

# Application of two-dimensional gel electrophoresis to detect proteins associated with harvest maturity in stonefruit

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## Abstract

The correct assessment of harvest maturity stage of stonefruit is an important determinant of their quality when they reach the consumer. However, the current indices of ripening used (e.g. skin colour, firmness and soluble solids concentration) vary with cultivar, rootstock, growing conditions and seasonal climatic factors. To develop an index that is independent of environmental factors, total proteins were extracted from fruit and separated by 2D-PAGE. Four proteins (Z1, Z2, Y and X) that were synthesised in fruit a few days before optimum harvest maturity were identified in Japanese-type plums (*Prunus salicina*), peaches and nectarines (*Prunus persica*), and European plums (*Prunus domestica*). These proteins are first expressed a few days before optimum harvest date and their expression increases as the fruit mature. The N-termini of the three most prominent proteins from plums (Z1, Z2 and Y) were sequenced. There appears to be close homology between these proteins and those from a range of species including almond, grape and rubber. It appears that these proteins belong to a family of allergens common to plants whose function may be to confer some form of protection to the plant during periods of stress. These proteins may be used for the development of a test kit that will assist growers to determine optimum harvest maturity for cultivars of plums, peaches and nectarines. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Fruit ripening; Harvest maturity; *Prunus*

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## 1. Introduction

Traditionally, the characteristics used to judge commercial harvest maturity in stonefruit include combinations of changes in ground colour of the skin, softening, titratable acidity, soluble solids concentrations and aroma volatiles. These charac-

teristics are not reliable because they vary with fruit size, production area, climatic conditions and crop load (Kader and Mitchell, 1989; Abdi et al., 1997b). The consequences of incorrect judgement of maturity are economically serious. Harvesting too early results in a significant loss of yield, as stonefruit grow rapidly during the last few days of maturation (DeJong and Johnson, 1989) and often do not develop acceptable flavours. On the other hand, harvesting too late may mean that the fruit have a short shelf-life and

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are unable to withstand normal retail distribution. Therefore, a more reliable index of maturity is required that is closely linked to the genetically controlled, physiological changes in fruit and that is independent of environmental factors.

It has been demonstrated that fruit ripening is accompanied by changes in protein synthesis and the appearance of new enzymes that catalyse various ripening changes (Brady and O'Connell, 1976; Dilley et al., 1993a). A few proteins synthesised during ripening have been characterised, such as polygalacturonase, ACC synthase and ACC oxidase, but the functions of many remain unknown (Dilley et al., 1993a). Since the ripening of plums and related stonefruit is genetically programmed, it should be possible to detect changes in ripening-related proteins that are correlated with commercial maturity.

Plant cells contain many components that may interfere with protein extraction, separation and purification (Granier, 1988; Meyer et al., 1988). Despite this potential problem, two-dimensional gel electrophoresis (2D PAGE), pioneered by O'Farrell (1975), has been used widely in plant biology to study changes in gene expression during development. This technique has been used by Dilley et al. (1993b) to survey changes in total extractable proteins in maturing apples. This group found proteins whose expression coincided with ideal commercial maturity.

To date, however, there are no protein extraction and separation methods for use with plums, nectarines or other stonefruit. Therefore, the objectives of this study were to: (1) develop procedures that allowed changes in protein expression to be followed using 2D PAGE; and (2) use this methodology to identify proteins from plums whose expression correlated with commercial harvest maturity.

## 2. Materials and methods

### 2.1. Source of fruit

The plum cultivars used in this study were hybrid Japanese-types derived from crosses with *Prunus salicina* (Lindl). Fruit of the cultivars

'Beauty', 'Shiro' and 'Rubyred' were harvested from three matched, 10 year-old trees grown at Bilpin, NSW. Fruit from the cultivar 'Gulfruby', were harvested from the orchard at the Centre for Horticulture and Plant Sciences, UWS, as were 'Elberta' peaches. 'Silver King' nectarines and 'Prune d'Anjou' plums were obtained from a commercial orchard at Picton, NSW. The cool storage behaviour of the plum cultivars has been described previously (Abdi et al., 1997a; McGlasson and Holford, 2002). Individual fruit of all types were pitted, sliced, snap-frozen and stored at  $-80^{\circ}\text{C}$ . Proteins were extracted from tissue that had been finely ground in a pestle and mortar using liquid nitrogen.

### 2.2. Protein extraction

Fruit tissue (2–4 g) was frozen in liquid nitrogen, ground and suspended in 10 ml of protein extraction buffer (Barent and Elthon, 1992). An equal volume of water-saturated phenol was then added, and the tube was shaken vigorously for 5 min at room temperature after which the phases were separated by centrifugation at  $7000 \times g$  for 10 min. The phenol phase was recovered and re-extracted with an equal volume of protein extraction buffer and 1 ml of water-saturated phenol; this was repeated until a clean interface was obtained. The proteins were then precipitated overnight at  $-20^{\circ}\text{C}$  by the addition of five volumes of 0.1 M ammonium acetate in methanol. The following day, proteins were collected by centrifugation at  $7000 \times g$  for 10 min and the protein pellets washed three times with ammonium acetate in methanol and once with acetone. The pellet was dried under vacuum for 5 min and solubilised in a 350  $\mu\text{l}$  of O'Farrell lysis buffer (O'Farrell, 1975) containing 0.7% (v/v) Bio-Lyte 5/7 and 1.3% (v/v) Bio-Lyte 3/10 (Bio-Rad). Each extraction procedure was repeated twice for each sample of fruit. At least four gels were obtained from one extract of fruit from each maturity stage of each cultivar. In later extractions, 2 mM PMSF and 1% (w/v) DTT were added to the O'Farrell lysis buffer. The protein concentration of each sample was determined using a modified Bradford protein assay (Ranagli and Rodriguez, 1985).

### 2.3. Assessment of quality

For chemical analysis, sections of fruit tissue were frozen, then thawed and homogenised using a Waring blender. Aliquots of the homogenate were centrifuged ( $6000 \times g$  for 6 min) and the supernatant solutions were used to measure SSC using a hand-held refractometer (Atago, Japan). TA was also measured by titrating 5 ml of juice diluted with 5 ml of distilled water to pH 8.1 with 0.1 N NaOH (Robertson et al., 1991).

