VG427
Flavour improvement in tomatoes

Jim Spiers and **Elizabeth Lee**
CSIRO Division of Horticulture

Know-how for Horticulture™

VG427

This report is published by the Horticultural Research and Development Corporation to pass on information concerning horticultural research and development undertaken for the vegetable industry.

The research contained in this report was funded by the Horticultural Research and Development Corporation with the financial support of Zeneca Seeds.

Cover price: $20.00
HRDC ISBN 1 86423 712 0

Published and distributed by:
Horticultural Research & Development Corporation
Level 6
7 Merriwa Street
Gordon NSW 2072
Telephone: (02) 9418 2200
Fax: (02) 9418 1352
E-Mail: hrdc@hrdc.gov.au

HRDC

HORTICULTURAL RESEARCH & DEVELOPMENT CORPORATION

Partnership in horticulture

TABLE OF CONTENTS

Summary

In the course of the project we have accomplished the following:

* Produced transgenic tomatoes with modified levels of alcohol dehydrogenase (ADH2) activity in the fruit.

* Shown that modification of ADH2 activities in the fruit results in modification of aldehyde/alcohol ratios for 6-carbon and 5-carbon flavour/aroma volatiles.

* Undertaken a small-scale taste test which selected (with a high degree of probability) fruit from a high-ADH2 transgenic plant as having improved flavour characteristics.

* Found that improved flavour characteristics are also present in the transgenic fruit at early stages of ripening.

* Found that preliminary analyses of acid levels in the transgenic fruit suggested some modification of citrate 'associated' with modification of ADH2 expression.

* Found an enhanced perception of sweetness in fruit from high-ADH2 transgenic plants.

* Have taken out applications for patent coverage of intellectual property generated in the course of the work.

* Have monthly telephone conferences with Zeneca UK to discuss progress and future directions.

INTRODUCTION

The tomato is a universally important food, its popularity deriving, at least in part, from its attractive colour, flavour and versatility. A recent survey of the nutritional status of commonly eaten foods in Australia found tomato to be the most balanced single food of all, contributing >2% of the total amount of over a dozen nutrients important to humans (P.Clifton, CSIRO Division of Human Nutrition, personal communication). It is also the primary dietary source of lycopene, a potent antioxidant associated with resistance to several forms of human cancers, in particular that of the prostate (cf. Levy et al., 1995; Caperle et al., 1996; Clinton et al., 1996).

Tomatoes are used either fresh or as a range of processed products. An important quality of both the fresh and processing fruit is flavour, comprised mainly of sugars, acids and, of particular importance in the fresh fruit, volatile compounds. Despite the efforts of tomato breeders, fresh tomatoes often do not meet the high standards of flavour required by the consumer. Many breeders are now concentrating on improving sugar and acid levels and vine ripened fruit are becoming increasingly available. However, little attention is being given to improving the flavour and aroma characters produced by the volatile compounds.

The development of flavour and aroma volatiles in the ripening tomato fruit has been extensively studied (Kazeniac and Hall, 1970; Buttery et al., 1971; Dirinck et al., 1976; Buttery et al., 1987; McGlasson et al., 1987; Buttery et al., 1988; Buttery et al., 1989; Baldwin et al., 1991; Linforth et al., 1994). Approximately 400 volatile compounds have been found in the ripening fruit (Baldwin et al., 1991) but of these only a small number have been identified as important components of flavour and aroma. These include Z-3-hexenal, Z-3-hexenol, 2-E-hexenal, hexanal, 3-methylbutanal, 3-methylbutanol, β-ionone, 1-penten-3-one, 2-isobutylthiazole, 6-methyl-5-hepten-2-one, methyl salicylate, geranylacetone, E-2-heptenal, isobutyl cyanide and 2-phenylethanol (Dirinck et al., 1976; Buttery et al., 1987; Buttery et al., 1989). These flavour volatiles are formed by several different pathways such as the deamination and decarboxylation of amino acids (3-methylbutanal/ol (Yu et al., 1968)) and lipid oxidation of unsaturated fatty acids (hexanal/ol and the hexenals/ols (Galliard et al., 1977; Hatanaka et al., 1986)). This latter pathway is shown in Figure 1 and is of particular interest to

us because of the role played by the enzyme alcohol dehydrogenase in interconversion of the alcohol and aldehyde forms of the 6-carbon volatiles.

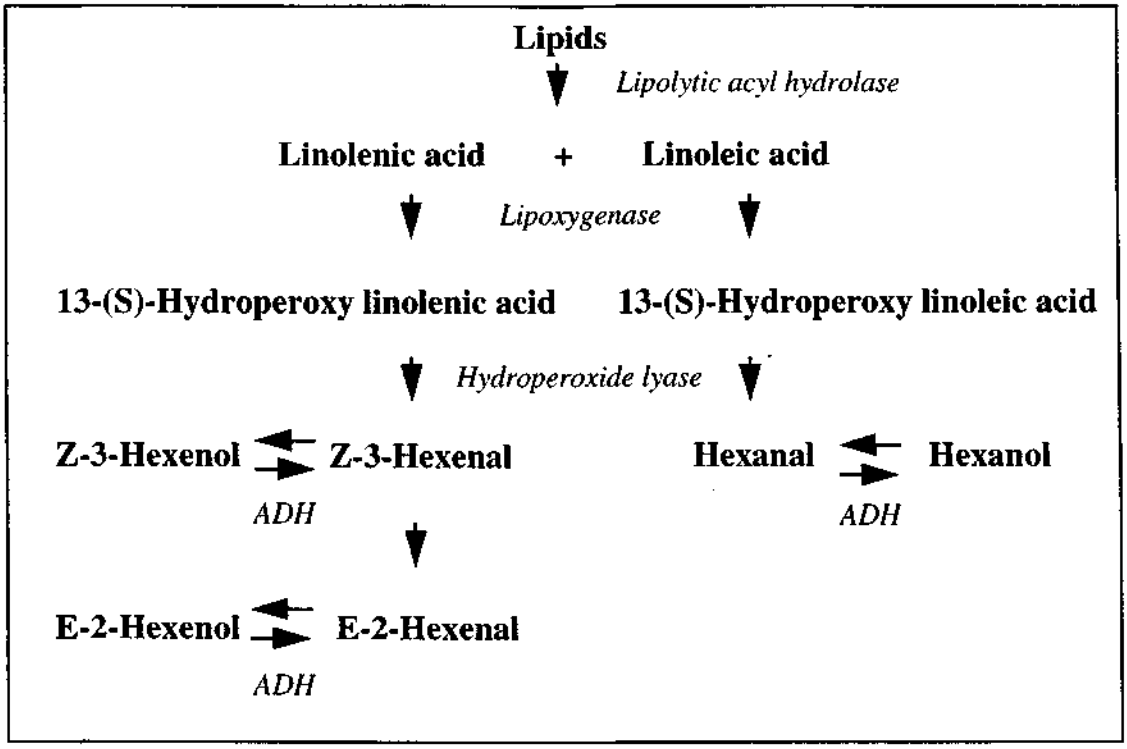

Figure 1. Pathway of formation of important flavour volatiles from the breakdown of lipid.

The tomato alcohol dehydrogenase II enzyme (ADH 2 ; alcohol:NAD^+ oxidoreductase; EC 1.1.1.1) is one of two ADH enzymes described in tomato. The ADH 2 enzyme has been implicated in the interconversion of the aldehyde and alcohol forms of flavour volatiles (Sieso et al., 1976; Bicsak et al., 1982). It has been shown to accumulate in the fruit during ripening (Bicsak et al., 1982; Longhurst et al., 1990; Chen and Chase, 1993) and to have appropriate substrate specificities *in vitro* (Bicsak et al., 1982) but direct proof of its role has not been obtained. The tomato ADH 1 enzyme is found only in pollen, seeds and young seedlings (Tanksley, 1979) and apparently is not associated with functions in the ripening fruit.

The accumulation of the ADH 2 enzyme late in ripening, combined with the coincident large increase in flavour volatiles in the fruit and the enzyme's putative role in interconversion of the volatile aldehydes and alcohols has led to the suggestion that ADH may play an important role in flavour development (Longhurst et al., 1990).

