VG97093

Flavour Improvement in Tomatoes: Genetic Manipulation and Breeding for Improved Flavour

J Speirs
CSIRO Plant Industry, Horticulture Unit

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VG97093

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Flavour Improvement in Tomatoes: Genetic Manipulation and Breeding for Improved Flavour

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HRDC Project VG97093

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Media Summary.

Most fresh market tomatoes are bred for robustness, attractiveness and long shelf life but lack the intensity of flavour of fresh-picked, garden grown varieties. The use of firm, slow ripening cultivars is a major factor contributing to the lack of flavour, although there are other reasons including some harvesting, storage, transport and marketing practices. The characteristic ripe fruit flavour is due partly to the balance of sugars and acids in the ripe fruit but is also critically dependent on a balance of flavour volatiles.

- We have identified an enzyme which is involved in the development of flavour in the ripe tomato fruit and which is produced in response as the fruit soften during ripening. In firmer fruit less of this enzyme is produced with the result that less intense flavour is developed in the fruit.
- We have increased the activity of this enzyme in the ripening fruit of one variety of tomato by introducing extra copies of the tomato's own gene for the enzyme into the plant. The increased enzyme activity in these fruit is independent of the softening of the fruit and is linked to a significant improvement in flavour in the fruit.
- We have transferred the genetic improvement into six tomato cultivars which have been grown commercially in Australia for the fresh table market. In all six cases, activity of the enzyme was significantly increased in the ripening fruit.
- The one modified cultivar, Floradade, which has been trialed by a taste panel was found to have significantly improved flavour in its fruit.
- It is planned to field trial and taste trial the modified Floradade cultivar in 2000.

Firmness in tomatoes makes for easier picking, handling and marketing, ensures that the tomatoes will survive the supermarket trolley and the shopping bag and gives the fruit a longer shelf life. Until now the disadvantage has been a lack of flavour in the tomato. We believe we have broken the link between firmness and lack of flavour and that application of this technology will make a significant contribution to improving the flavour of fresh market tomatoes.

Technical Summary.

Most fresh market tomatoes are bred for robustness, attractiveness and long shelf life but lack the intensity of flavour of fresh-picked, garden grown varieties. The use of firm, slow ripening cultivars is a major factor contributing to the lack of flavour, although there are other reasons including some harvesting, storage, transport and marketing practices.The characteristic ripe fruit flavour is partly due to the balance of sugars and acids in the ripe fruit but is also critically dependent on a balance of flavour volatiles. We have identified a tomato enzyme, *Alcohol Dehydrogenase 2* (ADH2), which is involved in the production of several of the important flavour volatiles and is produced as the fruit soften during ripening. The ADH2 enzyme is known to play an important role in the production of a number of important flavour volatiles, in particular the 6-carbon aldehydes and alcohols derived from lipids via the lipoxygenase pathway, and the 5-carbon aldehyde/alcohol, 5-methylbutanal/ol, derived by deamination and decarboxylation of leucine. By uncoupling ADH2 activity from the softening of the fruit, it may be possible to improve the flavour characteristics of the firm tomato fruit cultivars favoured by the tomato industry.

In a previous project we have increased the activity of the ADH2 enzyme in the fruit of one cultivar of tomato (Ailsa Craig) by introducing into the plant extra copies of the tomato's own gene encoding the enzyme. Taste trials have shown that the increased enzyme activity was linked with a significant improvement in flavour in the fruit.

In the course of this project we have shown that:

- The activity of the enzyme alcohol dehydrogenase 2 (ADH2) in ripening tomato fruit is strongly correlated with the softening of the fruit. Commercially favoured firm fruit have less of the enzyme than soft fruit.
- Genetically modified fruit with increased levels of the ADH2 enzyme throughout development have:
  - significantly improved ripe-fruit flavour and sweetness as determined by taste panels.
  - increased accumulation of the 6-carbon alcohols hexanol and hexenol which are important flavour compounds in the ripening fruit.
  - significantly higher levels of the sugars glucose and fructose.
  - lower levels of isobutylthiazole, which can be an off flavour at higher concentrations.

We have transferred the genetic improvement , by traditional breeding, into six tomato cultivars which have been grown commercially in Australia for the fresh table market. In ripening fruit of all six of the primary crosses, activity of the ADH2 enzyme was significantly increased. Seeds were collected from the primary crosses and one cross (Floradade x transgenic Ailsa Craig) was selected for further analysis and for backcrossing in preparation for field trials and taste trials in 2000.

In a recent taste trial, fruit from the Floradade x transgenic Ailsa Craig cross was found to have significantly improved flavour relative to both Floradade and to Floradade x non-transgenic Ailsa Craig controls.

Backcrossing of the Floradade x transgenic Ailsa Craig cross is under way with BC1 fruit continuing to show enhanced levels of the ADH2 enzyme.

Introduction.

Alcohol dehydrogenase (ADH; alcohol:NAD oxidoreductase; E.C. 1.1.1.1.) of tomato fruit is a relatively nonspecific oxidoreductase capable of using as substrate a range of primary alcohols or aldehydes (1,2). Sieso et al., (3) have demonstrated that tomato fruit ADH can catalyse the interconversion of hexanal and hexanol, and of trans-hexen-2-al and trans-hexen-2-ol. They proposed that, in conjunction with lipoxygenase, ADH was involved in the formation of these important flavour volatiles from linoleic acid although hexanal and hexanol have since been shown by Hatanaka et al., (4) to be the products of lipoxygenase activity on linolenic acid. Bicsak et al., (2) also suggested that ADH may play an important role in producing significant aroma volatiles such as 3-methylbutanol (5) in the ripening fruit. A number of organoleptic and biochemical studies have identified the volatiles hexanal/ol, hexenal/ol and 3-methylbutanal/ol as important flavour and aroma compounds in tomato fruit (6-10).

Studies undertaken in this laboratory (HRDC project VG427 and Speirs et al., 1998 [11], Prestage et al., 1999 [12]) have shown that the modification of ADH activity in ripening fruit results in changes in the levels of the 5-carbon and 6-carbon aldehydes and alcohols as described above.

Analyses of ripening tomato fruit show an increase in messenger RNA for the ADH enzyme and a corresponding increase in activity of the enzyme when flavour development is at a maximum. There is also an observation that ADH levels are lower in fruit of less flavoursome tomato cultivars (13,14).

In this study we confirmed a correlation between ADH activity and ripe-fruit flavour and also demonstrated a correlation between the softness of the fruit and the enzyme activity. We tested the genetic and phenotypic stability of the modification used to increase ADH activity in the modified tomato lines and, by traditional breeding, transfered the most promising genetic modification into 6 commercial cultivars of tomato, commonly field grown in Australia, in order to field trail and taste trial the modification early in 2000.

We have also carried out a storage trial on control and high ADH fruit, to test the effects of cold storage on the fruit, and have noted additional effects of ADH activity on other flavour components of the tomato fruit, namely on the volatile isobutylthiazole and on the accumulation of reducing sugars.

Material & Methods

Plant Growth and Crossing.

Tomato (*Lycopersicon esculentum* Mill.) 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. Day/night temperatures were regulated at 26°C and 18°C respectively. 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.

Ailsa Craig and a transgenic line of Ailsa Craig, C20, containing two tomato *Adh2* genes coupled with constitutive promoters and with high ADH activity in the fruit,

were the primary experimental tomato lines. The transgenes were crossed into 6 near-isogenic tomato cultivars which have been commercially field grown in Australia:
From Queensland: Floradade, Hayslip, Walter and Tristar.
From Victoria: Goulburn and Arcadia.

For crosses, the best period for fertilising the flowers of the recipient plants was 1 –3 days before pollen shed, when there was a gradual change in colour of the flower from green to yellow. Pollen was obtained from fully open flowers of the Ailsa Craig donor plants.

Selection of Plants Containing Transgene Inserts.

Seeds from transgenic fruit were germinated, transferred to individual containers approximately 1 week after emergence and one cotyledon from each seedling was removed for DNA typing. DNA was extracted from each leaf (approximately 200mg of leaf tissue) by the method of Hamilton et al. (1998) (15). 1μg of the DNA was used for PCR (Polymerase Chain Reaction) amplification using primers designed for the amplification and detection of DNA sequences encoding the ADH2 enzyme. Amplified DNA was fractionated on 1% agarose gels where the endogenous gene product was recognised as a 400 bp fragment while the presence of the transgene was evidenced by a 200 bp fragment.

Analysis of Number of Transgenes Inserted in Plant Lines.

The number of transgenes inserted in each plant line was determined by genomic Southern blot analyses as described in Speirs et al. (1998) (11). Genomic DNA was extracted from young leaves by the method of Thomas et al., (1993) (16). 7μg of each of the genomic DNAs was digested with either *Hind* III 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 digested DNA). 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.

Analysis of Alcohol Dehydrogenase (ADH) Activity.

Alcohol dehydrogenase was extracted from fruit pericarp tissue and assayed according to the method of Longhurst et al. (14) using ethanol as the substrate and monitoring the convertion of NAD to NADH. The protein concentration was measured using a protein quantification Kit II (BioRad).

Analysis of Flavour Volatiles.

Fruit were harvested 7 days post breaker. 10g of pericarp tissue 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. 1µl of uniformly labelled, deuterated Hexanol (80nmoles /µl) was added as an internal standard. 6.6g of the mixture were 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 with 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.

Analysis of Sugars and Acids.

Fruit pericarp tissue was prepared for sugar and acid analysis as follows. Two 400-500mg samples of pericarp tissue were taken from each fruit. The samples were each soaked in 500µl of 100% ethanol in sealed eppendorf tubes for a *minimum* of 24 h at –20°C. The tissues were then ground in the ethanol until fully homogenised and centrifuged at 10,000 g for 2 min after which the supernatants were transferred to fresh eppendorf tubes. A further 100µl of ethanol was added to each tissue pellet and the pellets were resuspended by grinding briefly. The tubes were again centrifuged at 10,000 g for 2 minutes and the supernatants for each sample were pooled and stored at –20°C until required.

