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Carbon isotope ratios and the variation in the diurnal pattern of malate accumulation in aerial roots of CAM species of *Phalaenopsis* (Orchidaceae)

H. MOTOMURA[*, **], O. UENO[***], A. KAGAWA[+], and T. YUKAWA[*]

Tsukuba Botanical Garden, National Science Museum, Tsukuba, Ibaraki 305-0005, Japan[*]
Composite Materials Group, National Institute for Materials Science, Tsukuba, Ibaraki 305-0047, Japan[**]
Laboratory of Plant Production Physiology, Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan[***]
Forestry and Forest Products Research Institute, Tsukuba Norin PO Box 16, Ibaraki 305-8687, Japan[+]

Abstract

We investigated the carbon isotope ratios and the diurnal pattern of malate accumulation in leaves and aerial roots of eight species of *Phalaenopsis* grown in greenhouses. The leaves of all the species showed carbon isotope ratios and the diurnal patterns of malate content typical of CAM plants. However, the aerial roots exhibited a large variation in the diurnal pattern of malate content among species and even among plants within the same species, although carbon isotope ratios were always CAM-like values. Some aerial roots showed the typical diurnal pattern of CAM, but others maintained high or low malate contents during a day without fluctuation. In order to characterize more strictly the nature of the malate variation in the aerial roots, we further investigated a possible variation of the diurnal pattern of malate among different aerial roots within an individual for *Phalaenopsis amabilis* and *P. cornu-cervi*. The diurnal pattern of malate content was varied even among different aerial roots within the same plant. Thus the photosynthetic carbon metabolism in aerial roots of orchids is fairly complex.

Additional key words: epiphyte; photosynthetic metabolism; species differences.

Introduction

Crassulacean acid metabolism (CAM) plants are characterized by CO_2 uptake at night and depression of CO_2 uptake with stomatal closure during the daytime. This confers CAM plants the ability to acquire carbon in water-limited environments (Winter *et al*. 2005). Atmospheric CO_2 is primarily fixed by phospho*enol*pyruvate carboxylase generating oxaloacetate, which is further converted to organic acid, mainly malate. The malate is temporarily stored in the vacuoles. It is decarboxylated by C_4-acid decarboxylase during the daytime, and the released CO_2 is re-fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase. As a result, CAM plants accumulate organic acids at night and consume them to generate internal CO_2 during the daytime. Thus, the diurnal consumption of malate is characterized only in CAM photosynthesis (Ting 1985, Silvera *et al*. 2005, Motomura *et al*. 2008).

The occurrence of CAM plants and their ecophysiological characteristics have been extensively studied (reviewed in Kluge and Ting 1978, Avadhani *et al*. 1982, Zotz and Hietz 2001, Keeley and Rundel 2003, Lüttge 2004). CAM plants generally have thick succulent leaves and exhibit distinct ecological characteristics, reflecting their particular biochemical and physiological function. They generally grow in arid regions and under other dry environments, and are also common among epiphytes occurring in tropical and subtropical regions. Thus, CAM photosynthesis is one of the important adaptable characteristics for succulents and epiphytes to survive in environments with water stress (Kluge and Ting 1978, Ehleringer and Monson 1993, Keeley and Rundel 2003, Lüttge 2004, Motomura *et al*. 2008).