### 2.4. Protein separation

#### 2.4.1. First-dimension

IEF gels were prepared using the formulation of Hochstrasser et al. (1988) either using bisacrylamide or diacrylolo-piperazine both at 0.8% w/v as the cross-linking agent. Different mixtures of Bio-Lyte ampholines (Bio-Rad) were trialed: (a) 0.2% 3/10; 0.8% 5/7; (b) 0.4% 3/10; 0.6% 5/7; and (c) 0.8% 3/10 and 0.2% 5/7. In later extractions, 2 mM DTT and 2 mM PMSF were added to the phenol extraction buffer. About 25–40  $\mu$ g protein sample was loaded at the acidic end of the IEF gel, with or without 10  $\mu$ l of 2D SDS-PAGE standard proteins (Bio-Rad). The proteins were separated using an upper catholyte solution consisting of 20 mM NaOH and lower anolyte solution of 10 mM  $\text{H}_3\text{PO}_4$  either: (a) for 3 h at 300 V, 15 h at 700 V and then at 1000 V to give 35 000 V h or (b) 13 h at 400 V, 5 h at 800 V then 1000 V to give 35 000 V h.

#### 2.4.2. Second-dimension

IEF gels were equilibrated prior to SDS-PAGE according to the method of Bjellqvist et al. (1993) by soaking in IEF equilibration buffer containing 2% (w/v) DTT for 5 min and then IEF equilibration buffer containing 2.5% (w/v) iodoacetamide plus 1% (w/v) bromophenol blue for a further 5 min. For the second separation, six 12% (acrylamide/bis) resolving gels were cast in a Multicell-II-xi-Protean chamber (Bio-Rad). These were then overlaid with a 4% stacking gel. Each IEF gel was placed directly onto the slab gel, straightened and any air bubbles removed.

Electrophoresis was performed using lower ( $1 \times$ ) and upper tank ( $2 \times$ ) electrolyte buffer solution (0.025 M Tris base, pH 8.3, 0.192 M glycine, 1% (w/v) SDS) at a constant current of 40 mA/gel until the dye front was approximately 10 mm from the bottom of the gel.

#### 2.5. Silver-nitrate staining procedure (after Rabilloud et al., 1994)

Gels were fixed in 38% (v/v) ethanol, 10% (v/v) acetic acid, 52% (v/v)  $\text{ddH}_2\text{O}$  for 60–90 min then soaked overnight in incubation solution (30% (v/v) ethanol 95%, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 0.5% (v/v) glutaraldehyde 25%). The incubation solution was then removed and the gel washed for  $3 \times 15$  min in distilled water. The gels were then stained with 0.2% (w/v) silver nitrate dissolved in deionised water for 60 min. After staining, the gels were washed with distilled water for 5 min and the stained proteins were visualised by soaking in developing solution (3.6% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde). After 15–20 min development, the visualisation reaction was stopped by the addition of 1.5% acetic acid.

#### 2.6. Analysis of polypeptide patterns

For comparative analysis, the distance travelled by each standard with respect to the edge of the gel was correlated with the  $M_r$  and  $pI$  of the protein using linear regression. From the regression equations, the  $M_r$  and  $pI$  of individual polypeptides were determined. Estimates of  $M_r$  and  $pI$  were made on all proteins of interest from each cultivar at each maturity stage. Differences in these attributes of each polypeptide were determined between cultivars using analysis of variance and Duncan's multiple range test. The relative expression of proteins of interest was visually assessed on at least four gels from each combination of maturity and variety using the following scale: 0 = no expression; + = polypeptide just visible; ++ = low expression; +++ = moderate expression; and ++++ = high expression.

### 2.7. Protein sequencing

For protein sequencing, 2D gels stained with Coomassie Brilliant Blue R were sent to the Australian Proteome Analysis Facility located at Macquarie University, Sydney, Australia. The proteins of interest were blotted on to polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd, UK) and their N-terminal amino acid sequences determined by automatic Edman degradation using an Applied Biosystems 494 Procise automated sequencing system.

### 2.8. Protein characterisation

Homology to nucleotide and amino acids sequences recorded in the GenBank and EMBL sequence databases was determined using programs of the BLAST package (Altschul et al., 1990) via either the Australian National Genome Information Service (University of Sydney, 1996) or National Center for Biotechnology Information (National Library of Medicine, 2001) servers. Multiple sequence alignments were determined using PILEUP (Feng and Doolittle, 1987). Comparisons of amino acid content and protein molecular weights and *pI*s were made using PEPSTATS accessed through ANGIS.

## 3. Results and discussion

In this study, a number of extraction protocols were trialed for protein purification and a modified version of the method of Barent and Elthon (1992) was found to be most suitable for stonefruit tissues. This method uses a two-phase phenol/buffer system where proteins partition into, and are recovered from, the organic phase. This method has several advantages over other methods. Firstly, the phenol and urea in the protein lysis buffer break non-covalent hydrogen bonds and hydrophobic interactions ensuring that a wide range of proteins are extracted (Hurkman and Tanaka, 1986). Secondly, the phenol also reduces proteolysis (Hurkman and Tanaka, 1986) resulting in a greater recovery of proteins of a higher quality. And, lastly, nucleic acids and car-

bohydrates, which can interfere with isoelectric focusing, are removed in the aqueous phase.

To further improve the quality of the protein extracts, 0.002 M PMSF was added to the extraction buffer and to the O'Farrell lysis buffer in which the extracted proteins were dissolved for separation. PMSF inhibits serine proteases, such as trypsin, and the reduced proteolysis resulted in greater yields and more tightly focused protein spots during 2D-PAGE as has been reported by Damerval et al. (1987), Hurkman and Tanaka (1986) and Meyer et al. (1988). Approximately 25–40  $\mu\text{g g}^{-1}$  fresh weight of protein were extracted and could be separated by 2D-PAGE without significant smearing or streaking.

Initial analysis of protein expression during ripening concentrated on four cultivars of Japanese-type plums. 'Gulfruby' and 'Beauty' exhibit a typical, climacteric pattern of ripening whilst 'Shiro' and 'Rubyred' are suppressed-climacteric cultivars (Abdi et al., 1997a). These latter types have lower peak ethylene levels during the climacteric and ripening attributes are less coordinated. 2D-PAGE revealed similar patterns of protein in the fruit of these cultivars in spite of the significant differences in their ripening behaviour; the protein profiles for 'Gulfruby' and 'Shiro' are shown in Figs. 1 and 2, respectively.

In all four cultivars studied, differences were observed in the levels of certain proteins during maturation. At least four proteins (X, Y, Z1 and Z2) could be first detected between 4 and 7 days before optimum harvest time. The optimum harvest time was determined by comparison of composition changes during maturation and their response to cool storage (Abdi et al., 1997b). The expression of the proteins also occurred before the onset of the climacteric and continued to increase as the fruit matured. Their expression in relation to optimum harvest time, the ethylene and respiratory climacterics and softening are shown in Table 1. In the typical climacteric cultivars, harvested fruit entered their climacteric 4–7 days after these proteins started to be expressed. However, any association between the expression of these proteins and the climacteric in the suppressed-climacteric cultivars was unclear. In 'Shiro' fruit, the climacteric commenced 9 days

after these proteins were expressed. However, high levels of these proteins were found in fruit from ‘Rubyred’ without a respiratory or ethylene climacteric occurring. Associations can also be found between the expression of these proteins and other parameters of ripening. Table 1 shows a positive relationship between softening and expression of X, Y, Z1 and Z2. A positive relationship also exists between expression and SSC and a negative relationship with TA (data not presented).

