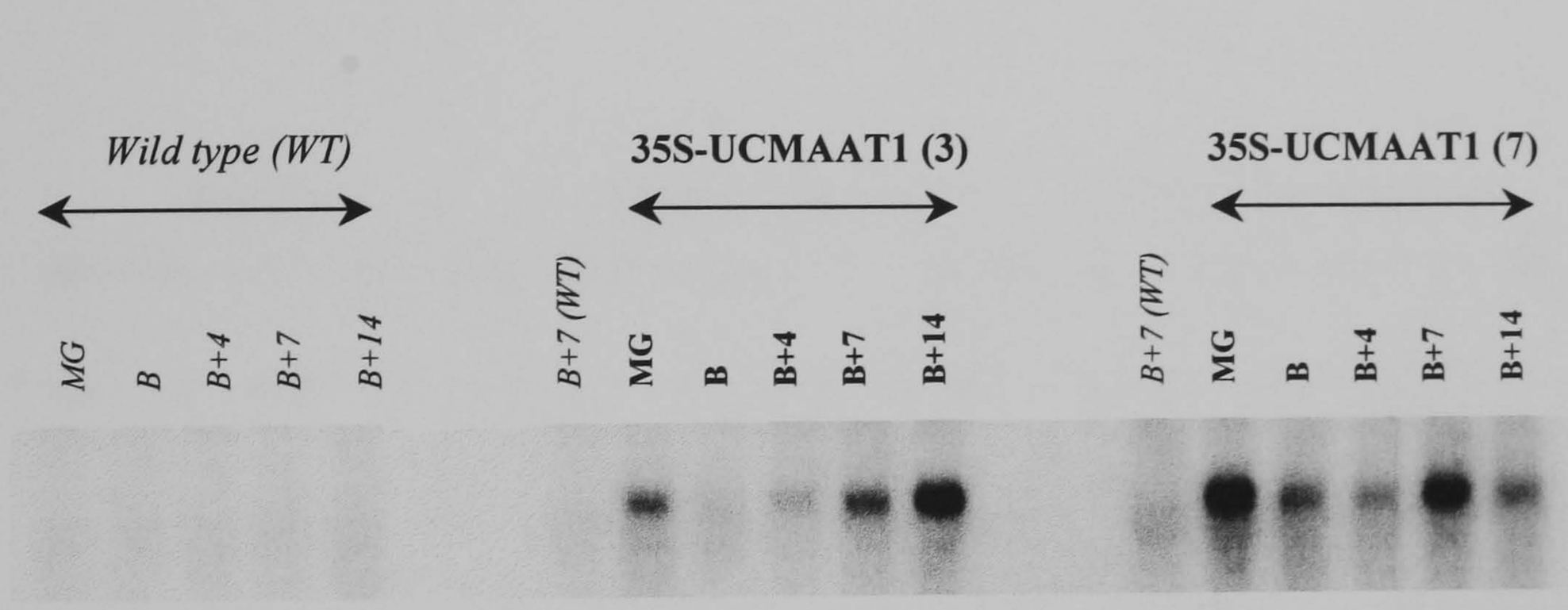


Figure 3.36 *CM-AAT1* accumulations in transgenic tomato fruit during ripening

Northern blot analysis of total RNA (25 μg), from different stages of fruit ripening in wild type and the ACOP-UCMAAT1 Line 7 and Line 15, probed with the *CMAAT1* probe. The photograph below the northern blot shows the corresponding rRNA in the gel stained with ethidium bromide.

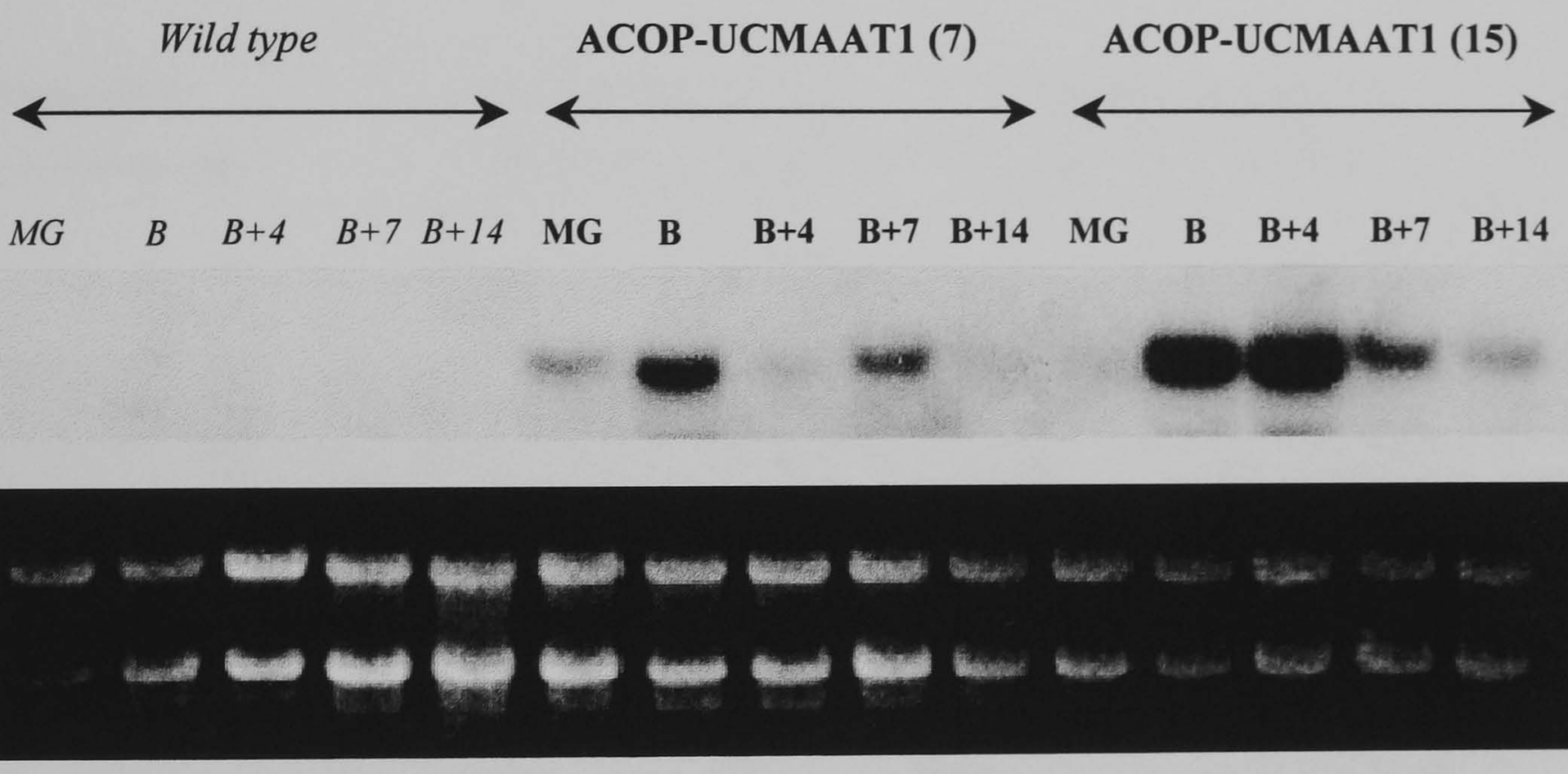
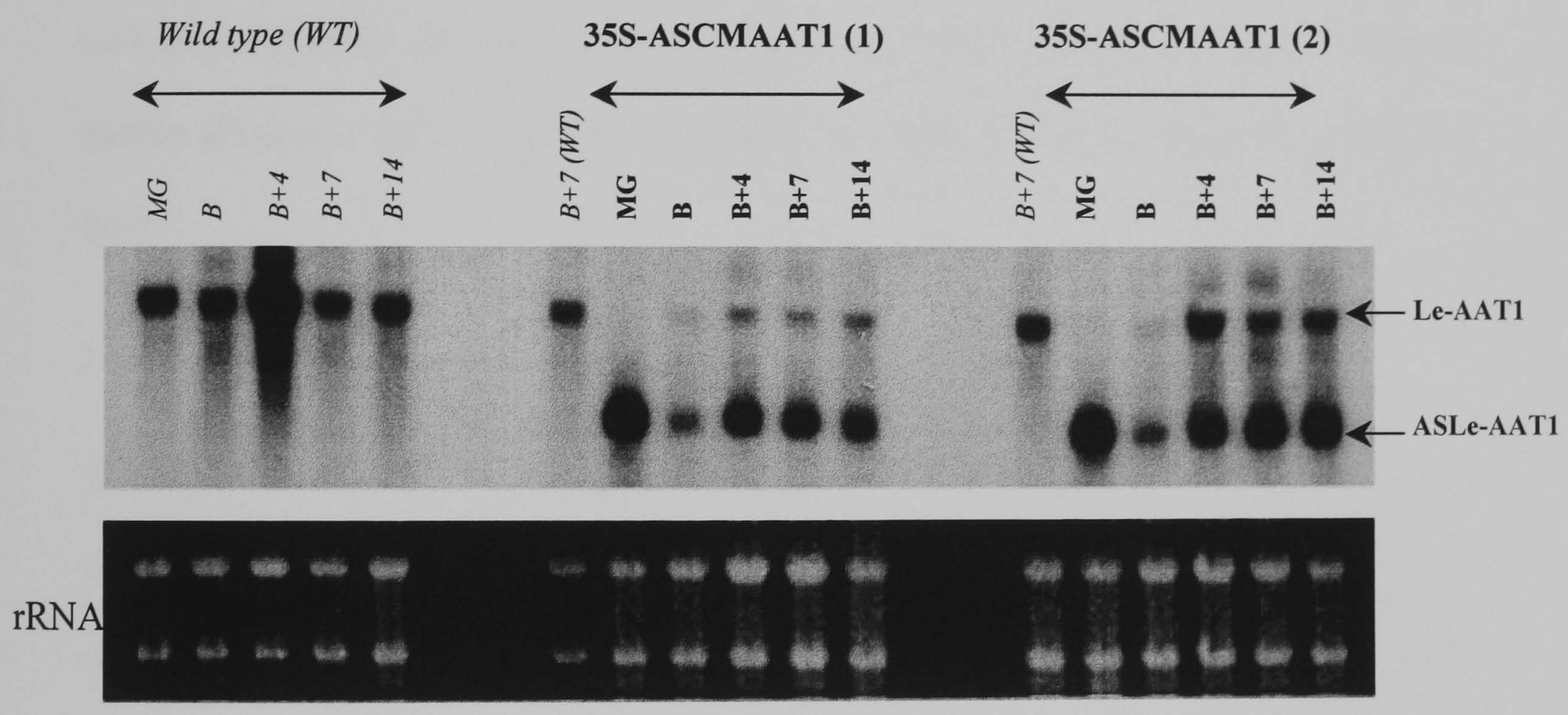


