PERGAMON

Phytochemistry 52 (1999) 275–280

PHYTOCHEMISTRY

Biosynthesis of carotenoids in bittermelon at high temperature

T. Lan Huong Tran[a], Leoncio C. Raymundo[b,*]

[a]*Department of Storage and Processing of Agricultural Products, Faculty of Crop Science, Hanoi Agricultural University, Viet Nam*
[b]*Institute of Food Science and Technology, College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines*

Received 2 August 1998; received in revised form 25 January 1999; accepted 25 January 1999

Abstract

Detached mature green bittermelon fruit were ripened at 25 and 35° to determine the influence of temperature on the biosynthesis of carotenoids in the fruit tissues. Carotenoid biosynthesis was inhibited in the seed aril at 35°. The production of α-carotene, α-cryptoxanthin, β-cryptoxanthin, zeaxanthin and violaxanthin in the pericarp was not significantly affected at high temperature while β-carotene and the total carotenoids increased during the later stages of ripening at 35°. Carotenogenesis in the aril and in the pericarp responded differently to high temperature probably because the chromoplasts in the tissues metamorphose from different plastid types. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Momordica charantia; Cucurbitaceae; Bittermelon; Biosynthesis; Aril; Carotenoids; Inhibition; Pericarp; Plastids; Temperature

1. Introduction

The ripe fruit of bittermelon, *Momordica charantia* L. contains carotenoids that are typically found in chloroplasts as well as in chromoplasts (Rodriquez-Amaya, Raymundo, Lee, Simpson & Chichester, 1976). In this respect, it is similar to tomato and pepper fruits. However, in *Momordica* there appear to be two different tissues in the fruit where carotenoids are synthesized. The seed aril changes from white to scarlet while the green pericarp becomes orange on ripening. Because of this unique characteristic, the fruit may be a good material for elucidating the nature of the carotenoid biosynthetic pathway. In the tomato fruit, for example, high temperature inhibits lycopene but not β-carotene synthesis (Goodwin & Jamikorn, 1952) during ripening thus precluding a precursor–product relationship between the two pigments. This was later attributed to the synthesis of β-carotene via another pathway (Goodwin, 1971; Raymundo, Griffiths & Simpson, 1967) which does not involve lycopene. Lycopene, however, can be converted to β-carotene (Wells, Schelble & Porter, 1964; Hill, Calderwood & Rogers, 1971) by isolated plastids. In addition, in the *high-beta* tomato strain (Porter & Lincoln, 1950; Tomes, Quackenbush & Kargl, 1958; Tomes, Quackenbush & McQuistan, 1954) β-carotene is formed at the expense of lycopene. The formation of the additional β-carotene, however, is inhibited at high temperature (Tomes, 1963). The biosynthetic origin of the β-carotene in this strain, therefore, may be different from that found in the *normal red* tomato (Goodwin & Jamikorn, 1952; Tomes, 1963; Tomes et al., 1958) where β-carotene formation is not affected by ripening at temperatures above 30°.

It has been suggested (Rodriquez-Amaya et al., 1976) that the bittermelon fruit could serve as a most instructive model for the study of carotenogenesis as a function of ripening due to the large number of carotenoids it synthesizes during the process. The use of a carotenogenic system with the propensity to accumulate both β-carotene and lycopene in separate tissues while ripening could unravel the biosynthetic connection between the two pigments.

This study was conducted to determine the response of the two tissues to high temperature during post-harvest ripening.

* Corresponding author.

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00174-0

Table 1
Carotenoid composition of bittermelon paricarp as influenced by ripening at 25 and 35°, μg/g dry wt[a]