In order to test the putative role of the enzyme in the reduction of some flavour aldehydes to alcohols, and to examine its possible involvement in the development of flavour in the tomato fruit, we have produced a number of transgenic tomato plants with modified levels of ADH 2 activity in the ripening fruit. Analysis of volatiles from fruit with enhanced, normal, or reduced levels of ADH 2, was carried out by gas chromatography / mass spectrometry to determine the effects on relative amounts of volatile aldehydes and alcohols and the flavour of fruit from representative plants was determined by a taste panel. The results indicate that ADH is involved in the interconversion of aldehydes and alcohols in tomatoes and this in turn affects the flavour of the fruit.

MATERIALS AND METHODS

Plant material

Tomato (*Lycopersicum esculentum* Mill cv Ailsa Craig) plants were grown under controlled conditions in a glasshouse and in compliance with regulations for the contained growth of transgenic plants as specified by the Australian Genetic Manipulation Advisory Committee. Harvesting of fruit was randomised so that environmental or positional effects such as slight variations in light intensity or position of fruit on the vine, were minimised.

Construction of *ADH* transgenes

Two genes were constructed for insertion into the tomato plant. Both contained the tomato *Adh2* gene sequence but were coupled with differring regulatory sequences. The first was designed to be active constantly in all tissues. For this purpose the tomato *Adh2* gene sequence was coupled with a constitutive promoter (the cauliflower mosaic virus 35S- protein promoter; CaMV 35S-promoter) and with a termination sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Nos-3' terminator). The second gene was designed to be active only in ripening fruit. For this purpose the tomato *Adh2* gene was coupled with the promoter and termination sequences from the tomato polygalacturonase gene (PG-promoter and terminator). The gene constructs are described in Figure 2

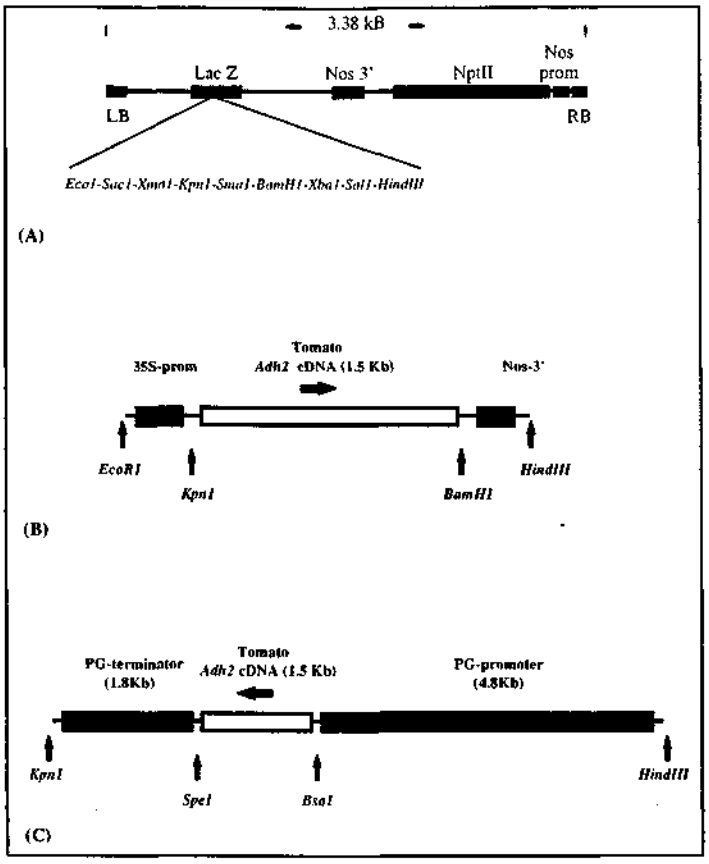

Figure 2 Constructs PJR-ADH and PRD-ADH

A. General map of the T-DNA region of the binary vector Bin 19 (Bevan, 1984; Frisch et al., 1995) showing the *Npt* II selectable marker gene and the polylinker site into which was ligated either gene construct B or gene construct C.
B. For construction of the vector PJR-ADH, the tomato *Adh2* cDNA was ligated between the CaMV 35S promoter and Nos 3' terminator which were inserted into the polycloning site of the Bin 19 vector.
C. For construction of the PRD-ADH vector, the tomato *Adh2* cDNA was ligated between the promoter and 3' regions of the tomato polygalacturonase which were inserted into the polycloning site of the Bin 19 vector.

Tomato Transformation

Tomato (*Lycopersicum esculentum* Mill cv. Ailsa Craig) transformations were carried out via *Agrobacterium tumefaciens* according to Bird et al., (1988). Southern hybridisation analyses of DNA from young leaf tissue was used to estimate the number of *NptII* and *Adh 2* inserts integrated into each plant and plants were allowed to grow to maturity and set fruit. Fruit were harvested at first colour change (Breaker, Br) or two days post Breaker (Br+2) and 7 days post breaker (Br+7) and ADH activity in the pericarp tissue was determined.

For maintainance of the primary transformants and to provide sufficient fruit for subsequent analyses, multiple cuttings of selected plants were vegetatively propagated.

Genomic Southern Hybridisation Analyses

Genomic DNA was extracted from young leaves by the method of Thomas et al., (1993). 7µg of each of the genomic DNAs was digested with either *Hind* I or *Xmn* I and was fractionated by electrophoresis on 0.7 % agarose-TBE gels. The DNA was transferred onto nylon membranes (Zetaprobe, BioRad) as described by the manufacturer. The filters were hybridised sequentially with ^{32}P- labelled probes corresponding to the *Npt* II and *Adh 2* cDNA regions of the transgene. Hybridisation was according to the procedure recommended for Zetaprobe, at 65°C for 16 h. The filters were washed twice with 2XSSC, 0.1% SDS at 65°C for 15 min each, followed by two washes of 10 min with 0.1XSSC, 0.1% SDS at 65°C. The membranes were blotted dry and analysed by phosphoimaging. The numbers of *Npt* II and *Adh 2* inserts were estimated from the number of bands of hybridisation with the respective probes together with their intensities (*Hind* III). Digestion with *Xmn* I excised the introduced *Adh 2* cDNA from the inserted gene construct(s). In this case, the number of copies of the *Adh 2* gene inserted was estimated from the intensity of hybridisation to the inserted gene(s) relative to the intensity of hybridisation to the endogenous gene.

Extraction and Assay of ADH

Tomato pericarp tissues, and whole fruit tissues were extracted and assayed by spectrometric quantitation of the reduction of NAD to NADH, with ethanol as substrate, according to the method of Longhurst et al., (1990). Protein concentration was measured using a protein quantification Kit II (BioRad). Enzyme activities are given in Units mg^{-1} protein where 1 Unit is the amount of enzyme required to produce 1 µmole NADH min^{-1}.

Volatile Analyses

The method used for isolation of headspace volatiles was modified from that of Buttery et al., (1987) and included a short, room temperature incubation of the macerated tomato tissue prior to inactivation of endogenous enzymes by the addition of $CaCl_2$. This step was included

to simulate the development of volatiles in sliced and chewed tomato. SPME absorption was used to collect headspace volatiles for fractionation and analysis by GC/MS.

Fruit were harvested 7 days post breaker. Pericarp tissue (10g) was taken from freshly harvested fruit, sliced and then briefly macerated using a Polytron PT2000 homogeniser (Kinematica AG, Switzerland). The slurry was allowed to stand at room temperature for exactly 3 min after which 3.3g solid $CaCl_2$ were added to inhibit further enzyme activity. Uniformly labelled, deuterated Hexanol (1µl of 80nmoles /µl) was added as an internal standard. An aliquot of the mixture (5.5g) was transferred to a 20ml headspace vial which was sealed with a silicon/teflon septum. The vial was incubated at 40°C for 30 min. Sampling of the headspace was carried out by insertion of a Solid Phase MicroExtraction (SPME, Supelco) fiber (65µm Carbowax-Divinylbenzene), for 30 min while continuing incubation of the vial at 40°C. The absorbed sample was analysed by gas chromatography on an HP-GC series 6809 fitted with a capillary DB-wax column (30m x 0.25mm ID x 0.25µm) and individual peaks were identified by mass spectrometry. Peak areas were measured by integration and were normalised against the internal deuterated hexanol standard.