The 2D-PAGE technique described here has shown that the polypeptides produced by ripening plum fruit can be adequately separated allowing proteins of interest to be unambiguously iden-

tified. This technique has also shown that the same four proteins always increased in each individual fruit of each cultivar examined and in the same pattern in relation to optimal harvest time. Such changes in protein expression are to be expected as ripening is a distinct developmental stage during which new proteins are synthesised, as has been shown in apples (Lay-Lee et al., 1990), banana (Dominguez-Puigianer et al., 1992), cherimoya (Montero et al., 1993) and avocado (McGarvey et al., 1992). This study is the first to show such changes in plums.

The use of 2D-PAGE to detect these ripening-related proteins was extended to other species of stonefruit. Fig. 3 shows that, in general, the pat-

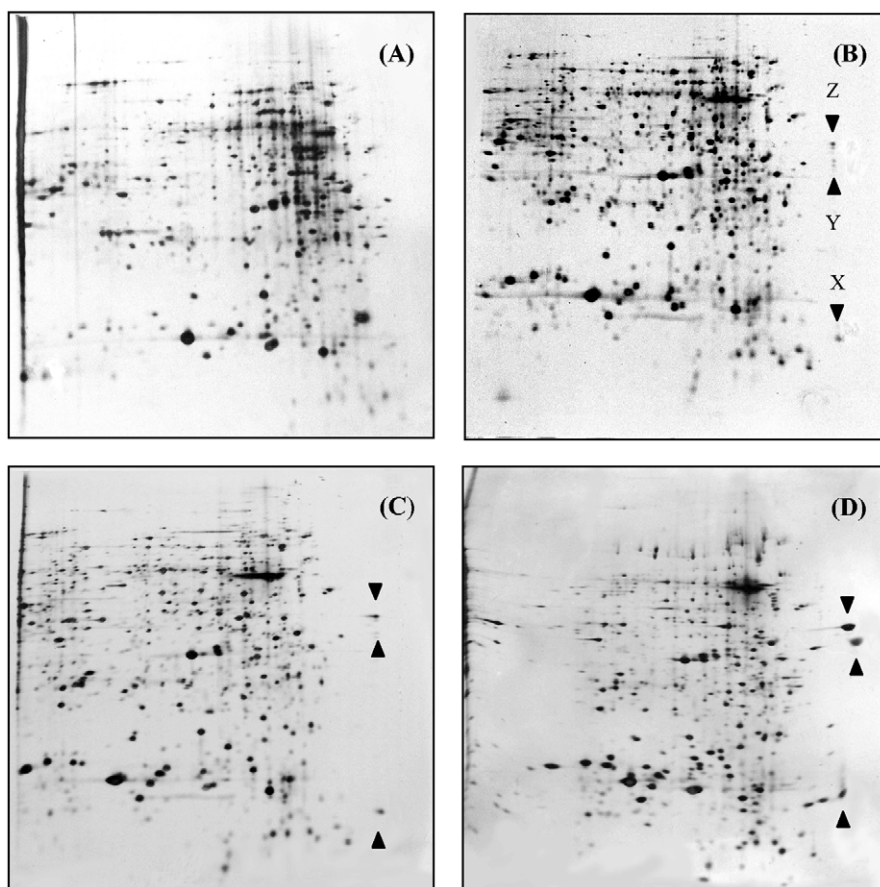


Fig. 1. Separation by 2D-PAGE of proteins extracted from ‘Gulfruby’ plums harvested 14 (A), 21 (B), 25 (C) and 32 (D) days after pit hardening (DAFH). The arrows indicate the location of proteins Z, Y and X. Optimal harvest time for cool storage was 25 DAFH.

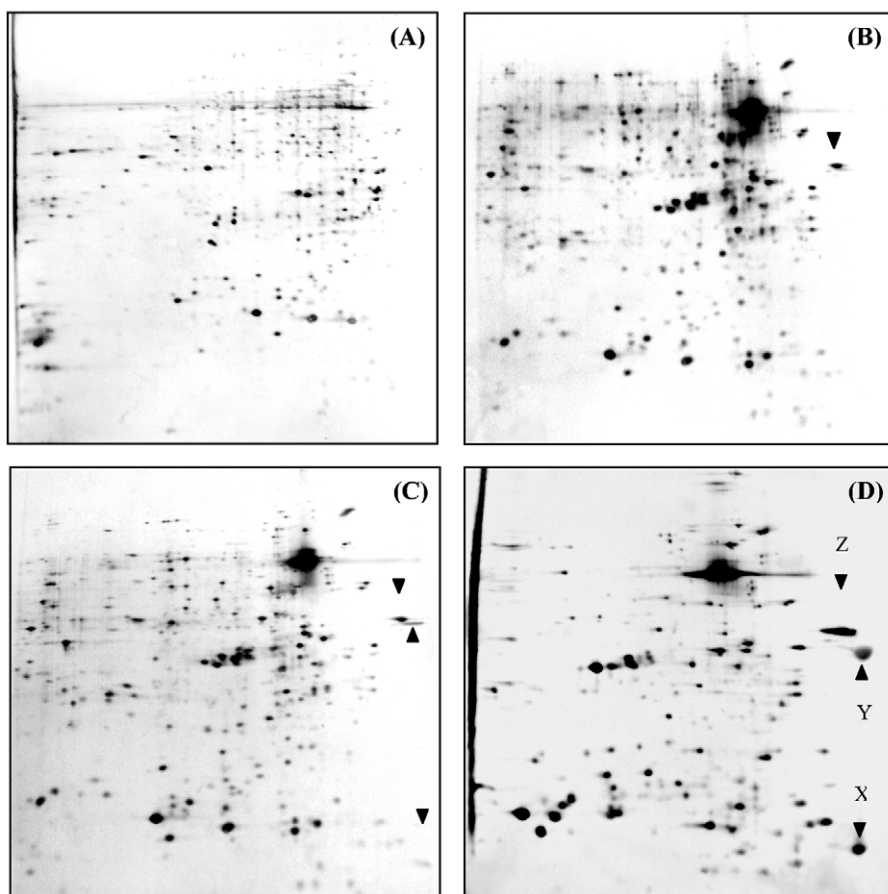


Fig. 2. Separation by 2D-PAGE of proteins extracted from 'Shiro' plums harvested 28 (A), 40 (B), 45 (C) and 49 (D) DAPH. The arrows indicate the location of proteins Z, Y and X. Optimal harvest time for cool storage was 45 DAPH.

terns of protein expression in peaches and nectarines (*P. persica*) and European plum (*P. domestica* cv. Prune d'Anjou) were similar to that in Japanese-type plums. The presence of proteins homologous to Z1, Z2, Y and X was detected in these species. However, the intensity of protein Y on these gels was variable and was either difficult to detect or appeared to be absent. Similar variation in the expression of this protein was found in plums.

