Analysis of Polyamines, 1-Aminocyclopropane-1-carboxylic Acid and their Conjugated Forms in Floral Organs of *Hibiscus syriacus* L.

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A convenient method was devised for the fractionation of aliphatic polyamines (PAs), 1-aminocyclopropane-1-carboxylic acid (ACC), and their conjugated forms using a cation exchange resin, and applied to the floral organs of *Hibiscus syriacus* L. 'Diana'. A batch-wise use of the cation exchange resin to the acid extracts of the *Hibiscus* flower effectively separated the ACC-conjugate from free ACC, free PAs, and PA-conjugates. Good recovery rates, showing over 90% for PAs and 76–97% for ACC, were obtained when known amounts of ACC and PAs were added to the tissue extract. The amounts of these cellular compounds were determined in the petal, sepal, ovary, and style with stigma (+stamen) collected at two different stages (flower opening and flower senescence showing complete petal in-rolling). Both ACC and ACC-conjugate, which are generally associated with tissue senescence, were consistently detected in all organs even immediately after flower opening, but their concentrations, especially that of the ACC-conjugate in the ovary, greatly increased in the senescent flowers. As regards the free PA levels, a high concentration of spermidine was found in the ovary, and its level was maintained even when the petals wilted. PA-conjugates bound to small molecules decreased in the ovaries of senescent flowers, while the PA-conjugates bound to macromolecules remained very low in all organs at the two different flower stages. The present method seems applicable to a quantitative analysis of these physiologically important compounds in a variety of plant tissues, despite the fact that their extracts contain highly viscous materials that generally reduce the recovery rate of ACC.

Key Words: 1-aminocyclopropane-1-carboxylic acid, conjugated polyamine, floral organ, *Hibiscus syriacus* L., polyamine, senescence.

Introduction

The physiology and biosynthesis of ethylene and polyamines (PAs) in higher plants have been extensively studied and reviewed (Kushad and Dumbroff, 1991; Lieberman, 1979; Pandey et al., 2000; Yang and Hoffman, 1984). Aliphatic PAs such as putrescine (PUT), spermidine (SPD), and spermine (SPM) play an important role in plant cell growth, differentiation, and the retardation of senescence, while ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) have a plant-aging function leading to the promotion of senescence. Thus, PAs and ethylene have diametrically opposed functions. Not only the physiological actions but also the biosynthesis of these cellular

molecules are closely linked to each other through their common precursor, S-adenosyl-L-methionine (Kushad and Dumbroff, 1991; Pandey et al., 2000; Ravel et al., 1998).

Both ACC and PAs are also known to occur in plant cells as conjugated forms (Hoffman et al., 1982; Martin-Tanguy, 1985, 1997; Yang and Hoffman, 1984). The presence of malonyl-ACC, the conjugated end-product of ACC, was first demonstrated in wheat leaf by Hoffman et al. (1982). They suggested that malonyl-ACC may be a good stress indicator in higher plants, since this conjugate accumulates under stress conditions such as water stress (Hoffman et al., 1983). On the other hand, PAs can be linked with hydroxycinnamic acids by an amide bond, forming hydroxycinnamic amides. These PA-conjugates bound to small molecules (SM-PAs) are found in large quantities in flowers, but not in leaves or other vegetative tissues (Martin-Tanguy, 1985, 1997). Pérez-Amador et al. (1996) reported the presence of

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another type of SM-PA, N⁴-hexanoyl-SPD, which accumulates during the senescence process of the ovary and petal in peas. The true functional role or physiological meaning of such ACC- and PA-conjugates in the process of anthesis and flower senescence, however, remains unknown.

