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Changes in β-Cyanoalanine Synthase Activity during Stratification of Japanese Apricot Seeds

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Summary

Changes in β-cyanoalanine synthase (CAS) activity and amygdalin content of Japanese apricot (*Prunus mume* Sieb. et Zucc.) seeds were monitored during stratification at different temperatures. When seeds were kept under moist conditions at 5, 10, 18, 24 and 30°C, β-CAS activity increased at 5 and 10°C from the start to 20 and 40 days, respectively, followed by slight declines. The lower temperature was more efficient in the elevated activity. However, β-CAS activity was gradually decreased at temperatures higher than 18°C. The higher the temperatures, the greater the activity declined. The content of amygdalin, major seed cyanogenic glycoside of Japanese apricot, seemed to be slightly declined at 5 and 10°C for a long stratification, but tended to increase slightly and then decreased at 24 and 30°C. Seeds germinated only at 5°C. Furthermore, whenever seeds kept at 24°C were transferred to 5°C, the β-CAS activity increased and seeds germinated. Upon the transfer, amygdalin content seemed to be lowered compared with the control. Benzylaminopurine (BA) enhanced both seed germination and β-CAS activity. Therefore, the β-CAS activity is associated with cyanide metabolism in dormancy breaking of Japanese apricot seeds.

Introduction

Prunus species are cyanogenic and contain cyanogenic glycosides in their tissues[1,9]. Prunasin is detected in whole plant parts but amygdalin is localized in maturing seeds[9,10,13]. Prunasin in the root residues in peach replant sites causes soil sickness, because its hydrolysates and derivatives such as hydrogen cyanide, benzaldehyde and benzoic acid repress the growth of peach seedlings[9]. β-CAS is the enzyme that catalyzes the formation of β-cyanoalanine from hydrogen cyanide and L-cysteine[2,3,4,6]. Thus it is considered that it plays a role for detoxification of free cyanide in the tissues. β-CAS activity increases concomitantly with the increase of amygdalin content in Japanese plum seeds at late developmental stages[10]. We found that β-CAS activity in peach flower buds remains low during dormant period but gradually increases with the release of dormancy[11]. Prunasin content in peach flower buds increased concomitanly with the increasing β

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-CAS activity[11]. It is well known that most fruit seeds requires wet chilling conditions (stratification) for dormancy breaking[15]. We investigated how β-CAS activity and amygdalin, major seed cyanogenic glycoside, change in Japanese apricot seeds during stratification.

Materials and Methods

Seeds

Japanese apricot (*Prunus mume* Sieb. et Zucc.) fruit were obtained in late June from a local market and pits were taken out from the fruit. The flesh was thoroughly removed from the pits in order to prevent pathogenic infection during stratificaiton. The pits were mixed with sand and vermiculite (1 : 1) and kept at 5, 10, 18, 24 and 30°C. Pits stored at different temperatures were taken out at intervals and seeds were removed from the pits for analysis. In another experiment, Japanese apricot pits including seeds obtained from a nursery store in Nagano Prefecture in late October. This means that the pits had passed several months after harvest under ambient temperature fluctuations before the stratification began. The pits were similarly mixed with sand and vermiculite (1 : 1) and stored at 24°C and 5°C. Part of the pits stored at 24°C were transferred to 5°C 60 days and 138 days after the start of stratification.

BA treatment

Pits inluding seeds that were stored at 24°C for about 8 months were used. Seeds were taken out from the pits and treated with 10% NaOCl solution (effective chloride, about 1%) for 15 min, followed by several rinses in sterile water. Seeds were soaked in 200 ppm Bezylaminopurine (BA) solution (pH 5.5) for 24 hours at 25°C, whereas control seeds similary soaked in distilled water. The seeds were placed on moist filter paper in petri dishes and kept at 25°C. β-CAS activity and germination were monitored at intervals.