The reason for this variation is most likely due to the staining method used. Silver ions were used to detect the proteins separated on these gels. This stain was chosen as it is more sensitive and gives a lower level of background staining than Coomassie blue (Monroy and Schwartzbach,

1983; Hurkman and Tanaka, 1986). The degree of staining of an individual protein by silver is dependent on its amino acid content. Silver binds to basic amino acids and those with sulphur groups (Nielsen and Brown, 1984; Heukeshoven and Dernick, 1985). It is likely that protein Y does not contain amino acids with sulphur groups and has a low percentage of basic amino acids (see below and Tables 2 and 4). Therefore, it will not stain intensely and, in conjunction with differences in the amount of the protein loaded onto the gels, may explain much of the apparent variability in its appearance. This assumption is supported by the appearance of gels stained with Coomassie blue (not shown) where the intensity of expression of protein Y was similar to that of proteins Z1 and Z2.

An attempt to visualise the proteins produced by cherries (*P. avium*), a non-climacteric member of the Rosaceae, was also made (Fig. 3). The pattern of proteins produced in ripe fruit appears to be different from that of the other species studied. In particular, there is a heavy expression of a protein with an approximate  $M_r$  of 30 kDa (arrowed) that stains yellow/brown with silver. This staining reaction may indicate that the protein is glycosylated. Also, none of the ripening-related proteins can be found; however, this result needs to be confirmed.

The molecular weights and  $pI$ s of the proteins are given in Table 3. Proteins Z1 and Z2 have similar  $M_r$ s and  $pI$ s, are often poorly resolved from each other, have identical N-termini (Table 2) and appear to have identical patterns of expres-

sion. Therefore, it is assumed that they are variants of the same protein. As Table 3 shows, significant differences could be detected in the molecular weights (as estimated from 2D gels) of each of the proteins from the four cultivars. Estimates of the size of protein X gave two values for the  $M_r$  of this protein, approximately 17 kDa from 'Gulfruby' and 'Shiro' and 20 kDa from 'Beauty' and 'Rubyred'. Estimates of the size of proteins Z1 and Z2 also suggests that there may be two forms of this protein as 'Beauty', 'Shiro' and 'Rubyred' all had sizes of approximately 40 kDa whilst the size for 'Gulfruby' was 35.7 kDa. Estimates of the size of protein Y exhibited the greatest variability among the cultivars with the possibility of three isoforms existing. The  $pI$ s of each of the three proteins did not show any

Table 1

Correlations between expression of the ripening-related proteins with ethylene, carbon dioxide production and flesh firmness in the four cultivars of plums

| Maturity stages (DAFH) | Ripening-related proteins |      |        | C <sub>2</sub> H <sub>4</sub> (μM/kg h) | CO <sub>2</sub> (mM/kg h)<br>(N) | Flesh firmness (N) |
|------------------------|---------------------------|------|--------|-----------------------------------------|----------------------------------|--------------------|
|                        | X                         | Y    | Z1 & 2 |                                         |                                  |                    |
| <i>Gulfruby</i>        |                           |      |        |                                         |                                  |                    |
| 14                     | 0                         | 0    | 0      | nm                                      | 0.31                             | no sample          |
| 21                     | ++                        | +    | +      | nm                                      | 0.36                             | 42.0               |
| 25*                    | +++                       | ++   | ++     | 0.09                                    | 0.36                             | 30.5               |
| 32                     | +++                       | +++  | +++    | 0.80                                    | 0.85                             | 6.5                |
| <i>Beauty</i>          |                           |      |        |                                         |                                  |                    |
| 28                     | 0                         | 0    | 0      | nm                                      | 0.54                             | 62.0               |
| 35                     | +++                       | ++   | +++    | nm                                      | 0.51                             | 30.0               |
| 42*                    | ++++                      | +++  | ++++   | 0.11                                    | 0.58                             | 18.0               |
| 49                     | ++++                      | ++++ | ++++   | 0.23                                    | 0.56                             | 6.0                |
| <i>Shiro</i>           |                           |      |        |                                         |                                  |                    |
| 35                     | 0                         | 0    | 0      | nm                                      | 0.51                             | 38.5               |
| 40                     | ++                        | +    | ++     | nm                                      | 0.49                             | 29.0               |
| 45*                    | +++                       | ++   | +++    | nm                                      | 0.47                             | 20.0               |
| 49                     | ++++                      | +++  | ++++   | 0.02                                    | 0.54                             | 11.0               |
| <i>Rubyred</i>         |                           |      |        |                                         |                                  |                    |
| 56                     | 0                         | 0    | 0      | nm                                      | 0.30                             | 61.5               |
| 80                     | +                         | 0    | +      | nm                                      | 0.33                             | 43.0               |
| 85*                    | +++                       | ++   | +++    | nm                                      | 0.31                             | 33.0               |
| 95                     | ++++                      | ++   | ++++   | nm                                      | 0.36                             | 21.5               |

The data for protein expression are the means of three replicate gels with 0 representing no expression, +, low expression and ++++ the highest level of expression. Data for ethylene and  $CO_2$  were taken from three fruit per three trees (i.e.  $n = 9$ ) and for flesh firmness from 10 fruit from three trees (i.e.  $n = 30$ ). The rates of ethylene and  $CO_2$  production were measured in fruit held at 20 °C 24 h after harvest. \* = optimum harvest time, nm = levels not measurable.