Taste Trial

In order to comply with the requirements of the Australian Genetic Manipulation Advisory Committee, seeds were removed from the tomatoes prior to taste trialing. Tomatoes from several vegetatively propagated clones of each of the primary transformed plants of interest, were harvested at between 7 and 9 days into ripening. The tomatoes were matched on the basis of size. Each tomato was quartered and the locular tissue, containing the seeds, removed. Each quarter was used for each of the four attributes (ripe flavour, green flavour, sweetness and acidity) by a single panellist. Four sets of samples were presented to each panellist, each in a different random order. Each set was used to rank only one of the attributes. The order of assessment of the attributes was the same for each panellist (ripe flavour, green flavour, sweetness and acidity). Panellists were asked to rank the samples on each of the four attributes. Samples with the greatest intensity of the attribute were given a rank of 1, whilst the least intense was ranked 4. An orthogonal latin square design was used

to balance out carry-over effects. This design required 12 assessments (panelists). However, due to insufficient samples, only 11 were made in this trial.

RESULTS

Transformation with *Adh 2* constructs and initial screening.

Tomato explants were transformed with constructs containing the tomato *Adh 2* cDNA as described in Figure 2, and 24 and 22 transformed plants from each experiment were selected for analysis.

Two types of transformation construct were used. Both contained the tomato *Adh 2* cDNA in a sense orientation relative to the construct promoter. In one set of experiments the CaMV 35S-promoter was used to provide constitutive expression of the cDNA. In the other the tomato polygalacturonase gene promoter (PG-promoter ; Bird et al., 1988; Nicholass et al., 1995) was used to provide fruit ripening-specific expression of the cDNA.

ADH activity was measured in pericarp, locular and whole fruit tissues. Activity was found to vary in the different tissues but was highest in pericarp tissue (data not shown). Because of this, and because of the uniform composition of the tissue, only pericarp activities are presented here.

Constitutive expression of the introduced cDNA(s) resulted in both enhanced and inhibited accumulation of the ADH 2 enzyme in the ripening fruit (Fig 3). Enhanced levels of accumulation were most evident in Breaker (Br ; first colour change) fruit but a number of fruit continued to show significantly ($P < 0.05$) enhanced levels at Br+7 (Breaker + 7 days). About 40% of the plants analysed had fruit in which ADH 2 expression was completely inhibited.

In the second set of experiments, in which the introduced cDNA(s) were expressed in a fruit ripening-specific manner, ADH activities at Br + 2 ranged from approximately that in control fruit to 6-7 times control levels, while activities at Br + 7 ranged from approximately control fruit levels to 2-3 times control levels (Fig 3). With the fruit-specific promoter none of the transgenic fruit had significantly lower ADH activity than the controls (Fig 3).

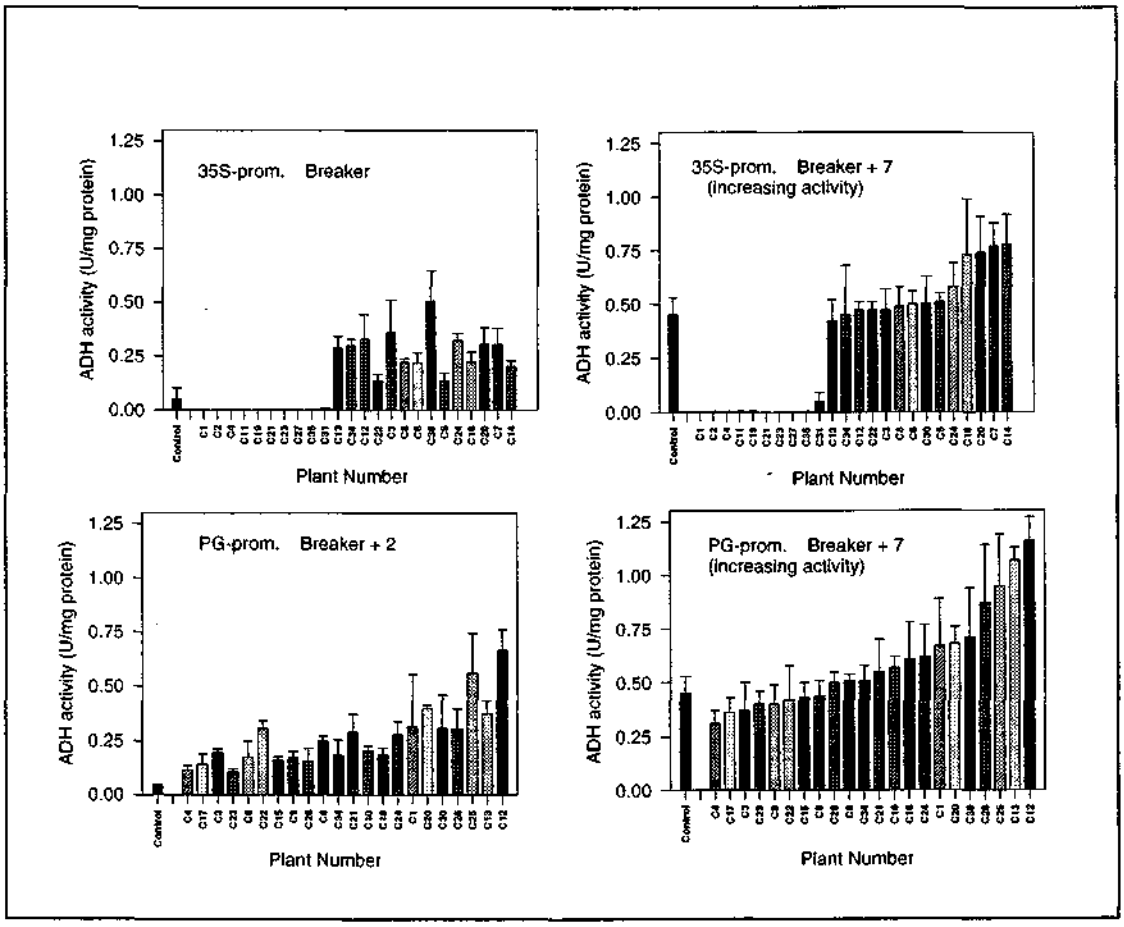

Figure 3 ADH specific activities in fruit from transformed plants

ADH activities in pericarp from breaker or 2 days post breaker (Breaker + 2) and 7 days post breaker (Breaker + 7) fruit from transformed tomato plants. The top two histograms show results for plants transformed with the PJR-ADH construct containing the tomato *Adh 2* cDNA coupled with the constitutive CaMV35S-promoter. The bottom two histograms show results from plants transformed with the PRD-ADH construct containing the tomato *Adh 2* cDNA coupled with the tomato fruit/ripening specific PG-promoter. Results are arranged in order of increasing activity in Breaker + 7 fruit (right hand histograms), and are indicated in the same order in the left hand histograms. Three fruit were averaged for each data point. Control in each histogram is a mean ± S.D. obtained from 3 fruit (each control point) from separate, untransformed plants.