Carotenoid	Ripening time, days									
	0		2		4		6		8	
	25°	35°	25°	35°	25°	35°	25°	35°	25°	35°
α-Carotene	9.97ax	9.97ax	12.57ax	9.44ax	9.00ax	11.84ax	5.28ax	7.93ax	4.69ax	7.52ax
α-Cryptoxanthin	0.80ax	0.80ax	1.13ax	1.61ax	0.66ax	1.88ax	4.53ax	5.14ax	3.83ax	2.21ax
Lutein	8.31ax	8.31ax	24.99bx	19.66bx	22.14bx	0.0	0.0	0.0	0.0	19.32bx
Lutein 5,6-epoxide	0.51ax	0.51ax	1.40ax	2.30ax	0.76ax	0.0	0.0	0.0	0.0	0.0
β-Carotene	15.56ax	15.56ax	28.20ax	14.02ay	28.44ax	26.25ax	27.78ax	58.44by	26.55ax	42.06bx
β-Cryptoxanthin	0.0	0.0	0.0	0.0	0.0	9.10ax	21.86ax	29.74bx	32.25bx	25.98bx
Zeaxanthin	0.0	0.0	0.0	0.0	0.0	31.27bx	8.38ax	8.08ax	7.18ax	5.25ax
Antheraxanthin	7.24bx	7.24bx	0.0	0.0	0.0	0.0	0.0	0.29ax	0.0	0.39ax
Violaxanthin	0.0	0.0	0.0	0.08ax	0.0	0.04ax	0.0	0.02ax	0.10ax	0.04ax
Total	42.39ax	42.39ax	68.29ax	47.09ax	61.00ax	80.46abx	67.63ax	109.66bcy	74.52ax	102.75bcx

[a] The letters, x and y are designated to compare mean values at 25 vs 30°. The letters, a, b, and c are used to compare mean values within the designated days of ripening. In both cases, means with the same letter are not significantly different at $\alpha = 0.05$ using DMRT.

2. Results

The mature fruit of bittermelon was uniformly green from the blossom-end to the stem-end. The seed cavity of the fruit was filled with a white cotton-like mass in which the seeds were embedded.

There was no change in the surface color on the 2nd day at 25° but a slight yellow tinge appeared on both the external and internal surfaces of the pericarp at the stem-end on the 4th day of ripening. The inner surface of the seed aril appeared pinkish while the outer surface was still white. Six days after harvest the longitudinally rounded ridges were mostly yellow-green while the furrows between them turned orange-yellow. Most of the seeds in the cavity were scarlet. On the 8th day, the fruit was fully ripe and had a yellow-orange color. The fruit also split open somewhat irregularly at the blossom-end, its three sections curling back and exposing the scarlet seeds.

Exposure to 35° accelerated the rate of ripening and color development in both the pericarp and seed aril by ca 2 days. The outer surface of the pericarp was yellow-orange after only 4 days at 35°. The seed arils, however, were yellow instead of pinkish. Six days after harvest, the pericarp had a more intense yellow-orange color but the surface of the ridges was still yellow-green. Some of the arils were pale red. The color of the whole fruit was uniformly yellow-orange on the 8th day. The arils were almost of the same color as the pericarp. The fruit did not rupture at 35° unlike those ripened at 25°.

The yellow color of the pericarp during the early stages of ripening at 25° was due mainly to the accumulation of α-carotene, β-carotene, lutein and antheraxanthin (Table 1). The color of the seed cavity changed from white-opaque to orange-yellow 6 days after harvest. This color change was caused by a significant increase in the synthesis of β-cryptoxanthin and zeaxanthin in addition to β-carotene. The most dramatic change was seen in β-cryptoxanthin which was absent in 0-, 2- and 4-day samples but became one of the principal pigments in the pericarp of the fruit ripened for 6 and 8 days; about 42% of the total carotenoids in the pericarp of the 8-day sample was β-cryptoxanthin. A similar trend, though to a much lesser magnitude, was observed with zeaxanthin. Lutein and lutein 5,6-epoxide completely disappeared from the pericarp after 4 days. In contrast the synthesis of β-cryptoxanthin, zeaxanthin, antheraxanthin and violaxanthin fluctuated as ripening progressed. α-Carotene, β-carotene and α-cryptoxanthin persisted throughout the ripening period suggesting that a preset level of these carotenoids is being constantly maintained in the system.

Lycopene was not detected in the pericarp of ripe bittermelon fruit contrary to the report of Rodriquez-Amaya et al. (1976). In the earlier study no attempt was made to separately examine the carotenoid composition of the pericarp and the seed aril.