Hibiscus syriacus L., the national flower of Korea, has been planted in broad areas of Japan not only as an ornamental tree in gardens but also as a familiar tree in parks, on roadsides, and in the central reservations of freeways. Many cultivars having a variety of colors and diverse flower forms have now been released. *H. syriacus* L. has a very short flower life, but there have been few physiological and biochemical studies on the senescing process of this ephemeral flower as compared with those on the vase life of various cut flowers. In the course of a study on the senescence of *H. syriacus* L. flower, we encountered the difficulty of the accurate measurement of ACC and its conjugate due to the presence of a large amount of viscous materials, possibly polysaccharides, in the tissue extract of the flower. Furthermore, it was necessary to use a sequential fractionation method to determine the cellular compounds in the reproductive organs of the flower due to the very small sample size of the organs collected from each flower, especially the ovary. In this paper, we report a convenient method enabling the separation and estimation of both free ACC and ACC-conjugate together with free PAs and their conjugates in the same sample extract. We determined the concentrations of these compounds in the floral organs of *H. syriacus* L. collected at two different flower stages, flower opening and flower senescence showing complete petal in-rolling.

Materials and Methods

Plant materials

Hibiscus syriacus L. 'Diana' plants were grown in a greenhouse at $25 \pm 3^\circ\text{C}$ under a natural day length. Flowers were cut from the plants at the bud stage 12 h before full bloom, transferred to 20-mL vessels containing distilled water, and placed in a growth chamber under a 14-h photoperiod ($25^\circ\text{C}/22^\circ\text{C}$; light/dark, $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) regime at 65% relative humidity. Flowers in the early morning (AM 6:00 to 7:00) that had just achieved full bloom and flowers 48 h after full bloom that showed complete petal in-rolling were collected, immediately frozen in liquid nitrogen, and lyophilized with a freeze dryer. The freeze-dried flowers were then separated into four floral parts, the petal, sepal, ovary, and style with stigma (+ stamen), and ground using a pestle and mortar.

Extraction and fractionation of ACC, polyamines, and their conjugates

The method for sequential fractionation of these compounds by the batch-wise use of an ion exchange

resin is diagrammatically shown in Figure 1. Dried powder of floral organs (100 mg) was extracted with 0.25 M HCl in 50% aq. methanol (MeOH) and centrifuged at $35000\times g$ for 25 min. An aliquot of the supernatant (referred to as supernatant A in Figure 1) was subjected to the determination of free ACC, ACC-conjugate, free PAs, and PA-conjugates bound to small molecules (SM-PAs). The pellet was washed with the above acid and hydrolyzed with 6 M HCl at 110°C for 15 h. After filtration using filter paper (Advantec No. 3, Toyo, Tokyo, Japan), the filtrate was evaporated to dryness and dissolved in 5% perchloric acid (PCA). This fraction is referred to as FR-1 containing PA-conjugates bound to macromolecules (MM-PAs).

Four mL of the clear supernatant A was transferred to a 15-mL polystyrene conical tube, mixed with 2.2 mL of a cation exchange resin (Dowex 50W-x 8, H⁺ form, 200–400 mesh, Dow Chemical Co., USA), and shaken with a reciprocal shaker for 20 min. After centrifugation at $170\times g$ for 5 min, the supernatant containing ACC-conjugate (referred to as supernatant B) was adjusted to 3 M HCl with conc. HCl and hydrolyzed to free ACC at 100°C for 3 h, filtrated with a filter paper, evaporated to dryness, and finally dissolved in distilled water. This fraction is referred to as FR-2. The above-mentioned ion exchange resin was washed with distilled water four times, eluted with 15 mL of 6 M HCl, and divided into three fractions. One aliquot of the solution was hydrolyzed at 110°C for 15 h, evaporated to dryness, and dissolved in 5% PCA (FR-3 containing both free- and SM-PAs). Other aliquots, after evaporation, were dissolved in 5% PCA (FR-4) and distilled water (FR-5) for the determination of free PAs and free ACC, respectively. The amount of SM-PAs was calculated by subtracting the analytical value of FR-4 from that of FR-3.

Analysis of PAs

The PA contents in FR-1, FR-3, and FR-4 were analyzed by high performance liquid chromatography (HPLC: LC-10A, Shimadzu, Kyoto, Japan) according to Flores and Galston (1982) with slight modifications. Derivatization of the PAs was carried out with benzoyl chloride using 10 nmol of 1,6-diaminohexane as an internal standard. The sample solution (0.5 mL) was mixed with 1 mL of 2 M NaOH and 10 μL of benzoyl chloride. The mixtures were vigorously vortexed and incubated for 40 min at room temperature. The reaction was terminated by the addition of 2 mL of saturated NaCl, and the benzoyl-PAs were extracted with 2 mL of cold diethyl ether. After centrifugation at $170\times g$ for 5 min, 1 mL of the ether phase was evaporated to dryness under an N₂ flow, re-dissolved in 300 μL of MeOH, and filtered through a chromato-disc (pore size: 0.45 μm , GL Science Inc., Tokyo, Japan). Each PA derivative was separated on a $4.6 \times 250 \text{ mm}$ C₁₈ reverse-phase column (COSMOSIL 5C₁₈-ARII, Nacalai Tesque Inc., Kyoto,