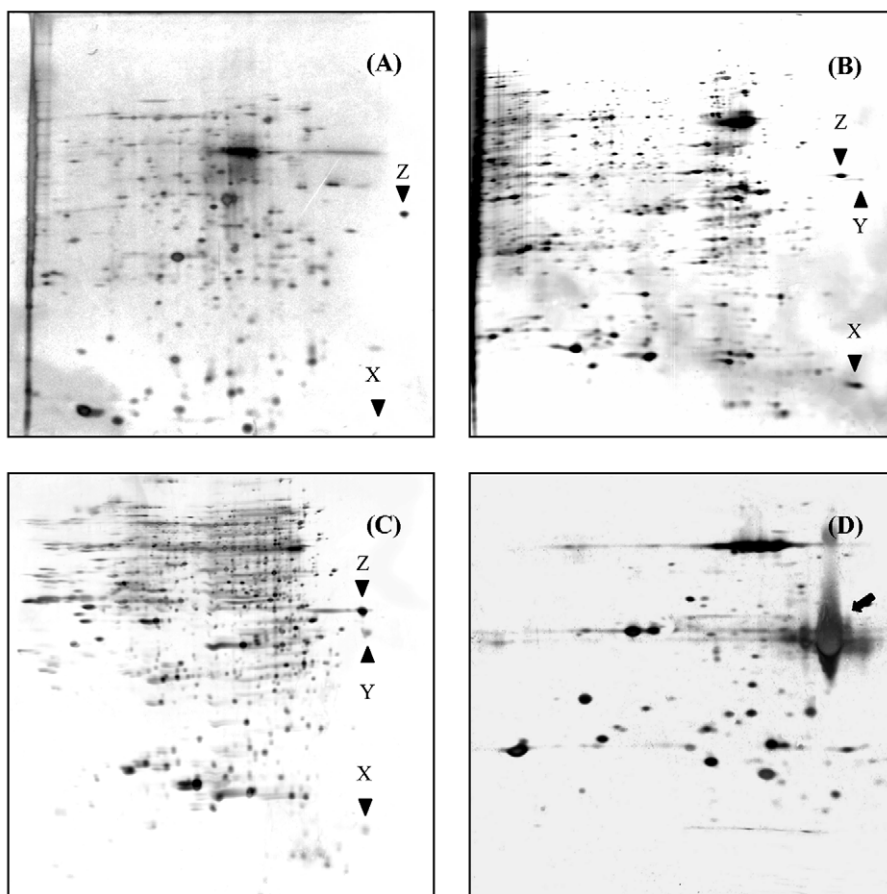


Fig. 3. Separation by 2D-PAGE of proteins extracted from ripe fruit of (A) 'Elberta' peaches; (B) 'Silver King' nectarines; (C) European plums (*Prunus domestica* cv. Prune d'Anjou) and (D) cherries (cv. unknown). The arrows in panels A, B, and C indicate the location of proteins Z, Y and X, respectively, and in panel D, the position of a highly expressed protein not seen in other stonefruit.

variation among cultivars (Table 3). Further work is required to confirm whether the differences in  $M_r$  found within proteins X, Y, Z1 and Z2 are due to different amino acid compositions, variation in glycosylation or random differences due to the conditions of the electrophoretic separation.

The results of N-terminal amino acid sequencing of the ripening-related proteins are presented in Table 3. Protein Z1 yielded reliable data for its first 11 residues. Protein Z2 gave reliable data for ten out of eleven residues; these were identical with protein Z1. Residue 9 was found to be either glycine or proline and, as the equivalent residue in Z1 was glycine, it was assumed that this residue

was also glycine. These sequence data, together with the estimates of  $M_r$  and  $pI$  determined from 2D PAGE, suggests that the two proteins are isoforms of each other. Analysis of protein Y yielded 23 residues that could be unambiguously identified. The sequence information obtained from protein X was ambiguous with only six out of the first eleven residues being reliably identified (data not presented).

The sequence data from the Z and Y proteins were used to determine if homologous sequences could be found in the EMBL and GenBank databases. No homologous sequences could be found when the data from the Z proteins were used as

Table 2  
Amino acid sequence data from proteins X, Y, Z1 and Z2

|                   |    |    |    |    |    |    |    |    |     |    |    |    |
|-------------------|----|----|----|----|----|----|----|----|-----|----|----|----|
| <i>Protein Y</i>  |    |    |    |    |    |    |    |    |     |    |    |    |
| Residue           | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9   | 10 | 11 | 12 |
| Major             | V  | A  | P  | A  | A  | T  | L  | Q  | E   | N  | E  | A  |
| Residue           | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21  | 22 | 23 |    |
| Major             | K  | T  | V  | E  | V  | I  | K  | T  | E   | E  | K  |    |
| <i>Protein Z1</i> |    |    |    |    |    |    |    |    |     |    |    |    |
| Residue           | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9   | 10 | 11 |    |
| Major             | S  | K  | E  | E  | V  | V  | V  | K  | E   | E  | E  |    |
| <i>Protein Z2</i> |    |    |    |    |    |    |    |    |     |    |    |    |
| Residue           | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9   | 10 | 11 |    |
| Major             | S  | K  | E  | E  | V  | V  | V  | K  | E/P | E  | E  |    |

Key: alanine, A; arginine, R; asparagine, N; glutamine, Q; glutamic acid, E; glycine, G; isoleucine, I; leucine, L; lysine, K; proline, P; serine, S; threonine, T; tryptophan, W; valine, V.

the query sequence. This lack of homology could be due to either: (1) genes for similar proteins from other species have not been cloned or their sequences entered in the databases; (2) homologous proteins do not exist in other species; or (3) insufficient data from the Z proteins exists for the search programs to detect homology with other proteins. Further work on this protein will determine which of these possibilities is correct.

The sequence data from protein Y (Table 2) revealed homology with a protein from apricots (Fig. 4). The sequence was obtained using mRNA extracted from the mesocarp and endocarp of ripe fruit. The authors (Mbeguie-a-Mbeguie and Fils-Lyacon, 1999) stated that the protein is a putative allergen: no further information on this protein is available. When either the N- or C-terminal re-

gions of the apricot gene were used as query sequences, searches of the genetic databases revealed homology with genes from a further ten species: *Hevea brasiliensis* (Slater et al., 1996), *Vitis vinifera* (Davis and Robinson, 2000), *Actinidia chinensis* (Ledger and Gardner, 1994), *Ribes nigrum* (Woodhead et al., 1998), *Solanum tuberosum* (Taylor et al., 1992), *Arabidopsis thaliana* (Guan et al., 1997), *Fagopyrum esculentum* (Fujino et al., 1996), *Ricinus* sp. (Van der Loo et al., 1995), *Alnus glutinosa* (Guan et al., 1997) and *Lotus japonicus* (Szczyglowski et al., 1997).

Alignments of these protein sequences (Fig. 4) show that substantial homology exists in their N- and C-termini. As pointed out by Akasawa et al. (1996), these proteins have a high number of proline and glutamic acid residues with the latter residues often occurring in doublets or triplets of

Table 3  
Molecular weights ( $M_r$ ) and isoelectric points (pIs) of three proteins of interest (X, Y and Z) in four cultivars studied

| Cultivar | Protein X    |             | Protein Y     |             | Protein Z    |             |
|----------|--------------|-------------|---------------|-------------|--------------|-------------|
|          | $M_r$        | pI          | $M_r$         | pI          | $M_r$        | pI          |
| Gulfruby | 17.3 ± 0.43a | 3.4 ± 0.10a | 35.0 ± 0.57a  | 3.6 ± 0.12a | 35.7 ± 0.66a | 3.6 ± 0.16a |
| Beauty   | 20.1 ± 0.90b | 4.0 ± 0.57a | 38.3 ± 0.70b  | 3.9 ± 0.55a | 39.3 ± 0.66b | 4.1 ± 0.55a |
| Shiro    | 17.0 ± 0.28a | 3.7 ± 0.21a | 40.3 ± 0.32c  | 3.8 ± 0.25a | 40.1 ± 0.10b | 3.9 ± 0.20a |
| Rubyred  | 20.0 ± 0.57b | 3.5 ± 0.25a | 39.2 ± 0.58bc | 3.6 ± 0.17a | 39.4 ± 0.40b | 3.7 ± 0.28a |

The data are the means and standard errors of three gels from three harvest maturities of each cultivar (i.e.  $n = 9$ ). Means followed by the same letter are not significantly different according to Duncan's multiple range test at  $P \leq 0.05$ .