ADH activities during ripening

Seven plants showing significant modifications in pericarp ADH activities were selected for more extensive analysis during fruit ripening and for analysis of fruit flavour volatiles. Cuttings from the selected plants were propagated to provide sufficient material and fruit of appropriate ages. Plants C20 and C23 contained constructs with constitutive expression of the inserted

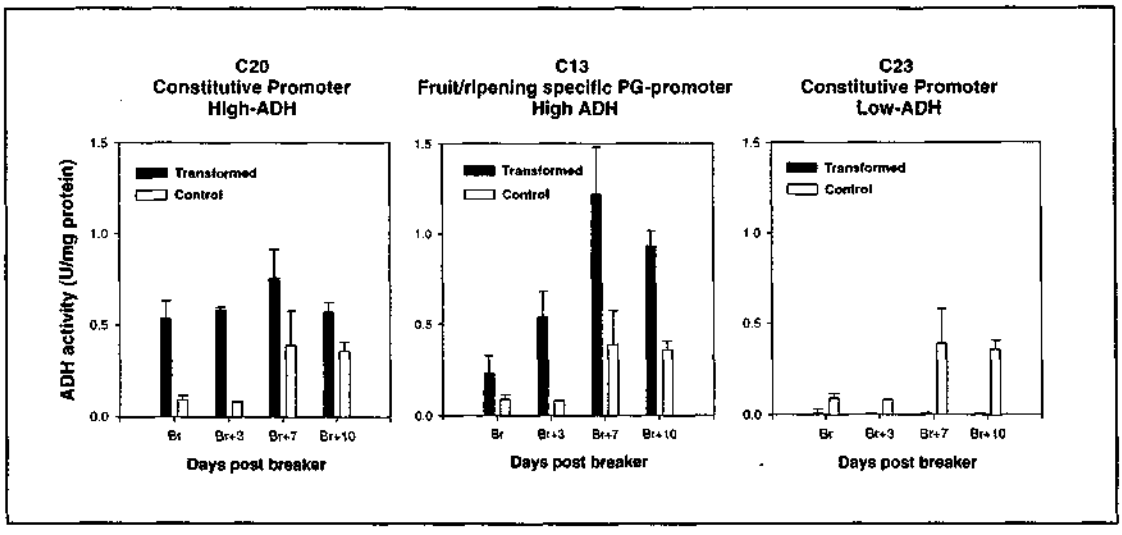

Figure 4. ADH specific activity in ripening fruit from transformed plants.

ADH activities in pericarp tissue are shown for ripening fruit from three transgenic plants, and are compared with ADH activities in fruit from an untransformed plant. Plant C13 was transformed with the *Adh 2* cDNA coupled with the fruit/ripening specific PG-promoter. Plants C20 and C23 were transformed with constructs containing the CaMV 35S-promoter. Control, untransformed fruit data are shown as open histograms. Data from transformed plants are shown as hatched histograms. Three fruit were averaged for each data point.

cDNA(s). Fruit from these plants showed respectively either enhanced ADH activity or minimal activity in fruit throughout ripening relative to levels in fruit from untransformed control plants (Fig 4). Plant C13 contained *Adh 2* cDNAs regulated by the fruit ripening-specific PG-promoter. Fruit from the C13 plant showed significantly enhanced levels of ADH activity which continued to increase up to 7 days post breaker then declined. In a separate series of experiments, ADH levels and volatiles were determined for breaker + 7 fruit from these plants and five other transformed plants (Table 1).

Analysis of *Adh* mRNA

Hybridisation analysis was used to examine gene activity relative to ADH activity in one of the plants in which ADH activity was suppressed. Hybridisation analysis of *Adh* mRNA in pericarp tissue of fruit from one of the untransformed plants (Ac#2), showed mRNA abundance increasing in the fruit up to about 10 days post breaker, then declining slightly (Fig 5). In contrast, no *Adh* mRNA was detectable in ripening fruit from the C23 plant in which

	Constitutive promoter High-ADH			PG-prom. High-ADH	Controls untransformed	Constitutive promoter Low-ADH		
Plant number	**C7**	**C14**	**C20**	**C13**	**AC#1,2 & 3**	**C4**	**C11**	**C23**
Estimated number of genes inserted (*Adh2*/NptII)	1/1	1/1	2/2	5/7	-	4/3	3/3	3/5
Number of fruit sampled	n=4	n=3	n=5	n=3	n=10	n=3	n=4	n=3
ADH specific activity (U/mg protein)	$0.67 \pm 0.16^{*}$	$0.56 \pm 0.04^{**}$	$0.76 \pm 0.15^{**}$	$1.26 \pm 0.31^{*}$	0.375 ± 0.15	$0.01 \pm 0.016^{***}$	$0.006 \pm 0.01^{***}$	$0.011 \pm 0.02^{***}$
Hexanal	8.48 ± 1.60^{ns}	9.13 ± 0.31^{ns}	9.94 ± 1.98^{ns}	$6.95 \pm 0.89^{*}$	9.43 ± 1.44	9.62 ± 0.85^{ns}	8.45 ± 0.44^{ns}	10.09 ± 2.27^{ns}
Z-3-hexenal	8.42 ± 1.07^{ns}	7.28 ± 0.40^{ns}	7.73 ± 1.28^{ns}	7.67 ± 1.42^{ns}	7.76 ± 1.57	9.28 ± 1.22^{ns}	7.57 ± 0.72^{ns}	8.71 ± 2.46^{ns}
E-2-hexenal	14.38 ± 3.24^{ns}	13.57 ± 1.76^{ns}	$15.99 \pm 1.72^{**}$	12.92 ± 2.78^{ns}	12.37 ± 2.03	11.68 ± 2.62^{ns}	$15.88 \pm 1.42^{**}$	10.82 ± 3.23^{ns}
Hexanol	0.25 ± 0.09^{ns}	$0.40 \pm 0.05^{**}$	$0.52 \pm 0.17^{*}$	0.29 ± 0.08^{ns}	0.22 ± 0.07	$0.02 \pm 0.025^{***}$	$0.04 \pm 0.02^{***}$	$0.02 \pm 0.002^{***}$
Z-3-hexenol	$3.08 \pm 0.28^{*}$	4.12 ± 0.79^{ns}	$6.02 \pm 1.58^{**}$	4.48 ± 1.27^{ns}	2.51 ± 0.46	$0.15 \pm 0.08^{***}$	$0.065 \pm 0.08^{***}$	$0.057 \pm 0.05^{***}$
Geraniol	0.72 ± 0.34	0.71 ± 0.16	0.62 ± 0.14	0.49 ± 0.10	0.89 ± 0.25	0.81 ± 0.13	0.6 ± 0.09	0.92 ± 0.15
E-2-octenal	0.43 ± 0.01	0.50 ± 0.09	0.57 ± 0.06	0.45 ± 0.03	0.61 ± 0.09	0.56 ± 0.1	0.52 ± 0.05	0.54 ± 0.20
1-octen-3-ol	0.09 ± 0.01	0.07 ± 0.02	0.11 ± 0.02	0.08 ± 0.01	0.10 ± 0.03	0.12 ± 0.03	0.10 ± 0.02	0.09 ± 0.03
Citral	0.30 ± 0.10	0.90 ± 0.14	0.69 ± 0.28	0.38 ± 0.10	0.70 ± 0.22	0.6 ± 0.01	0.35 ± 0.07	0.44 ± 0.15

Table 1. Number of introduced genes, ADH specific activities and relative abundances of some volatiles in fruit from transformed and control plants.

Number of inserted genes was estimated by quantitating the hybridisation to genomic DNA of probes for *Npt* II and for the *Adh 2* cDNA, as detailed under Materials and Methods. Headspace volatiles were extracted from Br+7 fruit, collected by SPME absorption, and analysed by GC/MS. Peak areas were determined by integration and were normalised against the area of a deuterated hexanol standard introduced during maceration of the tissue. Numbers of fruit sampled are indicated (n=). Averaged ADH activities from pericarp tissue and volatile abundances were derived from the same sets of fruit, and were derived separately from the data presented in Figure 2. Data from individual transformed plants were compared with pooled data from the control plants using Welch's t-test which does not assume equal variances: *** extremely significant ($P < 0.0001$), ** very significant ($P < 0.01$), * significant ($P < 0.05$). Statistical analyses of geraniol, E-2-octenal, 1-octen-3-ol and citral are not included as no correlation with ADH activity was evident.