Acid analysis: One supernatant from each fruit was evaporated to dryness in an Aquavac (vacuum centrifuge) and was resuspended in Acid Running Buffer (0.1% H_3PO_4) at a rate of 1µl ARB per µg of original sample (i.e. 200mg tissue/200µl H_3PO_4). The dissolved pellet was centrifuged at 10,000g for 2 min and 100µl removed for HPLC analysis. The sample was diluted 1:10 with ARB and was analysed by HPLC on a Supelcogel™ G610H column (Supelco) which had been calibrated with citric and malic acids at a range of loadings from 10 – 25 µg. 100µl of diluted sample was loaded and was eluted at 30°C with ARB. Sample elution was detected at 210 nm.

Sugars analysis: The second supernatant from each fruit was deionized by treating with 100µ Amberlite MB3 beads (Sigma A-7518) for 30 min after which the beads were rinsed twice with 75µl of 60% ethanol. The pooled supernatant and washes were dried under vacuum, redissolved in 0.1mM $CaNa_2EDTA$ and centrifuged, as above, prior to analysis. Analysis was carried out by HPLC on a Sugar-Pak 1 column (Waters Corp.) which had been calibrated with glucose and fructose at a range of loadings between 50 – 250 µg. 15µl of sample were loaded and were eluted with the 0.1mM $CaNa_2EDTA$ buffer at a column temperature of 80°C. Sample elution was detected with an IR detector.

Measurement of Fruit Softness.

Fruit softness was measured with a compression meter that indicated deformation (mm) under a load of 500g for 5 sec (17).

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 the plants of interest were harvested at between 7 and 9 days after breaker (first colour change). 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, while the least intense was ranked 4. An orthogonal latin square design was used to balance out carry-over effects. Panel sizes varied and were made up of some untrained panellists (about 50%) and some partially trained panellists with extensive experience in wine flavour and acid balance assessment (mean experience=10years). While all panelists knew the general purpose of the tasting, i.e. a comparison of flavour modified tomatoes, none were aware of the exact nature of the samples.

Statistical Treatments.
Biochemical data from the 3rd taste trial were analysed using a one-way analysis of variance. The residuals were examined for each analysis. Where the usual assumptions for the analysis of variance were not met (this applied especially to the ratios), a log transformation was used. The experimental data in the Storage Experiment were analysed as a two-way analysis of variance using Genstat (Payne *et.al.* 1993) (22). The residuals were examined for each analysis. Where the usual assumptions for the analysis of variance were not met (for example with hexenol), a log transformation was used.

Single lsd^s for many of the graphs were calculated after consideration of the various statistical assumptions required. In several cases the assumptions were not found to be met. In these cases the data were subdivided in an attempt to overcome the problem. Where the conditions were approximately fulfilled, a single lsd was provided – this was done knowing that the approximation is still good even if the variances are slightly different, providing the group sizes are comparable. In the case of Isobutylthiazole, the distribution was very skew. Furthermore the data included zeros. By using a transform of the type $y = \log(x + k)$, where x was the measured concentration and k was set to 0.01, the assumptions were satisfied.

Results

#1 Correlation between softening and the activity of the enzyme alcohol dehydrogenase 2 (ADH2) in tomato fruit.

The enzyme ADH2 is associated with the development of flavour in tomato fruit. To demonstrate that there is a correlation between the softening of the tomato fruit and the actvity of the enzyme in the fruit (and hence the development of flavour in the fruit), six commercial cultivars of tomato with a range of fruit softnesses were selected for analysis. Four plants of each cultivar were grown to maturity in a controlled glasshouse environment and fruit were harvested at various stages of ripeness for analysis of softness and ADH2 activity. A number of commercial cultivars were selected on the basis of fruit firmness or softness. The selected cultivars were as follows:

Firm:	MH1	Intermediate:	Celebrity	Soft:	Rouge de Marmande
	Goulburn				Sioux
	Floradade				

Ripening stages analysed were Br (first colour change), Br + 3 days, + 7 days, + 10 days and + 15 days.

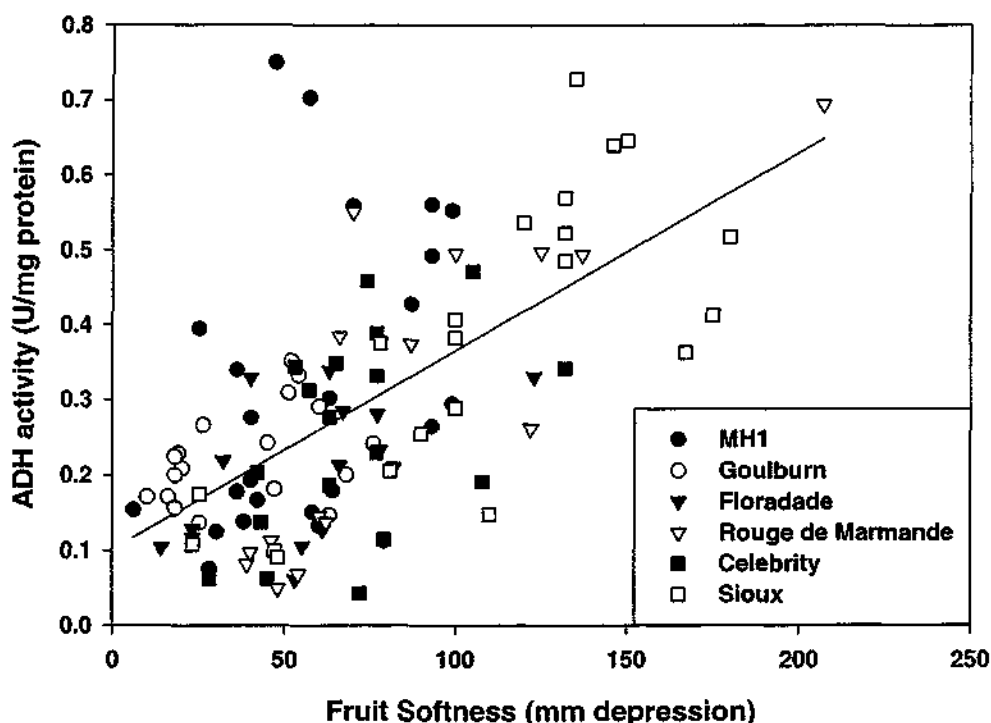

Figure 1. ADH activity in tomato fruit relative to the softness of the fruit.
Data points are individual fruit obtained from 4 plants of each variety.

Results.
There is a strong correlation between ADH activity and softness as shown in Figure 1. The correlation coefficient of 0.625 is clearly significant as it exceeds the critical value of 0.254 for a $P<0.01$.

#2 Taste trialing of tomato fruit with modified levels of ADH activity.

Taste trials: Three formal taste trials have been conducted, two small scale trials with 11 and 10 panelists, and one large scale with 33 panelists. In the two smaller trials the panelist were mainly people with training and extensive experience in wine flavour and acid balance assessment. In the larger trial, about half of the panelists were experienced tasters and the remainder were not. In this trial reference samples of a very ripe tomato and of a green tomato with artificially enhanced levels of the 'green-flavour' volatile hexanal, were provided. Prior to tasting the test samples, the panelists were instructed to taste the former and smell the latter to familiarise themselves with the concepts of 'ripe' and 'green' tomato character.

Samples included in the trials were as follows:

Ac#2 Ailsa Craig - untransformed control fruit
C20 Genetically modified Ailsa Craig fruit with ADH activity increased constitutively.
C13 Genetically modified Ailsa Craig fruit with ADH activity increased in a fruit-specific manner.
C23 Genetically modified Ailsa Craig fruit in which alcohol dehydrogenase (ADH) activity was constitutively suppressed. (1st trial only)

	Control Ailsa Craig	Constitutive High ADH C20	Fruit-specific High ADH C13	Constitutive Low ADH C23	p	LSD 5%
Ripe tomato flavour	28^{b}	14^{a}	34^{b}	34^{b}	0.002	10
Green leaf character	28^{a}	34^{a}	23^{a}	25^{a}	0.288	10
Sweetness	24^{ab}	18^{a}	36^{b}	32^{b}	0.014	10
Acidity	30^{a}	25^{a}	29^{a}	26^{a}	0.819	10

Table 1. Taste trial #1 (11 panelists)
High intensity of character scored 1; Low intensity of character scored 4. Rank sums with different superscripts are significantly different at $\propto$ = 5%.

	Control Ailsa Craig Ripe	Control Ailsa Craig Unripe	Constitutive High ADH C20 - Ripe	Constitutive High ADH C20 - Unripe	p	LSD 5%
Ripe tomato flavour	21^{ab}	37^{c}	17^{a}	25^{ab}	0.004	9.5
Green leaf character	28^{a}	18^{a}	28^{a}	26^{a}	0.253	9.5

Table 2. Taste trial #2 (10 panelists)
High intensity of character scored 1; Low intensity of character scored 4. Rank sums with different superscripts are significantly different at $\propto$ = 5%.

	Control Ailsa Craig	Constitutive High ADH C20	Fruit-specific High ADH C13	Constitutive Low ADH C23	p	LSD 5%
Ripe tomato flavour	99^{b}	67^{a}	84^{b}	82^{ab}	0.033	17
Green leaf character	78^{a}	88^{a}	87^{a}	77^{a}	0.607	17
Sweetness	102^{c}	55^{a}	83^{b}	90^{bc}	<0.001	17

Table #3. Taste trial #3 (33 panelists)
High intensity of character scored 1; Low intensity of character scored 4. Rank sums with different superscripts are significantly different at $\propto$ = 5%.

In the first taste trial, fruit from the C20 plant, with constitutive high ADH levels, were ranked as having a significantly higher ripe fruit flavour than the other fruit in the trials, and marginally higher sweetness. In the second and third taste trials, the differences in the characters of the various fruits were not as marked as in the first trial but the C20 (constitutive high-ADH) fruits were still ranked as having improved (Trial #2) and significantly improved (Trial #3) ripe fruit flavour with respect to the control, untransformed fruit. Sweetness was not measured in Trial #2 but, in Trial #3, was again ranked as significantly higher ($p<0.001$) in the C20 (constitutive high-ADH) fruit compared to other fruit in the trial.