Plants contain chlorophyll in various organs as well as

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[**]Corresponding author; fax: +81-29-859-2401, e-mail: MOTOMURA.Hiroyuki@nims.go.jp
Abbreviations: AR – aerial root; CAM – crassulacean acid metabolism; FM – fresh mass; SR – subterranean root.
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leaves (Aschan and Pfanz 2003). CAM photosynthesis has been well characterized for leaves but scanty for other chlorophyllous organs. In epiphytic orchids, the aerial root (AR), which is exposed to atmosphere, contains chloroplasts and is green (Benzing and Ott 1981, Ho *et al*. 1983, Hew *et al*. 1984, Aschan and Pfanz 2003), and thus has a photosynthetic capability. Actually, gas exchange experiments on the ARs of orchids demonstrated the fixation of atmospheric carbon dioxide (Dycus and Knudson 1957, Erickson 1957, Goh *et al*. 1983, Hew *et al*. 1984). The carbon isotopic ratios in ARs are within the range of values indicating the occurrence of CAM activity (more than −20 ‰; Winter *et al*. 1983, Hew *et al*. 1984, Kluge *et al*. 1995, Zotz and Ziegler 1997). Thus, the ARs in orchids usually perform CAM (Aschan and Pfanz 2003). However, although some researchers observed the conspicuous diurnal consumption of malate in ARs (Benzing *et al*. 1983, Hew *et al*. 1984, Cockburn *et al*. 1985), Endo and Ikushima (1989) failed to find it. Thus the diurnal pattern of malate content in ARs may vary among species and even among plants, but the actual nature remains unclear.

Phalaenopsis Blume is a genus of Orchidaceae including both epiphytes and terrestrial plants. Some species in *Phalaenopsis* have thick-leaves with high CAM activity (Endo and Ikushima 1989, Ota *et al*. 1991, Kawamitsu *et al*. 1995). The ARs are greenish and thus expected to exhibit the CAM activity. In this study, we investigated the carbon isotope ratios and the diurnal pattern of malate accumulation in ARs in eight species of *Phalaenopsis* and found that the photosynthetic carbon metabolism is more complex than known previously.

Materials and methods

Eighteen plants of eight *Phalaenopsis* species cultivated in the Tsukuba Botanical Garden of the National Science Museum were used for two different experiments of August 2005 (Exp. 1) and November 2007 (Exp. 2). In the Exp. 1, all plants were grown in naturally irradiated glasshouses and/or shade houses under 50−90 % shade. The minimum and maximum temperatures in the glasshouses and the shade houses were 18/35 ºC and 13/36 ºC, respectively (June to August, 2005). In the Exp. 2, plants were grown in naturally irradiated glasshouses. The minimum and maximum temperatures in the glasshouses were 15/34 ºC (September to November, 2007). Plants were watered immediately after drying of the surfaces of the potting media and barks. Although the growth conditions for each species correspond approximately to its natural environmental conditions, less-stressed conditions than those in the original habitats may occasionally affect expression of photosynthetic characteristics.

Exp. 1: To investigate general features of CAM activity in ARs and leaves of *Phalaenopsis* plants, tip portions of ARs and leaves were sampled from 14 plants of eight species (Table 1). Fully expanded young leaves and vigorous ARs were selected for the experiment. In *P. pulcherrima*, subterranean roots (SR) were also sampled. The root diameter and leaf thickness were measured for these root and leaf samples. In the ARs, the tip region is younger and more active than the middle and base regions. Therefore, AR tissues were sampled at 3−7 cm from the tips. For determination of malate, one AR and one leaf were sampled from one plant at 05:00 (the end of night), and then another AR and leaf were taken from the same plant at 17:00 (the end of day). Thus, the leaf and AR sampled at the end of night differed from those sampled at the end of day. For determination of $\delta^{13}C$ values, the third root and leaf were sampled from the same plant between 11:00 and 13:00. In a plant, therefore, two ARs and two leaves were examined for malate contents, and further different one AR and one leaf were examined for $\delta^{13}C$ values.

For determination of malate contents, leaf and AR samples were frozen in liquid nitrogen immediately and stored at −80 ºC until analysis. Samples (0.2 g fresh mass, FM) were ground using a mortar and pestle with 5 cm^3 of 5 % (v/v) $HClO_4$ and incubated for 20 min on ice. The homogenate was then adjusted to pH 5 with 2 M KOH and centrifuged at 4 000×*g* for 10 min at 4 ºC. The pellet was re-suspended in 2 cm^3 of water and centrifuged again. The combined supernatants were used for determination of malate contents by the method of Möllering (1974).