Figure 3.37 *Le-AAT1* down regulation in antisense transgenic tomato fruit during ripening

Northern blot analysis of total RNA (25 μg), from different stages of fruit ripening in wild type and the 35S-AsLeAAT1 Line 1 and Line 2, probed with the *Le-AAT1* probe. The photograph below the northern blot shows the corresponding rRNA in the gel stained with ethidium bromide.



3.4.4 Volatile Profiles

The pH value of the MG fruit was 3.9 and this dropped slightly at the breaker stage, and then gradually rose to approximately 4.2 at the B+14 fruit (Figure 3.36). There was no difference between the pH of fruit extracts from control and transgenic fruits at similar stages. Production of all volatile compounds greatly increased during fruit ripening (Figure 3.39). The volatile compound profiles of wild type fruit showed that the amount of hexanal (Figure 3.39E), *E*-2-hexenal (Figure 3.39F), 3-methylbutanal (Figure 3.39G), hugely increased in homogenates of ripe tomatoes. Ester compounds such as phenylethyl acetate (Figure 3.39A), hexyl acetate (Figure 3.39B), *Z*-3-hexenyl acetate (Figure 3.39C) were rarely produced in green fruit, but increased in the ripe fruit.

3.4.4.1 Ester Compounds

The amounts of ester compounds measured from fruit extracts were variable and showed no difference by statistic analysis between control, *CM-AAT1* sense overexpressed, and *Le-AAT1* antisense expressed transgenic fruit compared in each fruit developing stage. Phenylethyl acetate seemed to be such an indicator for fruit ripening (Figure 3.40). In green fruit, less than 0.02 mg/m^3 of the compound was generated and the production rose by 25 fold during ripening, but below 0.5 mg/m^3 in all fruit. Hexyl acatate was produced at an average of 0.05 mg/m^3 in mature green fruit, increased sharply in breaker fruit (0.14 mg/m^3), and rose to 0.18 mg/m^3 in fully ripe fruit (Figure 3.41). *Z*-3-hexenyl acetate, which was at 0.23 mg/m^3 at the MG stage, exhibited the highest amount in ripe fruit (1.12 mg/m^3) of all esters. (Figure 3.42). 3-methylbutyl acetate in fruit was generated at 0.22 mg/m^3 the MG stage, rose quickly to 0.59 mg/m^3 at the breaker and was at 0.67 mg/m^3 at the B+14 stage (Figure 3.43).

Figure 3.38 Changes of pH in fruit homogenates at different stages of ripening

The pH of blended tomato fruit extracts was measured during ripening. Averages and SD of 6 replicates are given.

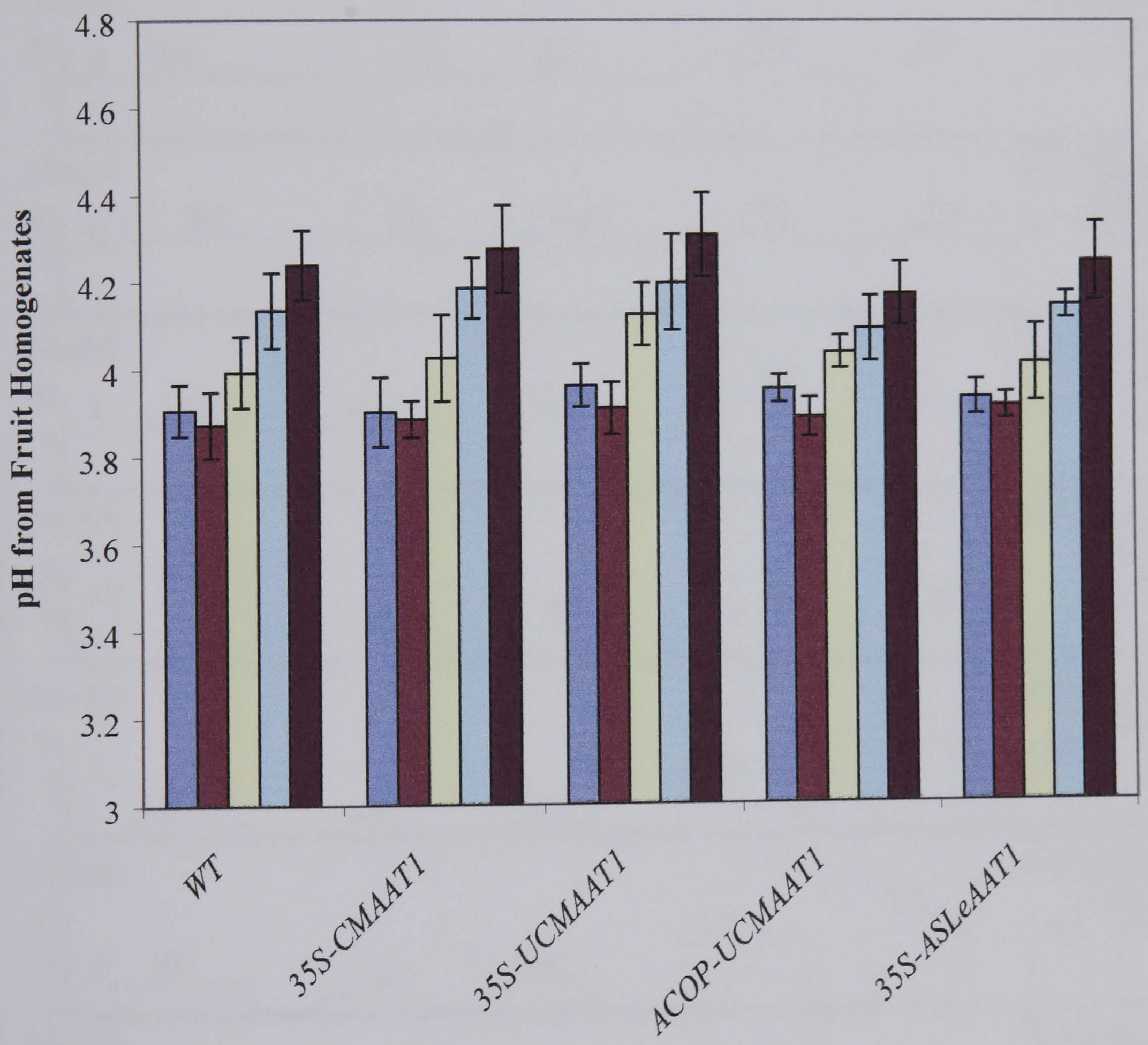


Figure 3.39 Volatile compound profiles detected from APCI

Volatile profiles are shown for selected compounds released from blended fruit at mature green (MG) and 14 days after breaker (B+14) stages of wild type. The compounds are phenylethyl acetate (A), hexyl acetate (B), *Z*-3-hexenyl acetate (C), 3-methylbutyl acetate (D), hexanal (E), *E*-2-hexenal (F), 3-methylbutanal (G).