In Trial #1 the C13 fruit (fruit-specific high ADH) ranked in ripe-fruit flavour with the control Ailsa Craig fruit and was significantly lower than the constitutive high-ADH C20 fruit despite having high levels of ADH activity. In Trial #3 the ripe-fruit flavour of C13 fruit was intermediate between the untransformed Ailsa Craig fruit and the high-ADH fruit from plant C20. The different effects of constant high ADH during fruit development and high ADH only during fruit ripening may explain this observation. In Trial #1 C23 fruit (constitutive low-ADH), ranked with the control fruit. In informal tasting, in the laboratory, C23 was always scored as bland and was considered less tasty than the control fruit. The overall conclusions from the taste trials were that the fruit with constitutively enhanced ADH levels had an enhanced ripe fruit flavour and enhanced sweetness in comparison with unmodified fruit and with the other genetically modified fruit in the trials, while green leaf character and acidity were not significantly different.

Biochemical analyses: Ripe fruit (at breaker + 7days) from the fruit harvested for Taste Trial #3, were taken for analysis of ADH activity and volatile composition, with particular attention to the relative abundances of aldehydes and alcohols affected by ADH (see Fig 2).

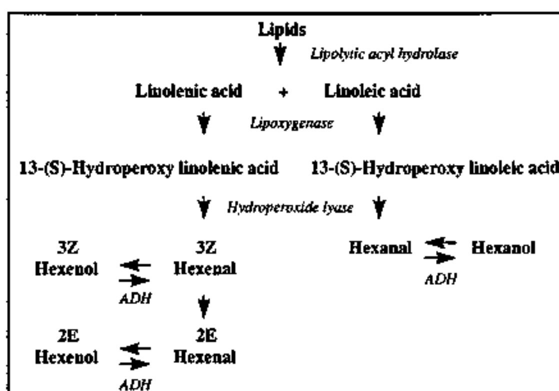

Figure 2. Pathway of synthesis of some flavour aldehydes and alcohols affected by ADH activity.

Additionally, tissues from the same fruit were analysed for the relative abundances of acids (citrate and malate) and sugars (glucose and fructose), as determined by HPLC.

Results:

ADH activity: Analysis of the ADH activity in fruit, randomly selected from Taste Trial #3, indicated significant differences in the activity of the enzyme between the genetically modified fruit and the control, Ailsa Craig fruit (Table 4).

	Control Ailsa Craig	Constitutive High ADH C20	Fruit-specific High ADH C13	Low ADH C23	p	LSD 5%
ADH2 activity U/mg protein	0.23 ± 0.02^{a}	0.51 ± 0.08^{b}	0.80 ± 0.12^{c}	0^{d}	>>0.001	0.143

Table 4. ADH2 activity in Br+7 fruit from Taste Trial #3
No ADH was detected in the C23 fruits, whereas the enzyme was present in all other cases. This alone indicates a difference between the genotypes that is significant at the $P<0.001$ level. Analyses of variance were performed omitting the C23 genotype, and this showed high significance ($P<<0.001$).

Aldehydes and Alcohols: Conversion of the 6-carbon aldehydes (hexanal and hexenal) to their respective alcohols (hexanol and hexenol) (Table 5) was enhanced by increased ADH activity resulting in lower aldehyde to alcohol ratios in the C13 and C20 fruit and was decreased in the low ADH fruit resulting in higher aldehyde to alcohol ratios in the C23 fruit. The conversion of octenal to octenol was not affected by variations in the ADH2 enzyme. This interconversion is believed to be mediated by an $NADP^{+}$-preferring terpene-ADH (Bicsak et al., 1982 [2]; Hoffman, 1962 [18]).

	ADH activity U/mg protein	Hexanal: Hexanol	Hexenal: Hexenol	Octenal: Octenol
Control Ailsa Craig	0.23 ± 0.02^{a}	300 ± 72^{a}	91 ± 43^{a}	5.8 ± 0.2^{a}
C20 Constitutive High ADH	0.51 ± 0.08^{b}	117 ± 27^{b}	28.2 ± 2.6^{b}	5.5 ± 0.4^{a}
C13 Fruit-specific High ADH	0.80 ± 0.12^{c}	67.8 ± 24.7^{c}	22.6 ± 7.5^{b}	6.5 ± 1.8^{a}
C23 Constitutive Low ADH	0^{d}	∞	∞	6.0 ± 0.7^{a}

Table 5. Ratios of aldehydes to alcohols.
There were significant differences between hexanal/ol and hexenal/ol ratios of all four tomato types at the $P<0.001$ level, but no difference in the C8-octenal/ol ratios. Analysis of variance, carried out on the log of the ratios, is shown in Table 6 below.

Genotype	AC	C13	C20	C23	LSD
Log(Hexanal/Hexanol)	2.248	1.72	1.966	2.441	0.181
Log(Hexenal/Hexenol)	1.682	1.267	1.352	1.963	0.184
Log(Octenal/Octenol)	0.562	0.53	0.546	0.563	0.079

Table 6. Statistical comparison of aldehyde to alcohol ratios among the 4 tomato types.

Sugars and Acids: The constitutive high-ADH (C20) fruit had marginally higher ($P<0.1$) levels of sugars (glucose + fructose) than the control AC fruit (Table 7) and the low-ADH (C23) fruit significantly lower ($P<0.05$) levels than the control AC fruit. No differences were detected between the levels of sugars in the control AC fruit and the fruit-specific high-ADH (C13) fruit.
The constitutive high-ADH (C20) fruit had significantly higher levels of total acids (citrate + malate) than the control, Ailsa Craig, fruit. Acid levels in the other modified fruits were not significantly different from the control.

	Sugar glucose + fructose mg/g F.Wt.	Acid malate + citrate mg/g F.Wt.
Control Ailsa Craig	26.96[a]	1.918[a]
C20 Constitutive High ADH	31.37[a]	2.320[b]
C13 Fruit-specific High ADH	26.89[a]	2.142[a]
C23 Constitutive Low ADH	20.00[b]	2.090[a]
LSD ($P<0.05$)	4.889	0.319

Table 7. Sugars and acids in pericarp tissue of Br+7 fruit from Taste Trial #3. Means of 5 fruit together with Least Significant Difference ($P<0.05$).

Summary of organoleptic and biochemical analyses of fruit from Taste Trial #3.

Increasing the activity of the enzyme ADH2 in tomato fruit significantly improved the ripe-fruit flavour and sweetness of the fruit according to the results of a taste trial with a panel of 33 people. Coincident with the improved flavour of the high-ADH fruit were significantly increased abundances of the 6-carbon alcohols hexanol and hexenol and a marginally increased abundance of the sugars (fructose and glucose). A corresponding decrease in the acids (malate and citrate), which might have been expected, was not evident, indeed, the acid level in the high ADH fruit was found to be significantly higher than in the control fruit.

#3 Analysis of segregating (selfed) populations of genetically modified plants.

A number of genetically modified plants were selfed and second generation (T_1) plants analysed for inheritance of transgenes and of ADH activity phenotype (Tables 8 and 9). T_1 offspring from the single insert, high ADH plants C7 and C14, showed Mendelian inheritance of the transgene and segregation of the high-ADH phenotype along with the transgene. The complement of two transgenes in the homozygous T_1 plants did not result in higher fruit ADH activities than were found in hemizygous, single insert fruit. The two transgenes in the C20 plant always segregated as a single Mendelian factor indicating linkage within the genome. Homozygous C20 T_1 plants, containing 4 transgenes, had suppressed ADH activity in their fruit which was consistent with observations on several of the other T_0 plants, that insertion of more than two genes caused supression of ADH activity. Backcrossing homozygous C20 plants with untransformed plants resulted in a reversion to the high-ADH phenotype indicating that suppression of activity was related to transgene dosage and was reversible. Inheritance of transgenes in the other genetically modified plants reported in Table 8 was complicated as their multiple inserts were not linked. The ADH-phenotype was only determined in detail in offspring from the three plants, C7, C14 and C20, with transgene inserts at single genetic loci.

Homozygous T_1 offspring of the C7, C14 and C20 plants were selected for breeding purposes because of their simple genetics. In addition, a number of other low transgene T_1 plants were selected for further study. Some plants with linked multiple transgenes were also selected in order to provide low ADH (suppressed) plants for comparison purposes.

Parent T_o	Inserts in T_o	ADH phenotype	No of T_1's analysed	T_1 segregation No.of inserts /No. of T_1's
C7	1	high-ADH	25	0/5, 1/12, 2/8
C14	1	high-ADH	25	0/8, 1/11, 2/6
C20	2	high-ADH	39	0/9, 2/23, 4/7
C13	6	high-ADH	16	3/1, 4/3, 6/3, 7/4, 8/3, 12/2
C4	4	low-ADH	39	0/2, 1/9, 2/1, 3/2, 4/10, 5/6, 6/3, 7/2, 8/4
C11	8	low-ADH	40	0/7, 6/3, 7/3, 8/5, 9/6, 10/3, 11/2, 12/3, 13/2, 14/3, 16/3
C23	3	low-ADH	30	0/1, 1/9, 2/10, 3/3, 4/3, 5/2, 6/2

Table 8. Segregation of T_1 plants. The number of transgene inserts in the T_1 plants was determined by Southern analysis and quantitation of the number and intensity of bands of hybridisation relative to hybridisation to the endogenous genes.

Genetic and biochemical stability of transgenes.

Analysis of the patterns of inheritance of single and multiple transgenes in the various transgenic plants indicated stability of the transgenes at a genetic level. To determine stability at a phenotypic level the ADH activities were measured in fruit from segregating populations of T_1 plants derived from selfing the three high-ADH T_0 plants, C7 (single insert), C14 (single insert) and C20 (linked double insert), (see Tables 8 and 9).

It had been observed that T_o plants with more than two constitutively expressed transgenes were cosuppressed for ADH activity. This was also observed with the T_1 plants which had inherited more than two transgenes (C20 – homozygous, Table 9). In addition, we observed suppression in some older C20 hemizygous T_o clones (two linked inserts) and in 25% of the C20 hemizygous T_1^s. It is believed that these incidences of cosuppression were due to the plants being stressed by high temperatures in the glasshouse. We saw no evidence of cosuppression in C13 T_o or T_1 plants containing multiple ADH transgenes regulated by a fruit-specific promoter.

Inheritance of transgenes was further studied in a T_2 population from one of the single transgene plants C7, and in BC1 plants from C7 and from C20 (two linked transgenes). As with T_1 populations, inheritance of the transgenes was consistent with inheritance of single genetic loci and the integrity of the transgenes appeared stable. Biochemical activity of the transgenes in the T_2 plants was stable although there was a tendency for ADH activities to be slightly lower in both T_2 and BC1 fruit than in T_o fruit.