Fig. 1. Diagram of sequential fractionation for the analysis of plant tissue PAs, ACC, and their conjugates.

Japan) at a $0.8 \text{ mL} \cdot \text{min}^{-1}$ flow rate at 35°C , and monitored at 254 nm. As a gradient solvent system, MeOH-water changing from 55% to 90% MeOH (v/v) was used for the elution of benzoyl-PAs.

Analysis of ACC and ethylene

The ACC contents in FR-2 and FR-5 were determined by chemical conversion of ACC to ethylene according to the method of Lizada and Yang (1979). The sample solution (generally 0.8 mL) was mixed with 100 mL of cold 10 mM HgCl_2 in a 10-mL glass vial, sealed with a rubber serum stopper, and stored on ice. Then, 0.1 mL of a mixture of 5% NaOCl and saturated NaOH (2 : 1, v/v) was injected into the vial through the stopper. The mixture was vortexed for 5 s, incubated on ice for 2.5 min, and vortexed again for 5 s. After incubation at room temperature for 30 min, 1 mL of the gas phase was injected into a gas-chromatograph (GC-17A, Shimadzu) equipped with a glass column packed with Unipak S (GL Science Inc.). Ethylene gas was analyzed under the following GC conditions: oven temperature, 60°C ; injection temperature, 120°C ; detector temperature, 120°C ; carrier gas flow rate, N_2 $50 \text{ mL} \cdot \text{min}^{-1}$, and H_2 and air pressure, 60 and 50 kPa, respectively.

Ethylene production from the *H. syriacus* L. flower was also determined. The flowers collected at the stages of full bloom and senescence were individually enclosed in a 1.3 L vessel fitted with a silicon septum. After a 2-h incubation, 1 mL of the gas phase was withdrawn and ethylene was determined as described above.

Estimation of recovery rates for ACC and PAs

For the evaluation of quantification throughout the

present procedure, *Hibiscus* petals (100 mg DW) at full bloom were extracted with 0.25 M HCl in 50% MeOH, and recovery rates for ACC and PAs were examined as follows.

The petal acid extract and supernatant B after treatment with an ion exchange resin (Fig. 1) were fortified with 1 and 2.5 nmol of ACC, respectively. ACC contents in FR-5 (free ACC fraction) and FR-2 (ACC-conjugate fraction) prepared according to the fractionation procedure were quantified by the analytical method described above. ACC recovered in FR-2 or FR-5 was estimated by subtracting the value without addition of the authentic ACC. The ACC recovery rate was calculated from the ratio of recovered ACC in each fraction to added ACC.

Recovery rates for PAs were similarly determined using a standard PA mixture containing equal amounts of PUT, SPD, and SPM. The petal acid extract, the pellet after centrifugation, and the eluate from a cationic resin prior to the acid hydrolysis (Fig. 1) were fortified with 200, 40, and 200 nmol of a standard PA mixture, respectively. PA contents in FR-4 (free PAs fraction), FR-1 (MM-PAs fraction), and FR-3 (free + SM-PAs fraction) were quantified and the recoveries for PUT, SPD, and SPM in each fraction were calculated by subtracting the values without addition of the standard PA mixture.