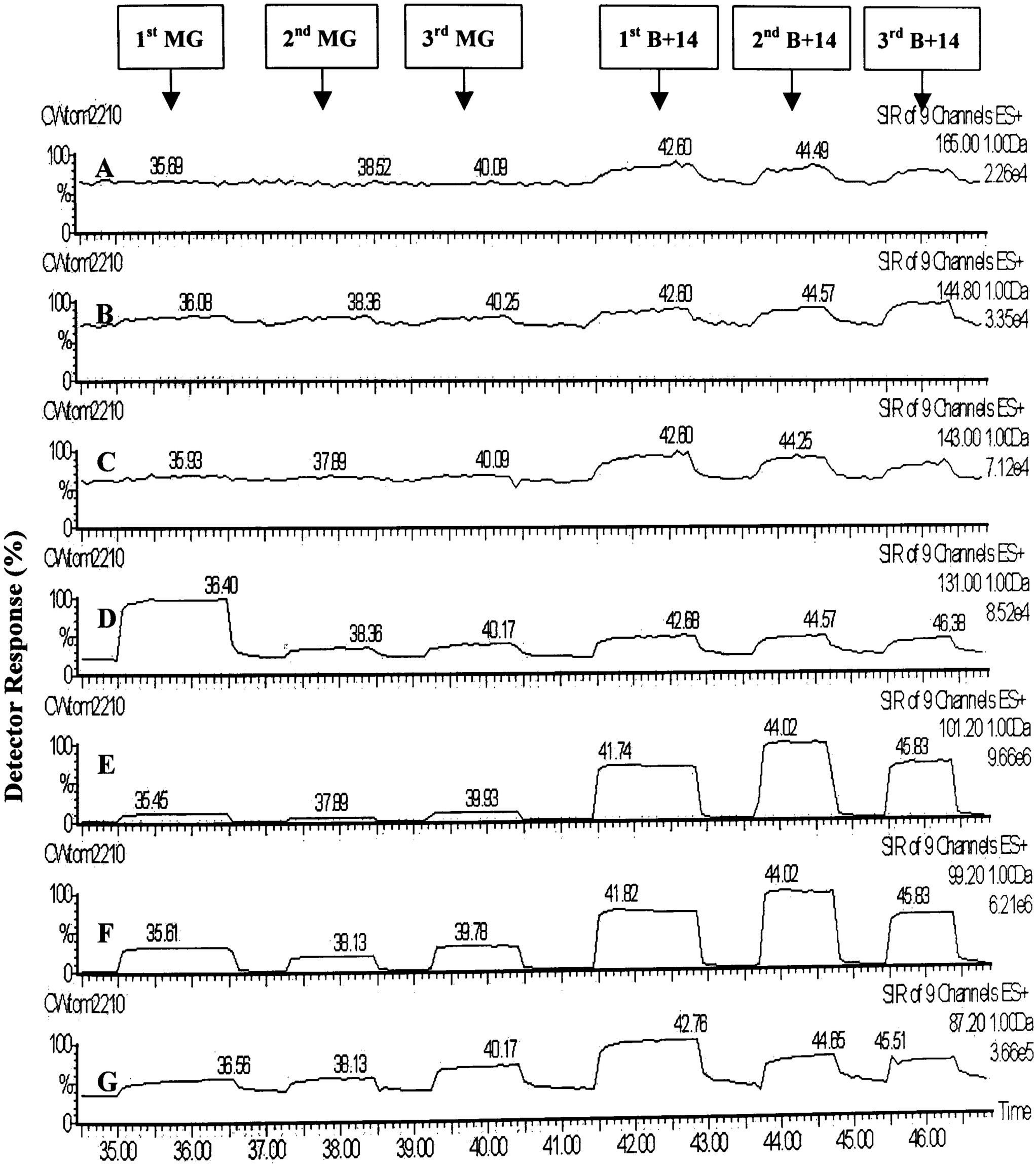


Figure 3.40 Phenylethyl acetate production by homogenates of transgenic tomato during ripening

Concentrations of phenylethyl acetate (mg/m^3), released from macerated fruit of transgenic lines at different stages of ripening were compared to wild type. Averages and SD of 4-5 replicates are given.

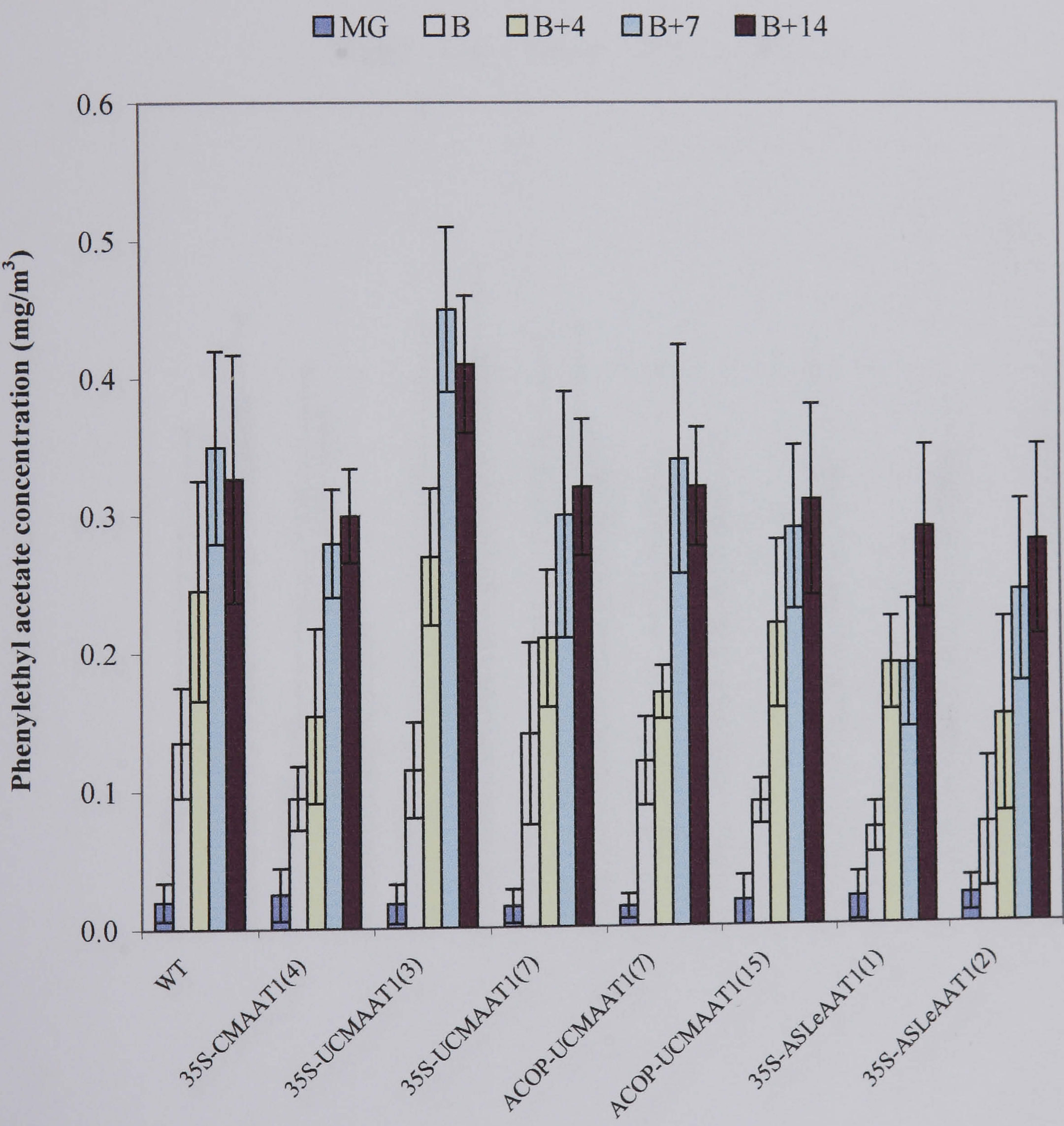


Figure 3.41 Hexyl acetate production by homogenates of transgenic tomato during ripening

Concentrations of hexyl acetate (mg/m^3), released from macerated fruit of transgenic lines at different stages of ripening, were compared to wild type. Averages and SD of 4-5 replicates are given.

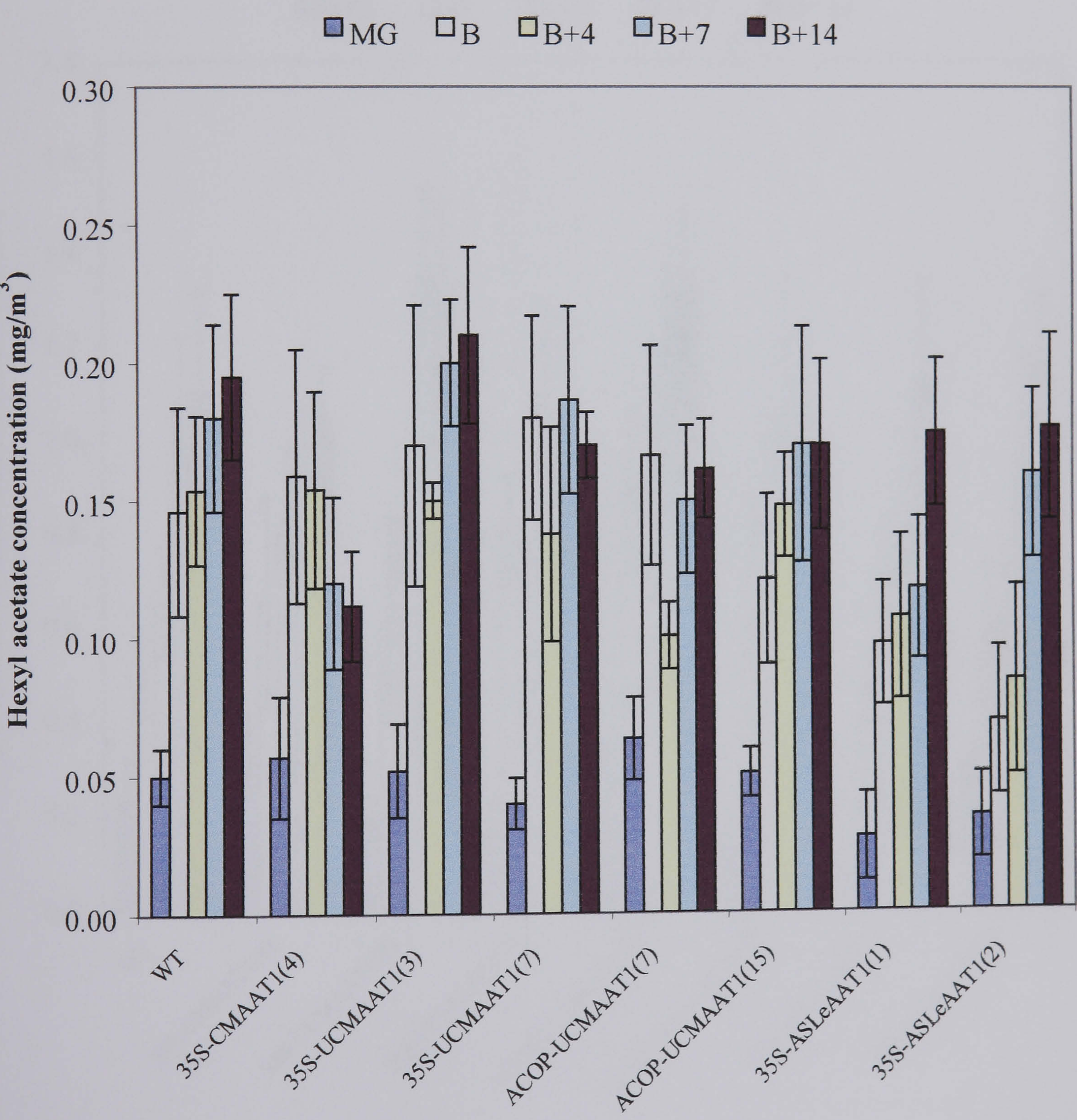


Figure 3.42 *Z*-3-Hexenyl acetate production by homogenates of transgenic tomato during ripening

