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Phenylalanine Ammonia-lyase (PAL) Activity and its Relationship to Anthocyanin and Flavonoid Levels in New Zealand-grown Apple Cultivars

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Abstract. Phenylalanine ammonia-lyase (PAL) activity was measured in a range of New Zealand-grown apple (*Malus domestica* Borkh.) cultivars at three stages of fruit development. Anthocyanin and total flavonoid levels were also measured (by HPLC) in the same fruit. There was wide variation in the level of PAL activity, anthocyanin and total flavonoid levels in different apple cultivars and at different stages of development. There was no apparent correlation between average PAL activity over the three developmental stages and final anthocyanin concentration ($r = 0.34$, $P > 0.1$), but there was significant correlation between average PAL activity over the three developmental stages and the final concentration of total flavonoids ($r = 0.75$, $P < 0.02$). An inhibitor, PAL-IS, was also assayed in the same fruit but no correlation was found between PAL-IS and final anthocyanin levels ($r = -0.30$, $P > 0.1$) or total flavonoid levels ($r = 0.15$, $P > 0.1$). These results suggest that PAL activity has an influence on total flavonoid levels in the fruit but that PAL-IS does not. Anthocyanin levels are likely controlled at a point in the flavonoid pathway other than PAL or PAL-IS.

The color and consumer appeal of ripe apples results from the accumulation of anthocyanins, compounds that belong to a class of plant secondary metabolites called flavonoids. The type and amount of flavonoids in plants depends on genotype and developmental stage (Hahlbrock and Grisebach, 1979). During early development, apple skin contains high levels of flavonoids such as flavonols and proanthocyanidins, and in some cultivars anthocyanins accumulate during ripening (Lister et al., 1994). An understanding of the biosynthesis of flavonoids is important because they influence fruit quality and because of their role in developmental physiology and defense mechanisms (Cheng and Breen, 1991).

All flavonoids derive their carbon skeletons from two basic compounds: malonyl-CoA and *p*-coumaroyl-CoA from the phenylpropanoid pathway (Heller and Forkmann, 1988). The first committed step for biosynthesis of the phenylpropanoid skeleton in higher plants is the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia. This reaction is catalyzed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and is often regarded as a key step in the biosynthesis of the flavonoids. The level of PAL activity depends on the genotype and also the age and developmental stage, the organ, and even tissue type of the plant (Camm and Towers, 1973). PAL activity is affected by a number of factors including light, temperature, growth regulators, inhibitors of RNA and protein synthesis, wounding, and mineral nutrition (Jones, 1984).

Concomitant increases in the levels of PAL and flavonoid compounds have been demonstrated in many tissues (Jones, 1984). In many cases this is also coordinated with the appearance of the other enzymes associated with the flavonoid biosynthetic pathway (Camm and Towers, 1977). The synthesis of anthocyanin in several plant tissues has been associated with increased PAL activity (Tan, 1979). Although anthocyanin production is often associated with an increase in PAL activity, there are many examples of PAL activity without anthocyanin production (Camm and Towers, 1977; Jones, 1984). This is because PAL is active in the biosynthesis of a wide range of phenylpropanoid compounds, such as substituted cinnamic acids and their CoA-esters, and conjugates of these such as chlorogenic acid, coumarin, and lignin (Jones, 1984).

PAL activity in apple fruit has been studied by a variety of workers (Aoki et al., 1970; Arakawa et al., 1986; Blankenship and Unrath, 1988; Faragher, 1983; Faragher and Chalmers, 1977; Kubo et al., 1988; Tan, 1979). Large variations in the levels of enzyme activity have been reported, which probably relate to the number of factors that affect PAL. Aoki et al. (1970) demonstrated PAL activity only in the red sections of the skin and concluded that PAL activity was closely related to the formation of anthocyanin. Although there have been many studies on the effect of light on PAL activity, there are fewer reports on the developmental changes in PAL activity in apples. Wide variation in the levels of PAL activity between cultivars has been reported, but it is difficult to compare these since they were probably grown under different environmental conditions that would affect PAL. Despite these differences in PAL activity, the patterns of change during development were similar. PAL activity was highest in the immature fruit, and dropped to very low levels during growth followed by a rise during ripening, which in red cultivars coincided with an increase in anthocyanin concentration (Blankenship and Unrath, 1988; Kubo et al., 1988). However, cultivars such as 'Golden Delicious' that did not accumulate anthocyanins still showed a rise in PAL activity (Kubo et al., 1988).