Results and Discussion

In a preliminary study, the presence of highly viscous materials in the acid extract of *Hibiscus* flowers interfered with the estimation of free ACC, since the recovery rate of ACC added to the tissue extract showed

an exceedingly low value. In order to reduce the viscosity of the tissue extract in the present study, 0.25 M HCl in 50% MeOH was substituted for sulfosalicylic acid or trichloroacetic acid, which is usually used in the extraction of ACC from various plant tissues (Bartoli et al., 1997; Lizada and Yang, 1979; Quan et al., 2002; Serrano et al., 1991; Zapata et al., 2004). Furthermore, as shown in the diagram of the procedure (Fig. 1), free ACC was separated from its conjugate by a batch-wise treatment with a fine mesh cation exchange resin added directly to supernatant A after centrifugation of the acid extract. The cationic resin completely adsorbed not only free ACC, but also free PAs and PA-conjugates bound to small molecules (SM-PAs). Because the ACC-conjugate had an acidic nature, it was recovered in supernatant B after centrifugation and washing of the cationic resin.

A good recovery rate of ACC was obtained using the

present method (Table 1), although the recovery rate of ACC in FR-2 was somewhat lower than that in FR-5, possibly because a small amount of viscous materials remained in supernatant B. Likewise, authentic PAs (a mixture of PUT, SPD, and SPM) added to the acid-insoluble pellet, acid extract, or eluate from a cationic resin prior to acid hydrolysis were satisfactorily recovered in FR-1, FR-3, and FR-4, respectively (Table 2).

Free ACC and ACC-conjugate in floral organs of H. syriacus L.

Both free ACC and ACC-conjugate, which are closely associated with tissue senescence (Hoffman et al., 1982, 1983; Ketsa and Luangsuwalai, 1996; Lizada and Yang, 1979; Yang and Hoffman, 1984), were detected in all floral organs even at the full bloom stage (Fig. 2). In fact, the generation of ethylene gas from the *Hibiscus*

Table 1. Recovery rates for ACC added to *H. syriacus* L. extract.

Sample	FR-5	FR-2
	ACC (nmol·100 mg ⁻¹ DW)	
Petal	0.32 ± 0.03 ^x	2.82 ± 0.08
Petal + ACC (1 nmol, 2.5 nmol) ^z	1.28 ± 0.04	4.74 ± 0.03
Recovery rate (%) ^y	96.9	76.7

Petals (100 mg DW) were extracted with 0.25 M HCl in 50% MeOH and subjected to the fractionation procedure shown in Figure 1.

^z ACC was added to the acid extract (1 nmol) or supernatant B (2.5 nmol) in Figure 1.

^y Recoveries of ACC in FR-5 (free-ACC fraction) and FR-2 (ACC-conjugate fraction) were calculated by subtracting the values without addition of ACC.

^x The values are the means ± SE of triplicate measurements.

Table 2. Recovery rates for PAs added to *H. syriacus* L. extract.

Sample	FR-1		
	PUT	SPD	SPM
	(nmol·100 mg ⁻¹ DW)		
Petal	0.0 ± 0.0 ^x	0.2 ± 0.3	0.0 ± 0.0
Petal + PAs (40 nmol) ^z	39.5 ± 0.4	37.5 ± 0.4	37.0 ± 0.4
Recovery rate (%) ^y	98.8	93.2	92.5
	FR-3		
	(nmol·100 mg ⁻¹ DW)		
Petal	23.5 ± 1.6	85.9 ± 3.1	12.7 ± 0.5
Petal + PAs (200 nmol) ^z	223.7 ± 4.8	282.6 ± 6.4	203.8 ± 3.9
Recovery rate (%)	100.1	98.4	95.6
	FR-4		
	(nmol·100 mg ⁻¹ DW)		
Petal	17.2 ± 0.3	68.4 ± 4.8	9.9 ± 1.0
Petal + PAs (200 nmol) ^z	213.1 ± 7.3	261.0 ± 6.5	190.4 ± 2.4
Recovery rate (%)	96.6	96.3	90.2

Petals (100 mg DW) were extracted with 0.25 M HCl in 50% MeOH and subjected to the fractionation procedure shown in Figure 1.

^z A mixture of PUT, SPD, and SPM was added to the acid-insoluble pellet (40 nmol), acid extract (200 nmol), or the eluate from a cationic resin prior to hydrolysis (200 nmol) in Figure 1.

^y Recoveries of PAs in FR-1 (MM-PAs fraction), FR-3 (free and SM-PAs fraction), and FR-4 (free PAs fraction) were calculated by subtracting the values without addition of a standard PA mixture.