Concentrations of *Z*-3-hexenyl acetate (mg/m^3), released from macerated fruit of transgenic lines at different stages of ripening, were compared to wild type. Averages and SD of 4-5 replicates are given.

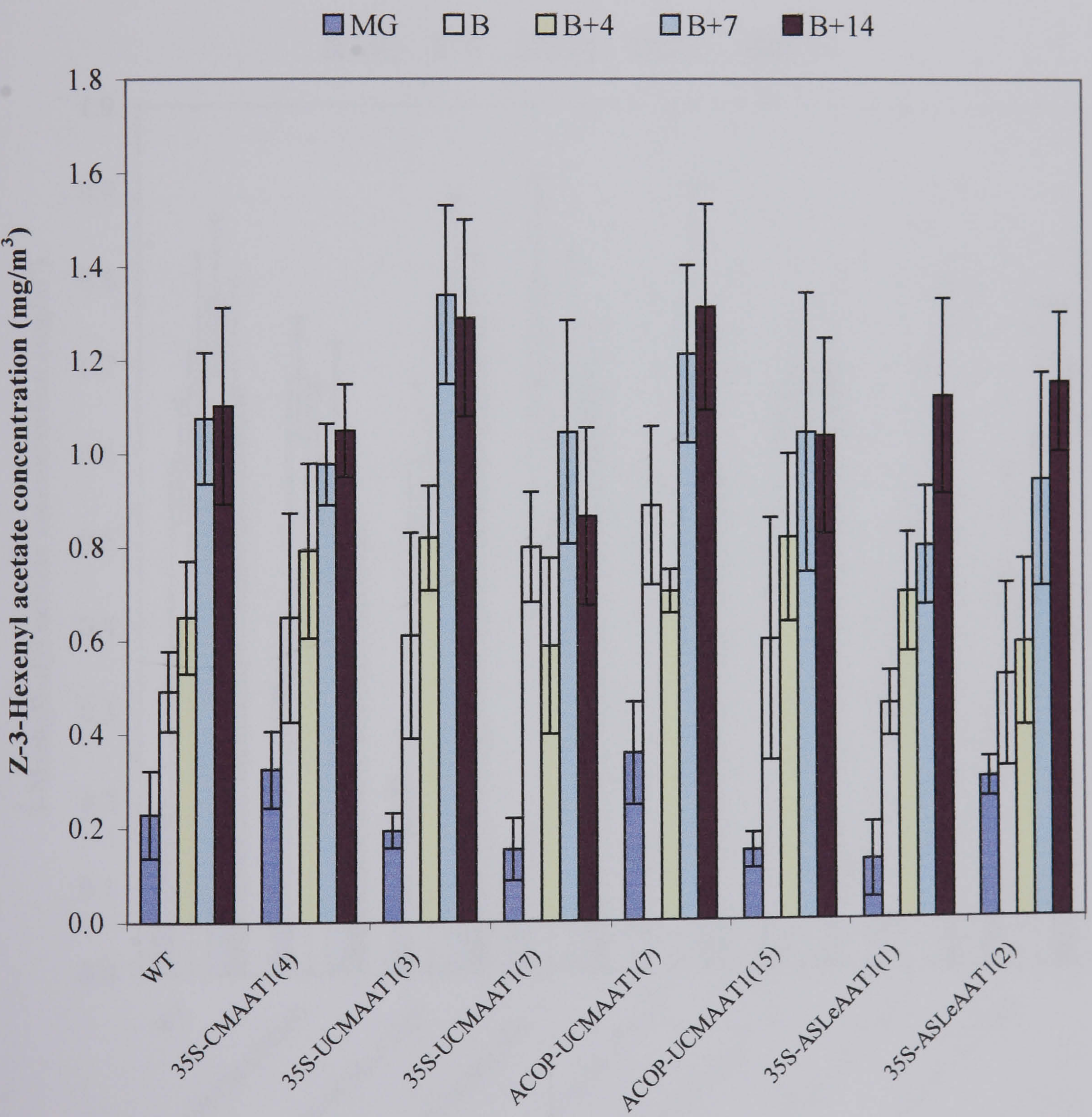
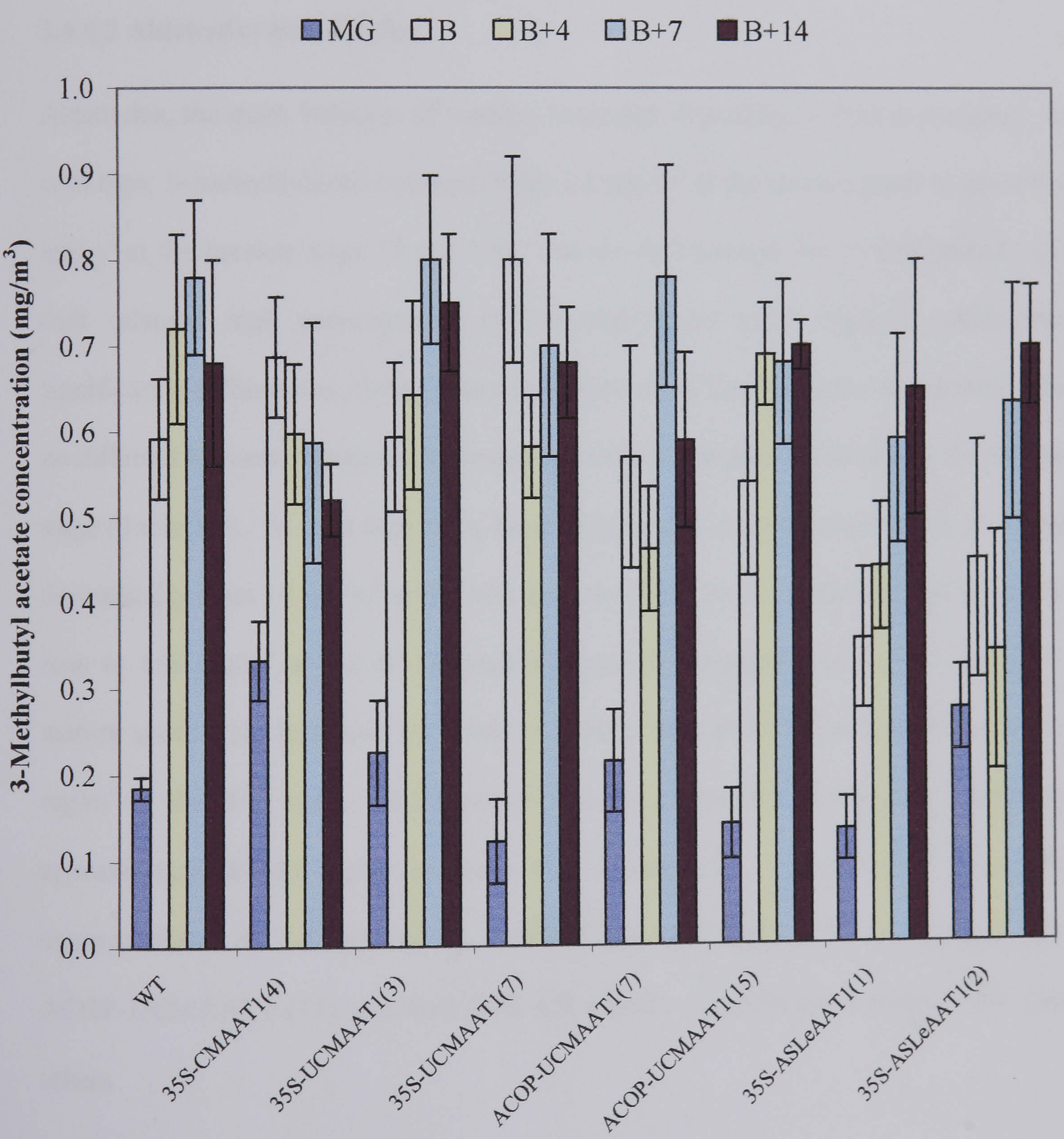


Figure 3.43 3-Methylbutyl acetate production by homogenates of transgenic tomato during ripening

Concentrations of 3-methylbutyl acetate (mg/m^3), released from macerated fruit of transgenic lines at different stages of ripening, were compared to wild type. Averages and SD of 4-5 replicates are given.