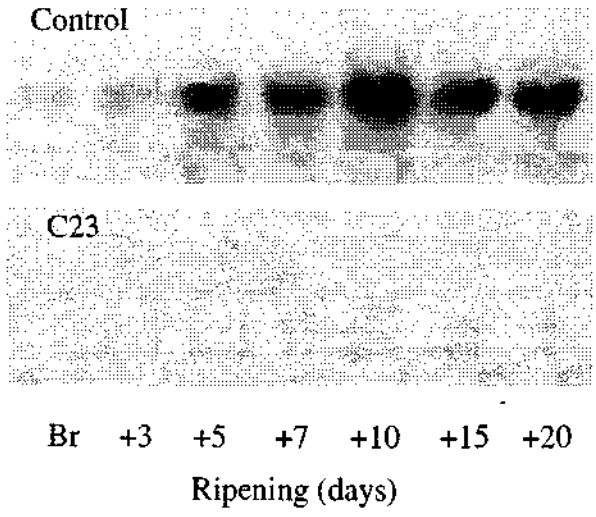

Figure 5. Hybridisation of ^{32}P-labelled tomato *Adh2* cDNA to RNA from pericarp of ripening fruit from a control plant and a low ADH transgenic plant (C23).

Each lane contains 10µg total RNA. Fractionation, transfer to membrane and hybridisation were as described in Longhurst et al. (1994).

ADH activity was minimal. This observation indicates that inhibition of ADH activity in this plant is a result of supressed gene activity, or cosuppression (Jorgensen, 1990)

Analysis of volatiles

Seven transformed plants, with the most pronounced variations in fruit ADH activities, were selected for analysis of the volatiles produced in their ripening fruit. Solid Phase Micro Extraction (SPME) was used as a quick and convenient method for measuring headspace volatiles from individual fruit. This method revealed some 15 major peaks of volatiles with many minor peaks (Fig 6). A number of the volatiles identified in Figure 6 are amongst those

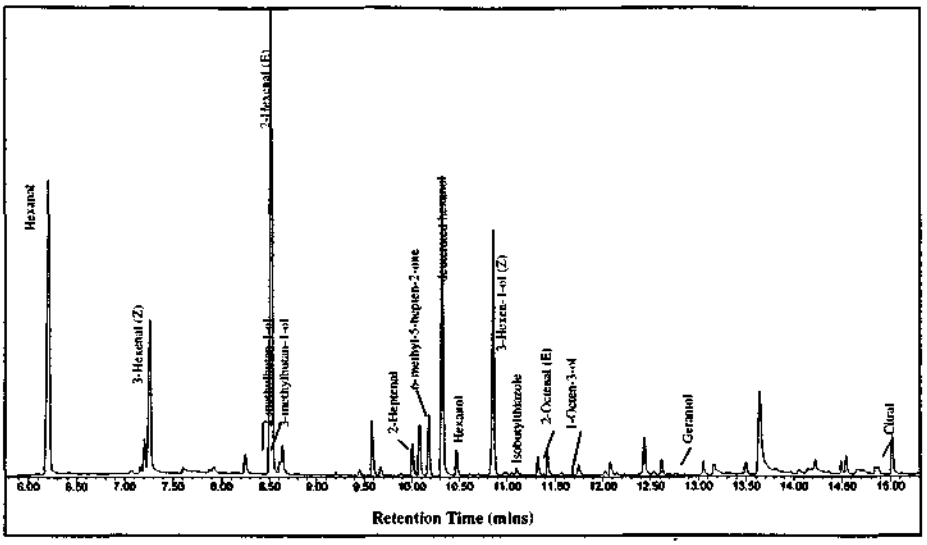

Figure 6. Volatiles isolated from headspace above macerated whole fruit tissue.
Typical elution profile of compounds detected by GC analysis of headspace volatiles.

considered to be important to flavour and aroma development. Of particular interest to us were the hexanal/ol and hexenal/ol compounds, peak areas of which are listed in Table 1, together with areas of some other aldehydes and alcohols detected.

Comparison of areas of individual peaks between tissue from control, high ADH and low ADH fruit showed, in general, little variation. Peak areas of the aldehyde Z-3-hexenal did not vary greatly between the various plants, while variations in the levels of the E-2-hexenal were detected only in fruit from the C20 and C11 plants. . Similarly the area of the aldehyde hexanal varied little between plants except in the case of the C13 plant where it was significantly lower relative to controls (Table 1). In contrast, levels of the alcohols, hexanol and Z-3-hexenol, did show significant variation between plants with differing ADH levels (Table 1). Relative to levels in control fruit, hexanol levels were significantly higher in fruit from two of the high ADH plants, C14 and C20. Z-3-hexenol levels were also higher in fruit from two of the high ADH plants, but in this case the significantly increased levels were in C7 and C20. Both hexanol and hexenol levels were significantly lower in fruit from all three low ADH plants. E-2-hexenol was not detected in any of the fruit.

Ratios of the fruit aldehydes to alcohols have been calculated for the three plants C20, C13 and C23 and are shown plotted as histograms in comparison with ratios in control fruit (Figure 7). As Z-3-hexenal isomerises to E-2-hexenal (Kazeniac and Hall, 1970), the ratio of

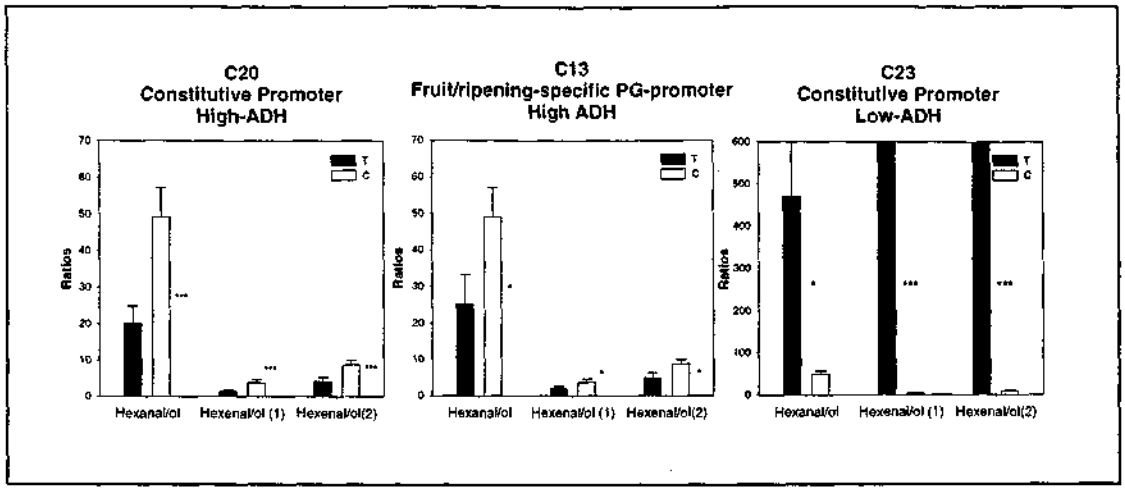

Figure 7. Aldehyde:alcohol ratios for a number of headspace volatiles from fruit of three ADH-modified plants, compared with ratios for control plants.

Ratios of aldehydes to alcohols were determined for individual fruit from the three representative transgenic plants (T) and from control plants (C) and means ± S.D. were calculated. The number of fruit sampled in each case is shown in Table 1. Control fruit ratios were determined for fruit from three control plants, AC#1, AC#2 and AC#4 (total fruit n=10), the ratios were pooled and the mean ± S.D. calculated . Hexenal/ol (1) is the ratio of Z-3-hexenal : Z-3-hexenol. Hexenal/ol(2) is the ratio of Z-3-hexenal + E-2-hexenal : Z-3-hexenol. As no hexenol was detectable in some of the C23 fruit, the composite ratios Hexenal/ol(1) and Hexenal/ol(2) for this plant are both infinity. The various ratios were compared with ratios from control fruit using Welch's t-test which does not assume equal variances. *** extremely significant ($P < 0.0001$), ** very significant ($P < 0.01$), * significant ($P < 0.05$).

(Z-3- + E-2-)hexenal : Z-3-hexenol has been included. In the two high ADH plants, C20 and C13, the ratios of fruit hexanal/ol , Z-3-hexenal/Z-3-hexenol and (Z-3- + E-2-)hexenal/Z-3-hexenol are all significantly lower than those from control fruit, while the low ADH plant, C23, has ratios significantly higher than those of the control.

Flavour testing - Taste Trial.

