Changes in L-Phenylalanine Ammonia-lyase Activity and Anthocyanin Synthesis during Berry Ripening of Three Grape Cultivars

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Summary
The changes in L-phenylalanine ammonia-lyase (PAL) activity were investigated during berry ripening in relation to the coloration of 3 grape cultivars: 'Muscat of Alexandria' (white grapes), 'Kyoho' (purple grapes) and 'Super Hamburg' (black grapes).

In all cultivars, higher PAL activity in the skin was observed at the earlier stages of berry development, followed by a rapid decline toward veraison to a considerably lower level.

In 'Kyoho' and 'Super Hamburg', PAL activities gradually increased again with the beginning of berry coloration. 'Super Hamburg' had more anthocyanin content and higher PAL activity than 'Kyoho'.

The sugar content in the skin rose rapidly in the ripening period in all cultivars, preceding the increases in both PAL activity and anthocyanin content in 'Kyoho' and 'Super Hamburg' grapes.

Free-abscisic acid (ABA), also, rapidly accumulated in the early stages of ripening in each cultivar and maintained a considerably higher level. However, there was no much difference in the maximum ABA content among 3 cultivars.

Introduction
Skin color of grape berry varies widely with cultivars, being classified into white, red and black. Even within a cultivar, color development is profoundly affected by various cultural and environmental factors(11, 14, 20).

Anthocyanins, the major pigments of the red and black cultivars, are the flavonoid compounds derived from shikimic acid pathway. L-phenylalanine ammonia-lyase (PAL) (EC. 4. 3. 1. 5.) catalyzing the conversion of L-phenylalanine to trans-cinnamic acid in this pathway has been considered to play an important role as a key enzyme in the biosynthesis of phenolics(2,12).

This paper deals with the changes in PAL activity during berry ripening in relation to the coloration of 3 grape cultivars; 'Muscat of Alexandria' (white grapes), 'Kyoho' (purple grapes) and 'Super Hamburg' (black grapes). In addition, the levels of sugar and abscisic acid (ABA) in the berry skin which are assumed to be involved in the anthocyanin formation (15,16) were compared among these cultivars.

Materials and Methods
Mature vines of cvs. Muscat of Alexandria (Vitis vinifera L.), Kyoho (V. vinifera L. x V. labruscana Bailey.) and Super Hamburg (V. labruscana Bailey. x V. vinifera L.) growing in the experimental farm of Kyoto University, Kyoto, were used.

Fifty berries were collected from each cultivar at weekly intervals starting on June 11 until August 24, 1982. Ten berries were immediately used for PAL and anthocyanin determinations and the rest were stored in a freezer for further analyses.

PAL extraction and assay: PAL was extracted by the method based on that of Faragher and Chalmers(6). Two g of berry samples were ground in a mortar and pestle with 10 ml of 0.1 M borate buffer, pH 8.8,
containing 2-mercaptoethanol (20 mM) and Polyclar AT (0.25 g per g of sample). A further 20 ml of the buffer was added to the homogenate which was then centrifuged at 15,000 g for 30 min. To the 20 ml of the supernatant, ammonium sulphate was added to 70% saturation to precipitate protein and the mixture centrifuged at 15,000 g for 15 min. The precipitate was resuspended in 5 ml of the borate buffer and used as the crude enzyme solution. All procedure was carried out at 4°C.

The activity of PAL was assayed by measuring the rate of formation of trans-cinnamic acid as increase in absorbance at 268 nm according to the method of Koukol and Conn (12). The reaction mixture contained 1 ml of 25 µM L-phenylalanine and 2 ml of the enzyme preparation and was incubated at 37°C for 3 h with shaking. In the reference mixture, L-phenylalanine solution was replaced by distilled water. The reaction was stopped by the addition of 0.1 ml of 6 N-HCl. After the extraction with 5 ml of ethyl ether, the ethereal phase was evaporated to dryness. The residue was dissolved in 4 ml of 0.05 N-NaOH and optical density at 268 nm was measured with a spectrophotometer. PAL activity was expressed as pmoles of t-cinnamic acid formed per g of fresh weight per h.

The absorption spectrum of the reaction product was identical with that of authentic t-cinnamic acid (Fig.1). The reaction product was also identified on TLC by co-chromatography of authentic t-cinnamic acid using 2 solvent systems indicated in Table 1.

**Anthocyanin concentration**: Anthocyanin was extracted by immersing 2 disks of skin, 8 mm in diameter, taken from each berry in 10 ml of 1% HCl-methanol at 4°C in the dark for 12 h. The concentration was expressed as O.D. at 530 nm.