The concentration of ester production from ground leaves (only wild type and *CM-AAT1* transgenic tomatoes expressed under the CaMV 35S promoter; Figure 3.44A) and blended fruit when added alcohols and acetyl CoA (Figure 3.44B) in the conditions showed higher production of ester compounds. However, there was no significant difference between transgenic and control tomatoes. 3-Methylbutyl acetate rose greatly with added substrates in both ground and macerated fruit.

3.4.4.2 Aldehydes and Alcohol

Aldehydes, the main volatiles of tomato, increased dramatically during ripening. In wild type, 3-methylbytanal increased from 2.2 mg/m^3 at the mature green stage to 9.4 mg/m^3 at the breaker stage (Table 3.9). At the B+14 stage, the *35S-UCMAAT1* (3) fruit released high concentration of 3-methylbutanal (11.9 mg/m^3), which was significantly different to others. The concentrations of hexanal and *E*-2-hexenal were no different between tomato fruit homogenates when compared at each fruit developing stage (Table 3.9). In wild type fruit, hexanal produced in abundance, which increased dramatically from 19 mg/m^3 at the MG stage to 70.56 mg/m^3 at the breaker stage and rose to 158 mg/m^3 at the B+14 stage whereas *E*-2-hexenal was at 1.17 mg/m^3 in mature green fruit, increased by 2-fold in breaker fruit (3.58 mg/m^3), and rose to 5.3 mg/m^3 at the B+7 stage. *Z*-3-Hexenol was also increased in ripe fruit, which is approximately 8-fold higher to green fruit (Table 3.9). At the B+7 stage, the concentrations of *Z*-3-hexenol in the35S-CMAAT1 (4), ACOP-UCMAAT1 (7), ACOP-UCMAAT1 (15) and both 35S-ASLeAAT1 lines revealed to be lower than others.

Figure 3.44 Ester production from tomato leaves and fruit with added substrates

Concentrations of selected esters, released from ground leaves (A) and macerated B+7 fruit (B), were measured after adding 2 mM of alcohols (3-methyl butanol, hexanol, *Z*-3-hexenol, and phenyl ethanol) and 5 μM acetyl CoA. Averages and SD of 4 replicates are given. WT: wild type, WT-Sub: wild type without substrates.

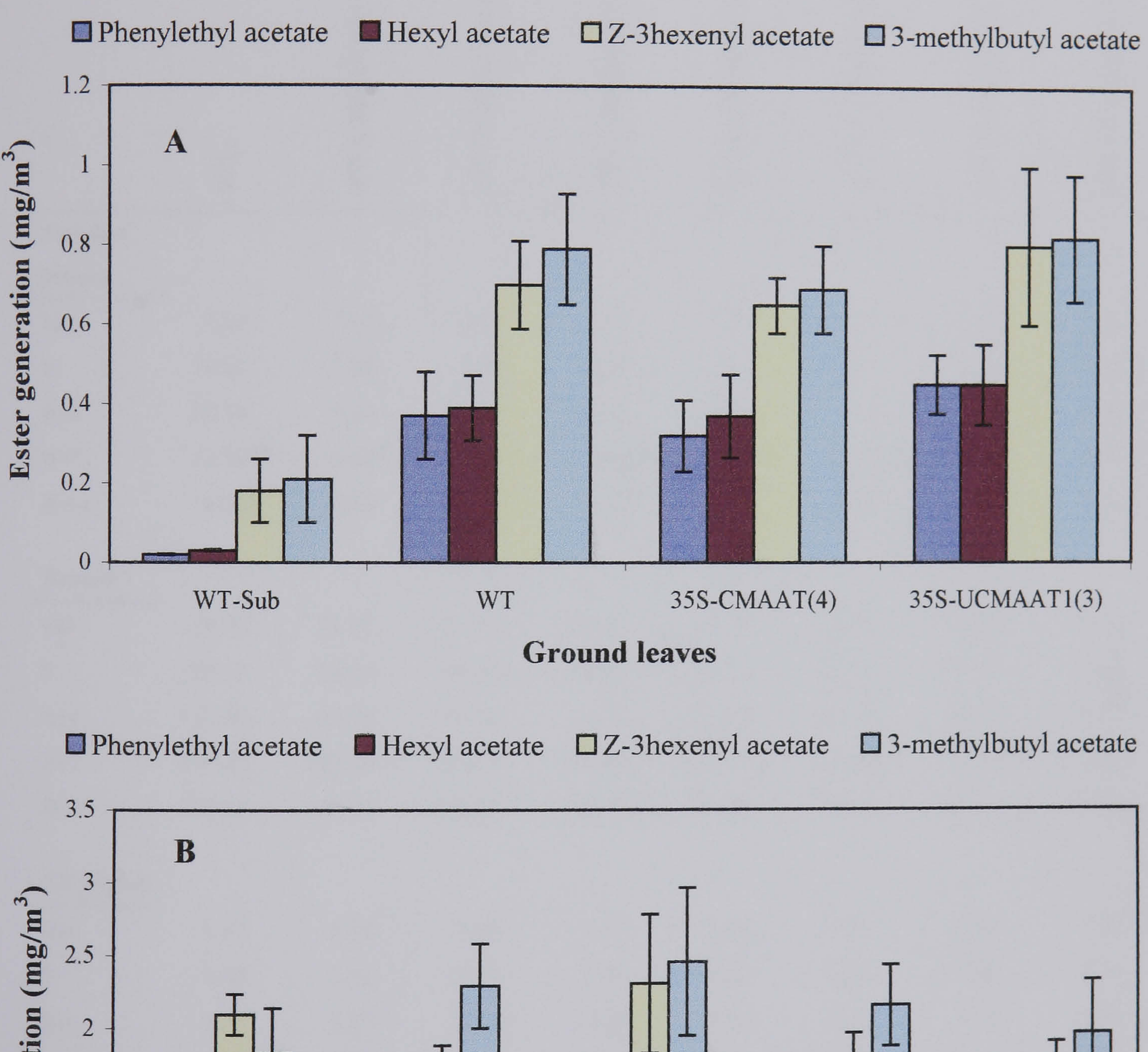


Table 3.9 Flavour volatiles released from transgenic tomato fruit.

Concentrations of 4 major tomato volatiles in mg/m^3,were measured from fruit of transgenic lines in different stages of ripening. Means of volatiles from each developing stage were statistically compared (4-5 replicates) between tomato varieties. N : non-significance, * : significance at $P<0.05$, Means with the same letter in a row were not significantly different.

	WT	35S-CMAAT1(4)	35S-UCMAAT1(3)	35S-UCMAAT1(7)	ACOP-UCMAAT1(7)	ACOP-UCMAAT1(15)	35S-ASLeAAT1(1)	35S-ASLeAAT1(2)	
3-Methyl butanal									
MG	2.23	3.05	2.64	1.27	2.21	2.07	1.81	2.86	N
B	9.42	7.88	7.75	9.28	7.74	6.97	6.92	8.17	N
B+4	10.34	8.24	10.14	10.43	8.36	8.66	8.75	8.24	N
B+7	10.51^{AB}	9.98^{B}	12.82^{A}	11.95^{AB}	10.13^{AB}	10.28^{AB}	7.84^{B}	10.79^{AB}	*
B+14	8.75^{C}	8.85^{C}	11.87^{A}	9.32^{C}	8.58^{C}	8.48^{C}	10.19^{B}	9.36^{C}	*
Hexanal									
MG	18.78	21.12	16.67	14.33	25.49	16.52	16.83	17.36	N
B	70.56	68.02	86.30	88.06	66.25	81.72	71.74	76.82	N
B+4	113.96	96.76	97.24	110.13	91.92	109.69	90.43	92.07	N
B+7	136.42	127.21	125.77	115.83	112.96	119.18	112.12	127.21	N
B+14	157.64	152.71	146.48	143.41	134.36	139.80	136.67	147.10	N
***E*-2-hexenal**									
MG	1.17	1.59	1.02	1.50	1.01	1.13	0.98	1.31	N
B	3.58	3.36	3.73	3.39	4.06	2.80	2.88	3.27	N
B+4	5.13	3.65	4.31	3.29	3.38	4.35	3.60	3.66	N
B+7	5.30	4.82	5.41	5.06	4.87	4.71	4.88	4.84	N
B+14	4.55	4.40	5.02	4.94	4.42	4.34	4.79	4.87	N
***Z*-3-Hexenol**									
MG	4.65	5.75	3.06	3.99	3.18	4.2	3.89	4.95	N
B	15.40	14.32	15.02	15.52	16.69	16.93	12.97	11.78	N
B+4	25.07^{AB}	18.98^{ABC}	21.13^{ABC}	16.60^{BC}	18.64^{ABC}	26.77^{A}	17.76^{BC}	14.05^{C}	*
B+7	37.54^{A}	25.88^{B}	35.69^{A}	36.24^{A}	30.12^{B}	27.87^{B}	29.30^{B}	30.91^{B}	*
B+14	38.05	35.22	37.22	37.66	39.72	35.85	36.07	32.04	N