De-seeded fruit tissue from the selected plants was taste trialed by a panel of eleven individuals. The panel found a significant increase in ripe-fruit flavour in fruit from the C20 plant in which ADH levels were constitutively enhanced relative to fruit from other transgenic plants and from control plants (Table 2). Other attributes did not vary significantly between fruit from the various plants however a slight correlation between lowered green tomato character and increased sweetness of the C20 fruit, relative to the other fruit, was noted. The flavour characteristics of fruit from the C13 plant did not differ from those of control fruit.

	C20 constitutive high-ADH	C13 fruit specific high-ADH	Control untransformed	C23 constitutive low-ADH	P
Ripe tomato flavour	14[a]	34[b]	28[b]	34[b]	0.002
Green tomato leaf character	34[a]	23[a]	28[a]	25[a]	0.288
Sweetness	18[a]	36[b]	24[ab]	32[b]	0.014
Acidity	25[a]	29[a]	30[a]	26[a]	0.819

Table 2. Taste trial of transgenic tomatoes.

Tomatoes from the selected plants were harvested at optimal ripeness, between 7 and 9 days post breaker. Pericarp tissue from the fruit was tasted by eleven panellists as described under Materials and Methods. Samples with greatest intensity of attribute were given a ranking of 1, while those with least were ranked 4. Summed scores are shown with low rank sums implying a high intensity of that attribute. In a row, scores followed by a common letter are not significantly different (probability shown in column 'p').

The lowest rank sum for Ripe Tomato Flavour consisted of ten "1" rankings and a single "4" ranking.

DISCUSSION

The object of the three year project was to investigate the role of the enzyme alcohol dehydrogenase in flavour development in ripening tomato fruit. The abundance of the ADH enzyme appears to be related to the softness of the ripening fruit and a correlation between the enzyme and flavour development might provide a connection between the firmness of current commercial varieties and the percieved lack of flavour of the varieties. The project set out to accomplish the following three outcomes:

* Make transformation vectors containing the tomato *Adh2* cDNA in sense and antisense orientations, coupled with a CaMV 35S-promoter or the *Adh2* gene promoter, and a Nos 3' end.

* Transform tomato tissue with the constructs via *Agrobacterium* infection. Select transformed callus. Regenerate mature tomato plants and produce second generation plants by selfing.

* Determine insertion of *Adh2* gene, and gene dosage, by Southern analysis. determine gene expression by Northern analysis. Analyse ADH enzyme and activity levels. Analyse ethylene, acetaldehyde and flavour volatiles, hexanal/hexanol and 3-methoxybutanal/butanol. Flavour test if permission obtained from GMAC.

An early decision was made to concentrate the study on primary transgenic plants, so that plants with significantly modified phenotypes could be selected for production of second and subsequent generations. Also, the volatile compounds that were studied differed somewhat from those suggested as objectives primarily because of the methodology used and the relative importance of the various volatiles that we could detect accurately.

By introducing tomato *Adh 2* cDNA constructs, coupled to either a constitutive promoter or a fruit/ripening specific promoter, we produced a number of primary transgenic tomato plants with modified levels of ADH 2 activity in their ripening fruit. The introduction of the *Adh 2* cDNA under the control of the constitutive promoter resulted in a spectrum of transgenic plants including those with enhanced levels of ADH 2 activity in the ripening fruit (and in other tissues - unpublished result) and plants with barely detectable levels of ADH 2 activity in the ripening fruit. Hybridisation analysis (Fig 5) related the suppressed ADH activity in fruit from one of the transgenic plants to an absence of *Adh* mRNA in the fruit, indicating that the introduced transgene had induced cosuppression (Jorgensen,1990). Transgenic plants containing constructs with the tomato PG-promoter produced fruit showing enhanced levels of ADH 2 activity. In fruit from these plants, ADH 2 activity increased as the fruit ripened (C13, Fig 4), consistent with the fruit/ripening specificity of the PG-promoter (Bird et al., 1988; Nicholass et al., 1995). Unlike the observed incidence of cosuppression in plants containing the constitutively expressed transgene(s), no plants containing the fruit specific construct were found with suppressed ADH 2 activity in the ripening fruit. One explanation for this difference may be that, if cosuppression occurs in the ripening fruit of the PG-promoter plants, it occurs

subsequent to the synthesis of active enzyme whose residual activity throughout the sampling period masks the more long term effects.

The production of tomato plants with fruit which have significantly different ADH activities in their tissues has allowed us to examine the role of ADH in flavour development in the ripening fruit. We found some quantitative variation in headspace volatiles between fruit, but we also found consistent differences in the balance between some of the volatile aldehydes and alcohols in fruit from plants with differing ADH levels. Of particular interest was the interconversion of the 6-carbon alcohols and aldehydes which has been linked indirectly with the ADH 2 enzyme (Sieso et al., 1976; Bicsak et al, 1982; Longhurst et al., 1990; Chen and Chase, 1993).

Constitutive promoter - high-ADH plant C20.

Associated with the increased ADH activity in fruit from plant C20 were increases in the alcohol forms of the hexanal/ol and hexenal/ol volatiles (Table 1) giving rise to reduced ratios of aldehyde to alcohol as seen in Figure 7. These low ratios are consistent with an increased conversion of the C-6 aldehydes to their alcohols by the increased level of ADH in the transformed fruit and is the first direct evidence that the tomato ADH 2 enzyme mediates interconversion of hexanal/ol and the Z-3- form of hexenal/ol in the ripening fruit.

Fruit specific promoter - high-ADH plant C13.

In fruit from the C13 plant ADH activity was marginally higher during development, relative to control fruit (unpublished observation), but increased to approximately 3x the control fruit level, during ripening (Fig 4). As with the constitutive high-ADH plant C20 an increase in Z-3-hexenol was found in fruit from this plant, resulting in a decrease in hexenal/ol ratios (Fig 7). However, no increase in hexanol was observed while a decrease in hexanal was evident (Table 1). While this results in a similar, but less significant, reduction in aldehyde to alcohol ratio as that evident in the C20 plant, the mechanism clearly differs, differing also from that mediating the balance between the hexenals and hexenols in both plants. Assuming that ADH is involved, the high ADH activity attained in the fruit, or the specific timing of its increase in the tissues, may be affecting the mechanism. Possibilities include feedback from the alcohol end of the pathway to the primary events associated with the conversion of lipid to linoleic acid, or some mechanism for increased efflux of the alcohol product. It may also be that the

high ADH levels are influencing some remote pathway which is having an indirect influence on the levels of hexanal and/or hexanol.

Constitutive promoter - low-ADH plant C23.

ADH activity was reduced to barely detectable levels in fruit of plant C23. Also barely detectable in these fruit were hexanol and hexenol (Table 1) with the resulting aldehyde to alcohol ratios for C23 fruit differing grossly from those of control fruit (Fig 7) . Reduction of ADH 2 activity has therefore resulted in inhibition of the conversion of hexanal and hexenal to their alcohols providing further direct evidence of at least one of the roles of ADH 2 in the ripening fruit.

All the transformed plants showing marked modifications of phenotype contained more than one introduced *Adh 2* construct. The two plants C7 and C14 contained only single insertions (Table 1) and showed only marginal modifications of ADH activity and aldehyde : alcohol ratios. While this suggested a correlation between the number of genes inserted and the magnitude of their effect, the correlation did not hold in all cases. Gene silencing was only observed in plants transformed with constructs regulated by the constitutive promoter and only in plants containing more than one inserted gene (Table 1).

Our results indicate that modification of ADH 2 activity in the tomato fruit affects the balance between the 6-carbon aldehydes and alcohols. The effects are most marked when ADH 2 activity is reduced. The less pronounced consequences of increasing ADH 2 activity may indicate an upper limit to the tolerated concentration of the alcohols, regulated by feedback or by further metabolism. It may also reflect limitations in the reducing potential of NADH. While the levels of the alcohols differed significantly in the various classes of fruit, corresponding changes in the levels of the aldehydes were not evident. In all the transgenic plants except C13, the ADH levels were modified constitutively and the fruit could presumably reach an equilibrium during development. In the C13 plant, increased ADH activity only occurred in the ripening fruit (unpublished observations). In this case a significant decrease in the level of the hexanal was evident, but the Z-3- and E-2- hexenals did not differ significantly from the levels in control fruit. In all these cases the actual mechanism of regulation is unknown at this time.