**Total phenol content**: Total phenols in the skin were determined by Folin-Denis method (18) and expressed as mg D-catechin per g of fresh weight.

**Total soluble solids and total acidity**: Percent soluble solids of the juice were determined with a hand refractometer. Total acidity was measured by titrating with 0.05 N-NaOH, using phenolphthalein as an indicator. Results were expressed as percent tartaric acid in the juice.

**Total sugar content**: One g of skin sample was extracted in 80% ethanol. After an appropriate dilution of the extract, total sugars were measured using anthrone reagent.

**ABA content**: Free-ABA contents in the skin were determined by GLC after processing methanol extracts according to the procedure shown in Fig.2. The condition of GLC (ECD) was described previously (20). The methyl ester of ABA in the extracts was identified tentatively by both comparison

**Table 1. Rf values of t-cinnamic acid and reaction product on TLC*.**

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Authentic t-cinnamic acid</th>
<th>Reaction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene : Methyl formate : Formic acid</td>
<td>0.51</td>
<td>0.50</td>
</tr>
<tr>
<td>Benzene : Methanol : Acetic acid</td>
<td>0.72</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Plate : Silica gel 60 F254
Changes in *L*-phenylalanine ammonia-lyase activity and anthocyanin

Homogenize materials in 80% methanol
Extract for 48 h at 0°C in the dark
Evaporate the extract to aqueous phase
Adjust pH to 3.0
Add 200 mg of PVP, mix and filter after 5 min
Partition against ethyl acetate
Organic phase
Partition against carbonate buffer, pH 10.0
Adjust pH to 3.0
Partition against ethyl acetate
Organic phase: Free ABA
Fig. 2. Procedure of processing the extract for GLC.

of retention times of the authentic *cis*, *trans*-ABA methyl ester and its conversion to *trans*, *trans*-ABA methyl ester with the UV irradiation (13).

Results

Anthocyanin development: In 'Kyoho' and 'Super Hamburg', anthocyanin development in the skin proceeded rapidly from July 23, 1–2 weeks after the onset of veraison, lasting until August 24. In the final sample on August 24, anthocyanin level was threefold higher in 'Super Hamburg' than in 'Kyoho'. There was no appearance of anthocyanin pigment in the skin of 'Muscat of Alexandria' (Fig. 3).

Total phenol content: In 'Kyoho' and 'Super Hamburg', higher phenol contents were observed in the early stages of berry development, followed by the gradual declines until 1–2 weeks after veraison. The content, however, increased again toward the final harvest and was much higher in 'Super Hamburg' than in 'Kyoho'.

In 'Muscat of Alexandria', relatively higher level of phenols in the skin was found in the early stages of berry development, while there was no increase in the ripening period. 'Muscat of Alexandria' consistently had much less phenol contents in the skin than 'Kyoho' and 'Super Hamburg' (Fig. 4).

PAL activity: Using 'Kyoho' grapes, PAL activities in different parts of a berry were

examined at various growing stages (Fig. 5). PAL activity was highest in the skin on June 18, then declined rapidly toward veraison to an undetectable level. The activity, however, increased again in the ripening stage.

In the seed, a higher activity was found
from June 25 to July 2, followed by a rapid decline to a considerably lower level. The activity remained at a low level during the berry ripening. The pulp tissue, however, had no detectable PAL activity throughout berry development and ripening.

PAL activity in the skin was compared among 3 cultivars (Fig. 6). During berry development, a similar pattern of PAL activity was noted in the skins of 'Super Hamburg' and 'Muscat of Alexandria' as with 'Kyoho'. In the ripening stage, however, 'Super Hamburg' exhibited much higher PAL activity than 'Kyoho' and no activity was detectable with 'Muscat of Alexandria'. These changes in PAL activity in the ripening stage took place quite parallel with the anthocyanin development.

**Total soluble solids content and total acidity:** Total soluble solids content in the juice rose gradually in the ripening period of each cultivar, lasting until the final harvest (Fig. 7). The gradual increase in total acidity proceeded toward veraison of each cultivar, followed by a sharp decline thereafter (Fig. 7).

**Sugar content:** The sugar content in the skin as well as total soluble solids content rose rapidly in the ripening period and the sugar accumulation preceded the increases of PAL activity and anthocyanin content in 'Kyoho' and 'Super Hamburg' (Fig. 8).