CHAPTER 4

DISCUSSION

4.1 Screening of Fruit cDNA Libraries

4.1.1 Screening the Tomato cDNA Library

4.1.1.1 CM-AAT1 Homologue

CM-AAT1, which was originally identified as the ripening-specific melon fruit cDNA *MEL2* (Aggelis *et al.*, 1997b), showed sequence similarity with a benzyl alcohol acetyl-transferase published subsequently (Dudareva *et al.*, 1998) and also some acyl-transferases from genetic database alignment. This suggested that it might play an important role in ester generation in melon during ripening. This was shown subsequently to be correct (see below section 4.3.1; Yahyaoui *et al.*, 2002). Accordingly, *CM-AAT1* was used to screen a tomato cDNA library. Any clone in the library showing strong hybridisation with the probe should have some homology with *CM-AAT1* and could have biological functions related to *CM-AAT1*. From Southern analysis and sequence alignment, *TOMM7* was the only clone identified having some homology with *CM-AAT1* (Figure 3.1). *TOMM7* was highly identical to the previously published sequences of TC98820 (TIGR) and *TOM36* (Accession No A16155; Davies and Grierson, 1989) as shown by DNA sequence alignment (Figure 3.7). The nucleotide differences between *TOMM7*, *TOM36* and TC98820 (Figure 3.7) may be due to error arising from Taq PCR amplifying or might represent different genes encoding similar sequences. Because *TOM36* lacked an adenosine (A) at the sequence position 815 (Figure 3.7), the reading frame after this position was therefore shifted resulting in different amino acids near the C-terminus compared to the

others (Figure 3.8). However northern analysis, using the probe identical to the all clones, from a range of tomato tissues revealed that the positive band (1.6 Kb), showing only during fruit ripening (Figure 3.18B), is longer than the sequencing clone *TOMM7* (1.3 Kb) and *TOM36* (1.05 Kb). This evidence is consistent with the mRNA size of *TOM36,* reported by Davies and Grierson (1989), when expressed during fruit ripening and leaf senescence. As a result, *TOMM7* and *TOM36* should be the same gene and are not full-length clones.

The predicted protein (442 aa) encoded by TC98820 contains two important conserved regions, having some similarities to many plant AATs (Figure 3.9). One region is the 'H-x-x-x-G' motif, corresponding to a conserved consensus motif for the transferase family, and the other one is the 'DFGWG' group, a conserved consensus motif for many *O*-acyl-transferases (Aharoni *et al.*, 2000; D'Auria *et al.*, 2002; Lavid *et al.*, 2002; St Pierre *et al.*, 1998; Yahyaoui *et al.*, 2002). This suggests that the TC98820 protein may play a role in ester generation in tomato fruit. As results of the homologous screening, the northern analysis and the AAT-sequence similarity, TC98820 is then considered to be a good candidate for further research on tomato flavour generation. Nevertheless, the sequence of TC98820 (Tentative Consensus) sequence is theoretically combined from EST sequences, which are derived from partial cDNA clones. Consequently, we isolated the clone from total mRNA of ripe tomato through the RT-PCR procedure (see section 2.9.1.1.2) and the complete sequence was renamed as *Le-AAT1* (Figure 3.24).

4.1.1.2 Non-CM-AAT1 Homologues

The other 6 clones from the screening revealed no similarity with the *CM-AAT1* sequence. Weak bands that showed up in the Southern analysis after long exposure

time might be due to either distantly related sequences or nonspecific hybridising bands (Figure 3.1). The *TOMM10* clone was identical to tomato *ADH2*, encoding alcohol: NAD^+ oxidoreductase, but is not similar to the 'short chain' *ADH*, the *ERT10* clone (Accession No X72730; Picton *et al.* 1993). Alcohol dehydrogenase, a dimer-subunit enzyme, is responsible for interconversion of alcohol and aldehyde forms with Zinc as a cofactor (Bicsak *et al.*, 1982). *ADH2* plays a role in tomato flavour generation (Speirs *et al.*, 1998) since ripe tomato fruit contains high contents of aldehydes and alcohols (Buttery, 1993; Buttery *et al.*, 1987; Riley *et al.*, 1996; Tandon *et al.*, 2000).

TOMM1U is remarkably similar to catalytic subunit of the serine/threonine protein phosphatase PP2A (EC 3.1.3.16), which is a group of enzymes removing the serine-or threonine-bound phosphate group from a wide range of phosphoproteins (White *et al.*, 2002). *TOMM1L* has homology to the pepper *Sn-1* encoding a placental specific vacuolar membrane protein in *Capsium annum*, Yolo Wonder protein (Pozueta-Romero *et al.*, 1995). *TOMM1L* might play a defensive role in tomato as *Sn-1* is similar to the opium-poppy major latex protein, a substance believed to have a protective function in the plant (Nessler and Burnett, 1992), and *MEL7* melon predicted protein (Aggelis *et al.*, 1997b). *TOMM4* is similar to many putative pyrophosphate-fructose-6-phosphate 1-phosphotransferases, which might be involved in cell glycolysis and/or gluconeogenesis. *TOMM5* is a tomato actin gene. Actins are highly conserved proteins involved in various types of cell motility and are essential component of the cell cytoskeleton and playing an important role in cytoplasmic streaming, cell division, cell shape determination, organelle movement and extension

growth (Page *et al.*, 1998). *TOMM9* is identical to the ripening E8 protein, which is induced by ethylene (Lincoln *et al.*, 1987).

4.1.2 Screening the Avocado cDNA Library

None of selected avocado cDNA clones showed strong hybridisation with the *CM-AAT1* probe (Figure 3.12). Three clones were isolated and sequenced. There was no significant homology of any of these proteins with any known proteins clones except *AVOM2*, which was shown to have some similarity to *Arabidopsis* cyclin *2b* protein (Accession No Z31401). Avocado (*Persea americana* Mill.) fruit contains a lot of unsaturated fatty acids in the pericarp such as 52% oleic acid (C18:1), 14.9% linoleic acid (C18:2), 8.9% palmitoleic acid (C16:1), and 1.8% linolenic acid (C18:3) (Moreno *et al.*, 2003). It has been reported that the main volatile is β-caryophyllene (60.2%), followed by α-humulene (5.9%), caryophyllene oxide (4.8%), α-coaene (4.5%) (Sinyinda and Gramshaw, 1998). Nevertheless, avocado fruit consists of small amounts of some esters such as farnesyl acetate (Sinyinda and Gramshaw, 1998), 2-ethylheyl propionate, 2-methyl-3-hydroxy-2, 4, 4-trimethylpentyl propionate (Moreno *et al.*, 2003). Hence there might be some genes involved in ester production in ripe avocado fruit, although none was detected during the screening.

4.2 AAT mRNA Expression

CM-AAT1 is a fruit ripening specific gene, which can be induced by exogenous ethylene (Aggelis *et al.*, 1997b) in both wild type and *ACO1*-antisense fruit (Figure 3.17). The expression was partially inhibited in ripe *ACO1*-antisense fruit and the ripening processes are delayed. Consequently, *CM-AAT1* exhibited the expression pattern of an ethylene-dependent gene as suggested by Aggelis *et al.* (1997b).

Wounding of fruit tissue could, however, reduce by up to 7-fold the amount of *CM-AAT1* mRNA, compared to the control (Aggelis *et al.*, 1997b). *ACO1* antisense melon fruit smelt less aromatic and Bauchot *et al.* (1998) reported a 60-85% reduction of total volatiles in the fruit, which greatly decreased some esters (Flores *et al.*, 2002). These investigations showed that ethylene is a regulator for the *CM-AAT1* expression and is required for ester volatile production in melon.

Le-AAT1 is also a fruit ripening specific gene in tomato and is inducible by exogenous ethylene (Figure 3.18A). However, the gene might not be sensitively regulated by ethylene as described by Harpham *et al.* (1996) due to no responsive expression when were treated with ethylene at low concentration (10 ppm) for 16 h (Figure 3.18B). The expression is apparently complicated since it was still high in low ethylene *ACO1* co-suppression tomatoes, T4B+11 and V11B+7, and slightly reduced in the *Nr* mutant (Figure 3.19). The *Le-AAT1* expression is similar to the PG mRNA expression, which is reported to be developmentally regulated through the ethylene-independent transduction pathway (Oeller *et al.*, 1991; Theologis *et al.*, 1993). Nevertheless the accumulation of *TOM36*, which is identical to *Le-AAT1* (see section 3.1.5), was greatly reduced when the ethylene production was inhibited by silver sulphate ($Ag(S_2O_3)_2$), an inhibitor of ethylene perception or action (Davies and Grierson, 1989). In addition, as Sirit and Bennett (1998) clarified subsequently that the PG mRNA accumulation is ethylene regulated, further study of the *Le-AAT1* expression would be investigated in low ethylene fruit with ethylene treatments.

The *ADH2* clone, which was isolated and sequenced after screening by the *CM-AAT1* probe, was found to be expressed in seed, root, stem, and fruit (Figure 3.20), including low O_2 conditions (Longhurst *et al.*, 1994; Van Der Straeten *et al.*, 1991). The

expression could not be induced by exogenous ethylene (Figure 3.20) and was slightly reduced in low ethylene production fruit (Figure 3.21). ADH2 protein was reported to be involved in alcohol generation in ripe tomato (Longhurst *et al.*, 1994; Speirs *et al.*, 1998; Van Der Straeten *et al.*, 1991). As the accumulation of *ADH2* rose up highly during late ripening stages (Figure 3.20), we expected that it would contribute with *Le-AAT1* to produce some esters in the fully ripe fruit.