The enhancement of color development in the pericarp elicited by ripening at 35° (Table 1) was due mainly to the increase in the levels of β-carotene, β-cryptoxanthin and zeaxanthin. The total carotenoid content also increased significantly on ripening at 35° for 6 and 8 days. The initial increase may be traced to zeaxanthin and β-cryptoxanthin synthesis, then later to the accumulation of β-carotene and β-cryptoxanthin. Zeaxanthin comprised ca 39% of the total carotenoids in the pericarp of bittermelon fruit ripened at 35° for 4 days. In the fruit ripened for 6 days, 80% of the total carotenoids was due to β-carotene and β-cryptoxanthin.

Table 2
Carotenoid composition of bittermelon aril as influenced by ripening at 25 and 35°, μg/g dry wt[a]

Carotenoid	Ripening time, days									
	0		2		4		6		8	
	25°	35°	25°	35°	25°	35°	25°	35°	25°	35°
α-Cryptoxanthin	2.5ax	2.5ax	25.98bx	8.32ay	0.0	11.41bx	20.92bx	5.49ay	28.88bx	11.19by
β-Cryptoxanthin	0.0	0.0	20.68ax	3.88ay	22.07ax	13.40ax	39.98abx	29.26bx	69.55bx	24.69by
Fraction 3	0.0	0.0	0.0	0.92ax	0.0	3.74bx	7.11ax	3.96bx	9.10ax	4.27bx
Fraction 4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.04ax	26.13ax	7.14ay
Lycopene	14.57ax	14.57ax	47.06abx	8.04ay	54.43abx	39.29ax	322.92cx	102.17by	411.30cx	25.02ay
Fraction 6	1.59ax	1.59ax	1.66ax	0.0	1.27ax	0.0	0.0	0.0	6.21bx	0.0
Total	18.66ax	18.66ax	95.37bx	21.17aby	77.76bx	67.84bx	390.93cx	148.93cy	551.20cx	72.31by

[a] The letters, x and y are designated to compare mean values at 25 vs 30°. The letters, a, b, and c are used to compare mean values within the designated days of ripening. In both cases, means with the same letter are not significantly different at $\alpha = 0.05$ using DMRT.

The increase in the concentration of β-carotene, β-cryptoxanthin and zeaxanthin is considerably greater than what could be accounted for by changes in the α-carotene and lutein fractions or, corollarily, by the interconversion of α-ionone and β-ionone rings. In any case this type of transformation is generally considered unlikely (Walton, Britton & Goodwin, 1969).

Antheraxanthin reappeared on the 6th day at 35°. The production of violaxanthin was also induced by ripening at 35°. Lutein, 5,6-epoxide, on the other hand, disappeared 2 days after exposure to 35°, at 25° it was still present in the pericarp on the 4th day of ripening. The reappearance of lutein on the 8th day at 35° cannot be explained.

The change in the color of the aril as the fruit ripened (Table 2) was due to the accumulation of substantial amount of lycopene in the tissue. A steady increase in the lycopene content occurred throughout the period as the aril turned from white to scarlet on ripening at 25°. Although the major carotenoids initially present in the aril are α-cryptoxanthin, β-cryptoxanthin and lycopene, the latter became the dominant pigment starting on the 6th day.

Among the six bands obtained on chromatographic separation of the carotenoids in the crude extract of the aril only lycopene, α-cryptoxanthin and β-cryptoxanthin were identified. The least polar band which ran with the solvent front was probably a mixture of α- and β-carotenes together with the other more saturated precursors of lycopene. The two other bands that were more polar than lycopene absorbing at 447, 475 nm and at 456, 486 nm but less polar than either β-cryptoxanthin or α-cryptoxanthin were unidentified. Nonetheless the concentration of these unidentified fractions either decreased or remained essentially unchanged in response to ripening at 35°.

The effect of high temperature on the carotenoid distribution during ripening (Table 2) of bittermelon fruit was most striking in the seed aril. At 35°, the synthesis of all carotenoid fractions was partially inhibited such that the aril did not turn red as it normally would have if the fruit were ripened at 25°. Instead β-cryptoxanthin and α-cryptoxanthin were the predominant pigments on the 8th day at 35°. Together they imparted a yellow-orange color to the aril.

The total carotenoid content of the aril of the fruit ripened at 35° decreased by 83% from 551.2 μg/g to 72.3 μg/g dry wt because of the overall inhibition of carotenogenesis in the tissue. The degree of inhibition of the xanthophylls, however, was not as drastic as that of lycopene.