Amino terminus

|           |            |            |            |                       |
|-----------|------------|------------|------------|-----------------------|
| Hevea     | .....      | ..MASVEVES | AATALPKNET | .PEVTKAEET            |
| Actinidia | .....      | ..MATVEVTP | AVTALPENET | ADEVTKPQEP            |
| Vitis     | .....      | ..MATVEVAS | VTTALPENEK | TEELNKVEEG            |
| Prunus    | .....      | ..MATVEVAP | AATLQENEAK | TVEVIKTEEK            |
| Fagopyrum | .....      | ..MAAVEVEP | NTTTLQENEK | S.EVAQVEEV            |
| Alnus     | .....      | ..MATVEVVS | AKTALPEETI | EEPIKVHETI            |
| Ricinus   | .....      | .....      | .EKPKEEETT | EE.....               |
| Lotus     | .....      | ..MATAQVAS | ANSALHDDTT | KASLKDHEEK            |
| Ribes     | .....      | ..MATVEVVS | AQTAFQEEKK | HDQ.EVITTK            |
| Solanum   | FAPSISSFIF | LFPIFLFFNF | IPMASVEVES | APVAAVETTT PAEVE..... |

Carboxy terminus

|           |            |            |             |                           |
|-----------|------------|------------|-------------|---------------------------|
| Hevea     | .....      | .....EK    | PITEAAETAT  | TEVPVEKTEE                |
| Actinidia | EPTNAPEAPA | VGEEPEAKEG | KPDEAVEEAS  | TEVPVDKTEE                |
| Vitis     | ..APAEAAAA | KEEE....EA | KPAEAAEEAS  | TEVPVEKIEE                |
| Prunus    | AAPPAAE... | .....      | ...EKAAEEAT | TDVPVEKTEE                |
| Fagopyrum | .....      | .....      | VVEEKAAEEAT | TEAAVEKAE                 |
| Alnus     | PEPKPEPEAE | VPKKAEVPEE | VAAEVPKEEV  | VVKEEEKPVE AEEKVGTEAP     |
| Ricinus   | ..PAAEPEA. | .....E     | PAVEAPKDEV  | KEEVEEKKE.                |
| Lotus     | EEKVAEPEA. | EAPDEKKEEE | KLDTEGSDEK  | KEEEEEVIKVA PAEKTE        |
| Ribes     | EEKVAVEEVE | V.....     | ....KVETEE  | GEVTEEKTE.                |
| Solanum   | EASPVSEEP. | .....      | .....       | EKVEE IIQWLMDNGF FFIIIEVW |

Fig. 4. Alignment of the amino acid sequence from protein Y with the predicted amino acid sequences from *Hevea brasiliensis* (Slater et al., 1996), *Actinidia chinensis* (Ledger and Gardner, 1994), *Vitis vinifera* (Davis and Robinson, 2000), *Prunus armeniaca* (Mbeguie-a-Mbeguie and Fils-Lyacon, 1999), *Fagopyrum esculentum* (Fujino et al., 1996), *Alnus glutinosa* (Guan et al., 1997), *Ricinus* sp. (Van der Loo et al., 1995), *Lotus japonicus* (Szczyglowski et al., 1997), *Ribes nigrum* (Woodhead et al., 1998) and *Solanum tuberosum* (Taylor et al., 1992). The underlined section of the *Prunus* sequence indicated the region of identity between the amino acid sequence from *Prunus salicina* and the predicted amino acid sequence from *Prunus armeniaca*.

Table 4  
Spectrum of amino acid types found in the proteins with sequence homology to the ripening-related protein from *Prunus armeniaca*

|                                     | <i>Prunus</i> | <i>Actinidia</i> | <i>Hevea</i> | <i>Vitis</i> | <i>Ribes</i> | <i>Fagopyron</i> | <i>Lotus</i> | <i>Solanum</i> | Average |
|-------------------------------------|---------------|------------------|--------------|--------------|--------------|------------------|--------------|----------------|---------|
| Small (Ala Gly)                     | 23.8          | 25.0             | 19.2         | 25.7         | 13.3         | 25.8             | 12.0         | 21.8           | 20.8    |
| Hydroxyl (Ser Thr)                  | 14.9          | 8.7              | 15.2         | 9.2          | 14.5         | 5.7              | 17.7         | 7.6            | 11.7    |
| Acidic (Asp Glu)                    | 31.0          | 30.4             | 31.8         | 33.6         | 31.2         | 34.0             | 33.3         | 21.3           | 30.8    |
| Acid/Amide (Asp Glu Asn Gln)        | 32.1          | 33.2             | 33.8         | 34.9         | 33.5         | 38.4             | 37.0         | 22.7           | 33.2    |
| Basic (His Lys Arg)                 | 7.1           | 5.4              | 9.3          | 7.9          | 9.2          | 5.0              | 12.5         | 2.4            | 7.4     |
| Charged (Asp Glu His Lys)           | 38.1          | 35.9             | 41.1         | 41.4         | 40.5         | 39.0             | 45.8         | 23.7           | 38.2    |
| Small hydrophobic (Ile Leu Met Val) | 10.7          | 11.4             | 7.2          | 14.5         | 19.1         | 17.6             | 14.1         | 21.8           | 14.6    |
| Aromatic (Phe Trp Tyr)              | 0             | 0                | 0            | 0            | 0.6          | 0                | 0            | 10.9           | 1.4     |
| pI                                  | 3.74          | 3.55             | 3.86         | 3.76         | 3.87         | 3.52             | 3.97         | 3.39           | 3.71    |
| Mr (kDa)                            | 17.53         | 18.92            | 16.09        | 15.92        | 18.77        | 16.67            | 21.00        | 22 64          | 17.84   |

The figures represent the percentage of each type of residue on a molar basis.

the form X<sub>1</sub>EE(E)X<sub>2</sub>, where X<sub>1</sub> is most often alanine or tryptophan and X<sub>2</sub> is commonly tryptophan or proline. This motif is also found in proteins Z1 and Z2. The proteins of this group also have similar amino acid spectra, molecular weights and pIs (Table 4).