4.3 Fusion Protein Analysis

4.3.1 AAT Activity

CM-AAT1 protein, expressed in yeast, exhibited AAT activity using a wide range of alcohols and acyl-CoAs as substrates (Yahyaoui *et al.*, 2002; Table 3.5). Le-AAT1, although sharing only 43% identity to CM-AAT1 at the amino acid level (Figure 3.9), also showed AAT activity (Table 3.7). The overnight *CM-AAT1*and *Le-AAT1* transformed yeast culture released a banana smell, because of the synthesis of endogenous 3-methyl butanol and 2-phenyl ethanol (Malcorps and Dufour, 1992; Yahyaoui *et al.*, 2002), which are substrates for the proteins and 3-methyl butyl acetate is an indicator of ripe banana smell (Mayr *et al.*, 2003; Myers *et al.*, 1970). Surprisingly, the CM-AAT2 protein had 86% identity to CM-AAT1, but did not show AAT activity (Figure 3.26; Yahyaoui *et al.*, 2002). However we have been informed recently (March 2003) by Professor Jean-Claude Pech that errors may have been introduced to the *CM-AAT2* sequence during PCR amplifying, although it contains both conserved regions, 'H-x-x-x-D' and 'DFGWG' motifs, found in many plant *O*-acyl-transferases. The altered sequence might have affected the active sites of CM-AAT2 protein, causing a lack of enzymatic activity. Accordingly CM-AAT2 when compared to CM-AAT1 (Table 3.4) lacks the *N*-glycosylation site and some *N*-

myrislation sites, which are reported to be responsible for the subunit specificity of many enzymes of eukaryotes (Grand, 1989; Towler *et al.*, 1988). Another possibility is that *Saccharomyces cerevisiae* might not be a good host to produce appropriate CM-AAT2 protein.

The AAT function of the CM-AAT1 protein probably requires specific posttranslational modifications because there was no AAT activity of the fusion protein, expressed in *E. coli* (Yahyaoui *et al.*, 2002), but in yeast. Yeast are single-celled eukaryotic microorganisms and the protein producing system performs posttranslational modifications and subcellular localizations as more complex eukaryotes, resulting in more accuracy of the structure of heterologous eukaryotic protein expressed in yeast (Walker, 1998). For example, the *Arabidopsis ETR1* protein, expressed in yeast, was shown to have an appropriate binding site for ethylene (Schaller and Bleecker, 1995), which is better than when first expressed in bacteria (Chang *et al.*, 1993). However, the activity of some plant AATs had been satisfactorily achieved in *E. coli* (D'Auria *et al.*, 2002; Dudareva *et al.*, 1998; Dudareva *et al.*, 2000; Yang *et al.*, 1997).

The yeast-expressed proteins of *CM-AAT1* and *Le-AAT1* revealed bigger Mws (Figure 3.25B) than those estimated. As some enzymes are active as a dimer *in vivo* such as ADH, the fusion proteins were treated with dithiothreitol, but the western analysis showed the same result of Mws (see section 3.3.2.3). As a result, there is no disulfide linkage between the protein subunits. Surprisingly, the AAT protein, extracted from 'Gemsung' melon, has been reported to have a Mw of 400 kDa (Ueda *et al.*, 1997), which is approximately 7 times larger than the CM-AAT1 fusion protein expressed in yeast (Figure 3.25A).

4.3.2 Effects of pH and Ion strength on the Fusion AATs

CM-AAT1 and Le-AAT1 fusion proteins were active from the pH range 6.0 to 8.0 (Yahyaoui *et al.*, 2002; Figure 3.27) consistent with the previous studies of partially purified proteins from banana (Harada *et al.*, 1985), yeast (Malcorps and Dufour, 1992), strawberry (Pérez *et al.*, 1993), and melon (Ueda *et al.*, 1997). While Mg^{2+} is a preferred metal ion for CM-AAT1, Na^{+} also greatly stimulated AAT activity of Le-AAT1. The activity of the proteins encoded by *CM-AAT1* and *Le-AAT1* decreased sharply above 50 and 40 mM $MgCl_2$, respectively (Yahyaoui *et al.*, 2002; Figure 3.28). This is similar to a partial inhibition by Mg^{2+} on ester formation, reported in AAT of brewer's yeast (Yoshioka and Hashimoto, 1981). In strawberry, Mg^{2+} had no inhibitory effect on AAT (Pérez *et al.*, 1993), but 1 to 100 mM $MgCl_2$ and NaCl show an inhibitory effect on banana AAT (Harada *et al.*, 1985).

4.3.3 Substrate Specificity

In general, the CM-AAT1 protein exhibited higher AAT activity than the Le-AAT1 protein when the fusion proteins were compared roughly at the same amounts by western analysis (Figure 3.25B). When the best substrate *E*-2-hexenol was calculated to be 100% relative activity of acetylation for CM-AAT1, Le-AAT1 had only 44% with the best substrate, hexanol (Table 3.5 and 3.7). CM-AAT1 could modify 2-phenyl ethanol and 3-methyl butanol at 95% and 57% relative activity, but only 20% and 29%, respectively, for Le-AAT1. This is consistent with the stronger aromatic volatiles released from the overnight culture with *CM-AAT-* than with *Le-AAT1*-transformed yeast.

While CM-AAT1 exhibited more flexibility utilising a range of acyl-CoAs (Table 3.5), Le-AAT1 showed high activity only with short acyl-donors such as acetyl- or propionyl-CoA, but not with hexanoyl CoA (Table 3.7). Regarding to the amino acid sequence of Le-AAT1, 'LSKTLVFY' residues near the *N*-terminus at positions 68 to 75 of the protein are similar to the carboxyl transferase β subunit of acetyl CoA carboxylase, which is reported to be important for reactions using acetyl CoA as co-substrate (Aharoni *et al.*, 2000), in the green alga *Chlorella vulgaris* (Accession No BAA57908). This might elucidate why Le-AAT1 have affinity on acetyl-CoA.

CM-AAT1 showed higher AAT activity with longer carbon chain alcohols (Table 3.5), consistent with SAAT (Aharoni *et al.*, 2000; Pérez *et al.*, 1993). Alterations of a group residue in alcohol structures influenced activity of CM-AAT1 and Le-AAT1 that acylation of 2-methyl butanol or 2-phenyl ethanol was higher than of 3-methyl butanol or 1-phenyl ethanol, respectively. Changes in the nature of the chemical structure of the alcohol have been reported to affect greatly the activity of some AATs (Aharoni *et al.*, 2000; D'Auria *et al.*, 2002). The size of the aromatic residue has an effect on acylation of AATs as 2-phenyl ethanol was a better substrate for CM-AAT1 than benzyl ethanol, as well as SAAT (Aharoni *et al.*, 2000) and melon AAT (Shalit *et al.*, 2001), but in contrast to Le-AAT1, BEAT (Dudareva *et al.*, 1998), and BEBT (D'Auria *et al.*, 2002).

The data from the Km values between the acyl-CoAs from both AATs were similar, while the Km towards alcohols were much more variable (Table 3.6, 3.8). This suggests that the affinities for alcohols were pivotal in terms of the level of activity. In tomato fruit, alcohols are much lower than those were used in the experiments. Thus, concentrations of alcohols in the system may lead generation of ester compounds. In

ripe tomato, there is only 7 ppb hexanol compared to 12,000 ppb *Z*-3-hexenal or 2000 ppb hexanal (Butter, 1993), while the Km value of Le-AAT1 for hexanol is 6.5 mM (Table 3.8). Ester composition in tomato fruit might be limited by low AAT activity and/or the availability of substrates, for example, lipid or carotenoid breakdown, or amino acid metabolism.

During melon ripening, *CM-AAT1* could play a major role in generating a number of esters derived from aliphatic, branched, and aromatic alcohols. However *CM-AAT1* failed to acylate ethanol (table 3.5) despite the fact that some ethyl esters such as ethyl acetate, ethyl propionate, ethyl 2-methyl propionate, ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate are major ester volatile compounds of ripe melon (Bauchot *et al.*, 1998; Horvat and Senter, 1987; Shalit *et al.*, 2001; Wyllie and Leach, 1990; Yabumoto and Jenning, 1977). The Southern analysis data from Aggelis *et al.* (1997b) showed that there is more than one corresponding gene for *CM-AAT1* and some genes have low homology at the DNA level. Moreover western analysis showed the different sizes of the CM-AAT1 proteins and a purified melon AAT by Ueda *et al.* (1997). These suggest that there may be other AAT genes, involved in ester production in ripe melon.

4.4 Phylogenetic Relationship

The melon *CM-AAT1* and tomato *Le-AAT1* are members of a large acyl-transferase multifunctional gene family. In *Arabidopsis*, for instance, more than 70 members encode enzymes for acyl-transferases involved in the synthesis of secondary metabolites such as various scent, pigment, and defensive compounds (Pichersky and Gang, 2000).

When amino acid sequence relationships were compared (Figure 3.30), CM-AAT1 had strong homology with CM-AAT2 (84% identity), and was close to MaAAT (apple AAT) (47% identity) and also had homology to Le-AAT1 (43% identity) or even to 5AT (anthocyanin-5-aromatic acyl-transferase) involved in the colour biosynthesis of *Gentiana triflora*. However, although it was separated into another subgroup by the evaluated relationship analysis, strawberry AAT (SAAT) is close to CM-AAT1 (only 24% identity) and Le-AAT1 (only 21% identity) in terms of its enzyme activity, which can modify a board range of alcohols, including methanol and ethanol (Aharoni *et al.*, 2000). Moreover despite without similarity to CM-AAT1and Le-AAT1, the yeast AAT, AFT1P, can syntheses short-chain and medium-chain aliphatic esters in *Saccharomyces cerrevisiae* (Fujii *et al.*, 1994; Malcorps and Dufour, 1992).

The molecular evolution could be manipulated by certain adaptable mechanisms, resulting in the great diversity of plant secondary metabolites (Pichersky and Gang, 2000). Since new genes almost always arise by gene duplication mechanisms such as unequal crossing-over, translocation between non-homologous chromosomes, or replicative transposition of transposable elements (Pichersky, 1990), developmental and metabolic diversity still exits (Somerville and Somerville, 1999). The movement and duplication of DNA sequences in plant genomes can be followed by divergence, for example, *CM-AAT1* and *CM-AAT2* (in case of no error during PCR amplifying) in melon or *Le-AAT1* and *CaAT* in the *Solanaceae*.