There are undoubtedly two distinct carotenogenic tissues in the bittermelon fruit, namely, the pericarp which accumulates cyclic carotenoids, and the seed aril where lycopene and α- and β-cryptoxanthin are the major carotenoid components.

3. Discussion

The inability of high temperature to block the pathway for cyclic carotenoids in the pericarp (Table 1) and the susceptibility of the carotenogenic system in the aril (Table 2) to inhibition were both apparent in detached bittermelon fruit ripened at 35°. Several studies (Chang, Raymundo, Glass & Simpson, 1977; Cheng, Flores, Shewfelt & Chang, 1988; Goodwin & Jamikorn, 1952; Tomes, 1963) have shown that lycopene synthesis decreased when ripening tomato fruits are exposed to temperatures above 30°. Furthermore the lycopene content of tomatoes increases with storage time (Yang, Cheng & Shewfelt, 1990) as long as the temperature is maintained at 21°. Exposing the fruits to 30° causes a partial inhibition of lycopene production and at 37°, complete inhibition occurs. High temperature does not affect β-carotene synthesis (Goodwin & Jamikorn, 1952) in the tomato fruit. In the bittermelon pericarp there was substantial accumu-

lation of β-carotene during the later stages of ripening at 35°.

There is general agreement that sequential desaturation of phytoene leads to the formation of lycopene. However, the concensus differs as to whether lycopene is the direct precursor of the cyclic carotenes or whether the diversion occurs at the neurosporene level. The latter is unsaturated at the 7,8 position while lycopene has an additional double bond at the 7,′8′-position. In the usual pathway lycopene should have been the precursor for β-carotene (Tomes et al., 1954; 1958) and since lycopene synthesis is inhibited at high temperature, β-carotene should respond accordingly. Based on this observation other investigators (Goodwin, 1980; Raymundo et al., 1967) suggested that β-carotene is being synthesized via a different pathway. Alternative pathways (Goodwin, 1980, 1971) for the conversion of acyclic carotene to β-carotene were proposed. One pathway goes through lycopene where β-carotene is formed at the expense of the former; another pathway is via neurosporene → β-zeacarotene. A third option is the cyclization of the ζ-carotene isomer 7′,8′,11′,12′-tetrahydrolycopene to 7′,8′,11′,12′-tetrahydro-γ-carotene → β-zeacarotene.

In *Phycomyces blakesleeanus* 2-(4-chloropenylthio)-triethylamine HCl (CPTA) and its analogues 2-(4-chlorophenoxy)-triethylamine HCl and 2-(4-chlorophenylthio)-ethylamine-*N*-ethyl HCl promote the sequential dehydrogenation steps from phytoene to lycopene (Elahi, Glass, Lee, Chichester & Simpson, 1975) while at the same time inhibiting cyclase activity. Consequently lycopene and γ-carotene accumulate while β-carotene decreases and β-zeacarotene invariably disappears. Since the conversion of β-zeacarotene to γ-carotene is a dehydrogenation step, the process is enhanced by CPTA resulting in the loss of β-zeacarotene. The synthesis of β-carotene from γ-carotene, on the other hand, requires cyclization to form the second 5,6-cyclohexene ring. The process is inhibited by CPTA causing γ-carotene to accumulate. The same rationale can explain the increase in lycopene concentration at the expense of β-carotene in tomatoes (Chang et al., 1977) treated with CPTA. It has been suggested (Kirk & Tilney-Bassett, 1978) that tertiary amines like CPTA bind with the regulator protein controlling the synthesis of the lycopene-forming enzyme. At the same time it forms an inactive complex with the protein regulating cyclase synthesis, effectively displacing the natural inducer and stimulating lycopene synthesis at the expense of β-carotene.

Although the inhibition of the carotenogenesis in the aril at 35° was not specific, a wide disparity in response was observed between lycopene and the cyclic carotenoids. The latter behaved as if they are formed via a pathway not involving lycopene. If lycopene is the major substrate for the cyclase, a 96% decrease in the lycopene content should have resulted in a reduction more substantial than the ca 60% inhibition of α- and β-cryptoxanthin. Thus the possibility that lycopene and the cyclic carotenoids are formed independently in the tissue either via parallel pathways or through another scheme cannot be ruled out entirely.