Finding that a PAL inactivating system (PAL-IS) was capable of inactivating PAL in vitro led to increased study of its possible role as a regulator of PAL in the plant (Tan, 1979). PAL-IS has been demonstrated in a variety of plants including leaf disks of sunflower (Creasy, 1976), sweet potato root (Tanaka et al., 1977) and also apple skin and leaves (Tan, 1979). Tan (1980) studied the

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relationship between PAL and PAL-IS in apple and concluded that low temperature reduced the level of PAL-IS leading to an increased accumulation of PAL which, in turn, increased anthocyanin accumulation in the skin of whole apples.

The objectives of this study were to compare PAL activity in a range of apple cultivars to see if enzyme activity accounted for the differences in anthocyanin and flavonoid levels that have been observed (Lister, 1994). The levels of PAL-IS were also measured to see if they were related to the different pigment levels.

Materials and Methods

Plant material. All apple cultivars were obtained from the Lincoln Univ. Research Orchard (Christchurch, New Zealand). Flowering occurred in early November and samples of each apple cultivar were collected early, in the middle, and at the end of the growing season (middle of December, February, and April 1992–93). Nine apples showing typical coloration were collected at each sampling point from the outside of the tree. The skin was removed from the whole of the apple, to eliminate any bias due to differential light exposure between the sun and shade sides of the fruit and we carefully removed all underlying flesh. Samples were frozen in liquid N_2 and, if not used immediately, stored at –80C.

Fruit colors were as follows: 'Cox's Orange'—yellow fruit, during ripening developed orange red flush with light red stripes over 50% of fruit; 'Fuji'—bright green with slight bronzy red on 25% of fruit in both immature and mature fruit; 'Gala'—yellow fruit during ripening developed bright orange red flush and light red striping over 75% of fruit; 'Golden Delicious'—greenish yellow turned gold; 'Granny Smith'—bright green became yellowish green; 'Jonagold'—developed light orange-red flush on ripening; 'Red Delicious'—immature fruit light red which darkened throughout development, 100% red color; 'Royal Gala'—yellow fruit turned mid red with dark red striping over 100% of fruit on ripening; 'Splendour'—light yellow green fruit developed bright red flush over 80% of fruit during ripening.

Extraction of PAL activity. The skin from three apples (usually 2–5 g) was ground to a fine powder in a mortar and pestle using liquid N_2. The powder was then extracted with 20 ml 50 mM phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.0 containing 5% PVP (MW 44,000), 50 mM Na ascorbate, 18 mM mercaptoethanol and 0.1% Triton X-100. The homogenate was filtered and centrifuged at 20,000× *g* for 10 min. Ammonium sulfate was added to the supernatant (to 35% saturation) which was then centrifuged for 20 min at 20,000× *g* to remove the PVP. More ammonium sulfate was added to this supernatant to reach a final saturation of 80%. This fraction was centrifuged at 20,000× *g* for 20 min and the pellet resuspended in 1–2 ml extraction buffer (without PVP and Triton). This solution was then dialyzed overnight against the same buffer to give a partially purified extract that was used for PAL assays. All these procedures were carried out at 4C. Three enzyme preparations were prepared for each apple cultivar. Protein was estimated by the method of Bradford (1976) using BSA as a standard.

Assay of PAL activity. PAL activity in the partially purified enzyme extracts was assayed by an adaptation of the method of Zucker (1965) as reported by McCallum and Walker (1990). The assay mixture consisted of 0.06 M borate buffer (875 µl) and crude enzyme (250 µl). The reaction was initiated by the addition of L-phenylalanine (250 µl of 10 mg·ml^{-1}, to give a final concentration of 11 mM). Tubes were incubated at 30C for 30 min (or one h when activity was low) and the reaction stopped by addition of 35% w/v trifluoroacetic acid (125 µl). Tubes were then centrifuged for 5 min at 5000× *g* to pellet the denatured protein. PAL activity was determined from the yield of cinnamic acid, estimated by measuring A_{290} of the supernatant in 10 mM quartz cuvettes. Triplicate assays were performed for each extract, both with and without substrate to compensate for increases in absorbance in the absence of added phenylalanine.