We were unable to detect the E-2-hexenol volatile in SPME-headspace analyses of any of the tomato extracts despite confirming that the compound could be detected by our sampling procedure. Low levels of the aldehyde E-2-hexenal relative to its Z-3-isomer have been reported in tomato fruit (Dirinck et al., 1976; Buttery et al., 1987, 1988, 1989; Baldwin et al., 1991) and an absence or low activity of the cis-3-trans-2 isomerase has been postulated as a reason for this (Galliard et al., 1977) . The relatively high levels of E-2-hexenal observed in all our samples therefore are unexpected and may have resulted from non-enzymic isomerisation during volatile collection and processing. The apparent absence of E-2-hexenol would then result from the accumulation of the E-2-aldehyde subsequent to the inactivation of the ADH enzyme.

Also missing in the headspace analyses was 3-methylbutanal. In this case however, standards of the volatile were also undetectable by our methods. Direct analysis of headspace volatiles has been undertaken and does show ADH related modulation of the ratio of 3-methylbutanal/ol (A.Taylor, Nottingham University, U.K., personal communication).

It was of interest to determine the specificity of ADH 2 on other aldehydes and alcohols readily detectable in headspace analyses of the various fruit. While some inter- and intra-fruit variation was observed in the relative abundances of octenal and octenol, there was no evidence of ADH 2 being involved in their interconversion. Production of the alcohol from the unstable free radical of linoleic acid, and an unstable hemi-acetal intermediate, has been postulated (Hoffman, 1962), and it is possible that dehydrogenases are not involved in regulating the balance between this alcohol and its aldehyde.

Another aldehyde / alcohol combination of interest was citral (*trans*-3,7-dimethyl-2,6-octadien-1-al) / geraniol (*trans*-3,7-dimethyl-2,6-octadien-1-ol). Bicsak et al., (1982) reported the tomato ADH enzyme as having "appreciable activity" *in vitro* on the terpene geraniol, and suggested that the NAD^+- dependent tomato enzyme was associated with interconversion of citral and geraniol in the tomato fruit, with the equilibrium being in favour of aldehyde formation. In strawberry (Yamashita et al., 1978), pea (Eriksson, 1968; Leblová and Mancal, 1975), potatoes and oranges (Potty and Bruemmer, 1970), this interconversion is believed to be mediated by an $NADP^+$ -preferring terpene alcohol dehydrogenase (Bicsak et al., 1982). In addition, Kazeniac and Hall (1970) reported an absence of geraniol in extracts of tomato,

although its isomer, linalool, was found. In contrast, we detected significant amounts of geraniol in the tomato fruit, and, although there is considerable variation in ratios of citral : geraniol between individual fruit and between plants, there is no evidence of the ratio being influenced by variations in the activity of the ADH 2 enzyme.

The tomato ADH 2 enzyme accumulates naturally during the ripening/softening of the fruit (Bicsak et al., 1982; Longhurst et al., 1990), being most abundant late in ripening. Associated with the increase in enzyme is an increase in abundance of its mRNA (Chen and Chase, 1993; Longhurst et al., 1994). The mechanism for these increases is as yet unclear. It has been speculated (Longhurst et al., 1990) that changes in cytoplasmic pH accompanying changes in cytoplasmic ion concentrations (Vickery and Bruinsma, 1973), which in turn result from membrane leakage in the softening fruit, might be responsible. Another suggested mechanism (Longhurst et al., 1994) is enhanced transcription of the *Adh 2* gene in response to a slight lowering of internal O_2 concentrations, again as a consequence of the softening process. Analysis of ADH activity levels in firm fruit varieties (Sunny and Floradade) and soft fruit varieties (83G38 , Momotaro and Castlemart) has suggested a loose connection between fruit softness and ADH activity (Longhurst et al., 1990). If fruit softness affects ADH activity which in turn influences, as shown here, the natural balances of flavour associated aldehydes and alcohols in the ripening fruit, then a correlation between fruit firmness and flavour development can be postulated.

Modification in flavour

Fruit from the C20 high-ADH plants were evaluated by a taste panel as having significantly enhanced ripe-fruit flavour relative to control fruit (Table 2). Interestingly, the C20 fruit also tended to a lower intensity of the green fruit attribute and higher intensity of the sweetness attribute, but these trends were not statistically significant. In the taste trial, the C13 high-ADH fruit and C23 low-ADH fruit did not appear to differ in flavour characteristics from control fruit. Both the C20 and C13 fruit have raised levels of ADH activity relative to control fruit, approximately 2.0 times and 3.4 times respectively. However, the resulting increase in levels of the 6-carbon alcohols, hexanol and hexenol, is greater in the C20 fruit than in the C13 fruit. As discussed above, this may be due to the enhanced ADH levels being constitutive in the

C20 plant while being specific to the ripening fruit in the C13 plant. The improved flavour characteristics of the C20 fruit therefore appear to be related to increased levels of the alcohols. Kazeniac and Hall, (1970) underlined the importance of the aldehydes to the fresh tomato flavour and suggested that reduction in the aldehydes allowed the contribution of the alcohols to predominate and resulted in development of a 'processed' or 'enzymic' flavour. In contrast, our findings stress the importance of the alcohols and suggest that a balance between aldehydes and alcohols is essential to the development of the ripe fruit flavour.

While the taste panel did not rank the low-ADH fruit as having significantly poorer taste compared to control fruit, our observations in the laboratory are that the fruit are bland. Dr Richard Gawel, who ran the taste trial for us, commented on the subjective nature of taste trials and that low levels of significance were common. The "improved ripe fruit flavour" of the C20 fruit was of much greater significance than would have been expected for such trials.

Wang et al., (1996) have reported the introduction of a functional yeast Δ-9 desaturase gene into tomato. The introduced gene affected unsaturation of fatty acids in the fruit which resulted in increased abundances of several of the volatiles discussed above. In fruit from one transgenic plant, hexanal and *cis*-3-hexenal concentrations were 2.7 and 2 fold higher respectively than levels in control fruit while hexanol and *cis*-3-hexenol concentrations were both increased about 4 fold relative to controls.

The increasing ability to modify individual stages of this important pathway greatly increases the prospects for improving the flavour characteristics of tomato and possibly other fruits.

EXTENSION/ADOPTION BY INDUSTRY.

This project has taken the research close to proof of concept, and the final stages of the next project should be proof of concept. This proof includes incorporating the genetic modification into Australian varieties by backcrossing, in collaboration with QDPI and the AgVic. Some of the issues involved in the extension and adoption of this work by industry are covered in the project currently under consideration by HRDC and others are part of CSIRO plans for follow-on projects together with HRDC and Zeneca and the tomato industry, assuming proof of concept is complete. There will be two issues to be addressed:

Firstly how better to handle existing tomatoes if it is definitely shown that alcohol dehydrogenase activity, flavour development and tomato softening are inextricably linked.

The second issue is how to bring this material to market. This will involve education on public issues and grower issues in the use of transgenics and will necessitate a financial plan by the partners in order to ensure that the intellectual property rights necessary to bring this project to the market are protected in such a way as to be accessible to the Australian tomato industry.

FUTURE RESEARCH.

We have shown that the enzyme ADH plays an important role in regulating the abundances of various aldehydes and alcohols associated with the ripe fruit flavour in tomatoes. Our findings suggest that a critical level of ADH activity is required for optimal flavour development. We also have evidence that ADH activity is a function of fruit softness, with softer fruit attaining a higher ADH activity than firmer fruit. Therefore we see a linkage between poor fruit flavour and fruit firmness that may not be approachable using traditional breeding methods, but may be amenable to genetic engineering. In the course of the above project we have increased the ADH activity in one tomato variety by genetic manipulation and have demonstrated a significant increase in ripe-fruit flavour in the modified fruit relative to fruit from control, unmodified plants.

The next step of taking these improvements through to a commercial application is to test whether the beneficial genetic modifications can be successfully transferred into other tomato lines. This will be tested with two current Australian commercial lines. Transfer will be by standard breeding and backcrossing and the BC2 fruit will be analysed for volatiles, sugars and acids, ADH activity and flavour (taste testing). Concurrent with the breeding, the biochemical and genetic stability of three of our modified lines will be examined by selfing for two generations and analysis of the progeny, and by backcrossing to the untransformed parental line and testing of the progeny.