The inhibition of carotenogenesis in the aril may be due to some other cause such as the inability of the tissue to form chromoplasts. It may not be a direct effect of high temperature on the carotenogenic enzymes as previously assumed (Goodwin, 1971; Raymundo et al., 1967), since the total carotenoid content of the pericarp (Table 1) did not decrease on ripening at 35°. The implication is that the activities of these enzymes are not impeded at temperatures as high as 35°.

Chromoplasts may develop from chloroplasts or originate from proplastids in carotenogenic plant tissues. Carotenogenic systems where chloroplasts metamorphose directly to chromoplasts include ripening fruits of *Sambucus racemosa, Physalis alkekengi*., *Sorbus aucuparia* as cited by Kirk and Tilney-Bassett (1978), *Solanum capsicastrum, Capsicum annuum* (Steffen & Walter, 1958; Frey-Wyssling & Kreutzer, 1958; Spurr & Harris, 1965), Navel orange peel (Thompson & Spurr, 1969), and *Lycopersicon esculentum* (Harris & Spurr, 1969; Rosso, 1968; Steffen, 1964).

The transformation is associated with substantial changes in size, shape and pigment composition of the plastid (Rosso, 1968). Chlorophyll degradation is accompanied by the disappearance of granal disks leaving only stromatic thylakoid materials followed by the accumulation of osmiophilic globules. Small amounts of stroma remain in the mature chromoplasts. Lycopene formation is associated with the remaining membrane material which eventually forms crystalloids or plates.

Chromoplasts, however, do not necessarily develop from chloroplasts. The chromoplasts in the epidermis of the orange-red berries of *Convallaria maialis* (Steffen, 1964), for example, arise from colorless proplastids. Amyloplasts become chromoplasts during the growth and maturation of carrot roots (Gronegress, 1971). Chromoplasts, on the other hand, develop from chloroplasts instead of amyloplasts or proplastids (Steffen & Reck, 1964) in carrot roots exposed to sunlight while growing in the field. If carrot roots, however, are illuminated for 48 h after harvest, the chromoplasts change into chloroplasts (Gronegress, 1971). The carotene crystals of the chromoplasts breakdown in the process. Reversible plastid metamorphosis in the mesophyll cells of Golden Saxifrage (*Chrysoplenium*) is responsible for the shift in color from green to yellow and back to green (Sitte, 1924). In regreening Valencia oranges (Thompson, Lewis & Coggins, 1967) chromoplasts revert to chloroplasts.

The biosynthesis of carotenoids in some fruits is not susceptible to inhibition at high temperature probably because the carotenoid-forming system is already operating prior to the transformation of chloroplasts to chromoplasts (Kirk & Tilney-Bassett, 1978). Such may be the case in the bittermelon pericarp where carotenogenesis was normal at 35° (Table 1).

The seed aril chromoplasts, on the other hand, do not develop from chloroplasts, but most likely from proplastids since the aril is devoid, or very nearly so, of chloroplasts. The transition from proplastids may be the stage susceptible at high temperature. Since carotenoids form around vesicles in developing chromoplasts (Guilliermond, 1942; Kirk & Tilney-Bassett, 1978), a deficiency in these structures or the presence of some anatomical aberrations induced at high temperature may interfere with carotenogenesis in the organelles. Failure of the regulator protein (Kirk & Tilney-Bassett, 1978) to activate the synthesis of the carotenoid-forming system in the seed aril at high temperature may equally result in the inhibition of the carotenoid biosynthetic pathway.

There is evidence that plastid development is sensitive to high temperature. Cells of *Euglena gracilis* grown at 34° (Kirk & Tilney-Bassett, 1967; Pringsheim & Pringsheim, 1953; De Deken-Grenson & Messin, 1958) lose the ability to form chloroplasts. The cells consequently become colorless. The formation of chromoplasts may be similarly sensitive to high temperature considering that chloroplasts and chromoplasts as well as the other types of plastids are ultimately derived from proplastids (Pringsheim & Pringsheim, 1953). The fact that the synthesis of chlorophyll in the chloroplasts and carotenoid pigments in the chromoplasts can be blocked at high temperature suggests that a mechanism common to both plastid types may be responsible for the biochemical disorder.