Assay for β-CAS activity

A seed was weighed and homogenized with a cold mortar and pestle which had been previously kept in a freezer. The homogenate was taken in 12.5 ml cold Tris-HCl buffer (50 mM, pH 8.5) and 300 mg insoluble PVP was added. After centrifugation at 12,000 x g at 4°C for 10 min, the resultant supernatant was used for the enzyme assay. NaCN and L-cysteine were dissolved in 100 mM Tris-HCl buffer (pH 8.5) to a final concentration of 50 and 100 mM, respectively. To the crude enzyme extract (1.0 ml) was added 0.5 ml of the buffered NaCN, followed immediately by 0.5 ml of the buffered L-cysteine solution. After one-hour incubation at 30°C, the reaction was stopped by adding 0.5 ml of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 0.5 ml of 0.03 M ferric chloride in 0.2 N HCl. The samples were then centrifuged at 1,050 x g for 5 min to remove precipitated proteins and the optical density at 650 nm was recorded with a spectrophotometer. Identical assays lacking substrates and containing boiled enzyme were used as control. Sodium sulfate was used as the standard reference. The enzyme assay was triplicated and the result was expressed as the mean ± SE.

Analysis of amygdalin

Freeze-dried Japanese apricot seeds were ground in a mill and a 50 mg sample was taken in a 3-ml vial, to which 1 ml pyridine containing 1,3,5-triphenylbenzene (1 mg/ml) as an internal standard was added. The vial was subjected to an ultrasonic generator for several times during a 48-hour extraction period at room temperature. A 20 μl aliquot of the supernatant of extract was taken in a 1-ml reacti-vial and dried in air. To the vial, 20 μl pyridine, 20 μl hexamethyldisilazane and 10 μl trimethylchlorosilane were added successively and the sealed vial heated at 60°C for 30 min. A 2.0 μl of the reaction mixture was injected into a Hitachi 063 gas chromatograph equipped with FID. Gas chromatographic conditions were as follows : column, 2 m x 3 mm ϕ glass column packed with 1.5 % SE-30 coated on Chromosorb WAW DMCS (80-100 mesh) ; oven temperature, 280°C ; carrier gas, N_2 ; flow rate, 11.5 ml/min.

Results and Discussion

It is well known that seeds of many fruit tree species require moist chilling conditions for dormancy breaking. In this experiment, pits including seeds were mixed with sand and vermiculite (1 : 1) and stored at different temperature regimes. Germination occurred only at 5°C 100 days after the start of stratification. We can often see seed germination under continuous low temperatures. Effect of temperatures on β-CAS activity is shown in Fig. 1. Temperatures higher than 18°C decreased the enzyme activity, but 5°C and 10°C enhanced it up to 20 and 40 days of treatment, respectively, followed by slight declines. The lower the temperature, the greater the elevated activity. At 30°C amygdalin content in seeds gradually increased and peaked aftor 40 days followed by a slight decline ; at 24°C a similar but rather delayed increase was observed with a peak after 60 days ; at 18°C a slight and steady gradual increase was noted. On the other hand, at 5°C and 10°C only slight decrease in amygdalin content was found after 100 days

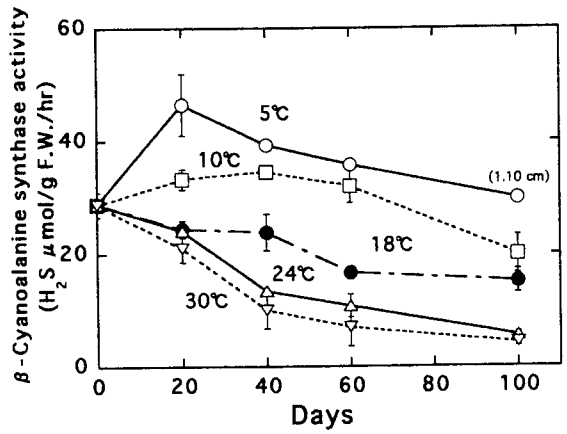

Fig. 1 Effect of temperatures on β-CAS activity in Japanese apricot seeds during stratification. (The stratification began soon after fruit were harvested. The numerical in the parenthesis in the figure is the mean root length of germinated seeds.)