For determination of $\delta^{13}C$ values, samples were dried at 60 ºC, and grinded with a steel ball mill (*Wig-L-Bug* model *30*, *International Crystal Laboratories*, Garfield, NJ, USA). Part of each grinded sample (2 mg) was taken for measurement of ^{12}C and ^{13}C contents. The carbon stable isotope ratios of leaf tissue were determined using the combined system of an elemental analyzer (*NC-2500*; *CE Instruments*, Milan, Italy) and an isotope ratio mass spectrometer (*MAT-252*; *Thermo Electron*, Bremen, Germany). We expressed the isotope ratio in the delta notation in per mil units [‰] with respect to the Pee Dee belemnite standard. The standard deviation for replicate combustions of our internal standards (DL-alanine) was 0.04 ‰.

Exp. 2: Possible variations of malate contents and the diurnal pattern of malate accumulation among ARs within an individual plant were more strictly examined for *Phalaenopsis amabilis* and *P. cornu-cervi* (Table 2). For each individual plant, we sampled six vigorous ARs and one fully expanded young leaf. At the end of night, the tips of the leaf and the ARs (No. 1 and 2) were sampled, and the cut ends were sealed with parafilm to avoid water loss. The lengths of the leaf and AR samples were 1.5−2.0 cm. At the same time, two different ARs (No. 3

and 4) were sampled. At the end of day, the tip regions of the same leaf and AR (No. 1 and 2) were sampled again. Two different ARs (No. 5 and 6) were also sampled. Malate contents for each sample were determined spectrophotometrically as described in the Exp. 1. The root diameter and leaf thickness were also measured for these samples. The $\delta^{13}C$ values were also determined for some representative samples.

Results

Exp. 1: General features of CAM expression in *Phalaenopsis*: The leaf thickness in *Phalaenopsis* plants ranged from 0.3 to 2.1 mm, but most plants had relatively thick leaves (Table 1). Most *Phalaenopsis* plants had thick ARs, but plants of *P. pulcherrima* had relatively thin ARs. *P. pulcherrima* is a terrestrial species and has both ARs and SRs. The SRs of this species were also thin (Table 1). The diameters of roots sampled at the end of night and the end of day were almost the same.

Among 14 plants of *Phalaenopsis*, $\delta^{13}C$ values ranged from −15.7 to −11.7 ‰ in leaves and −15.4 to −12.9 ‰ in ARs (Table 1). These values were within the range of strong CAM plants (Motomura *et al*. 2008) and generally stable within single species.

Table 1. Diurnal changes of malate contents [mmol kg^{-1}(FM)] and $\delta^{13}C$ values [‰] in leaves and aerial roots (ARs) of eight species of *Phalaenopsis*. Exp. 1: Different leaves (L) and ARs were sampled from a plant at the end of night and at the end of day, respectively. SR – subterranean roots. *TBG* series indicate accession numbers in living collection database at Tsukuba Botanical Garden.