^x The values are the means ± SE of triplicate measurements.

flower was observed at this stage, although the ethylene gas concentrations were exceedingly low compared with those from senescent flowers, with the concentrations at 5.5 ± 0.9 nL/flower/h at the full bloom stage and 20.1 ± 2.0 nL/flower/h at the senescence stage.

The free ACC level was lowest in the petal and highest in the ovary at the full bloom stage, and its concentration increased in the senescent flowers, especially in the sepal and ovary (Fig. 2A). The concentration of ACC-conjugate in each organ was 5 to 10 times higher than that of free ACC (Fig. 2B). The ACC-conjugate also increased in all organs of the senescent flowers, showing a similar pattern in the case of free ACC. The most striking accumulation of ACC-conjugate in the senescent flowers was observed in the ovary, indicating a rapid conversion of free ACC to ACC-conjugate in this organ.

Free PAs in floral organs of *H. syriacus* L.

It has generally been accepted that PAs, especially SPD, are abundant in young and metabolically active tissues (Kaur-Sawhney and Galston, 1991; Mad Arif et al., 1994). We also reported that in the cucumber plant, younger leaves contained much higher levels of free PAs, especially SPD, than older ones (Fujihara and Yoneyama, 2001). The compositions of free PAs in *Hibiscus* floral organs were similar to each other, with SPD showing the highest concentration amongst free PAs except in the sepal, which contained a relatively high concentration of PUT (Fig. 3). The ovary, a reproductive organ, contained high levels of free PAs, especially SPD. In the petal and style with stigma (+stamen), the SPD concentrations decreased in the senescent flowers while high SPD levels were maintained both in the ovary and sepal. Roberts et al.

Fig. 2. Determination of free ACC and ACC-conjugate in floral organs of *H. syriacus* L.

Flowers at full bloom (□) and at senescence showing complete petal in-rolling (■) were collected and separated into four parts. Free ACC (A) and ACC-conjugate (B) were separated and determined according to the diagram shown in Figure 1. Bars indicate means $\pm$ SE of triplicate measurements of each floral organ collected from fifteen flowers.

Fig. 3. Determination of free PAs in floral organs of *H. syriacus* L.

Flowers at full bloom (□) and at senescence showing complete petal in-rolling (■) were collected and separated into four parts, petal (A), style with stigma (+stamen) (B), ovary (C) and sepal (D). Free PAs were determined according to the diagram shown in Figure 1. Bars indicate means $\pm$ SE of triplicate measurements of each floral organ collected from fifteen flowers.

(1984) reported a marked accumulation of PUT above SPD levels during the senescence of carnation flowers. The life span of the *Hibiscus* flower is far shorter than that of the carnation. The PUT concentration in *H. syriacus* L. somewhat decreased in the flowers at senescence, apparently indicating the difference of PA metabolism during flower aging between the carnation and *H. syriacus* L.

MM-PAs and SM-PAs in floral organs of *H. syriacus* L.

Plant PAs are also known to occur in conjugated forms bound to small or macromolecular compounds. It is now apparent that in many cases, these can represent a substantial portion of the metabolic pool. Mossetti et al. (1987) reported that half of the PAs in dormant tubers of the Jerusalem artichoke are PA-conjugates covalently bound to high molecular weight compounds (MM-PAs). They postulated the storage function of MM-PAs from the evidence that after the release of tuber dormancy, free PAs markedly increased and this increase was accompanied by a reduction of MM-PAs. In the present study, the MM-PAs in the acid-insoluble fraction (FR-1 in Fig. 1) were consistently detected in all floral organs of *H. syriacus* L., especially in reproductive organs such as the style with stigma (+stamen) and ovary (Table 3). However, their concentrations were exceedingly low compared with those of free PAs. Furthermore, the flower stage did not significantly influence the levels of

these MM-PAs. Thus, the present data exclude the storage function of MM-PAs that supply free PAs (Mossetti et al., 1987), at least concerning the process of *Hibiscus* flower senescence.