	N=No. different plants n=No. of fruit/plant (N x n)	No. of inserts	ADH sp.act. U/mg
Control AC#2	(1 x 5)	0	0.381 ± 0.10
C7	T_o (1 x 3)	1	$0.602 \pm 0.06^{*}$
	T_1 - azyg (5 x 3)	0	0.342 ± 0.10^{ns}
	T_1 - hemi (12 x 3)	1	$0.528 \pm 0.10^{*}$
	T_1 - homo (8 x 3)	2	$0.652 \pm 0.13^{**}$
C14	T_o (1 x 8)	1	$0.598 \pm 0.10^{**}$
	T_1 - azyg (8 x 3)	0	0.402 ± 0.06^{ns}
	T_1 - hemi (11 x 3)	1	$0.581 \pm 0.09^{**}$
	T_1 - homo (6 x 3)	2	$0.642 \pm 0.09^{**}$
C20	T_o (1 x 5)	2	$0.583 \pm 0.11^{*}$
	T_1 - azyg (9 x 3)	0	0.291 ± 0.09^{ns}
	T_1 - hemi (18 x 3)	2	$0.575 \pm 0.07^{*}$
	T_1 - hemi (6 x 3)	2	$0.003 \pm 0.003^{**}$
	T_1 - homo (7 x 3)	4	$0.002 \pm 0.003^{**}$

Table 9. ADH activities in Br+7 fruit from T_o and T_1 plants.
ADH activity was determined in pericarp tissue from 3 Br+7 fruit of each plant. Pooled activities for each class of plant were compared with the activity of control (AC#2) Br+7 fruit grown and harvested during the same period of time. Statistical comparison used Welch's t-test: ** very significant ($P < 0.01$), * significant ($P < 0.05$).

Where analysed, C7 T_1, T_2 and BC1, and C20 T_1 (hemizygous) and BC1 fruit had consistently higher levels of ADH activity than in control Ailsa Craig fruit or azygous T_1 fruit (see Table 9) and correspondingly higher abundances of the alcohols hexanol

and hexenol (data not included here) indicating stability of the transgenes and their immediate and subsequent biochemical activities.

Inheritance of transgenes in plants with multiple loci of insertion (C13, C4, C11 and C23), was complex and was not studied in detail. Where identified, T_1 offspring from these plants which had inherited a single transgene were kept for later analysis as they would differ genetically (presumably) and possibly phenotypically from the C7 and C14 T_0 plants. Similarly T_1 plants derived from the C13 T_0 parent (multiple inserts regulated by a fruit-specific promoter) and containing one or more transgenes at a single genetic locus were kept for future analysis.

Many of these results are included in the attached publication – Speirs J, Lee, E, Holt K, Yong-Duk K, Steel Scott N, Loveys B and Schuch W (1998). Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit affects the balance of some flavor aldehydes and alcohols. *Plant Physiology* **117**: 1047-1058.

#4 Introduction of Genetic Modification into (Australian) Commercial Tomato Lines.

So far the work has concentrated on modifying ADH activity in one tomato cultivar (Ailsa Craig) and examining the effects of the modifications on the biochemical and organoleptic properties of the fruit. Ailsa Craig is not a cultivar of tomato that can be field grown in Australia. Indeed, it has also proved difficult to grow consistently well in a glasshouse environment with the result that the fruits in the taste trials have been of less than optimal quality. To overcome this problem and to work towards field trials and a commercial product it was decided to transfer the beneficial gene modification into one or more cultivars of tomato that can be (and have been) field grown commercially in Australia. Six near isogenic tomato cultivars, four from Queensland and two from Victoria, were selected for modification. The insertion of the gene modifications was undertaken using traditional breeding methods.

Recipient commercial lines (selected on the basis of being essentially isogenic lines still in common commercial production) :

Victoria -

- Arcadia (firm, desirable agronomics, current)
- Goulburn

Queensland-

- Floradade
- Walter
- Hayslip
- Tristar

Transgenic lines used as pollen donors:

x C20P7	Homozygous T_1 of C20 - 2 x 2 inserts, high ADH as a hemizygous, cosuppressed as homozygous.
x C11T_0	Hemizygous (6-7 inserts), cosuppressed as hemizygous, contains a cluster which may confer cosuppression.
x C7P35	Homozygous of single insert (ie 2 x 1insert), high ADH.
x C13P6	Fruit specific, hemizygous with 3 (clustered) inserts.
x C13P13	Fruit specific, hemizygous, 6 inserts.
x Ac	Ailsa Craig - control, untransformed

All crosses were made and seeds collected.

To rationalize the numbers of plants to be grown and assays to be performed, only crosses into the four Queensland tomato cultivars were further analysed. Seedlings from the four crosses were germinated and one of each pair of cotyledons from each seedling was taken for polymerase chain reaction (PCR) analysis to check for the presence or absence of the transgene from the donor parent (see Materials and Methods). Crosses from the homozygous C20P7 and C7P35 were all positive (hemizygous) for the C20 or C7 inserts, as would be expected. Crosses from the other transgenic donors (hemizygous) constituted segregating populations from which seedlings containing the transgenes were selected.

Crosses with C20 (high-ADH).

Six Floradade x C20 plants and two plants of each of the other three C20 crosses were grown to maturity and fruit harvested at progressive stages of ripening for ADH activity analysis. Fruit from crosses of Ailsa Craig x commercial cultivar were used as controls as were selfed Ailsa Craig and selfed commercial cultivars.

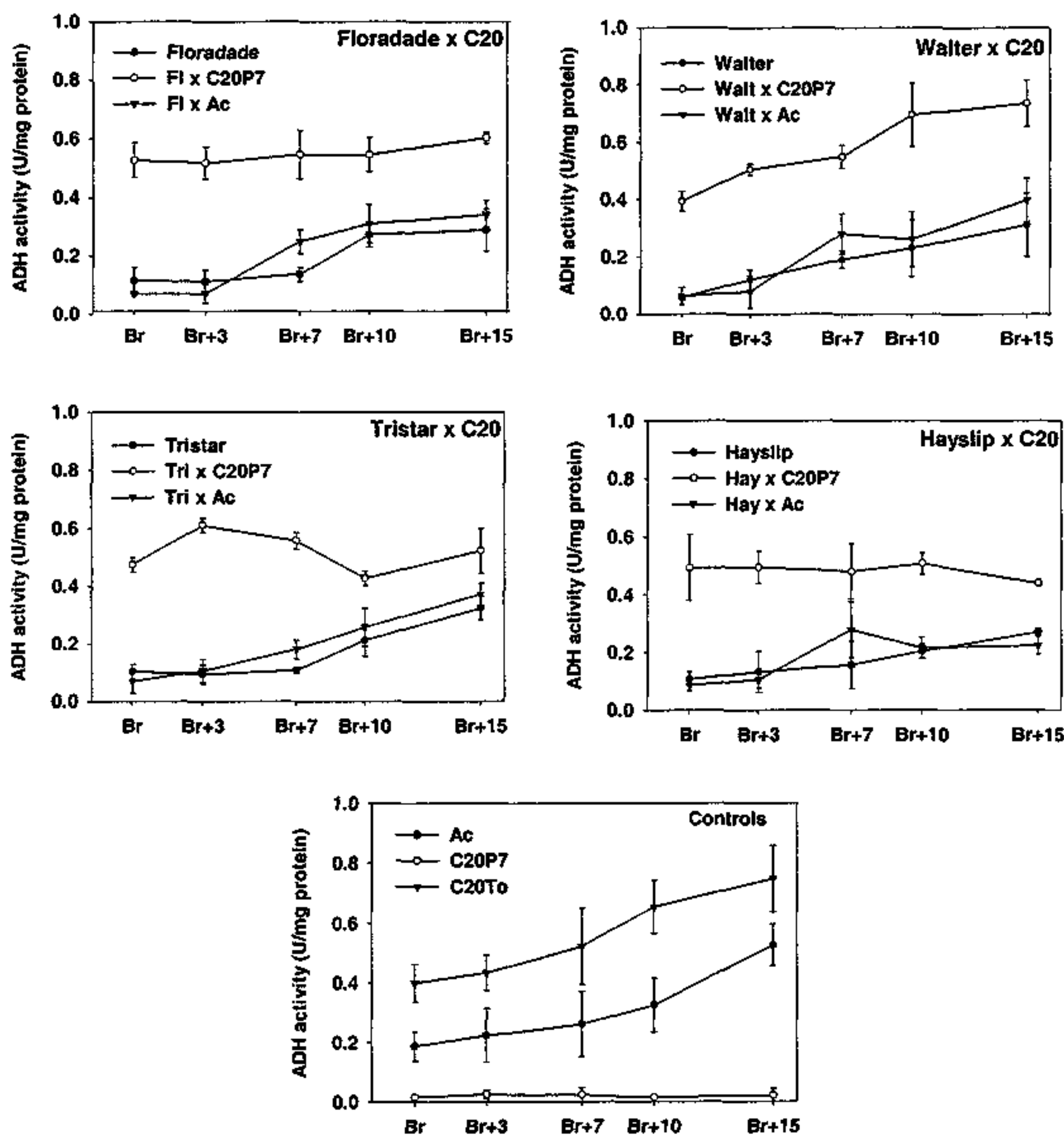

Figure 3. ADH Actvity (U/mg protein) in Fruit from Crosses of the Transgenic C20 (High ADH) into 4 Commercial Tomato Varieties. Means and standard deviations, derived from 3 fruit per point from 6 plants (Floradade crosses) or 2 plants (other crosses).

Fruit from the four Queensland cultivars examined, and the two Victorian cultivars (data not shown), had consistently low levels of ADH activity supporting the contention that firmness relates to low activity. Ailsa Craig, a softer variety, had moderate levels of ADH activity, which rose as ripening progressed, while the T_0 transgenic C20 (hemizygous, constitutive high-ADH) fruit had constant high levels of ADH throughout ripening. The homozygous C20 fruit, which contained 4 transgenes, were cosuppressed for ADH.

Fruit from the primary crosses of C20 into the four Queensland cultivars all had significantly increased levels of ADH activity which were relatively constant throughout ripening except in the case of the Walter x C20 cross where the ADH activity was seen to rise progressively with ripening.