Species		Organ	Thickness, diameter [mm]	$\delta^{13}C$	Malate End of night	day	Δ(night − day)
P. amabilis	*TBG 145847*	L	1.1, 1.2	−13.6	45.0	15.3	29.7
		AR	3.3, 3.6	−14.6	20.1	24.6	−4.4
	TBG 145848	L	1.3, 1.5	−11.7	49.7	14.8	35.0
		AR	3.5, 3.5	−14.7	4.6	3.8	0.8
	TBG 145849	L	1.3, 1.4	−13.0	44.4	8.7	35.8
		AR	3.1, 3.9	−14.9	23.5	25.0	−1.5
P. chibae	*TBG 118396*	L	0.6, 0.5	−15.5	36.1	11.6	24.4
		AR	1.4, 2.6	−14.9	21.1	19.6	1.5
P. cornu-cervi	*TBG 118541*	L	1.4, 1.4	−13.4	47.6	3.8	43.9
		AR	4.4, 3.5	−13.6	2.5	2.7	−0.3
	TBG 144567	L	0.7, 1.4	−15.1	51.2	11.4	39.8
		AR	4.0, 3.7	−14.3	12.0	2.8	9.2
	TBG 145696		1.8, 1.4	−15.2	28.5	1.7	26.8
			3.4, 3.6	−15.5	14.3	5.4	8.8
P. parishii	*TBG 144570*	L	1.0, 1.1	−15.2	47.8	15.7	32.1
		AR	2.0, 2.4	−15.4	33.6	31.9	1.7
P. pulcherrima	*TBG 118344-1*	L	0.3, 0.3	−13.2	60.2	18.2	41.9
		AR	0.3, 0.3	−14.4	20.0	12.6	7.4
		SR	0.3, 0.5	−13.0	4.2	0.6	3.7
	TBG 118344-2	L	1.3, 1.0	−13.1	51.2	11.2	40.0
		AR	1.1, 0.9	−12.9	5.7	6.1	−0.4
		SR	1.1, 1.0	−13.7	0.3	0.6	−0.3
	TBG 145647	L	2.1, 1.6	−13.9	58.8	15.2	33.7
		AR	1.6, 1.8	−14.4	9.5	12.5	−3.0
		SR	0.3, 0.3	−12.8	0.4	4.6	−4.3
P. venosa	*TBG 145711*	L	0.6, 1.0	−15.7	31.5	4.9	26.6
		AR	4.0, 4.3	−14.5	11.0	0.5	10.5
P. violacea	*TBG 145785*	L	1.1, 0.7	−12.9	46.6	4.7	42.0
		AR	4.0, 4.4	−15.0	3.6	4.5	−0.9
P. wilsonii	*TBG144576*	L	1.0, 0.8	−15.6	52.8	13.1	39.7
		AR	2.9, 2.6	−14.8	35.0	30.1	4.9

The accumulation of malate was more and less detected in all the samples. In leaf samples, the malate contents ranged from 28.5 to 60.2 mmol kg^{-1}(FM) at the end of night and from 1.7 to 25.2 mmol kg^{-1}(FM) at the end of day (Table 1). The leaves at the end of night consistently showed high malate accumulation, and the malate contents were always higher than those at the end of day. Thus, diurnal consumption of malate occurred in leaves of all the plants of *Phalaenopsis*. This shows the occurrence of CAM photosynthesis in the leaves of *Phalaenopsis* species.

In the ARs, the malate contents ranged from 2.5 to 35.0 mmol kg^{-1}(FM) at the end of night and from 0.5 to 31.9 mmol kg^{-1}(FM) at the end of day (Table 1). The malate contents at the end of night in the ARs were generally lower than those in the leaves, while the malate contents at the end of day in the ARs were somewhat higher than those in the leaves (Table 1). However, the diurnal pattern of malate accumulation in the ARs showed a great variation among species and plants, although in this experiment we compared different ARs at the end of night and at the end of day. The diurnal pattern of malate accumulation in the ARs was generally classified into the following three types: (*1*) typical CAM pattern with high contents at the end of night and low content at the end of day; (*2*) latent CAM pattern (Lee and Griffiths 1987) with always high contents at the both sampling times; (*3*) C_3 pattern with always low contents at the both sampling times. For example, the ARs of *P. venosa* showed the typical CAM pattern, while those of *P. chibae*, *P. parishii*, and *P. wilsonii* showed the latent CAM pattern. The ARs of *P. violacea* represented the C_3 pattern. In other three species in which three different plants were examined per species, the diurnal pattern of malate accumulation in the ARs was varied among plants within the same species (Table 1). In the ARs of *P. amabilis*, two plants showed the latent CAM pattern, but one plant showed the C_3 pattern. In the ARs of *P. cornu-cervi*, two plants showed the typical CAM pattern, while one plant showed the C_3 pattern. In those of *P. pulcherrima*, one plant had the typical CAM pattern, while two plants showed the latent CAM pattern but the malate contents were relatively low (Table 1).

The SRs from three plants of *P. pulcherrima* had low contents of malate at the both sampling times [less than 4.6 mmol kg^{-1}(FM)].