In the tomato fruit where chromoplasts are known to arise from chloroplasts (Harris & Spurr, 1969; Rosso, 1968), lycopene in the *normal red* (Goodwin & Jamikorn, 1952) and the β-carotene fraction in the *high-beta* (Tomes, 1963) strains are vulnerable to inhibition at high temperature (Chang et al., 1977) because these pigments are produced by the chromoplast-type system. The genes responsible for the lycopene and for β-carotene synthesis in each strain are derepressed (Kirk & Tilney-Bassett, 1978). The associated structural reorganizations in transforming chloroplasts occur during the transition to chromoplasts. The changes may be the points that are compromised at high temperature rather than the activity of the lycopene-forming enzyme or that of the cyclase and could explain the inhibition of the CPTA-induced synthesis of lycopene (Chang et al., 1977) in these tomato strains at 32° as well as the results in Table 1. Lycopene synthesis in the *yellow* tomato genotype (Harris & Spurr, 1969; Le Rosen, Went & Zechmeister, 1941), in addition, is almost totally suppressed; phytoene does not accumulate. The presence of residual β-carotene in the ripe fruit infers that further carotenoid synthesis does not occur during ripening. These findings suggest that the structural β-carotene in the chloroplast and the chromoplast-type carotenoids are formed separately. It is possible that in higher plants carotenoids are synthesized in chromoplasts that are ontogenetically different. This is clearly the case in ripening bittermelon fruit where only the cyclic carotenoids accumulate in the pericarp whose chromoplasts develop from chloroplasts while lycopene as well as cyclic carotenoids are synthesized in chromoplasts that probably develop from proplastids in the aril.

The current findings did not resolve the lycopene-β-carotene paradox. In the bittermelon it is evident that there is a delineation of the pathway for the α-carotene series, the β-carotene series, and lycopene in the fruit tissues.

4. Experimental

4.1. Material

Freshly picked mature, green bittermelon fruits cv. ''Jade Star X' used in the study were purchased from a farmer in Looc, Calamba, Laguna. The fruits were disinfected by dipping in 2% solution of sodium hypochlorite for 2 min, rinsed then air-dried.

4.2. Ripening conditions

The fruits were ripened in storage chambers with a RH of 75–80% and at temperatures of 25° (control) and 35° for 8 days.

4.3. Experimental design

Each treatment had two replications laid out in a completely randomized design (CRD). There were two fruits per sample taken at 2-day intervals, i.e. 0, 2, 4, 6 and 8 days. The samples were frozen and held at −17° until analyzed.

4.4. Pigment extraction and chromatographic analysis

The extraction and separation procedures followed were essentially those described previously (Rodriquez-Amaya et al., 1976) with some modifications. The seeds were carefully separated from the pericarp. Only the outer surface of the pericarp was used for analysis in order to avoid contamination with aril carotenoids. The crude extract of the pericarp was initially run in a 22 × 220 mm column of MgO–Hyflo SuperCel (1:1, w/

w) and developed with increasing concentrations of acetone in petroleum ether (PE) to separate the α-carotene and β-carotene. The unresolved bands were eluted from the adsorbent and purified by thin-layer chromatography (TLC) on silica gel G-60, pH 7.0. The developing solvents were 3% MeOH in benzene for pericarp extract and either 0.5 or 2.5% Et_2O in PE for the aril extract. The crude aril extract was not pre-separated by column chromatography.

4.5. Identification of carotenoids

The pigments were identified according to (1) order of elution from the column, (2) TLC Rf values, (3) visible absorption spectra in PE, (4) chemical reactions such as iodine catalyzed isomerization and epoxy tests (Rodriquez-Amaya et al., 1976), and (5) co-chromatography.

4.6. Quantitative determination

The visible absorption spectrum in PE of each fraction was obtained by scanning from 550 and 350 nm using a Hitachi UV-Visible Recording Spectrophotometer Model U3200. The concentration of each carotenoid was calculated according to Davies (1999).

4.7. Solvents

The solvents used in the extraction, chromatographic separation and purification of the carotenoid pigments were all analytical grade. The solvents were distilled prior to use.

Acknowledgements

Mrs Tran wishes to thank the Southeast Asian Regional Center for Research in Agriculture (SEARCA) for the scholarship that enabled her to pursue the M.S. in Food Science degree at the University of the Philippines Los Baños (UPLB).

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