The concentrations of SM-PAs in the acid-soluble fraction (FR-3 in Fig. 1) were always higher than those of MM-PAs in all floral organs (Table 3). At the time of flower initiation, SM-PAs are known to move from leaves to the young floral tissue (Havelange et al., 1996), and are reported to accumulate with flowering (Martin-Tanguy, 1985). In fact, PA-conjugates with small molecules have been widely found in flowers (Ponchet et al., 1982), pollen (Meurer et al., 1986), and ovaries (Pérez-Amador et al., 1996). Thus, it is generally accepted that SM-PAs are universal constituents of reproductive organs and are closely associated with sexual organogenesis in different plant species. In the *Hibiscus* flower, the ovary and style with stigma (+stamen) at the stage of flower opening contained high amounts of SM-PAs, especially SPD- and SPM-conjugate (Table 3). In the tobacco plant, a remarkable reduction of PA-conjugates such as caffeoyl-PUT and caffeoyl-SPD after pollination has been reported (Martin-Tanguy, 1985). Although the partner molecules of polyamines in SM-PAs were not determined in this study, the levels of SPD-conjugate and SPM-conjugate in the ovary significantly declined in the senescent flowers, suggesting the involvement of these PA

Table 3. Determination of PAs bound to small and macro molecules in floral organs of *H. syriacus* L.

Floral organ	Stage	MM-PAs			
		PUT	SPD	SPM	Total PAs
		(nmol·g ⁻¹ DW)			
Petal	Full bloom	0.0±0.0 ^z	3.0±2.8	0.0±0.0	3.0
	Senescence	0.0±0.0	1.1±1.9	0.7±1.1	1.8
Style with Stigma (+ Stamen)	Full bloom	9.2±1.3	13.4±0.3	2.4±0.6	25.0
	Senescence	11.4±1.4	13.5±1.4	3.7±1.3	28.5
Ovary	Full bloom	5.2±1.0	20.7±1.1	8.3±0.8	34.3
	Senescence	7.9±0.9	23.7±4.1	9.2±2.1	40.8
Sepal	Full bloom	0.8±1.4	7.1±0.9	3.0±0.5	10.9
	Senescence	0.0±0.0	3.7±6.4	4.0±0.7	7.7

Floral organ	Stage	SM-PAs			
		PUT	SPD	SPM	Total PAs
		(nmol·g ⁻¹ DW)			
Petal	Full bloom	26.5± 8.3	49.5±19.2	6.9± 3.1	82.9
	Senescence	32.6±20.3	44.2±38.0	9.9± 2.3	86.7
Style with Stigma (+ Stamen)	Full bloom	43.2±38.2	58.6±39.3	37.6±17.5	139.3
	Senescence	55.7± 7.7	67.1± 7.9	25.4± 2.3	148.1
Ovary	Full bloom	45.3±39.9	104.2±48.3	72.8±17.7	222.3
	Senescence	37.7±40.1	37.5±43.6	34.6± 9.6	109.8
Sepal	Full bloom	28.8±15.6	21.9±20.8	6.7± 9.6	57.3
	Senescence	35.9± 9.8	11.8±19.0	4.6± 7.5	52.3

^z The values are the means±SE of triplicate measurements of each floral organ collected from fifteen flowers.

conjugates in sexual organogenesis, as postulated by Martin-Tanguy (1997).

As indicated above, the ovary contained a high concentration of free SPD and maintained its level even when the petals wilted (Fig. 3). In addition, a striking accumulation of ACC-conjugate during flower aging was observed in this reproductive organ (Fig. 2). The ovary after pollination may perhaps require PAs, particularly free SPD, to support meristematic activities, and may activate a metabolic conversion of ACC to ACC-conjugate, a physiologically inactive form of ACC (Yang and Hoffman, 1984), to avoid the senescence of fertilized tissue.

Remarkable changes in free PAs, ethylene, or ACC during flower aging or fruit ripening have been reported so far in a wide variety of plants (Aziz et al., 2001; de Dios et al., 2006; Ketsa and Luangsuwalai, 1996; Llop-Tous et al., 2000; Serrano et al., 1991). Relatively little information is, however, available on the physiological interrelationship or mutual metabolic regulation of these cellular compounds including their conjugated forms. More detailed studies on the metabolism of PAs, ACC, and their conjugates in the anthesis and senescence processes of the *H. syriacus* L. flower are currently under way.

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