Extraction and assay of PAL-IS. Extraction and assay of PAL-IS in apple skin was carried out by the method of Tan (1979 and 1980). Skin samples were ground to a fine powder with liquid N_2, 100 mg PVPP and 5 ml 0.1 M MES buffer (pH 5.5) added, and the homogenate centrifuged at 2,000× *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 20,000× *g* for 15 min. Sodium borate was added to the supernatant until the pH reached 8.8 followed by centrifugation at 1,000× *g* for 10 min to remove excess Na borate. This final supernatant, containing no PAL activity (as determined by the method above), was used immediately for assay of PAL-IS. PAL-IS was assayed by adding a known amount of PAL (from a Camellia flower extract) to 50 µl of the supernatant plus 40 µl of 0.1 M Na borate buffer pH 8.8. The tubes were incubated at 30C and samples taken at time 0 and 15 min. The control consisted of the PAL extract from Camellia flowers and buffer only. A milliunit (mU) of PAL-IS was defined as a 1% loss in PAL activity per minute.

Quantification of flavonoids. A small sample of apple fruit skin (0.5 g) was taken from each enzyme sample and ground to a fine powder in liquid N_2 and then extracted with 10 ml 15% acetic acid in methanol. The residue was re-extracted at least twice, to remove all the color, and the combined extracts centrifuged at 5000× *g* for 10 min. The extract was concentrated to almost dryness at 40C and taken up in 0.5–1.0 ml 15% (v/v) acetic acid in methanol and centrifuged at 10,000× *g* for 5 min before injecting directly onto a high-performance liquid chromatograph (HPLC). The HPLC system comprised a solvent delivery system with an automatic sample injector and a variable wavelength UV detector (models 600, WISP 712, and 490; Waters, Milford, Mass.) The RP-18 column (220 × 4.6 mM) was fitted with a guard column (15 × 3.2 mM) (Aquapore RP-18; Applied Biosystems, Foster City, Calif.). Chromatographic traces were recorded using the Waters/Dynamic solutions maxima program. Samples (5 µl) were injected onto the column which was maintained at 30C and eluted with a flow rate of 1.0 ml·min^{-1}. A linear 20 min solvent gradient from 0% to 20% acetonitrile in 10% (v/v) acetic acid in water with a 10 min hold at the final concentration was used. The column was returned to initial solvent composition over 1 min and re-equilibrated for 10 min before the next analysis. Eluted components were monitored at 280 nm for proanthocyanidins, 350 nm for flavonols and 530 nm for anthocyanins. The individual compounds were identified and quantified by comparison with standard solutions of known concentration as detailed by Lister et al. (1994). Total flavonoid concentration was calculated from all proanthocyanidins, flavonols and anthocyanins.

Statistical analysis. Enzyme activity was expressed in pKat/g fresh weight so that activity could be compared directly to changes in total flavonoid and anthocyanin concentrations. Correlation coefficients were calculated for PAL and PAL-IS activity versus anthocyanin and flavonoid concentrations. The enzyme assay data were paired with the appropriate anthocyanin and total flavonoid data for analysis. Standard ANOVA established differences among cultivars over time and also differences between cultivars at a given stage. Least significant differences (LSD) are given at the 5% significance level. Variation between assays on the same extract were less than 1% and only the average of these was used for calculation of the LSD 5%.

The theoretical amount of flavonoids that could be formed from

the PAL activity was calculated by determining how much cinnamic acid was formed and then converting this to an amount of flavonoid assuming that one molecule of cinnamic acid produced one flavonol or anthocyanin molecule, and two molecules of cinnamic acid were required for each proanthocyanin molecule.