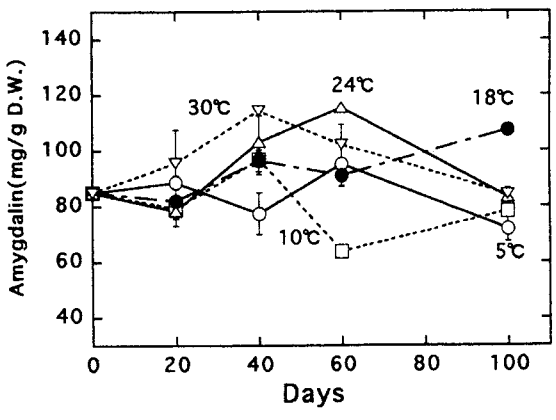

Fig. 2 Effect of temperatures on amygdalin content in Japanese apricot seeds during stratification. (See the legend in Fig. 1)

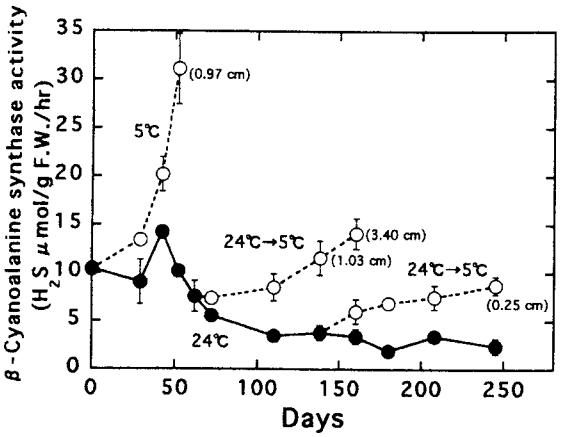

Fig. 3 Effect of temperature changes from 24°C to 5°C on β-CAS activity in Japanese apricot seeds during stratification. (The pits including seeds were used after exposed under ambient temperature fluctuations about four months after fruit were harvested. The numericals in the parentheses in the figure are the mean root length of germinated seeds.)

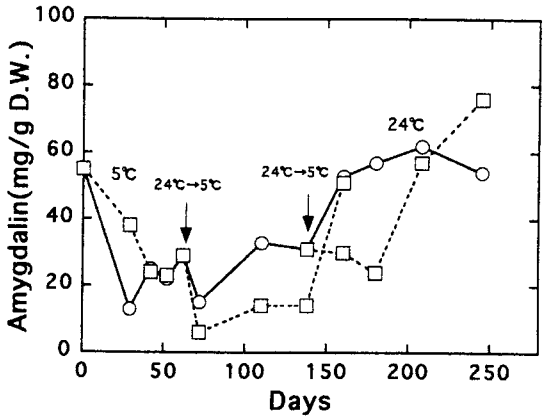

Fig. 4 Effect of temperature changes from 24°C to 5°C on amygdalin content in Japanese apricot seeds during stratification. (See the legend in Fig. 3)

(Fig. 2). Amygdalin is not detected in young *Prunus* seeds but appears in late developmental stages and the content increases as the seeds mature[9,10,13]. Because Japanese apricot seeds in the above experiment were employed soon after harvest, it seems that the ability to biosynthesize amygdalin still functioned and was favored by high stratification temperatures.

In the second experiment, the pits including seeds were obtained in late October from a nursery store in Nagano Prefecture. Therefore several months had passed under ambient temperature fluctuations before the stratification started. It is considered that the β-CAS activity had been declined because seeds had been exposed under ambient temperture fluctuations and relatively dried (not wet) conditions. In fact at the start of stratification β-CAS activity was lower than that of the first experiment, in which rather freshly harvested seeds were employed. But the exact comparison is impossible due to cultivar difference. However we confirmed this point by using peach seeds (data not shown). Whenever seeds kept at 24°C were transferred to 5°C, β-CAS activity began to increase and the seeds eventually germinated (Fig. 3). On the other hand, amygdalin content seemed to be lowered compared with the control, when seeds were transferred to 5°C after 60 days and 138 days (Fig. 4).