Exp. 2: Variation of the diurnal pattern of malate accumulation among different ARs within an individual plant: In the Exp. 1, we found a great variation in the diurnal pattern of malate accumulation in ARs even among different plants within a species. In the Exp. 2, therefore, we more strictly investigated this phenomenon for *P. amabilis and P. cornu-cervi* (Table 2).

In leaf samples, the malate contents ranged from 5.1 to 27.4 mmol kg^{-1}(FM) at the end of night and from 1.7 to 23.6 mmol kg^{-1}(FM) at the end of day (Table 2). The

Table 2. Diurnal changes of malate contents [mmol kg^{-1}(FM)] in leaves and aerial roots (ARs) of *Phalaenopsis amabilis* and *P. cornu-cervi*. ND – not determined. Carbon isotopic values [‰] from samples collected at end of *night, **day.

Species		Organ	Thickness, diameter [mm]	$\delta^{13}C$	Malate End of night	day	Δ(night − day)
P. amabilis	Plant 1 (*TBG 145663*)	L	1.7, 1.2	−15.9**	22.0	22.0	0.0
		AR1	4.4, 4.4	ND	18.0	15.4	2.6
		AR2	4.5, 4.2	ND	19.4	18.6	0.8
		AR3	3.5	ND	2.4	ND	−
		AR4	4.1	−16.6*	1.2	ND	−
		AR5	6.0	ND	ND	2.0	−
		AR6	5.8	−16.5*	ND	1.8	−
	Plant 2 (*TBG 145662*)	L	1.0, 1.3	−16.1*	20.9	15.7	5.2
		AR1	3.8, 3.8	ND	9.5	6.4	3.1
		AR2	3.1, 3.1	ND	11.9	7.6	4.3
		AR3	3.7	ND	9.7	ND	−
		AR4	3.7	ND	8.5	ND	−
		AR5	4.4	ND	ND	7.6	−
		AR6	3.5	ND	ND	2.2	−
P. cornu-cervi	Plant 1 (*TBG 145695*)	L	1.2, 1.2	−15.6**	27.4	23.6	3.8
		AR1	3.3, 3.5	ND	6.6	3.4	3.2
		AR2	5.3, 5.2	ND	7.4	5.4	2.0
		AR3	4.7	ND	10.6	ND	−
		AR4	4.9	ND	7.8	ND	−
		AR5	2.7	ND	ND	1.1	−
		AR6	4.6	ND	ND	2.9	−
	Plant 2 (*TBG 118877*)	L	1.1, 1.2	−16.1**	5.1	1.7	3.4
		AR1	4.0, 3.5	ND	6.7	3.2	3.5
		AR2	4.9, 5.1	−12.7**	11.7	5.6	6.1
		AR3	5.0	ND	8.2	ND	−
		AR4	4.4	ND	10.2	ND	−
		AR5	4.5	ND	ND	11.9	−
		AR6	4.7	−12.4**	ND	9.0	−

malate contents at the end of night were lower than those in the Exp. 1. The differences in malate contents between at the end of night and at the end of day were also much smaller than those found in the Exp. 1. These different patterns of malate contents between the two experiments were caused by the difference in the growth season: the Exps. 1 and 2 were made in summer and autumn, respectively. Nevertheless, the $\delta^{13}C$ values of the leaf samples ranged from −16.1 to −15.6 ‰ (Table 2), which were typical of CAM plants.

Most ARs also accumulated substantial contents of malate (Table 2). In the ARs of both the plant 2 of *P. amabilis* and the plant 1 of *P. cornu-cervi*, there was a tendency that the malate contents decreased at the end of day as compared with those at the end of night, although the degree of the malate consumption was small (Table 2). Thus, it appears that these 2 plants co-ordinately express a weak CAM pattern in all leaves and ARs. In the aerial roots of the plant 1 of *P. amabilis* and the plant 2 of *P. cornu-cervi*, however, the diurnal pattern of malate content was greatly varied among different ARs within a plant (Table 2). In the plant 1 of *P. amabilis,* the ARs 1 and 2 showed a weak and a latent CAM pattern, respectively. The ARs 3 and 4 showed very low contents of malate at the end of night, indicating the lack of CAM. On the other hand, the ARs 5 and 6 showed low contents of malate at the end of day. In the plant 2 of *P. cornu-cervi*, the ARs 1 and 2 showed a weak CAM pattern. The ARs 3 and 4 accumulated much malate at the end of night. However, the ARs 5 and 6 also maintained high malate contents at the end of day, implying the existence of the latent CAM. Despite such variation in the diurnal pattern of malate accumulation, the ARs showed $\delta^{13}C$ values typical of CAM plants (Table 2).