Results

Analysis of PAL activity There was a wide variation in the level of PAL activity in different apple cultivars (although not as great as previously reported in the literature) and there were significant changes in activity during development (Table 1). In all cultivars, PAL activity was highest in the immature fruit, dropped to low levels during development, and then rose on ripening. Final levels of activity were, however, lower than the initial levels. The same pattern of change in PAL activity was observed when expressed in terms of pKat/mg protein. The observed decrease in PAL activity did not appear to be caused by inhibition of PAL since activities were additive when extracts from immature fruit were mixed with those from the middle stage of development or from mature fruit.

Levels of PAL-IS. There was variation in PAL-IS levels between the different apple cultivars (Table 2). The highest levels were present in the immature fruit dropping in the middle of the season and remaining relatively constant in most cultivars. There were no significant changes in PAL-IS during ripening when the anthocyanins increased and no correlation between PAL-IS and final anthocyanin levels ($r = –0.30$, $P > 0.1$) or PAL-IS and total flavonoid levels ($r = 0.15$, $P > 0.1$).

Table 1. PAL activity in New Zealand-grown apple cultivars at different developmental stages (flowering occurred in early November and samples were taken in the middle of December, February, and April).

	PAL levels (pKat/g fresh wt)			
Apple cultivar	Early	Mid	Late	LSD 5%
Cox's Orange	14.41	1.84	3.46	0.57
Fuji	30.23	2.49	3.16	1.63
Gala	25.08	1.37	14.47	1.26
Golden Delicious	9.01	0.00	5.69	0.58
Granny Smith	10.26	0.00	2.22	0.50
Jonagold	9.23	0.97	1.68	0.39
Red Delicious	14.41	6.43	10.12	0.77
Royal Gala	18.06	1.33	10.46	0.99
Splendour	38.57	2.20	7.32	1.61
LSD 5%	1.44	0.21	0.46	

Changes in flavonoid levels. Wide variation in the levels of both total flavonoids and anthocyanins in the different apple cultivars was observed (Table 3). Flavonoid levels showed a similar, although less pronounced, pattern of change to PAL activity with the highest levels present in the immature fruit, apart from 'Splendour'. On ripening, flavonoid levels in 'Splendour' rose to a higher level than in the immature fruit. Anthocyanins were generally absent in the immature fruit and in the middle of the season, increasing only at the end of the season. There were two exceptions, 'Red Delicious' and 'Royal Gala', where there was a steady accumulation of anthocyanins throughout development.

Correlation of PAL activity with flavonoid levels. At all stages there was no correlation between PAL activity and anthocyanin level ($r = 0.01$, $P > 0.1$) but there was a significant correlation between PAL activity and total flavonoids ($r = 0.525$, $P < 0.01$). Similarly, there was no correlation between average PAL activity for a cultivar during development and the final anthocyanin concentration ($r = 0.34$, $P > 0.1$), but there was a significant correlation between average PAL activity and final concentration of total flavonoids ($r = 0.75$, $P < 0.02$).

Table 2. PAL-IS activity in New Zealand-grown apple cultivars at different developmental stages (flowering occurred in early November and samples were taken in the middle of December, February, and April).

	PAL-IS levels (mU/g fresh wt)			
Apple cultivar	Early	Mid	Late	$LSD_{0.05}$
Cox's Orange	21	12	11	2.3
Fuji	27	12	14	3.1
Gala	19	12	13	2.8
Golden Delicious	21	8	5	2.8
Granny Smith	24	13	16	2.3
Jonagold	31	15	17	2.8
Red Delicious	12	5	7	2.5
Royal Gala	25	14	16	2.8
Splendour	28	17	19	3.5
LSD 5%	2.8	2.2	2.1	

At all stages of development the level of PAL activity would have allowed the production of much higher levels of flavonoids than actually accumulated. Where PAL activity was absent there was no accumulation of flavonoids, with the concentration of flavonoids actually dropping.