Collaboration with QDPI and VicAg will help with planning and evaluation of the breeding program. Taste trialing will be undertaken in association with the University of Adelaide but

application will be made to GMAC for commercial growing of fruit for the taste trials, as difficulty has been encountered in providing sufficient fruit of suitable quality in previous trails.

Correlation of results from taste trials, with biochemical data, is expected to provide a biochemical basis for screening fruit from breeding trials for enhanced flavour characteristics.

FINANCIAL/COMMERCIAL BENEFITS.

A key to obtaining direct benefits from this research is to ensure that the intellectual property protection is in place and the correct licence arrangements have been achieved. The current agreement between CSIRO, HRDC and Zeneca addresses the principles behind these issues. In terms of direct commercial benefits to the Australian industry successful production of these tomatoes would generate a more valuable product for sale by Australian growers, both on the domestic and on the export market. On the domestic scene, the industry is already concerned about a declining market related to quality issues, and in particular to flavour. In terms of exports, if it is possible to separate firmness and flavour, then firm but flavoured tomatoes would be a potential export product.

Commercialisation and adoption of this research would also ensure that the Australian tomato industry remains in the forefront of the best products available in the tomato market.

Acknowledgements.

The authors are grateful to Dr Richard Gawel, University of Adelaide, for conducting the taste trial, to Dr Rachel Drake for advice on analysis of transformants, to Sue Maffei for helping to establish the system for volatile analyses and to Dr Ray Correll for help with statistical analyses. Thanks go also to the Australian Horticultural Research and Development Corporation for financial support.

LITERATURE CITED

Baldwin EA, Nisperos-Carriedo MO, Moshonas MG (1991) Quantitative analysis of flavour and other volatiles and for certain constituents of two tomato cultivars during ripening. J Amer Soc Hort Sci **116(2):** 265-269

Bevan MW (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res **12:** 8711-8721

Bicsak TA, Kann LR, Reiter A, Chase TJr (1982) Tomato alcohol dehydrogenase: purification and substrate specificity. Arch Biochem Biophys **216:** 605-615

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

Buttery RG, Seifert RM, Guardagni DG, Ling LC (1971) Characterization of additional volatile components of tomato. J Agric Food Chem **19:** 524-529

Buttery RG, Teranishi R, Ling LC (1987) Fresh tomato aroma volatiles: A quantitative study. J Agric Food Chem **35:**540-544

Buttery RG, Teranishi R, Ling LC, Flath RA, Stern DJ (1988) Quantitative studies on origins of fresh tomato volatiles. J Agric Food Chem **36:** 1247-1250

Buttery RG, Teranishi R, Flath RA, Ling LC (1989) Fresh tomato volatiles. *In:* Flavour Chemistry: Trends and Developments. Roy Teranishi, Ron G Buttery and Fereidoon Shahidi (eds). ACS Symposium Series 338. American Chemical Society, Washington DC. Pp 213-222

Caperle M, Maiani G, Azzini E, Conti EMS, Raguzzini A., Ramazzotti V, Crespi M (1996) Dietary profiles and anti-oxidants in a rural population of central Italy with a low frequency of cancer. Eur. J. of Cancer Prevention **5(3):** 197-206

Chen A-RS, Chase TJr (1993) Alcohol dehydrogenase 2 and pyruvate decarboxylase induction in ripening and hypoxic tomato fruit. Plant Physiol Biochem **31:** 875-885

Clinton SK, Emenhiser C, Schwartz SJ, Bostwick DG, Williams AW, Moore BJ, Erdman JW (1996) Cis-trans lycopene isomers, carotenoids, and retinol in the human prostate. Cancer Epidemiology Biomarkers & Prevention **5(10):** 823-833

Dirinck P, Schreyen L, van Wassenhove F, Schamp N (1976) Flavour quality of tomatoes. J Sci Food Agric **27:** 499-508

Eriksson CE (1968) Alcohol: NAD oxidoreductase (E.C.1.1.1.1.) from peas. JFood Sci **33:** 525-532

Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC (1995) Complete sequence of the binary vector Bin 19. Plant Molec Biol **27:** 405-409

Galliard T, Matthew JA, Wright AJ, Fishwick MJ (1977) The enzymatic breakdown of lipids to volatile and non-volatile carbonyl fragments in disrupted tomato fruits. J Sci Food Agric **28:** 863-868

Hatanaka A, Kajiwara T, Sekiya J (1986) Fatty Acid Hyperoxide Lyase in Plant Tissues. Volatile Aldehyde Formation from Linoleic and Linolenic Acid. In: *Biogeneration of Aroma.* ACS Symposium Series 317. Parliment TH and Croteau R eds. American Chemical Society, Washington DC. Pp. 167-175

Hoffman G (1962) 1-Octen-3-ol and its relation to ther oxidative cleavage products from esters of linoleic acid. J Amer Oil Chemists' Soc **39:** 439-444

Jorgensen R (1990) Altered gene expression in plants due to *trans* interaction between homologous genes. Trends in Biotechnology **8:** 340-344

Kazeniac SJ, Hall RM (1970) Flavour chemistry of tomato volatiles. J Food Sci **35:** 519-530

Leblová S, Mancal P (1975) Characterization of plant alcohol dehydrogenase. Physiol Plant **34:** 246-249

Levy J, Bosin E, Feldman B, Giat Y, Miinster A, Danilenko M, Sharoni Y (1995) Lycopene is a more potent inhibitor of human cancer cell proliferation than either alpha-carotene or beta-carotene. Nutrition and Cancer - An International Journal **24(3):** 257-266

Linforth RST, Savary I, Pattenden B, Taylor AJ (1994) Volatile compounds found in expired air during eating of fresh tomatoes and in the headspace above tomatoes. J Sci Food Agric **65:** 241-247

Longhurst TJ, Tung HF, Brady CJ (1990) Developmental Regulation of the Expression of Alcohol Dehydrogenase in Ripening Tomato Fruits. J Food Biochem **14:** 421-433

Longhurst T, Lee L, Hinde R, Brady C, Speirs J (1994) Structure of the Tomato *Adh 2* Gene and *Adh 2* Pseudogenes, and a Study of *Adh 2* Gene Expression in Fruit. Plant Molec Biol **26:** 1073-1084

McGlasson WB, Last JH, Shaw KJ, Meldrum SK (1987) Influence of the non-ripening mutants *rin* and *nor* on the aroma of tomato fruit. HortSci **22(4):** 632-634

Nicholass FJ, Smith CJS, Schuch W, Bird CR, Grierson D (1995) High Levels of Ripening-Specific Repoter Gene Expression Directed by Tomato Fruit Polygalacturonase Gene-Flanking Regions. Plant Mol Biol **28:** 423-435

Potty VH, Bruemmer JH (1970) Oxidation of geraniol by an enzyme system from orange. Phytochem **9:** 1001-1007

Sieso V, Nicolas M, Seck S, Crouzet J (1976) Constituants volatils de la tomate: mise en evidence et formation par voie enzymatique du *trans*-hexene-2-ol. Agric Biol Chem **40:** 2349-2353

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

Tanksley SD (1979) Linkage, chromosomal association and expression of *Adh-1* and *Pgm-2* in tomato. Biochem Genet **17:** 1159-1167

Thomas MR, Matsumoto S, Cain P, Scott NS (1993) Repetitive DNA of grape wine: classes present and sequences suitable for cultivar identification. Theor Appl Genet **86:** 173-180

Vickery RS, Bruinsma J (1973) Compartments and permeability for potassium in developing fruits of tomato (*Lycopersicon esculentum* Mill.). J Exp Bot **24:** 1261-1270

Wang C, Chin C-K, Ho C-T, Hwang C-F, Polashock JJ, Martin CE (1996) Changes in fatty acids and fatty acid-derived flavour compounds by expressing the yeast Δ-9 desaturase gene in tomato. J Agric Food Chem **44:** 3399-3402