**ABA content:** Free-ABA, also, rapidly
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accumulated in the skin after veraison in 'Kyoho' and 'Super Hamburg'. In 'Muscat of Alexandria', ABA gradually accumulated well before veraison, but it rapidly increased after the onset of veraison. There were no great differences in the maximum ABA content among 3 cultivars (Fig. 9).

**Discussion**

In all cultivars used for this study, PAL activity was high in the early stages of berry development, but thereafter it rapidly declined toward veraison. Such higher activity in the skin of young berry is probably associated with the high level of phenols in the tissue. These results agree with the observations in several other species (1,8).

In 'Kyoho' and 'Super Hamburg', however, PAL activity gradually increased again in the ripening period parallel with the beginning of coloration. 'Super Hamburg' accumulated more anthocyanin and phenols in the skin and higher PAL activity than 'Kyoho' in this period. Such a close relationship between PAL activity and anthocyanin accumulation has been observed with other fruit species; e.g. strawberries(7), peaches(1) and apples(6,19).

In the seeds of 'Kyoho' berries, markedly higher levels of PAL activity were observed from June 25 to July 2. This seems to be related to lignification of the seed proceeding in that period. In this respect, Aoki et al. (1) reported that the higher PAL activity was detected in the peach endocarp tissue just before the beginning of lignification.

In the studies with 'Shiraz' and 'Cabernet Sauvignon' grapes, Pirie and Mullins(14) reported that the closest correlations of sugar vs. anthocyanin and sugar vs. total phenols in the skin were found in the first 5 weeks after veraison, and that there was, however, a poor correlation between soluble solids and polyphenolic substances in the skin at all stages of ripening. Furthermore, they postulated that sugar flux to berries, especially to skin tissue, is one of the main factors which govern the rate of phenol accumulation (17). In fact, in culturing leaf disks of strawberry or skin disks of grape berry in vitro, addition of sugars to the medium induced PAL activity or anthocyanin synthesis (4,5,16).

In this experiment, total sugar accumulation in the skin as well as the increase of the total soluble solids in the juice preceded the rises of PAL activity and anthocyanin content in the ripening period. In the earlier stages of berry development, however, higher PAL activity was noted in the skin of 3 cultivars in spite of lower sugar levels. This suggests that higher sugar concentration is not necessarily a prerequisite for the
induction of PAL activity, at least in the early period of berry development.

ABA is now known to be a possible trigger of ripening process in grape berries (3, 9). Anthocyanin synthesis is markedly stimulated by ABA application to excised berries or leaf disks of grapes (15). Previously, we reported that exogenous ABA could enhance anthocyanin accumulation in 'Kyoho' berries on the vine even with total defoliation (10). In this experiment, there were great differences in PAL activity and anthocyanin content between 'Kyoho' and 'Super Hamburg' grapes during ripening period, but no difference was present in the maximum ABA content.

In conclusion, PAL activity in the skin is assumed to be directly involved in the phenolic synthesis of grape berries during ripening period, showing a close relationship to the anthocyanin accumulation in purple and black grape cultivars. It will be needed to investigate further how PAL activity is subject to modification under the various environmental conditions affecting the anthocyanin synthesis.

Literature Cited

3 品種のブドウ果粒の成熟期における L-フェニルアラニンアンモニアリアーゼ活性及びアントシアニン生成の変化

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摘要

3 品種のブドウ果粒（‘マスカット・オブ・アレキサンドリア’, ‘巨峰’及び‘スーパー・ハンブルグ’）について、成熟に伴う L-フェニルアラニンアンモニアリアーゼ（PAL）活性の消長とアントシアニン生成の関係を調査した。

全品種において、果粒発育の初期に高い PAL 活性が認められたが、その後ペレゾーン期まで急速に低下した。‘巨峰’及び‘スーパー・ハンブルグ’では、果粒の着色開始とともに再び果皮中の PAL 活性が増大した。

‘スーパー・ハンブルグ’では、‘巨峰’と比較して、アントシアニン蓄積量が多く、PAL 活性も高かった。これに対して、‘マスカット・オブ・アレキサンドリア’では、成熟期には、果皮中に PAL 活性は認められなかった。

一方、全品種において、果皮中の糖含量及び果汁中の可溶性固形物含量は成熟期に急速に増加した。また、‘巨峰’及び‘スーパー・ハンブルグ’では、これらの増加開始は、アントシアニン含量や PAL 活性の上昇開始に先立って起こった。

果皮中の遊離型アブジン酸もまた、各々の品種の成熟期に急速に増加したが、最大蓄積量には品種間での差異は認められなかった。