Discussion

PAL activity Since all the apples used in this study were obtained from the same location the variation can be attributed to genetic differences and not environmental influences. The levels of PAL activity measured in the skin of New Zealand-grown apples were similar to those reported elsewhere for intact fruit (Arakawa et al., 1986; Faragher, 1983; Faragher and Brohier, 1984; Kubo et al., 1988). However, activities were generally lower than those reported for skin disks or light induced tissues where PAL activities of up to 874 pKat/g fresh weight have been reported (Arakawa et al., 1986; Faragher and Chalmers, 1977). This is not surprising because PAL is induced by a number of factors such as wounding (which occurs when skin disks are cut) and light, leading to more rapid accumulation of flavonoids than in normal tissue. Blankenship and Unrath (1988) reported much lower levels of PAL activity during apple fruit development, more that 10-fold lower than reported in this study for the same cultivar. This suggests that either flavonoid and anthocyanin levels were extremely low, or they may have lost some activity since their levels of PAL activity would not be sufficient to allow for even 70% of normal pigment production. When PAL activity was absent or very low there was no synthesis of the flavonoids. Thus, it seems that under normal conditions there are no pools of intermediates (available after PAL) for use in flavonoid synthesis and PAL activity is required for flavonoid accumulation.

Results obtained in the present study are similar to those obtained by Kubo et al. (1988) regarding changes in PAL levels during the development of intact apple fruit. These workers also reported that, in a non-red apple ('Golden Delicious'), PAL activity increased on ripening as was observed in this study for both 'Golden Delicious' and 'Granny Smith'. Similar patterns of change in enzyme activity have been observed in developing strawberry (*Fragaria ×ananassa* Duch.) fruit where there were two peaks in PAL activity, one in immature green fruit, coinciding

Table 3. Anthocyanin and total flavonoid levels in New Zealand-grown apple cultivars at different developmental stages (flowering occurred in early November and samples were taken in the middle of December, February, and April).

	Anthocyanin ($mg \cdot g^{-1}$ fresh wt)				Total flavonoid ($mg \cdot g^{-1}$ fresh wt)			
Apple cultivar	Early	Mid	Late	$LSD_{0.05}$	Early	Mid	Late	LSD 5%
Cox's Orange	0.07	0.00	0.32	0.03	9.43	7.04	8.27	0.61
Fuji	0.08	0.00	0.09	0.02	13.03	9.97	10.56	0.79
Gala	0.00	0.00	0.49	0.04	8.64	6.15	7.46	0.62
Golden Delicious	0.00	0.00	0.00	0.00	3.76	2.86	3.14	0.24
Granny Smith	0.00	0.00	0.00	0.00	8.09	5.15	5.69	0.47
Jonagold	0.00	0.00	0.37	0.03	7.34	4.59	5.42	0.49
Red Delicious	0.64	1.48	2.59	0.13	9.09	8.13	8.92	0.63
Royal Gala	0.14	0.38	2.31	0.12	8.46	6.04	8.14	0.62
Splendour	0.06	0.03	0.97	0.13	8.76	7.84	9.56	0.68
LSD 5%	0.03	0.04	0.10		0.53	0.48	0.50	

with an increase in flavonoids other than anthocyanins, and a second peak concomitant with the rise in anthocyanin (Cheng and Breen, 1991). The rise in activity during ripening was due to *de novo* enzyme synthesis (Given et al., 1988).

PAL-IS. It appears that PAL-IS was not involved in the regulation of anthocyanin biosynthesis by control of PAL activity since there was no drop in level on ripening when the anthocyanins increased. Nor did it appear to be a controlling factor in the biosynthesis of other flavonoids since the only drop in PAL-IS occurred early in development when flavonoid concentrations were also dropping but when there was still appreciable PAL activity (Lister, 1994).

The regulation of in vivo PAL activity by PAL-IS is questionable (Blankenship and Unrath, 1988). PAL-IS may have no effect in intact tissue, for example, if PAL and PAL-IS are located separately in the plant cell. Studies by Blankenship and Unrath (1988) also suggested that PAL-IS was not closely involved with the regulation of anthocyanin synthesis in apple skin since similar PAL-IS levels were observed in 'Red Delicious' and 'Golden Delicious' apples that had markedly different anthocyanin concentrations. Tan (1979) has shown that PAL-IS may regulate the activity of PAL in the skin of whole apples although these studies involved wounding and light treatments and no reference was made to the effects during development. Thus, PAL-IS may be involved in controlling PAL levels in stress responses but does not appear to be the ultimate controlling factor in influencing the pattern of change in flavonoids or anthocyanins during development.