Crosses with the other transgenic plants.

Floradade x C13 (fruit ripening-specific high-ADH):

Crosses of the C13 transgenic (fruit ripening-specific) Ailsa Craig with Floradade were disappointing. Three primary crosses were selected for analysis. One, Fl x C13P6, contained 3 transgenes while two from the cross between Floradade and C13P13 contained 2 transgenes [Fl x C13P13] and 4 transgenes [Fl x C13P13(1.3)].

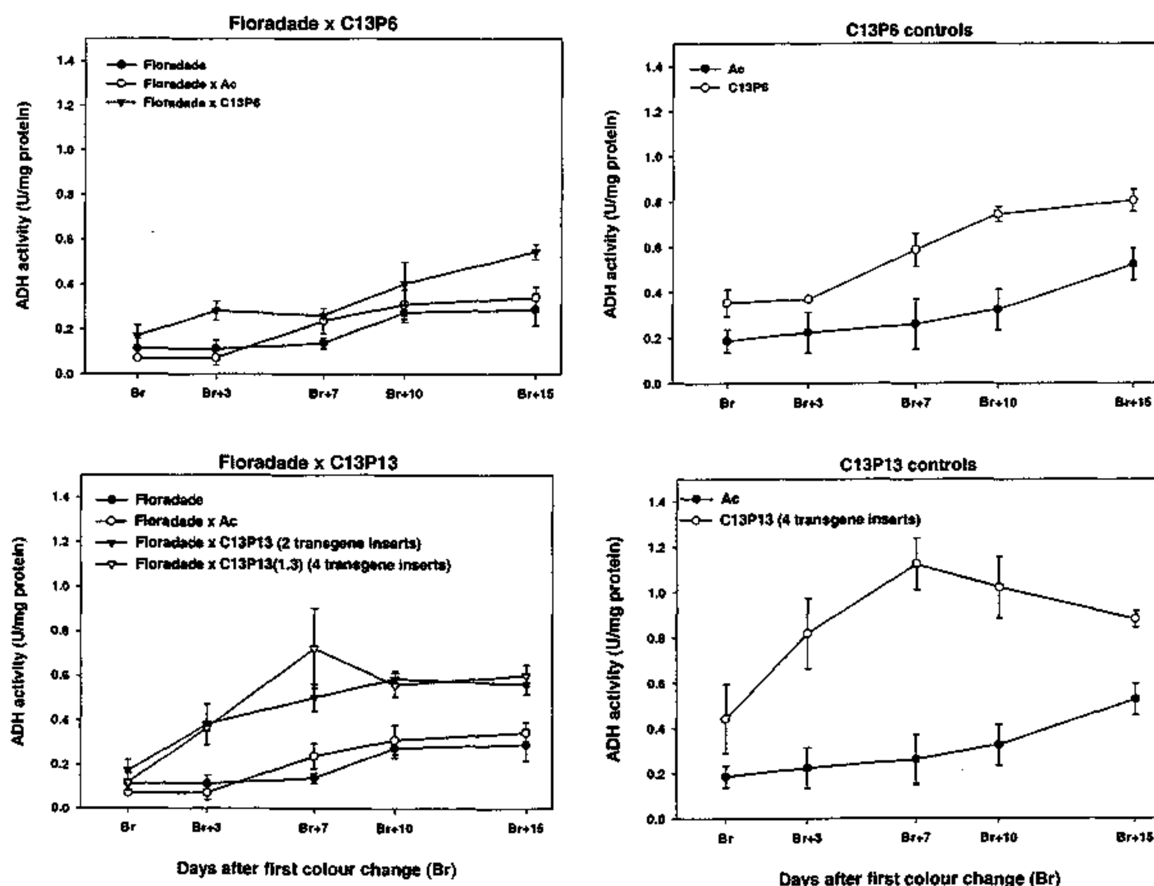

Figure 4. Floradade x C13 (fruit ripening-specific high-ADH).

While fruit from the C13P13 control plant had very high levels of ADH activity (Figure 4) none of the crosses approached the control level. Seeds of these crosses were kept but, as taste trials on the primary C13 transgeneic fruit did not indicate significant improvement in flavour, no further analyses on the crosses were undertaken.

Floradade x C7 (single transgene, constitutive high-ADH):

C7 Ailsa Craig contains a single, constitutively expressed ADH transgene. Its homozygous T_1 C7P35 was used for crossing with Floradade giving a uniform population of primary crossed plants all hemizygous for the transgene, i.e. each containing a single transgene.

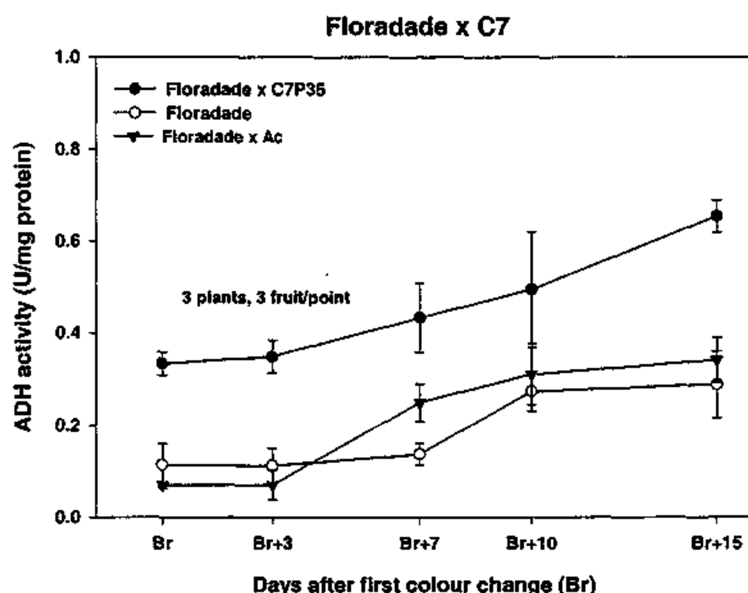

Figure 5. Floradade x C7 (single transgene, constitutive high-ADH).

Fruit from the Fl x C7 cross showed significantly higher levels of ADH activity throughout ripening compared with the control Floradade or Fl x Ac fruit Figure 5). The initial levels of ADH in the Fl x C7 fruit, however, were lower than in the Fl x C20 fruit, rising during the course of ripening. Informal taste trials did not rank the Fl x C7 fruit as flavoursome as the Fl x C20 fruit.

Floradade x C11 (multiple transgenes, constitutively cosuppressed for ADH):

The transgenic C11 (multiple inserts, constitutively cosuppressed for ADH) was used as a donor for the production of crosses with commercial tomato cultivars in an attempt to generate cosuppressed crosses for use as ADH null controls for field trialing and biochemical and organoleptic analyses. However, we did not observe any

cosuppression in the fruit from the Floradade x C11 crosses despite the crosses having multiple transgene inserts, as determined by Southern analysis. The fruit of the crosses were, in fact, indistinguishable in ADH activity from the Floradade x Ailsa Craig control fruit (data not shown here) suggesting that the multiple transgenes had been inactivated – possibly by methylation. Seeds of these crosses have been stored but further analysis has been postponed.

Taste Trialing of the Floradade x C20 Primary Cross:

In a taste trial with 8 panelists, all the panelists ranked ripe fruit flavour intensity in Br+7 fruit as follows:

Floradade x C20 (high ADH) > Floradade x Ailsa Craig > Floradade

Crosses of all the listed transgenic Ailsa Craig plants were made with all six commercial tomato cultivars and seeds were collected. Analysis of fruits from the crosses was limited to those detailed above. In light of the results with crosses into Floradade it was decided to limit further work to the production of backcross2 (BC2) or BC3 Floradade x C20 plants for field trialing and formal taste testing.

Floradade x C20 – Backcross 1.

BC1 seeds from six Floradade x C20 fruit (from different backcrosses) were germinated. Cotyledons from the seedlings were taken for PCR analysis. Twelve seedlings containing the C20 transgenes were selected and grown to maturity along with 3 seedlings from the segregating population which were azygous for the transgenes, to act as control Floradade x Ailsa Craig BC1 plants. During maturation the plants became heavily infested with whitefly and suffered some fungus infection. As a result 7 of the 12 transgenic plants were stressed and their fruit became cosuppressed for ADH. All 12 transgenic plants were used as pollen donors for crossing with Floradade to produce BC2 seeds, care being taken to note the pollen donor from which each fruit was derived. ADH analyses were performed on fruit from all the BC1 plants but results are shown only for the five trangenic plants which had not become cosuppressed.

ADH activity was consistently high throughout ripening in the fruit from the 5 transgenic BC1 plants (Table 10), with levels similar to those in the ripening fruit of the C20T_o plants. ADH activity in the control (Fl x Ac) BC1 fruit slowly increased with ripening but reached only about half the activity found in the transgenic fruit.

BC2 seeds were collected from fruit from this set of crosses. Seeds were germinated and seedlings with or without the transgene have been selected by PCR analysis as before. Seedlings derived from crosses with the cosuppressed plants have been included in order to determine whether cosuppression has been transmitted via the seeds.

	Br	Br+3	Br+7	Br+10
Fl x C20 BC1 #1	0.703 ± 0.127	0.715 ± 0.049	0.562 ± 0.038	0.639 ± 0.063
Fl x C20 BC1 #2	0.544 ± 0.047	0.808 ± 0.048	0.624 ± 0.068	0.546 ± 0.067
Fl x C20 BC1 #5	0.630 ± 0.080	0.551 ± 0.026	0.458 ± 0.049	0.435 ± 0.072
Fl x C20 BC1 #10	0.561 ± 0.032	0.458 ± 0.034	0.501 ± 0.010	0.413 ± 0.030
Fl x C20 BC1 #11	0.600 ± 0.045	0.547 ± 0.073	0.557 ± 0.093	0.506 ± 0.062
(Fl x Ac) BC1	0.089 ± 0.034	0.116 ± 0.054	0.169 ± 0.010	0.225 ± 0.041
$C20T_0$ (hemizygous)	0.571 ± 0.077	0.533 ± 0.097	0.585 ± 0.092	0.570 ± 0.102

Table 10. ADH activity (U/mg protein) in ripening fruit from 5 Floradade x C20 BC1 plants together with BC1 Fl x Ac and $C20T_0$ controls.