Discussion

In the Exp. 1, we found that the diurnal patterns of malate content in the ARs were largely varied among species and plants. In the Exp. 2, therefore, we strictly investigated possible variation of the diurnal pattern of malate accumulation among ARs within the same plant, with the four plants of *P. amabilis* and *P. cornu-cervi*. The two plants of *P. amabilis* and *P. cornu-cervi* generally showed a CAM-like pattern of malate contents in all ARs, although the degree of CAM expression was low. On the other hand, other two plants of these species showed the variation in the degree of CAM expression among different ARs within a plant. Especially in the plant 1 of *P. amabilis*, the diurnal pattern of malate contents showed a great variation among different ARs. Thus, it would be impossible to decide a photosynthetic mode for the ARs of this plant. For example, if we examined the malate content at the end of night for the AR 1 and that at the end of day for the AR 5, we would conclude that the ARs of this plant perform a typical CAM. In contrast, if we examined the malate content at the end of night for the AR 3 and that at the end of day for the AR 5, we would conclude that the ARs of this plant perform the C_3 mode but not CAM. In order to study the photosynthetic carbon metabolism of the ARs, therefore, it is required to examine the diurnal pattern of malate contents in the same AR, using several aerial roots of the same plant. Thus, the data shown in the Exp. 1 may not represent the actual nature of photosynthetic carbon metabolism in the ARs of respective *Phalaenopsis* species.

In the strict comparative study with *P. amabilis* and *P. cornu-cervi* (the Exp. 2), we found some novel features about photosynthetic carbon metabolism of the ARs. The most important finding is that these orchids are capable to express different patterns of malate accumulation among different ARs within a plant as well as among different plants of the same species. The degree of CAM expression is influenced by various environmental and endogenous factors such as temperature, irradiance, moisture, growth stage, and leaf age (Kluge and Ting 1978, Osmond 1978, Ota *et al*. 1991, Lüttge 2004). In this experiment, we examined vigorous ARs of plants grown under the same environment. All these plants were also not in the flowering period. At present, it remains unknown why the ARs express different patterns of malate accumulation within a plant.

The AR 2 in the plant 1 of *P. amabilis* exhibited relatively high malate content with no diurnal consumption of malate. Such feature is similar to that of the 'latent CAM' (Lee and Griffiths 1987), which keeps a high content of malate without the diurnal consumption. In the ARs of a hybrid of *Phalaenopsis* grown hydroponically, a high content of malate without the diurnal consumption was also observed (Endo and Ikushima 1989). Lee and Griffiths (1987) showed that leaves of *Sedum telephium* (Crassulaceae) change from the latent CAM state to the ordinary CAM state in response to water stress. Thus, the ARs with high malate contents during the daytime may change to a typical CAM form when subjected to water stress. On the other hand, the ARs 3 and 4 in the plant 1 of *P. amabilis* accumulated low malate contents at the end of night. Nevertheless, the ARs and leaves in the plant 1 of *P. amabilis* exhibited $\delta^{13}C$ values typical of CAM plants. These facts suggest that the growth of ARs may depend on saccharides translocated from leaves with CAM. In other words, in this plant most atmospheric CO_2 is fixed through CAM photosynthesis by the leaves, and the CO_2 fixation by the ARs may not contribute to the formation of ARs themselves.

Our data suggest that the photosynthetic carbon metabolism in the ARs is more complex than previously considered. The ARs of orchids provide an intriguing subject for future studies on the regulatory mechanism of CAM expression.

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