Relationship between PAL activity and flavonoid levels. It has often been postulated that PAL is the main limiting factor in the biosynthesis of flavonoids, cinnamic acids, and other phenylpropanoids (Camm and Towers, 1973; Zucker, 1972). In contrast, Margna (1977) suggested that the level of phenylalanine may be the rate-limiting factor. Feedback inhibition and enzyme repression may also be more efficient ways to turn off or reduce an enzyme activity. The sequestration of important substrates, such as phenylalanine, from active metabolic sites of a sequence could be another mechanism. Conversion of phenylalanine to 'expensive' secondary pathways without regulation does not seem likely since it would be inefficient for the plant (Stafford, 1990).

PAL activity in apples at all stages was much higher than expected for the amounts of flavonoids synthesized but there are many cases where the potential capacity of available PAL to deaminate phenylalanine was much higher than would be expected from the amounts of phenolics synthesized (Ahmed and Swain, 1970; Laanest and Margna, 1972; Maier and Hasegawa, 1970; Swain and Williams, 1970). In strawberry there was a parallel rise in PAL activity and anthocyanin level, although the apparent excess of PAL indicated this may not have been the rate-limiting enzyme (Cheng and Breen, 1991). However, it was postulated that PAL may be a key factor controlling the channelling of phenylalanine into phenolic synthesis and hence flavonoid biosynthesis. One explanation was that differences between in vivo and in vitro PAL activity may have been responsible. The measured activity may not be the true rate in vivo since factors such as inhibitors and the pH may lower the actual activity. There are a number of other factors to take into consideration when examining PAL activity in relation to flavonoid accumulation rates. Metabolic turnover of flavonoids is another possibility, although these compounds have been shown to be relatively stable and in most cases catabolism has been reported to be relatively low, if occurring at all (Dangelmayr et al., 1983; Zenner and Bopp, 1987).

There was no obvious correlation between PAL activity and anthocyanin level, which is not unexpected, since these represent only a small fraction of the flavonoids. However, there was a correlation between average PAL activity and total flavonoid concentrations. Since PAL is also involved in the biosynthesis of a range of other secondary products, and only part of its activity may be available for flavonoid biosynthesis, a better correlation may have been observed between PAL activity and total phenolics. PAL is involved in the synthesis of chlorogenic acid which is present in high concentrations in apple skins (Burda et al., 1990; Coseteng and Lee, 1987; Walker, 1962). Evidence is increasing that different isoenzymes may be involved in the separate pathways of phenylpropanoid metabolism (Heller and Forkmann, 1988) and different PAL isoenzymes have been reported from *Phaseolus vulgaris* cell cultures (Bolwell et al., 1985). In molecular genetic studies, different genomic clones have been detected for PAL (Heller and Forkmann, 1988). Thus, in apples there may be a specific isoenzyme for the flavonoid pathway and more specifically for pathways to individual flavonoid groups, such as the anthocyanins, and activity of these may be limiting.

Despite these considerations it is possible that changes in PAL activity do not actually influence the rate of accumulation of flavonoids and other polyphenols. It is probable that the deaminating capacity of the plant is always sufficient to consume all the phenylalanine surplus to protein synthesis (Margna, 1977). Therefore, if there is a shift in the accumulation of flavonoids it may not be the result of the controlling action of PAL but may be due to a change in the supply of the primary substrate, i.e. phenylalanine, to the enzyme. Without further investigation it is not possible to determine whether the level of phenylalanine is the rate-limiting step in the biosynthesis of flavonoids in apples. Other enzymes in

the flavonoid biosynthetic pathway may also be rate-limiting.

Apples commonly have high levels of flavonoids during development and some cultivars accumulate anthocyanins only near maturity; these changes are matched by changes in activity of PAL. Other factors, such as inhibitors like PAL-IS, do not appear to be responsible for the changes occurring in flavonoid levels during development or the variation between cultivars but may still have an influence on the actual concentration of the flavonoids. PAL activity is a prerequisite for anthocyanin accumulation but it does not guarantee that synthesis will occur, since green fruit, like 'Granny Smith', also have appreciable PAL levels. However, PAL is essential for accumulation of the other flavonoids which do increase on ripening in all cultivars studied.

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