Analysis of the BC1 plants was complicated by the stressing of the plants and the analysis is currently being repeated with a second set of BC1 plants.

Floradade x C20 – Backcross 2.

BC2 Floradade x C20 plants were selected together with BC2 (Fl x Ac) controls and are currently being used as pollen donors for the production of BC3 seeds. Fruit for analysis of ADH activity will be available by late October, early November.

#4 Storage Experiment: Quality of transgenic and control fruit ripened on and off the vine and stored at 4°C and 10°C.

Storage of tomatoes at 4°C and, to a lesser extent 10°C , is known to be detrimental to the flavour of the fruit yet many transport and marketing proceedures include periods of cold storage. Similarly vine ripened fruit are rated as having better flavour than fruit ripened off the vine. Both cold storage and ripening off the vine may be affecting flavour by changing levels of sugars/acids and/or volatiles. In this experiment we set out to examine the effects of cold storage and ripening on and off the vine on levels of sugars and some volatile compounds in the fruit. Included in the experiment were fruit from the transgenic C20 plant to determine whether high levels of ADH may help to overcome some of the adverse effects on flavour volatiles.

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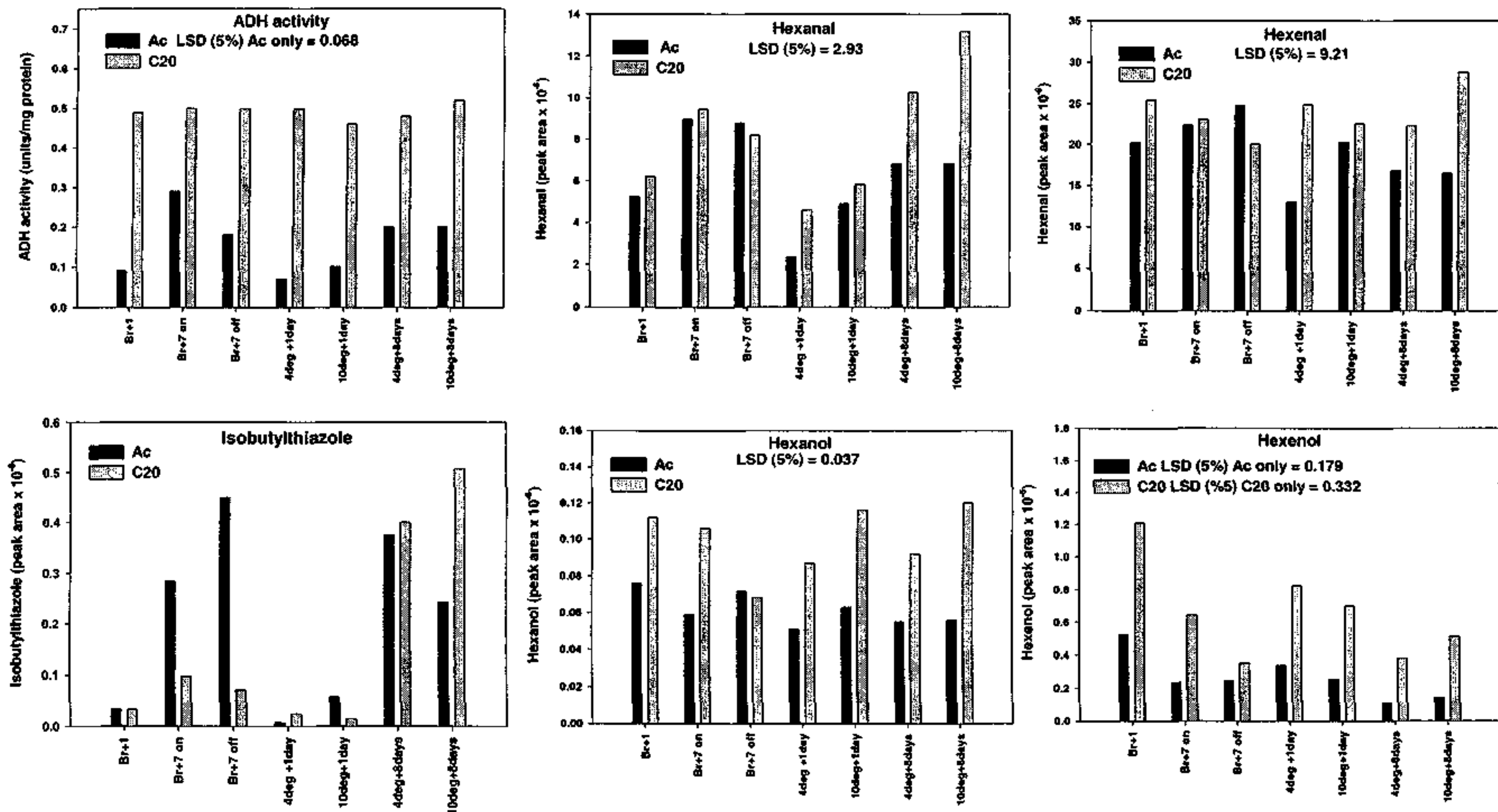

Figure 6. Levels of ADH activity and some volatiles in tomato fruit under different conditions of storage and/or ripening.
Ailsa Craig (Ac) and high-ADH transgenic (C20) fruit were harvested either 1 day after first colour change (Br+1) or 7 days after first colour change (Br+7). Br+1 fruit were subjected to a number of different storage and/or ripening regimes as follows:-

- Br+7 off – ripened for an additional 6 days at RT off the vine
- 4deg+1day – stored at 4°C for 5 days + 1 day at RT
- 10deg+1day – stored at 10°C for 5 days + 1 day at RT
- 4deg+8days – stored at 4°C for 5 days + 8 days at RT
- 10deg+8days – stored at 10°C for 5 days + 8 days at RT.

Statistical Methods:

Five fruit were analysed for each data point. The data were analysed as a two-way analysis of variance using Genstat (Payne *et.al.* 1993). The residuals were examined for each analysis. Where the usual assumptions for the analysis of variance were not met (for example with hexenol), a log transformation was used.

Results:

ADH: Genome modification significantly increased ADH activity in pericarp tissue of the transgenic C20 fruit (P<<0.001) (Figure 6). The various treatments did not affect ADH activity in the C20 fruit but did have a very significant effect on the activity in the control AC fruit (P<0.001, LSD(5%) + 0.068).

Hexanal: Little difference was seen in hexanal levels between C20 and AC fruit harvested at Br+1, ripened on the vine and ripened off the vine (Figure 6). Hexanal levels increased significantly (P=0.035) in C20 and AC fruit between harvest (Br+1) and ripened, either on and off the vine, with no significant difference between fruit ripened on or off the vine. Low temperature storage appeared to affect the levels of hexanal in the 4 deg + 1 day treatment probably resulting from slowing of the ripening of the fruit. Large increases in hexanal were observed in the C20 fruit, but less so in the AC fruit, exposed to 4 deg or 10 deg then ripened for 8 days at ambient temperature. The reason for these differences is not known.

Hexanol: A significantly increased level of hexanol was found in the pericarp of the C20 fruit compared with the AC fruit (P<0.001) in all the treatments but the various storage treatments did not have an effect on the levels (Figure 6).

Hexenal: While there was no significant effect of storage treatment on levels of hexenal in fruit from the two tomato types, there was a significant difference (p<0.05) between the C20 and AC which was most evident in the fruit subjected to cold storage (Figure 6).

Hexenol: Significant increases (P<<0.001) in hexenol levels were associated with the C20 fruit relative to the AC fruit in all the treatments (Figure 6). In addition, the various treatments caused significant changes in hexenol levels in all the fruit (LSD for AC = 0.179, LSD for C20 = 0.332).

Isobutylthiazole: The Isobutylthiazole data (in Figure 6) were extremely skew and required a logarithmic transformation (Table 11). The residuals were then almost normally distributed with a similar variance among the treatments.
Levels of isobutylthiazole, which can become an off-flavour at certain concentrations (Kazeniac SJ and Hall RM , 1970). (20) were significantly reduced (P<0.05) in the C20 fruit relative to the AC fruit when not subjected to cold storage treatments. After

storage at 4 deg or 10 deg followed by 1 day at ambient temperature, the levels of isobutylthiazole remained similar to those in Br+1 day fruit, presumably because ripening had been delayed. However, both C20 and AC fruit subjected to cold treatment and then allowed to ripen for 8 days; 4 deg + 8 days and 10 deg + 8 days; had high levels of isobutylthiazole equivalent to the levels found in ripe AC fruit and much higher than those in ripe C20 fruit.

Treatment	AC	C20
10° + 1day	-1.184	-1.497
10° + 8days	-0.585	-0.374
4° + 1day	-1.566	-1.504
4° + 8days	-0.446	-0.423
Br+1	-1.276	-1.324
Br+7 off	-0.46	-1.085
Br+7 o/v	-0.571	-0.95

Table 11. Treatment means for isobutylthiazole on a log (base 10) scale. There is a significant interaction. The appropriate lsd (5%) is 0.35.The table shows that there is little effect of increased ADH activity in the C20 fruit except in the Br+7 on and Br+7 off fruit. There were also clear differences in the applied treatments.

Sugars: The C20 fruit, with high levels of ADH activity, had very significantly higher levels of sugars (P<<0.001) in the overall experiment than did the control AC fruit (Figure 7). While sugar levels were always higher in C20 fruit than in AC fruit, the differences were significant at the P<0.05 level in 4 of the 7 treatments. No significant interaction or effects of treatments were observed.

Acids: The C20 fruit, with high levels of ADH activity, had significantly lower levels of acids (P=0.023) in the overall experiment than did the control AC fruit (Figure 7). There was also an effect of the various treatments on the levels of acids (P=0.013) which was most apparent in the fruit ripened on and off the vine (Br+7 o/v and Br+7 off).