BA was effective in promoting germination (Fig. 5) and enhanced β-CAS activity (Fig. 6). Cytokinin is known to be effective to break dormancy in seeds and buds. Ethylene can evoke β-CAS activity in citrus fruit[14] but not effective in dormancy breaking of grapevine buds[7,8]. In our previous paper, we reported that β-CAS in peach flower buds increased with releasing dormancy. The present results show that β-CAS in Japanese apricot seeds also activated by chilling treatment. It seems that the activity level itself is not so important for dormancy breaking bacause the activity is greatly different at the time visible germination occurs (Fig. 3). Cyanide is another effective chemical in breaking dormancy[5,12,17]. Thus it is interesting to know how

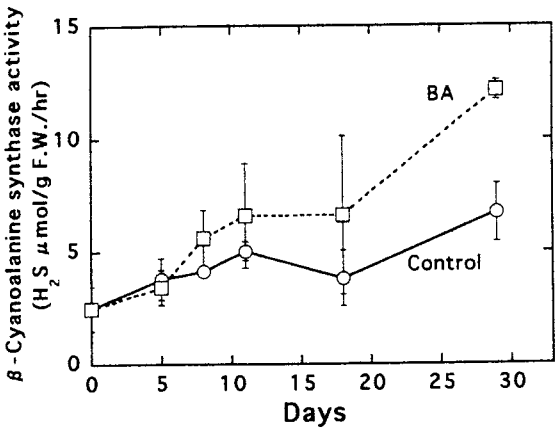

Fig. 5 Effect of BA on β-CAS activity in Japanese apricot seeds.

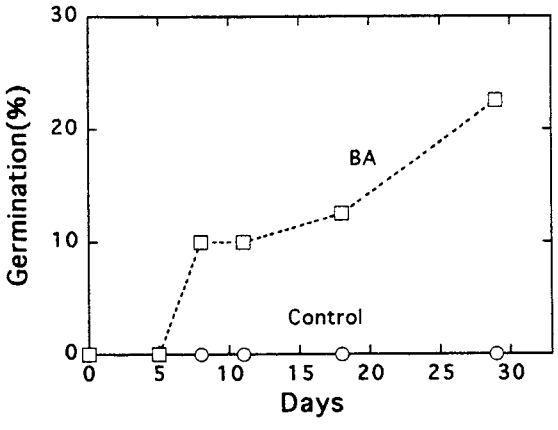

Fig. 6 Effect of BA on germination percentage of Japanese apricot seeds.

cyanogenic glycosides and free cyanide in cyanogenic plants like Japanese apricot function in regulation of breaking dormancy. For this end, further research for accurate measurement of free cyanide levels in seeds and buds in relation to their dormancy is needed.

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ウメ種子の層積期間中のβ－シアノアラニン合成酵素活性の変化

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摘　　要

異なる温度で層積したウメ種子のβ－シアノアラニン合成酵素（CAS）活性とアミグダリン含量の変化を調査した。5、10、18、24及び30℃で層積すると、5℃と10℃ではβ-CASの活性はぞれぞれ処理開始後20日目と40日目までは増加し、その後は少し減少した。10℃に比べて5℃のほうが活性が高くなった。しかしながら、18℃以上で層積した種子では活性が低下し、温度が高いほど低下の程度も大きかった。ウメ種子の主要な青酸配糖体であるアミグダリン含量は5℃と10℃では層積期間が長くなるに連れて少し減少するようであったが、24℃と30℃では増加してピークに達した後減少する傾向が見られた。層積中に発芽が見られたのは5℃のみであった。さらに、24℃に層積していた種子を5℃に移すといつもβ-CAS活性は増加し、種子発芽が起こった。24℃から5℃に移すとアミグダリン含量は減少する傾向が見られた。ベンジルアデニン（BA）を処理すると発芽を促進し、β-CAS活性を高めた。以上のことから、β-CAS活性はウメ種子の休眠打破、種子発芽過程での青酸代謝に関係していると思われた。