The patterns of expression of this family of proteins vary during plant development. Genes for four of these proteins are expressed in ripening fruit although they each have differing patterns of expression. The gene (*pKIWI501*) in *Actinidia* is expressed at low levels in immature fruit, expression then increases in fruit up to 8–10 days after anthesis and then declines. The gene is also expressed at a low level in roots and shoots (Ledger and Gardner, 1994). The gene from blackcurrant is expressed during all stages of berry ripening with expression increasing from low levels in young fruit to a maximum in ripe fruit (Woodhead et al., 1998). The transcripts of the grape gene also accumulate in ripening fruit (Davis and Robinson, 2000). In contrast, however, transcripts of the *Ricinus* gene are only found in immature fruit (Van der Loo et al., 1995).

Little work has been performed on ripening-related gene expression in *Prunus*. Callahan et al. (1993) found three classes of genes in peaches whose pattern of expression changed during fruit development, one class accumulated during the softening phases, the second increased during development to a maximum in ripe fruit and the third had maximum expression during the middle of fruit development. However, the sequences of these genes have not been published; therefore it is not possible to determine if they are related to the proteins this study has found in peaches, plums and nectarines.

The other proteins found through searches of the databases that show homology with protein Y have patterns of expression that are not associated with fruit ripening. The protein from *Alnus*, *ag13*, is produced during nodule development caused by the actinomycetous bacterium, *Frankia* (Guan et al., 1997). Its expression is confined to the pericycle of the vascular system of the younger parts of the nodule and to the senescent zone where nitrogen fixation has stopped and the vesicles (terminal swellings of the hyphae of the actinomycete) in which fixation occurs have started to degrade. The *Arabidopsis* homologue, *Ara21371* (Guan et al., 1997), is expressed in stems, flowers and siliques. The protein from *Hevea* was purified from latex and a cDNA representing the gene encoding it was identified in a library of genes expressed in latex

(Akasawa et al., 1996). Therefore, it seems that this family of genes related to protein Y must have a diverse set of functions.

Davis and Robinson (2000) suggested that the protein in grape might be involved in changes in cell wall properties and that these changes may help to prevent attack by pathogens. Bradley et al. (1992) have also proposed that proteins such as the ones found by Davis and Robinson have a role in strengthening cell walls. A function for the proteins related to protein Y involving the modification of plant cell walls is quite plausible as many of the processes detailed above where this protein family are expressed are associated with changes in cell wall properties. However, Atkinson et al. (1996) found a ripening-related protein that is also expressed in response to stress and pathogen attack. Therefore, it is likely that the proteins behave like heat shock proteins, are produced as a general response to environmental (e.g. pathogenesis and wounding) or developmental stresses (e.g. ripening and senescence) and provide some kind of protection to the plant.

Protein Y shares homology with a putative allergenic protein from apricot (Mbeguie-a-Mbenguie and Fils-Lyacon, 1999) and a known allergen from rubber (Slater et al., 1996). The proteins identified in this study are typical of allergenic proteins in that they have molecular weights between 10 and 70 kDa (Taylor et al., 1987) and an acidic *pI* (Taylor and Lehrer 1996). It has been noted that individuals showing allergies to rubber products may also present reactions to fruits such as avocado, chestnut, banana papaya, fig, celery, passion fruit, kiwifruit and peach (Blanco et al., 1994). The cross-reaction between rubber and peach supports the assumption of Mbeguie-a-Mbenguie and Fils-Lyacon (1999) that the apricot protein is an allergen; therefore, protein Y is also likely to be allergenic. The above list of cross-reactions also supports the idea that this family of proteins is common to a wide range of plants.

This study has found four proteins whose changes in expression can be correlated with the ripening process. These proteins were detected in three species of stonefruit (European and Japanese plums, peaches and nectarines). It appears that three of these proteins (Z1, Z2 and Y) may belong

to a family of allergenic proteins common to plants whose function may be to confer some form of protection to the plant during periods of stress. These proteins have the potential for development into an assay system that growers can use to assist with the judgement of optimal harvest time.

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## References

- Abdi, N., McGlasson, W.B., Holford, P., Mizrahi, Y., 1997a. Ripening behaviour and responses to propylene in four cultivars of plums. *Postharvest Biol. Technol.* 12, 21–34.
- Abdi, N., Holford, P., McGlasson, W.B., 1997b. Effects of harvest maturity on the storage life of Japanese-type plums. *Aust. J. Exp. Agric.* 37, 391–397.
- Akasawa, A., Hsieh, L.-S., Martin, B.M., Liu, T., Lin, Y., Brian, M., 1996. A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterization. *J. Biol. Chem.* 271, 25389–25393.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment tool. *J. Mol. Biol.* 215, 403–410.
- Atkinson, R.G., Perry, J., Matsui, T., Ross, G.S., Macrae, E.A., 1996. A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also ripening related. *N. Z. J. Crop Hortic. Sci.* 24, 103–107.
- Barent, R.L., Elthon, T.E., 1992. Two-dimensional gels: an easy method for large quantities of proteins. *Plant Mol. Biol. Rep.* 10, 338–344.
- Bjellqvist, B., Sanchez, J.C., Pasquali, C., Ravier, F., Paquest, N., Frutiger, S., Hughes, G.J., Hochstrasser, D.F., 1993. Micropreparative two-dimensional electrophoresis allowing the separation of samples containing milligram amounts of proteins. *Electrophoresis* 14, 1375–1378.
- Blanco, C., Carrillo, T., Castillo, R., Quiralte, J., Cuevas, M., 1994. Latex allergy: clinical features and cross-reactivity with fruits. *Ann. Allergy* 73, 309–314.
- Brady, C.J., O'Connell, P.B.H., 1976. On the significance of increased protein synthesis in ripening banana fruits. *Aust. J. Plant Physiol.* 3, 301–310.
- Bradley, D.J., Kjellbom, P., Lamb, C.J., 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70, 21–30.
- Callahan, A.M., Morgensm, P.H., Cohen, R.A., 1993. Isolation and initial characterization of cDNAs for mRNAs regulated during peach fruit development. *J. Am. Soc. Hortic. Sci.* 118, 531–537.
- Damerval, C., Hebert, Y., de Vienne, D., 1987. Is the polymorphism of protein amounts related to phenotypic variability? A comparison of two-dimensional electrophoresis data with morphological traits in maize. *Theor. Appl. Genet.* 74, 194–202.
- Davis, C., Robinson, S.P., 2000. Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. *Plant Physiol.* 122, 803–812.
- DeJong, T.M., Johnson, R.S., 1989. Growth and Development. In: LaRue, J.H., Johnson, R.S. (Eds.). *Peaches, Plums and Nectarines: Growing and Handling for Fresh Market*. Univ. Calif. Div. Agr. Nat. Res. Publication No. 3331, pp. 32–37.
- Dilley, D.R., Kuai, J.P., Poneleit, L., Zhu, Y., Pekker, Y., Wilson, I.D., Burmeister, D.M., Gran, G., Bowers, A., 1993a. Purification and characterization of ACC oxidase and its expression during ripening in apple fruit. In: Pech, J.C., Latché, A., Balagué, C. (Eds.), *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. In: *Current Plant Science Biotechnology in Agriculture*, vol. 16. Kluwer, Dordrecht, pp. 46–52.
- Dilley, D.R., Wilson, I.D., Kuai, J., Poneleit, L., Zhu, Y., Pekker, Y., 1993b. Molecular biology of pome fruit ripening and senescence: concepts, status, perspectives and future prospects. *Acta Hortic.* 326, 325–334.
- Dominguez-Puigjaner, E., Vendrell, M., Ludevid, M.D., 1992. Differential protein accumulation in banana fruit during ripening. *Plant Physiol.* 98, 157–162.
- Feng, D.F., Doolittle, R.F., 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* 25, 351–360.
- Fujino, K., Funatski, H., Inada, M., Shimono, Y., Kikuta, Y., 1996. Expression and sequence analysis of cDNAs corresponding to genes differentially expressed during seed development of buckwheat (*Fagopyrum esculentum* Moench). GenBank accession D87983.
- Granier, F., 1988. Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis* 9, 712–718.
- Guan, C., Akkermans, A.D.L., van Kammen, A., Bisseling, T., Pawlowski, K., 1997. *ag13* is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle. *Physiol. Plant.* 99, 601–606.
- Heukeshoven, J., Dernick, R., 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6, 103–112.