Discussion:

There were a number of differences in the biochemical composition of the fruits that may be affecting fruit flavour in this experiment. The sugar and acid components increased and decreased respectively during ripened on the vine but not in fruit ripened off the vine or subjected to storage treatments. This was unexpected as it was widely thought that the differences in sugar content between vine-ripened and non vine-ripened fruit was a function of sugar metabolism. In contrast, these results suggest that the difference is caused more by continuing assimilation of photosynthate by fruit on the vine which stoped as soon as the fruit were harvested. There was no clear evidence of any of the aldehyde/alcohol flavour volatiles examined being

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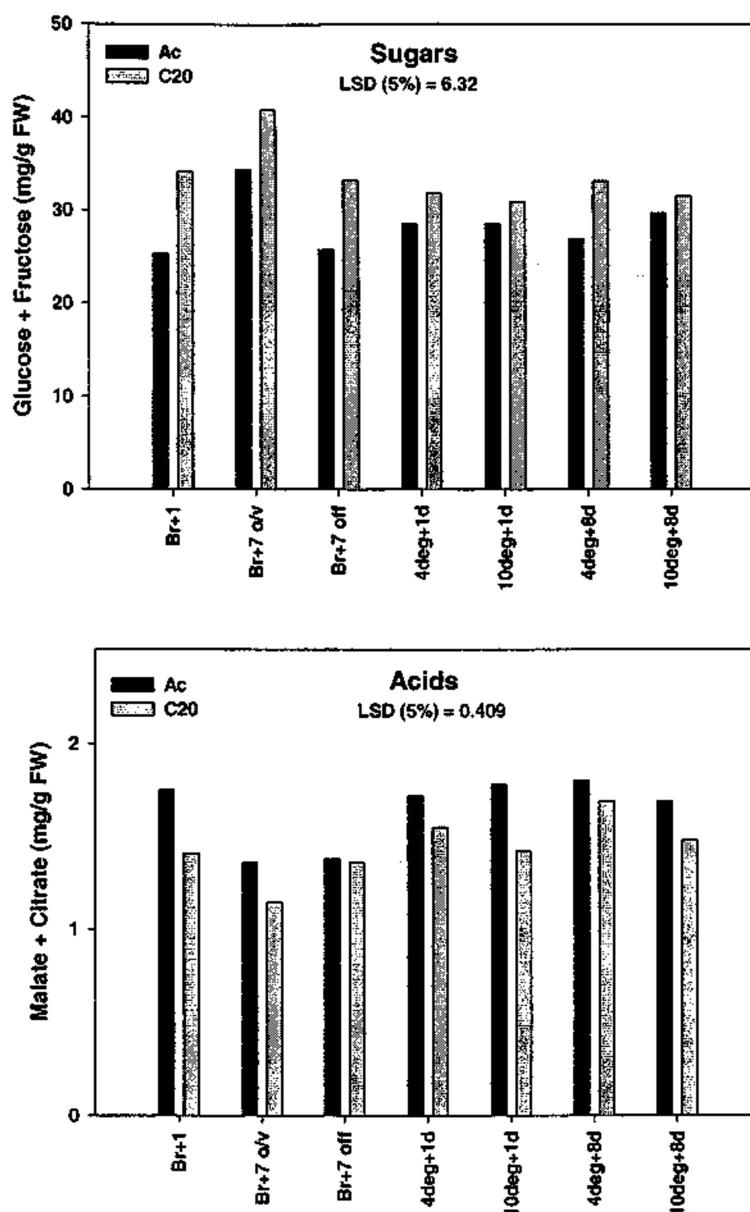

Figure 7. Sugars and acids in tomato fruit under different conditions of storage and/or ripening.
Experimental conditions are detailed in caption to Figure 2.

influenced by early harvesting but hexanal, hexenal and hexenol levels were lower in control Ailsa Craig fruit subjected to cold storage treatments both as an effect of the slowing of ripening and as a longer term effect in fruit subsequently allowed to ripen at room temperature. Enhanced ADH activity in the C20 fruit tended to counteract these effects and may be of particular importance in the case of hexenol, levels of which were very low in Ailsa Craig fruit after 4deg+8days and 10deg+8days treatments, relative to fruit ripened on or off the vine, but were much higher in C20 fruit after the same treatments.

Possibly of greater significance are the differences in abundance of isobutylthiazole, which is an important flavour component but can be an off-flavour when present in excess. Further examination of the effects of storage treatments, and of modified ADH activities, on levels of isobutylthiazole in the ripening fruit are warranted.

This experiment has indicated that there are measurable differences in biochemical components associated with flavour in fruit subjected to different storage and/or ripening regimes. It also indicated that enhanced levels of ADH activity in fruit can compensate for some of these differences. Any future extension of this work should include analysis of fruit ripened either on or off the vine and stored at 4°C until just before analysis (to mimic common domestic practice) and would be greatly enhanced by coupling with taste testing. There are no plans for such an extension at present.

General Discussion.

There are strong pressures, primarily from growers, transporters and retailers, for commercial fresh market tomatoes to be firm, free of blemishes and with a long shelf life. Unfortunately the firmer cultivars that are being produced to meet these requirements are generally considered to be lacking in flavour although some cultivars with smaller fruits, such as cherry tomatoes, still retain good flavour. Breeders are making efforts to improve the flavour of the fruit by increasing the sugar content however sugar content is not the full story. In a previous HRDC project (VG427) we proposed that the apparent lack of flavour in firm fruit is connected with low activity in the fruit of the enzyme alcohol dehydrogenase which plays a role in the production of several compounds associated with flavour. Using genetic manipulation, we produced several modified tomato lines with increased or decreased activity of the enzyme in their ripening fruit. Analysis of volatiles in the ripe fruit confirmed the importance of the enzyme for the production of several of the flavour volatiles and a small scale taste trial confirmed that fruit with higher ADH activity had a more intense ripe-fruit flavour.

In this current project we demonstrated a correlation between fruit softness and the activity of the ADH enzyme in the fruit, by analysing a number of firm and soft commercial tomato cultivars. We undertook further 'formal' and 'informal' taste trials of the genetically modified fruits together with biochemical analyses of ADH activity, volatiles and sugars and acids in fruits from one of the trials.. We also examined in detail the stability of the modified genotypes and phenotypes in 1st and 2nd generation plants generated from the primary modified plants, and in populations of backcross 1 (BC1) and BC2 plants generated by backcrossing to the unmodified parental line, and have transferred the gene modifications into 6 commercial lines of tomato with a view to field trialing and taste trialing at least one of the modified lines prior to commercialization. Finally we examined the differences in volatiles, sugars and acids in fruits ripened on and off the vine or subjected to storage and ripening at different temperatures. Included in the storage experiment were genetically modified fruits with constitutively high ADH activity, to determine whether high ADH activity was beneficial under storage conditions.

Fruit softening and alcohol dehydrogenase. It has been our contention that the accumulation of the alcohol dehydrogenase enzyme in the tomato fruit (and its activity, associated with flavour development) are functions of the softening of the fruit. Comparisons of ADH activity in ripening fruit from 'firm' and 'soft' tomato cultivars demonstrated a strong correlation between fruit softness and ADH activity (Figure 1). The relationship was further strengthened by the low ADH activities found throughout ripening in the firm fruit of all of the 6 commercial tomato cultivars selected for crossing with the transgenic Ailsa Craig plants (Figure 3).

Taste trialing and biochemical analysis: In all three taste trials the C20 fruit (constitutive high-ADH) were ranked as having a higher intensity of ripe-fruit flavour than control, unmodified fruit (Ailsa Craig) or fruit with enhanced ADH activity only during ripening (C13). Analysis of volatiles in the fruit confirmed that the high ADH activity in the C20 and C13 fruit was associated with greater conversion of the aldehydes hexanal and hexenal into their respective alcohols hexanol and hexenol than was evident in the control fruit, all four compounds being important to flavour development. Why the raised levels of the alcohols, hexanol and hexenol, in the C13 fruit did not, evidently, result in enhanced flavour is not clear but C20 fruit do have higher levels of the aldehyde hexanal relative to the C13 fruit and it may be that the relative proportions of aldehydes and alcohols are of greater importance than the levels of the alcohols alone.

No significant differences were seen in the levels of sugars and acids in the various fruit types in the taste trial although sugar levels did tend to be higher in the constitutive high-ADH (C20) fruit than in the control unmodified fruit or the ripening specific (C13) fruit. In a separate set of experiments, "Storage Trial", the levels of reducing sugars were seen to be significantly higher ($P<<0.001$) in C20 fruit than in control fruit, in all harvesting and storage treatments, with the higher sugar abundances being most pronounced in breaker fruit and in fruit ripened for 7 days on or off the vine. It has been shown (Paz et al., 1982) (19) that exposure of tomato fruit to atmospheres of low concentrations of acetaldehyde for 3 – 4 days resulted in significant increases in the abundance of reducing sugars in the fruit, although why this should be is not known. In our C20 fruit, the high ADH activity may be perturbing the balance between ethanol and acetaldehyde and this may be affecting the accumulation and/or metabolism of the reducing sugars, but this has not been tested so far. Clearly, a genetic modification, or a treatment, which resulted, consistently, in significant increases in reducing sugars in ripe tomato fruit would be valuable and further work needs to be done to investigate this observation.

Stability of modified genotypes and phenotypes: The genetic modifications were passaged through T_1 and T_2 selfed generations and BC_1 and BC_2 generations without any indication of instability. The high-ADH phenotype passaged through various generations in a stable manner in plants carrying either single constitutively expressed transgenes (C7 and C14) or in fruit-ripening specific transgenes (C13) but showed some instability in the C20 plant carrying two, tandemly linked constitutively expressed transgenes, where older plants or plants under stress tended to become suppressed for ADH activity. Although the mechanism of supression of gene activity in this plant is not clear, we believe it to result from a threshold level of ADH activity being exceeded (stress induces the endogenous ADH gene) and feel that it will not be

a problem when the modified genes are transferred to commercial lines of tomato with naturally low levels of ADH activity.

Transfer of modified genes into commercial tomato lines: A number of the modified genes were transferred into 6 commercial cultivars of tomato in order to test the modifications in commercial lines, to field trial one or more of the modified commercial lines and to undertake taste trials on the field grown lines. The constitutive single (C7 and C14) and double (C20) modifications transferred the high-ADH phenotypes to all six commercial cultivars, as predicted. The fruit-ripening specific (C13) modifications also transferred the increased ADH phenotype to the one commercial line analysed, Floradade, but the resulting increases in ADH activity in the Floradade crosses with 2, 3 or 4 C13 genes, were lower than expected and no further work was undertaken on this series of crosses.