Peppers (*Capsicum* sp.) are in the family *Solanaceae* as is tomato so that the CaAT protein, the acyl-transferase 2, from a placental-specific gene of habanero chile, showed highly genetic relationship to Le-AAT1 with 80% identity (Figure 3.30). The

CaAT protein also contains both 2 important conserved regions for *O*-acl-transferase. Therefore, the CaAT clone could play a role of ester generation in placental tissue of the pepper. However, in bell pepper, aldehydes, ketones and alcohols are the main volatiles in fruit with methyl acetate and ethyl acetate in small amounts (Ruth *et al.*, 2003). Recently, we found from an alignment searching in the EMBL database that *CM-AAT1* had 54.11% identity over 401 nucleotides to an unknown ripening-related clone in pulp of ripening banana (*Musa acuminata*, Accession No MAZ93116). This clone might have some relations with the banana AAT protein, reported by Harada *et al.* (1985).

4.5 Volatile Compounds from Transgenic Tomatoes

There was no significant difference of ester generation between control and transgenic fruit, even when exogenous alcohols and acetyl-CoA were added into the conditions (Figure 3.44). The failure of transgene *CM-AAT1* over-expression on higher generation of ester compounds might be because of faults in some steps of the protein expression. The transgenes, although incorporated into the nuclear genomes (Figure 3.33) and expressed in fruit (Figure 3.34, 3.35, 3.36, 3.37), revealed weak effects on ester generation during ripening of transgenic fruit. In this experiment, we did not check the protein expression *in vivo* by western analysis. Consequently it is still unclear whether the trans-proteins were produced in the tomatoes. Theologis *et al.* (1990) reported that antisense tomato fruits accumulated normal levels of PG, but it was not translated that might be because of problems in posttranslation. However it was eventually clarified that PG mRNA translation occurs behind the accumulation by at least 24 h (Sitrit and Bennett, 1998). Another possibility of transgene inactivity is that if the proteins were expressed, they might not function properly in tomato cells

or tomato fruit might be limited by the availability of substrates. Matsui *et al.* (2001) reported the failure of transgene function *in vivo* that the tomatoes, transformed with the gene encoding 9-hydroperoxide lyase (9-HPL), generated the derivative products only when exogenous fatty acids were added. While the pH values of tomato fruit homogenates were approximately 4.0 (Figure 3.38), the fusion CM-AAT1 protein are active between pH 6.0 and 9.0 (Yahyaoui *et al.*, 2002). The pH of fruit homoginates was adjusted to be about 6.0 and exogenous alcohols and acetyl-CoA were then mixed into the homogenates (see section 2.12.1), but there was still no difference of ester compounds in all treatments (Figure 3.44). The failure of ester generation from the homogenates might be caused from improper pH, buffer strength, or less incubation time.

Since we selected only 2 lines from each transgenic tomato for flavour analysis due to load-work with many gene constructs, more transgenic lines should have been selected for the analysis. However the *CM-AAT1* fused with 0.2 Kb 5'UTR of *Le-AAT1* constructs, the 35S-UCMAAT (3) and ACOP- UCMAAT1 (7) showed a trend to produce higher phenylethyl acetate (Figure 3.40) and *Z*-3-hexenyl acetate (Figure 3.42) and might relate to the alteration of some intermediate substrates shown in Table 3.9. The transgenes may show more effects in transgenic plants homozygous (Smith *et al.*, 1990) since the transformants under CaMV 35S promoter were found to be hemizygous in Southern analysis (Figure 3.33).

Although the Le-AAT1 enzyme can manipulate a wide range of alcohols and acyl-CoAs, ripe tomato comprises very low ester compounds (Buttery and Ling, 1993) and *LeAAT1* may play only a minor role in the generation of aroma. It is supported by the experiment with the antisense *Le-AAT1* fruit, which showed a slight effect on a reduction

of ester compounds. However there might be more than one gene corresponding to tomato AAT, because the Le-AAT1 protein had a weak activity on ethanol (Table 3.7) while ethyl acetate was reported to be in ripe tomato (Buttery and Ling, 1993). Furthermore there may be another corresponding gene for *Le-AAT1* with low homology at the DNA level as suggested by the appearance of the weak band expressing above *Le-AAT1* in the northern blot analysis (Figure 3.18).

Feeding exogenous linolenic acids to tomatoes prior to maceration influenced the rapid formation of large amount of C6 aldehydes produced due to lipoxigenase/ hydroperoxide lyase activity (Boukobza *et al.*, 2001; Boukobza and Taylor, 2002). While at all stages of fruit development, hydroperoxide lyase did not show any significant change in activity as the fruit ripened, tomato lipoxygenases showed the greatest change in activity with fruit development, increasing by 69% between the mature-green and breaker stages of development and declining again as the fruit turned red (Riley *et al.*, 1996). This is consistent with the information from Griffiths *et al.*, (1999) that *TomloxB* and *TomloxC* exhibited maximum expression at the breaker to 3 days post-breaker stage and then the expression was reduced. Furthermore, although silencing of *TomloxA* and *TomloxB* by antisense genes failed to reduce aldehyde volatiles in ripe fruit (Griffiths *et al.*, 1999), when the *TomloxC* gene was knocked out this caused a high reduction of total volatiles especially C6 aldehydes and their derivatives in fruit (Dr Gouping Chen, this laboratory, personal communication). These results suggest that lipoxygenases in particular *TomloxC* may play a crucial role in generation of aroma volatiles in tomato fruit by providing substrates for ADH and AAT enzymes.

The main ripe tomato aroma is a combination of 10 major volatiles, and a mixture consisting of *Z*-3-hexenal, *E*-2-hexenal, hexanal, 1-penten-3-one, 3-methyl butanal, *Z*-3-hexenol, 6-methyl-5-hepten-2-one, methyl salicylate, 2-Isobutylthiazole and β-ionone, was considered to very similar to the aroma compounds of a sliced fresh ripe tomato (Buttery, 1993). Nevertheless with very low levels in green fruits (Figure 3.40), phenylethyl acetate shows to be as a ripening aromatic indicator for tomato. Apart from aldehyde and alcohol generation, some biological pathways of acyl-CoA production in plants might be involved in improved flavour generation. For instance, pyruvate decarboxylase (PCD), which converts pyruvate to acetyl-CoA, is highly expressed during fruit development (Aharoni *et al.*, 2000) and in fruit stored at low O_2 (Imahori *et al.*, 2002; Lara *et al.*, 2003) and the contribution of other enzymes to flavour generation should not be over looked.

CONCLUSIONS

1. Only one clone, the tomato *Le-AAT1*, showed strong hybridisation with the melon *CM-AAT1* probe from screening of a tomato cDNA library. Similar screening of an avocado library failed to identify a clone with similarity to *CM-AAT1*. *Le-AAT1* is identical to the tomato *TOM36* (Davies and Grierson, 1989) and is similar to many plant genes in the acyl-transferase family.

2. Northern blot analysis of mRNA from different tissues indicated that *CM-AAT1* and *Le-AAT1* behave as fruit ripening specific genes and were induced by exogenous ethylene. In keeping with this, the *CM-AAT1* mRNA was highly reduced in accumulation in *ACO1* antisense fruit, but the *Le-AAT1* expression was still high in low ethylene production lines. ADH2, which converts aldehydes to alcohols, was expressed in many tomato organs and highly expressed at the late stages of fruit ripening, but was not induced by exogenous ethylene.

3. Both CM-AAT1 and Le-AAT1 fusion proteins, expressed in *Saccharomyces cerevisiae*, showed alcohol acyl-transferase activity, which modified many alcohols and acyl-CoAs to ester compounds. No AAT acitivity of CM-AAT1 protein could be detected when expressed in *E. coli*. The yeast-expressed CM-AAT2 protein, which is 84% identical to the CM-AAT1 protein, did not show any activity. This may be explained by the recent finding that there are errors (probably caused by PCR) in the sequence (Professor J-C. Pech, personal communication)

4. All volatile compounds were increased dramatically as ripening proceeded. Ester volatiles also increased during tomato ripening, but were still low compared to

other volatiles and there was no statistical significance between control and transgenic fruit. The over expression of the transgene, *CM-AAT1*, in transgenic fruit showed weak effects on ester generation. Further investigation may be required for more information.

FUTURE WORK

1. Due to the lack of the activity of CM-AAT1 with ethanol as a substrate, further screening of melon fruit cDNA library may need to be carried on to identify more AATs, including one predicted to produce ethyl esters.

2. The search for tomato varieties, producing a high alcohol content, or for pathways of amino acid or terpene generation might be useful for further study on fruit flavour improvement.

3. Growing either the homozygous (F1) of the 35S-UCMAAT and ACOP-UCMAAT, or *Le-AAT1* sense constructed tomatoes might help us to clarify the transgene function. Fruit volatiles should be analysed and the aroma panel test should be done.

4. Because *ACO1*-antisense melon produce low ester concentration during ripening, *CM-AAT1* over-expression constructs should be introduce to these melons to ensure good flavour and exploit their good keeping qualities. The *CM-AAT1* over-expression lines will backcross to *ACO1*-antisense melon background. The *ACO1*-antisense melon containing *CM-AAT1* over-expression would be selected, grown, and checked for ester volatile production in slow ripening fruit.

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