- Hochstrasser, D.F., Harrington, M.G., Hochstrasser, A.C., Miller, M.J., Merrill, C.R., 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* 173, 424–435.
- Hurkman, W.J., Tanaka, C.K., 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* 81, 802–806.
- Kader, A.A., Mitchell, F.G., 1989. Postharvest Physiology. In: LaRue, J.H., Johnson, R. (Eds.). *Peaches, Plums and Nectarines: Growing and Handling for Fresh Market*. Univ. Calif. Div. Agr. Nat. Res. Publication No. 3331, pp. 158–164.
- Lay-Lee, M., Dellapenna, D., Ross, G.S., 1990. Changes in mRNA and protein during ripening in apple fruit (*Malus domestica* Borkh. cv. 'Golden Delicious'). *Plant Physiol.* 94, 850–853.
- Ledger, S.E., Gardner, R.C., 1994. Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var *deliciosa*). *Plant Mol. Biol.* 25, 877–886.
- Mbenguie-a-Mbenguie, D., Fils-Lyacon, B.R., 1999. Molecular cloning and nucleotide sequence of a putative allergen protein from apricot (*Prunus armeniaca* cv. Bergeron). GenBank accession GI4887128.
- McGarvey, D.J., Sirevag, R., Christoffersen, R.E., 1992. Ripening-related gene from avocado fruit: ethylene-inducible expression of the mRNA and polypeptide. *Plant Physiol.* 98, 554–559.
- McGlasson, W.B., Holford, P., 2002. Detection of ripening-related proteins in stonefruit. *Acta Hort.* (in press).
- Meyer, Y., Grasset, J., Chastier, Y., Cleyet-Marel, C., 1988. Preparation by two-dimensional electrophoresis of proteins for antibody production: antibodies against proteins whose synthesis is reduced by auxin in tobacco mesophyll protoplasts. *Electrophoresis* 9, 704–712.
- Monroy, A.F., Schwartzbach, S.D., 1983. Photocontrol of the polypeptide composition of *Euglena*. *Planta* 158, 249–258.
- Montero, L.M., Escribano, M.I., Zamorano, J.P., Merodio, C., 1993. Two-dimensional protein patterns of cherimoya fruits during ripening. In: Pech, J.C., Latché, A., Balagué, C. (Eds.), *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. In: *Current Plant Science Biotechnology in Agriculture*, vol. 16. Kluwer, Dordrecht, pp. 156–157.
- National Library of Medicine, 2001. National Center for Biotechnology Information [www.ncbi.nlm.nih.gov/entrez/query.fcgi](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (accessed 4.6.01).
- Nielsen, B.L., Brown, L.R., 1984. The basis for colored silver–protein complex formation in stained polyacrylamide gels. *Anal. Biochem.* 144, 311–315.
- O'Farrell, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007–4021.
- Rabilloud, T., Vuillard, L., Gilly, C., Lawrence, J.J., 1994. Silver staining of proteins in polyacrylamide gels: a general overview. *Cell. Mol. Biol.* 40, 57–75.
- Ranagli, L.S., Rodriguez, L.V., 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6, 559–563.
- Robertson, J.A., Meredith, S.O., Forbus, W.R., 1991. Changes in quality characteristics during peach (cv. *Majestica*) maturation. *J. Food Qual.* 14, 197–207.
- Slater, J.E., Vedvick, T., Arthur-Smith, A., Trybul, D.E., Kekwick, R.G.O., 1996. Identification, cloning, and sequence of a major allergen (Hev b5) from natural rubber latex (*Hevea brasiliensis*). *J. Biol. Chem.* 271, 25394–25399.
- Szczyglowski, K., Hamburger, D., Kapranov, P., de Bruijn, F.J., 1997. Construction of a *Lotus japonicus* late nodulin expressed sequence to library and identification of novel nodule-specific genes. *Plant Physiol.* 114, 1335–1346.
- Taylor, S.L., Lehrer, S.B., 1996. Principles and characteristics of food allergens. *Crit. Rev. Food Sci. Nutr.* 36(S), S91–S118.
- Taylor, S.L., Lemanske, R.F., Bush, R.K., Busse, W.W., 1987. Food allergens: structure and immunologic properties. *Ann. Allergy* 59, 93–99.
- Taylor, M.A., Arif, S.A., Kumar, A., Davies, H.V., Scobie, L.A., Pearce, S.R., Flavell, A.J., 1992. Expression and sequence analysis of cDNAs induced during the early stages of tuberisation in different organs of the potato plant (*Solanum tuberosum* L.). *Plant Mol. Biol.* 20, 641–651.
- University of Sydney, 1996. Australian National Genomic Information Service. [www.angis.org.au/copyright.html](http://www.angis.org.au/copyright.html) (accessed 4.6.00).
- Van der Loo, F.J., Turner, S., Somerville, C., 1995. Expressed sequence tags from developing castor (*Ricinus communis* L.) seeds. *Plant Physiol.* 108, 1141–1150.
- Woodhead, M., Taylor, M.A., Brennan, R., McNicol, R.J., Davis, H.V., 1998. Cloning and characterization of the cDNA clones of five genes that are differentially expressed during ripening in the fruit of blackcurrant (*Ribes nigrum* L.). *J. Plant Physiol.* 153, 381–393.