Fruit from crosses of C11 (constitutive ADH-suppressed) with Floradade were expected to be suppressed for ADH as they contained more than 2 transgenes, which has been shown with primary transformants to result in cosuppression. It was planned to use fruit with no ADH activity from this cross as a null control in field trials, however, the crossed fruit did not differ significantly from control fruit in ADH activity and this crossing was abandoned. As there was no discernable difference between the Floradade x C11 fruit containing several transgenes and Floradade x Ailsa Craig containing no transgenes, it was assumed that the transgenes had been inactivated during transfer, possibly by methylation.

Fruit of backcross 1 (BC1) of the Floradade x C20 cross retained a constitutively high ADH activity and were ranked, in 'informal' taste trials, as having improved flavour relative to both the Floradade x Ailsa Craig BC1, (the unmodified control) and to the Floradade fruit. BC2 Floradade x C20 fruit will be available for biochemical and organoleptic analysis in the near future and BC3 is currently underway.

Directions for future research: Currently we are working towards large scale field and taste trials of one of the modified commercial tomato cultivars. In collaboration with the Queensland Department of Primary Industry we propose to grow three lines of tomato at their Research Station at Ayr : (a) an unmodified Floradade cultivar, (b) a backcross 3 line of Floradade x Ailsa Craig – obtained by crossing with genetically modified lines but lacking the transgene, and (c) a backcross 3 line of Floradade x C20. Fruit from the field trial will be taste trialed at the Centre for ChemoSensory Research, University of New South Wales with a smaller trial at the University of Adelaide tasting facility. GMAC application has been made for both field and taste trials. Analysis of ADH activity, sugars and acids and (possibly) volatiles will be undertaken concurrently with the taste trials.

It is planned to conduct the trials over two consecutive seasons, 2000 and 2001 after which the tomato line will be made available for commercial application.

Extension/adoption by industry: Once we have proved the commercial potential of the genetically modification we would seek assistance in commercialization from large national distributors and retailers such as Coles or Woolworths and from seed brokers such as Austseed. We see the need for proof of concept before we will be able to interest such companies. Should the first field and taste trials of the modified Floradade cultivar prove successful in 2000 we would invite industry involvement in the 2001 trials, particularly in the taste trials.

Our existing contract with our current industry collaborator, Zeneca Plant Science, specifies an Australian advantage in any commercialization and is being modified to accommodate the new work that is planned. Zeneca has experience in production and

marketing of transgenic tomato products world wide and will provide that experience to aid in commercialization in Australia and world wide.

Patents covering the genetic modification are in process.

Publication will be primarily in international journals but will also be via State Departments of Agriculture and QFVG to breeders and growers.

Financial commercial benefits of adoption of research findings: A benefit cost analysis has been undertaken based on two possible senarios - i) the genetically improved tomato fruit cost no more to produce than standard tomatoes but can command an increase in farm gate value of at least $30 per tonne; or ii) a 5% increase in sales of fresh market tomatoes results from the introduction of the 'improved flavour' tomato.

Variable	Based on $30 per tonne premium on existing production	Based on increased farm sales of 5%
Year research begins	1999	1999
Year research ends	2001	2001
Discount Rate	8%	8%
Total External Funding	$295,575	$295,575
Total Internal Funding	$667,620	$667,620
No. of tonnes affected	370,990	18,500
On-farm benefits/tonne/yr	$30.00	$1,123.00
On-farm costs/tonne/yr	$0.00	$935.00
Total Weighted Benefit	$8,623,425	$2,695,450
Yr farmers begin to adopt	2004	2004
Max. % adoption	50%	50%
Year max adopt reached	2014	2014
No more benefits after	2030	2030
PV of Costs	$826,000	$826,000
PV of Benefits	$16,594,000	$5,187,000
NPV	**$15,768,000**	**$4,361,000**
BCR to 1	**20**	**6**

Program used to calculate BCA and do the sensitivity analysis: **REVS – The Research Evaluation Spreadsheet.** Produced by the Dept of Ag WA

Total weighted benefits based on following probabilities

PROBABILITY WEIGHTED BENEFIT SCENARIOS based on 5% increase in sales

Verbal Description	PROPORTION OF FULL BENEFITS	ESTIMATED BENEFIT per tonne	UNITS AFFECTED tonne	PROBABILITY	WEIGHTED BENEFIT
Project Fails	0%	$0	18,500	0.00	$0
Project partially succeeds	50%	$94.00	18,500	0.30	$521,700
Project partially succeeds	75%	$141.00	18,500	0.30	$782,550
Project achieves goal	100%	$188.00	18,500	0.40	$1,391,200
Project exceeds goal	0%	$0.00	18,500	0.00	$0
			TOTAL:	1.00	$2,695,450

The 20:1 BCR is based on 50% adoption by 2014 and a $30 per tonne premium on sales with no additional costs. Even with a $20 per tonne premium and only 30% adoption by 2010 the BCR is 8:1. This does not include any other benefits that may happen like additional exports or additional retail profits.

The figure of 370,990 tonnes per year for fresh market tomatoes was obtained from The Australian Bureau of Statistics. However, their figure for the value of the fresh market crop, $176.2m, seems very low. Using a monthly average value of $11.32 per 10kg carton or $1,132 per tonne (Brisbane Markets) the value of the fresh market crop would be closer to $416.5m. We have used the value of $1,132 per tonne to calculate the second senario.

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HRDC FINAL REPORT CHECKLIST

Project No VG97093 **Title:** Flavour improvement in tomatoes: genetic manipulation and breeding for improved flavour

Milestone No 6 **Date Received** 29/10/99 **PM** ~~MS~~ ST/PP

Criteria

Chief Investigator Jim Speirs **Agency** CSIRO Plant Industry

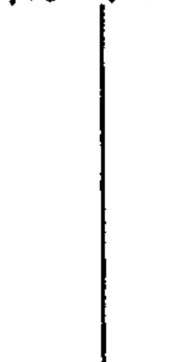
Life of Project Milestones

Stone	Date	Description	Payment Due	Achievement Date	Payment Date	Payment Amount
1	30/09/97	HRDC Research Agreement signed, and if relevant voluntary contribution received. Intellectual Property arrangements in place.	$39,238	1/03/99	5/03/99	$39,238
2	31/01/98	Results obtained from full taste test and biochemical analysis of fruit from three transgenic plants. Homozygous transgenic plants suitable for breeding and backcrossing, produced.	$39,238	18/12/98	18/03/99	$39,237
3	1/08/98	Inheritance and stability of ADH transgene(s) determined. Quality of transgenic and control fruit ripened on and off the vine and stored at 4degC and 10degC determined.	$42,669	18/12/98	25/05/99	$42,669
4	31/01/99	Introduction of genetic modification into two (Australian) commercial tomato lines completed. Completion of initial market analysis by Zeneca to CSIRO and HRDC.	$42,669	2/02/99	25/05/99	$42,668
5	1/08/99	Taste testing and biochemical analysis of selected genetically modified commercial lines completed. Correlation between taste testing and biochemical parameters of flavour completed.	$3,886			
6	31/10/99	Final Report submitted to HRDC				$0
7	30/11/99	Final Report accepted by HRDC	$3,885			$0
		TOTAL	$171,585			

SECTION A: Receptionist (to be completed within 1 week of receipt)

Action to be Taken — **Initials & Date**

Generate Checklist & enter **Industry &/or Technical Summary** into database — NC 29-10-9
Enter **Due Date** into database (Publish section)
Enter **Date Received** into database (Publish section)
Enter **Category** into database (Publish section)
Enter **Category Description** into database (Publish section)
Enter **Status** into database (Publish section)
Enter **Date Received** into database (milestone section)
Generate an acknowledgement of receipt letter & post to the Chief Investigator (cefn/winword/work/final rep/fr_acknl)
Forward Checklist & final report copies to Publications Manager



HRDC FINAL REPORT CHECKLIST

SECTION B: Publications Manager (to be completed within 3 days of receipt)

Before Publication Process

Is the Project No & Title on report?	(YES)	NO
Have 5 copies of the report plus 1 unbound copy been included?	YES	NO
Has the report ever had a Decision Code of 6666 (Confidential)? (Dates section)	YES	* (NO)
If confidential, has a public report also been submitted?	YES	(NO)
Has the Technical/Industry Summary been included?	(YES)	NO

Comments

IMPORTANT

* This report should be referred to Les Baxter given the sensitive nature of the research from the consumer perspective prior to consideration for publication.

Action

Action to be Taken — **Initials & Date**

Forward Checklist & copy of final report(s) to the relevant PM — NK 29.10.9[illegible]

HRDC FINAL REPORT CHECKLIST

ECTION C: Program Manager (to be completed within 3 weeks of receipt)

Have the milestone criteria been achieved?	(YES)	NO
Is the final report adequate for payment?	(YES)	NO
I recommend that the final report be accepted?	(YES)	NO
I authorise for the milestone payment to be made	(YES)	NO
Have you checked the final report confidentiality status in the database? (Dates)	(YES)	NO
Is the final report **confidential?**	YES	(NO)
If confidential, has a public report also been submitted?	YES	(NO)
Is the final report suitable for general library lodgement?	(YES)	NO
Is the final report suitable for publication?	(YES)	NO
I have entered the **Achieved Date** into the database		(YES)

PM signature P.G. Peale Date 14/12/99

Comments (must be completed, include standard of final report, sales potential etc)

Care should be taken regarding the provision of this report to the media given the consumer perceptions of this research (any issue to the media should be considered after consulting with relevant Program Managers)

Action

This checklist includes signing off Milestone 7.

Action to be Taken	**Initials & Date**
Forward report & Checklist to Publications Manager	

ECTION D: Publications Manager (Rejected Final Reports only) (to be completed within 7 days of receipt)

Action to be Taken	**Initials & Date**
Select the appropriate criteria in the Status field of the database	
Forward report & Checklist to Information Management Officer	

ECTION D: Publications Manager (Accepted Final Reports only) (to be completed within 7 days of receipt)

Action to be Taken	**Initials & Date**
Allocate an ISBN number	
Select the appropriate criteria in the **Format** field of the database	
Select the appropriate criteria in the **Status** field of the database	
Select the appropriate criteria in the **For Sale** field of the database	
Select the appropriate criteria in the **Library** field of the database	
Hold report and forward Checklist to Information Management Officer	